JOURNAL OF **Pharmaceutical Sciences**

January 1968 volume 57, number 1

Review Article

Topical Toxicity and Testing

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THE PHARMACEUTICAL or cosmetic chemist, involved with the problems of topical formulations, is often concerned with the potential of his product for skin irritation and sensitivity and confused by the number of possible local toxicity tests. This review is an attempt to outline the nature of the topical toxicities and the principal test methods for irritation and sensitivity. The vital problems of systemic toxicities, such as acute and subacute studies, as well as special tests (*e.g.*, teratogenesis) are not the province of this review.

INCIDENCE OF SKIN REACTIONS

Considering the widespread use of toiletries and cosmetics the incidence of skin reactions is remarkably low (1–4). This is primarily due to the efforts of reputable manufacturers to detect potential hazards before a product is marketed and thus minimize the possibility of adverse side reactions in normal use. The ultimate goal of toxicity studies is to insure safety or harmlessness under the proposed use conditions (5). Calculated risk is sometimes necessary. This must be based on knowledge of compound sensitivities, such as the use of bromoacid dyes in lipsticks or certain resins in nail polishes. The potential toxicities of cosmetics and toiletries have been discussed in several excellent reviews (3, 6–12).

Statistics on the incidence of skin reactions from cosmetics and toiletries are mainly derived from studies by dermatologists on clinical patients (2, 6, 7, 13, 14). Yet dermatitic skin exhibits about 10–50 times the reaction incidence of intact skin (15, 16). Complaint letters to manufacturers vary from 0.2–1.2 per 100,000 units sold (2, 17, 18), while dermatologists report 2–4% reactions (3). It must be remembered that the data on complaint letters assume each unit sold represents a separate user. This is a fallacy since one consumer may actually use multiple packages (19), which would raise the complaints per user. The most valid procedure is to conduct consumer tests in which the product is placed with a relatively large number of users, and each subject is questioned as to irritation side effects as well as product performance.

Judgments of safety and size of human test panels are based on the prediction of 95% certainty of safety. There has been an assumption that the results with 200 test subjects will apply

Received from the Pharmaceutical Research and Development Department, Hoffmann-LaRoche, Inc., Nutley, NJ 07110

to the population at large (20). Yet if 200 subjects contained no reactors, this would denote 95% confidence that not more than 1.5% of the general public will become sensitized. Even in the absence of an adverse reaction on 30,000 persons tested, it is 95% certain that 1 in 10,000 would still be liable to skin reactions (21, 22). Thus, if there are 10 million users, 1,000 cases of dermatitis will probably arise. The prediction of 99.9% safety in a mass population would require an impossibly huge number of subjects (23). The degree and type of use is the cardinal factor. A 0.5% frequency of sensitization (1:200) might be tolerated if the material is in limited distribution and its efficacy is great. However, in a mass distribution cosmetic, 1 in 200 reactions would necessitate further prerelease studies. An incidence of 1 or less in 100,000 is the desirable goal for mass distributed products.

Primary irritation is rather unusual in a modern commercially available product, although new agents are of concern. Agents capable of inducing skin reactions in 1 in 100 or even 1 in 1,000 subjects are readily detected, but it becomes increasingly difficult to predict the irritation or sensitization potential of a preparation causing skin reactions in 1 in 10,000 or even 1 in 100,000. (This is particularly true for topical agents having weak photosensitizing properties.) It is this particular type of agent that concerns those who are responsible for passing on the safety of products that are used by millions of people.

TOPICAL TESTING

No ideal single test procedure exists which will adequately measure irritation and sensitivity potential. There is rather a spectrum of tests to be selected by the trained toxicologist who can secure data and translate it into probable effects in man, and balance these effects against the intended use and possible misuse of the product.

Simpler and less expensive tests are first performed to determine the range of the problems. Usually, animal eye and primary skin irritation tests must first prove favorable before more complex tests are undertaken. The further experimental design should be geared to indicate the possible type injury, the extent or seriousness of the injury, and the margin of safety that prevails under the most extreme conditions of use (24). Yet, even with these considerations it must be understood that tests which are absolutely predictive are, for the present, beyond practical accomplishment (20).

While opinions concerning the value of animal studies vary (25–27), no responsible person would dispense with animal skin tests. If properly judged, on the basis of degrees of variability and not on a "yes–no" basis, animal tests help predict dangers of severe human irritation and, to some degree, the concentrations that may safely be tolerated by man. The tests have their greatest value as comparative tools. The data, with adequate controls, are related to other substances whose hazards have been defined by time and experience. In addition, toxic doses provide an estimate of the margin of safety under normal usage (24). Additional animal studies may be necessary even after a product has been introduced into clinical usage. As the major effects of a preparation are characterized by studying it in detail in man, a more suitable laboratory model, to simulate more closely these actions in man, may be sought by rescreening a wide variety of animals.

Argument continues as to the great differences between human and animal skin. No one can doubt this. Yet it appears more profitable to be concerned with what, if any, correlations can be obtained between animal testing and human clinical experiences, than to be concerned with differences. The mounting costs of clinical tests virtually dictate initial animal tests as an aid in screening and predicting toxicity potential.

Whenever possible, the intended marketing product and package should be studied. If feasible, the program should include parallel studies of a similar product of known safety. If any question of an individual component's safety or "newness" is involved, it should be examined separately. Ultimately, tests must be performed on man under conditions of actual use. Kennon has discussed many of these usage tests in a recent review of toiletry technology (28).

IRRITATION REACTIONS

Primary irritants are substances which damage skin by direct cytotoxic action (134). Reactions are divided according to severity. A strong irritant has been defined as one which will cause 100% of subjects to react in 24 hr. at a definite reasonable concentration (29).

Mild primary irritants exert their reactions after repeated exposure or overexposure. These include soaps, detergents, and solvents that also present a more serious problem of possible sensitization. Mild irritation may become chronic or cumulative after a number of exposures resulting in "skin fatigue." Conversely, after daily exposure to mild irritants, the irritated skin often adapts to these changes, and recovers from and

becomes resistant to further irritation, resulting in "skin hardening" or "skin accommodation" (132).

Generally speaking, agents that cause primary irritation are not selective in their action and will ultimately affect any normal skin if allowed to act, in sufficient concentration, for a long enough period of time. A precise frame of reference is essential for valid assessments of safety. Unfortunately, there is no universal statement that can be made about the type of chemical which will irritate healthy skin. While the pH of normal skin is mildly acid, there is little reason to believe that mildly alkaline products are irritating to the skin. Excellent tests (30) have demonstrated that except for relatively high alkaline or acid materials, pH *per se* is an unreliable criterion for predicting irritancy to skin. Substances which are easily oxidized or reduced are usually more likely to irritate; *e.g.*, hair dyes with strong reducing agents or depilatories containing sulfides. The products most commonly associated with reactions of the primary irritant type are antiperspirants, depilatories, and permanent wave preparations. Many liquid "automatic" or "roll on" mascaras and an occasional cream-type mascara produce false positive primary irritant reactions when patch tested under occlusive coverings. These misleading irritant reactions are apparently caused by solvents and can be avoided by using nonocclusive patch tests (31).

IRRITATION TESTING

Eye Irritation—There are few materials that are unlikely to enter the eye accidentally. Consequently, investigation of animal eye irritancy is the starting point of virtually all safety evaluation programs. The most widely used procedure has been and is the "Draize" test and its modifications (32–53), intended to provide a measure of the irritancy of a product by exposing rabbits under conditions which would resemble an accidental human exposure, such as splashing a substance into the eye. Many descriptions, modifications, and critiques of the test have been published (26, 32, 53–60).

The Draize test (32) scores the effects on the cornea, iris, and conjunctiva of rinsed and unrinsed material after 24, 48, and 72 hr., and 4 and 7 days. The emphasis is on corneal and iris lesions. Eyes that do not clear by the seventh day are considered to have contained severe irritants. For substances covered under the Federal Hazardous Substances Act, a modified procedure has been proposed (61). To aid in interpretation, a colored set of photos are available from FDA (62).

Variabilities in Draize results may stem from personal error, sample size, time of release of an irritant from a formulation, or sample drainage (54). The most viscous samples tend to remain in the eye longer and are more likely to localize in one area, increasing the possibility of irritation. The competence of the trained observer is always the most important factor. While there is considerable variability between laboratories testing the same sample by the "same" Draize techniques (26, 60), the tests have served quite well in delineating moderate and severe irritants. Their main value may lie in comparing an unknown material with control substances whose potential for producing human eye injuries is known through experience. As with other tests, problems arise with nonirritating or mildly irritating substances, or comparison of related formulations in product development.

Variant scoring systems of the Draize test and modifications have yielded greater reliability for comparisons between products. Kay and Calandra (55) score the extent of irritation, its persistency, and the over-all consistency of the data. Test materials are given tentative ratings on the basis of the scores obtained from all 3 tissues (cornea, iris, and conjunctiva) within the first 96 hr. These tentative ratings are adjusted according to the mean total scores at various specified times. Gaunt and Harper (63) judge the conjunctival reaction that is accompanied by persistent and severe injury to the cornea and iris persisting for more than 5 days. The arbitrary scoring system ranges from 1 for a nonirritant to 5 for a severe irritant. S. P. Battista (54) varies exposure conditions to the irritant quantitatively between no-effect and maximum-effect levels. Interpretation and comparison of data are reduced to a single number which represents a time measurement for achieving a level of response. In addition, it enables an estimate of the maximum exposure time that will be tolerated for a given irritant. Time–response curves have been plotted to estimate the time required for a product to produce a given degree of opacity, iritis, or conjunctivitis. When the procedure is repeated with different sample concentrations, a comparative formulation study can have significance.

Several investigators have noted difficulty in correlating rabbit eye irritation with the experience found in humans (2, 26, 57, 60, 64). The rabbit eye is considered more sensitive than that of man. Nevertheless, Rieger and G. W. Battista (26), in their review of the correlation be-

tween animal and human tests, have cited the example of the hair neutralizer which was indicated to be safe according to the rabbit eye test, but which proved to be irritating in human usage.

Study of records of eye accidents in a liquid detergent factory provide a check against the predictive value of the standard rabbit eye irritation tests (21). The animal and human sources were compared on the basis of the number of days required for the eyes to recover completely. Irritation was more severe in the rabbit eyes than in the eyes of the factory workers subjected to actual accidental exposure. In rabbits there was a high incidence of corneal opacity, with some eyes requiring up to 91 days to recover. The effects in humans were limited to conjunctivitis, and all cases recovered within 2 days. The degree and type of irritation produced in the Rhesus monkey eye appeared to more closely approximate that which resulted from exposure of the workers' eyes. Buehler and Newmann (56) studied the response of rabbit and monkey eyes to surfactant solutions and recommend use of the monkey for eye irritancy tests.

In testing some shampoos consideration must be given to the nature of the active ingredients. They may act as anesthetics when placed in the eye. This is dangerous since the user may not be aware that the shampoo has entered the eye and damage may result before it is washed out. To test for anesthetic effects the guinea pig blinking test is used (65).

Aerosol sprays represent a difficult area in which there is lack of standardization for eye tests. Some laboratories apply sprays directly into the rabbit eye. The direct spray may cause eye damage due to the physical impingement of the particles as well as the cooling due to the propellant. This response may mask the eye damage which may be due to the active ingredients. Other workers allow the propellant to evaporate and instill the residue in the eye. This removes the possibility of damage that may be produced by pressure or chilling and allows the experimenter to test only the active ingredients.

The eye irritation test has become so important that further development of a prospective product with excellent sales potential is often arrested purely on the basis of conclusions drawn from eye irritation studies. In fact, some cosmetic firms use ophthalmic irritancy routinely as *the* principal evaluation criterion for checking all new products early in their developmental stages. Products should not be rejected solely on the basis of a questionable Draize, or any other eye test, unless gross injury is detected. The question is to resolve irritancy *versus* possible injury (59). If the product is desirable on a market basis, it would be well to try another species of animal, such as the dog or monkey. If the one or two other species show positive reactions, it is likely that human eye irritation would result.

Skin Irritation—Methods devised to measure skin irritancy are adequate for screening out stronger and moderate irritants, but are virtually insensitive in the low irritancy range. Materials are either placed in single contact with human or animal skin for varied time periods, at varied concentrations, or repeatedly used. These tests determine not only primary irritation but also "skin fatigue," when the skin reacts due to a succession of insults of a subthreshold intensity.

The skins of the mouse, rat, guinea pig, rabbit, miniature pig, and sheep have been used to screen compounds for primary irritant activity. Histologically, their skins do not closely resemble human skin, but from a responsive point of view, they all show changes when irritated. The rabbit and guinea pig are most frequently chosen (66). Materials which cause simple primary irritation of the skin of rabbits generally can be expected to cause a similar response in humans, but not necessarily in all humans. If appreciable edema occurs in rabbit skin, the material should be suspected as a possible vesicant to human skin.

Draize (32) has also proposed the most widely used skin irritation test. Patch tests are read of suspected irritants on the abraded and intact skin of rabbits. A "primary irritation index" is reached. An index of 0–2 indicates mild irritation potential; 2–6, moderate; and over 6 is rated severe, with 8 a maximum.

Roudabush (67) compared the dermal effects of a large number of diverse organic compounds in the guinea pig and rabbit. The results of primary irritation tests showed that the intact guinea pig skin test is as sensitive as, or more sensitive than, the rabbit skin test in eliciting skin reactions. Levenstein and Wolven (65) claim a close correlation between the response to graded patch tests in the rabbit and observations noted in man.

Carter and Griffith (21) point out the pitfalls in placing sole reliance on animal data for assessing primary irritant hazard. They compared a number of unrelated household products by the Draize rabbit irritation index and human patch tests. The products included toilet soap, a general purpose granular detergent, a light-duty liquid detergent, isopropyl alcohol, and a hair dressing. Agreement between the two methods was poor. The product that gave the least reaction in the

human test, the general purpose granular detergent, gave the most severe reaction on the rabbit, and would be classified as a severe irritant on the basis of the animal data.

The Draize rabbit test (32) or Roudabush guinea pig t[illegible](67) are adequate for screening out stronger ir[illegible]ts but are insensitive in the low irritancy range. To obtain responses to moderate or low irritants, exaggerated conditions of exposure of the skin are required to get an incidence of reaction which may be used as a base for extrapolation. These procedures are comparative to decide whether an irritancy is greater than, equal to, or less than some reference substance. The most practical product reference is one whose irritancy potential has become known through widespread use. The detection of moderate and low irritants usually involves increasing the visual sensitivity of the erythema response by injecting a dye substance, which concentrates in the injured areas as a result of increased vascular permeability. Trypan blue has been most widely used (64, 68–71). Recently, use of sulphan blue has been advocated for greater sensitivity (72, 73).

Finkelstein, Laden, and Miechowski (69) have combined the trypan blue technique with formaldehyde presensitization and occlusive patch testing of varied animal skin to give a fairly sensitive and reliable indicator of the irritancy of agents which fall in the low range of the Draize test. The test, omitting presensitization and dye injection, has been adapted for humans (64). Results of the tests indicate whether it is worthwhile to proceed to sensitivity studies and usage trials with a new formulation. With this technique and all similar procedures, selection of proper control formulation is important.

Kligman (29, 134) has critically discussed quantitative measurement of irritants on human skin. He has attempted to use statistical analysis to find the concentration of agent to reach an irritant response in 50% of subjects (ID_{50}). For strong irritants, this value is read directly from a curve of a percentage of reactors *versus* concentration. For weak irritants, patches are applied to the same site for a minimum of 10 days to reach a cumulative frequency plot which estimates the number of days required to cause 50% of the sampled population to develop a threshold irritant response. By inspecting the curves it is usually possible to judge whether 2 agents differ significantly.

Studies of very minor states of irritation or "mildness" use all available tests (21, 74–77). The procedures of Justice, Travers, and Vinson (76) use human arm immersion (HAI), human patch tests, repeat mouse patch tests (RAP), and water transmission through rat and rabbit excised skin. Both the RAP and HAI tests utilize the principle of eliciting a response by repeated exposures of the same area to the test material. In the HAI test, the quantitative difference between two products lies in the number of exposures necessary to achieve a prescribed level of irritation. In the RAP test, the number of exposures is kept fixed and a quantitative measure of the effect of these exposures is made by histological examinaion of the excised skin. The concentration of the test material can also be varied in the RAP test to obtain additional information about the effect of a product on the skin. The most sensitive test for revealing relatively small differences in mildness between the products appeared to be the HAI. The RAP test served best as a preliminary screening of topical products. Materials showing a significant irritation in the RAP test, at standard testing levels, are not given further consideration for use in products. Should the mildness ratings be favorable, studies are continued, employing the other tests. The water transmission test correlates well with the other mildness tests (120, 121). Opdyke and Burnett (77) immerse guinea pigs in products up to their axillae for 4 hr. on 3 successive days, grading mildness on a 0–10 scale.

SENSITIVITY REACTIONS

Allergic sensitization reactions form by far the largest proportion of toiletry and cosmetic reactions (3). An eczematous allergen has been defined as a substance that is not primarily irritating on first exposure but which, in animals or human beings of appropriate genetic constitution, causes the development of a sensitization. Subsequent contact with concentrations that are not irritating to unexposed or nonsensitized individuals produces a reaction (78). An individual may use a cosmetic for years without reacting and then suddenly acquire an allergenic hypersensitivity to the particular material (79). An antigen–antibody system is required for sensitization reactions.

Cross-sensitization is a special form of sensitization which occurs fairly frequently. People affected are not only sensitive to the substance to which their skin was originally exposed, but also to other substances that are chemically related. The closer the relationship and the longer the contact, the greater the incidence and degree of sensitization. Cross-sensitization may be provoked equally by substances which form a related compound as the result of metabolic changes in

the body (9). Kinmont (80) has compiled a list of varied products which are involved in cross-sensitization. Oxidation hair dye cross-sensitivity is fairly common in sensitive individuals (81, 82).

There is hardly a cosmetic or toiletry product to which someone has not become allergic at some time or other. The most frequent sensitizers are the paraphenylenediamine oxidation type of hair dye and the formaldehyde resin in nail lacquers. Perfumes, lipsticks, and sunscreening agents are also among the more frequent offenders. Artificial colorants, principally the halogenated fluoresceins (eosins) in lipsticks, are probably the most frequent single cause of allergic reactions to cosmetics (83). Paraphenylenediamine hair dyes are undoubtedly potent sensitizers to the majority of the population. Yet considerably fewer than 1% of women who dye their hair develop contact allergy. This is probably explained by the fact that the dye is mainly applied to the hair and not to the scalp. While perfumes are often strong sensitizers, the number of skin reactions is small. Perfumes composed of easily oxidizable aldehydes, phenols, and ketones are more liable to cause reactions than those with the more difficultly oxidizable alcohols and esters. With surface-active agents there is great variance in dermal toxicity. Yet, in general, their order of toxicity is cationic > anionic > nonionic (32, 33).

SENSITIVITY TESTING

Delayed type sensitivity reactions are rarely seen if an agent or product is applied to exposed intact skin. Patch tests in humans and animals and intradermal injections in animals aid in provoking the signs of sensitivity.

Animal testing is useful for preliminary screening before any human sensitization testing. Calnan (84) has reviewed the role of animals in contact sensitivity studies. The guinea pig has proved to be the most useful. The most widely accepted animal technique is that of Landsteiner (85), using the guinea pig. Instead of a patch technique, the material is injected intradermally every other day for 10 applications. Following a 2-week rest a challenging dose is injected and the resulting response is compared to the preceding reactions. Sensitization is present if the challenging dose causes a greater skin response than that evoked by any earlier injection. The major limitation of guinea pig testing is the range of sensitivity. Strong sensitizers may be picked up earlier in the guinea pig than in man, but the weak reactors may not be eliminated. These latter must be studied employing human patch test techniques. While humans can react to 1 p.p.m. of a potent sensitizer, such as dinitrochlorobenzene, it is not normally possible to make guinea pigs react to dilutions greater than 1:25,000 (84). However, with current guinea pig sensitization procedures, one may conclude that if a material produces a significant number of reactions, it is also likely to produce sensitizations in human testing (86, 87). Eczematous changes, similar to those occurring in man, have been produced in guinea pigs by first treating their skin with squalene, sodium lauryl sulfate, sorbitan monolaurate[1] (88), and polysorbate 80[2] (89), all of which stimulate acanthosis.

Patch tests, usually with occlusion, enhance sensitivity testing and are universally used, both with animals and humans (12, 16, 20, 26, 90–95). Human patch tests are the province of the dermatologist. Yet, virtually all products are tested by patch test, so it is important to be aware of the varied tests and their limitations. All predictive procedures are designed around the basic technique of insulting the skin of a group of test subjects one or more times and then, after a lapse of time to permit a reactive state to develop, challenging with another application of the material. Substantially increased reactivity to the challenge is taken as evidence of sensitization.

The Schwartz-Peck (S-P) test (96, 97) is the pioneer "prophetic patch test." New products are tested, using a closed patch, on at least 200 subjects. A control of an old formula with a known record of safety is used. If the new formula shows more reactions than the control, it is deemed unsafe. To secure usage data, a 4-week paired-comparison use test of the cosmetic on the same 200 subjects is recommended before trial sale. Trial sale, the final step by S-P definition, is the sale and use of 5,000–10,000 units in one community. The test has certain inherent defects. False positives may occur, since borderline primary irritants can sometimes produce reactions which can be confused with sensitizations when only a patch test reading is made. More important are the false negatives, which are due to the fact that the single application of a small amount of the product is often inadequate to produce sensitization except in the case of strong allergens. Most of the predictive burden is shifted to the use test, but this part of the procedure is probably numerically inadequate to reveal low reaction rates. Also, some products are used only once weekly, or every 4 to 6 weeks,

[1] Marketed as Span 20 by Atlas Chemical Industries, Wilmington, Del.
[2] Marketed as Tween 80 by Atlas Chemical Industries, Wilmington, Del.

and cannot be evaluated for sensitization in a 4-week use test.

The Traub-Tusing-Spoor (T-T-S) (98) and the Brunner-Smiljanic (B-S) (99) tests are extensions or completions of the S-P to widen usage, increase frequency of applications, and use of a larger area of contact with the test substance. Regardless, all these tests suffer in that trial periods are too short, since many chemicals do not cause sensitization until used for many months.

Draize (100) and Shelanski (101) independently published methods which have come to be known as the "repeated insult" test or the Draize-Shelanski test. The test substance is applied every other day for 10 or 15 exposures to 200 subjects. Two weeks after the last exposure the subjects are challenged at a new site. Draize randomizes the exposure. Shelanski applies the occlusive repetitive applications to the same site to give a higher yield of sensitized subjects but tends to magnify the irritant effects of the test formulation. The resulting so-called "skin fatigue," due to summation of irritations under the test conditions, may make the differentiation between sensitization and irritancy more difficult.

Neither the Draize nor the Shelanski procedures specifies the anatomical site to which the patch should be applied or uses a high enough concentration to allow prediction of sensitization in a small panel of volunteers. Maibach and W. Epstein (93) apply the patches to limbs, since the lymph nodes are crucial to development of allergic sensitization of the contact type. The highest nonirritating concentration is used. With these modifications it was possible to detect the marked sensitization and potential of tetrachlorosalicylanilide (TCSA), which would not be predicted with Draize use concentrations.

A judgment of the value of these tests can be made using agents whose sensitizing capacities have become rather well-defined through extensive use. Kligman (20) evaluated a large series of topical drugs and concluded that the S-P, T-T-S, and Draize procedures were all almost useless. The Shelanski test, while still inadequate, was somewhat better.

The basic problem with all the discussed predictive tests is the attempt at utilizing larger and larger numbers of subjects in lieu of a sensitive method of detection (84). The requirements of large numbers of subjects restricts evaluation to those investigators with access to such populations, so that fewer preparations can receive adequate testing. Furthermore, the same subjects are retested too often, which results in false positive reactions because of conditioned irritability (102). What is needed is to enhance the sensitizing capacity of weak allergens to the point where smaller groups of subjects yield more positive responses.

The judicious use of irritation will improve the sensitivity of any animal or human test. Simultaneous use of mild irritants, the allergen, and occlusion appear to aid penetration and enhance sensitization. These combined techniques are used in the triple-freeze (16) and maximization procedures (20, 91, 103, 133).

The triple-freeze technique consists of irritation of the patch test site by freezing for 3 sec. with dichlorodifluoromethane.[3] Occlusion is maintained for 48 hr. after application of a maximal nonirritating concentration of the test compound. The procedure is repeated an additional two times at 5-day intervals (for three exposures). The challenge or eliciting patch test is applied 10 days after the last freeze. While the triple-freeze method has considerable merit, yielding greater sensitivity results than the previously discussed prophetic methods, it does not give adequate reproducible results (84).

The "maximization" provocative patch test of Kligman (20, 91, 103, 133) was primarily designed to yield allergenicity ratings for individual substances, not complex mixtures, finished products, or formulations. It does not seek to predict the ultimate evidence of sensitization in the consuming public, but rather classifies substances according to the sensitization capabilities they exhibit under an arbitrarily defined set of experimental conditions. Essentially, the test consists of a course of five 48-hr. exposures of an allergen to a single skin site which has been previously inflamed by treatment with aqueous 5% sodium lauryl sulfate (SLS). Chemical irritants, such as SLS or dimethylsulfoxide (DMSO) appeared superior to physical agents such as ultraviolet radiation or Scotch tape. Potential sensitizers are divided into 5 grades ranging from weak to extreme allergenic potential (133). A failure to sensitize a single subject demonstrates almost with certainty that the compound is not a significant sensitizer.

The maximization procedure was used to test a large group of diverse substances including cosmetics, drugs, and industrial contactants. There was good correlation between usage and the maximization test results. Sensitization, within limits, was found to be proportional to the surface concentration of the allergen and not to the total amount of allergen. High concentrations are required for weak allergens. The optimal concen-

[3] Freon 12. E. I. du Pont de Nemours & Co., Wilmington, Del.

tration for the challenge patch testing is the highest nonirritating amount, up to a maximum of 10%. The vehicle was an important factor. Petrolatum was the most generally useful and effective. There is some concern that use of occlusion and other methods of facilitating skin damage, such as use of SLS, may create a false impression of allergenicity (95). There is little danger of this if proper judgment is used and further testing is done. Grief (109) utilized the maximization procedure to study a series of compounds used in fragrance formulation. A panel of 25 adult volunteers was tested with 5% sodium lauryl sulfate for 24 hr., and a 48-hr. occlusive patch was applied with high concentrations of the test material. No sensitization was noted.

Most screening series consist of numerous tests, since the greater the number the greater the likelihood of detecting the allergen. This on the whole renders patch testing expensive, tedious, and time consuming. Patch test screening could be simplified by using mixtures which permit screening for several allergens with a single patch. Mixtures sharply reduce the number of patches required in a screening series without curtailing the number of chemicals screened. E. Epstein (104) has demonstrated the usefulness of patch testing mixtures of topical medicaments, rubber additives, and antiseptic agents. Topical medicaments were most suitable for combination. In general, the irritancy of mixtures seems to be related to the sum of irritancy of their components.

Results of predictive tests, as now constituted, serve as guides rather than absolute criteria of sensitization and irritancy. The tests function best when the test substance is used on a comparative basis against control formulations, with known behavior, in actual usage. Positive reactions do not invariably signify sensitization and negative reactions do not rule it out. False positive reactions may be caused by substances which do not produce dermatitis under normal conditions of use but become primary irritants under closed patch test conditions. This is particularly true of depilatories, permanent wave preparations, shampoos, hair tonics, certain hair preparations, and cosmetics containing volatile solvents (11). These should be tested by the "open" method in which the compound is simply rubbed on a small area of the forearm. False negative reactions may also be encountered when the degree of sensitivity is low and when the area involved by the dermatitis is composed of thin skin, such as the eyelid. If the product in question produces a negative patch test result, it should be retested by actual use at the normal site of application.

Further study of the basic mechanisms of sensitization is required before the tests can be significantly improved. Alterations in current test procedures can increase or decrease the number of reactors but do not help in answering the crucial question of the relationship between sensitization results in laboratory test and in consumer usage. Properly supervised consumer use tests are still required to supplement the laboratory studies. The perfect predictive patch test for allergic sensitization has not been devised. This will ideally involve an *in vitro* test easily employed in the laboratory. Until there is additional information on the pathogenesis of delayed hypersensitivity, this approach is improbable. In the interim, predictive patch testing remains a useful tool. Hopefully, in the years to come, we will learn more about the molecular basis for allergic sensitization. It may then be possible to predict sensitizers on the basis of molecular structure (93).

PHOTOSENSITIVITY REACTIONS

Exposure to light of a particular wavelength will evoke allergic photosensitivity in a predisposed individual (39–51, 105, 106). The phototoxic reactions may be manifested as an exaggerated sunburn or as an eczematous response with pigmentation. The wavelength of light to set off a response closely corresponds to the absorption peak of the offending agent and is referred to as the "action spectrum." Most of the phototoxic materials have an action spectrum in the ultraviolet band between 2800 to 4300 Å. (3, 105). Once an individual becomes photoallergenic the reaction time becomes shorter with subsequent exposures. The eruption usually subsides in a few days leaving no residual pigmentation.

The mechanisms involved in photosensitivity are poorly understood (107). Photosensitizers probably act by virtue of photodynamic action or interaction of light and a photosensitizer in the presence of oxygen to cause a destructive photochemical reaction (39). They also may act as free radical reaction initiators. The free radicals produced can cause damage to the intracellular membrane (108). *In vitro* biochemical and biophysical studies strongly suggest that ultraviolet irradiation of halogenated salicylanilides leads to formation of free radicals.

If topically applied, the penetrability of the sensitizer is of decisive importance (110). A photosensitizer must be imbibed by the Malpighian layer of the skin before hypersensitivity

to light can occur (111). Lipid or aqueous solubility, or both, and the type of vehicle are important factors. The furocoumarins, anthracene, and benzpyrene are fat soluble, penetrate well, and are potent photosensitizers when applied topically (112, 113). Penetration of substances that are water soluble only (eosin, rose bengal, acridine preparations, and sulfanilamide) is slight, as is their phototoxic potentiality after external application (114). A number of drugs taken internally also evoke phototoxicity. The chief offenders are sulfonamides, sulfonylurea, diuretics, phenothiazines, chlorothiazides, tetracyclines, and griseofulvin (42).

The dyes used in lipsticks have been reported to cause an occasional photosensitization (38). Considering the vast number of lipsticks sold, the incidence of cheilitis is very low. An example of photosensitization associated with certain perfume ingredients or essential oils is the so-called Berloque dermatitis usually caused by oil of bergamot, which results in skin pigmentation after exposure to sunlight. The photosensitizing agent in oil of bergamot is not defined, although 5-methoxypsoralen may be responsible (115). In spite of the widespread use of such perfumes, few people have Berloque dermatitis because a number of other circumstances must coincide to produce the pigmentation (41).

The reports of photosensitivity to sunscreen agents would seem paradoxical. Yet, monoglyceryl *p*-aminobenzoate (116) and digalloyl oleate (48) have shown sensitivity effects. In these cases the subject patients were shown to have prior sensitizations to a number of related compounds, or were cross-photosensitized.

The halogenated salicylanilides and bithionol are examples of antibacterial compounds that can produce allergic photocontact reactions. Tetrachlorosalicylanilide, particularly, caused a serious flare-up several years ago in England, and to a lesser extent in the United States, and was withdrawn promptly from the market (118–120). Tribromosalicylanilide has been widely used in soap products for several years without any reported cases of photosensitization until quite recently (49, 121). Bithionol is a bacteriostatic agent related to hexachlorophene. It can produce not only ordinary allergic contact dermatitis but photoallergic reactions as well (80, 121–125). Cross reactions may occur between the polyhalogenated salicylanilides and bithionol. Hexachlorophene itself is apparently only a rare sensitizer.

The major sensitivity activity of the salicylanilides resides in the salicylic acid ring with a carboxyl and hydroxyl group being essential. At least one halogen atom is also required (126). The mechanism appears to be a photoactivation of the salicylanilide ring by 3600 Å. radiation, followed by free radical formation at the 3 or 5 carbon–halogen bond. Ensuing loss of free radical activity is associated with a chemical binding of the free radical (hapten) to protein (complete antigen formation).

PHOTOSENSITIVITY TESTING

Suspected agents are applied as photopatches in the same concentrations as used for ordinary patch tests (50). Tests are particularly indicated if an agent is present which has a chemical structure resembling known photosensitizers, such as chlorpromazine, promethazine HCl,[4] bithionol, tribromosalicylanilide, dibromosalicylanilide, and tetrachlorosalicylanilide.

Natural sunlight is the ideal source for photosensitization testing, but it is usually not regularly available at a given time. Artificial light sources are useful and have been advantageously substituted (35, 43, 46, 105).

Until recently, attempts to produce drug photosensitization in laboratory animals were generally unsuccessful. Guinea pigs (43, 72, 107, 127, 128), rabbits (32), rats (129), and mice (129, 135) have been reasonably satisfactory for some photosensitization substances. In some instances, it has not been possible to elicit photosensitization in animals to substances that regularly produce this response in man.

Vinson and Borselli (127) use stress conditions to elicit skin responses in guinea pigs to marginal sensitizers. If the results of this test and the Landsteiner-Jacobs test are both negative the test material can probably be tested on humans with good assurance that no photosensitization problem will develop. If an agent passes both tests, but shows cross-photosensitivity, additional tests on the preparation in the intended vehicle should be conducted. Wolven and Levenstein (72) used the Vinson-Borselli test, adding intraperitoneal injection of sulphan blue to better visualize the inflamed area and increase the sensitivity of the test. Sams and J. Epstein (107) use guinea pigs to test phototoxicity of systemic drugs. Sunlight, initially used, proved unreliable because the intensity of light varies and because the survival of guinea pigs exposed to sunlight is dependent on the temperate climate. The phototoxic reactions could be produced with either a bank of fluorescent lamps or a mercury vapor lamp (43, 130), but only when Mylar transparent plastic (duPont) was interposed between

[4] Phenergan. Wyeth Laboratories., Philadelphia, Pa.

the light source and the experimental animals. The plastic absorbs all wavelengths below 3100 Å. It prevents normal erythema from developing in control animals but has no effect on the phototoxic response. This represents a means of enhancing the contrast between the control and experimental animals. Using Mylar, phototoxicity of chlorpromazine (43) and demethylchlortetracycline (131) could be demonstrated. Recently, hairless mice have been used in a test which may have predictive phototoxic value, especially for new drugs (129).

The Curwen-Jillson (106) technique is in chief human use to differentiate photoallergic from phototoxic reactions. The ordinary patch test, using the suspected photosensitizer, is first performed in triplicate. Twenty-four hours later a sub MED (minimal erythema dose) is applied to one uncovered test site and a DED (delayed erythema dose), which is 8 times the MED, is applied to the second uncovered test site. The third patch site is a control. A phototoxic reaction is present when there is a sharply demarcated erythema at the sub MED irradiated patch test site. This is usually most marked 24 hr. after irradiation. A photoallergic reaction is present when there is an eczematous or papular response at the DED irradiated test site. This usually appears 48 hr. after irradiation (47).

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Keyphrases

Topical toxicity—review
Skin reactions—incidence
Topical testing
Irritation reactions
Irritation testing
Sensitivity reactions
Sensitivity testing
Photosensitivity reactions
Photosensitivity testing

Toxicological Studies on Certain Medical Grade Plastics Sterilized by Ethylene Oxide

By R. K. O'LEARY and W. L. GUESS

Ethylene oxide gas sterilization of plastics used in medical and pharmaceutical applications presents a toxicity problem which at the present time remains largely unsolved due to the increasing complexity of the plastic itself. A series of preliminary experiments was conducted to survey the problem and to present evidence of the toxic potential of plastics having a variety of chemical and physical properties. It was demonstrated that residual ethylene oxide caused extensive blood hemolysis and death to cells grown in culture.

ETHYLENE OXIDE appears to be an ideal sterilant for many heat sensitive items, but as a chemical agent it is recognized as being toxic in both its liquid and vapor phases or when prepared as a solution (1). In recent years ethylene oxide sterilization has become an important tool to biomedical personnel engaged in duties where sterilization by autoclaving is either impractical or, as in the case of many of the new plastic devices, is destructive to the product. The introduction of gas sterilization of plastics has raised questions as to the possible entrapment of ethylene oxide in a plastic, which may cause a toxic effect when placed in contact with living tissues, and the effect sorbed ethylene oxide will have on possible changes in the physical-chemical properties of the medical plastic. Certain aspects of these questions have been only partially answered in the medical literature.

Hirose *et al.* (2) and Clarke *et al.* (3) have found that plastic tubings which have been ethylene oxide sterilized can cause significant hemolysis when placed in contact with human blood. This condition was found not to exist if the materials were aerated for at least 5 days after sterilization. Similarly, Royce and Moore (4) have demonstrated that, on human volunteers, vesicular lesions occurred on the fingers, hands, and forearms when the ethylene oxide concentration in sterilized rubber gloves was greater than 2 mg./Gm. of rubber. It has been demonstrated (5) that higher concentrations of the epoxide can be taken up by polyethylene, gum rubber, and plasticized polyvinyl chloride (PVC) which reemphasizes the problem of gas entrapment in plastics. Bain and Lowenstein (6) in 1967 found that when mixed leukocyte cultures are incubated in disposable plastic tubes sterilized with ethylene oxide, the cell survival is severely affected by a toxic residue left on the plastics. The residue dissipated after 4 or 5 months' storage at room temperature, and the cell survival returned to the value obtained with ultraviolet sterilized control tubes. There have been other reports of the persistence of toxic effects after sterilization with ethylene oxide (7–12), as well as many references to the over-all procedures to be used in an effective sterilization of pharmaceutical and medical supplies (13).

This paper presents data on the ability of ethylene oxide to remain entrapped within a nonclosed system, such as a surgical tubing, gas washing bottle, plastic syringe, or plastic bottle at various temperatures above the boiling point, and also relates the hemolyzing ability of known amounts of ethylene oxide to that of freshly gas sterilized plastic pharmaceutical products. Some preliminary data on the effects of ester type plasticizers upon the sorption of ethylene oxide into PVC products are also introduced.

EXPERIMENTAL

The Effect of Plasticizer Concentration on the Uptake of Ethylene Oxide by Polyvinyl Chloride—Because literature references suggested that plasticized polymers were producing the greatest number of toxic responses following ethylene oxide sterilization, the following flexible PVC formulations were prepared for sterilization.

Received June 21, 1967, from the Drug-Plastic Research and Toxicology Laboratories, College of Pharmacy, University of Texas, Austin, TX 78712

Accepted for publication August 29, 1967.

This research was supported in part by grant CA-06120 from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

The authors are grateful to Mrs. Beverly C. Schmidt for valuable technical assistance with the blood hemolysis and cell culture toxicity studies.

Sample A—One hundred grams polyvinyl chloride,[1] 3 Gm. dibutyltin maleate heat stabilizer (M and T Chemical Co., Rahway, N. J.).

Sample B—One hundred grams PVC, 52 Gm. di-2-ethylhexyl phthalate plasticizer (Eastman Chemical Co., Bristol, Tenn), 5 Gm. epoxidized soya oil (Rohm & Haas, Philadelphia, Pa.), 2 Gm. calcium-zinc soap stabilizer (Advance Chemical Co., New Brunswick, N. J.).

Sample C—One hundred grams PVC, 20 Gm. di-2-ethylhexyl phthalate, 5 Gm. epoxidized soya oil, 2 Gm. calcium-zinc soap.

The above formulations were then processed in the following manner. The polymer and the additives (in the desired concentration) were placed into a 1-gal. laboratory Hobart mixer and mixed for 30 min. The films of the polymers were prepared by milling them on a Farrell-Birmingham mill with a chromium-plated, variable-speed roller. Prior to milling each film, the rollers were carefully cleaned with practical grade stearic acid. The temperature of the rollers was maintained at 175° C., and the duration of the milling was 5 min. During the final phase of the operation, the separation of the rollers was adjusted so as to furnish films having thicknesses in the range of 0.040–0.045 in. Cut samples $5^7/_8 \times 5^7/_8$ in. from the milled stock were then press polished on the Dake compression press. The molds containing the samples were then heated for 3.5 min. at 35,000 lb. of pressure and rapidly cooled to 120° before removing the test sample from the mold. All samples were finally allowed to condition at room temperature and 50% relative humidity for 48 hr. prior to their use. The 3 PVC plastics, containing 0, 15.8, and 32.7% di-2-ethylhexyl phthalate, were sterilized in a 10 × 16-in. Cryotherm experimental gas sterilizer.[2] The specific sterilization involved prehumidifying the test samples in the chamber at 50% relative humidity, 57° C., under 660 mm. of Hg vacuum for 1 hr. A mixture of 11.0% ethylene oxide, 78.3% trichlorofluoromethane[3] (CCl_3F), 10.7% dichlorodifluoromethane[4] (CCl_2F_2) was added to the chamber until a pressure of 16 p.s.i.g. was reached. The plastics used were then sterilized for 4 hr. At the end of this time interval, a vacuum of 660 mm. of Hg was placed on the system in order to remove the ethylene oxide–CCl_3F–CCl_2F_2 mixture. The samples were analyzed by a procedure previously described by Gunther (5). A plot of the concentration of ethylene oxide detected *versus* the concentration of plasticizer is presented in Fig. 1. These data were obtained on the plastics after they had been aerated at room temperature for 24 hr.

Ethylene Oxide Solubility Studies of a Series of Dialkyl Benzene Carboxylate Plasticizers—Because the experiments described in the preceding section suggested that the presence of an ester-type plasticizer in PVC permits a greater degree of ethylene oxide sorption to occur, an investigation of the solubility of this gas in a series of phthalic acid esters was undertaken. It was hoped that this information would help in the rationalization of the fact that plasticized PVC plastics produce a greater degree of toxicity following sterilization.

[1] Geon 101EP. B. F. Goodrich Co., Cleveland, Ohio.
[2] American Sterilizer Co., Erie, Pa.
[3] Freon 11. E. I. du Pont de Nemours and Co., Wilmington, Del.
[4] Freon 12. E. I. du Pont de Nemours and Co., Wilmington, Del. (commonly known as Cryoxide).

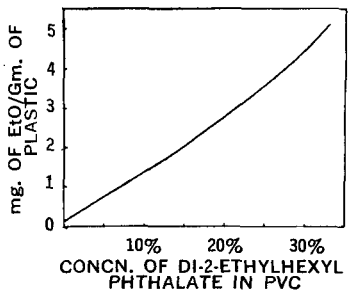

Fig. 1—A plot of di-2-ethylhexyl phthalate concentration in polyvinyl chloride versus *sorption of ethylene oxide after 24 hr. aeration.*

Description of Test System—Pure ethylene oxide vapor was distilled into a 125-ml. gas washing bottle containing an accurately weighed sample. The bottle was stoppered with a ground-glass cap which was provided with a venting tube connected to a desiccator. After approximately 5 ml. of liquid ethylene oxide had accumulated in the bottom of the container, it was removed from the ice bath and placed in a constant-temperature bath. Twenty minutes were allowed for the ethylene oxide–plasticizer mixture to reach equilibrium at the desired temperature. A glass-coated stirring bar provided agitation for the system. The bottle was then removed from the bath and titrated rapidly with a standardized HBr–glacial acetic acid reagent according to the method described by Gunther (5). The indicator was 0.1% crystal violet in glacial acetic acid. The concentration of ethylene oxide was calculated by:

$$\frac{\text{mg. of ethylene oxide}}{\text{Gm. of plasticizer}} = \frac{\text{ml. HBr} \times \text{normality} \times \text{equivalent wt.}}{\text{wt. of sample}}$$

Control Value for System—Before investigating the solubility of ethylene oxide in dimethyl, dipentyl, and dinonyl phthalate, it was necessary to determine if any ethylene oxide would remain in the test system in the absence of a sample. Five determinations were made at 45.5, 57.0, and 71.5° C. for the ethylene oxide which would remain in the gas washing bottle under the test conditions described above. Table I summarizes the data and presents the mean values obtained as well as the probable deviation of the mean (14).

Prior to testing the aforementioned plasticizers,

TABLE I—EFFECT OF TEMPERATURE ON RESIDUAL ETHYLENE OXIDE IN GLASS BOTTLES

Experiment	Wt., mg. 45.5° C.	57.0° C.	71.5° C.
1	459	399	382
2	473	456	391
3	481	452	371
4	482	413	399
5	466	399	439
Mean values	472	424	396
S. E.	3.0	8.5	7.8

TABLE II—SOLUBILITY OF ETHYLENE OXIDE IN A SERIES OF PHTHALATE ESTERS AT 45.5° C.

Dimethyl Phthalate		Dipentyl Phthalate		Dinonyl Phthalate	
mg./-Gm.	Gm./-mole	mg./-Gm.	Gm./-mole	mg./-Gm.	Gm./-mole
Mean Values					
224.1	43.5	170.0	52.1	118.7	49.6
5.1	1.0	3.9	1.2	5.9	2.5

it was imperative to know that these esters would not interfere with the titration method of analysis. An evaluation of the amount of ethylene oxide which could be detected in the presence and absence of the esters showed that these plasticizers did not contribute to the titers.

Ethylene Oxide Solubility in Phthalates—Various amounts of dimethyl, dipentyl, and dinonyl phthalate were accurately weighed into the gas washing bottle previously described; they were then saturated with ethylene oxide prior to being placed in the constant temperature bath at 45.5° C. In this study only this one temperature (45.5° C.) was evaluated. Five replicate experiments were performed for each of the esters. The data obtained from the titration were corrected for the value of ethylene oxide obtained from the standard values in Table I. The corrected data appear in Table II, and are expressed both as Gm. of ethylene oxide per Gm. of ester and as Gm. per mole of ester. Probable deviations of the mean values were also calculated.

Application of Raoult's Law of Partial Pressure—Once an evaluation of the number of moles of ethylene oxide present in the phthalate plasticizers had been made, it was possible to evaluate the vapor pressure of ethylene oxide over this solution. Raoult's law states:

$$P = P_0 X_0$$

where P is the vapor pressure of the solvent or volatile solute over its solution, X_0 is the mole fraction, and P_0 is the vapor pressure of the pure solvent or the volatile solute at the same temperature as the solution. The mole fraction was obtained by dividing the number of moles of ethylene oxide in the plasticizer at 45.5° C. by the number of moles of plasticizer and ethylene oxide. Before carrying out this operation it was necessary to evaluate the vapor pressure of pure ethylene oxide with the Moor-Kanep formula which states that (15):

$$\text{EtO log vapor pressure [mm. Hg]} = 6.839 - \frac{1410}{T°\text{K.}}$$

DISCUSSION

For the sake of clarity and continuity, the discussion that follows will coincide with the sequence of topics covered under *Experimental*.

Ethylene Oxide Solubility Studies in Phthalate Esters—One of the most interesting observations made in the solubility experiments of ethylene oxide in phthalic acid esters was that this gaseous vapor has the ability to remain in a nonclosed system in high equilibrium concentrations at relatively high temperatures. Since ethylene oxide boils at 10.7° C., it was not anticipated that any significant amount of ethylene oxide would remain in the gas washing bottle system (previously described) at 71.5° C. However, at 7 times the boiling temperature, there was still almost 0.5 Gm. of residual sterilant (see Table I). If one considers the porous nature of certain plastic devices sterilized for medical use, it immediately becomes obvious that a thorough degassing technique must be employed following sterilization. It is not known at this time whether the ethylene oxide is sorbed in much the same manner as some tin compounds onto the surface of a glass system. In light of this observation, a possible explanation of the toxicity found in certain tubing systems following ethylene oxide sterilization might be better rationalized in terms of sorption processes into the polymeric matrices. One immediate example of where this residual ethylene oxide phenomenon could present a hazard would be in the sterilization of such devices as syringes. If the barrel and plunger of the syringe were engaged too soon after sterilization, the residual gas might produce a toxicity from the oxidation of the epoxide to a glycol, which in turn could be further oxidized to glyoxal, glyoxalic acid, glycolic acid, or even oxalic acid. All of these compounds could potentially produce a toxic response. As for the effect of the gas or its oxidation products upon the syringe contents, it is difficult to begin to even enumerate the many novel toxicities that could be produced.

In addition to the possibility of absorption of the ethylene oxide gas, it has been found that this relatively polar ether does have significant ability to solvate such nonpolar hydrocarbon esters as dinonyl phthalate. Considering that PVC tubing will contain approximately 35% of dioctyl phthalate, and that dinonyl phthalate has a solubility of 119.7 mg./Gm., as well as the fact that as the length of the hydrocarbon ester moiety decreases the EtO solubility increases (see Table II), it thus becomes possible that a flexible tubing weighing 1 Gm. might contain as much as 40 mg. of ethylene oxide. It has been shown that this is a sufficient amount to cause significant hemolysis in human blood. At the present time it is not known whether the plasticizer in the polymer will attain the same solubility limits for ethylene oxide as it does in the pure state. This information will be investigated in future studies.

The application of Raoult's law and the Moor-Kanep formula proves to be rewarding intuitively and practically. It was learned that the partial vapor pressure of ethylene oxide in the presence of dialkyl phthalates decreases as a linear function of the length of the dialkyl chain. This suggests

TABLE III—PARTIAL PRESSURES AND MOLE FRACTIONS OF ETHYLENE OXIDE IN A SERIES OF PHTHALATE ESTERS AT 45.5° C.

Plasticizer	Moles of EtO	Moles of Phthalate	Moles of EtO and Ester	Mole Fraction	Vapor Pressure, mm. Hg
Dimethyl phthalate	27.66	121.39	149.05	0.18558	490.3
Dipentyl phthalate	28.58	162.58	191.16	0.14951	395.0
Dinonyl phthalate	31 72	256.52	288.24	0.11005	290.75

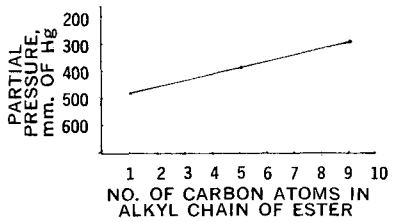

Fig. 2—Plot of the partial pressure of ethylene oxide in phthalic acid esters at 45.5°C. versus *length of alkyl moiety of the ester.*

that the lower esters would degas at a faster rate than their higher molecular weight congeners. (See Table III and Fig. 2.) However, as can be seen from Table II, it appears that this is not the only major consideration. The solubility of the ester in the epoxide also rises with the decrease of the number of carbon atoms in the hydrocarbon moiety. Thus one might rationalize that the higher alkyl esters of the phthalate type would be the preferred plasticizers for ethylene oxide sterilized plastics. If the solubility of the ester in the epoxide is to be the predominant consideration, then it can be said that for every four carbons added to the alkyl chain there will be a 25% decrease in the ethylene oxide solubility.

Table III presents the mole fractions, vapor pressure, and partial pressures just described. Figure 2 illustrates the linear function of the partial pressure of ethylene oxide to the length of the alkyl ester moiety.

A Dose Response Study of Ethylene Oxide Induced Hemolysis—Whole human blood, 27 days old, type O, Rh positive, was centrifuged and the plasma removed. The packed cells were washed with 0.85% saline and then recentrifuged and decanted. The procedure was repeated 8 times until the fragile cells and plasma had been thoroughly removed. A final 10% suspension of red blood cells was then prepared. This suspension was then added in varying volumes to a gas washing bottle which was known to contain the amount of ethylene oxide desired for the test. At 30, 60, and 120 min. after the red blood cell suspension was exposed to the ethylene oxide, the sample and the supernatant solutions were examined with the aid of a Spectronic 20 (Bausch & Lomb) spectrophotometer. The wavelength suggested for hemoglobin determinations is 540 mμ. The amount of hemoglobin as indicated by the percent absorption from the spectrophotometer was plotted against the time the suspension was exposed to the ethylene oxide. Figure 3 is a plot of the typical dose–hemolysis responses obtained for different concentrations of ethylene oxide at three time intervals of exposure.

An Evaluation of the Hemolysis Liability of Pharmaceutical Plastics Sterilized with Ethylene Oxide—Fourteen different plastic devices which may or may not be used to store blood were sterilized by a standard ethylene oxide sterilization technique which is presently used in many hospitals. The procedure involved exposing the devices to 7 p.s.i. (600 mg./L.) of ethylene oxide (12%) and CCl_3F–CCl_2F_2 (88%) for 4 hr. at 50% relative humidity and 57° C., followed by degassing the plastics 15 min. at 26 mm. of mercury vacuum at 57° C. The actual devices studied are given in Table IV, along with the percent absorption obtained on a Spectronic 20 from the development of hemolysis in a 10% red blood cell suspension. The devices were filled with the suspension immediately after sterilization and evaluated at varying time intervals. The controls were the same devices given the same treatment except that no ethylene oxide was allowed in the sterilization chamber. No attempt was made to control the surface-to-volume ratio.

Cell Culture Studies on the Toxicity of Ethylene Oxide Sterilized Polymers—Eighteen plastics (10-mil films) listed in Table V were sterilized by the procedure described in the previous section. The sterilized plastics were tested by a cell culture method which has been previously described (16, 17).

Table V presents the results obtained when the freshly sterilized plastics were placed on cell culture plates using the agar overlay techniques and observed 24 hr. later. It should be pointed out that all plastics listed in Table V were previously evaluated by the same cell culture technique and all were found to be nontoxic to the two cell systems utilized prior to sterilization.

TABLE IV—HEMOLYSIS LIABILITY OF SELECTED PHARMACEUTICAL PLASTICS STERILIZED WITH ETHYLENE OXIDE

Name of Device	Polymer Type	Time Blood Exposed to Plastic, min.	% Absorption at 540 mμ
Venopak disposable venoclysis set (Abbott lot No. 758-3518)	Flexible PVC	60 120	68 87
Blood administration set with plastic filter (Abbott, lot No. 740-3515)	Flexible PVC	60	22
Monoject 535S-C disposable syringe (Roehr Products Co.)	Polypropylene	40 140	18 18
Jelco disposable syringe (Jelco, lot No. 3165 JMB 130)	Polypropylene	45 130	2 8
Tomac disposable syringe (Amer. Hosp. Supply No. 4775 C2L106)	Polystyrene	85 195	15 18
Hemovial disposable blood collection unit (E. H. Wilburn Corp.)	Polyethylene	95 190	18 24
ACD—whole blood container (Pharmachem Corp.)	Polyethylene high density	75 135	33 33
Rilsan bottle (Wheaton Plastics)	Polyamide	80 150	3 14
Teflon—FEP bottle (E. I. du Pont)	Fluoroethylene Propylene	30 143	0 30
Acrylic bottle (Wheaton Plastics)	Methacrylate	100 170	22 26
Bellow's bottle (Wheaton Plastics)	Polyethylene	50 180	4 15
Natural Droptainer (Alcon)	Polyethylene	115 170	16 20
Bottle 4 oz. (Alcon)	Polyethylene	100 175	10 16
Bottle 2 oz. (Wheaton Plastics)	Blend of polypropylene and linear polyethylene	100	8

TABLE V—CELL CULTURE TOXICITY RESULTS FOR 18 PHARMACEUTICAL PLASTICS STERILIZED WITH ETHYLENE OXIDE[a]

Plastic (10-mil Film)	"L" Cell Culture	Chick Cell Culture
Cellulose acetate	Cell death	Cell death
Polycarbonate	No effect evident	No effect evident
Polyethylene	Cell death	No effect evident
Teflon 7	No effect evident	No effect evident
PVC with 35% dioctyl phthalate	Cell death	Not tested
Nylon 6,6	No effect evident	No effect evident
Polyphenylene oxide	Cell death	Cell death
Polypropylene	No effect evident	No effect evident
PVC with 15% dioctyl phthalate	Cell death	Cell death
Rigid PVC	No effect evident	No effect evident
Cellulose triacetate	Cell death	Cell death
Rigid polyurethane	Cell death	No effect evident
Flexible polyurethane	Not tested	No effect evident
Cellulose acetate–butyrate	Cell death	No effect evident
Polymonochlorotrifluoroethylene	No effect evident	No effect evident
Polybutylene	No effect evident	No effect evident
Acrylonitrile–butadiene styrene	Cell death	No effect evident
Polyvinyl acetate	Cell death	Cell death

[a] Plastics were placed on the culture plates within 15 min. after the sterilization procedure was completed. Toxicity observations were made with the unaided eye, after 24 hr. exposure to the culture plates.

Toxicity Studies of Ethylene Oxide—The data submitted in Fig. 3 indicate that ethylene oxide is a rapid acting hemolytic agent. Independent of the dose involved, this gas will perform the major portion of its hemolyzing effect in the first 30 min. of exposure to blood. After this peak time is reached, the effect becomes asymptotically parallel with respect to the time axis. The importance of the plot in Fig. 3 is not as a direct read-out of the degree of percentage of hemolysis, but rather as a standard curve for the effect of a particular dose of ethylene oxide on human erythrocytes as compared to the same effect achieved with a sterilized plastic. For instance, in Table IV, it can be seen that Abbott's venoclysis set produced a hemolysis absorption read-out of 68% in 1 hr. According to the standard curve, it would take a concentration greater than 78.6 mg. of ethylene oxide per ml. of human blood cells (10% suspension) to produce this same hemolysis. The eye can detect this amount of hemolysis since it is so extensive. It is the opinion of the authors that the cause for the severe hemolysis liability of the Abbott devices is

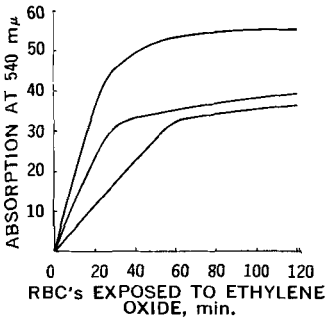

Fig. 3—Plot of ethylene oxide concentration in human blood versus *hemoglobin absorption at 540 mμ. Key: top, 78.6 mg. EtO/ml. RBC; middle, 47.2 mg. EtO/ml. RBC; bottom, 23.6 mg. EtO/ml. RBC.*

the result of the higher solubility of ethylene oxide in a plasticized PVC polymer as compared to other nonplasticized or crystalline polymers. This device also demonstrated the same phenomenon as was reported for the previously described gas washing bottle. This former device is a hollow tubular system in which residual gas pockets can develop as the ethylene oxide is desorbed from the polymer. Perhaps future studies of the effects of vacuum, temperature, and time upon the degassing of these plastics might reveal that no hemolysis liability does exist for these devices.

The different absorption readings obtained for the three syringes sterilized with Pennoxide (see Table IV) is not a reflection of the plastic used to make the barrel of the syringe, but rather the composition of the hub on the plunger. Once blood is drawn into the sterilized syringe it can be visually recorded that the hubs of the Monoject and Tomac syringes release bubbles of a gas. In the undisturbed syringe filled with the blood, hemolysis was extensive in the area next to the hub, but no noticeable difference was recorded in the rest of the barrel. The Jelco syringe did not produce this same effect and concomitantly less hemoglobin absorption was recorded.

It is difficult to pin down the differences in absorption readings obtained for the various polyethylene devices. It might be said that the narrow openings of the Pharmachem products might have permitted a greater residual ethylene oxide content and build-up as desorption occurred in the device. But the bottles with the wider openings allow for a lower final equilibrium concentration after the samples were removed from the sterilizer. The variation in the densities of the individual devices might also be reflected in the different absorption values because the higher density polyethylenes would be more impermeable to sorption and diffusion of the gas. It is felt that this same type of phenomenon was responsible for the delayed hemolyzing ability of the fluoroethylene propylene produced by du Pont. This product has a high degree of crystallinity and would resist the uptake of gas. However, some gas diffusion would occur into the polymer during the 4 hr. of sterilization under pressure. Once the pressure was released and temperature was dropped, the degassing would thus occur slowly over the 1.5-hr. period. The first 30 min. failed to show hemolysis because desorption had not progressed enough to produce a toxic dose.

The cell culture toxicity evaluation of the gas sterilized plastics gave somewhat erratic results, as noted in Table V. Certain explanations may be noted, however, to at least partially account for this variation. First, the mouse fibroblasts ("L" cells) are somewhat more sensitive to toxicants than are chick embryo cells in culture, and the toxic results shown in Table V reflect this difference in sensitivity. It should be noted that the more plasticized formulations caused cell death, again reflecting a higher degree of ethylene oxide solubility in the plasticizer and a more rapid release of the gas from this material. The cell deaths noted with the nonplasticized plastics are related to the complex desorption relationships resulting from different degrees of crystallinity, branching, and molecular weights of the polymers.

In conclusion, it appears that the blood saline suspension technique is a sensitive and effective

method for evaluating hemolysis liabilities of sterilized plastics. From the results of these tests, the polypropylene devices would have to be considered the best candidates for making a medical product which would undergo ethylene oxide sterilization. However, techniques must be evaluated to prove the effectiveness of longer degassing times, higher temperatures, and the effects of vacuum. It would appear from these results that further testing is necessary to establish the actual percentage of hemolysis that occurs for a particular concentration of ethylene oxide. In any event, it is sufficient to say that hemolysis liabilities do exist from the sterilization of medical plastics of many different types.

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Glass—entrapment
Toxicity of ethylene oxide
Tissue culture
Hemolysis—induced

Surface Films of Soybean Lecithin II

Interactions Between Lecithin and Lipid Substances in Mixed Monomolecular Films

By G. TOROSIAN* and A. P. LEMBERGER

Mixed films of lecithin with the fatty acids (stearic, elaidic, and oleic) as well as with the glycerol esters of mono-, di-, and triglyceride have been investigated. Evaluation of these systems has been made utilizing two properties of surface films, their mean molecular areas and their collapse points. It would appear from this investigation that in mixed film systems, lecithin–stearic acid and lecithin–monoglyceride are miscible, but without a significant degree of interaction and as such, form ideal two-dimensional liquids. Lecithin–oleic and lecithin–elaidic acids have been shown to be miscible in mixed film systems but with a significant degree of interaction so that they may be considered to be nonideal two-dimensional liquids. The data for the lecithin–di- and triglyceride systems did not conclusively show whether these systems were ideal or nonideal two-dimensional liquids in mixed surface films.

THE IMPORTANCE of phospholipids in biological membranes has been discussed by Bangham (1). Among the phospholipids, lecithin is considered particularly important since it aggregates into sheet-like micelles which are capable of further accommodating lipids, phospholipids, and sterols (2, 3). The mixed film system of lecithin–cholesterol, first investigated by Dervichian (4), showed the ability of cholesterol to condense lecithin to a smaller cross-sectional area than it exhibits alone. Van Deenen (5) indicated the molecular proportions of cholesterol to phosphotides of animal red cell membranes

Received April 3, 1967, from the School of Pharmacy, University of Wisconsin, Madison, WI 53706
Accepted for publication August 8, 1967.
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This investigation was supported in part by grant A2649 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
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to be 1:1, thus increasing the biological significance of Dervichian's lecithin–cholesterol mixed film observations. Van Deenen (5) verified the ability of cholesterol to condense lecithin using synthetic lecithin containing saturated fatty acid moieties alone and lecithin with unsaturated fatty acid moieties. The condensing effect of cholesterol was more pronounced when in a mixed film in conjunction with the synthetic lecithin containing unsaturated acyl groups.

Lipid materials with which lecithin may be associated in nature include fatty acids and glyceryl esters. Dervichian (6) and Desnuelle (7) have observed interactions between phospholipids and other lipid substances. Yeadon *et al.* (8) studied the emulsification properties of lecithin. They were able to show that purified lecithin alone was an inefficient emulsifier while purified lecithin in conjuction with other lipids in some cases led to improved emulsions. The implications of mixed film interactions between lecithin and these materials, both in nature and in pharmaceutical systems such as emulsions, are of broad interest. Hence this study was undertaken to examine carefully the nature and degree of lecithin interactions with several fatty materials.

EXPERIMENTAL

Materials—Lecithin used in this study was prepared from soybean concentrate[1] and purified after the method of Saunders and Perrin (9, 10). Stearic and oleic acids, obtained from Mann Research Laboratories, were chromatographically pure and were used without further treatment. The elaidic acid used in this study was obtained from K and K Laboratories and was further purified by recrystallization twice from ethanol–water mixture. The pure elaidic acid had a melting point range from 44–45°. The saturated (primarily stearoyl with some palmitoyl moieties) mono-, di-, and triglycerides were obtained from K and K Laboratories as a mixture and were purified and separated after the chromatographic method of Higuchi *et al.* (11).

Apparatus and Experimental Procedure—The surface balance employed was that developed by Poulsen and Lemberger (12). Concentrated solutions of the pure components for a particular mixed film series were prepared separately using dried redistilled reagent benzene as the solvent. Specific volumes of these stock solutions were combined, maintaining the final volume constant, so that the mole fraction of the second component, any compound mixed with lecithin, increased for each mixed film determination within a particular system. These solutions containing the specified mole fraction of each component were then layered on the surface of double-distilled water contained in the trough of the film balance by using a Hamilton syringe with a Chaney adapter. The volume of the injection was held constant throughout this study at 0.1 ml. Temperature was held constant throughout a particular compression and all runs were made at 25 ± 0.5°.

Layering of the material was performed slowly to avoid dissolution of the wax coating on the trough by the benzene. The resulting mixed film was compressed manually until the film collapsed, with readings taken at specific trough areas. This allowed the construction of the π–A isotherms, where π equals the film pressure and A equals the area occupied by the molecules. The time intervals between individual compressions were constant throughout the course of a single run, but did vary with different mixed films from 1–5 min., depending upon the components in the mixed films. Molecular cross-sectional areas for lecithin, obtained by extrapolation to $\pi = 0$, were reproducible within less than 2% for a given lot of lecithin. In general, the study of each system was completed with the same lot of lecithin. Molecular cross-sectional areas for mixed film systems were reproducible within 1–2 Å.^{2}. This allowed the construction of the π–A isotherms, where π = the film pressure, and A = the area occupied by the molecules.

RESULTS AND DISCUSSION

Construction of π–A Isotherms—Figures 1 and 2 are representative families of curves which graphically indicate what occurs as the mole fraction of lecithin is decreased while increasing that of the second component. While systems containing lecithin with stearic acid, oleic acid, elaidic acid, mono-, di-, and triglycerides were investigated, only representative isotherms for lecithin–stearic acid and lecithin–oleic acid are presented since they show what typically occurs in these systems.

In general, it can be seen that as the mole fraction of the second component increases, the curve for the mixed film shifts along the area/molecule axis. Reading the plots from right to left, each succeeding curve represents a further increase in the mole fraction of the second component, until on the extreme left is represented the pure second component. Films of pure natural lecithin exhibit an expanded liquid character, which when diluted

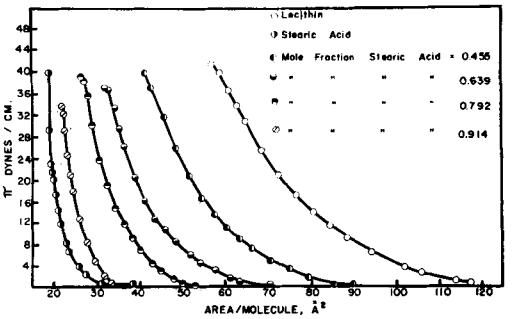

Fig. 1—Representative compression isotherms for lecithin–stearic acid systems. Area/molecule axis based on total number of molecules.

[1] Azolectin, Associated Concentrates, Inc., Woodside, N. Y.

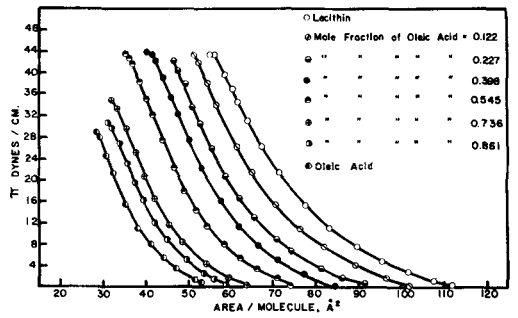

Fig. 2—Represenative compression isotherms for lecithin–oleic acid system. Area/molecule axis based on total number of molcules.

with a second component whose mono-molecular film exhibits a more condensed solid state, results in a mixed film which is a hybrid in its character with respect to the two pure components. The degree to which this hybridization occurs is dependent upon the mole fraction of the second component and on the specific characteristics of the second component. This is demonstrated in Fig. 1, which shows the lecithin–stearic acid system in which the gradual progression of the expanded liquid state of pure lecithin to the more condensed solid state of component 2, stearic acid, takes place. These rather general observations hold equally well for those other more condensed components when mixed with lecithin such as the mono-, di-, and triglycerides.

On the other hand, when 2 components are mixed, both of which when pure show an expanded liquid character, the resulting mixed film retains its expanded liquid characteristic from pure component 1 all the way to pure component 2. This can be seen in Fig. 2 for the lecithin–oleic acid system and is also true for lecithin–elaidic acid system.

In addition to the change in the shape of the curve and the progression of the curves toward smaller cross-sectional areas as the mole fraction of component 2 is increased, there is another property of these films which may change and that is the point at which the mixed film collapses. The collapse point is experimentally taken to be the highest film pressure attainable. In Fig. 1, the lecithin–stearic acid system shows little change of the collapse point, while Fig. 2 for lecithin–oleic acid system shows a gradual reduction in the collapse pressure from the high point exhibited by pure lecithin to the low collapse pressure indicated for pure oleic acid, particularly above the mole fraction of 0.55. This property is again observed in the systems containing elaidic acid, di-, and triglycerides. The pure monoglyceride, like pure stearic acid, has a collapse pressure as high as pure lecithin itself, and one observes little change in the collapse pressure for the mixed film systems of lecithin–stearic acid and lecithin–monoglyceride.

In an attempt to characterize the systems of lecithin with stearic acid, oleic acid, elaidic acid, mono-, di-, and triglycerides, plots of the extrapolated mean cross-sectional area *versus* mole fraction of the second component were constructed. The extrapolated mean cross-sectional areas were obtained by extrapolating the linear portions of the isotherms to $\pi = 0$ on the area/molecule axis. The extrapolated mean cross-sectional area obtained in this manner represents the average area occupied by the molecule in the particular system at zero pressure. The mean cross-sectional areas, on the other hand, were obtained by reading the area/molecule on the x-axis at the specified pressure. This results in a mean cross-sectional area for the molecule at the pressure specified and reflects the average area occupied by the molecules at that specific pressure for that specific system.

When these properties are plotted against the mole fraction of the second component, a linear relationship results if the cross-sectional areas are additive implying the existence of an ideal two-dimensional liquid. This is seen to be the case in Fig. 3 for the lecithin–stearic acid system. This relationship is shown to hold as well for the mean cross-sectional area at film pressures of 10–30 dynes/cm. as seen in Fig. 4. Figures 5 and 6 for the lecithin–monoglyceride system also seem to be linear and closely resemble the lecithin–stearic acid system. This is not, however, the case for the lecithin–oleic acid and the lecithin–elaidic acid systems. Figures 7 and 8 show that most of the points fall below the theoretical line. This is further demonstrated in Figs. 9 and 10 for the film pressures from 4 to 20 dynes/cm. This implies that the cross-sectional areas are less than additive for these systems.

Apparent Linearity of Lecithin–Di- and Triglyceride—The lecithin–di- and triglyceride

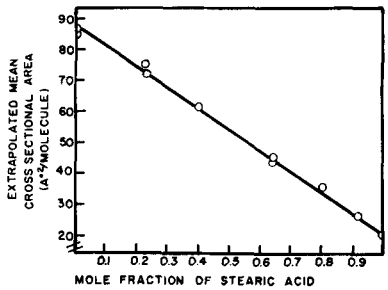

Fig. 3—Extrapolated mean cross-sectional area versus *mole fraction of component 2, stearic acid, of lecithin–stearic acid system. Key: ——, theoretical; ○, experimental.*

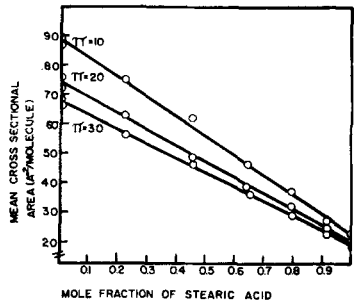

Fig. 4—The mean cross-sectional areas taken at different π values versus *mole fraction of stearic acid for lecithin–stearic acid system. Key: ——, theoretical; ○, experimental.*

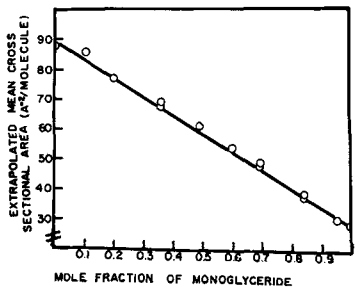

Fig. 5—Extrapolated mean cross-sectional area versus *mole fraction of monoglyceride for lecithin–monoglyceride system. Key: ——, theoretical; ○, experimental.*

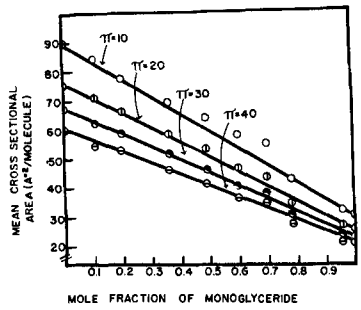

Fig. 6—Mean cross-sectional area at different π values versus *mole fraction of monoglyceride for lecithin–monoglyceride system. Key: ——, theoretical; ○, experimental.*

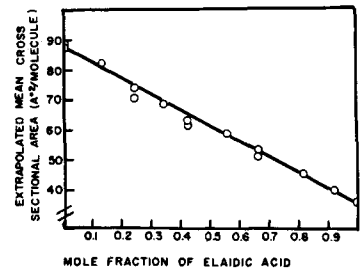

Fig. 7—Extrapolated mean molecular cross-sectional areas versus *mole fraction of elaidic acid for lecithin–elaidic acid system. Key: ——, theoretical; ○, experimental.*

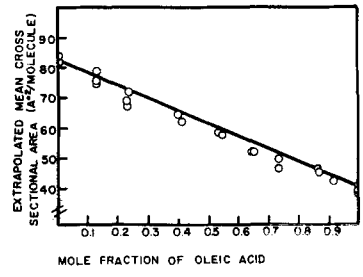

Fig. 8—The extrapolated mean cross-sectional area versus *the mole fraction of oleic acid for lecithin–oleic acid system. Key: ——, theoretical; ○, experimental.*

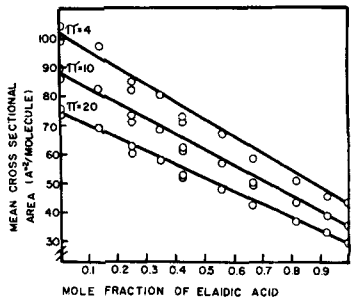

Fig. 9—Mean cross-sectional areas at different π valves values versus *mole fraction of elaidic acid for lecithin–elaidic acid system. Key: ——, theoretical; ○, experimental.*

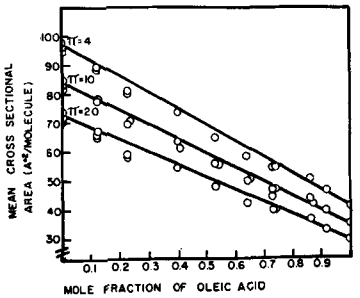

Fig. 10—Mean cross-sectional area at different π values versus *the mole fraction of oleic acid in the lecithin–oleic acid system. Key: ——, theoretical; ○, experimental.*

systems show a linear relationship between the extrapolated mean cross-sectional area and the mean cross-sectional area *versus* the mole fraction of the second component similar to that observed for the lecithin–monoglyceride system. This would seem to indicate that the mean molecular cross-sectional area for the lecithin–diglyceride and triglyceride mixed films are also additive. However, it will be shown that this evidence alone is insufficient to confirm this first impression.

According to Crisp (13), two components of a mixed film are immiscible if the components remain separate and distinct from each other, when they form as for example, surface micelles, then the following equation is obeyed:

$$A_T = A_1N_1 + A_2N_2 \qquad \text{(Eq. 1)}$$

where A_T = total area, A_1 = area of component 1, N_1 = the mole faction of component 1, A_2 = area of component 2, and N_2 = the mole faction of component 2. Molecular interaction and miscibility result when Eq. 1 is not obeyed, yet agreement with Eq. 1 does not absolutely indicate immiscibility since components may be miscible with each other yet have little or no interaction between them, and Eq. 1 may still be observed for these ideal two-dimensional solutions. Most mixed film systems may have some range of miscibility and according to Crisp (13) one may determine the limiting solubility of component 2 in component 1, as well as the partial molar area of component 2, the value of which serves to give some indication as to the ex-

tent of the interaction. Crisp (13) defines the partial molar area as:

$$\bar{A} = -RT \frac{d \ln N'_s}{d\pi'} - RT \frac{d \ln f_s'}{d\pi'} \quad \text{(Eq. 2)}$$

where $\bar{A}$ = partial molar area, R = gas constant, T = absolute temperature, N_s' = mole fraction of the second component being squeezed out of the films. π' = film pressure at which the second component is being squeezed, and f_s' = activity coefficient of the second component at the point of being squeezed out. If the activity coefficient is reasonably constant, one may plot $\ln N_s'$ *versus* π'. The partial molar area may be calculated from the slope of the line, and limiting solubility of component 2 in component 1 may be obtained from the point at which the linear portions at high N values, as reported by Crisp, intersect.

Evaluation of Partial Molar Areas—Figures 11–14 are examples of plots of the film pressure at the collapse point *versus* log N_2. These plots, made after the method of Crisp (13), do not show an immiscible region at high N_2 values. There is, however, a linear segment in these plots corresponding to the region in which collapse film pressure increases as N_2 decreases up to a limiting value. The slope of the first portion allows the calculation of the partial molar area, $\bar{A}$, of component 2 in the system. $\bar{A}$ values for the various additives are listed in Table I along with their extrapolated cross-sectional areas and the actual molecular area at the collapse pressure of these components obtained when their films were cast alone. There is a reduction in the partial molar areas with respect to the extrapolated values, indicating that the interaction between lecithin and these second components does occur, and hence the mixed films appear to be condensed with respect to the areas of either component alone. Crisp describes this region as the region where component 2 is miscible with component 1, consequently the film pressure at the collapse point increases. In the systems reported here the mole fraction of component 2 was observed to reach a critical value at which the enhancement of collapse pressure was altered. At N_2 levels below the critical value in oleic and elaidic acid systems the collapse point became essentially constant and was essentially the same as for pure lecithin. In the lecithin–triglyceride system, the collapse points seemed to increase gradually with a reduction of N_2 until the collapse point of lecithin was reached while the diglyceride system demonstrated a maximum collapse pressure at the critical value. Since, according to Gaines (14) Eq. 2 holds only in the region where

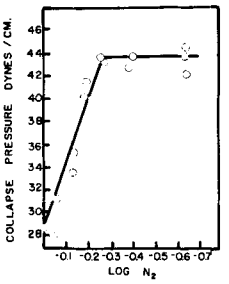

Fig. 11—A plot of the logarithm of the mole fraction of oleic acid versus *the film pressure at the collapse point for the lecithin–oleic acid mixed film system.*

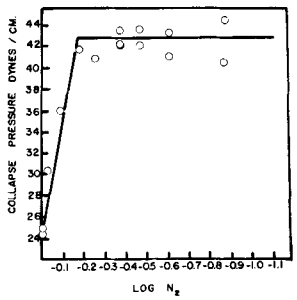

Fig. 12—A plot of the logarithm of the mole fraction of elaidic acid versus *the film pressure at the collapse point for the lecithin–elaidic acid mixed film system.*

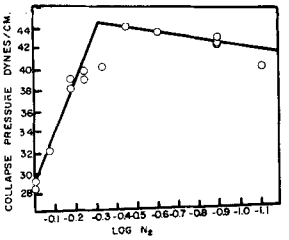

Fig. 13—A plot of the logarithm of the mole fraction of diglyceride versus *the film pressure at the collapse point for the lecithin–diglyceride mixed film system.*

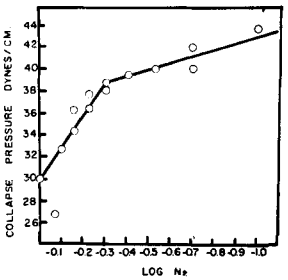

Fig. 14—A plot of the logarithm of the mole fraction of triglyceride versus *the film pressure at the collapse point for the lecithin–triglyceride mixed film system.*

TABLE I—TABULATION OF PARTIAL MOLAR AREAS OF COMPONENT 2 IN MIXED LECITHIN FILMS

Mixed Film System	Molecular Areas of Component 2, Å.² Partial Molar Areas in Mixed Film	at Collapse[a]	Extrapolated[a] to π = 0
Lecithin–oleic acid	17	27.0	39
Lecithin–elaidic acid	8.7	26.0	35.6
Lecithin–diglyceride acid	19.8	39.5	47.5
Lecithin–triglyceride acid	33	59	65.8

[a] Averages of 2 or more values.

$N_2 \rightarrow 1$, the significance of these observations at low N_2 values remains clouded.

The lecithin–stearic acid and the lecithin–monoglyceride systems cannot be evaluated in the same manner, since the collapse pressures for stearic acid and monoglyceride films were both very similar to that of lecithin films. However, they do obey Eq. 1, which supports the hypothesis that these systems are either immiscible or miscible but devoid of any specific interaction.

GENERAL DISCUSSION

The linear relationships existing between the extrapolated mean molecular area, the mean molecular area at the various π values, and the collapse points *versus* the mole fraction of component 2 of the mixed films of the lecithin–stearic acid, the lecithin–monoglyceride systems—together with the fact that stearic acid, monoglyceride, and lecithin are similar types of compounds—would seem to indicate that these mixed films are miscible. However, they do not manifest a significant degree of interaction and as such may be considered to form ideal two-dimensional solutions.

The negative deviations from linearity seen in Figs. 7–10 for the lecithin–oleic acid and lecithin–elaidic acid would seem to indicate, along with the data in Table I, that these systems do form miscible films. They further show the existence of interactions between the lecithin molecules and those of oleic and elaidic acid. This is corroborated in the case of the lecithin–oleic acid system by Dervichian in a preliminary note (6), indicating that oleic acid condensed lecithin films of unknown purity by approximately 18%.

Interactions between film components similar to those seen at the air–water interface may also occur at the oil–water interface in emulsion systems. Where this is the case, we would expect better stabilizing effects for those film systems which manifest a significant degree of interaction. To some extent, this is supported by the work of Yeadon *et al.* (8), who found that purified lecithin mixed with oleic acid formed better emulsions than purified lecithin alone.

In the case of the lecithin–diglyceride and lecithin–triglyceride system, the plots of the extrapolated mean cross-sectional area and the mean cross-sectional area *versus* the mole fraction for component 2 appear to be linear indicating an ideal two-dimensional liquid. However, Figs. 13 and 14 would imply that di- and triglyceride both tend to cause a condensation of the lecithin film. Table I shows the partial molar areas for these compounds to be smaller than the extrapolated molecular cross-sectional area for either diglyceride or triglyceride alone, which indicates the presence of a significant amount of interaction between lecithin and the glycerides. Desnuelle (7), using phospholipids obtained by precipitation of their ether solutions into acetone and a crude extract of unsaturated triglyceride, showed that the unsaturated triglyceride caused a condensation of the phospholipid film. His work tends to corroborate the authors' findings with lecithin–triglyceride systems —although the triglyceride in our system contained saturated acyl groups.

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Keyphrases

Lecithin surface films
Soybean lecithin lipids-interaction of
Mean molecular area
Partial molar area
Film pressure
Collapse points

Equilibrium Phenomena in Solid Dosage Forms

By J. THURØ CARSTENSEN*, J. B. JOHNSON, D. C. SPERA, and M. J. FRANK

Certain aspects of the mechanics of drug decay in solid dosage forms are discussed. Apparent equilibration often takes place in solid dosage forms. In order to obtain data that are amenable to detection of equilibria and can be subjected to mathematical analysis, it is necessary, in the case of fairly stable drugs, to assay for degradation products. Van't Hoff plots should be used in this case.

THE AUTHORS are presently engaged in the study of stability behavior in solid dosage forms.

Theoretical treatment of reaction through moisture interfaces should lead to zero-order behavior (1). To our knowledge only Leeson and Mattocks (2) and Lachman (3) have touched on this in a quantitative manner. Garrett (4, 5) has paved the way for use of extrapolatory procedures and Higuchi (6), Tardif (7), and Carstensen (8, 9) have reported on first- and zero-order behavior of vitamin A in solid dosage forms.

Nevertheless, the literature is poor in reports on this important aspect of stability evaluation, and some of the authors' findings are presented with special emphasis on (*a*) the occurrence of apparent equilibria and (*b*) the importance of following degradation product formation rather than evaluating only percent active drug retained.

THEORETICAL

It is a general practice to evaluate stability by percent active component retained.[1] For short term evaluation, however, it is more meaningful, and in general much more instructive, to gauge degradation by the amount of decomposition products formed.

In general, a good analytical procedure for the intact drug exists at the time pharmaceutical product development is initiated, but breakdown products may be either unknown or poorly quantitated.

It has been the authors' experience that application of TLC in evaluating compatibility (9) as well as stability, gives insight far beyond simply testing for percent intact drug retained, even though analytical procedures for the latter may be good to ± 2% and the former only to ± 20%.

Received September 30, 1966, from the Pharmaceutical Research Department, Hoffmann-LaRoche, Inc., Nutley, NJ 07110

Accepted for publication April 25, 1967.

Presented in part to the Philadelphia Chapter, APhA, February 10, 1966, and the Pharmaceutical Research Discussion Group, East Orange, N. J., September 8, 1966.

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[1] It is also popular practice to treat log linear relations by the method of least squares directly. This, however, is quite fallacious if the data are very scattered (10).

EXPERIMENTAL

Vitamin A tablets were made by mixing vitamin A acetate beadlets (17.5 mg. per tablet of 500 unit/mg. beadlets), spray-dried lactose (427.5 mg. per tablet), and calcium stearate (5 mg. per tablet), and compressing the tablets on a conventional tablet machine using a $^3/_8$-in. deep concave punch. The tablets were stored at 25, 37, 45, 55, 70, and 85° in sealed containers, and samples were removed at regular intervals. The tablets were assayed by a modified USP method[2] using Morton Stubbs correction factor.

Stability of isopropanolic solutions of vitamin A acetate were studied in the absence of air and oxygen in the following fashion.

A solution of vitamin A acetate in isopropanol was prepared under nitrogen and transferred to the vessel shown in Fig. 3. Nitrogen was bubbled through the vessel through stopcock B, with stopcock A open. Both stopcocks were then closed, and the vessel stored at 25°. Samples were taken at various intervals by opening stopcocks A and B, and applying nitrogen pressure at position A, and collecting the sample at position B.

Vitamin E succinate tablets were made in the same fashion as described for vitamin A acetate tablets. They contained 6 mg. vitamin E succinate, 192 mg. lactose, and 2 mg. magnesium stearate. Storage conditions and sampling procedure paralleled those for vitamin A acetate tablets.

The tablets inherently contain sufficient moisture for complete de-esterification. Even though this would imply a second or higher order reaction, it will be seen that the data lend themselves to first-order kinetics and that meaningful extrapolations can be made.

The melting point of vitamin E succinate is 68°, but addition of additives[3] (lactose, magnesium stearate) sufficiently lowers the melting point so that the physical state of the vitamin E succinate is the same at all temperatures studied.

Vitamin E succinate potency was determined spectrophotometrically by measurement of the absorbance at 285 mμ of an ethanol extract of the tablet mass. The absorptivity, at 285 mμ in 1-cm. cells, is 3.78 for *dl*-α-tocopherol succinate.

A portion of finely ground tablet mass equal to approximately 10 mg. of vitamin E succinate was accurately weighed, transferred to a 100-ml. volu-

[2] USP method employs four washings with ether, whereas the modified method (which in our hands gives identical results) employs two extractions with petroleum ether.

[3] If vitamin E succinate and magnesium stearate are melted together and cooled, the following congealing points may be observed: vitamin E succinate 99%–magnesium stearate 1%, 45°; vitamin E succinate 95%–magnesium stearate 5%, 30°; vitamin E succinate 90%–magnesium stearate 10%, 5°.

metric flask, and 50 ml. of 95% ethanol added. The liquid was shaken for 10 min. and then brought to volume with ethanol, filtered through Whatman No. 12 paper, and absorbance of this solution then was measured at 285 mμ on a suitable spectrophotometer.

The free tocopherol was determined by thin-layer chromatography. An amount of tablet mass equal to approximately 10 mg. of vitamin E succinate was triturated with 1 ml. of chloroform. Fifty microliters of this solution (equal to 500 mcg. of vitamin E succinate) was then applied to a Silica Gel G thin-layer chromatographic plate, and the following thin-layer chromatographic system was used: adsorbent, Silica Gel G; sample, 500 mcg. from chloroform solution; solvent, cyclohexane–diethyl ether (80:20); development, 15 cm. ascending; visualization, 20% phosphomolybdic acid in 95% ethanol.

RESULTS AND DISCUSSION

Sufficient information may be extracted by studying percent retained in the case of lactose tablets containing vitamin A, since in this case, losses are of fair magnitude. Figure 1 shows the percent retained of vitamin A in lactose–magnesium stearate base tablets as a function of time. The assay is modified USP with Morton Stubbs correction, and although data pertaining to degradation products are absent (except for the quantitative Morton Stubbs correction), they show that the assay will level off.

The vitamin A used was vitamin A acetate containing substantial amounts of antioxidants. In the absence of antioxidants the effect is noticeable only after long periods at room temperature. Three batches of vitamin A acetate, containing no antioxidants, when incorporated into uncoated multivitamin tablets, retained vitamin A potency at room temperature in the pattern shown in Fig. 2. Statistical evaluation, as well as visual observation, shows the potency to level off, but long periods at room temperature are necessary to observe the effect.

That equilibration should take place is not unreasonable, since isopropanolic solutions of vitamin A acetate show a similar behavior, if care is taken to exclude air, for example by use of a vessel such as shown in Fig. 3. Figure 4 shows the results of such a study.

The vitamin A beadlets may be viewed as an isolated system in the tablet matrix, and as long as compression of the tablet is kept reasonably gentle the fall-off of potency is fairly independent of the surrounding mass. The reason for introducing vitamin A at this point is to demonstrate that equilibrium conditions do occur in solid dosage forms, and that, if the equilibrium point is sufficiently in favor of the degradation product(s), it is possible to follow the pattern by determining percent retained as a function of time.

Since this is the exception rather than the rule in good tablet formulations, it would be more appropriate to study a compound which would be relatively stable.

It is obvious, however, that in such a case much longer storage times would be necessary to attain meaningful results, if assays of the intact molecule were followed. If individual assay tolerances were ±2%, either a great many assays would have to be

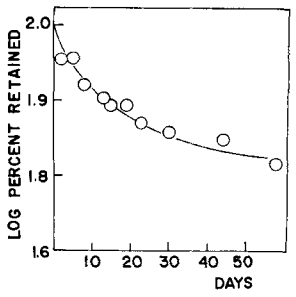

Fig. 1—Stability pattern at 45° of vitamin A acetate beadlets (containing antioxidants) in lactose base tablets.

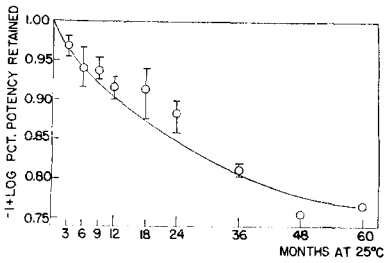

Fig. 2—Long term room temperature stability of vitamin A beadlets (without antioxidants) in a multivitamin tablet formulation.

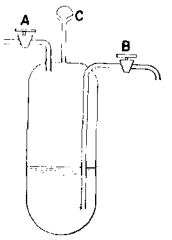

Fig. 3—Storage and sample vessel used for studying stabil ty patterns of vitamin A acetate in isopropanol solutions. The vessel allows sample storage and transfer under nitrogen.

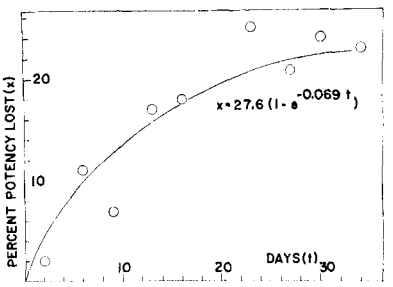

Fig. 4—Stability pattern at 25° of vitamin A acetate dissolved in isopropanol.

performed to distinguish between, *e.g.*, 100% and 99.5% content of active component, or sufficient time would have to elapse in storage to allow the content of active component to drop to a level which would be significantly different from initial content to allow differentiation by triplicate (or other reasonable multiplicate) assays. If the rate of degradation product formation is followed, however, even a crude assay will be more meaningful for this purpose. The example of vitamin E succinate tablets (6 mg.) shown in Table I demonstrates this.

TABLE I—VITAMIN E SUCCINATE DE-ESTERIFICATION AT 55°

Storage Time, 55°	mg./Tablet	% Free Tocopherol[a]
0 days	5.92	0
3 days	...	0.5–1.0
5 days	5.96	1.0
7 days	5.93	1.5
10 days	5.92	1.5
20 days	6.11	2.0

[a] As mentioned, the content of free tocopherol here was evaluated by comparing thin-layer chromatograms of the tablet with standard chromatograms of tocopherol with various amounts of tocopherol applied. In the amounts shown, the assay of "decomposition product" is good to ±25 relative percent in amounts above 1% decomposition product present.

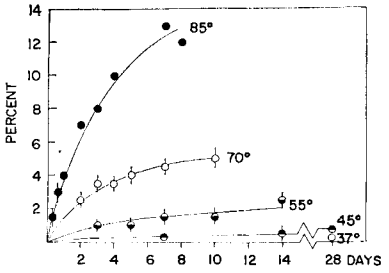

Fig. 5—Stability patterns at various temperatures of vitamin E succinate in lactose base tablet . For estimation of asymptotic values, see text.

It is obvious that the tablet and assay variance here does not allow stability evaluation by column 2 (intact molecule). The data regarding decomposition product formation as they stand are not impressive, but considering the fact that the data concerning percent retained can give no quantitative information at all, and viewed in light of the 85°-data described in the following, they lend themselves to mathematical analysis.

The product formation curve at 85° is shown in Fig. 5 and is indicative of equilibration. Considering the simplest form, $A \rightleftarrows B$, and denoting forward and backward rate constants by k_+ and k_-, and equilibrium concentration by X_e, would lead to the rate equation for content of decomposition product (X):

$$X = X_e[1 - e^{-(k_+ + k_-)t}] = X_e(1 - e^{-kt}) \quad \text{(Eq. 1)}$$

where $k = k_+ + k_-$. X then is the concentration of the component denoted by B above, and $(1 - X)$ is the content of intact molecule A.

Drawing a crude curve through the points of Fig. 5 and recording the X-values at time 2, 4, and 6 yield equations (*i*), (*ii*), and (*iii*) below. It will be noted, that for arithmetic handling any arithmetic series (*e.g.*, $t = 1, 2$, and 3 or $t = 3, 6$, and 9) could have been used equally well. However, the highest numbers, compatible with the data, give the best estimates.

k and X_e are then evaluated by the procedure shown:

$$(i) \quad X_2 = X_e - X_e \cdot e^{-2k}$$

$$(ii) \quad X_4 = X_e - X_e \cdot e^{-4k}$$

$$(iii) \quad X_6 = X_e - X_e \cdot e^{-6k}$$

Subtraction now yields:

$$(ii) - (i) \quad X_e(e^{-2k} - e^{-4k}) = X_4 - X_2$$

$$(iii) - (ii) \quad X_e(e^{-2k} - e^{-6k}) = X_6 - X_2$$

Division of $(ii) - (i)$ by $(iii) - (i)$ then eliminates X_e and yields:

$$(X_4 - X_2)e^{-6k} - (X_6 - X_2)e^{-4k} + \{(X_6 - X_2) - (X_4 - X_2)\}e^{-2k} = 0$$

or, providing that $(X_4 - X_2)$ is different from zero:

$$(iv) \quad (X_4 - X_2)e^{-2k} \times \left\{(e^{-2k})^2 - \frac{X_6 - X_2}{X_4 - X_2}(e^{-2k}) + \frac{X_6 - X_4}{X_4 - X_2}\right\} = 0$$

The equation is satisfied when the quadratic equation in brackets is zero, *i.e.*, when:

$$(v) \quad (e^{-2k})^2 - \frac{X_6 - X_2}{X_4 - X_2}(e^{-2k}) + \frac{X_6 - X_4}{X_4 - X_2} = 0$$

or

$$(vi) \quad (X_4 - X_2)(e^{-2k})^2 - (X_6 - X_2)(e^{-2k}) + (X_6 - X_4) = 0$$

One of the roots of the quadratic equation (*v*) is an estimate of e^{-2k} (and hence an estimate of k) and insertion of this value in, *e.g.*, (*i*), will yield X_e.

It will be noted that the factoring of $(X_4 - X_2)$ in (*iv*) requires it to be different from zero, but $X_4 - X_2 = 0$ only if $k = 0$. In this case $e^{-k} = 1$, and $e^{-kt} = 1$ for all t, and Eq. 1 degenerates to $X = 0$. For this case $e^{-2k} = 1$ and $X_6 = X_4 = X_2 = 0$, and the quadratic equation (*v*) is satisfied by this value.

The equation hence always has the superfluous root $e^{-2k} = 1$, and the other root is the value determining k. Since, in a quadratic equation the two roots ($e^{-2k'}$ and e^{-2k}) add up to the negative coefficient of the first power term, when the equation is normalized, and since, here, $e^{-2k'} = 1$, then:

$$e^{-2k'} + e^{-2k} = 1 + e^{-2k} = \frac{X_6 - X_2}{X_4 - X_2}$$

or

$$e^{-2k} = \frac{X_6 - X_2 - (X_4 - X_2)}{X_4 - X_2} = \frac{X_6 - X_4}{X_4 - X_2}$$

The 85°-data obtained with vitamin E succinate yield the following values (*iv*):

$$0.039\, e^{-2k}\{(e^{-2k})^2 - 1.54\, e^{-2k} + 0.537\} = 0$$

The sum rule gives: $1 + e^{-2k} = 1.54$ or $e^{-2k} = 0.54$. A check on how good the crude curve is, can be obtained by solving for e^{-2k} in conventional fashion and ascertaining that the one root is (or is close to) 1.

In the case above, the roots are $e^{-2k} = 0.523$ and $e^{-2k'} = 1.037$, *i.e.*, the latter is sufficiently close to 1.0 to justify the initial estimate of X_2, X_4, and X_6, and the ensuing k value. Having a first estimate, a best estimate can be found by computer, using Taylor series and iteration as described by Wylie (10).

Inserting $e^{-2k} = 0.54$ in (*i*) yields:

$$X_2 = 0.066 = X_e(1 - 0.54)$$

so

$$X_e = 0.143 \text{ or } X_e = 14.3\%$$

TABLE II—DEGRADATION OF VITAMIN E SUCCINATE IN TABLETS AT VARIOUS TEMPERATURES

Time, Days	Temp., °C.	Free Tocopherol, %
0.33	85	1.5
0.67	...	3.0
1	...	4.0
2	...	7.0
3	...	8.0
4	...	10.0
5	...	12.0
7	...	13.0
10	...	12.0
2	70	2.5
3	...	3.5
4	...	3.5
5	...	4.0
7	...	4.5
10	...	5.0
7	45	0.25
14	...	0.50
28	...	0.75
28	37	0.25

is indicated in this case, since equilibrium values, rather than rate constants are being studied. Stability prediction should indeed be done by this equation rather than by Arrhenius plotting, when equilibrium conditions prevail. The data are presented graphically in Fig. 6. The rate constants, in this case, may or may not follow an Arrhenius relationship. Figure 7, however, shows that they conform fairly well in this case to this type equation. However, for prediction of stability, the rate constants are of no importance if equilibrium is ultimately reached.

The important features then are (*a*) that it becomes apparent that equilibrium is reached and that Van't Hoff rather than Arrhenius plotting is indicated, and (*b*) that use of percent retained data is of no value for extrapolation when compounds have fair stability.

In such cases a crude assay of the degradation product is not only more meaningful, but also more informative and accurate.

TABLE III—VAN'T HOFF CORRELATION OF VITAMIN E SUCCINATE TABLETS

Temp., °C.	$10^3/T$ (°K^{-1})	X_e, %	K (Range)	$\ln_e K$ (Range)	k (Days^{-1}) (Range)	$\ln_e k$ (Range)
85	2.79	13.9–14.3	0.16 –0.17	(−1.83)–(−1.77)	0.27 –0.54	(−1.309)–(−0.616)
70	2.92	5.8– 8.0	0.062–0.087	(−2.78)–(−2.44)	0.15 –0.31	(−1.897)–(−1.171)
55	3.05	2.5– 3.6	0.026–0.037	(−3.65)–(−3.30)	0.11 –0.17	(−2.207)–(−1.772)
45	3.15	1.0– 1.4	0.010–0.014	(−4.61)–(−4.27)	0.049–0.067	(−3.016)–(−2.700)

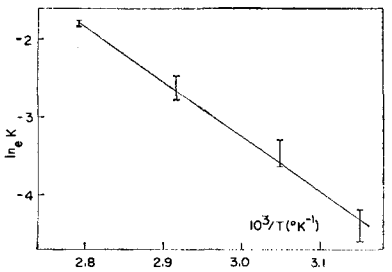

Fig. 6—Van't Hoff plot of apparent equilibrium values derived from asymptotes in Fig. 5.

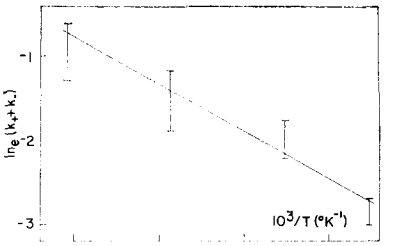

Fig. 7—Arrhenius type plot of the sum of rate constants of vitamin E succinate in lactose base tablets. Values are derived from the curves shown in Fig 5.

The theoretical curve then is $X = 0.143\ (1 - 0.735^t) = 0.143\ (1 - e^{-0.31t})$ as a first estimate.

The theoretical curve can then easily be constructed, and is shown in Fig. 5. Plots at 70° and 55° can similarly be run in a span of 20 days, and fairly good values of X_e may be obtained; longer periods are required for lower temperatures.

Results from storage at other temperatures are shown in Table II and graphically in Fig. 5. The ranges of k and X_e values, as well as of the apparent equilibrium values [$K = X_e/(100 - X_e)$] are shown in Table III. Van't Hoff plotting, *i.e.:*

$$\ln_e K = \frac{-\Delta H}{RT} + \alpha$$

SUMMARY

Cases of equilibrium in solid dosage forms are presented. Such cases should be handled by Van't Hoff plotting rather than Arrhenius plotting.

The virtues of following breakdown product formation as opposed to sole reliance on assays of intact drug are presented.

A rapid method for establishing crude estimates of sum of rate constants and equilibrium value is presented.

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Keyphrases

Drug stability in solid dosage forms
Vitamin A decay
Vitamin E decay
Equilibrium phenomena
UV analysis
TLC determination of free tocopherol

p-Methoxycinnamate and Its Metabolite in Rabbit Serum

By WON SICK WOO

A method of *p*-methoxycinnamate determination in serum is described. *p*-Methoxycinnamate, following intravenous injection, disappeared very rapidly from the serum with a half-life of 0.4 hr. due to metabolic alteration. When orally administered to fasted rabbits, it is rapidly absorbed and maximum concentration is reached within 1 hr. Maximum concentration proportionally increases with increasing dose. The metabolite of *p*-methoxycinnamate in serum was identified as *p*-methoxybenzoate.

SCHROPHULARIAE RADIX, a medicinal plant cultivated in Korea, has long been used as an antipyretic and anti-inflammatory drug. In this laboratory, *p*-methoxycinnamic acid (*p*-MCA) was isolated from this plant, and its antipyretic and analgesic properties have been reported (1, 2). It was also shown that *p*-MCA decreased the ascorbic acid contents of adrenals of rats (3) and in unpublished experiments it has been shown that it inhibits the edema formation by irritants such as yeast, formalin, croton oil, and dextran. When administered to a human and rabbits, it is oxidized to *p*-methoxybenzoic acid (*p*-MBA) which is excreted in the urine as conjugates of glycine and glucuronic acid (4).

The present paper describes the rate of decline of the serum *p*-MCA concentration in rabbits following a single dose, and its metabolite in serum. A rapid and convenient method for the measurement of *p*-MCA in serum is included.

EXPERIMENTAL

Estimation of p-Methoxycinnamate in Serum—The determination method of *p*-MCA has not yet been reported. The following UV spectrophotometry for measurement of *p*-MCA in serum was established.

Serum sample (0.2 ml.) was pipeted into a 5-ml. volumetric flask, brought to exactly 5 ml. by adding a 5% solution of $HClO_4$, and thoroughly mixed. After heating at 60–70° for 10 min., the mixture was cooled and centrifuged at 3000 r.p.m. for 5 min. The clear supernatant was poured into a 1-cm. silica cell and the absorbance was measured at a wavelength of 308 mμ in a Beckman DU spectrophotometer. The test solution was read against a 5% solution of $HClO_4$. The mean absorption of serum was subtracted from the absorbance readings. The mean absorption was determined on a series of sera from normal untreated animals.

Figure 1 shows the standard curve obtained by this method as applied to standard solution of sodium *p*-MCA. Linear function is clearly demonstrated over the range studied.

Table I illustrates the recovery of added amounts of sodium *p*-MCA to rabbit serum. The amounts recovered vary from 96 to 112%, averaging 100.5%.

When serum contained more than 20 mg. % sodium *p*-MCA, the analysis was carried out using a smaller aliquot of sample plus water, and diluting

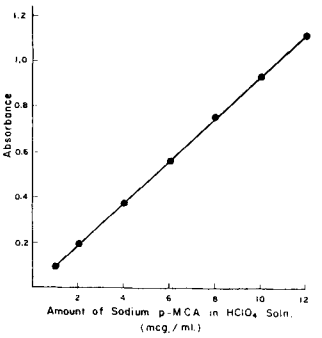

Fig. 1—Calibration curve for Na p-MCA.

Received May 29, 1967, from the Drug Research Institute, Seoul National University, Seoul, Korea.
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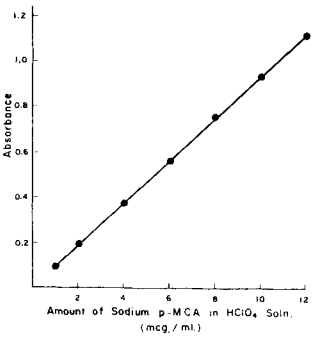

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TABLE I—RECOVERY TEST OF SODIUM *p*-MCA IN SERUM

Serum No.	Added, mg./100 ml.	Found, mg./100 ml.	Recovered, %
1	12.750	12.500	98.04
2	12.750	13.000	101.96
3	12.750	13.125	102.94
4	12.750	12.250	96.08
5	12.750	12.750	100.00
6	6.375	6.250	98.04
7	6.375	7.125	111.76
8	6.375	6.425	100.78
9	6.375	6.325	99.22
10	6.375	6.150	96.47

supernatant with 5% $HClO_4$ if necessary, in order to make final concentration in $HClO_4$ solution less than 8 mcg./ml. The results were calculated from the following formula:

$$G \times V/v \times 0.1 = \text{mg. Na } p\text{-MCA/100 ml. serum}$$

where G = amount (mcg.) found in Fig. 1 (after subtraction of serum absorption); V = total volume of the solution after adding $HClO_4$; v = volume of serum used.

Experimental Animals—All rabbits, weighing 2.0–2.5 Kg., were maintained on a constant diet for 1 week prior to the experiments and randomly divided into groups of 4–6 rabbits each. The indicated amounts of sodium *p*-MCA in water solution were administered intravenously and orally to rabbits. In the case of oral administration, animals had been fasted overnight. No food was ingested by animals until at least 5 hr. after drug administration.

Extraction of Metabolites from Serum and Paper Chromatography—A portion of the serum, at a definite interval after drug administration, was adjusted to pH 1 by adding dilute HCl, and extracted with ether. The ether extract was shaken with saturated aqueous solution of $NaHCO_3$. The aqueous phase was made to pH 1 again, and extracted with ether. After drying over anhydrous Na_2SO_4, ether was evaporated under reduced pressure from the extracts. The UV absorption spectrum of the residue in a 5% solution of $HClO_4$ was quite similar to that of the unextracted deproteinized serum.

The residue was dissolved in ethanol to use as a sample for paper chromatography. R_f values of reference compounds are given in Table II.

TABLE II—CHROMATOGRAPHIC BEHAVIOR OF *p*-MCA AND RELATED SUBSTANCES[a]

Compd.	R_f	Color with Reagent A	B	C
p-Methoxycinnamic acid	0.65	Bl	Br	...
p-Methoxybenzoic acid	0.58	Bl	...	...
p-Methoxycinnamoyl glycine	0.57	Bl	Br	Or
p-Methoxybenzoyl glycine	0.53	Bl	...	Or
p-Hydroxycinnamic acid	0.35	Bl	Br	...
p-Hydroxybenzoic acid	0.25	Bl	Br	...

[a] The ascending method was used with Whatman No. 4 paper at room temperature. Solvent mixture: isopropyl alcohol–NH_4OH–H_2O–toluene (80:5:15:20). Detecting reagents: *A*, 0.4% bromophenol blue; *B*, iodine vapor; *C*, 4% *p*-dimethylaminobenzaldehyde in acetic anhydride. Abbreviations: Bl, blue; Br, brown; Or, orange.

p-Methoxycinnamoyl glycine (*p*-MCG), m.p. 161°, and *p*-methoxybenzoyl glycine (*p*-MBG), m.p. 172°, were prepared by reaction of corresponding acid chlorides with glycine. *p*-MCA and *p*-hydroxycinnamic acid (*p*-HCA) were prepared according to Knoevenagel (5) and Zincke and Leisse (6), respectively, and *p*-MBA by oxidation of anisaldehyde. *p*-Hydroxybenzoic acid (*p*-HBA) was obtained commercially.

RESULTS AND DISCUSSION

The Decline of Serum p-Methoxycinnamate Concentration After Intravenous Administration—Figure 2 shows the decline of the serum *p*-MCA concentration following intravenous administration at dose levels of 100 mg. and 200 mg./Kg. Serum *p*-MCA concentrations were about 41 mg. and 80 mg./100 ml. 3 min. after injection. It can be seen that *p*-MCA disappeared very rapidly from serum and this elimination was first order. Half-life time for excretion was about 0.4 hr. But this first-order process was preceded by an initial apparent lag phase.

Variation of the Serum p-Methoxycinnamate Concentration After Oral Administration—The results obtained in this study are given as plots of log concentration against time in Fig. 3. The peaks of serum concentration were obtained at around 30–60 min. after drug administration. This result indicates that *p*-MCA was rapidly absorbed. Since *p*-MCA could not be detected in feces excreted within 24 hr. after drug administration (7), it is suggested that *p*-MCA was almost completely absorbed from the digestive tract. The elimination of *p*-MCA from serum was found to proceed by first-order kinetics at a small dose (100 mg./Kg.). At higher doses, *p*-MCA elimination did not follow first-order kinetics (nor a zero-order process), until the amount remaining in the serum was decreased to about 17 mg./100 ml. When serum *p*-MCA concentration has fallen below this level, the first-order elimination seemed to occur. But the rate constants were essentially the same, regardless of

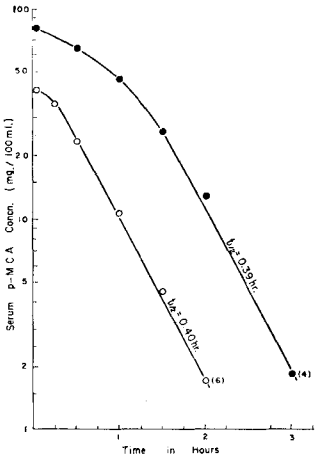

Fig. 2—Elimination of p-*MCA from serum following its intravenous injection of 100 mg./Kg.* (○) *and 200 mg./Kg.* (●). *Figure in parentheses at the right of each line is number of rabbits used.*

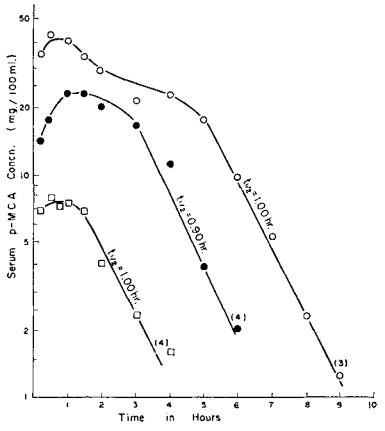

Fig. 3—Serum p-*MCA levels after its oral administration. Key:* □, *100 mg./Kg.;* ●, *300 mg./Kg.;* ○, *500 mg./Kg. Figure in parentheses at the right of each line is number of rabbits used.*

the amount of drug administered. This observed phenomenon is similar to that of salicylate elimination at higher doses (8).

It is of interest that half-life for first-order elimination when administered orally differed from that when administered intravenously. In the case of administration of 100 mg./Kg., the *p*-MCA level was higher (10.85 mg. ± 2.0/100 ml.) 1 hr. after intravenous treatment than when orally given (7.38 mg. ± 0.41/100 ml.). After 2 hr., the *p*-MCA level was higher (3.97 mg. ± 0.8/100 ml.) by oral administration than by intravenous (1.68 mg. ± 0.51/100 ml.).

Maximum Serum p-Methoxycinnamate Concentration as Function of Dose—By representing the average value of observed serum concentration at 30–60 min. after oral administration of drug as a mean maximum serum concentration, over-all results are given in Fig. 4, which indicate that there is a linear relationship between mean maximum concentration and dose.

Metabolite of p-Methoxycinnamate in Serum—Figure 5 shows the spectra of deproteinized serum with $HClO_4$ at various times after an intravenous injection of 100 mg./Kg. Absorption peak at 308 mμ might represent that of *p*-MCA. Although *p*-MCG and *p*-HCA have the absorption peaks at around 292 mμ and 306 mμ, respectively, as shown in Fig. 6, they were not detected in serum by paper chromatography. It is, therefore, evident that this peak was reflected neither by *p*-MCG nor by *p*-HCA. *p*-MBA, *p*-MBG, and *p*-HBA all have no absorbance above 300 mμ. These facts reconfirm that UV spectrophotometry of *p*-MCA is suitable for its determination in serum.

This absorption peak at 308 mμ decreased considerably as time passed. However, the secondary peak at around 258 mμ developed, which also decreased with advance of time. From this phenomenon it is clear that *p*-MCA was converted into another substance. The fact that *p*-MBA has an absorption peak at 258 mμ as shown in Fig. 6 and that *p*-MBA was detected in serum by paper chromatography while neither *p*-MBG nor *p*-HBA was detected indicates that the secondary peak might be identical with that of *p*-MBA. These results show that *p*-MCA is *in vivo* rapidly metabolized to *p*-MBA. This fact coincides with the previously reported communication (4).

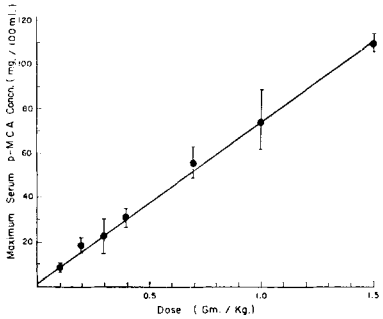

Fig. 4—Linear relationship between mean maximum serum concentration of p-*MCA and oral dose.*

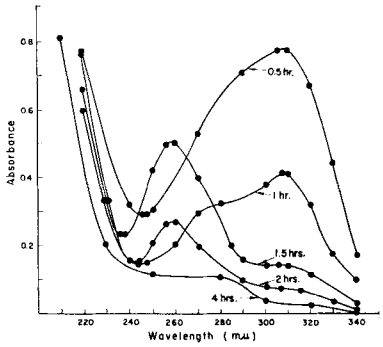

Fig. 5—Typical UV absorption spectra of deproteinized serum at various times after an intravenous injection of 100 mg./Kg. of p-*MCA.*

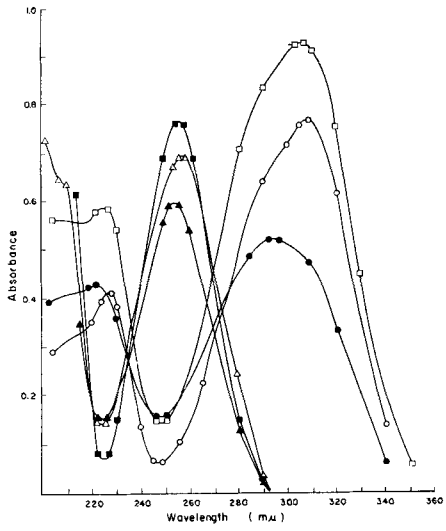

Fig. 6—UV absorption spectra of p-*MCA and its related compounds in 5% $HClO_4$. Key:* ○, p-*MCA (8.3 mcg./ml.);* ●, p-*MCG (8.8 mcg./ml.);* □, p-*HCA (9.6 mcg./ml.);* ■, p-*HBA (8.0 mcg./ml.);* △, p-*MBA (8.0 mcg./ml.);* ▲, p-*MBG (8.0 mcg./ml.).*

SUMMARY

A rapid and convenient method was developed for the measurement of *p*-methoxycinnamate. With this method, the serum *p*-methoxycinnamate concentration has been measured after oral and intravenous administration to rabbits. *p*-Methoxycinnamate disappeared very rapidly from serum with a half-life of 0.4 hr. when injected intravenously. When orally administered, it was rapidly absorbed into the blood stream, and blood levels peaked within 1 hr. but then declined slowly. Average maximum concentrations were strictly proportional to the oral doses. Its elimination from serum was also first order, but the apparent half-life was more than 2 times that when administered intravenously. At higher doses, however, the elimination was not an exponential process in the initial stage. The metabolite of *p*-methoxycinnamate was identified as *p*-methoxybenzoate by ultraviolet spectrophotometry and paper chromatography.

REFERENCES

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(3) Woo, W. S., *Seoul Univ. J.*, **14**, 119(1963).
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Keyphrases

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p-Methoxybenzoate identified as metabolite
UV analysis
Paper chromatographic analysis
Half-life of *p*-methoxycinnamate

Opium Alkaloids VI

Isolation of *N*-Methyl-14-*O*-desmethylepiporphyroxine

By E. BROCHMANN-HANSSEN, K. HIRAI, B. NIELSEN, S. PFEIFER, I. MANN, and L. KÜHN

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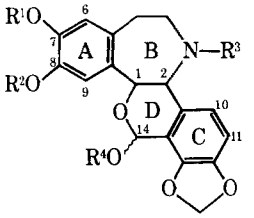
ALKALOIDS of the general structure (I) are widely distributed in the genus *Papaver* and actually constitute the principal alkaloids of several species (1–3). Many compounds of this type have been isolated from natural sources and differ with regard to their substitution pattern, their conformation, as well as the stereochemistry at the three asymmetric centers. Four opium alkaloids belonging to this group have been reported, namely porphyroxine = papaverrubine D (I; $R^1 = R^4 = CH_3$, $R^2 = R^3 = H$) (4–8), papaverrubine B = *O*-methylporphyroxine (I; $R^1 = R^2 = R^4 = CH_3$, $R^3 = H$) (6, 7, 9, 10), papaverrubine C = epiporphyroxine (I; $R^1 = R^4 = CH_3$, $R^2 = R^3 = H$) (12), and glaudine = *O,N*-dimethylporphyroxine (I; $R^1 = R^2 = R^3 = R^4 = CH_3$) (9, 11). They have a *trans*-



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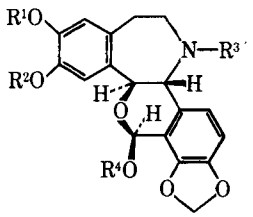

II

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Accepted for publication August 14, 1967.

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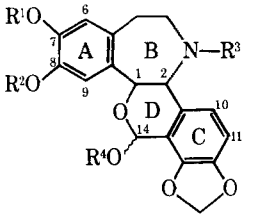

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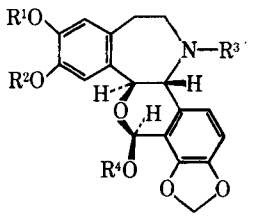

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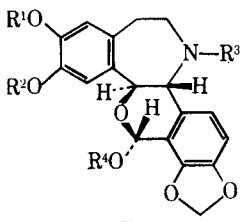
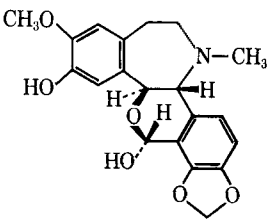
P. rhoeas (13) and oreodine from *P. oreophilum* (14, 15) have the C-1 and C-2 protons in *cis*-configuration. There is convincing evidence that the stereochemistry at the 14-position of porphyroxine, papaverrubine B, and glaudine is as shown in II (8). This configuration is thermodynamically unstable and refluxing with methanol containing trace amounts of acid will cause epimerization to III (12, 16). Papaverrubine C already has this configuration (12). However, it may be an artifact formed from porphyroxine during the extraction (12). Treatment with 1 *N* HCl will invert the *trans*-configuration at the B/D ring junction to *cis* (1, 16, 17). Gentle hydrolysis of the methyl acetal glaudine (II; $R^1 = R^2 = R^3 = R^4 = CH_3$) will give the hemiacetal glaucamine (III; $R^1 = R^2 = R^3 = CH_3$, $R^4 = H$) which has been isolated from *P. glaucum* (18). During this hydrolysis, epimerization occurs at the 14-position, and methylation of glaucamine does not produce glaudine but epiglaudine (1, 16) (III; $R^1 = R^2 = R^3 = R^4 = CH_3$).



III



IV

A new phenolic opium alkaloid related to glaucamine has now been isolated simultaneously by research groups at the University of California, San Francisco,[1] and Humboldt University, Berlin, Germany.[2] The present paper is a joint report of the isolation and structure elucidation of this substance. The mass spectrum and accurate mass determinations of the major fragments indicated that the alkaloid was a lactol of structure I in which $R^4 = H$. This was confirmed by NMR spectrometry which showed the presence of one *O*-Me group and a *N*-Me group. The coupling constant $J_{1,2}$ placed the alkaloid as a member of the B/D-*trans*-series. The position of the phenolic hydroxyl group was established by observing the changes in the chemical shifts of the C-6 and C-9 protons in going from the phenol to the phenolate ion. This made it possible to assign structure IV to the new compound. Confirmation of this structure was achieved by methylation of the hydroxyl group in the 14-position to give *N*-methylepiporphyroxine (III; $R^1 = R^3 = R^4 = CH_3$, $R^2 = H$). *O*-Methylation of the new alkaloid with diazomethane produced glaucamine which was further methylated with methanol and acid to epiglaudine. This substance was converted to epiglaudine methiodide with methyl iodide. Glaucamine obtained by methylation of the new alkaloid was refluxed with 1 *N* hydrochloric acid to give the *cis*-isomer (oreogenine) which on methylation produced oreodine. The various derivatives were identified by NMR spectrometry, thin-layer chromatography, melting points, and mixed melting points with authentic materials.

[1] F. Brochmann-Hanssen, K. Hirai, and B. Nielsen.
[2] S. Pfeifer, I. Mann, and L. Kühn.

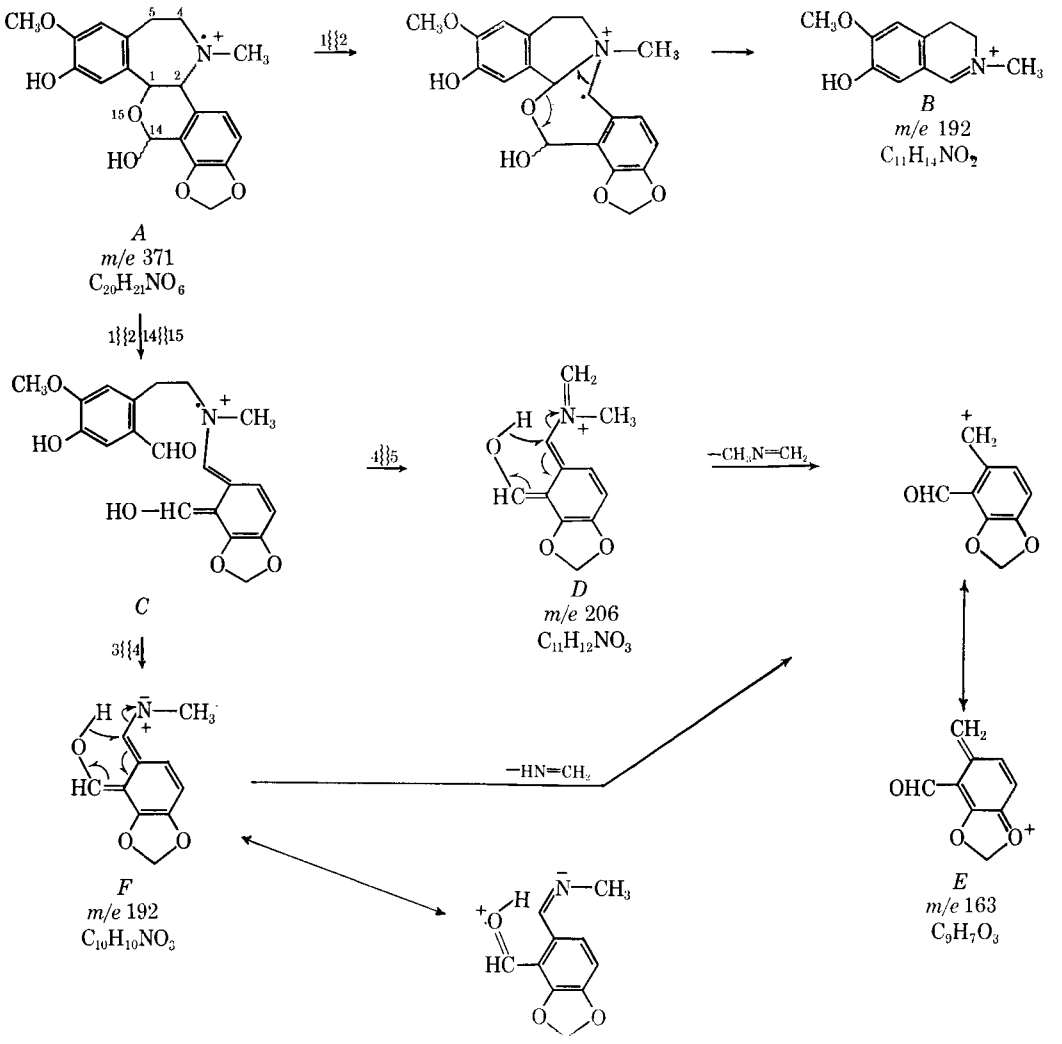
EXPERIMENTAL

Isolation—Dried and powdered opium (1,755 Gm.) of Indian origin was extracted and a separation of the major alkaloid groups carried out by liquid–liquid extraction techniques as described in an earlier publication (19). Preparative thin-layer chromatography of the phenolic alkaloid fraction on silica gel (20) revealed reticuline, isoboldine, scoulerine, and a broad band consisting of several alkaloids. This alkaloid mixture was eluted from the silica gel with warm methanol, the solution evaporated to dryness *in vacuo*, and the residue chromatographed on a column of neutral alumina (Merck, activity 4) with chloroform. Twenty-milliliter fractions were collected with an automatic fraction collector. The first fractions contained porphyroxine (8) followed by scoulerine and several unknown alkaloids. One of these (100 mg.) referred to in the following as A-4 crystallized from methanol as colorless prisms, m.p. 217–218° (micro m.p.K.), $[\alpha]_D^{20} = +340$ (c 0.2% in methanol). It gave a single spot by thin-layer chromatography in two different solvent systems (21) and a single, somewhat broad peak by gas chromatography on silicone rubber SE-30 (3.8%, 4 ft., 200°). When the thin-layer plate was sprayed with potassium iodoplatinate, the orange-brown spot turned to a stable red color after several hours.

Anal.[3]—Calcd. for $C_{20}H_{21}NO_6$: C, 64.73; H, 5.70; N, 3.78. Found: C, 64.32; H, 5.78; N, 4.00.

Elucidation of Structure—The IR spectrum[4] (in Nujol) showed broad bands at about 3270 and 3450 cm.$^{-1}$ due to hydroxyl groups The UV spectrum[5] (in 95% ethanol) had maxima at 238 and 288 mμ which shifted to 242 and 293 mμ on addition of sodium hydroxide, indicating the presence of a phenolic hydroxyl group. The mass spectrum[6] gave a molecular ion with mass 371 and major fragments at *m/e* 206, 192, and 163. The M-1 ion was very small while there was a significant M-18 peak. Metastable ions appeared at *m/e* 138.4 and 129. Accurate mass determinations[7] gave a molecular weight of 371.136908 for the molecular ion

[3] Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, Calif.
[4] Unicam SP-200 infrared spectrophotometer.
[5] Cary model 11 spectrophotometer.
[6] Associated Electrical Industries, MS 9.
[7] The accurate mass determinations were made by the Robert Robinson Laboratories, University of Liverpool, England.



Scheme I

($C_{20}H_{21}NO_6$ = 371.136879). The *m/e* 206 peak gave a molecular weight of 206.081697 ($C_{11}H_{12}NO_3$ = 206.081712). The *m/e* 192 peak was a doublet: 192.065933 ($C_{10}H_{10}NO_3$ = 192.066063) and 192.101421 ($C_{11}H_{14}NO_2$ = 192.102447) with a 3:1 peak height ratio for the low and the high mass. The *m/e* 163 peak had a mass calculated to 163.039576 ($C_9H_7O_3$ = 163.039515). These results indicated that A-4 was a hemiacetal of the same general structure as glaucamine (22). There is a significant difference in the fragmentation pattern of the acetals (*i.e.*, glaudine and rhoeadine) and hemiacetals (*i.e.*, glaucamine and rhoeagenine). The acetals readily split off $CH_3\cdot$ and $CH_3O\cdot$ to give M-15 and M-31 peaks, whereas the hemiacetals give distinct M-18 ions by a pyrolytic removal of water. The proposed fragmentation of A-4 is illustrated in Scheme I (1, 6, 17, 22). The principal fragmentations occur according to the sequences $A \rightarrow C \rightarrow D \rightarrow E$ and $A \rightarrow C \rightarrow F \rightarrow E$, while the sequence $A \rightarrow B$ represents only a minor pathway. This is consistent with the results obtained by the accurate mass determinations as well as the appearance of metastable ions at *m/e* 138.4 and 129.

The NMR spectra[8] in deuterochloroform and hexadeuterodimethylsulfoxide (internal TMS standard) support the structure IV for A-4. The chemical shifts and the coupling constants are presented in Table I. The C-14 proton of the lactol ring gave a broad signal at 3.71 τ in $CDCl_3$, indicating coupling with an adjacent hydroxyl group. This was confirmed by the addition of D_2O which exchanged with the hydroxyl proton resulting in a sharp peak for the proton in position 14. In $(CD_3)_2SO$ this proton peak overlapped the methylenedioxy signal. The protons in positions 1 and 2 resonated as an AX-type quartet at 6.13 τ (2-H) and 4.26 τ (1-H) in $CDCl_3$ with a coupling constant J = 9 c.p.s. In $(CD_3)_2SO$ this quartet appeared at 6.23 τ and at 4.40 τ, J = 9 c.p.s. These results show that A-4 has a *trans*-configuration at the B/D junction

[8] Varian A-60A NMR spectrometer and C-1024 time averaging computer.

TABLE I—CHEMICAL SHIFTS (τ) AND APPARENT COUPLING CONSTANTS (c.p.s.) OF A-4 AND RELATED COMPOUNDS[a]

Compd.	Solvent	N—CH_3	14-OCH_3	ArOCH_3	14-$OCHO$—[a]	—OCH_2O—	6H	9H	10- and 11-H	2-H	1-H
A-4	$CDCl_3$	7.67	...	6.12	3.71	4.00d, 3.93d $J = 1.2$	3.32	(2.73)[b]	3.27, 3.13, 3.00, 2.87 AB-quartet $J = 8.5$	6.13d $J = 9$	4.26d $J = 9$
	$(CD_3)_2SO$	7.78	...	6.27	3.92	3.97	3.27	2.85	3.11	6.23d $J = 9$	4.40d $J = 9$
Glaucamine (23)	$(CD_3)_2SO$	7.76	...	6.28, 6.25	3.90d $J = 5$	3.98d, 3.90d	3.19	2.85	3.11	6.20d $J = 9$	4.31d $J = 9$
O-Me-A-4	$CDCl_3$	7.70	6.47	6.12	4.30	4.01d, 3.94d $J = \sim 1$	3.33	2.67	3.28, 3.14, 3.02, 2.89 AB-quartet $J = 8.5$	6.01d $J = 9$	4.50d $J = 9$
Epiglaudine (8)	$CDCl_3$	7.70	6.44	6.10 (6H)	4.25	4.02d, 3.95d $J = 1.7$	3.32	2.68	3.28, 3.14, 3.02, 2.88 AB-quartet $J = 8.5$	5.98d $J = 9$	4.43d $J = 9$
N-Me-porphyroxine (8)	$CDCl_3$	7.77	6.32	6.12	4.23	4.05d, 3.93d $J = 1.5$	3.33	2.63	3.27, 3.13, 3.00, 2.86 AB-quartet $J = 8.5$	5.93d $J = 9$	4.83d $J = 9$

[a] d, Doublet. [b] Overlapped with the peak of $CHCl_3$.

TABLE II—CHANGES IN THE CHEMICAL SHIFTS ON ADDITION OF SODIUM DEUTEROXIDE TO A SOLUTION OF A-4 IN $(CD_3)_2SO$ (τ)

	1	2	3	$\Delta\tau$
C-6	3.27	3.34	3.57	0.30 p.p.m.
C-9	2.96	3.06	3.37	0.41 p.p.m.
	phenol → phenolate			

because the coupling constant is in agreement with the values reported for glaucamine (23), isorhoeadine (13, 15), glaudine (15), and porphyroxine (8). A smaller coupling constant ($J_{1,2}$ = 2–2.5 c.p.s.) has been observed for the B/D *cis*-alkaloids (13, 15).

The position of the phenolic hydroxyl group in ring A was determined by measuring the chemical shifts of the aromatic protons as a function of pH as described previously for porphyroxine (8). Small amounts of sodium deuteroxide were added successively to a solution of A-4 in $(CD_3)_2SO$ until no further changes were observed in the chemical shifts of the C-6 and C-9 protons. The results are recorded in Table II. The C-6 proton shifted upfield 0.30 p.p.m. by conversion to the phenolate, and the C-9 proton shifted 0.41 p.p.m. in the same direction.

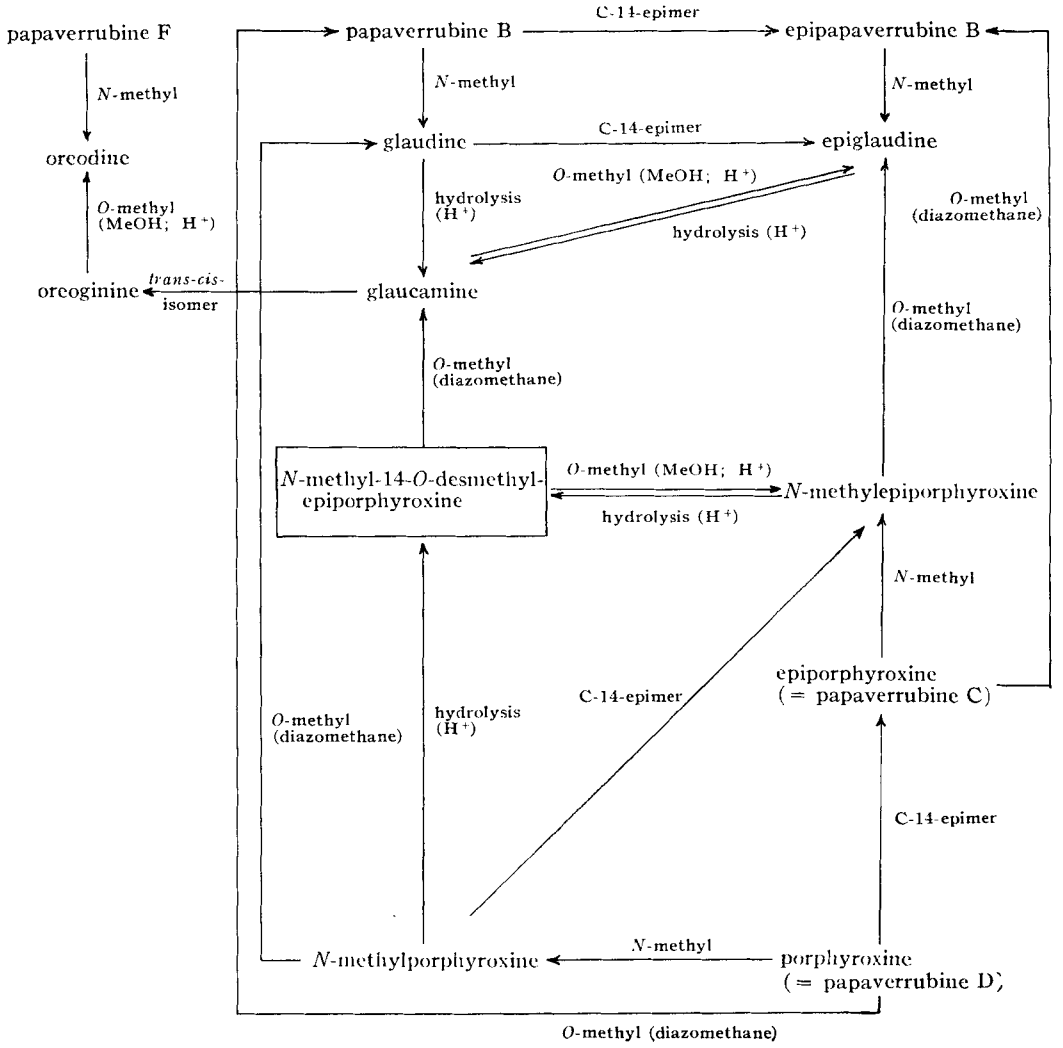
These values are in excellent agreement with those recorded for porphyroxine and show that these two alkaloids have the same substitution pattern in ring A, *i.e.*, the phenolic hydroxyl group is in position 8.

Synthesis of Derivatives

A—*14-O-Methyl-A-4*—Ten milligrams of A-4 was dissolved in 9 ml. of anhydrous methanol and 0.9 Gm. of dry hydrogen chloride was added at 0°. The mixture was allowed to stand at room temperature in the dark for 4 days. The solution was evaporated to dryness *in vacuo*, the residue dissolved in water, made alkaline with sodium bicarbonate, and extracted with chloroform. Evaporation of the chloroform gave an oil which was purified by preparative thin-layer chromatography on silica gel (20). An amorphous substance was obtained which gave a single spot by thin-layer chromatography on silica gel in two solvent systems (21). The R_f values of 14-*O*-methyl-A-4 were identical with those of authentic *N*-methylepiporphyroxine and slightly different from those of *N*-methylporphyroxine. The NMR spectrum of 14-*O*-Me-A-4 was very similar to that of epiglaudine, prepared as described previously (8), but it differed from that of *N*-methylporphyroxine (Table I). It may, therefore, be concluded that 14-*O*-Me-A-4 and *N*-methylporphyroxine only differ with respect to the configuration at the asymmetric center of position-14.

The acetal 14-*O*-methyl-A-4 was also prepared by refluxing for 5 hr. with methanolic hydrochloric acid (0.02 *N*).

B—*Preparation of Glaucamine from A-4*—To a solution of 15 mg. of A-4 in 2 ml. of methanol was added 3 ml. of diazomethane solution (from 0.1 mole of nitrosomethylurea in 100 ml. of ether), and the mixture was kept at 5° for 24 hr. The solution was evaporated to dryness *in vacuo* and the residue dissolved in ether. The solution was concentrated to 1 ml. and allowed to stand for several days to



Scheme II

crystallize. The crystals melted at 222–223° and gave no melting point depression in mixture with authentic glaucamine. The R_f values were identical with those of authentic glaucamine on Silica Gel G (benzene–acetone–methanol, 7:2:1) and on aluminum oxide (Merck) (heptane–chloroform–ether, 4:5:1 and 5:3:2).

C—*Preparation of Epiglaudine Methiodide from A-4*—Glaucamine prepared as described under *B* was refluxed for 5 hr. with methanolic hydrogen chloride (0.02 *N*). The solution was neutralized with ammonia and evaporated to dryness *in vacuo*. The residue was dissolved in a little 0.1 *N* sulfuric acid, immediately made basic with ammonia and extracted with ether. The ether extract was dried over anhydrous sodium sulfate and evaporated to dryness. The residue, which had the same R_f value as authentic epiglaudine, was converted to the methiodide with methyl iodide, m.p. 157–160°. [Lit. m.p. 159–161° (12).]

D—*Preparation of Oreodine from A-4*—Ten milligrams of glaucamine prepared from A-4 as described under *B* was dissolved in 3 ml. of 1 *N* hydrochloric acid and refluxed for 1 hr. After cooling to room temperature, the solution was made basic with ammonia and extracted several times with ether. The combined ether extracts were dried over anhydrous sodium sulfate and evaporated to dryness. The residue (= oreogenine) was dissolved in 5 ml. of methanol containing 0.1 ml. of 1 *N* hydrochloric acid and refluxed for 5 hr. After neutralization with ammonia, the solution was evaporated to dryness *in vacuo*. The residue was dissolved in a little 0.1 *N* sulfuric acid, made basic with ammonia, and extracted repeatedly with ether. The combined ether extracts were dried over anhydrous sodium sulfate and concentrated to 2 ml. The substance, which crystallized as colorless needles, melted at 184–186° and gave no melting point depression when mixed with authentic oreodine. It gave the same R_f values as oreodine by thin-layer chromatography on aluminum oxide (Merck) with two solvent systems prepared from heptane–chloroform–ether (4:5:1 and 5:3:2).

DISCUSSION

A new phenolic alkaloid has been isolated from opium and characterized as *N*-methyl-14-*O*-desmethylepiporphyroxine (IV) by mass and NMR spectroscopy and by chemical conversion to known compounds. It belongs to a group of plant bases of the general structure I which is widely distributed in the genus *Papaver*. *N*-Methyl-14-*O*-desmethylepiporphyroxine is the fifth such alkaloid isolated from opium. Like glaucamine, it is a lactol (hemiacetal) having a thermodynamically stable configuration at the 14-position, and a *trans*-configuration at the B/D ring junctions. The relationships of the new base to other compounds of this type have been verified experimentally and are illustrated in Scheme II. *N*-Methyl-14-*O*-desmethylepiporphyroxine may also be obtained by hydrolysis of *N*-methylporphyroxine. There is reason to believe that *N*-methylporphyroxine, having the less stable configuration at the 14-position (II) may occur in the opium poppy. It is, therefore, possible that *N*-methyl-14-*O*-desmethylepiporphyroxine may not exist as such in the fresh plant, but may be formed by acid hydrolysis and epimerization during the drying and storage of opium or in the course of the extraction and purification of the alkaloid fractions.

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Keyphrases

Opium alkaloids
N-Methyl-14-*O*-desmethylepiporphyroxine identification
TLC separation
Potassium iodoplatinate reagent
NMR spectrometry
Mass spectrometry
UV spectrophotometry—structure
IR spectrophotometry—structure
Synthesis of *N*-methyl-14-*O*-desmethylepiporphyroxine derivatives

Human Urinary Excretion of Orally Administered Anisotropine Methylbromide

By MORRIS PFEFFER, JOSEPH M. SCHOR, NORMAN GLUCK, MARTIN G. SEMMEL, and SOLOMON GRIBOFF

A procedure is described for the quantitative determination of anisotropine methylbromide in undiluted human urine. It has been used to follow the urinary excretion of this compound after oral administration of 20 mg. to six humans. A sustained excretion has been found in human volunteer subjects, in one case lasting up to 104 hr.

AN INVESTIGATION of anisotropine methylbromide[1] (8-methyltropinium bromide 2-propylpentanoate), a quaternary antispasmodic, required the development of a method sufficiently sensitive for its estimation in materials of biological origin.

A modification of the picric acid assay of Chin and Lach (1), itself adapted from a method reported by Slonecker *et al.* (2), was found to have definite advantages over the previously reported method of Mitchell and Clark (3) in ease and speed of operation, with a resultant gain in accuracy and precision, as well as permitting the analysis of human excretion of the quaternary ammonium compound (QAC) in undiluted urine.

EXPERIMENTAL

Reagents—Anisotropine methylbromide, methyltropinium bromide, and homatropine methylbromide originated at Endo Laboratories. Atropine methylbromide was of Mann assayed grade. Choline chloride, acetylcholine chloride, and phosphocholine chloride were obtained from Calbiochem, the first two of commercial grade and the last of A grade. Thiamine hydrochloride was a USP supplied standard. Chloroform (J. T. Baker Chemical Co.) was of spectrophotometric reagent grade. All other chemicals were commercial products of reagent grade.

Analytical Procedures—The analytical method is an ion-pair extraction of methyl anisotropinium picrate from an alkaline aqueous solution into a nonpolar medium. A 5-ml. aqueous QAC-containing solution or 5 ml. of urine was made alkaline (pH >11) with 0.05 ml. of concentrated ammonia and 1 ml. of 0.1% aqueous picric acid solution added. After addition of 5 ml. of chloroform, the mixture was extracted by agitation on a Vortex Genie mixer for 15 sec. The upper aqueous layer was removed by aspiration, and the absorbance at 365 mμ (A_{365}) of the chloroform layer was determined in a Hitachi Perkin-Elmer 139 spectrophotometer using 10-mm. silica cells. The spectrophotometer was set to zero absorbance with the chloroform layer derived from a reagent blank. Chloroform–urine emulsions were broken by centrifuging 10 min. at 1000 r.p.m. at ambient temperature.

The sensitivity of the method was increased roughly threefold by limiting it to a single extraction, thereby avoiding dilution of the absorbing material. Replicate alkaline solutions were extracted, in duplicate, with, respectively, one, two, or three 5-ml. portions of chloroform, and it was determined that 92% of all the methyl anisotropinium picrate extractable in three successive extractions came out in the first extraction.

The characteristics of the picric acid assay system are similar to those reported by Chin and Lach (1). Absorption spectra of the chloroform extracts of the alkaline QAC–picric acid solutions were determined. The QAC's used are listed above in the reagent section. Standard curves were determined by plotting A_{365} of the chloroform extracts *versus* the concentrations of the aqueous QAC solutions which were subjected to analysis. The absorption spectrum of the chloroform extract derived from the anisotropine methylbromide–picric acid reaction mixture is essentially identical to the absorption spectra reported for benzalkonium picrate and cetylpyridinium picrate by Chin and Lach (1). Chloroform extracts of alkaline aqueous or urine solutions of choline chloride, acetylcholine chloride, phosphocholine chloride, thiamine hydrochloride, methyltropinium bromide, atropine methylbromide, or homatropine methylbromide mixed with picric acid yielded negligible absorption spectra; the naturally occurring quaternaries, choline, its deriva-

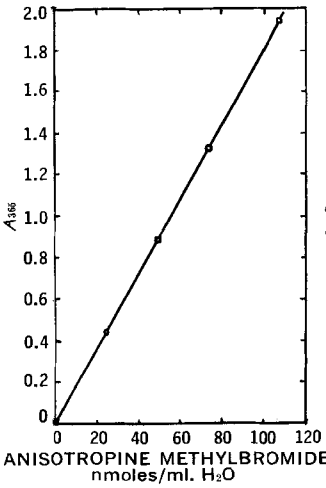

Fig. 1—Standard curve, methyl anisotropinium picrate in chloroform.

Received July 7, 1967, from the Department of Biochemistry and Department of Medicine, Endo Laboratories, Inc., Garden City, NY 11530
Accepted for publication August 21, 1967.
[1] Valpin, Endo Laboratories, Inc.

tives, or thiamine, will not interfere with the methyl anisotropinium assay when they are present in urine. Atropine methylbromide and homatropine methylbromide are several orders of magnitude more susceptible to alkaline hydrolysis than anisotropine methylbromide (4), both forming the unreactive quaternary alcohol, methyltropinium bromide. Presumably, thiamine hydrochloride produced no interaction due to prior destruction in the highly alkaline medium (5). At concentrations up to 110 nanomoles/ml. (nmoles/ml.), the only QAC which reacted to produce a standard curve was anisotropine methylbromide (Fig. 1).

When control, human urines from untreated individuals were compared to water blanks assayed at the same time, there was always a component of blank absorption. In addition, when human urines containing added anisotropine methylbromide were compared to water standards of the same concentrations, there was always an analytical loss. This loss could be due to the presence in urine of anions which have a greater affinity for methyl anisotropinium cation than does picrate. The blank absorptions and analytical losses were calculated by comparing quintuplicate samples of 0, 5, 10, and 15 nmoles/ml. anisotropine methylbromide in deionized water and in control urine, collected daily, over a period of 5 days. It was determined that the quantities recovered varied from one individual to another but were quite constant for the individual (Table I). The values for methyl anisotropinium ion in urine samples were determined by correcting the experimental A_{365} values for these factors.

Creatinine concentrations of urine specimens were determined by the method of Taussky (6) wherein ether removes reactive impurities while the creatinine is reacting with picric acid. The color of the picric acid–creatinine reaction product is developed with alkali. It was found that QAC's did not interfere with this assay in either aqueous solution or urine.

TABLE I—REPRODUCIBILITY OF BLANK VALUES AND ANALYTICAL RECOVERIES IN ANALYSIS OF URINE FOR ANISOTROPINE METHYLBROMIDE

Donor No.	Mean Blank A_{365} ± S.D.[a]	Mean Analytic Recovery,[b] % ± S.D.
1	0.019 ± 0.001	82.9 ± 5.5
2	0.024 ± 0.001	81.4 ± 3.5
3	0.021 ± 0.005	44.1 ± 7.2
4	0.018 ± 0.002	73.9 ± 7.4
5	0.041 ± 0.002	97.2 ± 1.7
6	0.014 ± 0.003	...
7	0.020 ± 0.002	...
8	0.043 ± 0.008	71.2 ± 5.8
9	0.038 ± 0.017	72.1 ± 5.2
10	0.018 ± 0.009	92.2 ± 5.3

[a] S.D. = standard deviation. [b] Losses are due to components normally present in urine. Expressed as percent of compound recovered after addition to urine from that donor.

TABLE II—CORRECTION VALUES AND DOSAGE FOR HUMANS

Subject	Mean Blank A_{365} ± S.D.	Mean Analytical Recovery, % ± S.D.	Anisotropine Methylbromide, Dose, mg./Kg.
A	0.015 ± 0.003	91.9 ± 6.7	0.264
B	0.016 ± 0.002	93.9 ± 3.5	0.304
C	0.020 ± 0.003	93.1 ± 4.6	0.284
D	0.026 ± 0.006	90.2 ± 6.4	0.244
E	0.021 ± 0.004	78.1 ± 10.3	0.200
F	0.021 ± 0.002	43.4 ± 6.6	0.310

TABLE III—ANISOTROPINE METHYLBROMIDE EXCRETION AS CUMULATIVE PERCENTAGE OF TOTAL DOSE (2 TABLETS, 20 mg.)

Subject	Total Excretion, %	Time, hr.	Time for ¾ Excretion, hr.
A	5.4	80[a]	48
B	10.7	96[a]	48
C	3.5	72[a]	60
D	7.6	104	72
E	4.1	96	24
F	11.4	96	64

[a] Excretion still continuing at termination of urine collection.

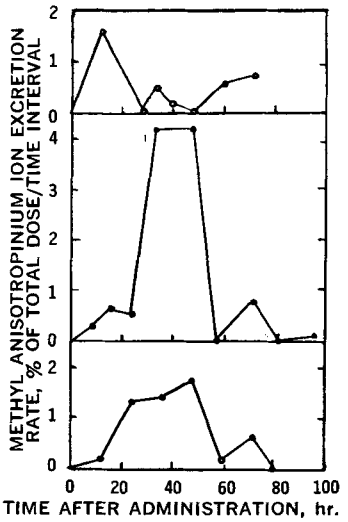

Fig. 2—Fractional urinary excretion of 20 mg. of anisotropine methylbromide administered as two 10-mg. tablets. Top, subject C; *middle, subject* B; *bottom, subject* A.

Excretion Study—After determining the blank value and the analytical loss in four separate 24-hr. urine samples per subject (Table II), six male subjects each took 20 mg. of anisotropine methylbromide (as two 10-mg. tablets) 0.5 hr. before breakfast. None of the subjects were taking any other medication at the time and none showed specific gravity abnormalities or any urine pathology; negative for blood, protein, and glucose and normal for pH as determined with Clinistix.[2] The mg./Kg. dosages of anisotropine methylbromide are listed in Table II. Urine collections from subjects *A*, *B*, and *C* were carried out for 80, 96, and 72 hr., respectively. Subjects *D*, *E*, and *F* were followed for 144 hr. The results for methyl anisotropinium ion excretion are presented in Table III and Figs. 2 and 3.

[2] Ames Co., Inc., Elkhart, Ind.

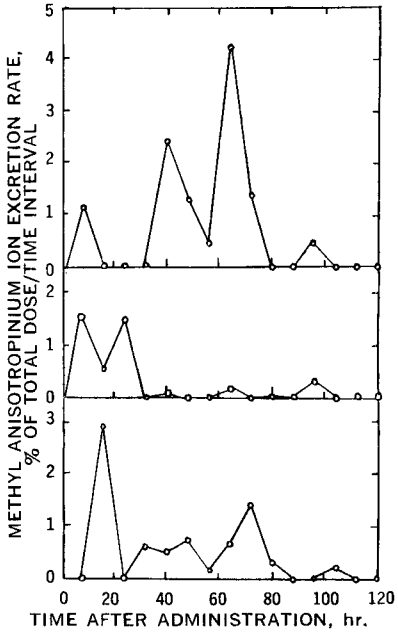

Fig. 3—Fractional urinary excretion of 20 mg. of anisotropine methylbromide administered as two 10-mg. tablets. Top, subject F; middle, subject E; bottom, subject D.

RESULTS AND DISCUSSION

Only methyl anisotropinium ion or nonhydrolyzed metabolic products would be likely to be detected by the picric acid assay method. If any such metabolites are present in urine, they are being included in the results reported as methyl anisotropinium ion excretion. Conversely, any drug broken down and being excreted as methyltropinium ion is being excluded.

The pattern indicated for methyl anisotropinium excreted by humans is most interesting. The data for fractional excretion (Figs. 2 and 3) indicate the presence of several excretion peaks over a period of 4 days. The magnitude of each of these peaks, and of the total cumulative excretion (Table III), varies with the subject. Previous studies of urinary excretion of QAC's after oral administration do not seem to have been carried out past 24 hr. (7–12). It is, as yet, unclear whether this sustained excretion is a common phenomenon in QAC excretion. The total excretions reported in Table III for anisotropine methylbromide show that anisotropine methylbromide, like previously studied quaternary compounds, is incompletely absorbed (7, 9, 11).

These results indicate not only absorption of anisotropine methylbromide, but also retention in the body of a portion of the absorbed material for an appreciable length of time. The compound may be stored for varying lengths of time in tissue depots, or perhaps recurring reabsorption of anisotropine methylbromide excreted in the bile has this effect. The periodicity of excretion would then be a function of the release of bile from the gall bladder. There are other explanations possible, but the present data do not permit definite conclusions to be drawn.

These results refer to only a limited number of human volunteers, and the variability which has previously been reported in QAC excretion (7–11) is also present here.

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Keyphrases

Anisotropine methylbromide
Urinary excretion
Ion-pair extraction
Picric acid reagent
Colorimetric analysis

Synthesis and Antibacterial Activity of Certain 3-Substituted Benzoxazolinones

By RAJENDRA S. VARMA and W. LEWIS NOBLES

The preparation of a series of 3-substituted 2-benzoxazolinones is described. These compounds were screened for their antibacterial properties; most compounds have shown significant antibacterial activity.

BENZOXAZOLINONES have been associated with various types of biological properties; Lespagnol and co-workers prepared and tested a number of derivatives of 2-benzoxazolinones for their anticonvulsive, hypnotic, antipyretic, and analgesic effects (1). 2-Benzoxazolinone and its 3-methyl, 3-ethyl, and 3-hydroxyethyl derivatives elicited the greatest anticonvulsant activity, whereas the 3-acetamido, 3-dihydroxyethyl, and 3-diethylaminoethyl derivatives exhibited marked analgesic activity. 2-Benzoxazolinone has also been reported to inhibit the growth of *Pseudomonas aeruginosa* (2), whereas its quaternary ammonium complexes have shown bactericidal properties (3).

The pronounced biological activity of many benzoxazole derivatives (4, 5) and the medicinal value of 5-chloro-2-benzoxazolinone (6) prompted the investigation of 3-substituted-2-benzoxazolinones. In this article the preparation of a number of 3-substituted-2-benzoxazolinones and their antibacterial activity are reported.

DISCUSSION

The compounds listed in Table I were prepared by the condensation of 2-benzoxazolinone, formaldehyde, and the appropriate secondary amine. The reaction usually occurred in the cold, but, in certain cases, warming for a few minutes resulted in improved yields. The source of formaldehyde was either formalin or paraformaldehyde; both gave satisfactory products. The condensations were performed in both ethanol or *p*-dioxane. Ethanol was usually preferred to *p*-dioxane.

Condensation of 2,6-dimethylpiperidine, as might be expected due to steric factors, with 2-benzoxazolinone did not yield the 2,6-dimethylpiperidinomethyl derivative, but gave instead a product whose analytical data, melting point, mixed melting point, and infrared spectra were identical with 3-hydroxymethyl-2-benzoxazolinone (XXIV). Under identical conditions 5-chloro-2-benzoxazolinone gave 3-hydroxymethyl-5-chloro-2-benzoxazolinone (XXV). While using 5-chloro-2-benzoxazolinone, 2,6-dimethylmorpholine also did not yield 3-(2,6-dimethylmorpholinomethyl)-5-chloro-2-benzoxazoline, but gave instead compound XXV.

The NMR spectrum (CCl_4) of compound III (Table I) was in complete agreement with the proposed structure. A multiplet representing protons (4H) of the aromatic ring and a singlet due to the $-N-CH_2-N-$ protons were observed at 6.9–7.2 δ and 4.5 δ, respectively. Four protons of the two methylene groups adjacent to the nitrogen were attributed to a doublet centered at 2.6 δ. A broad singlet observed at 1.5 was assigned to the methylene protons (6H) remote from the nitrogen.

Treatment of 2-benzoxazolinone with formaldehyde in the absence of amine furnished compound XXIV. The infrared spectrum of compound XXIV showed a band of medium intensity at 3400 cm.$^{-1}$ (OH, stretching) and a broad strong band at 1750 cm.$^{-1}$ (C=O, stretching). 5-Chloro-2-benzoxazolinone under identical conditions yielded compound XXV. The infrared spectrum of compound XXV exhibited a band of medium intensity at 3380 cm.$^{-1}$ (OH, stretching) and a strong band at 1755 cm.$^{-1}$ (C=O, stretching). In its NMR spectrum (CD_3CN) a complex multiplet was observed at 7 δ and a singlet at 5.16 δ which might be attributed to aromatic (3H) and methylene (2H) protons, respectively. The spectrum also showed a singlet at 4.18 δ which was assigned to the hydroxyl protons. The peak due to the hydroxyl group disappeared on D_2O exchange.

3-Hydroxymethyl-5-chloro-2-benzoxazolinone (XXV), on treatment with acetic anhydride, gave 3-acetoxymethyl-5-chloro-2-benzoxazolinone (XXVI). The infrared spectrum of this compound exhibited a doublet at 1785 cm.$^{-1}$ (ring carbonyl) and 1720 cm.$^{-1}$ (ester carbonyl). Compound XXV reacted with phosphorous tribromide to yield compound XXVII. Its infrared spectrum showed a strong band at 1780 cm.$^{-1}$ (C=O, stretching). The spectrum did not show hydroxyl absorption, thus indicating that the reaction has taken place. The NMR spectrum (CCl_4) of compound XXVII demonstrated singlets at 6.96 δ and 5.45 δ; these were assigned to aromatic (3H) and methylene (2H) protons, respectively.

The reaction of compound XXIV with 2-nitrophenylisocyanate furnished compound XXVIII. Its infrared spectrum showed characteristic absorptions at 3390 cm.$^{-1}$ (–NH–), 1790 cm.$^{-1}$ (ring carbonyl), and 1770 cm.$^{-1}$ (–C(=O)–NH–). Likewise compound XXV gave compound XXIX on treatment with 2-nitrophensyliocyanate. Its infrared

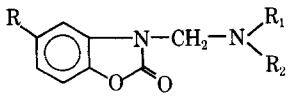
Received July 19, 1967, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Mississippi, University, MS 38677

Accepted for publication September 12, 1967.

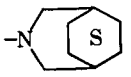
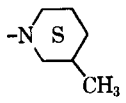
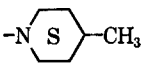
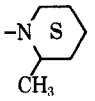
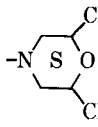
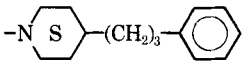
This investigation was supported in part by grant AI 04701 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

The authors thank Dr. Lyman Magee and Mr. Wayne Bing for biological data.

TABLE I—DIALKYLAMINOMETHYL-2-BENZOXAZOLINONES



R = H

Compd.	—N(R1)(R2)	Mol. Formula	Anal., % Calcd.	Anal., % Found	M.p., °C.	Yield, %[b]	Infrared cm.$^{-1}$ C=O
I[d,e]		$C_{16}H_{20}N_2O_2$	C, 70.56 H, 7.40 N, 10.29	70.80 7.53 10.17	159–160	94	1750
II[d,f]		$C_{10}H_{12}N_2O_2$	C, 62.49 H, 6.29 N, 14.57	...	125–126	76	1745
III[c,d,f]		$C_{13}H_{16}N_2O_2$	C, 67.22 H, 6.94 N, 12.06	66.93 6.85 12.26	139–140	60	1750
IV		$C_{14}H_{18}N_2O_2$	C, 68.27 H, 7.37 N, 11.37	68.52 7.55 11.47	150–152	62	1745
V[d]		$C_{14}H_{18}N_2O_2$	C, 68.27 H, 7.37 N, 11.37	68.55 7.50 11.59	107–108	67	1770
VI[d,]		$C_{12}H_{14}N_2O_3$	C, 61.53 H, 6.02 N, 11.96	...	148–150	83	1740
VII[d]		$C_{14}H_{18}N_2O_2$	C, 68.27 H, 7.37 N, 11.37	68.37 7.44 11.53	72–75	48	1765
VIII[d]	HMI[a]	$C_{14}H_{18}N_2O_2$	C, 68.27 H, 7.37 N, 11.37	68.50 7.50 11.42	110–111	73	1750
IX[d]		$C_{14}H_{18}N_2O_3$	C, 64.11 H, 6.92 N, 10.68	64.29 7.14 10.87	143–144	51	1765
X[d]		$C_{22}H_{26}N_2O_2$	C, 75.40 H, 7.48 N, 7.99	75.64 7.65 8.07	134–135	70	1745

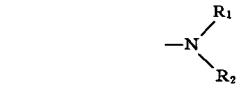
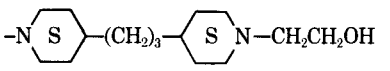
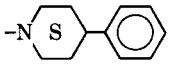
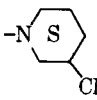
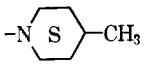
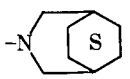
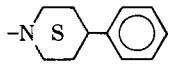
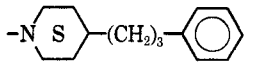
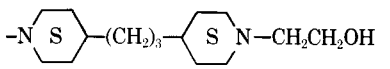
(*Continued on next page.*)

spectrum exhibited absorption bands at 3300 cm.$^{-1}$ (–NH–), 1790 cm.$^{-1}$ (ring carbonyl), and 1740 cm.$^{-1}$ (–C(=O)–NH–). Aniline in the presence of formaldehyde reacted with 5-chloro-2-benzoxazolinone to give compound XXX. Characteristic absorption bands were observed at 3380 cm.$^{-1}$ (–NH–) and 1750 cm.$^{-1}$ (C=O). 3,4,5-Trimethoxybenzoyl chloride in benzene solution successfully reacted with compound XXIV to yield compound XXXI. Its infrared spectrum showed a doublet at 1790 cm.$^{-1}$ (ring carbonyl) and 1720 cm.$^{-1}$ (ester carbonyl). Similarly, compound XXV yielded compound XXXII on treatment with 3,4,5-trimethoxybenzoyl chloride. A doublet was observed at 1800 cm.$^{-1}$ (ring carbonyl) and 1730 cm.$^{-1}$ (ester carbonyl) in the infrared spectrum of compound XXXII. The sequence of these reactions is described in Scheme I.

BIOLOGICAL DATA

All compounds reported here were subjected to preliminary antibacterial screening procedures except compounds XXIV, XXV, and XXVII. Filter paper disks (6.35 mm.), saturated with 2 drops of the solution or suspension of the test compound (20 mg./ml. in ethanol), were placed on the agar. The microbial spectrum consisted of four organisms: *Pseudomonas aeruginosa*, *Staphylococcus aureus* K257, *Mycobacterium smegmatis*, and *Klebsiella pneumoniae* ATCC8052. After 4 days of incubation, the zones of inhibition around the disks were measured. Out of the 29 compounds screened, 20 compounds inhibited the growth of *Ps. aeruginosa*, 19 compounds inhibited the growth of *S. aureus*

TABLE I—(*Continued.*)

Compd.		Mol. Formula	Anal., % Calcd.	Found	M.p., °C.	Yield, %[b]	Infrared cm.$^{-1}$ C—O
XI[d]		$C_{12}H_{14}N_2O_2$	C, 66.04 H, 6.47 N, 12.84	65.82 6.62 12.82	114–115	88	1750
XII[d]		$C_{22}H_{35}N_3O_3$	C, 68.80 H, 8.79 N, 10.46	68.50 8.90 10.60	107–108	56	1770
XIII[d]		$C_{19}H_{20}N_2O_2$	C, 74.00 H, 6.54 N, 9.08	74.17 6.73 9.08	174–175	84	1765
		R = Cl					
XIV[d]		$C_{12}H_{13}ClN_2O_2$	C, 58.53 H, 5.67 N, 10.51	58.76 5.84 10.31	108–109	56	1775
XV[d]		$C_{12}H_{13}ClN_2O_3$	C, 53.64 H, 4.88 N, 10.43	53.51 4.83 10.33	154–155	89	1775
XVI		$C_{14}H_{17}ClN_2O_2$	C, 59.88 H, 6.11 N, 9.98	60.04 6.28 10.02	89–90	48	1760
XVII[d]		$C_{14}H_{17}ClN_2O_2$	C, 59.88 H, 6.11 N, 9.98	60.01 6.23 10.04	91–93	60	1790
XVIII[d]		$C_{16}H_{19}ClN_2O_2$	C, 62.64 H, 6.24 N, 9.13	62.57 6.20 9.20	155	65	1780
XIX[d]	HMI[a]	$C_{14}H_{17}ClN_2O_2$	C, 59.88 H, 6.11 N, 9.98	59.73 5.91 9.81	68–69	53	1790
XX		$C_{19}H_{19}ClN_2O_2$	C, 66.57 H, 5.59 N, 8.17	66.76 5.70 7.96	173–174	70	1775
XXI[d]		$C_{22}H_{25}ClN_2O_2$	C, 68.64 H, 6.55 N, 7.28	68.48 6.41 7.15	87–88	73	1785
XXII[d]		$C_{12}H_{13}ClN_2O_2$	C, 57.12 H, 5.18 N, 11.09	57.02 5.32 10.86	74–78	40	1780
XXIII[d]		$C_{28}H_{34}ClN_3O_3$	C, 63.33 H, 7.86 N, 9.64	63.04 8.03 9.40	98–99	57	1780

[a] Hexamethyleneimino. [b] Yields are of the products obtained from first crystallization. [c] Prepared by method *A*. [d] Prepared by method *B*. [e] Prepared by method *C*. [f] Lit. (7) reports m.p. for II, III, VI, 126°, 110°, 148°, respectively.

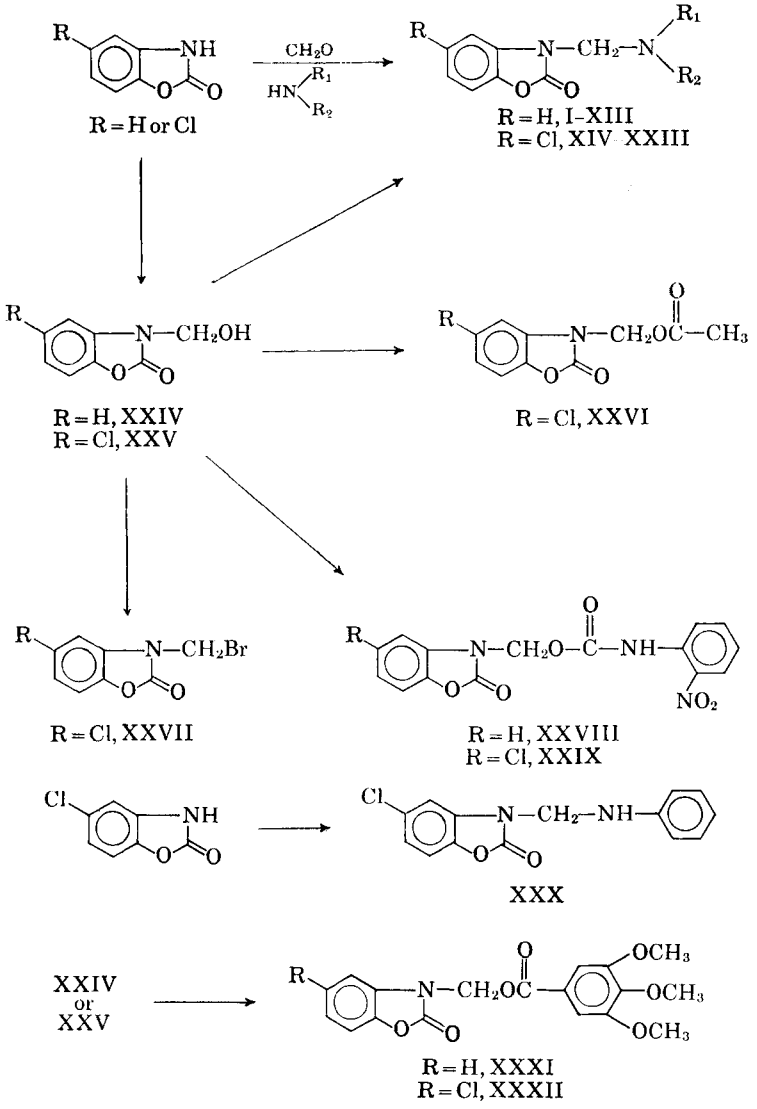
K257, 9 inhibited the growth of *M. smegmatis*, and 21 inhibited the growth of *K. pneumoniae* ATCC-8052. Zone sizes smaller than 6.35 mm. were considered to provide no inhibition of the growth of the organisms. A blank space indicates no activity.

The width of the zone of inhibition around a disk containing the compound depends to some extent on the rate of diffusion of the compound through the medium. A compound that diffuses very slowly through the agar may show a smaller zone of inhibition, but it might demonstrate a greater effect *in vivo* than another compound that showed a greater zone of inhibition *in vitro* because of the greater diffusion rate. This has been shown to be true with certain commercially available antibiotics. Because of this effect, even a narrow zone of inhibition *in vitro* may indicate a compound with potential usefulness. (See Table II.)

EXPERIMENTAL

Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. Infrared spectra were determined on a Perkin-Elmer model 137 Infracord spectrophotometer in Nujol mull. NMR spectra were charted on a Varian A-60 spectrometer using tetramethylsilane as the internal standard. Microanalyses were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

N-Dialkylaminomethyl-2-benzoxazolinones (I–XXIII, Table I)—*Method A*—2-Benzoxazolinone



Scheme I

(0.05 mole), paraformaldehyde (2.25 Gm.), ethanol (25 ml.), and appropriate secondary amine (0.05 mole) were mixed thoroughly in a 100-ml. round-bottom flask. The reaction mixture was refluxed for 4 hr. At the end of this time, the contents were cooled in a refrigerator, and the product thus separated was collected on a filter.

Method B—To a suspension of 2-benzoxazolinone (0.05 mole) in 20 ml. of ethanol, were added 7.5 ml. of 37% formalin and appropriate secondary amine (0.05 mole). During addition of amine, the reaction mixture became warm. It was kept at room temperature for 30 min. and then heated for another 30 min. on a water bath. The reaction mixture upon cooling furnished the desired product.

Method C—A mixture of 3-hydroxymethyl-2-benzoxazolinone (0.02 mole), amine (0.02 mole), and ethanol (50 ml.) was refluxed for 4 hr. The reaction mixture was cooled, and the product thus separated was recrystallized from a suitable solvent.

Dimethylamine was used as a 25% aqueous solution. When a compound was prepared by more than one method, its identity was always established by comparison of melting points, mixed melting points, and infrared spectra.

3-Hydroxymethyl-2-benzoxazolinone (XXIV)—2-Benzoxazolinone (6.75 Gm., 0.05 mole) was suspended in 100 ml. of hot distilled water. To this suspension 7.5 ml. of aqueous 37% formaldehyde solution was added, and the reaction mixture was set aside for 24 hr. The product thus separated was recrystallized from chloroform–petroleum ether (60–80°), m.p. 135–136°. [Lit. (7), m.p. 110°.] Mixed melting point with 2-benzoxazolinone, 108–110°; IR: 3400 (OH), 1750 cm.$^{-1}$ (carbonyl).

Anal.—Calcd. for $C_8H_7NO_3$: C, 58.18; H, 4.27; N, 8.48. Found: C, 58.36; H, 4.46; N, 8.58.

3-Hydroxymethyl-5-chloro-2-benzoxazolinone (XXV)—Formalin (9 ml.) was added to a suspension of 10.17 Gm. (0.06 mole) of 5-chloro-2-benzoxazolinone in 100 ml. of hot water. The reaction mixture after keeping at room temperature overnight yields

TABLE II—ANTIBACTERIAL ACTIVITY OF 3-SUBSTITUTED-2-BENZOXAZOLINONES

Compd.	*Ps. aeruginosa*	*S. aureus* K257	*M. smegmatis*	*K. pneumoniae* ATCC8052
I	8[a]	13	...	...
II	13[a]	13[a]	...	16
III	11	...	...	9
IV	8[a]	10[a]	...	9
V	12[a]	16	...	9[a]
VI	10[a]	16	...	9
VII	14[a]	16[a]	...	10
VIII	12[a]	15[a]	...	8[a]
IX	12[a]	13[a]	...	13
X	...	...	20[a]	10[a]
XI	15[a]	12[a]	...	9
XII	9[a]	14	18	10[a]
XIII	...	...	...	...
XIV	13	8	11[a]	11
XV	13[a]	15	...	9
XVI	11[a]	10[a]	15[a]	9
XVII	10[a]	...	20[a]	9
XVIII	9[a]	8	9[a]	8[a]
XIX	12[a]	...	...	11
XX	...	...	...	...
XXI	13	9	30[a]	18
XXII	10[a]	...	...	9
XXIII	9[a]	12	14	8
XXVI	...	8	9[a]	7
XXVIII	...	...	...	...
XXIX	...	8	...	...
XXX	...	10[a]	...	...
XXXI	...	...	...	...
XXXII	...	...	...	...

[a] Partial inhibition.

11.8 Gm. (98%) of the desired product. It was crystallized from ethanol, m.p. 138–143°. IR: 3380 (OH), 1755 cm.$^{-1}$ (carbonyl); NMR: 7 δ (aromatic, 3H), 5.16 δ (-CH_2-), 4.18 δ (OH).

Anal.—Calcd. for $C_8H_6ClNO_3$: C, 48.14; H, 3.03; N, 7.02. Found: C, 48.39; H, 3.22; N, 7.16.

3 - Acetoxymethyl - 5 - chloro - 2 - benzoxazolinone (XXVI)—Acetic anhydride (6 Gm.) was refluxed with 2 Gm. (0.01 mole) of 3-hydroxymethyl-5-chloro-2-benzoxazolinone for 3 hr. and then the whole was poured on crushed ice while stirring. The product thus obtained was crystallized from ethanol, m.p. 76–80°. IR: 1785 (ring carbonyl), 1720 cm.$^{-1}$ (ester carbonyl).

Anal.—Calcd. for $C_{10}H_8ClNO_4$: C, 49.70; H, 3.34; N, 5.80. Found: C, 49.48; H, 3.18; N, 6.02.

3 - Bromomethyl - 5 - chloro - 2 - benzoxazolinone (XXVII)—3 - Hydroxymethyl - 5 - chloro - 2 - benzoxazolinone (3.99 Gm., 0.02 mole) was suspended in 150 ml. of dry ether and allowed to cool in ice. Three milliliters of phosphorous tribromide was then added to it dropwise while stirring. The reaction mixture was stirred for 1 hr. in cold and another 1 hr. at room temperature. During this time the hydroxy compound dissolved and the reaction mixture became clear. Ether was removed under vacuum. The product was crystallized from benzene, m.p. 108–110°, yield 4 Gm. (76%). IR: 1780 cm.$^{-1}$ (C=O); NMR: 6.96 δ (aromatic, 3H), 5.45 δ (-CH_2-).

Anal.—Calcd. for $C_8H_5BrClNO_2$: C, 36.61; H, 1.91; Br, 30.44. Found: C, 36.82; H, 2.09; Br, 30.24.

3 - (2 - Nitrophenylcarbamylmethyl) - 2 - benzoxazolinone (XXVIII)—2 - Nitrophenylisocyanate (1.64 Gm., 0.01 mole) was added to 1.64 Gm. (0.01 mole) of 3-hydroxymethyl-2-benzoxazolinone in 25 ml. of anhydrous benzene. The reaction mixture was heated under reflux for 8 hr. At the end of this time, the reaction mixture was diluted with 50 ml. of petroleum ether (b.p. 60–80°) and refrigerated overnight. The solid product thus obtained was filtered and recrystallized from benzene, m.p. 142–143° (softens at 123°); yield, 2 Gm. (60%). IR: 3390 cm.$^{-1}$ (-NH-); 1790 cm.$^{-1}$ (ring carbonyl); 1770 cm.$^{-1}$ (-C(=O)-NH-).

Anal.—Calcd. for $C_{15}H_{11}N_3O_6$: C, 54.72; H, 3.37; N, 12.76. Found: C, 54.94; H, 3.51; N, 12.59.

3 - (2 - Nitrophenylcarbamylmethyl) - 5 - chloro-2-benzoxazolinone (XXIX)—3-Hydroxymethyl-5-chloro-2-benzoxazolinone (2 Gm., 0.01 mole) and 2-nitrophenylisocyanate (1.64 Gm., 0.01 mole) were refluxed overnight in 50 ml. of dry benzene. Benzene was removed under vacuum and the product was recrystallized from benzene, m.p. 177–178° yield, 1.8 Gm. (50%). IR: 3300 cm.$^{-1}$ (-NH-), 1790 cm.$^{-1}$ (ring carbonyl), 1740 cm.$^{-1}$ (-C(=O)-NH-).

Anal.—Calcd. for $C_{15}H_{10}ClN_3O_6$: C, 49.56; H, 2.77; N, 11.55. Found: C, 50.55; H, 2.85; N, 11.31.

3 - Anilinomethyl - 5 - chloro - 2 - benzoxazolinone (XXX)—Aniline (1.86 Gm., 0.02 mole) was added with stirring to 5-chloro-2-benzoxazolinone (3.39 Gm., 0.02 mole) suspended in 10 ml. of ethanol containing 3 ml. of 37% formalin. The reaction mixture was stirred for 1 hr. and allowed to remain at room temperature overnight. The product thus obtained was recrystallized from ethanol–acetone, m.p. 174–175°, yield, 4.2 Gm. (68%). IR: 3380 cm.$^{-1}$ (-NH-), 1750 cm^{-1} (carbonyl).

Anal.—Calcd. for $C_{14}H_{11}ClN_2O_2$: C, 61.20; H, 4.04; N, 10.20. Found: C, 60.92; H, 4.03; N, 10.09.

3 - (3,4,5 - Trimethoxybenzoyloxymethyl) - 2 - benzoxazolinone (XXXI)—Freshly prepared 3,4,5-trimethoxybenzoyl chloride (4.6 Gm., 0.02 mole) and 3-hydroxymethyl-2-benzoxazolinone (2.47 Gm., 0.015 mole) were refluxed in 50 ml. of dry benzene for 4 hr. At the end of this time, benzene was distilled off and residual semisolid was washed repeatedly with 10% aqueous sodium bicarbonate solution. The product on crystallization from acetone gave 2 Gm. (37%) of the ester, m.p. 171°. IR: 1790 cm.$^{-1}$ (ring carbonyl), 1720 cm.$^{-1}$ (ester carbonyl).

Anal.—Calcd. for $C_{18}H_{17}NO_7$: C, 60.17; H, 4.77; N, 3.90. Found: C, 60.31; H, 4.90; N, 3.88.

3 - (3,4,5 - Trimethoxybenzoyloxymethyl) - 5 - chlorobenzoxazolinone (XXXII)—This compound was prepared in a manner similar to XXXI from 2 Gm. (0.01 mole) of 3-hydroxymethyl-2-benzoxazolinone and 3.45 Gm. (0.015 mole) of 3,4,5-trimethoxybenzoyl chloride. Recrystallization from ethanol–acetone gave 2 Gm. (50%) of the product melting at 159°. IR: 1800 cm.$^{-1}$ (ring carbonyl), 1730 cm.$^{-1}$ (ester carbonyl).

Anal.—Calcd. for $C_{18}H_{16}ClNO_7$: C, 54.90; H, 4.10; N, 3.56. Found: C, 55.06; H, 4.25; N, 3.43.

Amine Exchange Reaction—3-Dimethylaminomethyl-2-benzoxazolinone (1.02 Gm., 0.01 mole), 3-methylpiperidine (0.99 Gm., 0.01 mole), and 2 ml. of ethanol were heated on a water bath for 3 hr. The product obtained after cooling the reaction

mixture was recrystallized from ethanol, m.p. 150°, mixed melting point with compound IV, 150°. Infrared spectrum, of this product was identical with IV.

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Keyphrases

Synthesis of 3-substituted benzoexazolinones
Condensation reaction
Antibacterial activity
Paper disk on agar plate
IR spectrophotometry
NMR spectrometry
Microanalysis

Influence of Cyclodextrins on Ester Hydrolysis

By TING-FONG CHIN, PING-HONG CHUNG, and JOHN L. LACH

The hydrolysis of various ethyl esters of aminobenzoic acids, acetylsalicylic acid, and atropine in alkaline solution was shown to be either accelerated or decelerated in the presence of α and β-cyclodextrin. It is also shown that the stereochemistry of binding and the degree of ionization of cyclodextrin are primarily responsible for these catalytic and inhibitory effects observed.

THE FACT that the cycloamyloses (cyclodextrins) are capable of forming inclusion complexes with various compounds in solution especially with molecules containing aliphatic and aromatic groups has been known for some time and was recently reviewed by Thoma and Stewart (1). Although this aspect of inclusion formation has received considerable study, the present interest in cycloamylose chemistry has been in the kinetic aspect, since these cycloamyloses or cyclodextrins also have been shown to impose both rate accelerations and decelerations on various organic reactions.

Cramer and co-workers (2–4) have studied the cyclodextrin catalyzed decarboxylation of various organic acids and the fission of pyrophosphate. With respect to carboxylic acids, Cramer assumes that the carboxylic acids, after inclusion in the cavity of cyclodextrin, are activated by nucleophilic attack of the ether and hydroxyl O atoms at the carboxyl C atom and that their decarboxylation is consequently accelerated.

Bender and co-workers (5–8) reported that the cyclodextrins accelerated the hydrolysis of the various esters studied and that the accelerations are often large and are markedly substituent dependent. They also stated that this acceleration effect was due primarily to nucleophilic attack on the included ester by alkoxide ion of the cyclodextrin.

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Received June 19, 1967, from the College of Pharmacy, University of Iowa, Iowa City, IA 52240
Accepted for publication August 23, 1967.
This work was supported in part by grant GM 06607 from the U. S. Public Health Service, Bethesda, Md.

mixture was recrystallized from ethanol, m.p. 150°, mixed melting point with compound IV, 150°. Infrared spectrum, of this product was identical with IV.

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Keyphrases

Synthesis of 3-substituted benzoexazolinones
Condensation reaction
Antibacterial activity
Paper disk on agar plate
IR spectrophotometry
NMR spectrometry
Microanalysis

Influence of Cyclodextrins on Ester Hydrolysis

By TING-FONG CHIN, PING-HONG CHUNG, and JOHN L. LACH

The hydrolysis of various ethyl esters of aminobenzoic acids, acetylsalicylic acid, and atropine in alkaline solution was shown to be either accelerated or decelerated in the presence of α and β-cyclodextrin. It is also shown that the stereochemistry of binding and the degree of ionization of cyclodextrin are primarily responsible for these catalytic and inhibitory effects observed.

THE FACT that the cycloamyloses (cyclodextrins) are capable of forming inclusion complexes with various compounds in solution especially with molecules containing aliphatic and aromatic groups has been known for some time and was recently reviewed by Thoma and Stewart (1). Although this aspect of inclusion formation has received considerable study, the present interest in cycloamylose chemistry has been in the kinetic aspect, since these cycloamyloses or cyclodextrins also have been shown to impose both rate accelerations and decelerations on various organic reactions.

Cramer and co-workers (2–4) have studied the cyclodextrin catalyzed decarboxylation of various organic acids and the fission of pyrophosphate. With respect to carboxylic acids, Cramer assumes that the carboxylic acids, after inclusion in the cavity of cyclodextrin, are activated by nucleophilic attack of the ether and hydroxyl O atoms at the carboxyl C atom and that their decarboxylation is consequently accelerated.

Bender and co-workers (5–8) reported that the cyclodextrins accelerated the hydrolysis of the various esters studied and that the accelerations are often large and are markedly substituent dependent. They also stated that this acceleration effect was due primarily to nucleophilic attack on the included ester by alkoxide ion of the cyclodextrin.

Lach and Chin (9) in their study dealing with hydrolysis of ethyl *p*-aminobenzoate in the presence of cyclodextrin, on the other hand, reported a deceleration effect. They attributed this deceleration in rate to a complete occlusion of the total ester in the void space of the cyclodextrin molecule and therefore shielding the ester linkage from hydroxyl ion attack and from alkoxide ion attack in the cyclodextrin molecules. They also stated that in this system the hydrolysis is due only to the free ester in solution resulting from the dissociation of the inclusion

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complex. Such a mechanism, however, cannot be applied to a reaction which exhibits a catalytic effect in that both the complexed ester and free ester in solution undergo hydrolysis. Since no detailed kinetic data or mathematic expressions have been reported dealing with this acceleration and deceleration reaction, the present study was undertaken in order that these two different mechanisms be better understood. Acetylsalicylic acid and the various ethyl esters of aminobenzoic acids were studied with respect to this cyclodextrin effect in alkaline solution.

EXPERIMENTAL

Reagents—Ethyl - *p* - aminobenzoate, recrystallized, m.p. 87–88°; ethyl-*o*-aminobenzoate, b.p. 266–268°; ethyl-*m*-aminobenzoate, b.p. 292–294°; acetylsalicylic acid, recrystallized, m.p. 135°; atropine, recrystallized, m.p. 114–115°; α-cyclodextrin, $[\alpha]_D^{25}$ in water = 150.5 ± .5°; β-cyclodextrin, $[\alpha]_D^{25}$ in water = 162.5 ± .5°.

Buffer Systems—pH range: 1.0–2.2, 5.8–7.8, 8.0–10.0 in HCl–KCl, KH_2PO_4–NaOH, and H_3BO_3–NaOH, respectively.

Analytical Procedure—The ethyl-*p*-aminobenzoate, ethyl-*o*-aminobenzoate, ethyl-*m*-aminobenzoate, and atropine are separated from their degradation product by extracting with chloroform in basic solution. The unhydrolyzed benzoates and atropine were then determined spectrophotometrically at the wavelengths of 285 mμ, 330 mμ, 316 mμ, and 257 mμ, respectively. Acetylsalicylic acid was determined by using two-component analysis determining the absorbance at wavelengths of 278 mμ and 308 mμ spectrophotometrically. The absorptivity of acetylsalicylic acid and salicylic acid at 278 mμ and 308 mμ were previously determined in different pH buffered solutions which were used in these studies. The presence of cyclodextrin in these solutions did not interfere with the spectrophotometric procedure in the concentrations employed for the analysis.

Kinetic Studies—Kinetic studies were carried out at specified temperatures and constant pH buffered solutions in a system with or without cyclodextrin. The reaction course was followed by measuring the absorbance at the given wavelength utilizing a Beckman DU spectrophotometer after proper dilution. An accurate weight of the ester was dissolved in 5 ml. of ethanol and an accurate amount of cyclodextrin was added in a system containing cyclodextrin and brought to exactly 100 ml. with buffer, separated into 10-ml. amber vials, sealed, and placed in the water bath. All these procedures were conducted under nitrogen. The hydrolysis was carried out in a Haake-Thermostate Unitherm constant-temperature water bath at a given temperature. Aliquots were withdrawn periodically from each vial and analyzed for the unhydrolyzed ester. In all cases first-order rate constants were observed at constant pH and constant temperature.

RESULTS AND DISCUSSION

For purpose of discussion some of the data dealing with the deceleration of the hydrolytic rate of ethyl-*p*-aminobenzoate in the presence of cyclodextrin and previously reported (9) are presented here. In this study it is shown that β-cyclodextrin and ethyl-*p*-aminobenzoate formed a 1:1 complex in aqueous solution and that the hydrolysis was due to the free or uncomplexed ester in solution resulting from the dissociation of the inclusion complex. This was verified mathematically and is presented, in part, in Scheme I,

$$(\text{BCD} \ldots \text{BZA}) \underset{K_F}{\overset{K_D}{\rightleftharpoons}} (\text{BZA})_f + (\text{BCD})_f, \quad (\text{BZA})_f \xrightarrow{k_h} \text{PABA} + \text{EtOH}$$

Scheme I

where K_D is the dissociation constant, K_F is the formation constant, k_h is the hydrolysis constant, $(\text{BZA})_f$ is the free ethyl-*p*-aminobenzoate, $(\text{BZA})_t$ is the total ethyl-*p*-aminobenzoate, $(\text{BCD})_f$ is the free β-cyclodextrin, (BCD . . . BZA) is the complex, PABA is the *p*-aminobenzoic acid.

$$K_D = \frac{(\text{BZA})_f \cdot (\text{BCD})_f}{(\text{BCD} \ldots \text{BZA})} \qquad \text{(Eq. 1)}$$

$$(\text{BZA})_f = \frac{K_D \cdot (\text{BCD} \ldots \text{BZA})}{(\text{BCD})_f} \qquad \text{(Eq. 2)}$$

Since in solution $(\text{BCD} \ldots \text{BZA}) = (\text{BZA})_t - (\text{BZA})_f$ therefore:

$$(\text{BZA})_f = \frac{K_D \cdot [(\text{BZA})_t - (\text{BZA})_f]}{(\text{BCD})_f} \qquad \text{(Eq. 3)}$$

Simplified Eq. 3 gives:

$$(\text{BZA})_f = \frac{K_D}{K_D + (\text{BCD})_f} (\text{BZA})_t \qquad \text{(Eq. 4)}$$

The rate equation for ethyl-*p*-aminobenzoate hydrolysis at constant pH is:

$$-d(\text{BZA})_f/dt = k_h(\text{BZA})_f \qquad \text{(Eq. 5)}$$

The rate equation for ethyl-*p*-aminobenzoate hydrolysis in solution containing β-cyclodextrin should be:

$$-d(\text{BZA})_t/dt = k_h(\text{BZA})_f = k_h \frac{K_D}{K_D + (\text{BCD})_f} (\text{BZA})_t \qquad \text{(Eq. 6)}$$

$$k_{\text{app.}} = k_h \frac{K_D}{K_D + (\text{BCD})_f} \qquad \text{(Eq. 7)}$$

$$k_h/k_{\text{app.}} = 1 + \frac{(\text{BCD})_f}{K_D} \qquad \text{(Eq. 8)}$$

From experimental data, K_D has been determined to be 2.34×10^{-3}. The apparent rate constants, evaluated for the reaction in a system containing β-cyclodextrin are thus related to the true rate constant by Eqs. 7 and 8. From Eq. 7 the apparent rate constants can be calculated and can be compared with the experimental rate constant data obtained in a system containing β-cyclodextrin. These comparisons are given in Table I.

The close agreement of the calculated and experimental apparent rate constants gives additional support to the assumptions that were made. Equation 8 shows a linear relationship between the ratio of $k_h/k_{\text{app.}}$ and the concentration of free β-

Table I—Comparison of Calculated and Experimental Apparent Rate Constants of Ethyl-*p*-aminobenzoate Hydrolysis in a System Containing β-Cyclodextrin in 0.04 *N* $Ba(OH)_2$ Solution at 30°

% of BCD	Apparent Rate Constants, hr.$^{-1}$/*M* Exptl.	Calcd.
0.00	0.666	
0.25	0.358	0.342
0.50	0.229	0.228
1.00	0.129	0.139

cyclodextrin. This linear relationship is shown in Fig. 1 which further substantiates our hypothesis.

The rate of hydrolysis of acetylsalicylic acid (ASA) in alkaline solution in the presence of cyclodextrin was accelerated indicating that the cyclodextrin exerted a catalytic effect. Previous study (10) has shown ASA and β-cyclodextrin interact in aqueous solution to form a 1:1 complex. It is believed that ASA and cyclodextrin undergo only partial inclusion with the phenyl portion of the organic acid occupying the void space of the cyclodextrin molecule, and being sterically fixed in this position, thus allowing a favorable approach of the carbonyl group to the hydroxyl group of the cyclodextrin. This catalytic effect then is due to the interaction of the ester with hydroxyl group of the cyclodextrin. The data also indicated that this catalytic effect only occurred at a pH where the hydroxyl group of the cyclodextrin existed as an alkoxide ion. The hydrolysis of ASA in alkaline medium and in the presence of cyclodextrin is due to the hydrolysis of the free ASA in solution resulting from the dissociation of this complex and to the hydrolysis of the ASA in the complex resulting from the catalytic effect due to the cyclodextrin, both of these reactions occurring simultaneously. A mechanistic scheme and the mathematical relationship for this hypothesis describing these reactions are shown in Scheme II,

$$\begin{array}{ccc} (BCD)_f + (ASA)_f & \overset{K_F}{\rightleftharpoons} & (BCD \ldots ASA) \\ \downarrow k_h & K_D & k_c \downarrow \\ SA + ACA & & (BCD)_f + SA + ACA \end{array}$$

Scheme II

where $(ASA)_f$ is the free acetylsalicylic acid, SA is the salicylic acid, ACA is the acetic acid, k_c is the constant of cyclodextrin catalysis, (BCD . . . ASA) is the complex.

$$K_F = \frac{(BCD \ldots ASA)}{(BCD)_f \cdot (ASA)_f} \quad \text{(Eq. 9)}$$

$$(BCD \ldots ASA) = K_F \cdot (BCD)_f \cdot (ASA)_f \quad \text{(Eq. 10)}$$

Rate of hydrolysis of ASA in alkaline solution containing cyclodextrin at constant pH should be:

$$\begin{aligned} -d(ASA)_t/dt &= k_h \cdot (ASA)_f + k_c \cdot (BCD \ldots ASA) \\ &= k_h \cdot (ASA)_f + k_c \cdot K_F (BCD)_f (ASA)_f \\ &= k_h[1 + k_c/k_h \cdot K_F (BCD)_f](ASA)_f \end{aligned} \quad \text{(Eq. 11)}$$

$$k_{app.} = k_h[1 + k_c/k_h \cdot K_F (BCD)_f] \quad \text{(Eq. 12)}$$

$$k_{app.}/k_h = 1 + k_c/k_h \cdot K_F (BCD)_f \quad \text{(Eq. 13)}$$

From experimental data, K_F has been determined to be 1.7×10^2; k_c to be 0.74 at pH 10 and at 35°.

From Eq. 12, one can then calculate the rate constants in systems containing cyclodextrin. Experimental rate constants and those calculated are given in Table II.

It is interesting to note here the close agreement obtained for these constants, indicating that the assumptions made are reasonable. Further verification of the assumptions is shown in Fig. 2, a plot of $k_{app.}/k_h$ against the concentration of free cyclodextrin should result in a straight line as indicated in Eq. 13.

Since the hydrolysis of ASA was carried out at pH = 10, it was felt that additional information with respect to this catalytic effect would be obtained if this reaction was carried out at various pH's.

An examination of Table III which lists these data indicated that this catalytic effect exhibited by the cyclodextrin is not operative at acid pH, and that this catalytic effect increased substantially with an increase in pH in the alkaline range. This suggested that the hydroxyl groups of the cyclodextrin must be in the ionized form in order that they act as a

Table II—Comparison of Calculated and Experimental Apparent Rate Constants of ASA Hydrolysis in Systems Containing β-Cyclodextrin in a Buffered Solution of pH = 10, at 35°

% of BCD	Apparent Rate Constants, hr.$^{-1}$/*M* Exptl.	Calcd.
0.00	0.258	
0.25	0.530	0.535
0.50	0.807	0.811
0.75	1.085	1.089

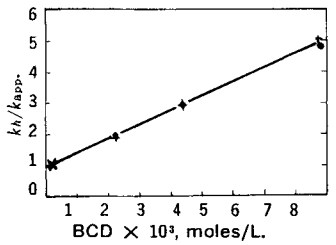

Fig. 1—Effect of β-cyclodextrin on the rate of ethyl-p-aminobenzoate hydrolysis in 0.04 N $Ba(OH)_2$ at 30°. Key: +, experimental; ●, calculated.

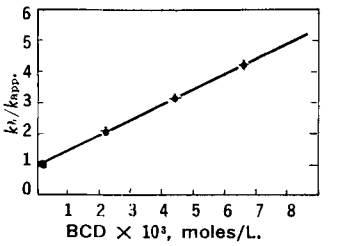

Fig. 2—Effect of β-cyclodextrin on the rate of acetylsalicylic acid hydrolysis at pH = 10 at 35°. Key: +, calculated; ●, experimental.

TABLE III—APPARENT RATE CONSTANTS OF HYDROLYSIS OF ASA AT DIFFERENT pH'S IN SYSTEMS WITH AND WITHOUT β-CYCLODEXTRIN

Temp.	pH	Apparent Rate Constants, hr.$^{-1}$/M			% Rate Increase
70°	1.0	0.5%	BCD	0.455	0.00
		No	BCD	0.454	
35°	7.0	0.5%	BCD	0.026	0.00
		No	BCD	0.026	
35°	8.0	0.5%	BCD	0.066	3.95
		No	BCD	0.063	
35°	9.0	0.5%	BCD	0.149	34.00
		No	BCD	0.110	
35°	10.0	0.5%	BCD	0.807	212.79
		No	BCD	0.258	

TABLE IV—HALF-LIFE OF ESTERS' BASIC HYDROLYSIS IN SYSTEMS WITH AND WITHOUT CYCLODEXTRINS

Compd.	Half-Life, hr. No CD[c]	0.5% ACD[d]	0.5% BCD[e]
Ethyl-*p*-aminobenzoate[a]	5.52	7.56	13.04
Ethyl-*o*-aminobenzoate[a]	37.06	23.40	41.25
Ethyl-*m*-aminobenzoate[a]	7.00	4.06	8.40
Atropine[b]	9.65	6.05	15.37
Acetylsalicylic acid[b]	2.67	0.956	0.855

[a] pH = 10.0, T = 70°. [b] pH = 10.0, T = 35°. [c] No CD = no cyclodextrin. [d] ACD = α-cyclodextrin. [e] BCD = β-cyclodextrin.

nucleophile. Cramer (3) also speculated as to this effect. For this reason a study was undertaken to investigate the ionization of cyclodextrin at different pH's. The pK value of β-cyclodextrin was found to be 12.2 which was in agreement with the previous reported value (6) and the percent of ionization was calculated using the following equation:

$$\% \text{ ionization} = \frac{100}{1 + \text{antilog}\,(pK_a - pH)} \quad \text{(Eq. 14)}$$

According to Eq. 14, the percent of ionization of β-cyclodextrin at different pH's is listed below:

pH	% of Ionization of BCD
12.2	50.000
11.0	5.940
10.0	0.627
9.0	0.063

Figure 3, which shows a plot of log percent of hydrolytic rate increase against pH and also a plot of log percent of ionization of β-cyclodextrin against pH, indicated that a direct relationship exists between the ionization of β-cyclodextrin and the rate increase of ASA hydrolysis in a system containing cyclodextrin. It seems reasonable therefore that the ASA undergoes only partial inclusion with cyclodextrin, is rigidly fixed with the void, and is catalyzed by the ionized hydroxyl groups of the cyclodextrin molecule and that this catalytic effect depends on the degree of ionization of these hydroxyl groups. In the case of deceleration effect noticed in the ethyl-*p*-aminobenzoate—β-cyclodextrin system, the ester linkage is totally shielded and therefore not subject to this nucleophilic attack. This was readily seen when Stuart models were prepared of these two systems. It should also

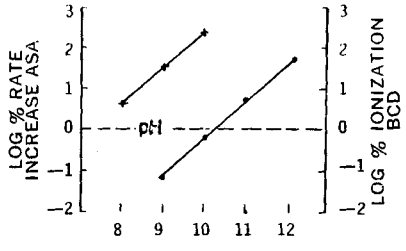

Fig. 3—A plot of log percent of hydrolytic rate increase of acetylsalicylic acid (+) hydrolysis in the system containing β-cyclodextrin against pH and log percent of ionization of β-cyclodextrin (●) against pH at 35°.

be pointed out here, that the cyclodextrin molecule exists in the C_1 conformation (11) with the hydroxyl groups existing on the outside of the cyclic structure. Since the pK value of some organic acids is decreased in the presence of cyclodextrin, and Henglein (12) also suggested that the catalytic effect may be due to this phenomenon, however, our own data with respect to this pK decrease or increase of various acids (acetic acid, hexanoic acid, benzoic acid, salicylic acid, acetylsalicylic acid, *p*-hydroxybenzoic acid, *p*-aminobenzoic acid, *m*-aminobenzoic acid, and *p*-nitrobenzoic acid) and the small pK change observed in the presence of cyclodextrin would not account for the large change in this hydrolytic rate and will be discussed later.

Additional information concerning the nature of the acceleration and deceleration effect was obtained in a study of the hydrolysis of various ethyl esters of aminobenzoic acids and atropine in the presence of α and β-cyclodextrin. Results of this study are given in Table IV.

These compounds were chosen in order to show that the rate of alkaline hydrolysis is determined primarily by the stereochemistry of the fit between guest and host in the relatively rigid cyclodextrin system. An examination of Table IV shows that the rates of hydrolysis of ethyl aminobenzoates (*para-*, *meta-*, and *ortho-*) were inhibited in the presence of β-cyclodextrin. This suggests that these molecules undergo total inclusion with the β-cyclodextrin and are shielded from nucleophilic attack by the alkoxide ion of the cyclodextrin and also by hydroxyl ion from solution. In the case of atropine due to its large size, total inclusion is not possible; however, it is highly likely that the phenyl and ester portion of the molecule is enclosed in the void space of the cyclodextrin and thus is shielded from attack. In these two cases the hydrolysis is due primarily to the free ester in solution resulting from the dissociation of the complexes. On the other hand, compounds, which do not totally fit into the void space of the cyclodextrin molecules and undergo only partial inclusion, exhibit an increase in hydrolytic rates due to the catalytic effect of the cyclodextrin in alkaline solution. For example, ASA which undergoes only partial inclusion with both α and β-cyclodextrin as seen in Table IV shows an increase in the rate of hydrolysis. Atropine, ethyl-*o*-aminobenzoate, and ethyl-*m*-aminobenzoate with α-cyclodextrin also show this catalytic reaction. Since the void space of β-cyclodextrin (8 Å.) is larger than the alpha void (6 Å.), it is readily seen by constructed molecular models that all of the ethyl esters of aminobenzoic acid can readily fit into the

void of β-cyclodextrin. On the other hand, only the *para* derivative readily fits into the void the of α-cyclodextrin molecule. The *meta* and *ortho* derivatives do not totally fit into the void of the α-cyclodextrin due to steric hindrance resulting from the amino group and thus are accelerated with respect to the rate of hydrolysis. With respect to ASA the large carbonyl group in the *ortho* position next to the leaving group prevents a total fit in both of the α and β-cyclodextrins and consequently it undergoes a catalytic reaction. In such cases the over-all hydrolytic rate is due not only to the free ester in solution but due also to the degradation of the ester in the complexed form.

CONCLUSION

The effect of cyclodextrins on the alkaline hydrolysis of various esters has been shown to be one of acceleration or deceleration depending on the nature of the complexes formed. Compounds which form a true inclusion compound or a compound in which the active center is included in the void of cyclodextrin exhibit a deceleration effect, and the hydrolytic rate is dependent primarily on the amount of free ester in solution resulting from dissociation of the complex. Compounds which do not totally fit into the void or are only partially included leaving the active center sterically fixed in close proximity to the hydroxyl group of cyclodextrin undergo an acceleration effect. In this case the hydrolytic rate is due not only to the free ester in solution resulting from the dissociation of the complex but also to nucleophilic attack of the ester linkage by the alkoxide ion of the cyclodextrin molecules. This catalytic effect is related to degree of ionization of these hydroxyl groups in the cyclodextrin molecule. Although it has been suggested that this catalytic effect may be due to a decrease in pK_a in the presence of cyclodextrin, the present data do substantiate this. For example, both α and β-cyclodextrin increase the pK_a value of *m*-aminobenzoic acid by approximately 0.1 unit, yet in the hydrolytic rate study, α-cyclodextrin exhibits an acceleration effect with respect to the hydrolysis of ethyl-*m*-aminobenzoate, whereas β-cyclodextrin has an opposite effect. This suggested that the stereochemistry of binding and the degree of the ionization of cyclodextrin are primarily responsible for the catalytic effect. This catalytic effect is absent when the hydroxyl groups are in the unionized form as in acid solution even though the ester is present in the complexed form.

The interest (13) in the kinetic aspect of these cyclodextrins has been due to the fact that these cyclodextrins have been used as models in the study of enzyme catalysis. Although such a model cannot explain completely the mechanism of enzyme action, kinetic data present in this investigation do illustrate some of the physical-chemical aspects involved in enzyme catalysis.

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Keyphrases

Ester hydrolysis
Cylodextrins influence on alkaline hydrolysis of esters
Acetylsalicylic acid hydrolysis catalyzed by cyclodextrins
Ethyl-(*para*, *ortho*, and *meta*) aminobenzoates hydrolysis decelerated by cyclodextrins
Atropine hydrolysis catalyzed by cyclodextrins
UV spectrophotometry—analysis

Sulfaethylthiadiazole (SETD) Release from Synthetic Wax Prolonged-Release Particles I

Effect of Dispersant Concentration

By IRA C. ROBINSON* and CHARLES H. BECKER

Synthetic wax particles containing sulfaethylthiadiazole (SETD) dispersed throughout and on the surface were made by a method of aqueous dispersion using various concentrations of polysorbate 80 USP and sorbitan monooleate each alone and in combination as dispersants. The *in vitro* release of SETD from 50–70 mesh wax particles was investigated with a rotating bottle dissolution apparatus, using simulated gastric and intestinal fluids. The dispersant factor was found to affect both the release rate and the proportion of total drug apparently constituting the prolonged-release fraction.

THE LITERATURE contains numerous examples of investigations of factors affecting the dissolution of drugs from both dosage forms and ideal experimental matrices effecting retarded drug release. Most of these studies were conducted on granules, pellets, or beads prepared by conventional means, by spray-drying, or by spray-congealing, or on dosage forms comprised of such particles.

In studies of drug release from various dosage forms Wagner (1) and Wiegand and Taylor (2) reported that the percent released time data followed apparent first-order rates. Lazarus *et al.* (3) observed the influence of drug particle size, drug concentration, and granule size on the *in vitro* release rate of drug from a compressed waxy granular tablet matrix and reported that drug concentration and crystal size exerted greater effects on the rate than matrix granule size.

Apparent first-order release rates for drug were found to be inversely proportional to drug–wax particle size in one investigation by Draper and Becker (4).

Higuchi attempted to relate the rate of drug release from one surface of sustained action solid matrices to physical constants based on simple laws of diffusion and obtained the following mathematical relation for the case where drug particles are incorporated into a granular matrix from which release is by a leaching action of penetrating solvent (5):

$$Q = \sqrt{\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s t} \qquad \text{(Eq. 1)}$$

where Q is the amount of drug released after time, t, per unit exposed area; D, the diffusivity of the drug in the permeating fluid; τ, the tortuosity factor; A, the total amount of drug present in the matrix per unit volume; C, the solubility of the drug in the permeating fluid; and ϵ, the porosity of the matrix.

Desai *et al.* (6) showed the applicability of the Higuchi theory in a study of drug release from planar surfaces of plastic matrices.

The effects of concentration of drug and water solubility on release rates were investigated by Sjorgren and Fryklof (7) with tablets prepared from granules. The use of water-soluble substances to increase the rate of release of water-soluble drugs from a compressed plastic tablet was suggested in a patent by Endicott (8).

Saski and Shah (9) studied the effects of three oxyethylene oxypropylene polymers on the availability of hexetidine and observed enhancement of activity with concentrations below the CMC's for two and a decrease in activity at the CMC for all three. Desai *et al.* (6) reported that the addition of benzalkonium chloride and sodium lauryl sulfate increased the rate of release of sodium salicylate from tablets, presumably by an increase in the effective porosity of the tablets.

In the present investigation, the authors prepared synthetic wax particles containing dispersed sulfaethylthiadiazole and dispersant, if used, by a method of aqueous dispersion and considered the effects of two nonionic surfactants, polysorbate 80 USP[1] and sorbitan monooleate,[2] when used singly and in combination as dispers-

Received November 22, 1966, from the College of Pharmacy, University of Florida, Gainesville, FL 32602
Accepted for publication August 29, 1967.
Abstracted in part from a thesis presented by Ira C. Robinson to the University of Florida, Gainesville, in partial fulfillment of Doctor of Philosophy degree requirements.
This investigation was supported in part by a grant from the University of Florida Computing Center, Gainesville, Fla.
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[1] Marketed as Tween 80 by Atlas Chemical Corp., Wilmington, Del.
[2] Marketed as Span 80 by Atlas Chemical Corp., Wilmington, Del.

ing agents, on the *in vitro* rates of drug release from the wax matrices. The procedure was to manufacture the particles under controlled conditions, characterize or examine, classify, and assay, and to use such particles in *in vitro* release studies.

DISCUSSION

The general method of aqueous dispersion consists of blending subdivided drug in a melted waxy material and dispersing this mixture by controlled agitation in either a heated or cooled aqueous bath, with or without the aid of a surface-active agent. The end product may be spherical or irregularly shaped particles, all containing drug over the surface as well as dispersed throughout the wax matrix. Drug dispersed on the surface would be expected to enter into the dissolution medium more rapidly than drug dispersed within the matrix since solvent must first penetrate the matrix and dissolve the medicament therein before diffusion from within can take place.

Factors which may affect matrix particle size and/or geometry include type and concentration of dispersant, temperature to which the initial melt and aqueous bath are heated, rate of cooling, and rate and type of agitation. Particle size and geometry and dispersant type and concentration would be expected to affect the drug release rate.

If the Higuchi relation (Eq. 1) applies to the systems under study, drug release data when plotted *versus* the square root of time should produce linearity for that portion of drug dispersed within the wax matrix. However, the fact that these percent released square root of time data plots are linear does not in itself prove that the equation applies to this study. Such proof is obtainable only from controlled studies specifically designed to test the effect of variables in Eq. 1.

It was theorized that altering the concentration of surfactant used as dispersing agent would result in altered release rates for SETD from within the synthetic wax–drug particles when tested in simulated digestive fluids. This result would presumably be due to a combination of the wetting effect of the dispersant and the consequent increase in availability of more channels to permit leaching to take place. Further, the linear portion of the release curve should not go through the origin when extrapolated to zero time.

EXPERIMENTAL

Official drug, or reagent grade materials were used throughout this work. Sulfaethylthiadiazole was obtained from American Cyanamid Co. Glyceryl tristearate[3] was supplied by Glyco Chemicals. The term SETD–glyceryl tristearate is employed to designate an approximate ratio of 1 part sulfaethylthiadiazole to 3 parts of glyceryl tristearate in completed products. Where reference is made to a mesh size range, it indicates that the particles passed through the lower-numbered sieve but were retained on the higher-numbered sieve.

[3] Glycowax S-932.

Table I—Dispersant Concentrations Employed in Preparation of SETD–Glyceryl Tristearate Particles

Product No.	% Dispersants Used[a] Sorbitan Monooleate	Polysorbate 80 USP
1	8.56[b]	0.07[g]
2	5.87[c]	0.11[g]
3	3.03[d]	0.23[g]
4	3.01[d]	0.63[e]
5	8.56	...
6	5.87	...
7	3.03	...
8	...	0.78[e]
9	...	0.47[f]
10	...	0.16[g]

[a] Used per 24 Gm. glyceryl tristearate and 8 Gm. SETD: [b] 3.0 ml. [c] 2.0 ml. [d] 1.0 ml. [e] 0.25 ml. [f] 0.15 ml. [g] 0.05 ml.

The USP XVII method of determining uniformity of fineness (10) was employed in a particle size analysis of the SETD powder reserved for this study, the mean diameter of which was determined to be 47 μ.

Preparation of SETD–Glyceryl Tristearate Particles—The basic formula for products manufactured was: glyceryl tristearate, 3 parts; SETD, 1 part; dispersing agent, a predetermined quantity (Table I); and distilled water, 50 parts. The glyceryl tristearate was melted in a glass beaker on a water bath and heated to a temperature of 75°. The SETD was uniformly dispersed in the melted glyceryl tristearate. In order to minimize loss, distilled water, generally containing a dispersant, was heated to 80° and added slowly to the SETD–wax dispersion with stirring provided by a "Lightnin" mixer, laboratory type, model F. Stirring was continued at an optimum rate until the temperature reached 45°. The SETD–glyceryl tristearate particles were separated from the aqueous phase by filtration and washing with three 250-ml. portions of distilled water to remove free SETD. These products were then air dried. A 50–70 mesh fraction was separated on a Ro-Tap sieve shaker by the official USP method (10) to represent a mean diameter of 203 μ, assuming spherical shape. Visual and microscopic examination of the particles was made. Bulk densities of the SETD–glyceryl tristearate particles were determined by the method of Butler and Ramsey (11) using the relationship:

$$\rho_b = W/V \qquad \text{(Eq. 2)}$$

where V is the volume in ml., W is the quantity of Gm. of material in the cylinder, and ρ_b, the bulk density expressed as Gm./ml. with the modification that the graduate was dropped 20 times/min. for better uniformity of results.

In the preparation of all drug–wax particles, it was desired that as many variables as possible be held constant. Therefore, the ratio of drug to wax (1:3), the volume of aqueous phase, the rate of addition of aqueous phase to melted wax–drug mixture, the temperature to which the wax–drug and the aqueous phases were initially heated, mixing container type and size, method of mixing, and position of stirrer in container were held constant throughout the manufacturing operations. Opti-

mum rates of stirring were determined and kept constant thereafter.

Assay for SETD Content—Duplicate samples of exactly 0.5000 Gm. of each air-dried product were placed in 25-ml. portions of chloroform heated on a water bath. These were then extracted with several 30-ml. portions of warmed 3.5% HCl using a separator. The combined extracts were made up to volume and an aliquot portion was assayed by the Bratton and Marshall colorimetric procedure (12). A Klett-Summerson colorimeter equipped with a No. 54 filter was employed.

In Vitro Release Studies—A rotating bottle dissolution apparatus[4] similar to that described by Souder and Ellenbogen (13) and constructed by the Bioelectronic and Instrument Department, J. Hillis Miller Health Center, was employed in release studies. It consisted of a rotating machine with spring clamps for holding sixteen 90-ml. screw-capped bottles in alternately perpendicular pairs along the rotating axis. A speed of rotation of 40 r.p.m. and a constant-temperature water bath were used.

The two dissolution fluids used were simulated gastric fluid (0.1 *N* HCl, pH 1.1) and simulated intestinal fluid (alkaline pancreatin solution, pH 8.3). For studies in the simulated gastric fluid, duplicate samples of each product equivalent to 120.0 mg. of SETD and one sample of an equivalent amount of blank glyceryl tristearate particles prepared in the same manner as the product were provided for each time interval and withdrawn for analysis after 15, 30, 45, 60, 90, and 120 min., and at appropriate intervals thereafter to determine when equilibrium of release was achieved. A similar procedure was followed with simulated intestinal fluid except that samples equivalent to 600.0 mg. of SETD were used and intervals after which withdrawals were made were 15, 30, 45, 60, 120, 180, 240, 360, 480, and 720 min., and additional ones to determine when equilibrium of release had been attained. With all samples, at withdrawal the contents of each bottle was filtered and 0.5 or 1 ml. of the filtrate was diluted to an appropriate volume with distilled water in a volumetric flask. One milliliter of the dilution was assayed for SETD by the colorimetric procedure previously mentioned.

RESULTS

Production of SETD–Wax Particles—Preliminary investigations showed the maximum workable ratio of sorbitan monooleate to wax to be 1:8 and higher proportions resulted in excessively soft masses. It was also found that a small proportion of polysorbate 80 USP to sorbitan monooleate gave particles with more desirable surface properties than particles prepared with larger proportions. Increasing the concentration of either dispersant when used singly or in combination with the other facilitated the production of finer particles.

In the preparation of particles from the dispersant mixtures employing the smallest amount of sorbitan monooleate along with polysorbate 80 USP, water-in-oil emulsions developed during the early stages of mixing. With cooling and further addition of aqueous phase containing dispersant, inversion took place. Resultant particles were less smoothly surfaced than were products made from formulas employing sorbitan monooleate alone in all proportions under investigation. Particles prepared with polysorbate 80 USP alone as dispersant had more irregular surfaces than those prepared with sorbitan monooleate alone. It was apparent from these results that the spherical and surface smoothness properties of completed products should be properly attributed to the surface activity of sorbitan monooleate. Observations by visual and microscopic examination of particles prepared and studied are presented in Table II along with the bulk densities for these products.

Increasing the concentration of sorbitan monooleate in combination with a constant amount of polysorbate 80 USP resulted in slightly increased bulk densities of the drug–wax particles. Products made with varying concentrations of each surfactant alone showed no trend with respect to bulk density differences. However, the bulk densities for these latter products were greater than for either product made with a combination of surfactants as dispersant.

SETD Release Rate Comparison—From plots of percent SETD released in alkaline pancreatin solution *versus* the square root of time (in min.), the slopes for the linear portions of the curves were calculated and compared for the various products. Most of the following discussion pertains to these results.

It was found that from 50–60% of the total SETD provided in the wax matrices was released in the simulated gastric fluid at equilibrium, and this amount was released quite rapidly during the first 15 min. as can be seen in Table III. The percentages of SETD released in the alkaline pancreatin solution during the first 15 min. was of the same relative order except for products 3 and 4 where the lowest percentage of sorbitan monooleate was used with polysorbate 80 USP. In the alkaline pancreatin solution, however, drug continued to be extracted from the wax particle after this initial period due not only to diffusion but also in part to a gradual disintegration of the wax matrix in this test fluid. Thus it appears that the SETD released in the simulated gastric fluid during the initial period (and at equilibrium) was drug from the particle surface, indicating negligible diffusion of drug from within the wax matrix. During dissolution studies in alkaline pancreatin solution, both the porosity and tortuosity factors were constantly changing. Nevertheless, the release data plots as square root of time results were reasonably well approximated.

Increasing the concentration of sorbitan monooleate approximately twofold in combination with constant amount of polysorbate 80 USP decreased the rate at which SETD was released in alkaline pancreatin solution by a factor of 4 (Fig. 1). The additional increase in sorbitan monooleate in a third product caused no further reduction in the SETD release slope, indicating that a minimum had been attained. Figure 2 shows that increases in sorbitan monooleate as singular dispersant promoted the release of SETD after the initial period. Rates are presented in Table III. Increased

[4] Subsequent to this work, this type of apparatus was made official in NF XII, 2nd supplement, March 1, 1967, p. 15.

TABLE II—PHYSICAL PROPERTIES OF SETD–GLYCERYL TRISTEARATE PARTICLES

Product No.	Bulk Density, Gm./ml.	Visual and Microscopic Examination
1	0.5611	Free-flowing, spherical particles, tan-colored with characteristic odor
2	0.5337	Free-flowing, spherical tan-colored granules with characteristic odor
3	0.5000	Free-flowing, nearly spherical granules of light tan color and characteristic odor
4	0.4939	Light tan-colored, free-flowing granules of slightly irregular shape and characteristic odor
5	0.5882	Free-flowing, spherical particles, cream-colored with faint characteristic odor
6	0.5574	Free-flowing, cream-colored spherical particles with faint characteristic odor
7	0.5864	Free-flowing, spherical cream-colored particles with faint characteristic odor
8	0.5769	Irregularly shaped, free-flowing, cream-colored granules with faint characteristic odor
9	0.5660	Free-flowing, irregularly shaped, cream-colored particles with faint characteristic odor
10	0.5882	Irregularly surfaced, free-flowing, cream-colored particles with characteristic odor

TABLE III—*In Vitro* DATA FOR SETD RELEASE FROM SETD–WAX PARTICLES

Product No.	Simulated Gastric Fluid —% SETD Released— 15 min.	Simulated Gastric Fluid —% SETD Released— Equilibrium	Simulated Intestinal Fluid —SETD Release Profile Data— Zero Intercept	Slope, min.$^{-1/2}$	% at Equilibrium
1	52.42	52.67	62.34	0.2648	91.19
2	52.67	52.67	63.09	0.2787	96.35
3	54.92	55.58	27.01	1.0631	82.23
4	57.17	56.00	17.00	1.8390	81.78
5	55.07	55.07	61.18	0.2053	65.65
6	57.10	57.10	63.98	0.1897	67.77
7	55.74	56.22	64.14	0.1780	67.88
8	51.08	52.00	56.51	0.3060	67.75
9	52.83	53.09	53.96	0.2389	60.95
10	49.25	50.60	53.52	0.1897	59.60

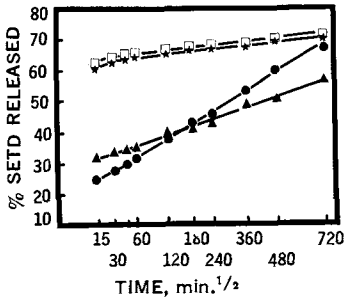

Fig. 1—Effect of dispersant concentration on SETD release in simulated intestinal fluid from SETD–wax particles. Key: ★, 8.56% sorbitan monooleate with 0.07% polysorbate 80 USP; □, 5.87% sorbitan monooleate with 0.11% polysorbate 80 USP; ▲, 3.03% sorbitan monooleate with 0.23% polysorbate 80 USP; ●, 3.01% sorbitan monooleate with 0.63% polysorbate 80 USP.

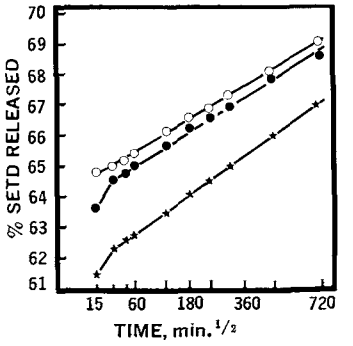

Fig. 2—Effect of singular dispersant sorbitan monooleate concentration on SETD release in simulated intestinal fluid from SETD–wax particles. Key: ★, 8.56%; ●, 5.87%; ○, 3.03%.

SETD release rates were observed with each raise in polysorbate 80 USP concentration when this dispersant was used both singly and in combination with constant amount of sorbitan monooleate (Figs. 1 and 3).

In addition to their effects on drug release rate and in the observable physical characteristics of the drug–wax particles, zero intercept values calculated from the linear portion of the dissolution curve for SETD in alkaline pancreatin solution suggest that variation in both the type and amount

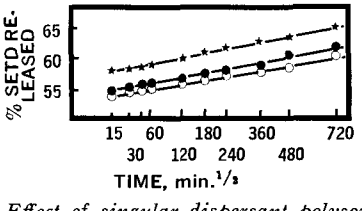

Fig. 3—Effect of singular dispersant polysorbate 80 USP on SETD release in simulated intestinal fluid from SETD–wax particles. Key: ★, 0.78%; ●, 0.47%; ○, 0.16%.

of dispersant influenced the amount of drug concentrating on or near the wax particle surface. A glance at the zero intercept values presented in Table III will show that more drug was apparently present on or near the surface of the drug–wax particles produced with the aid of sorbitan monooleate alone than on those prepared with polysorbate 80 USP alone; that increasing the concentration of sorbitan monooleate twofold with constant amount of polysorbate 80 USP gave a marked increase in this portion of drug rapidly released in the alkaline pancreatin solution; and that increasing the concentration of polysorbate 80 USP, while holding constant the amount of sorbitan monooleate in the formula, caused a marked decrease in the amount of SETD representing this value.

For all products except those prepared with use of polysorbate 80 USP singly as dispersant, where a larger portion of the total drug was apparently embedded within the wax matrix (total drug minus apparent surface drug), an increased rate of SETD release in the simulated intestinal fluid was observed.

SUMMARY

SETD–glyceryl tristearate particles were manufactured by a method of aqueous dispersion for prolonged-release application using various concentrations of polysorbate 80 USP and sorbitan monooleate singly and in combination as dispersants. The effects of variation in the type and concentration of surfactant used as dispersant were studied by *in vitro* release tests, physical testing, and visual and microscopic examination. Rates of release of SETD constituting the prolonged dissolution portion were calculated for results in simulated intestinal fluid using square root of time relationships and compared. Percent released square root of time plots gave reasonable linear approximations of prolonged-release SETD dissolution data.

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Keyphrases

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Sulfaethylthiazole–wax particle preparation
Surfactant effect on wax particle formation
Dispersant effect on wax particle formation
In vitro release studies
Colorimetric analysis

Complexation of Sodium Fluorescein with Polyvinylpyrrolidone

By RUSSELL E. PHARES, JR.

The complexation of sodium fluorescein (NaFluor) with polyvinylpyrrolidone (PVP) was studied at four pH values using a method similar to that of Benesi and Hildebrand. It was found that fluorescein can exist in four forms, each of which is capable of complexing. The results tend to indicate that at low pH values PVP exists in more than one form and that not all forms of the PVP are capable of complexing with NaFluor. When the ratio of NaFluor to PVP is small, a 1 : 1 complex is favored; but as the ratio increases, higher complexes predominate. The stability of the 1 : 1 complex seems to increase as the negative charge on the fluorescein decreases.

IT WAS recently discovered that polyvinylpyrrolidone (PVP), which for a long time has been known to complex with many substances, would form a sodium fluorescein complex possessing some interesting properties (1). By utilizing the unique properties of this complex, Krezanoski (1) has been able to formulate a stable and effective applanation tonometry diagnostic aid[1] con-

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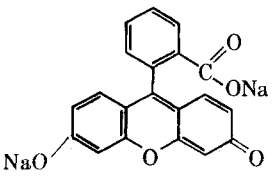
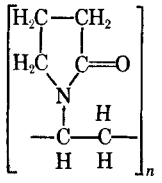
taining chlorobutanol, benoxinate HCl, and 0.25% sodium fluorescein in an isotonic boric acid buffer. It seemed desirable to collect more information about this complex in view of its practical applications and the fact that sodium fluorescein is a relatively simple molecule possessing the same basic structure found in many dyes which might be complexed with PVP to give other useful products. The structures of PVP and sodium fluorescein are given in I and II.



Sodium Fluorescein
I



PVP
II

EXPERIMENTAL

The sodium fluorescein (NaFluor) used was USP grade, and the PVP was pharmaceutical grade[2] having an average molecular weight of 30,000. All absorbance values were determined using a Beckman DK-2 spectrophotometer. A preliminary experiment was done to determine the spectral absorbance curves of a 1×10^{-5} *M* sodium fluorescein solution at pH values of 0.6 to 12.0. These curves are shown in Figs. 1 and 2. Another preliminary investigation was made using Job's method of continuous variation (2). A 3.7×10^{-5} *M* solution of PVP was mixed in various ratios with a 3.7×10^{-5} *M* solution of sodium fluorescein. Each solution was made in a pH 5.0 buffer adjusted to have an over-all ionic strength of 0.1. The absorbance of each mixture, as well as the absorbances of various concentrations of sodium fluorescein without PVP, was determined at 475 mμ. Figure 3 shows a plot of the differences between the expected and the observed absorbances for the various volume ratios of the PVP and sodium fluorescein solutions.

It was decided, based upon the results of the preliminary experiments, to study the complexation process at pH values of 1.5, 3, 5, and 8, using a method similar to that of Benesi and Hildebrand (3). The experiments conducted were as follows:

pH 1.5:

Sodium fluorescein held constant at 5×10^{-5} *M*, and the PVP varied from 1.66 to 13.28 $\times 10^{-4}$ *M*. Buffer system was HCl and KCl.

pH 3.0:

Sodium fluorescein held constant at 5×10^{-5} *M*, and the PVP varied from 1.66 to 13.28 $\times 10^{-4}$ *M*. Buffer system was phthalate.

PVP held constant at 1.8×10^{-5} *M*, and the sodium fluorescein varied from 1 to 36 $\times 10^{-5}$ *M*.

pH 5.0:

Sodium fluorescein held constant at 5, 25, and 50 $\times 10^{-6}$ *M*, and the PVP varied from 8.3 $\times 10^{-5}$ to 1.33 $\times 10^{-3}$ *M*. Buffer system was neutralized phthalate.

PVP held constant at 6.5×10^{-6} and 1.3×10^{-5} *M*, and the sodium fluorescein varied from 2.0×10^{-4} to 1.4×10^{-3} *M*.

[2] Plasdone C, General Aniline & Film Corp.

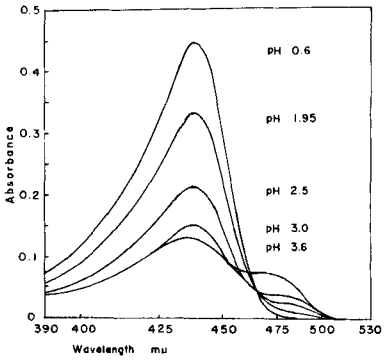


Fig. 1—Effect of pH on the spectral absorbance curve of sodium fluorescein.

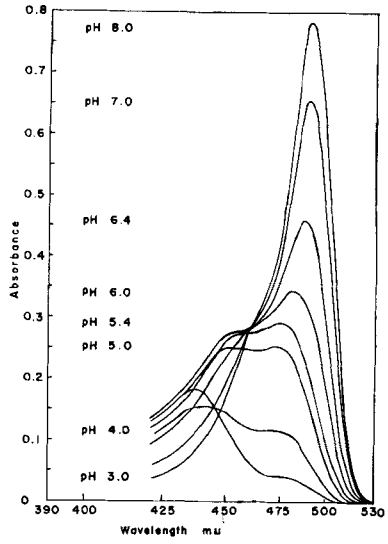


Fig. 2—Effect of pH on the spectral absorbance curve of sodium fluorescein.

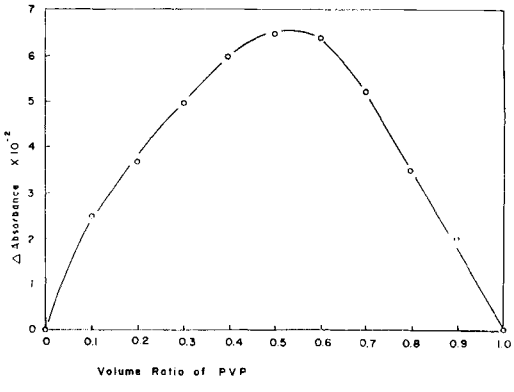


Fig. 3—Job plot of the NaFluor–PVP complex.

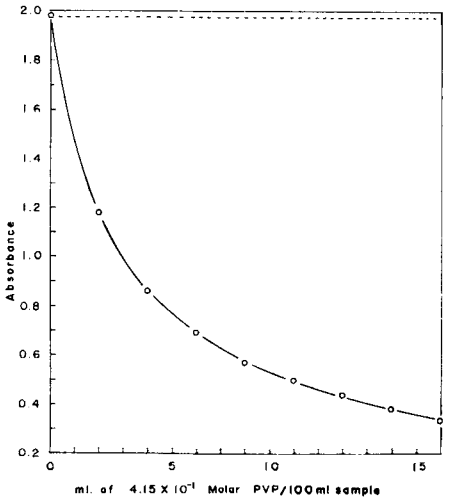


Fig. 4—Effect of PVP concentration on the absorbance of 5 × 10⁻⁶ M sodium fluorescein at pH 1.5.

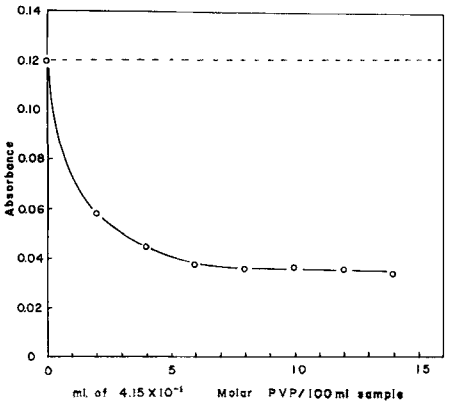


Fig. 5—Effect of PVP concentration on the absorbance of 5 × 10⁻⁶ M sodium fluorescein at pH 5.0.

pH 8.0:

Sodium fluorescein held constant at 5×10^{-5} *M*, and the PVP varied from 1.66 to 13.28 × 10^{-4} *M*. Buffer system was phosphate.
PVP held constant at 1.3×10^{-5} *M*, and the sodium fluorescein varied from 2×10^{-4} to 3.2×10^{-3} *M*.

The absorbances of the above solutions were measured at their spectral peaks, which were 437.5 mμ at pH values of 1.5 and 3.0, 475 mμ at pH 5.0, and 490 mμ at pH 8.0. Figures 4 and 5 are typical of the results obtained from the above experiments, and show the expected absorbances if no complexes were formed and the absorbances which were actually obtained.

DISCUSSION

The data summarized in Figs. 1 and 2 indicate that sodium fluorescein exists in several forms, much the same as phenolphthalein, which is quite similar structurally. Based upon the observed fact that the solubility of sodium fluorescein is much greater at the very low pH values than it is at a pH of about 2.5 to 3.0, and based upon the behavior of phenolphthalein at low pH values, it seems that the absorbance peak at 437.5 mμ is associated with a protonated form of the fluorescein. This form has a pKa of approximately 2.2. At about 465 mμ, there appears to be an isobestic point which is also associated with the protonation. The changes which take place between this isobestic point and the next one, which is also at 465 mμ, are probably associated with the singly charged anion, which appears to have a pKa of about 4.4. The peak at 490 mμ can be attributed to the doubly charged anion, which has a pKa of about 6.7. There were no indications of the formation of a triply charged anion such as is formed by phenolphthalein, even when the pH was raised to 12.0.

The Job plot shown in Fig. 3 has a maximum at about 0.5 parts of PVP by volume, which indicates the preferential formation of a 1:1 complex. It is interesting to note that the side of the curve representing relatively high concentrations of PVP is straight, as would be expected, while the side of the curve representing high concentrations of sodium fluorescein is curved. This curving is presumed to be a result of the formation of higher complexes which give way to the more stable 1:1 complex upon the addition of more PVP.

Assuming a 1:1 complex in dilute solutions of sodium fluorescein and recognizing the fact that any and possibly all forms of sodium fluorescein might be complexing with the PVP, the following equations were derived to describe the deviations of absorbances from those that would be predicted if no complexation were taking place.

The following equilibria are assumed:

$$\frac{[H^+][Fluor^-]}{[H\ Fluor^-]} = K_3 \text{ and } [H\ Fluor^-] = \frac{[H^+]}{K_3}[Fluor^-] \quad \text{(Eq. 1}a\text{)}$$

$$\frac{[H^+][H\ Fluor^-]}{H_2\ Fluor} = K_2 \text{ and } [H_2\ Fluor] = \frac{[H^+]^2}{K_3 K_2}[Fluor^-] \quad \text{(Eq. 1}b\text{)}$$

$$\frac{[H^+][H_2\ Fluor]}{H_3\ Fluor^+} = K_1 \text{ and } [H_3\ Fluor^+] = \frac{[H^+]^3}{K_3 K_2 K_1}[Fluor^-] \quad \text{(Eq. 1}c\text{)}$$

$$\frac{[C_1]}{[PVP][H_3\ Fluor^+]} = k_1 \text{ and } [C_1] = [PVP]k_1[H_3\ Fluor^+] \quad \text{(Eq. 2}a\text{)}$$

$$\frac{[C_2]}{[PVP][H_2\ Fluor]} = k_2 \text{ and } [C_2] = [PVP]k_2[H_2\ Fluor] \quad \text{(Eq. 2}b\text{)}$$

$$\frac{[C_3]}{[PVP][H\ Fluor^-]} = k_3 \text{ and } [C_3] = [PVP]k_3[H\ Fluor^-] \quad \text{(Eq. 2}c\text{)}$$

$$\frac{[C_4]}{[PVP][Fluor^-]} = k_4 \text{ and } [C_4] = [PVP]k_4[Fluor^-] \quad \text{(Eq. 2}d\text{)}$$

The following equation describes the measured absorbance (A_M) obtained when the sodium fluorescein is held constant and the absorbance due to PVP is negligible.

$$A_M = [C]_T Q_1 + [\text{Na}_2\text{ Fluor}]_{TF} Q_2 \quad \text{(Eq. 3)}$$

Q_1 and Q_2, defined in Eqs. 5 and 6, are constants for a given pH. The absorbance which would be expected (A_E) if no complex was formed should be:

$$A_E = [\text{Na}_2\text{ Fluor}]_T Q_2 \quad \text{(Eq. 4)}$$

The subscript TF stands for total free, T stands for total, and C stands for complex.

$$Q_1 = \frac{\epsilon_1 k_1[H^+]^3 + \epsilon_2 k_2 K_1[H^+]^2 + \epsilon_3 k_3 K_1 K_2[H^+] + \epsilon_4 k_4 K_1 K_2 K_3}{k_1[H^+]^3 + k_2 K_1[H^+]^2 + k_3 K_1 K_2[H^+] + k_4 K_1 K_2 K_3} \quad \text{(Eq. 5)}$$

$$Q_2 = \frac{\epsilon_5 K_1 K_2 K_3 + \epsilon_6 K_1 K_2[H^+] + \epsilon_7 K_1[H^+]^2 + \epsilon_8[H^+]^3}{K_1 K_2 K_3 + K_1 K_2[H^+] + K_1[H^+]^2 + [H^+]^3} \quad \text{(Eq. 6)}$$

where ϵ_1, ϵ_2, ϵ_3, ϵ_4, ϵ_5, ϵ_6, ϵ_7, and ϵ_8 are the absorptivity coefficients for C_1, C_2, C_3, C_4, H_3 Fluor$^+$, H_2 Fluor, H Fluor$^-$, and Fluor$^=$, respectively.

It can also be shown that when the above assumptions are true:

$$[A_E - A_M] = \Delta A = [C]_T(Q_2 - Q_1) \quad \text{(Eq. 7)}$$

and

$$\Delta A_{\max.} = [C]_{T\max.}(Q_2 - Q_1) = [\text{Na}_2\text{ Fluor}]_T(Q_2 - Q_1) \quad \text{(Eq. 8)}$$

An apparent stability constant (K_a) can be calculated as follows:

$$K_a = \frac{[C]_T}{[\text{PVP}][\text{Na}_2\text{ Fluor}]_{TF}} = \frac{\Delta A}{[\text{PVP}]_{TF}(\Delta A_{\max.} - \Delta A)} \quad \text{(Eq. 9)}$$

Equation 9 can be rearranged to give Eq. 10, which is more convenient for plotting. In plotting Eq. 10, it is assumed that $[\text{PVP}]_{TF} \simeq [\text{PVP}]_T$.

$$\frac{[\text{PVP}]_T}{\Delta A} = \left(\frac{1}{K_a\,\Delta A_{\max.}}\right) + \left(\frac{1}{\Delta A_{\max.}}\right)\left([\text{PVP}]_T\right) \quad \text{(Eq. 10)}$$

The data for each experiment in which sodium fluorescein was held constant are plotted in Figs. 6–9. The calculated K_a and the $\Delta A_{\max.}$ for each experiment are given in Table I. Although it appears that the stability constant for the complex at pH 5.0 may change as the amount of substrate changes, the variation may actually be a result of the inaccuracy associated with determining the absorbance values of small amounts of substrate. It is interesting to note that when the expected absorbance for each level of substrate is plotted *versus* the predicted $\Delta A_{\max.}$ a straight line which goes through zero, as would be expected, is obtained.

It can be shown that:

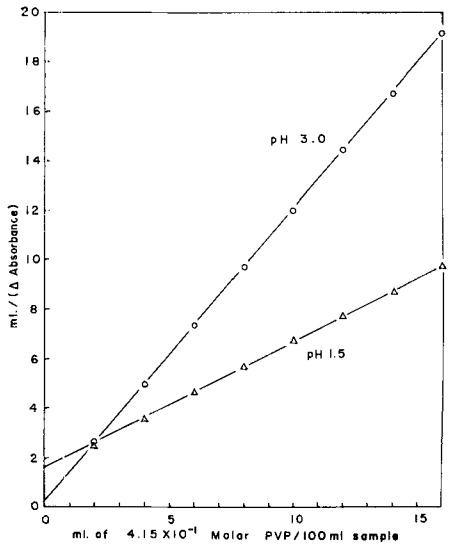


Fig. 6—The pH 1.5 and 3.0 data plotted according to Eq. 10.

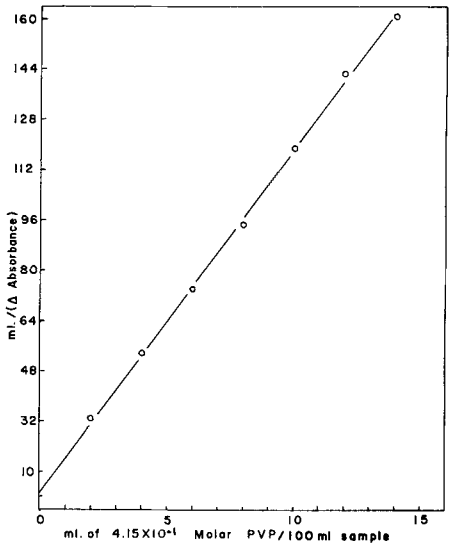


Fig. 7—The pH 5.0 data for 5 × 10^{-6} M substrate plotted according to Eq. 10.

$$K_a = \frac{k_1[H^+]^3 + k_2 K_1[H^+]^2 + k_3 K_1 K_2[H^+] + k_4 K_1 K_2 K_3}{[H^+]^3 + K_1[H^+]^2 + K_1 K_2[H^+] + K_1 K_2 K_3} \quad \text{(Eq. 11)}$$

By knowing the individual dissociation constants for sodium fluorescein and the apparent stability constants calculated at four different pH values, it should be possible to calculate the stability constant for the complex produced by each form of fluorescein. A large negative value was obtained for k_1 when the K_a values in Table I and the approximate dissociation constants for sodium fluorescein of 2.2,

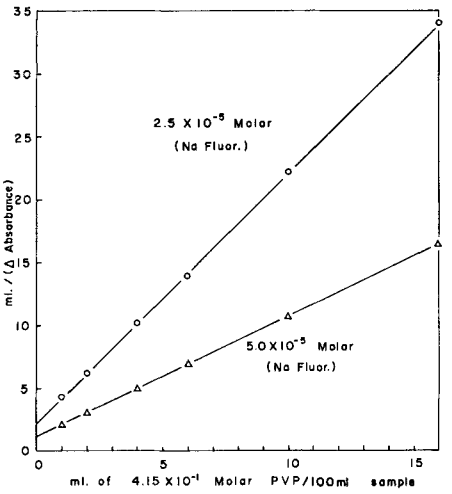


Fig. 8—The pH 5.0 data for 2.5 and 5.0 × 10^{-5} M substrate plotted according to Eq. 10.

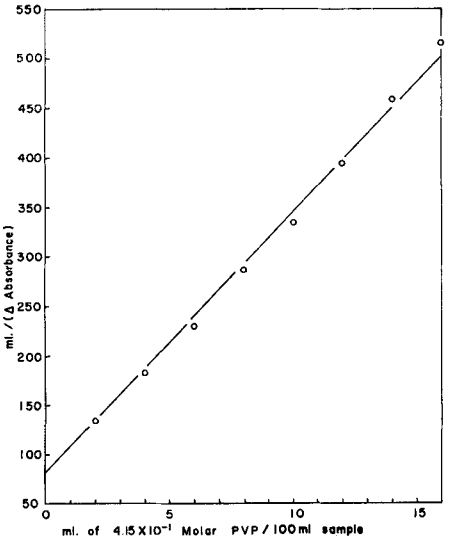


Fig. 9—The pH 8.0 data plotted according to Eq. 10.

TABLE I—SUMMARY OF BINDING DATA

pH	K_a × 10^{-4}	$\Delta A_{max.}$	ΔA_∞[a]	Concn. of Substrate × 10^{+5}
1.5	0.377	1.940	0.048	5.0
3.0	5.37	0.848	0.038	5.0
5.0	1.51	0.095	0.025	0.5
5.0	1.08	0.502	0.102	2.5
5.0	1.04	1.039	0.176	5.0
8.0	0.384	0.038	0.293	5.0

[a] ΔA_∞ is the measured absorbance at infinitely high concentrations of PVP and is obtained when all of the sodium fluorescein is complexed.

4.4, and 6.7 were used to calculate k_1, k_2, k_3, and k_4. This negative value was a result of the unusually

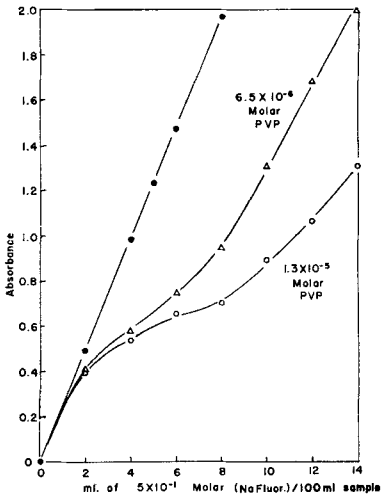


Fig. 10—Effect of sodium fluorescein concentration on the absorbance of 6.5 × 10^{-6} M PVP at pH 5.0.

small K_a observed at pH 1.5. A low value for K_a would result if the concentration of PVP free to complex were lower than expected. This would be the case at pH 1.5 if the complexing form of PVP was in equilibrium with a significant amount of a noncomplexing form of PVP. The literature states (4) that a PVP solution has its minimum viscosity at a pH of about 1.5, a statement which suggests that the PVP is in more than one physical form at this pH.

It was found that higher than 1:1 complexes were formed when the PVP was held constant and the sodium fluorescein varied. Although the stoichiometry and the stability constant of the higher complexes could not be determined, there was no doubt about their existence. The minimum number of moles of complexed sodium fluorescein required to produce the observed ΔA values were much larger than the number of moles of PVP which were present. The results at all three pH values were essentially the same as those shown in Fig. 10 for pH 5.0. None of the systems could be properly evaluated because of solubility problems, extremely high absorbances, or the need to make assumptions with questionable validity.

CONCLUSIONS

Based upon spectrophotometric data and qualitative solubility results, it appears that fluorescein can exist as a singly charged anion, a noncharged molecule, and a singly charged or doubly charged anion. Although each form of fluorescein has characteristic absorption properties, all four forms have a similar yellow color in solution. Each form of fluorescein can complex with PVP, but with varying strengths. This is probably a charged transfer type of complex. It appears that the higher the positive charge on the fluorescein, the weaker is the complex. When the ratio of fluorescein to PVP is about equal, a 1:1 complex is formed, but as the amount of fluorescein is increased, the order of the complex also increases. At low pH values anomal-

ous results are obtained which might well be due to an interaction of PVP with itself.

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(2) Job, P., *Ann. Chim.* **9**, 113(1928).
(3) Benesi, H. A., and Hildebrand, J. H., *J. Am. Chem. Soc.*, **71**, 2703(1949).
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Keyphrases

Complex formation
Sodium fluorescein–polyvinylpyrrolidone system
pH effect on complex formation
Colorimetric analysis

Comparative Pharmacokinetics of Coumarin Anticoagulants I

Unusual Interaction of Bishydroxycoumarin with Plasma Proteins—Development of a New Assay

By RENPEI NAGASHIMA, GERHARD LEVY*, and EINO NELSON†

A widely used method for the determination of bishydroxycoumarin (BHC) in plasma, which involves extraction of BHC from acidified plasma (pH <1) into an organic solvent, fails at high concentrations of BHC. Evidence is presented to show that this is not due to a limited solubility of the drug in the organic phase but rather to a concentration- and pH-dependent interaction of plasma protein with BHC. Complete extraction of BHC from the blood plasma of rats, guinea pigs, dogs, monkeys, and man is obtained readily only in the narrow pH range of 3.0 to 3.5. A new method of analysis for BHC in plasma has been developed on the basis of these findings and its specificity is demonstrated. It is shown that the development of analytical methods for drugs in plasma or serum, which involve extraction of the drug into an organic phase, cannot be based solely upon a consideration of the effect of pH on the distribution of the drug between the organic and aqueous phases. It is necessary also to consider the effect of pH (and possibly of other factors, such as the type of organic solvent, the buffer system, and ionic strength) on the physical-chemical properties of plasma proteins as they affect the type and magnitude of interaction of these proteins with the drug.

THE PHARMACOKINETICS of the coumarin anticoagulants has been and is the subject of intensive investigation in many laboratories (1–24). Studies have been concerned with the kinetics of elimination of various coumarin anticoagulants in several animal species (2–11) and in man (12–17), as well as with the interaction of these anticoagulants with various other drugs (*e.g.*, 18–23) and with plasma proteins (24). Of particular interest is the unusual, dose-dependent pharmacokinetics of bishydroxycoumarin (BHC) elimination in man, as well as the biologic fate of this drug which so far has eluded adequate characterization (25, 26).

The most widely used method for the determination of BHC is that of Axelrod and his associates (27), which involves extraction of the drug from plasma acidified with 3 *N* hydrochloric acid to pH <1 into *n*-heptane, followed by re-extraction into 2.5 *N* aqueous sodium hydroxide solution, and spectrophotometric measurement of BHC in the latter phase. In the course of studies in this laboratory on the comparative pharmacokinetics of BHC elimination, which will be described in subsequent reports (28, 29), it was noted that the assay method of Axelrod and his associates fails at relatively high concentrations of BHC. Therefore, a new analytical method for the determination of BHC in plasma or serum has been developed. This method and studies concerning its specificity are described in detail in view of the extensive interest in and continuing research related to the pharmacokinetics of the coumarin anticoagulants.

It will be shown that the recovery of BHC from plasma of various species, including man, is un-

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usually pH- and concentration-dependent. This phenomenon has wider implications in that it might be found also with other highly protein-bound drugs. It is evident on the basis of findings to be described here that the extractability of a drug from plasma is related not only to the degree of ionization and the intrinsic partition coefficient of the drug itself, but also to the physical-chemical state of plasma protein at a given pH and the degree and nature of the interaction of the drug with such protein(s) under given conditions.

EXPERIMENTAL

Materials—Heparinized blood was obtained from rats (Sprague-Dawley) and guinea pigs (Hartley) by cardiac puncture, and from dogs, rhesus monkeys, and humans by venipuncture. Plasma was obtained by centrifugation, pooled within the same species, and stored at −15° (for no longer than 1 month) until used. Bishydroxycoumarin[1] [m.p. 288–290°, uncorrected; lit. (30) m.p. 287–293°] was obtained from Nutritional Biochemicals Co., Cleveland, Ohio.

Preparation of Plasma Samples Containing Known Concentrations of BHC—A series of plasma samples containing various concentrations of BHC was prepared in the following manner. One-half milliliter of BHC solution, prepared by dissolving one part of bishydroxycoumarin and 10 parts of tris(hydroxymethyl)aminomethane (Nutritional Biochemicals Co.) in distilled water, was added to 2 ml. of plasma. The samples were maintained at 36–37° for 2 hr. and then stored at −15° (for no longer than 2 weeks) until used.

Micro-Adaptation of Axelrod's Method for the Determination of BHC (27)—Depending on the concentration of BHC, about 0.06 to 0.25 Gm. of plasma was pipetted into a tared 15-ml. capacity centrifuge tube (with a screw cap lined with polyethylene) which was then weighed. The sample was acidified with 0.03 ml. 3 *N* HCl, and the total volume of the aqueous phase was adjusted to approximately 0.35 ml. with distilled water. At this point the pH of the aqueous phase was found to be less than 1. Three milliliters of heptane was added and the tube was shaken vigorously for 10 min. by hand[2] and centrifuged. Two milliliters of the organic phase was transferred to another centrifuge tube, an exact volume of 2.5 *N* NaOH (0.5 to 2 ml.)[3] was added, and the tube was shaken for 5 min., by hand and centrifuged. The absorbances of the aqueous phase at 314 and 360 mμ were determined in a fused silica microcell, 10-mm. light path, with a Beckman DU spectrophotometer using a 0.3-mm. pinhole slit with 2.5 *N* NaOH in the reference cell. The absorptivity was determined with standard solutions of BHC in 2.5 *N* NaOH. Measurements at two wavelengths were necessary for plasma blank corrections, as described in a subsequent paragraph. Some studies were done using 0.5 ml. of suitably diluted plasma, in which case the following volumes of reagents were used: 0.2 ml. of distilled water, 0.06 ml. of 3 *N* HCl, and 6 ml. of heptane. All glassware was cleaned by immersion in an acid dichromate bath for at least 12 hr.

Extraction Efficiency as a Function of pH—BHC was extracted from plasma into *n*-heptane and assayed as described under *New Assay Method for BHC* except that the plasma was adjusted to various pH values by means of specified types and concentrations of buffering agents.

Determination of Rate of Extraction—Two and one-half milliliters of suitably diluted[4] BHC-containing rat plasma and 1 ml. of buffer solution were placed in a 60-ml. bottle furnished with a polyethylene cap. After a specified time, 20 ml. of heptane was layered over the aqueous phase. The bottle was then capped and agitated in an upright position on a reciprocating shaker (280 strokes per min., 5.9-cm. stroke travel). Three-milliliter aliquots of the organic phase were withdrawn 15, 30, 60, and 120 min. after the beginning of agitation, and an equal volume of *n*-heptane was added after each withdrawal. The BHC concentration in each 3-ml. aliquot of the heptane phase was determined by extraction of the drug into 2.5 *N* NaOH and spectrophotometric measurement at 314 mμ. The pH-adjusting agents used were 1.5 *N* HCl (for pH 0.6), 1.5 *M* citrate–phosphate buffers (for pH 3.0 and 4.9), and 1.5 *M* acetate buffer (for pH 4.9).

New Assay Method for BHC—*Small Scale Assay*—One-tenth milliliter of citrate–phosphate buffer (0.99 and 0.51 *M*, pH 3.0) and an appropriate volume of distilled water were added to 0.06 to 0.25 Gm. of plasma to yield a total volume of 0.35 ml. of sample with a pH of between 3.0 and 3.2. The same procedure as described for the micro-adaptation of Axelrod's method was then followed.

Large Scale Assay—This method was employed in the case of dog, monkey, and human plasma, where it is possible to obtain 0.6 ml. or more of plasma repeatedly from any one animal. Six-tenths milliliter suitably diluted plasma, 0.2 ml. citrate–phosphate buffer (1.5 *M*, pH 3.0), and 6 ml. of heptane were placed in a 15-ml. centrifuge tube, shaken for 10 min., and centrifuged. Four milliliters of the organic phase was transferred to another centrifuge tube and extracted with 1 to 5 ml. of 2.5 *N* NaOH, and the BHC concentration of the aqueous phase was determined at 314 and 360 mμ, using 2.5 *N* NaOH in the reference cell.

The lower limit of the sensitivity of the assay procedure is a function mainly of the contribution of the plasma blank to the total absorbance. One microgram of BHC/ml. of plasma can be determined readily in a plasma sample of 0.5 ml., but the plasma blank will be equivalent to almost one-third of the drug alone. The upper limit of the assay is determined by the solubility of BHC in heptane (approximately 14 mcg./ml. at room temperature). Thus, undiluted plasma containing up to 100 mcg./ml. can be assayed readily by the large scale assay method, although the volume of NaOH phase may

[1] The material showed a single spot when visualized with diazotized *p*-nitroaniline or under ultraviolet light, on a thin-layer chromatogram (Silica Gel G) developed with a solvent mixture of chloroform–heptane–acetic acid (10:15:4).

[2] This resulted in more rapid extraction than mechanical agitation as used by Axelrod *et al.* (27).

[3] One part of 2.5 *N* NaOH can quantitatively extract the drug from 4 parts of heptane.

[4] Dilution of plasma containing high concentration of BHC is necessary prior to extraction with *n*-heptane due to the limited solubility of BHC in the organic solvent.

have to be increased. Higher concentrations of BHC in the plasma require that the plasma sample be diluted prior to extraction. The Beer-Lambert law holds for BHC concentrations as high as 20 mcg./ml. in 2.5 *N* NaOH.

Correction for Plasma Blanks—The contribution of plasma blanks is negligible at BHC concentrations of 50 mcg./ml. and above. The blank contribution is, however, appreciable when BHC concentrations are quite low. In the small scale assay, when the drug is extracted from the organic phase into 0.5 ml. of 2.5 *N* NaOH, blank values ranged from 0.15 mcg./ml. to 0.30 mcg./ml. apparent BHC. This includes the contribution of plasma as well as organic solvent. While plasma blank values obtained from different animals vary appreciably, it was found that the ratio of the absorbances at 314 mμ (A_{314}) and 360 mμ (A_{360}) are quite constant. The average ratios, A_{314} to A_{360}, were 0.9 for the rat, 1.4 for the guinea pig, 1.0 for the dog, 0.6 for the monkey, and 0.9 for man. The plasma blanks for the large scale assay were approximately 0.15 mcg./ml. for the dog and monkey, and 0.35 mcg./ml. for man, in terms of apparent BHC.

For the determination of BHC in plasma, the absorbances of the sodium hydroxide extract are determined at 314 mμ and 360 mμ. The plasma blank value at 314 mμ is calculated from the absorbance of sample at 360 mμ and the known ratio of absorbance of plasma blanks at 314 and 360 mμ (R). The absorbance of BHC at 360 mμ is very small (about 1/110 of its absorbance at 314 mμ) and thus contributes only negligibly to the apparent plasma blank value. It may however be incorporated in the calculations in the following manner:

$$\text{plasma blank } A_{314} = R(A_{360} - 0.009 \times A_{314})$$

when A_{314} due to BHC plus blank $\gg A_{314}$ due to blank alone.

Specificity of the New Assay Method for BHC—The specificity of the new assay method for the determination of BHC, as opposed to metabolites of this drug, was determined by a partitioning method similar to that of Axelrod *et al.* (27). For this purpose, heparinized plasma samples were obtained from animals which had received BHC intraperitoneally at least 7 hr. earlier. Samples were also obtained from BHC-containing blood which had been perfused through an isolated rat liver since this was considered to be an effective means of obtaining plasma which contains both BHC and its metabolites. The procedures used in the partitioning study are outlined schematically in Fig. 1. In essence, the plasma was extracted with *n*-heptane, the organic phase was removed, and an appropriate volume of isoamyl alcohol was added to it.[5] This latter solution was then equilibrated with buffer solutions of pH 4.0 (0.5 *M* citrate-phosphate), 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 [tris-(hydroxymethyl)aminomethane–HCl; ionic strength, 0.1]. The amount of BHC remaining in the organic phase was determined by extracting the latter with 2.5 *N* NaOH, followed by spectrophotometry of that extract.

For purpose of comparison, other plasma samples were extracted with ethyl acetate following addition of solid sodium chloride. This is known to extract not only BHC but also its metabolites (7). The ethyl acetate extract was evaporated under reduced pressure, the residue was dissolved in *n*-heptane–isoamyl alcohol mixture,[6] and this solution was equilibrated with buffers of various pH values as described above. The results of the partitioning experiments are described in terms of the fraction of BHC (or apparent BHC) remaining in the organic phase.

RESULTS

Determination of BHC in Plasma by the Method of Axelrod et al. (27)—Known amounts of BHC were added to plasma samples obtained from rats, guinea pigs, dogs, and man to yield BHC concentrations ranging from 2 to 200 mcg. per ml. or Gm. of plasma. These plasma samples were analyzed by the micro-adaptation of the method of Axelrod *et al.* (27). The results, depicted in Fig. 2, show that all of the assay values were appreciably lower than theory at BHC concentrations above 50 mcg./ml.[7] The logarithmic scales of Fig. 2 tend to de-emphasize the magnitude of the deviation of the analytical results from theory; note that the assay results at BHC concentrations of 100 and 200 mcg./ml. were in some instances only 25 to 50% of theory. It was also found that there was appreciable intersubject variation in the results of an analysis of BHC in plasma of different healthy humans (Table I). Several modifications of the method of Axelrod and associates (27) were tried (Table II), but these did not result in any appreciable improvement in the recovery of BHC. The results listed in Table II rule out the possibility that the incomplete extraction is due to a possible saturation of the organic phase with BHC. It was noted in general that the recovery of BHC was more a function of the ratio of the concentration of BHC to that of plasma than of the concentration of BHC in the plasma *phase*.[8] This becomes apparent upon comparison of the results obtained by the unmodified assay method and the assay method which involves a thirteenfold dilution of the plasma prior to extraction. This degree of dilution produced a substantial decrease in the concentration of plasma proteins in the aqueous phase. Yet, neither the dilution of plasma proteins nor the decrease in the concentration of BHC produced a significant increase in the recovery of the drug.

Extraction Efficiency as a Function of pH—Figure 3 shows the effect of pH on BHC extraction efficiency, using rat plasma containing 200 mcg./ml., which was diluted to 2.5 times its volume with water, and *n*-heptane as the extracting solvent.

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[5] Note that isoamyl alcohol was added *after* the extraction The use of isoamyl alcohol was necessary because it was desired to compare the partitioning profiles of BHC and its metabolites. These metabolites do not dissolve in *n*-heptane alone.

[6] This is where the addition of isoamyl alcohol became necessary in order to dissolve the BHC metabolites.

[7] Note, however, that Axelrod *et al.* (27) made no claim for the usefulness of their method above this concentration. Therefore, nothing in our results or discussion should be interpreted as a criticism of the contribution made by these investigators.

[8] The plasma *phase* consists of plasma and aqueous diluent (*i.e.*, water, buffer, and/or HCl solution) in a ratio appropriate for effective extraction of the drug into the organic solvent; the terms "concentration in plasma phase" and "concentration in plasma" must be distinguished accordingly.

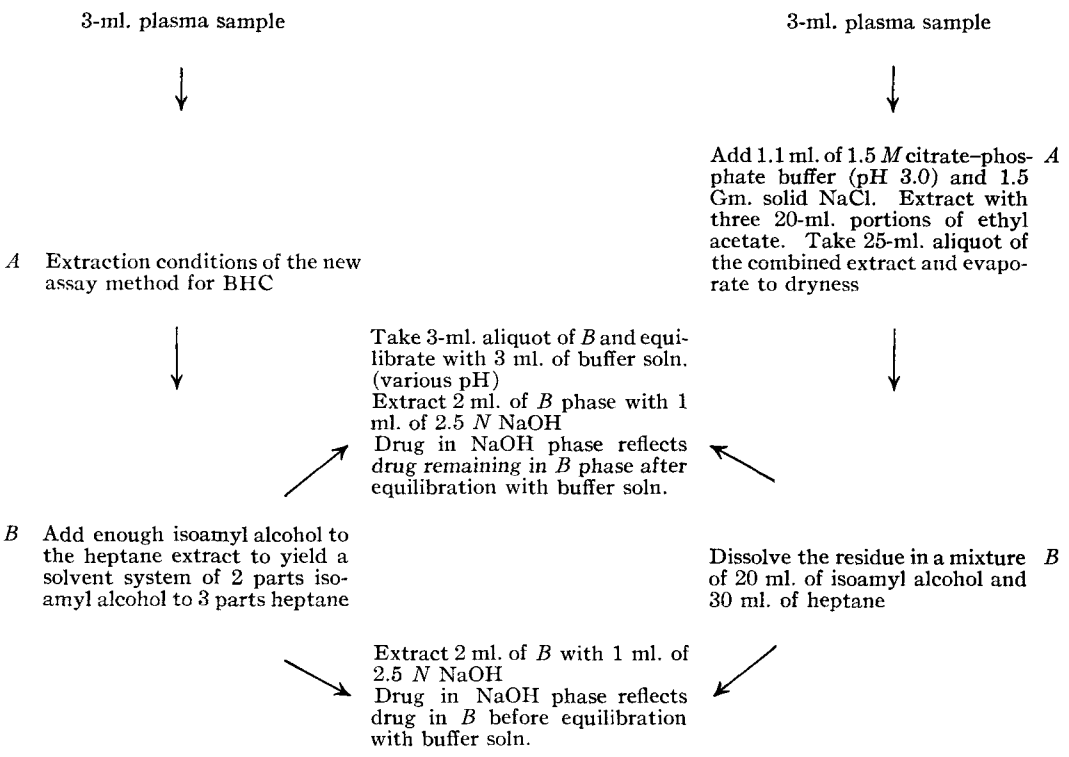

Fig. 1—Schematic diagram of the pH-partitioning procedure used to determine the specificity of the assay method for BHC. For further details see Experimental.

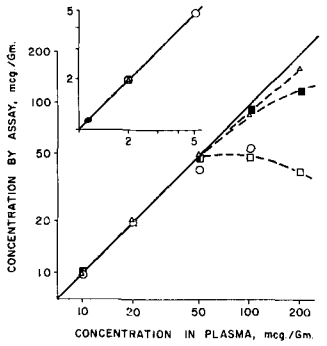

Fig. 2—Apparent concentration of BHC, as determined by the method of Axelrod et al., *plotted against the actual concentration of BHC in the plasma of rats* (■), *guinea pigs* (○), *dogs* (□), *and man* (△). *Average of at least 3 determinations for plasma from rat and guinea pig, and at least 2 determinations for plasma from dog and man. The inset represents the results obtained at concentrations lower than 10 mcg./Gm. In plasma from dog and man, the concentrations shown are expressed as mcg./ml. rather than mcg./Gm.*

TABLE I—RECOVERY OF BISHYDROXYCOUMARIN (BHC) FROM BLOOD PLASMA[a] OF SEVERAL HEALTHY HUMANS BY AXELROD'S METHOD

Subject[b]	Age, Yr.	Recovery, %[c]
A	25	63, 50
N	30	69, 64
K	35	86, 83
M	26	44. 49
R	26	57, 49
F	26	58, 44

[a] 200 mcg. of BHC/ml. of plasma. [b] All subjects were males. [c] Results of two separate analyses.

It can be seen that complete extraction of BHC is obtained only in a narrow pH range of pH 3.0 to 3.5. At pH <3 and >4.5 there is a precipitous decrease in the extractability of BHC from plasma. These effects are not due to specific buffers or differences in ionic strength. Complete extraction at pH 3 was obtained when the pH adjustment was made with either citrate–phosphate buffer or monochloroacetate buffer; a pronounced decrease in extraction efficiency was observed at pH 2.5 and below with monochloroacetate buffer as well as when hydrochloric acid was used to adjust pH. Similarly, though the ionic strength increased from 0.047 at pH 2.4 to 0.32 at pH 0.8 when HCl was used to adjust pH, the ionic strength decreased from 0.25 at pH 3.1 to 0.03 at pH 1.9 when monochloroacetate served as the buffer system. Nevertheless, the decrease in extraction efficiency at the lower pH was observed with both systems. It is possible that the magnitude of this decrease is affected by ionic strength. Variability of extraction efficiency in duplicate experiments at a given pH was very small

TABLE II—RECOVERY OF BHC FROM RAT PLASMA BY VARIOUS MODIFICATIONS OF AXELROD'S METHOD

Conditions	BHC Concn., mcg./ml. →	Recovery, mcg./ml.[a] 100	150	200
Axelrod's method, unmodified[b]		93	110	111
Thirteenfold dilution of plasma before extraction		100	121	118
Five repetitive extractions each with 3 ml. heptane		89	...	150
Single extraction with 3 ml. ethylene dichloride		85	...	106

[a] Average of 3 determinations except for the repetitive extractions where 2 determinations were made. [b] Additional data shown in Fig. 2.

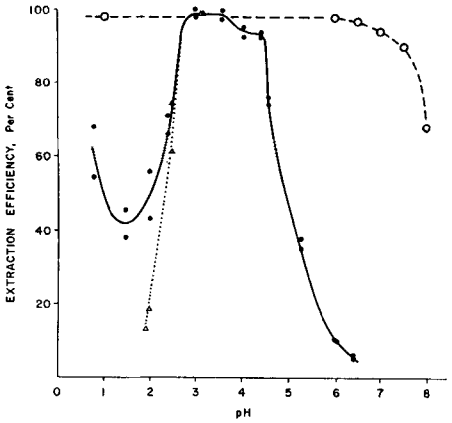

Fig. 3—Effect of pH on the efficiency of BHC extraction from rat plasma containing 200 mcg. of BHC/ml. Five-tenths milliliter of 2.5-fold diluted plasma was adjusted with 0.25–3 N HCl to pH <2.5 (●), with 1.5 M monochloroacetate buffers to pH ≤3.0 (△), and with 1.5 M citrate-phosphate buffers to pH ≥3.0 (●) prior to extraction with 7.5 volumes of n-*heptane. The concentration of buffers in the plasma phase was approximately 0.4 M. The data points represent individual values. Key: ---○---, BHC extraction efficiency from simple aqueous BHC solution as calculated from the partitioning data of Axelrod* et al. *(27).*

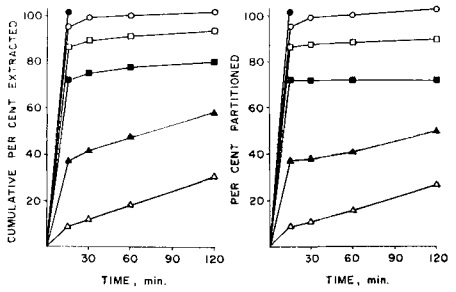

Fig. 4—Effect of pH and BHC concentration on kinetics of BHC extraction from rat plasma. Symbols are defined in Table III. (Further details under Experimental.)

in the pH range of 3.0 to 6.5, and rather pronounced at pH <2.5. This can be related to the kinetics of BHC extraction, as will be shown subsequently. The extraction efficiency *versus* pH profile of BHC from plasma differs appreciably from that of BHC from water (Fig. 3). The extraction of BHC from simple aqueous solution is practically complete at pH 6 and below.[9]

Kinetics of BHC Extraction from Plasma—The rate of BHC extraction from rat plasma was determined as a function of BHC concentration, pH of the plasma phase, and time interval between pH adjustment of the plasma and extraction with heptane. The various experimental conditions are listed in Table III and the results are shown in Fig. 4. In the case of plasma samples containing a low concentration (10 mcg./ml.) of BHC, complete extraction was obtained within 15 min., regardless of pH (<1 to 3.0) and time between pH adjustment and extraction. On the other hand, complete extraction of BHC from plasma containing 200 mcg./ml. was obtained within 15 min. only when the plasma pH was 3.0 and extraction was carried out immediately after pH adjustment. The increase in extraction of BHC with time, as shown in the lefthand graph of Fig. 4 for all other experiments involving 200 mcg. BHC/ml. plasma, could reflect either a slow rate of extraction of BHC or could be due to the fresh solvent which was added to replace that portion of organic phase which had been removed for assay (see under *Experimental*). A distinction between these two mechanisms can be made by expressing the amount of BHC in the heptane phase at any given time as a percent of the total amount of the drug in the system at that time (*i.e.*, sum of BHC in heptane phase and in plasma phase). This is shown in the righthand graph of Fig. 4 in terms of "percent partitioned" as a function of time.[10] At pH 4.9, the extraction of BHC from plasma containing 200 mcg./ml. was at its maximum within 15 min. and subsequent increases in the cumulative percent extraction as a function of time were due to addition of fresh organic solvent. The constancy of "percent partitioned" with time at a value of about 70% reflects the effect of plasma protein binding on the apparent partition coefficient of BHC at pH 4.9 since, in the absence of plasma proteins, extraction of BHC is complete within 15 min.

At low pH's (0.6 and 3.0) it is evident that the incomplete extraction of BHC at any given time

[9] The pKa_1 of BHC appears to be about 6.5 on the basis of Axelrod's partitioning data which have been verified in this laboratory. Other workers have reported a pKa_1 value of 5.7 based on potentiometric titration (31).

[10] The reader is cautioned to distinguish between "partition coefficient" (which is an equilibrium constant) and "percent partitioned." No assumption is made as to whether the latter reflects an equilibrium or a kinetic situation.

TABLE III—EXPERIMENTAL CONDITIONS FOR THE DETERMINATION OF THE KINETICS OF BHC EXTRACTION FROM PLASMA

Symbol in Fig. 4	BHC Concn., mcg./ml.	pH	Buffer System	Time Between pH Adjustment and Extraction, hr.
●	10	0.6	HCl 0.4 *N*	0 and 1
●	10	3.0	Citrate–phosphate, 0.4 *M*	0 and 1
●	200[a]	3.0	Citrate–phosphate, 0.4 *M*	0
○	200[a]	3.0	Citrate–phosphate, 0.4 *M*	1
□	200[a]	3.0	Citrate–phosphate, 0.4 *M*	3
■	200[a]	4.9	Citrate–phosphate, 0.4 *M*	0 and 1
■	200[a]	4.9	Acetate, 0.4 *M*	0 and 1
▲	200[a]	0.6	HCl, 0.4 *N*	0
△	200[a]	0.6	HCl, 0.4 *N*	0.5 and 1

[a] Diluted fourfold with distilled water (1 part plasma and 3 parts water) prior to pH adjustment.

is due to a kinetic effect. The rate of extraction decreased with decreasing pH, and decreased also at a given pH as the time interval between pH adjustment and extraction was increased. A detailed consideration of factors affecting the rate of extraction will not be undertaken here, but it should be pointed out that these factors include the time between pH adjustment and extraction, sampling interval, agitation intensity, volume of organic phase, and volumes of the aliquots removed for assay. In general, the time course of extraction consisted of an initial rapid phase followed by a slower exponential phase. Representative rate constants of the latter are 0.33 hr.$^{-1}$ at pH 3.0 after 3 hr. standing, and 0.14 hr.$^{-1}$ at pH 0.6 after 1 hr. standing. Thus the extraction efficiency *versus* pH profile shown in Fig. 3 reflects an equilibrium phenomenon at pH's above the optimum, and kinetic phenomena at pH's below the optimum pH for extraction. In fact, complete extraction of BHC may be attained at pH <3.0 by prolonged agitation. However, this would, for example, require more than 20 hr. in the case of plasma adjusted to pH 0.6 when the interval between pH adjustment and extraction is 1 hr. The adverse effect of low pH on BHC extraction from plasma is reversible as shown in Table IV.

New Assay Method for BHC in Plasma—The new assay method for BHC in plasma, which involves extraction of the drug with heptane from plasma adjusted to about pH 3.2, was used to determine the concentration of BHC in blood plasma obtained from rats, guinea pigs, dogs, monkeys, and man. These plasma samples were prepared by adding appropriate amounts of BHC to plasma obtained from unmedicated animals to yield BHC concentrations from 0.8 to 400 mcg./ml. The results, listed in Table V, show essentially complete recovery of the drug in all instances. The standard deviation of the analytical data was 1 to 2% except in the case of monkey plasma, where the possibility of a small and practically insignificant concentration-dependency of BHC recovery precluded the use of the combined data (*i.e.*, all concentrations of BHC) for the determination of a percent standard deviation.

TABLE IV—REVERSIBILITY OF ADVERSE pH EFFECT ON BHC EXTRACTION FROM RAT PLASMA

Conditions[a]	Extraction Efficiency,[b] %
Plasma adjusted to pH 3.0 and extracted immediately	99,99
Plasma adjusted to pH <1 and extracted after 30 min.	14,14
Plasma adjusted to pH <1 and extracted after 90 min.	10,12
Plasma at pH <1 for 30 min. then at pH 3.0 for 60 min.	61,70
Plasma at pH <1 for 30 min. and at pH ≃7 for 30 min. then adjusted to pH 3.0 and extracted immediately	99,101

[a] Plasma samples contained 200 mcg. BHC/ml. and were diluted 2.5-fold with distilled water prior to pH adjustment. [b] One ml. plasma phase was extracted for 10 min. with 8 ml. *n*-heptane. Results of two separate analyses.

Specificity of the New Assay Method for BHC—The specificity of the new assay method for BHC, particularly with respect to the lack of interference by biotransformation products of that drug, was investigated by means of the pH-partition profile (27). For this purpose, 20 mg. of BHC/Kg. of body weight was administered intraperitoneally to rats and guinea pigs, blood was withdrawn 7 to 8 hr. later [equivalent to about 1.5 biologic half-lives of BHC (28)] and the plasma thus obtained from animals of each species was pooled. The pH-partition profile for apparent BHC from rat plasma is shown in Fig. 5 and from guinea pig plasma in Fig. 6. There is excellent agreement between these profiles and that obtained by use of pure BHC in water. On the other hand, the use of an initial extraction procedure which is known to extract not only BHC but also some of its metabolites yields a quite different partition profile (stippled line in Figs. 5 and 6) and shows also that BHC metabolites are more polar than the parent drug. An even more rigorous determination of assay specificity was made by using plasma containing BHC which had been perfused for 3 hr. through an isolated rat liver (29). During the period of perfusion, the BHC concentration in the plasma decreased to less than one-third of its initial concentration. Thus, this sample is likely to have contained relatively high concentrations of BHC metabolites (except for the unlikely possibility that these metabolites were trapped totally in the liver and/or excreted completely in the bile).[11] The results of the partitioning experiment are shown in

[11] Christensen (7) has shown that the plasma of rats contains appreciable concentrations of BHC metabolites 6 and 24 hr. after intravenous administration of BHC.

TABLE V—RECOVERY OF BHC FROM PLASMA OF VARIOUS ANIMAL SPECIES BY THE NEW ASSAY METHOD

BHC Concn., mcg./ml.	Recovery, % Rat[a]	Guinea Pig[a]	Dog[b]	Monkey[a]	Man[a]
0.8	...	...	106	...	...
2	103	100	...	101	103
4.1	...	...	98	...	...
5	...	...	...	...	99
8	97	...	...	...	...
10	...	102	...	98	98
25	100	...	...	...	...
31.2	...	...	97	...	...
50	98	101	...	...	100
56.3	...	...	98	...	...
100	101	100	...	97	99
121.5	...	...	96	...	...
150	...	...	...	96	...
200	99	...	...	...	98
400	...	100	...	...	...
Mean ± S.D.[c]	99 ± 2[d]	101 ± 1[d]	97 ± 1[e]	[f]	99 ± 2
Total No. of samples	19	8	4	8	12

[a] Average of 2–4 determinations. [b] Single determinations. [c] Mean and S.D. of individual values irrespective of concentration. [d] Data from the 2 mcg./ml. samples were excluded due to pronounced variations within group (3 samples). [e] The value for the 0.8 mcg./ml. sample was excluded due to pronounced difference from those for the other concentrations. [f] Not calculated because relative recovery appears to be concentration dependent.

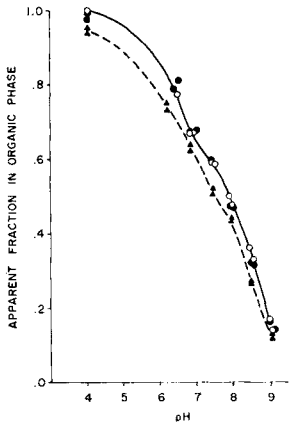

Fig. 5—Specificity of the new assay method for BHC in rat plasma, as determined by pH-partitioning. The plasma obtained from two rats 7 hr. after an i.p. dose of 20 mg. of BHC/Kg. was subjected to the new assay method (heptane extraction at pH 3) and to ethyl acetate extraction, and the pH-partition profiles of the substance(s) in each extract were determined. (See under Experimental *for details.) Pure BHC added directly to the organic phase used in the partitioning experiment* (○), *heptane extract (new assay method)* (●), *ethyl acetate extract* (▲).

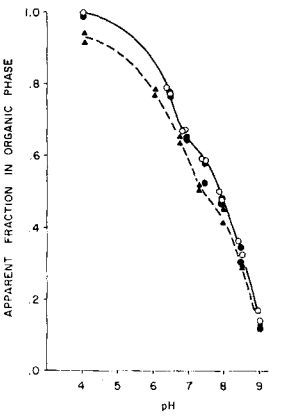

Fig. 6—Specificity of the new assay method for BHC in guinea pig plasma, as determined by pH-partitioning. The plasma was obtained from two guinea pigs 8 hr after an i.p. dose of 20 mg. BHC/Kg. (See Fig. 5 for other details.)

Table VI and indicate a very good agreement between results obtained with the plasma and with an aqueous solution of BHC. There was no noticeable interference by BHC metabolites in the assay.

DISCUSSION

Evaluation of the Method of Axelrod et al. (27) for the Determination of BHC in Plasma—The recovery of BHC from the plasma of rats, dogs, and man by the method of Axelrod *et al.* (27) is essentially quantitative at BHC concentrations up to 50 mcg./ml., but becomes incomplete and markedly concentration-dependent at BHC concentrations exceeding 50 mcg./ml. (Fig. 2). In guinea pig plasma, the recovery of added BHC was quantitative at 10 mcg./ml. but already incomplete at 50 mcg./ml. These results are not due to a limited solubility of BHC in *n*-heptane, nor to the precipitation of BHC in the acidified aqueous phase, since extraction of BHC from aqueous solution does not show the concentration dependence found with plasma. In addition to its incompleteness, the recovery of BHC in high concentrations from plasma shows poor reproducibility and appears to vary in

TABLE VI—SPECIFICITY OF THE NEW ASSAY METHOD FOR BHC AS TESTED BY pH-PARTITIONING USING PLASMA CONTAINING BHC AND METABOLITES DERIVED FROM PERFUSION OF ISOLATED RAT LIVER

	Fraction in Organic Phase	
pH	From Aqueous Soln. of BHC	From Plasma Containing BHC and Metabolites
4.2	0.99	0.99
6.4	0.79	0.76
6.8	0.68	0.70
7.4	0.58	0.56

the plasma samples obtained from different subjects (Table I).

The incomplete recovery of BHC from plasma at high concentrations can easily be overlooked. While it is standard practice to ascertain the adequacy of an analytical method for a drug in plasma at concentrations in the range suitable for instrumental analysis, it is usually taken for granted that plasma samples containing higher concentrations of drug may be diluted appropriately and that they can then be analyzed as readily as plasma samples containing lower concentrations of drug. Such is not the case with BHC (Table II). Apparently the extractability of this drug from plasma depends more on the BHC:plasma protein concentration ratio than on the absolute concentration of BHC. This phenomenon has some interesting implications and can lead to unusual artifacts resulting in the possibility of serious errors in pharmacokinetic interpretations. This is illustrated in the results obtained after intravenous administration of BHC in doses of 2 mg./Kg. and 20 mg./Kg. to guinea pigs, where the plasma concentrations of BHC were determined either by the method of Axelrod *et al.* (27) or by the new method which assures complete recovery of the drug (Figs. 7 and 8). The plasma concentrations determined by the method of Axelrod *et al.*, when plotted semilogarithmically, show a downward curvature with time. When plotted on linear coordinates, the data obtained with the 20 mg./Kg. dose yield essentially a straight line. It might be concluded, therefore, that the elimination of BHC is an apparent zero-order process, as had been suggested previously (12). [Note, however, that this hypothesis was rejected upon further investigation (17).] However, it is clearly evident from the results obtained with the new assay method (Fig. 8) that such conclusions are the result of an artifact due to the decreasing extraction efficiency of the method of Axelrod *et al.* at higher BHC concentrations.[12] In fact, BHC elimination follows apparent first-order kinetics in the guinea pig at doses of 2 and 20 mg./Kg. (Fig. 8). These observations led to a re-examination of published data on BHC elimination with the purpose of determining if pharmacokinetic analyses of others,

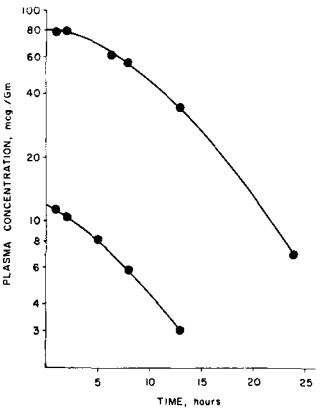

Fig. 7—Apparent plasma concentrations of BHC, as determined by the method of Axelrod et al., *in a guinea pig receiving intravenous doses of 2 mg./Kg. (lower curve) and 20 mg./Kg. (upper curve) of the drug. One-week interval between tests.*

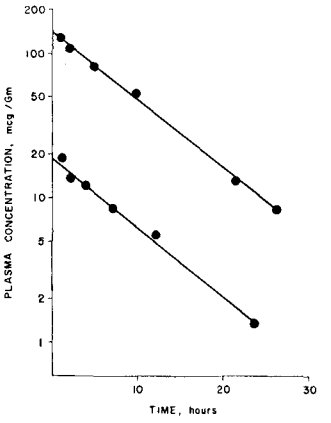

Fig. 8—Plasma concentrations of BHC, as determined by the new assay method, in a guinea pig receiving intravenous doses of 2 mg./Kg. (lower curve) and 20 mg./Kg. (upper curve) of the drug. One-week interval between tests.

and particularly the reported dose dependence of BHC elimination in man (12, 15), could be explained by the assay problems described in this report. It appears that this possibility can be excluded since published data are in a concentration range where assay problems are unlikely. Such problems will be encountered mainly in pharmacokinetic investigations with rather high doses of BHC (28, 29).

The Effect of pH on BHC Extraction from Plasma—The problems encountered in the determination of BHC in plasma by the method of Axelrod *et al.* led to an examination of the effect of pH on the extraction of BHC from plasma. It was found that complete extraction of the drug from plasma containing high concentrations of BHC was achieved only in a relatively narrow pH range of 3.0 to 3.5 (Fig. 3). The lower recovery in the pH range of 4 to 6 appears to be primarily due to a decreased apparent partition coefficient of BHC as a consequence of protein binding (Fig. 4). At higher pH

[12] The apparently incomplete recovery of BHC from plasma by the method of Axelrod *et al.* following the 2 mg./Kg. dose (Fig. 7) might appear to be quantitatively inconsistent with the recovery data shown in Fig. 2. However, this is due to the pronounced variability of BHC recovery at low pH as shown in Fig. 3. When, in another animal, BHC plasma levels were determined simultaneously by the method of Axelrod *et al.* and by our new method, similar differences in results were obtained, but the data derived from the Axelrod assay were not describable by either first- or zero-order kinetics.

the decrease in extraction efficiency is due to ionization of BHC (Fig. 3). The decreased extraction efficiency at pH's below 3 is a rate, rather than an equilibrium, phenomenon (Fig. 4 and Table IV). Quantitative extraction of BHC at pH <1 from plasma containing high concentrations of this drug would require almost 1 day. The slow rate of extraction at the low pH leads to poor reproducibility of results obtained by procedures involving a short extraction period and yielding incomplete recoveries (Fig. 3). This is probably why the data listed in Table I show poor reproducibility. While there apparently has not been a systematic study of the effect of pH on the extraction of BHC from plasma and other biologic tissues to date, it is of interest that Christensen (40) reported recently that the recovery of BHC from rat liver and muscle tissue was more complete at pH 4 and 5 than at pH <2 and >7. This is reasonably consistent with the findings of this study. The results described here may also have some relation to observations of others that the gastrointestinal absorption of BHC is very slow and prolonged in man, particularly at higher doses (12, 15). For example, O'Reilly *et al.* (15) noted that absorption of orally administered BHC in man occurred over about 65 hr. with a dose of 300 mg., 95 hr. with 600 mg., and 210 hr. after administration of 1200 mg. While the dissolution rate of BHC in gastrointestinal fluid will be very low due to the poor water solubility of the drug, it appears unlikely that undissolved particles could be retained in the gastrointestinal tract for a week or longer. What appears to be an unusually slow gastrointestinal absorption of BHC, when calculated by means of a single-compartment pharmacokinetic model, could actually be an unusual distribution phenomenon requiring the use of a multiple-compartment model. However, the pronounced affinity of BHC at high concentrations and low pH to plasma proteins, as observed in this study, suggests the possibility of a similar phenomenon in the gastrointestinal tract. BHC might be bound in the gastrointestinal mucosa for a prolonged period of time, as has been suggested by O'Reilly *et al.* (15). This could be the case particularly if the pH optimum for binding of BHC to mucosal protein is in the physiologic range.

Factors Affecting Drug–Albumin Interactions—BHC in the plasma is bound mainly, if not exclusively, to albumin (12, 15). An interpretation of the results obtained in this study requires, therefore, a consideration of factors affecting the interaction of drugs with plasma albumin. This in turn requires a consideration of the effect of pH on the conformation and physical-chemical properties of albumin. Plasma albumin undergoes a largely reversible structural alteration at pH $\simeq$4 (32). This involves a structural expansion at the low pH which is driven by electrostatic forces and which leads to formation of a species with greater electrophoretic mobility and higher viscosity (33). This expanded form of albumin is known as the F form as opposed to the more compact N form at neutral pH. A similar electrostatic expansion of albumin occurs above pH 9 (32). The N to F transition of albumin is accompanied by a pronounced decrease in the ability for hydrophobic binding of substances such as alkanes (33). It is believed that apolar binding sites are located in the interior of the albumin molecule and that they are therefore not in contact with polar solvent. N to F transition is thought to cause disruption of apolar clusters in the albumin molecule, resulting in the exposure of these regions to the aqueous solvent. This is accompanied by a drastic decrease in the capacity to bind alkanes such as butane and pentane (33) and drugs such as the xanthines (34). The N–F equilibrium is affected by ionic strength and specific counter ions and by interacting substances such as detergents (32, 35) and organic solvents used in extractions, including heptane (36). Detailed physical-chemical studies of bovine and human plasma albumin suggest that the N to F change actually involves a double transition, one at pH 3.6 and the other at pH 3.0 (39). Significantly, this coincides with the region of optimum pH for extraction of BHC from plasma (Fig. 3). It is evident, therefore, that the high extraction efficiency of BHC at pH's slightly below 4 as compared to higher pH's is due to the N to F transition of albumin at pH $\simeq$ 4 and the decreased binding of neutral molecules such as BHC by the F form of albumin. At pH 2.5, the positive charge on the protein is at its maximum and further lowering of pH appears to reduce the expansion of albumin molecules due to the increased ionic strength of the environment (37). There is evidence for slow aggregation of albumin below pH 3.2 (32), reflected among others by a slow increase in the viscosity of albumin solutions (38). The viscosity increase proceeds more rapidly at lower pH (38). The reversibility of this aggregation decreases with increased duration of exposure of albumin to low pH (32). The decreased extraction efficiency below pH 3 is apparently due to the conformational change of albumin at the low pH involving partial refolding of the expanded molecule (37) and/or aggregation (32). The decreased extractability of BHC with time at low pH (Fig. 4) is consistent with the relatively slow structural change of albumin at low pH. Specifically, the more rapid decrease in extractability of BHC at pH <1 as compared to pH 3 (Fig. 4) is paralleled by the more rapid increase in viscosity of albumin solutions at the lower as compared to the higher pH's (38). These effects are reversible by pH adjustment, as is evident from the data in Table IV.

Last, an explanation must be sought for the unusual concentration dependence of BHC extraction at low pH. O'Reilly (24), who studied the interaction of warfarin with human plasma albumin, concluded that the albumin molecule undergoes a reversible configurational alteration in the process of binding the drug, thereby yielding additional binding sites. It is likely that the same is true for BHC in view of its structural similarity to warfarin. One may then conceive of a series of events whereby BHC in low concentrations is bound to readily accessible peripheral binding sites on the albumin molecule, with increasing concentrations of BHC bringing about a partial unfolding of the albumin molecule and thereby causing the exposure of additional binding sites in the internal region of the macromolecule. If, during extraction at low pH, the peripherally bound BHC dissociates first, it is conceivable that the albumin molecules will contract and aggregate, and thus make it difficult for BHC molecules bound to internal sites in the albumin molecule to be extracted. Foster has recognized

the possibility of such a mechanism by suggesting that "A variety of substances, both ionic and nonionic, but particularly substances with considerable hydrophobic character, could become trapped within the postulated hydrophobic interfaces and be released only when the protein reaches an ionic environment favoring conversion to the F form" (32). In summary, BHC in high concentrations can be extracted readily from plasma only in a pH range where the interaction between albumin molecules (*i.e.*, aggregation) is minimal and where these molecules are maximally expanded.

New Assay Method for BHC in Plasma—The method of Axelrod *et al.* has been modified by changing the pH adjustment of the plasma from less than 1 to about 3.2 to assure maximum extraction efficiency. Essentially quantitative recovery of BHC in concentrations ranging from 2 to 400 mcg./ml. was obtained with plasma from rats, guinea pigs, dogs, monkeys, and man (Table V), and this assay method has been used successfully in a comparative pharmacokinetic study of BHC elimination in these species (28). The specificity of the new method, particularly in terms of distinguishing between BHC and its biotransformation products, has been demonstrated (Figs. 5 and 6; Table VI).

Conclusions—The findings of this study go clearly beyond the discovery of an unusual concentration dependence of a widely used method for the determination of BHC in plasma, the identification of the consequent possibility of interesting pharmacokinetic artifacts, and the development of a new assay method which is not subject to these shortcomings. Guttman and Gadzala (34), who studied the interaction of xanthine derivatives with bovine serum albumin, considered as their most interesting observation the sensitivity of the binding behavior of albumin to pH-dependent changes in its structure. This observation can now be extended to include the sensitivity in the extractability of a strongly albumin-bound drug to pH-dependent conformational changes of the albumin molecule. It is not unlikely that similar effects will be encountered with other strongly protein-bound drugs. The optimum pH for extraction of such drugs from plasma or other biologic materials may depend not solely on the pKa of the drug (as it affects the distribution of the drug between an organic phase and an aqueous phase of given pH), but also on the effect of pH on the physical-chemical properties of plasma proteins as they affect the type and magnitude of interaction of these proteins with the drug. The pH-extraction efficiency profile may be affected in some instances also by ionic strength and by the nature of the buffer system and organic solvent used in the extraction.

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Keyphrases

Coumarin anticoagulants
Bishydroxycoumarin in plasma—analysis
n-Heptane extraction
UV spectrophotometry—analysis
pH effect on extraction efficiency
Kinetics of extraction of bishydroxycoumarin from plasma
Albumin—bishydroxycoumarin interaction

Comparative Pharmacokinetics of Coumarin Anticoagulants II

Pharmacokinetics of Bishydroxycoumarin Elimination in the Rat, Guinea Pig, Dog, and Rhesus Monkey

By RENPEI NAGASHIMA, GERHARD LEVY*, and NATHAN BACK†

Low (1–2 mg./Kg.) and high (8–20 mg./Kg.) doses of bishydroxycoumarin (BHC) were administered intravenously in cross-over fashion to rats (Sprague-Dawley, male), guinea pigs (Hartley, male), dogs (beagle and mongrel, male and female), and monkeys (rhesus, male). BHC levels in the plasma declined exponentially with time in each species. The size of the dose had no significant effect on half-life in rats and guinea pigs. The half-life was variable in the dog and increased with increasing dose in the monkey. There was no pronounced change in the apparent volumes of distribution of BHC with dose in any of the animal species studied. The rat, guinea pig, and monkey eliminate BHC considerably more rapidly, while the dog eliminates the drug more slowly than man.

THE PHARMACOKINETICS of bishydroxycoumarin (BHC) in man are unusual in that the biologic half-life of the drug increases markedly with increasing dose, although plasma concentrations appear to decline exponentially at all dose levels (1, 2). It also has been suggested that the apparent volume of distribution of BHC in man decreases with increasing dose (2). These pharmacokinetic characteristics are encountered only infrequently, and a detailed study of the mechanism responsible for them is highly desirable. In view of the potential hazards associated with the use of BHC in man a search was instituted for a suitable animal species showing the same characteristics of BHC distribution and elimination as man. While there have been some studies of BHC elimination kinetics in the mouse (3–5), rat (6–8), rabbit (3–5, 9), and dog (4, 10), these investigations were carried out at only a single dose level of BHC and, therefore, provide no information concerning a possible dose dependency in the distribution and elimination kinetics of this drug. The distribution and elimination of BHC at several dose levels in rats, guinea pigs, dogs, and monkeys have been studied in this laboratory, and the results of this investigation are described in the present communication.

EXPERIMENTAL

Bishydroxycoumarin Solution for Injection—Bishydroxycoumarin was purchased from Nutritional Biochemicals Co., Cleveland, Ohio. One part by weight of the drug and 10 parts by weight of tris(hydroxymethylamino) methane (Nutritional Biochemicals Co.) were dissolved in a sufficient volume of freshly distilled water to yield a 0.5 or 1.0% (w/v) solution of BHC. The pH of the solutions was 9.2–9.3. They were stored in a refrigerator and were used within 12 hr. after preparation.

Animals—The following is a list of animal species, strain or its equivalent, sex, body weight, and the diet fed before and during the experiments: rat, Sprague-Dawley, male, 400–500 Gm., Big Red rat mouse diet;[1] guinea pig, Hartley, male, 400–500 Gm., Big Red guinea pig diet;[1] dog, mongrel male or female or beagle male, 10–25 Kg., Big Red dog foods expanded;[1] monkey, rhesus, male, 5–7 Kg., primate diet expanded.[2] The animals had unrestricted access to water and food before and during the experiments.

Drug Administration and Collection of Plasma Samples—The drug was administered intravenously in the morning and blood samples were withdrawn at appropriate intervals. Rats and guinea pigs were placed under light ether anesthesia, and blood was collected into a heparinized centrifuge tube[3] by retro-orbital puncture using a glass capillary tube (1.6–1.8 mm. o.d.). Blood withdrawal from the cephalic vein of dogs and the saphenous vein of monkeys was done without anesthesia, using a heparinized syringe.[4] After centrifugation of the blood at 1800 × g for 10 min., the plasma was separated and stored at −15° until assayed. Blank plasma was obtained from each animal immediately before every experiment. The volume of blood withdrawn from rats and guinea pigs did not exceed 0.6 ml. per sample and 3.0 ml. in 24 hr. Two-milliliter blood samples were obtained from dogs and monkeys.

Assay Method—BHC in the plasma was determined by a modification of the method of Axelrod *et al.* (11). The assay consists essentially of adjustment of plasma to pH 3.2, extraction with heptane, transfer of the drug into 2.5 *N* NaOH, and spectrophotometry at 314 mμ. BHC metabolites in the plasma do not interfere in the assay (12).

Determination of Apparent Volume of Distribution and Biologic Half-Life—The apparent volume of distribution (V_d) was calculated by dividing the intravenous dose by the apparent initial concentration of BHC in the plasma. The latter was determined by extrapolating the linear portion of a log BHC concentration *versus* time plot to zero time. The biologic half-life ($t_{1/2}$) was determined graphically from the linear portion of the semilogarithmic plot. The small volume of the plasma samples

Received April 6, 1967, from the Biopharmaceutics Laboratory, Department of Pharmaceutics, and the † Department of Biochemical Pharmacology, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication June 12, 1967.

Previous paper: Nagashima, R., Levy, G., and Nelson, E., *J. Pharm. Sci.*, **57**, 58(1968).

The competent technical assistance of Mr. R. P. Zendzian is acknowledged.

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[1] Country Best Foods, Agway, Inc., Syracuse, N.Y.

[2] Teklad, Inc., Monmouth, Ill.

[3] Prepared by placing 0.01 ml. sodium heparin solution (1000 units/ml., Upjohn Co.) in a centrifuge tube and allowing it to dry at room temperature overnight.

[4] Syringes were rinsed with sodium heparin solution (1000 units/ml.) and allowed to dry at room temperature overnight.

obtained from rats and guinea pigs made it necessary to use weighed rather than measured aliquots for analysis. Consequently, plasma concentration and V_d values for rats and guinea pigs are expressed in terms of mcg./Gm. and Gm./Kg., respectively. The specific gravity of the plasma averaged about 1.02.

RESULTS AND DISCUSSION

Figure 1 illustrates the elimination of BHC from the plasma in rats. Plasma concentrations of BHC declined exponentially and there was no pronounced difference in the apparent volume of distribution of BHC at the two dose levels. Unlike man, the rat did not show an increase in the biologic half-life of BHC with increasing dose.

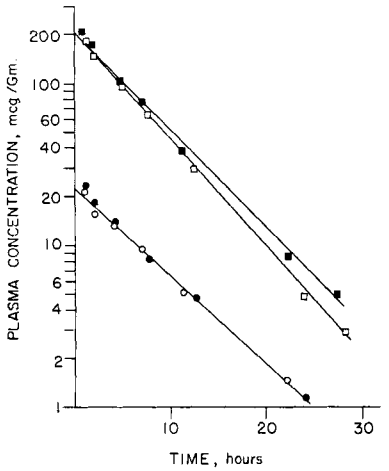

Fig. 1—Plasma concentration of bishydroxycoumarin (BHC) as a function of time after intravenous administration in rats. The dates listed in this and the other figure legends refer to the time of drug administration. Rat 1: □, 8-11-65, 20 mg./Kg.; ○, 8-4-65, 2 mg./Kg. Rat 2: ■, 8-4-65, 20 mg./Kg.; ●, 8-11-65, 2 mg./Kg.

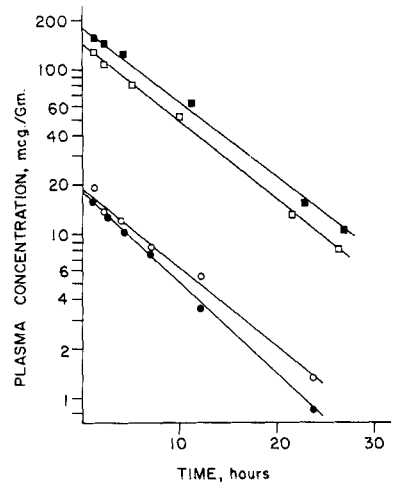

Fig. 2—Plasma concentration of BHC as a function of time in guinea pigs. Guinea pig 1: □, 8-18-65, 20 mg./Kg.; ○, 8-11-65, 2 mg./Kg. Guinea pig 2: ■, 8-11-65, 20 mg./Kg.; ●, 8-18-65, 2 mg./Kg.

TABLE I—PHARMACOKINETIC CONSTANTS FOR BISHYDROXYCOUMARIN ELIMINATION FROM PLASMA IN RATS AND GUINEA PIGS[a]

Animal No.	Body Wt., Gm.	Date Dosed	Dose, mg./Kg.[b]	$t_{1/2}$, hr.	V_d, Gm./Kg.[c]
			Rat		
1	470	8/4	2	5.6	89
	470	8/11	20	4.6	98
2	440	8/4	20	5.0	98
	430	8/11	2	5.6	89
			Guinea Pig		
1	450	8/11	2	6.3	106
	450	8/18	20	6.4	140
2	480	8/11	20	6.7	112
	500	8/18	2	5.5	111

[a] Rat, Sprague-Dawley, males; guinea pig, Hartley, males. [b] The drug was administered intravenously. [c] Apparent volume of distribution.

The elimination of BHC in the guinea pig followed a pattern similar to that found in the rat (Fig. 2). Drug elimination was exponential with the half-life slightly longer than in the rat. There was no apparent change in the half-life of BHC with increasing dose. The individual pharmacokinetic constants for BHC elimination in the rats and guinea pigs are listed in Table I.

The results of the study of BHC elimination in dogs are shown in Figs. 3, 4, and 5, and in Table II. The elimination followed apparent first-order kinetics, except for a small deviation from day 3 to day 7 after administration of the higher dose in dog *2*. The reason for this deviation is not known. There were appreciable differences in the plasma half-life of BHC between dogs. It appears that the half-life increased with increasing dose, but a definitive conclusion with respect to this apparent dose-dependency is not possible because of the occasional and unexplained pronounced changes in the half-life observed in dogs *4* and *5* (stippled lines in Figs. 4 and 5). These changes could not be ascribed to previous drug administration. There was no significant change in the apparent volume of distribution of BHC as a function of dose (Table II). Initial intensive blood sampling in one dog (No. *1*) during the first 2 hr. after BHC administration yielded an

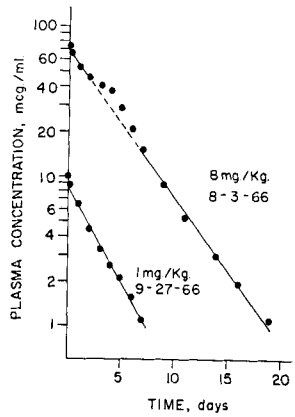

Fig. 3—Plasma concentration of BHC as a function of time in a female mongrel dog (No. 2) which received a high and a low intravenous dose of the drug.

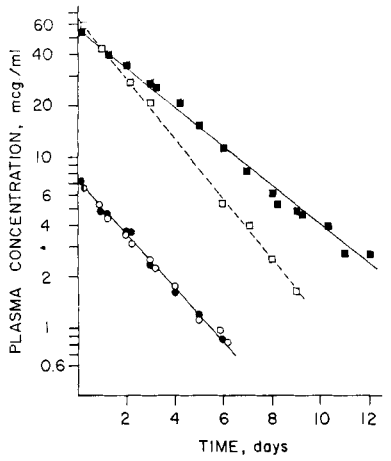

Fig. 4—Plasma concentration of BHC as a function of time in a male beagle dog (No. 4) which received two low and two high intravenous doses of the drug. Key: ●, 11-1-66, 1 mg./Kg.; ■, 11-29-66, 8 mg./Kg.; ○, 1-17-67, 1 mg./Kg.; □, 2-14-67, 8 mg./Kg.

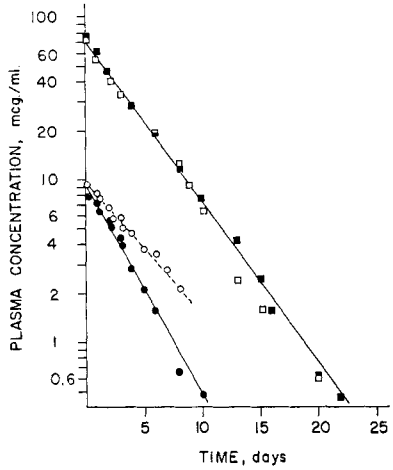

Fig. 5—Plasma concentration of BHC as a function of time in a male beagle dog (No. 5) which received two low and two high intravenous doses of the drug. Key: ○, 11-15-66, 1 mg./Kg.; ■, 12-13-66, 8 mg./Kg.; ●, 1-17-67, 1 mg./Kg.; □, 2-14-67, 8 mg./Kg.

apparent first-order distribution rate constant of 1.1 hr.$^{-1}$. Dog *3* expired due to massive internal hemorrhage on the 9th day after receiving a 10 mg./Kg. dose of BHC which had been administered 54 days after a 1 mg./Kg. dose. It is of interest that this dog exhibited the longest half-life for BHC (130 hr. at a 1 mg./Kg. dose) observed in all of the experiments (Table II).[5] Dog *1* had a considerably larger apparent volume of distribution for BHC than any of the other dogs; unfortunately, this animal was lost for reasons unrelated to BHC administration before additional experiments could be carried out.

The elimination of BHC by the monkeys showed consistent dose-dependency in that half-life increased with increasing dose (Figs. 6 and 7, Table II). There was no significant change in the apparent volume of distribution as a function of dose. An initial downward curvature in the elimination of BHC observed in monkey *1* after the first 1 mg./Kg. dose prompted a repetition of this experiment about 1 month later. Exactly the same phenomenon was observed in the second experiment. While a single occurrence of this type might be ascribed to an accidental extravascular injection of part of the dose, the exact reproduction of the plasma level curve in

[5] The $t_{1/2}$ and V_d for the 10 mg./Kg. dose were essentially the same as for the 1 mg./Kg. dose based on data collected up to the time of death.

TABLE II—PHARMACOKINETIC CONSTANTS FOR BISHYDROXYCOUMARIN ELIMINATION FROM PLASMA IN DOGS AND MONKEYS[a]

Animal No.	Body Wt., Kg.	Date Dosed	Dose, mg./Kg.[b]	$t_{1/2}$, hr.	V_d, ml./Kg.[c]
			Dog		
1	11.8	7/13	2	47	210
2	10.0	8/3	8	72	114
	10.9	9/27	1	57	118
3	23.9	8/4	1	130	106
4	10.0	11/1	1	45	133
	10.9	11/29	8	62.5	144
	12.7	1/17	1	45	133
	13.6	2/14	8	41	123
5	12.5	11/15	1	88	103
	12.5	12/13	8	72	113
	11.8	1/17	1	57	109
	12.0	2/14	8	72	113
			Monkey		
1	5.0	11/1	1	6.2	70
	5.0	11/15	4	7.4	81
	5.0	12/6	1	6.2	70
	5.0	12/13	10	10.0	76
2	6.4	12/28	1	5.5	83
	6.4	1/4	10	8.0	85
	6.4	1/17	1	5.5	83

[a] All the animals used were male except for dog *2*. Dogs *1* to *3*, mongrel; dogs *4* and *5*, beagle; monkeys, rhesus. [b] The drug was administered intravenously. [c] Apparent volume of distribution.

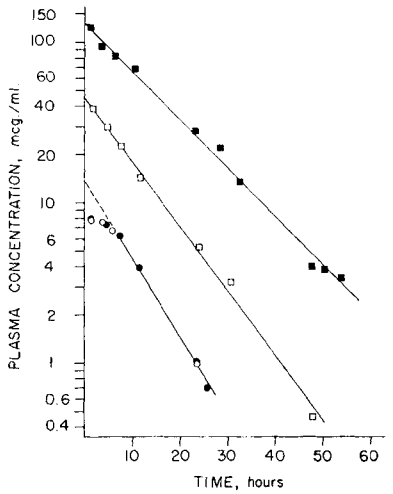

Fig. 6—Plasma concentration of BHC as a function of time in a male rhesus monkey (No. 1) which received one high, one intermediate, and two low intravenous doses of BHC. Key: ○, 11-1-66, 1 mg./Kg.; □, 11-15-66, 4 mg./Kg.; ●, 12-6-66, 1 mg./Kg.; ■, 12-13-66, 10 mg./Kg.

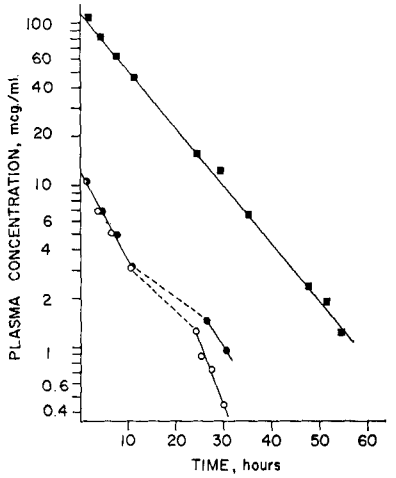

Fig. 7—Plasma concentration of BHC as a function of time in a male rhesus monkey (No. 2) which received one high and two low intravenous doses of BHC. Key: ●, 12-28-66, 1 mg./Kg.; ■, 1-4-67, 10 mg./Kg.; ○, 1-17-67, 1 mg./Kg.

TABLE III—AVERAGE PHARMACOKINETIC CONSTANTS FOR BISHYDROXYCOUMARIN ELIMINATION FROM PLASMA IN VARIOUS ANIMAL SPECIES

Species	Dose, mg./Kg.	Av. $t_{1/2}$, hr.	Av. Vd, ml./Kg.
Rat	2	5.6	89[f]
	20	4.8	98[f]
Guinea pig	2	5.9	109[f]
	20	6.6	126[f]
Dog[a]	1	58	119
	8	64	121
Monkey	1	5.9	77
	10	9.0	81
Man[b]	2.1	10	200
	8.6	32	130
Man[c]	2.0	23.6 ± 5.8	...
Man[d]	100 mg. total dose	22 ± 6	...
	150 mg. total dose	32 ± 7	...
Man[e]	5.0	40 (18–190)	~140

[a] Data on dogs *1* and *3* were excluded. [b] Data on one subject from O'Reilly *et al.* (2) after intravenous administration. [c] From Motulsky (15) after oral administration. [d] From Schrogie and Solomon (16) after oral administration. [e] From Weiner *et al.* (1) after intravenous administration. [f] Gm./Kg. (≃ml./Kg.).

the subsequent experiment makes this extremely unlikely.

Monkey *2* showed an interesting and reproducible discontinuity in the plasma concentration decline of BHC after administration of the lower dose (Fig. 7). Again, no explanation can be offered for this phenomenon other than to suggest the possibility of a postural effect, such as described recently by Levy (13). Diurnal variations were observed occasionally also in the dogs (see Fig. 4), although the long half-life of BHC in the latter makes these variations difficult to recognize. The value of intensive sampling and repetitive experiments is evident from the results of this study in that they illustrate the occurrence of interesting and apparently reproducible changes in individual elimination kinetics. A search for the mechanisms of such changes may lead to a better understanding of the effect of certain physiologic variables on drug elimination.

The findings of this study are summarized in Table III. The rat, guinea pig, and monkey showed similar half-lives for BHC elimination. The half-life of BHC in the dog is considerably longer. This is consistent with the previously observed unusually long half-life of another coumarin anticoagulant, biscoumacetate, in the dog (14). The limited number of animals studied in each group does not permit recognition of possible genetic or strain differences; the possibility of such effects should be recognized, particularly since a pronounced genetic effect has been noted in the rabbit (4, 5). Of the four species studied, only the rhesus monkey showed definite evidence of a dose dependency in the elimination kinetics of BHC, similar to that found in man. Differences in apparent volume of distribution as a function of dose were relatively minor in each species. In particular, there was no indication of a decreased volume of distribution for BHC with increasing dose, as reported for man (2). The possible reasons for this apparent difference are presently being studied.

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Keyphrases

Coumarin anticoagulants
Bishydroxycoumarin determination in plasma
Heptane extraction
UV analysis
Biologic half-life of bishydroxycoumarin
Volume of distribution—apparent
Pharmacokinetics of bishydroxycoumarin elimination

In Vitro Action of Mescaline

Possible Mode of Action

By EMMETT CLEMENTE and VINCENT DE PAUL LYNCH

Mescaline (TMPEA) elicited a motor response (dependent upon segment and concentration) from isolated guinea pig, rat, and cat ileum. It did not alter inotropic or chronotropic activity of isolated mammalian atria. Estrogen dominated uteri responded similarly. TMPEA antagonized serotonin-induced motor responses of rat and guinea pig uteri. High concentrations of ganglionic blocking agents potentiated TMPEA motor effects. Evidence suggests that TMPEA does not act *via* a cholinergic mechanism, but rather through catecholamine mechanisms. The end result varies in definite situations. The primary site of action of TMPEA appears to be at the α-adrenergic receptor site.

THE LITERATURE contains few reports concerning the effects of mescaline (trimethoxyphenylethylamine: TMPEA) on peripheral systems. This is especially true of investigations involving *in vitro* systems. Most of the available data seem to have been compiled from studies in which TMPEA was compared to other psychotomimetic compounds, rather than from research into the effects of TMPEA *per se.*

It was observed by Grace (1) that duodenal preparations from the cat and rabbit, as well as uteri obtained from the rat, rabbit, and guinea pig, did not respond to TMPEA. On the other hand, that investigator reported that TMPEA produced powerful contractions in the rabbit intestine *in situ* and that this effect could be abolished by intravenously administered atropine. Subsequently, Costa (2) demonstrated in *in vitro* studies that atropine did not block the effect of TMPEA on rat uteri. Costa (2) also reported that very low concentrations of lysergic acid diethylamide (LSD) and TMPEA synergized the effects of serotonin on uterine tissue from estrogen-primed rats.

There appears to be a consensus that TMPEA does not antagonize the response of rat uteri to serotonin. However, a number of investigators have reported the antiserotonin activity of LSD (2–5).

Serotonin produces contraction of umbilical vessels of human placenta, and TMPEA has been shown to cause constriction of the same preparation (4). The latter reported also that while LSD and tryptamine antagonized the effect of serotonin, TMPEA usually did not alter the response to either serotonin or epinephrine.

Received January 30, 1967, from the Department of Pharmacognosy, Pharmacology and Allied Sciences, College of Pharmacy, St. John's University, Jamaica, NY 11432

Accepted for publication August 15, 1967.

Abstracted in part from a thesis submitted by Emmett Clemente to the Graduate School, St. John's University, in partial fulfillment of Doctor of Philosophy degree requirements.

Grateful acknowledgment is made to the American Medical Association Education and Research Foundation for support of a major portion of this work.

In *in vivo* systems, Parker and Hildebrand (6) found TMPEA to cause a rapid rise in blood pressure when it was administered intravenously to anesthetized cats, and that this response could be blocked by dibenamine. Speck (7) reported similar vasoactive properties for TMPEA in the rat, but that investigator observed antagonism between TMPEA and epinephrine.

The structural similarity between TMPEA and epinephrine has been an important factor in the elaboration of theories which tend to explain psychotomimetic action as involving the catecholamines in some fashion. Nevertheless, studies of the interaction of TMPEA and the neurohormones are relatively rare. Thus, Giarman and Freedman (8) cited the work of Waser *et al.* (9) in stating that serotonin, norepinephrine, and histamine were affected similarly in blood and brain by both TMPEA and LSD. In view of reports of cross-tolerance between TMPEA and LSD in both humans and animals, these findings have been interpreted as suggesting a receptor site common to TMPEA and LSD.

Schopp and his co-workers (10) presented further evidence which demonstrates an interaction between TMPEA, epinephrine, and acetylcholine in peripheral nerve transmission.

In distribution studies of TMPEA in the dog, Cochin *et al.* (11) recovered that compound from ventricular tissue. This finding is especially interesting in light of reports that TMPEA has a variable effect on pulse and blood pressure (12, 13).

In view of the above findings, *in vitro* studies were undertaken to determine the effect of TMPEA on isolated structures from different species and to assess those effects by the use of agents which might aid in the elucidation of those activities. In addition, TMPEA was compared to drugs of known similar and dissimilar activities. In this way, it was expected that some knowledge of the mechanism of action would be

obtained, so far as peripheral effects were concerned.

EXPERIMENTAL

Various smooth muscle tissue from different species was used to observe the activity of TMPEA. Adult male and female guinea pigs weighing between 800 and 1000 Gm. were utilized. The animals were sacrificed by exsanguination. The selected tissue was removed (ileum, esophagus, aorta, trachea, duodenum, jejunum, and atria) and placed in a 20-ml. tissue bath containing Tyrode's solution.[1] When uterine tissue was used, De Jalon's solution[2] was employed as the bathing medium. The bath was aerated with a mixture of 5% CO_2 and 95% O_2 at a flow rate of 30 ml./min., for all tissues except the atria. In the latter case, flow rate was maintained at 60 ml./min.[3] The bath was thermostatically controlled at 37.5° (±0.5°). The tissue was connected to a light magnesium lever and the activity of the tissue was recorded on a slow-moving, smoked paper kymograph (0.791 or 5.18 cm./min.). Fasted and unfasted guinea pigs were used when uterine tissue was studied.

Guinea pig tracheal tissue was prepared by cutting the tissue into spirals. The responses to TMPEA and other compounds were recorded after a pilocarpine-induced spasm had reached a stable plateau (14), unless otherwise stated.

Uterine tissue from unfasted albino rats weighing between 200 and 250 Gm. (inbred strain, Food and Drug Research Laboratories, Maspeth, N. Y.) was challenged similarly to the tissue from guinea pigs. Tissue from rats previously mated and determined to be in estrus was used. It had been observed that tissue from these animals was able to withstand more challenges than virgin tissue. Determination of estrus was made according to Papanicolaou's method (15). The nictitating membrane, right atria, and ileum from fasted kittens (500–850 Gm.) were similarly challenged. Removal of these tissues was accomplished while the animal was anesthetized with sodium pentobarbital (35 mg./Kg.). Nictitating membrane and atrial tissue was allowed an adaptation period of 1 hr. prior to drug challenge. It was noted that atrial tissue required this period of time for stability. Challenges were performed at 15-min. intervals or when control activity was reestablished. Animals from which ileum or other intestinal tissue was removed had been fasted for approximately 16 hr. Some animals (guinea pigs in particular) were fasted for prolonged periods (36–48–60 hr.) in order to evaluate the effect of long periods of fast on the response to TMPEA. All intestinal tissue was allowed to equilibrate in the bath at least 30 min. before commencement of any challenge. In some experiments, guinea pig ileum from fasted animals was removed, quick frozen (dry ice and acetone), and placed in the cold (5°) for periods of 24 to 60 hr. before being challenged with TMPEA. Tissue which had been frozen was allowed to equilibrate for 2 hr. prior to experimentation. The object of these experiments was to observe the effect of TMPEA on denervated smooth muscle. The doses of all compounds are expressed as their salts. The drugs and their range of concentration (mcg./ml.), in terms of base, are given in Table I.

TABLE I—RANGE OF CONCENTRATION OF COMPOUNDS *In Vitro*

Compd.	Range of Dose Employed (Base mcg./ml.)
Mescaline sulfate	0.017–140.0
Phenoxybenzamine·HCl	2.2–8.8
Isopropylmethoxamine·HCl	0.86–43.0
Hexamethonium chloride	0.92–3.70
Tetraethylammonium chloride	4.35–43.5
Serotonin creatinine sulfate	0.0004–2.1
Nicotine (pure)	1.0
Ergotamine tartrate	0.44–2.20
l-Epinephrine-*d*-bitartrate	0.014–2.75
l-Phenylephrine·HCl	0.010–4.10
Acetylcholine chloride	0.009–0.045
Atropine sulfate	0.0083–4.15
Hydralazine·HCl	4.05–8.10
Reserpine	1.0–12.5
Isoproterenol·HCl	0.0043–0.043
Dichloroisoproterenol	0.0087–8.7
Pilocarpine·HCl	8.5–10.6
Histamine diphosphate	0.18
Lidocaine·HCl	43.5–261.0
l-Arterenol-bitartrate	0.013–2.65
Procaine·HCl	43.5–261.0
Diphenhydramine·HCl	0.08–0.44

RESULTS

Effect of TMPEA on the Intestinal Tissue of Several Species—During the course of this research, it became apparent that the action of TMPEA on the terminal section of ileum was concentration, species, and tissue dependent. Over a wide range of concentration, the effect of TMPEA on guinea pig and cat ileum was consistently motor in nature, while concentrations approaching the upper limit were necessary for responses of equal magnitude for the rat ileum. In general, in all species concerned, the lowermost portions of the ileum were more responsive to the motor stimulatory property of TMPEA than were portions adjacent to the jejunum. In fact, the terminal portion of the ileum was the segment of intestinal tissue most sensitive to the effects of TMPEA. It was noted that the motor response to TMPEA roughly paralleled the motor response to norepinephrine, epinephrine, and serotonin. The response to TMPEA of other intestinal tissue (*e.g.*, duodenum, jejunum, and upper portion of ileum) was considerably less than that observed for the terminal segment of the ileum in all species employed. The effect of TMPEA on isolated tissue from various sources, including the present study, is presented in Table II.

Effect of Diphenhydramine Pretreatment on the Response to Mescaline—Diphenhydramine, in concentration sufficient to block the motor response to histamine on guinea pig ileum, was ineffective against TMPEA. Similar results were obtained during an *in vivo* phase of this work (38).

Effect of Mescaline on Cholinergic Mechanisms—In order to demonstrate whether TMPEA could be

[1] NaCl, 120 Gm.; $CaCl_2$, 3 Gm.; KCl, 3 Gm.; $NaHCO_3$, 15 Gm.; glucose, 15 Gm.; $MgCl_2 \cdot 6H_2O$, 1.5 Gm.; and $NaHPO_3$, 0.75 Gm./15 L.

[2] NaCl, 60 Gm.; $CaCl_2$, 0.9 Gm.; KCl, 6.3 Gm.; $NaHCO_3$, 7.5 Gm.; glucose, 3.75 Gm.; $MgCl_2 \cdot 6H_2O$, 0.1597 Gm.; sucrose, 879 Gm./15 L.

[3] Brooks Sho rate flow meter, Brooks Instrument Co., Inc., Hatfield, Pa.

TABLE II—EFFECT OF MESCALINE (I), PRETREATMENT WITH DRUGS BEFORE MESCALINE (II), AND EFFECTS OF DRUGS ON MESCALINE ACTIVITY (III) ON SELECTED TISSUES OF VARIOUS SPECIES *In Vitro*[a]

Species	Tissue	I	mcg./ml.	II[c]	III[c]
Guinea pig	Duodenum	Sl. contraction	10–50	...	...
	Jejunum	Sl. contraction	10–50	...	...
	Ileum (terminal)	Contraction; magnitude depends upon initial tissue response	0.5–50	Acetylcholine (SA); L-epinephrine (A, R); L-norepinephrine (A, R); isoproterenol (NA); 5-HT (V; SA, A); nicotine (NA); phenylephrine (A)	Procaine (NA); lidocaine (NA); hexamethonium (SE); TEA (SA); atropine (NA); reserpine (NA, A); diphenhydramine (SA); DCI (NA); phenoxybenzamine (I); hydralazine (V); methoxamine (V); ergotamine (NA); nicotine (NA)
	Uterus	Variable (all or none type)	12.5–5	5-HT (I)	Reserpine (NA, I); DCI (V); hydralazine (V); methoxamine (V); ergotamine (NA)
		No effect (1)	10–200		
	Tracheal chain	Contraction	50–200	L-Epinephrine (SR)	...
	Aorta	Sl. contraction	50–100	L-Epinephrine (NA); L-norepinephrine (NA)	...
Rat	Ileum	Contraction	50	L-Epinephrine (SR); L-norepinephrine (SR); phenylephrine (SR)	DCI (V)
	Uterus	Contraction, increase in pendular movement	0.1–50	5-HT (NA) (3); 5-HT (S) (2) 5-HT (A); L-epinephrine (A, R); L-norepinephrine (A, R); ergotamine (NA); methoxamine (NA)	Atropine (NA) (2); hydralazine (V); methoxamine (NA); ergotamine (NA); DCI + TMPEA nullified the effect of epinephrine
		No effect	10–200		
		Contraction (3)	50		
		Contraction (2)	0.1–0.4		
Cat	Duodenum	No effect (1)	10–400	...	...
	Ileum	Contraction	12.5–50	...	Phenoxybenzamine (I)
	Uterus	No effect (1)	10–200	...	...
	Nictitating membrane	No effect	50–200	...	...
	Atria	No change in chronotropy or inotropy	12.5–50	Acetylcholine (SA); L-epinephrine (SE); L-norepinephrine (SE); isoproterenol (SE)	...
Rabbit	Duodenum	No effect (1)	10–400	...	...
	Uterus	No effect (1)	10–200	...	...
Human	Placental vessels	Constriction[b] (4)		5-HT (S) low dose 5-HT (no effect) high dose Epinephrine (no effect) high dose	...

[a] Concentration of compounds expressed as their salts. [b] Perfusion. [c] Key: A = antagonism, I = inhibition, V = variable, SA = slight antagonism, NA = no antagonism, SE = synergism (excitatory effect), SR = synergism (relaxatory effect), R = response reversal.

blocked by atropine, the tissue (guinea pig ileum) was first challenged with a submaximal concentration of acetylcholine (ACH), and when the response was maximal, atropine was added. After 2 or 3 min., another challenge to ACH was performed and if no response was elicited TMPEA was added to the system. On 30 occasions the characteristic motor response to TMPEA was observed. It has been reported (10) that TMPEA possesses a curare-like action. A series of experiments was performed to observe the action of TMPEA on ACH induced contractions. Tissue which did not actively respond to mescaline (25–50 mcg./ml.) did not block ACH contractions on the guinea pig ileum. If a tissue contracted in the presence of TMPEA, ACH produced a response which was less than control. However, if the heights of the induced TMPEA and ACH contractions were added; it equaled control response. The same phenomenon was observed with the catecholamines. Therefore, the specificity of the reaction is questionable.

Effect of Local Anesthetics, Lidocaine and Procaine, on the Activity of Mescaline—Concentrations of the above local anesthetics (50 to 250 mcg./ml.) not sufficient to entirely inhibit the action of ACH did not affect the response to TMPEA on the guinea pig ileum. Higher concentrations (300 mcg./ml.) were not observed to curtail the action of TMPEA, although these concentrations did block the action of ACH.

Effect of the Depolarizing Ganglionic Blocking Agent, Nicotine, on the Response to Mescaline in the Guinea Pig Ileum—Nicotine (1 mcg./ml.) did not antagonize the action of TMPEA (25 mcg./ml.) on the guinea pig ileum. Reversal of the procedure yielded the same results. In the majority of trials, nicotine was allowed a 3-min. contact period prior to the challenge with TMPEA.

Effect of TMPEA on the Adrenergic Receptor—Studies were initiated to observe the possible interaction between TMPEA and adrenergic receptors. Ergotamine, in sufficient concentrations (2.5–5.0 mcg./ml.) to block the motor response to a catecholamine (norepinephrine or epinephrine) was not observed to inhibit the motor response to TMPEA on guinea pig ileum and rat uterine tissue. It was noted that epinephrine and norepinephrine caused a greater degree of relaxation in the presence of *both* ergotamine and TMPEA, than in the presence of ergotamine only. Subsequently, it was observed that TMPEA *alone* could not only block the motor action of a catecholamine but also allow the relaxatory action to be manifested. In general, if the catecholamines produced weak to moderate motor responses, TMPEA was capable of antagonizing these responses. However, if a catecholamine caused a marked motor effect and TMPEA did not generate its characteristic action, the antagonism was diminished. It was apparent that sufficient incubation time and the concentration of TMPEA

was essential to its ability to effectively antagonize the catecholamines. An incubation period of 5 min. was found to be necessary for reproducible responses; however, other factors, such as the responsiveness of the tissue to both the catecholamines and TMPEA, were of equal importance. If shorter incubation periods were employed, the antagonism was proportionally diminished. Furthermore, it was demonstrated that when TMPEA was added after a "critical" period of time had elapsed following a stimulatory dose of norepinephrine, epinephrine, or phenylephrine (5 min.) an additional increase in tone was produced. If TMPEA was added before 5 min. had elapsed (1–3 min.) a decrease in tension was produced.

Interaction Between TMPEA and Serotonin, on Guinea Pig Ileum and Rat and Guinea Pig Uteri—TMPEA in very low concentration has been reported to synergize the motor action of serotonin on the rat uterus (2). Higher concentrations have been reported to have little effect on the activity of serotonin on rat uterus. The apparent synergistic action of low concentrations of TMPEA (0.1–0.4 mcg./ml.) observed during the present study on estrogen dominated uterus appeared questionable. Slightly higher concentrations of TMPEA (2.5 mcg./ml.) did antagonize the effect of serotonin. TMPEA was also observed to antagonize the effect of serotonin (100 trials) on guinea pig ileum. The degree of antagonism depended upon the sensitivity of the tissue to both compounds. Moderately stimulatory doses of serotonin could be almost completely blocked with very high concentrations of TMPEA. Ergotamine and TMPEA acted synergistically to antagonize the action of serotonin. In addition, moderate doses of TEA (15 mcg./ml.) plus TMPEA acted synergistically to antagonize the motor response to serotonin. TMPEA (25 mcg./ml.), after a 3-min. incubation period, was observed to block the motor response of guinea pig uterus to serotonin (0.25 mcg./ml.). Additional challenges were effectively antagonized.

Effect of TMPEA on Guinea Pig Tracheal Tissue—TMPEA alone (50–200 mcg./ml.) or following a pilocarpine (10 mcg./ml.)-induced spasm caused contraction. Epinephrine provoked relaxation alone or after either of the above procedures. The relaxation due to epinephrine appeared to be greater following mescaline than when the tissue was not pretreated. Unfortunately, a great deal of variability was encountered with this tissue, obviating definite conclusions.

The Interaction of TMPEA with Various Adrenergic Blocking Agents—A number of experiments were conducted to observe the possible interaction between hydralazine, isopropylmethoxamine, dichloroisoproterenol, and mescaline in various tissue. Concentrations of dichloroisoproterenol (10 mcg./ml.) were observed to inhibit or reduce the motor activity produced by TMPEA, while lower concentrations (0.5 mcg./ml.) had little effect. All tissue tested (guinea pig ileum and uterus and rat ileum and uterus) with the exception of cat atria reacted similarly. Hydralazine and isopropylmethoxamine had little modifying action on tissue response to TMPEA. However, it was noted that concentrations of TMPEA which were usually ineffective would produce a pronounced action on guinea pig uterus if the tissue were pretreated with either hydralazine or isopropylmethoxamine. In general, the results of the above procedures were difficult to assess because of the strong motor activities inherent to hydralazine and isopropylmethoxamine.

Concentration of DCI (10 mcg./ml.) and TMPEA (25–50 mcg./ml.) were effective in blocking the action of the catecholamine on rat uterine tissue. These results indicated that both α- and β-receptor sites were blocked. A more potent α-adrenergic blocking agent, phenoxybenzamine (10–20 mcg./ml.), was observed to completely block the motor activity due to TMPEA (25–50 mcg./ml.) on cat and guinea pig ileum. These results would suggest that TMPEA exerts its effect on the α-adrenergic receptor site.

In order to substantiate these results and to demonstrate the action of TMPEA on the β-adrenergic receptor site, cat atria (right atria) were selected as the test structure. TMPEA (12.5–50 mcg./ml.) had no demonstrable chronotropic or inotropic effect on this tissue, even after a 10-min. incubation period. Nevertheless, the tissue remained responsive to various catecholamines, isoproterenol, epinephrine, and norepinephrine. Pretreatment of the tissue with TMPEA (25–50 mcg./ml.) caused an apparent synergistic effect to the catecholamines. Phenoxybenzamine (10 mcg./ml.), incubated for the same period of time as TMPEA (3 min.), also seemed to synergize the action of the catecholamines. In contrast, DCI (10 mcg./ml.) completely blocked the action of isoproterenol, and to a lesser extent, epinephrine and norepinepheine. The synergistic effect of TMPEA was best observed if the water bath temperature was reduced from 37.5° to 32.0°.

It was of interest to observe the effect of TMPEA on ACH blockade of the right atria, in view of a report that TMPEA possesses a curare-like action. The effect of complete or partial blockade of normal rhythmicity of atrial tissue by ACH (0.25–0.5 mcg./ml.) was not affected by pretreatment with TMPEA (25–50 mcg./ml.). When slight antagonism was observed, the tissue usually responded in a different fashion to ACH following the washout of TMPEA. The very slight antagonism encountered in this series might be explained more logically as due to a change in response of the tissue to ACH, rather than specific antagonism by TMPEA.

DISCUSSION

Diphenhydramine, in sufficient concentration to block histamine, did not effectively antagonize TMPEA. There is some doubt as to how antihistamines actually antagonize histamine (16). This fact precludes a definitive conclusion that mescaline does not exert its effect *via* histaminic receptors. However, graded responses caused by TMPEA in the presence of diphenhydramine were not observed. These findings would suggest that a noncompetitive reaction due to the effect of TMPEA prevailed and the response elicited was distinct from the histamine effect.

Costa (2) reported that atropine *in vitro*, in sufficient concentration to block ACH, did not antagonize the effect of TMPEA in rat uterus. Grace (1) reported that atropine, *in vivo*, was capable of blocking the effect of TMPEA in the cat. The results obtained in this study using guinea pig

ileum are in agreement with those of Costa (2). Atropine in sufficient concentration to block submaximal stimulatory effects of ACH did not inhibit the motor response induced by TMPEA. Thus, TMPEA apparently does not produce its motor effects *via* a muscarinic action.

In order to determine if TMPEA stimulated nervous elements, local anesthetics were employed in concentrations which block ACH. The dose for both procaine and lidocaine, necessary to block ACH, caused a marked increase in the activity of the ileum. TMPEA was able to exert its effect in the presence of those compounds. These findings, in conjunction with those above, support the concept that TMPEA does not exert its effect on cholinergic receptors.

Ganglionic blocking agents (hexamethonium and TEA) did not antagonize the effect of TMPEA on guinea pig ileum. In the presence of both a ganglionic blocking agent and atropine, mescaline was observed to produce its characteristic effect. Thus it appears that TMPEA does not exert its action on ganglia. Moreover, large concentrations of TEA seemed to synergize the motor response exerted by TMPEA and serotonin. The latter findings are in agreement with those reported by Day and Vane (17) for hexamethonium.

Gaddum *et al.* (3), in an attempt to explain the antagonizing action of LSD on serotonin, postulated two distinct types of receptors in ganglia cells in the intestine. One of these was stimulated by ACH or nicotine and inhibited by excess nicotine or hexamethonium, while the other type was stimulated by serotonin and inhibited by excess serotonin. It is also possible that there exists two types of cells; one stimulated by nicotine, the other by serotonin. In light of this theory, TMPEA might conceivably be stimulating serotonin-sensitive receptors. However, serotonin was not observed to alter the action of TMPEA on guinea pig ileum. It is possible that the concentrations employed in these experiments were not sufficient to antagonize serotonin receptor sites.

Gaddum *et al.* (3) further differentiated between the response of smooth muscle of the ileum and rat uterus to serotonin. They stated that the receptors in the rat uterus are easily paralyzed by excessive serotonin, whereas those in the ganglia of the guinea pig ileum are not. They explained the antagonistic action of LSD on rat uterine tissue on the above hypothesis. TMPEA was not observed, in their studies, to antagonize the effect of serotonin on rat uterus. Costa (2) reported that mescaline acted synergistically with serotonin in causing increased activity on rat uterine tissue. Savini (18) reported that LSD was antagonistic to serotonin-induced constriction on perfused rabbit ear. Prior to Gaddum's theory concerning the site of action of serotonin, Rocha e Silva *et al.* (19) postulated the site to be the postganglionic cholinergic nerve fiber of the ganglia. Cocaine has been observed to effectively antagonize serotonin on isolated ileum, a finding which supports the proposal of Rocha e Silva and his collaborators.

Day *et al.* (17) have presented evidence demonstrating that serotonin is also capable of stimulating smooth muscle directly. However, they consider the blockade of the action of serotonin by phenoxybenzamine to be nonspecific and concluded that serotonin primarily exerts its effect indirectly through stimulation of cholinergic nerve fibers in guinea pig ileum.

The findings in this study involving the interaction between serotonin and TMPEA on guinea pig ileum and uterus and rat uterus are in agreement with the theory proposed by Gaddum *et al.* (3) and the findings of Day *et al.* (17). In the majority of experiments, there appeared to be some antagonism exerted by mescaline on the motor response to serotonin on the guinea pig ileum. These observations would indicate that the ganglionic component involved in the action of serotonin was not effectively antagonized, while the smooth muscle was affected.

Experiments conducted on rat and guinea pig uteri clearly demonstrated that TMPEA effectively antagonized the action of serotonin. These results are not in agreement with those reported by Gaddum *et al.* (3) and Astrom *et al.* (4). The latter authors stated that TMPEA had "no certain action against serotonin on human placental vessels." The findings in the present investigation would support the variation in receptor sites in different tissues sensitive to serotonin. They would also support the concept that TMPEA was primarily exerting its effect on the smooth muscle cell. These results support the clinical and experimental findings involving animals demonstrating cross-tolerance between LSD and TMPEA.

Costa (2) and Astrom (4) reported that low concentrations of TMPEA synergized the effect of serotonin on rat uterus and human placental preparations, respectively. Costa's (2) experiments were repeated precisely as he reported them during this investigation. After reproducible responses were obtained from challenges with serotonin by the method of Rocha e Silva (19), the tissue was challenged with TMPEA. When serotonin was added to this system, a synergistic effect was apparent. However, if this experiment was repeated without the addition of TMPEA, the same effect was observed. Thus the augmented response due to serotonin in the presence of TMPEA, in this investigation, appears to have been a function of variability in response to serotonin and not to a synergistic action of TMPEA.

Nicotine was not observed to antagonize the effect of TMPEA. It is generally agreed that nicotine depolarizes the postsynaptic neuron in ganglia, making it insensitive to acetylcholine (20, 21). Emmelin and Feldberg (22) demonstrated that nicotine also depressed smooth muscle directly. Evans and Schild (23) demonstrated that nerve-free preparations of intestine reacted to nicotine by contraction. This effect could be abolished by pretreatment with hexamethonium. The latter substance was reported to be the most specific ganglionic blocking agent known (24). The ganglionic blocking agents, *i.e.*, the depolarizing and nondepolarizing competitive types have, therefore, been shown to possess a number of properties which could have influenced the activity of TMPEA. However, if their major activities are considered, one would necessarily conclude that TMPEA does not possess characteristics of a substance which excites intramural ganglia cells responsive to ACH. Grace (1) reported that TMPEA caused an increase in tonic activity of uteri, *in situ*, from various species. The

effect was not abolished after pretreatment with nicotine.

TMPEA has been noted, in this study and others (1–3), to induce rhythmic movements in intestinal and uterine tissue. Various investigators have attributed the rate of spontaneous activity of uterine tissue to the level of catecholamine content (25, 26). Rudzik *et al.* (25) stated that the initial rate of spontaneous uterine contractions *in vitro* was inversely proportional to the quantity of epinephrine in the uterus. When the uterus from a rat in estrus was challenged with epinephrine, relaxation occurred—TMPEA caused contraction. The possibility exists that TMPEA produces uterine contraction by causing a release of endogenous epinephrine or directly stimulating excitatory sites, while epinephrine may relax the tissue by raising the endogenous level of epinephrine content or directly stimulating inhibitory sites.

The terminal segment of the ileum in a variety of species has been observed to contract in response to splanchnic nerve stimulation or to exogenous epinephrine (27–30). Dale (27) utilized this effect to demonstrate that the ergot alkaloids blocked the contracting properties of epinephrine on the terminal segment of the cat ileum. Munro (28) reevaluated this phenomenon and showed that various catecholamines were capable of causing similar responses (29). This motor effect produced by epinephrine on the intestinal tissue was not in accord with the theory proposed by Ahlquist (31). At that time α activity on intestinal tissue was classified as relaxatory. Some changes in this classification were proposed by Lands (32). He demonstrated that inhibitory and excitatory receptors were present in the terminal segment of the ileum and the response obtained was probably a function of the relative number and sensitivity of each type of receptor present. Furchgott (33) expanded upon the above classification and introduced the term "delta receptor" to explain intestinal inhibition mediated by a sympathomimetic amine. The subject, at present, is still unsettled (34).

In the present investigation, it was observed that when the terminal section of ileum was relaxed by a catecholamine, TMPEA had little or no effect. If a contraction was elicited in the presence of a catecholamine, TMPEA alone was also observed to produce a similar response. Moreover, the more sensitive the tissue appeared to be toward the motor effect of the catecholamines (contraction), the more sensitive it was to the effects of TMPEA. The above actions were interpreted as demonstrating that both receptors (α and β) or A_c (excitatory) and A_r (relaxatory) were present in the terminal segment of the guinea pig ileum. If a contraction was elicited, the α responses predominated, or if inhibition resulted, the β responses were manifested.

Ergotamine in sufficient concentration to antagonize the motor responses to epinephrine and norepinephrine did not inhibit the motor response to TMPEA on guinea pig ileum and rat uterine tissue. Phenoxybenzamine was capable of blocking the motor response of TMPEA on guinea pig and cat ileum as well as reversing a former stimulatory action of the catecholamines to a purely relaxatory one. The apparent ability of phenoxybenzamine and not ergotamine to effectively antagonize TMPEA may be attributed to the lower concentrations employed with the latter compound. Ergotamine and TMPEA appeared to possess synergistic properties in antagonizing motor responses to the catecholamines. The results would indicate that TMPEA was stimulating excitatory receptors in the terminal section of guinea pig ileum. Abolishment of this effect by phenoxybenzamine and the apparent synergistic effects of ergotamine and TMPEA on the blockade of motor responses to the catecholamines strongly suggest that TMPEA exerts its effect on α-adrenergic receptor sites.

Supporting this concept are the findings that TMPEA alone is capable of antagonizing the motor response to catecholamines in guinea pig ileum, rat ileum, and uterine tissue. Furthermore, simultaneous administration of dichloroisoproterenol (a β-adrenergic blocking agent) and TMPEA abolished the effect of epinephrine on rat uterus, thus demonstrating that both α- and β-adrenergic receptor sites were blocked. If TMPEA was stimulating α-adrenergic receptor sites, it should theoretically be possible to demonstrate competition between these compounds. This competitive phenomenon was observed to occur when TMPEA was added to a preparation which had reacted (motor response) to a catecholamine. It was also observed that TMPEA had to be introduced within a critical period of time, otherwise competition was not observed. These findings are in agreement with those reported by Speck (7) on the competitive nature existing between epinephrine and TMPEA on various parameters in the rat.

Experiments were conducted to observe a possible augmentation of the effect of mescaline with the use of β-adrenergic blocking agents. Of the three agents employed, isopropylmethoxamine, hydralazine, and dichloroisoproterenol, the latter was the most specific. The former compounds are also known to possess α-adrenergic stimulatory actions (34). The results would indicate that the β-adrenergic blocking agent, dichloroisoproterenol, did augment the action of TMPEA on guinea pig uterus. If isopropylmethoxamine was administered after TMPEA produced an excitatory response in rat uterus and ileum or guinea pig ileum, a marked reduction in tone was noted. These results would indicate that the former compound was exerting its effect on β-adrenergic sites, if the assumption is made that TMPEA had occupied the α-adrenergic sites. The variability encountered in these experiments, however, does not justify definitive conclusions. Powell and Slager (14) demonstrated that dichloroisoproterenol blocked the relaxatory response of epinephrine on tracheal tissue. Substituting TMPEA in place of dichloroisoproterenol did not alter the relaxatory response of epinephrine. These findings further substantiate the concept that TMPEA is not exerting an appreciable effect on β-adrenergic receptor sites. The fact that TMPEA increased the tone of the tracheal chain affords additional evidence that its activity is primarily on α-adrenergic receptor sites.

Reserpine, in low concentrations, was not observed to antagonize the action of mescaline on guinea pig ileum. Higher concentrations did effectively antagonize the motor activity of mescaline on guinea pig ileum and uterine tissue. The latter effect might be due to a direct action on the smooth muscle. The higher concentrations employed in

these studies have been observed to markedly reduce the response of guinea pig ileum to ACH and histamine (35). Reserpine is known to release catecholamines from isolated tissue (36). These effects might nonspecifically antagonize the action of TMPEA.

Chronotropic and inotropic changes were not observed to occur in isolated right atria (cat) in the presence of TMPEA. Similar results were obtained with phenoxybenzamine. The latter findings are in agreement with those of Nickerson and Chan (37). Changes in rate and force of contraction have been attributed to β-adrenergic stimulation (33, 34). Dichloroisoproterenol was observed, in this study and by Nickerson *et al.* (37), to antagonize the effect of various catecholamines on cardiac tissue. TMPEA did not, however, reduce catecholamine activity. There also appeared to have been some augmentation to the simulatory effects of epinephrine, norepinephrine, and isoproterenol in the presence of TMPEA. These results are supportive of the findings with TMPEA on other tissue used in this study; that is, TMPEA exerts its primary action on α-adrenergic receptor sites.

TMPEA has been reported to possess a curare-like action (10). In this study, TMPEA was observed to apparently antagonize the effect of ACH on smooth muscle of the guinea pig ileum and cat atria. The latter experiments were too variable to draw any conclusions. However, epinephrine and norepinephrine were also observed to diminish the response of smooth muscle to ACH. These results would indicate that the antagonizing effect of TMPEA on ACH-induced contractions was nonspecific. The fact that Schopp *et al.* (10) reported that epinephrine and prostigmine oppose the paralyzing action of TMPEA supports this concept.

CONCLUSIONS

Evidence is presented which demonstrates the ability of TMPEA to stimulate α-adrenergic receptor sites in various peripheral tissue. The fact that TMPEA alone can antagonize α-stimulatory agents, and in combination with specific β-blocking agents can annul the effects of epinephrine, strongly suggest that it possesses α-adrenergic blocking properties. Furthermore, since it is capable of contracting smooth muscle preparations containing excitatory receptors, and because its actions were inhibited by α-adrenergic blocking agents, the concept that TMPEA possesses α-stimulatory properties is demonstrated. Competition between TMPEA and various catecholamines provides additional evidence that TMPEA possesses agonistic and antagonistic properties. The β activity of TMPEA appeared to be slight. To clarify this action, TMPEA should be studied on target structures which possess a preponderance of β-adrenergic receptors.

Evidence is presented that TMPEA does not stimulate cholinergic receptors. Clarification of the action of TMPEA on receptors in the ganglia should be carried out with more specific ganglionic blocking agents and with techniques reported to reduce ganglionic sensitivity. TMPEA has been shown to antagonize effectively the action of serotonin on guinea pig and rat uteri, but not in guinea pig ileum. It is concluded that in the latter tissue serotonin exerts an effect on ganglionic receptors which cannot be blocked by TMPEA.

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Keyphrases

Mescaline (trimethoxyphenylethylamine)
In vitro action on smooth muscle
Cholinergic mechanism—effect on
Adrenergic receptor—effect on
Adrenergic blocking agents—interaction mescaline
Serotonin—mescaline interaction
Local anesthetic effect on mescaline activity
Nicotine effect on mescaline activity
Diphenhydramine effect on mescaline activity

Effect of Temperature Upon Solubilization by a Series of Nonionic Surfactants

By K. J. HUMPHREYS and C. T. RHODES

The solubilization of benzoic acid by four *n*-alkylpolyoxyethylene surfactants as a function of temperature has been examined by the dialysis and solubility techniques. In addition to their use in formulation work, it is shown that evaluation of the equilibrium constants controlling the solubilization process can be of use in elucidating micellar properties. The calculation of the thermodynamic parameters controlling the solubilization process is discussed and some difficulties indicated.

MICELLAR SOLUBILIZATION has been studied extensively in recent decades. To the pharmacist the topic is of particular interest because many pharmaceutical formulations utilize surfactants to enhance the solubility of sparingly soluble components.

One of the most important and illuminating ways of treating micellar solutions of surfactants is to regard the micelles as forming a definite phase which is at the same time dispersed throughout the system in submicroscopic particles of definite size, the micelles. The concentration of surfactant in the aqueous or continuous phase may be equated with the CMC. In systems where the CMC is very low, *e.g.*, aqueous solutions of many nonionic surfactants, the system is divisible into two essentially single-component phases, disregarding any solvent water bound to the micelles. If a third component, termed the "cosolute," is added to the system, it will be distributed between the 2 phases. When the distribution is in favor of the micellar pseudophase, the phenomenon is referred to as solubilization (1). When such surfactant solutions are brought into equilibrium with pure solid or liquid cosolutes, the system takes up appreciably more than an equivalent amount of pure water. Micellar solubilization is characterized by the enhanced solubility of cosolute only being apparent above the CMC of the ternary system. If the CMC itself is low, then the initial region of zero solubilization is not readily detectable.

In any L_1, isotropic aqueous liquid, surfactant system an equilibrium exists between micellar and nonmicellar cosolute.

$$(S_w) \overset{K}{\rightleftharpoons} (S_m) \qquad \text{(Eq. 1)}$$

where (S_w) and (S_m) represent the equilibrium activities of the nonmicellar and micellar species of cosolute and K is the equilibrium constant.

There is considerable evidence which suggests that the pharmacological activity of a drug in micellar systems is a function of (S_w), the micellar material being regarded as an inactive reserve of drug (1, 2). Two techniques are available for evaluating (S_w) and (S_m), equilibrium dialysis (3) and potentiometry (4). Unfortunately, comparatively little data have been collected using these methods. The results of most studies have been obtained by use of the conventional solubility technique which, of itself, does not allow differentiation between (S_w) and (S_m) except at saturation values. However, it is apparent from the results of those systems for which information about (S_w) and (S_m) is available that micellar solubilization can be accomplished by more than one mechanism (5). It is important to realize that, using the two-phase approach, since the micelles occupy a part of the total volume of the system, the over-all concentration of free cosolute may not necessarily be equated with the actual free concentration in the aqueous phase. However, considerable theoretical difficulties arise when attempts are made to estimate the volume of the micellar pseudophase. The phase could be considered to consist of (*a*) the hydrocarbon core of the micelle, (*b*) the whole micelle, or (*c*) the whole micelle plus bound and trapped water. At present insufficient evidence is available for a rational choice to be made.

The effect of temperature upon the properties of binary surfactant systems has been studied by many workers. Such measurements are particularly useful in that they allow evaluation of the thermodynamic parameters controlling the equilibrium in such systems (6, 7). Fewer studies of the effect of temperature upon equilibria in ternary surfactant systems have been reported, and little work has been done on the evaluation of the thermodynamic parameters controlling such equilibria. In the present

Received May 3, 1967, from the Portsmouth School of Pharmacy, Portsmouth, Hampshire, England.
Accepted for publication August 28, 1967.
The authors thank Dr. P. Molyneux for many stimulating discussions on the thermodynamics of solubilization, and Mr. P. Harger for technical assistance in the construction of dialysis cells. Thanks are also extended to the Smith Kline & French Foundation, Welwyn Garden City, England, for financial assistance during the course of this work.

paper some studies of the effect of temperature upon the solubilization of benzoic acid by a series of *n*-alkylpolyoxyethylene surfactants are reported and their practical and theoretical significance discussed.

EXPERIMENTAL

Materials—Analar benzoic acid,[1] four *n*-alkylpolyoxyethylene surfactants[2] all of the general formula C_nOE_mOH where n is the number of carbon atoms in the alkyl chain and m the number of polyoxyethylene groups. The mean molecular weights of these compounds have been previously determined by nuclear magnetic resonance spectroscopy (8).

Methods—Dialysis studies were performed using dialysis cells similar in design to those described by Patel and Foss (9). The cells were thermostatically controlled to ±0.1° of the required temperature. Each cell, compartment capacity 10 ml., contained a glass bead to ensure continuous mixing of contents. Nylon membranes[3] were shown by preliminary tests to be impermeable to the surfactants but to allow diffusion of the benzoic acid. The solvent used in the dialysis and solubility studies was 0.01 *N* hydrochloric acid, to depress ionization of the benzoic acid. The cells were shaken for 4 days, a time found to be sufficient for equilibrium to be reached. Samples from both sides of the membrane were removed by pipet, calibrated gravimetrically at the appropriate temperature, and after suitable dilution, the benzoic acid concentrations were determined by spectrophotometric assay at 273 mm. using a Uvispek.[4] The molar absorptivity of benzoic acid, 966, compares closely to the literature value of 970 (10).

Solubility determinations were made by shaking excess benzoic acid in various concentrations of surfactant solution for 4 days. Samples for assay were filtered through No. 3 filter sticks, and the benzoic acid content determined as described above.

Determinations of weight/ml. were made by use of pycnometers, calibrated gravimetrically at the appropriate temperature. The apparatus was thermostatically controlled to within ±0.1° of the required value.

RESULTS AND DISCUSSION

Figure 1 is typical of the results obtained from the dialysis studies. HA_m represents the amount of benzoic acid solubilized by the surfactant and $[HA_w]$ the concentration of nonbound acid. The linear nature of such plots indicates that the solubilization process in such systems is governed by a distribution coefficient, correlation coefficients calculated for all systems being above 0.95. Thus for these systems the solubilization mechanism is most readily interpreted in terms of distribution between an aqueous continuous phase and a micellar pseudophase.

$$K_d = [HA_m]/[HA_w] \qquad \text{(Eq. 2)}$$

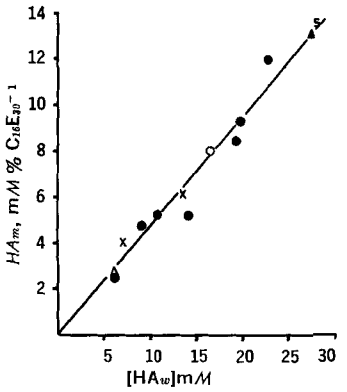

Fig. 1—Dialysis results for the interaction between benzoic acid and an n-*alkyl polyoxyethylene surfactant, $C_{16}E_{30}$, at 25°. Key: ▲, solubility point; ○, 1% w/v $C_{16}E_{30}$; ●, 2% w/v $C_{16}E_{30}$; ×, 3% w/v $C_{16}E_{30}$; △, 4% w/v $C_{16}E_{30}$.*

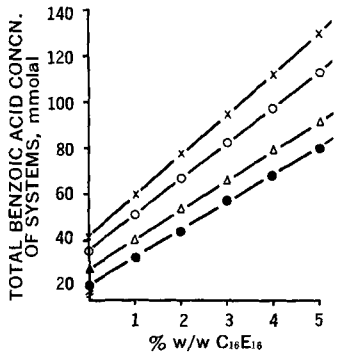

Fig. 2—Effect of temperature on the solubilization of benzoic acid by aqueous solutions of an n-*alkyl polyoxyethylene surfactant, $C_{16}E_{16}$. Key: ×, 37°; ○, 31°; △, 25°; ●, 18°.*

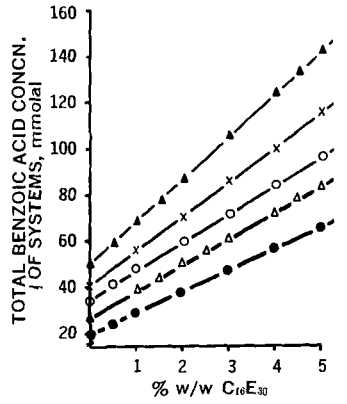

Fig. 3—Effect of temperature on the solubilization of benzoic acid by aqueous solutions of $C_{16}E_{30}$. Key: ▲, 45°; ×, 37°; ○, 31°; △, 25°; ●, 18°.

For unionized components such as benzoic acid the aqueous activity coefficient will be very close to

[1] Supplied by B. D. H. Ltd., Poole, England.
[2] Texofors, supplied by Glover's Ltd., Leeds, England.
[3] Supplied by Portex Plastics Ltd., Hythe, Kent, England.
[4] Supplied by Hilger & Watts Ltd., London, England.

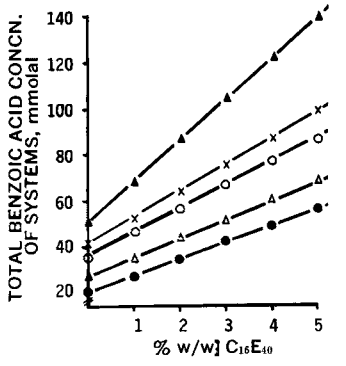

Fig. 4—Effect of temperature on the solubilization of benzoic acid by aqueous solutions of $C_{16}E_{40}$. Key: ▲, 45°; ×, 37°; ○, 31°; △, 25°; ●, 18°.

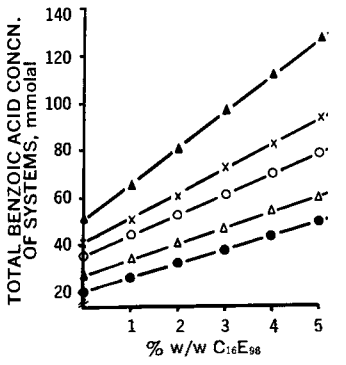

Fig. 5—Effect of temperature on the solubilization of benzoic acid by aqueous solutions of $C_{16}E_{96}$. Key: ▲, 45°; ×, 37°; ○, 31°; △, 25°; ●, 18°.

unity, and provided the activity coefficient of the micellar species also approximates to unity, the K_d values as defined in Eq. 2 will represent the thermodynamic equilibrium constant.

Figures 2 through 5 show the effect of temperature and surfactant concentration upon the solubilization of benzoic acid. In all cases the concentration of benzoic acid was calculated in molal terms by use of the appropriate weight/ml. value, though in fact the difference between molar and molal values was very small since in all cases the weight/ml. values were close to unity. The intercept values on the ordinates represent the solubility of benzoic acid in 0.01 *N* hydrochloric acid, $[HA_w^0]$. In all the systems examined the relationship between the total amount of benzoic acid present and surfactant concentration was found to be linear. This type of solubilization behavior is common, though in some systems both negative and positive changes of slope in such plots have been observed (11). Extrapolation of the graph in Figs. 2–5 to 100% w/w surfactant allows estimation of the solubility of benzoic acid in a "bulk micellar" surfactant phase, with properties exhibited by the surfactant when in 0–5% w/w aqueous solution. This value represents the solubility of benzoic acid in the micellar pseudophase. The K_d values for the various systems investigated in this work have been calculated by use of Eq. 3 and are listed in Table I.

$$K_d = [HA_m^0]/[HA_w^0] \qquad \text{(Eq. 3)}$$

This particular method of calculation of the K_d value is of considerable use since it does not involve estimation of micellar or nonmicellar phase volumes and thus avoids the difficulties described earlier. It is, of course, only applicable to systems in which it has been demonstrated that the solubilization process is governed by a form of the distribution law.

The effect of temperature upon the bactericidal activity of a preservative in a surfactant solution depends upon the temperature coefficient of the bactericide and the variation of the K_d value with temperature (12). Bean and his co-workers have emphasized that two-phase preparations containing bactericides must be formulated from a viewpoint that considers both the physical and microbiological properties of the system.

Using an extension of the two-phase model of micellar systems, it has been suggested that solubilization by surfactant micelles is due to the volume of the hydrocarbon core of the micelle, and that the solubilization process is directly comparable to solution in an organic bulk phase (13). If this hypothesis is valid, it may be predicted that the $[HA_m^0]$ values for the various surfactants studied in this investigation will be linear functions of the percentage of hydrocarbon, *i.e.*, alkyl chain H.

$$H = 225 \times 100/M \qquad \text{(Eq. 4)}$$

where M is the surfactant molecular weight, and 225 is the contribution to the molecular weight made by the cetyl chain. Thus the value $[HA_m']$, as defined in Eq. 5, should for any given temperature be the same for all the surfactants studied:

$$[HA_m'] = [HA_m^0] \times 100/H \qquad \text{(Eq. 5)}$$

Further, the values of $d[HA_m']/dT$ should be constant for the various surfactants. From the plots of $[HA_m']$ against temperature shown in Fig. 6, it can be seen that not only are the $[HA_m']$ values

Table I—Solubilization Parameters as a Function of Temperature

Surfactant Formula	H	K_d Temp., °K. 291.0	298.0	304.0	310.0	318.0
$C_{16}E_{16}$	23.30	59.51	50.07	43.75	44.11	...[a]
$C_{16}E_{30}$	14.26	47.80	45.55	35.42	38.23	38.66
$C_{16}E_{40}$	11.15	37.07	32.72	28.76	29.90	37.06
$C_{16}E_{96}$	5.02	31.22	27.43	25.43	27.46	32.25

[a] No K_d value determined at this temperature for $C_{16}E_{16}$ because the cloud point temperature was exceeded.

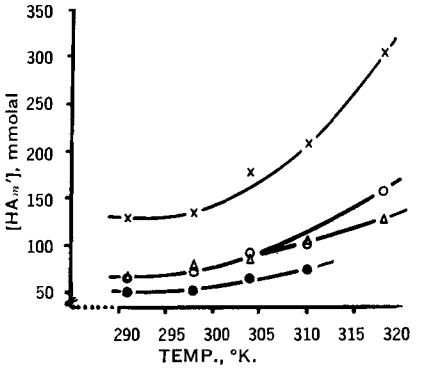

Fig. 6—Plot of [HA_m'] *as a function of temperature for four* n*-alkyl polyoxyethylene surfactants. Key:* ×, $C_{16}E_{96}$; ○, $C_{16}E_{40}$; △, $C_{16}E_{30}$; ●, $C_{16}E_{16}$.

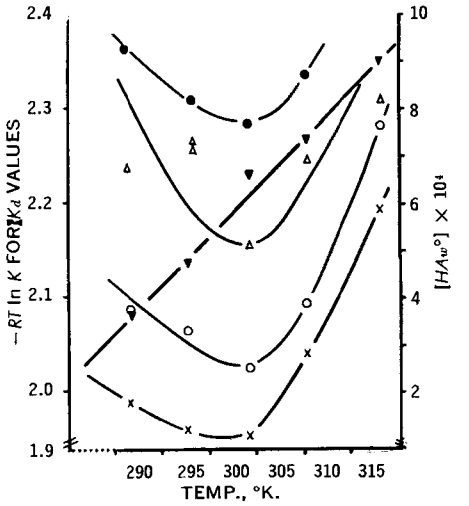

Fig. 7—Plot of −RT *ln* K *against temperature. Key:* ●, $C_{16}E_{16}$; △, $C_{16}E_{30}$; ○, $C_{16}E_{40}$; ×, $C_{16}E_{96}$; ▼, *benzoic acid, aqueous solubility.*

different for the various surfactants but also the rate of change of [HA_m'] with temperature varies. Thus the simple two-phase model appears insufficient to rationalize the solubilization phenomena in a quantitative manner. This failure may well be attributed to steric aspects of the solubilization process. Although the surfactant micelle is believed to be a loosely organized structure of transient existence, there are elements of rigidity not present in a hydrocarbon bulk phase. It has been suggested that benzoic acid and similar cosolutes are located at the junction of the hydrocarbon core and palisade layer of the micelle (14). Such an orientation provides a unique environment for the solubilized species; the hydrocarbon "tail" surrounded by the alkyl chains of the surfactant, and the carboxylic acid head group hydrogen bonded to either water molecules or polyoxyethylene groups in the palisade layer. This process cannot be envisaged entirely as a simple solution mechanism but rather as an interaction between surfactant and cosolute, *i.e.*, comicellization.

Although techniques for the calculation of the thermodynamic parameters controlling the micellization process in binary surfactant systems are well established, little attention has been directed to thermodynamic aspects of solubilization. When thermodynamic equilibrium constants for the solubilization process are available, it is possible to evaluate the free energy change of the process by use of Eq. 6:

$$\Delta G = -RT \ln K \qquad \text{(Eq. 6)}$$

Free energy changes may be resolved into two components, enthalpic and entropic values by means of Eq. 7:

$$\Delta G = \Delta H - T\Delta S \qquad \text{(Eq. 7)}$$

Thus a plot of $-RT \ln K$ against T should be linear with a slope of $-\Delta S$ and an intercept at 0°K. of ΔH. However, in order to substitute relevant ΔG values in Eq. 7, it is essential that the equilibrium being considered should exist between the same standard states. In the case of micellar solubilization it is doubtful if this criterion is fulfilled. As has been indicated previously, the uptake of solubilized material by the micelle must to some extent be a function of the micellar architecture. Since it is known that the aggregation number of surfactants, in both binary and ternary systems, varies with temperature (7), it may be concluded that micellar structure is temperature dependent and thus Eq. 7 may not be validly applied to such systems. Figure 7 shows the variation of $-RT \ln K$ as a function of T for the solubilization of benzoic acid by the four surfactants studied in this project. For comparison purposes the [HA_w°] values, solubility of benzoic acid in 0.01 N hydrochloric acid, expressed in terms of mole fraction are plotted in Fig. 7. However, plots of $-RT \ln K$, derived from the K_d values, against T show considerable curvature, all curves having a minimum at about 300°K. Though this curvature could be simply explained for equilibrium between the same standard states in terms of variation of ΔH or ΔS with temperature, it is felt that a more likely explanation is in terms of the variation of micellar structure as outlined above.

Relatively few attempts appear to have been made previously to estimate ΔH and ΔS for the micellar solubilization process from studies of the effect of temperature. Examination of the literature shows that studies of the effect of temperature upon solubilization processes made at several temperatures and over a considerable temperature range are comparatively rare. However, Bahal and Kostenbauder (15), who studied the interaction of chlorobutanol with polyvinylpyrrolidone, have shown that Eqs. 6 and 7 are obeyed by such a system.

Clearly, further studies of micellar solubilization, in particular the effect of cosolutes upon micelle size and shape, are required before a detailed theory rationalizing such interaction can be formulated.

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Keyphrases

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Equilibrium constants controlling solubilization
Micellar solubilization
Distribution coefficient
Dialysis studies
UV spectrophotometry—analysis

Synthesis and Properties of the Antileukemic Agent 5(or 4)-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide

By Y. FULMER SHEALY, CHARLES A. KRAUTH*, LEE B. HOLUM, and WILLIAM E. FITZGIBBON

The antileukemic agent 5(or 4)-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide (II, NSC-82196) is an unstable compound that is easily convertible to an isomeric transformation product. By taking appropriate precautions, however, the triazene may be prepared in good yield, with very little of the transformation product as contaminant, and may be stored for long periods at low temperatures. The quality of specimens of II may be estimated from its infrared spectrum and that of its transformation product.

In the mouse lymphoid leukemia L1210 test system (1), 5(or 4)-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide (II) has demonstrated excellent activity. Some of the animals treated with large single doses of this compound survived until the experiments were terminated 4–8 months after treatment. From the cell-kill kinetic studies of Skipper, Schabel, and Wilcox (2, 3), it may be concluded that complete eradication of leukemic cells must have occurred in these survivors. Compound II is unstable and is readily converted to an isomeric product that is not active against leukemia L1210. In a preliminary communication (1) the authors reported, without experimental details, the preparation of II from 5-diazoimidazole-4-carboxamide (I) and bis(2-chloroethyl)amine (III). In this report complete details are recorded on the preparation of II, including improvements; and some of its properties and the precautions that must be observed during the preparation, storage, and use of this compound are described more fully.

Received July 24, 1967, from the Southern Research Institute, Birmingham, AL 35205
Accepted for publication September 18, 1967.
This work was supported by contract PH43-64-51 from the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and by the C. F. Kettering Foundation.
The authors are indebted to Drs. W. J. Barrett, W. C. Coburn, Jr., and P. D. Sternglanz, Analytical and Physical Chemistry Division, of this institute for spectral determinations and elemental analyses and to Mr. H. W. Guin for technical assistance.
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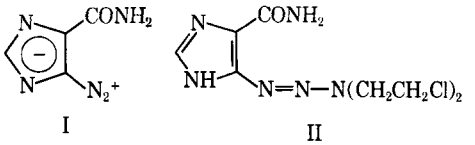

I II

$(ClCH_2CH_2)_2NH$ $[C_8H_{12}N_6OCl^+]Cl^-$

III IV

Bis(2-chloroethyl)amine free base (III) was extracted from an alkaline solution into either ethyl acetate or dichloromethane. In the procedure mentioned in the preliminary report (1) an ethyl acetate solution of III was diluted with methanol, the reaction of I and III was carried out in a 2 : 1 mixture of ethyl acetate and methanol, and the product was precipitated by the addition of a very large volume of petroleum ether (b.p. 30–60°). The preparative procedure has subsequently been improved by employing only methanol as the reaction medium (after removal of the extraction solvent from III) and by establishing an optimum temperature of about 15–20°. Compound II crystallizes directly from the reaction mixture and is obtained in higher yield (65–75%) and in improved quality without the need for large volumes of petroleum ether. Since dichloromethane is more easily volatilized, it is the preferred solvent for extracting III.

Although compound II is only slightly soluble in water and is sparingly soluble in methanol, prolonged stirring of a suspension of II in either solvent gives a solution from which a new compound is isolated. This transformation product (IV) has the same composition as II, but half of the chlorine is ionic as was shown by titration with silver nitrate. A suspension of the triazene (II) in a solution of the Bratton-Marshall reagent (4) gives a positive test upon acidification; under comparable conditions IV does not give a positive Bratton-Marshall test. Possible structures for the transformation product (IV) include aziridinium, *v*-triazolinium, and piperazinium salts formed by internal alkylation. The change of II to IV also occurs in dimethylsulfoxide solution and even in the solid state at room temperature. Infrared spectra showed, as explained below, that a considerable amount of IV had formed in a specimen of II kept at room temperature for 16 days and that II is, however, stable for at least 22 months at −15° to −20°.

Since IV has the same composition as II, elemental analyses are of value only for indicating gross contamination; however, infrared spectra of II and its potential contaminants constitute a reliable guide to the quality of the triazene product. If unreacted I is present, it can be detected by the diazo absorption band (which is very strong in pure specimens of I) at 2190 cm.$^{-1}$ (5). If the diazo band is absent but absorption is evident near 1700 cm.$^{-1}$, the product may contain 2-azahypoxanthine, a potential by-product that may be formed either directly from I (5) or from II by a light-catalyzed reaction (6). The most prominent differences between the infrared spectra of II (Fig. 1) and its transformation product (IV, Fig. 2) are as follows: the spectrum of IV shows strong bands at 1515, 1680 (C=O), and 3455–3460 (NH) cm.$^{-1}$; the spectrum of II has strong bands at 1655 (C=O) and 3380 (NH) cm.$^{-1}$. Other obvious differences are shifts of 10–15 cm.$^{-1}$ in the positions of 2 bands in the 1600–1540 cm.$^{-1}$ region, a medium band present at 742 cm.$^{-1}$ in the spectrum of II and absent from that of IV, a medium-strong doublet at 1145 and 1138 cm.$^{-1}$ in the spectrum of II, and a medium band at 768 cm.$^{-1}$ in the spectrum of IV.[1] Of these bands the first listed (1515 cm.$^{-1}$) is the most important for estimating the quality of II. Spectra of the best samples of II show very little, or no, absorption at 1510–1520 cm.$^{-1}$ (Fig. 1). In the spectra of specimens judged to contain only a very few percent of the transformation product, the 1515 cm.$^{-1}$ band can be observed as a very weak band or shoulder, whereas the 1680 cm.$^{-1}$ and 3460 cm.$^{-1}$ bands

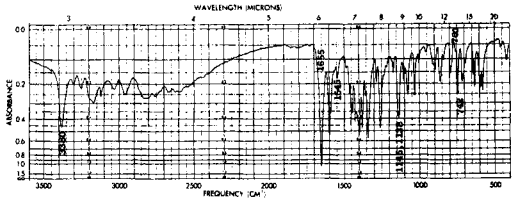

Fig. 1—Infrared spectrum of 5(or 4)-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide (II). Flattening of the curve in the 1520–1500 cm.$^{-1}$ region may indicate slight contamination with IV.

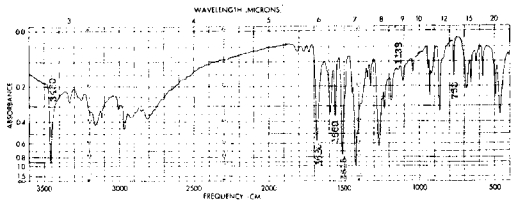

Fig. 2—Infrared spectrum of the transformation product (IV).

[1] However, weak bands at 1138 and 760 cm.$^{-1}$ in the spectra of IV and II, respectively, decrease the value of bands in these regions for detecting either IV or II in samples of the other.

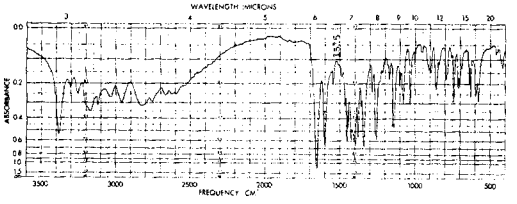

Fig. 3—Infrared spectrum of a specimen of II typical of those used for biological evaluation. The shoulder at 1515 cm.$^{-1}$ shows that a small amount of IV is present.

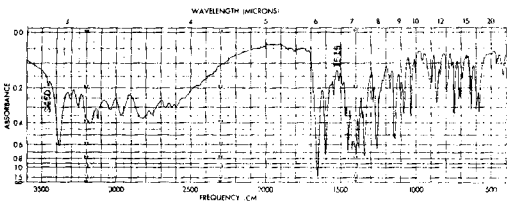

Fig. 4—Infrared spectrum of II showing further contamination with IV. The 3460 cm.$^{-1}$ band of IV appears after the 1515 cm.$^{-1}$ can be detected (cf. *Fig. 3*) *and before the carbonyl band of IV is perceptible. In this spectrum the 1545 cm.$^{-1}$ band of II has also begun to shift to the 1560 cm.$^{-1}$ band of IV.*

are not apparent. Most of the antitumor tests have been performed with samples of this type (Fig. 3). With increasing contamination, the 3460 cm.$^{-1}$ band appears on the side of the 3380 cm.$^{-1}$ band (Fig. 4). Shifting of the band near 1550 cm.$^{-1}$ and splitting of the carbonyl band are readily apparent only when considerable amounts of the transformation product are present.

Infrared spectra of crude products obtained from the ethyl acetate–methanol procedure showed that IV was usually present, the diazo compound was frequently present, and 2-azahypoxanthine was probably sometimes present. Brief and careful washing of these crude products (or of any specimens containing IV) with water at pH 7.3–7.5 reduced the amount of IV to a low concentration, as indicated by very slight absorption at 1515 cm.$^{-1}$, and removed the diazo compound (I) and 2-azahypoxanthine. This procedure may also be used, if necessary, to purify II obtained from the improved procedure. However, specimens from the improved procedure have not contained I detectable by infrared spectra, and, generally, IV has either not been detectable or has been present in quantities that produced only a slight shoulder at 1515 cm.$^{-1}$.

Specimens of II are routinely stored in brown jars that are kept in closed jars or desiccators containing a drying agent, and these containers are stored in a freezer at −15° to −20°. When specimens are to be transferred or weighed, the containers are allowed to come to room temperature before a specimen is removed in order to minimize condensation of atmospheric moisture, which might cause the formation of some of the transformation product on the surface. The infrared spectrum of a specimen stored in this way was essentially unchanged after 22 months. Solutions or suspensions for biological evaluation are prepared immediately before injection and are injected within 5 min., or less, after their preparation.

EXPERIMENTAL

Infrared spectra were recorded with a Perkin-Elmer model 521 spectrophotometer using samples in potassium bromide disks. Ultraviolet spectra were recorded with a Cary model 14 recording spectrophotometer.

5(or 4)-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4(or 5)-carboxamide (II)—*A. Preparation*—Compound II is protected, insofar as possible, from light and moisture during all operations involved in the preparation, handling, and storage. The starting material (I) is also protected from light and moisture (5).

A solution consisting of 625 Gm. of sodium chloride, 2.5 L. of distilled water, and 290 Gm., of bis(2-chloroethyl)amine hydrochloride in a 5-L. Morton flask was chilled in a salt–ice bath. Dichloromethane (1.25 L.) was added, and the mixture was stirred vigorously with a mechanical stirrer while a 50% aqueous sodium hydroxide solution was added until the pH of the mixture reached and became stable at 7.5–7.6. The sodium hydroxide solution was added at a rate that kept the temperature of the mixture below 4°. The mixture was stirred vigorously for 5–10 min., the dichloromethane layer was separated, and the water layer was extracted at the ice-bath temperature with a second 1.25-L. portion of dichloromethane. The two cold dichloromethane extracts were combined, washed quickly with 1 L. of cold saturated salt solution, and given a preliminary drying by stirring with magnesium sulfate in an ice bath for 15 min. The desiccant was removed by filtration, and the solution was dried further by stirring with calcium sulfate[2] for 1 hr. in an ice bath. Smaller additions of desiccant were made at 15-min. intervals. After the drying agent had been removed by filtration, the solvent was evaporated immediately under reduced pressure at 25–30°.

A solution of the bis(2-chloroethyl)amine free base in 1.25 L. of anhydrous methanol (which had been dried 24 hr. over calcium sulfate) was cooled to 15° in a 3-L. round-bottom flask wrapped with aluminum foil and equipped with a mechanical stirrer, a nitrogen inlet tube, and a drying tube. 5-Diazoimidazole-4-carboxamide (49.4 Gm.) was added slowly in small portions in a dry nitrogen atmosphere during 30 min., and the suspension was stirred vigorously for 3 hr. at 15–20°. The product was collected by

[2] Drierite.

filtration under a current of nitrogen, washed under nitrogen with 100 ml. of methanol and with two 100-ml. portions of acetone, and dried *in vacuo* at room temperature over phosphorous pentoxide: yield, 71 Gm. (71%). The absence of discernible absorption bands at 1515 and 2190 cm.$^{-1}$ indicated that the product contained very little, if any, transformation product (IV) or starting material (I).

Anal.—Calcd. for $C_8H_{12}Cl_2N_6O$:[3] C, 34.42; H, 4.33; N, 30.10 Found: C, 34.45; H, 4.30; N, 30.14.

The yields obtained from several experiments in which either dichloromethane or ethyl acetate was used to extract III were 65–75%. Usually absorption due to IV at 1515 cm.$^{-1}$ either was not observed or was very weak.

B. Purification—Specimens of II containing IV, the starting material (I), or 2-azahypoxanthine may be purified by washing the impure material with water adjusted to pH 7.5 with sodium hydroxide, provided that care is exercised to minimize the formation of additional IV. A mixture of the slightly basic solution and II (approximately 10 ml./Gm.) is stirred for 5–10 min. in the absence of light, and the triazene is then removed by filtration, washed with water and with 2 portions of acetone, and dried *in vacuo* over phosphorous pentoxide at room temperature. The purpose of washing with acetone is to hasten the removal of water, but most of the adhering water should be removed during the filtration because II appears to be more soluble in a mixture of acetone and water than in either solvent alone. Since the triazene is only slightly soluble in water, it is recovered in high yield from the washing operation.

Preparation of IV—A mixture of 2.5 Gm. of the triazene(II) and 250 ml. of absolute methanol was stirred in the dark for 44 hr. The mixture became homogeneous, and thin-layer chromatography (silica gel, chloroform–methanol, 1:1 by vol.) indicated that only a trace amount of II remained after 24 hr. (In this TLC system IV remains at the origin; II moves, but shows streaking owing to the formation of IV.) Although the total product from an earlier similar experiment gave satisfactory analytical data without recrystallization (1), the solution was treated with activated carbon, concentrated to about 125 ml., and diluted with an equal volume of chloroform. The yellow precipitate was recrystallized a second time from chloroform–methanol; it melted with decomposition at temperatures that varied with the determination procedure, *e.g.*, 197–199° dec., when inserted at 180° in a Mel-Temp apparatus.

Anal.—Calcd. for $C_8H_{12}Cl_2N_6O$: C, 34.42; H, 4.32; N, 30.10; total Cl, 25.4; ionic Cl, 12.7. Found: C, 34.41; H, 4.44; N, 30.02; total Cl, 25.3; ionic Cl, 12.6.

[3] Analysis of the product of an earlier experiment gave a satisfactory value for chlorine (Calcd.: 25.5%, found: 25.4%), as well as satisfactory data for carbon, hydrogen, and nitrogen (1).

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Triazene transformation product
TLC separation
IR spectrophotometry—structure
UV spectrophotometry—structure

Analysis of Data on Drug Release from Emulsions II

Pyridine Release from Water-in-Oil Emulsions as a Function of pH

By TAMOTSU KOIZUMI* and W. I. HIGUCHI

The practically important case of the release from an amine–amine hydrochloride solute mixture in a water-in-oil emulsion into an aqueous sink has been studied experimentally and theoretically. All of the parameters entering into the theory have been independently measured and put into the theory. The theoretically predicted release behavior was then determined employing the IBM 7090 computer. These results were then compared to the experimental data obtained over a wide range of conditions. The agreement between the experiments and the physical model was extremely good. These procedures should have extensive application in the future when complex solute–solute, solute–solvent, and solvent–solvent interactions are involved.

IN THE PREVIOUS paper (1), Fick's law of diffusion (Eq. 1) was used to predict medicament release from ointments:

$$\frac{\partial c}{\partial t} = \frac{\partial^2 c}{\partial x^2} \qquad \begin{matrix} t = 0 \text{ and } x > 0,\ c = C_0 \\ t > 0 \text{ and } x = 0,\ c = 0 \end{matrix} \qquad \text{(Eq. 1)}$$

Here C is the drug concentration, D is the diffusion coefficient, t is time, and x is the coordinate. Its solution (Eq. 2) was shown to agree with the experimental data in a satisfactory manner:

$$q = 2C_0\sqrt{\frac{Dt}{\pi}} \qquad \text{(Eq. 2)}$$

where q is the amount of drug released per unit area.

Among other assumptions involved in these equations is that D must be constant with respect to both time and position. This is a serious limitation, because in many situations involving emulsions the diffusion coefficient is not constant but varies with concentration.

This report deals with such cases, and an attempt is made to predict the drug release from emulsions in which the diffusion coefficient of the drug is a function of concentration. Studies such as this should be helpful in drug formulation practices.

THEORY

The Effective Diffusion Coefficient—It was reported (2) that approximate relationships between the effective permeability constant of a heterogeneous system and the permeability constants and the volume fractions of the individual phases can be obtained by considering the analogous electrostatic problem. The Wagner-Wiener equation (Eq. 3) and the Bruggeman equation (Eq. 4), which were derived originally to express the dielectric behavior of heterogeneous systems, may be employed to evaluate the effective diffusion coefficient:

$$\frac{P_e - P_c}{P_e + 2P_c} = \frac{P_i - P_c}{P_i + 2P_c} v_i \qquad \text{(Eq. 3)}$$

$$\frac{P_e - P_i}{P_c - P_i}\left(\frac{P_c}{P_e}\right)^{1/3} = v_c \qquad \text{(Eq. 4)}$$

and

$$P_j = K_j \times D_j \ (j = e, i, c) \qquad \text{(Eq. 5)}$$

where P is a permeability constant, K is a partition coefficient, D is a diffusion coefficient, and v is a volume fraction. The subscripts e, i, and c represent "effective," "internal phase," and "continuous phase," respectively. And K_e is given by Eq. 6:

$$K_e = K_i v_i + K_c v_c \qquad \text{(Eq. 6)}$$

where K_c is unity.

Solving Eqs. 3 or 4 for P_e and dividing by K_e, one obtains an expression for the effective diffusion coefficient, D_e, in the form of Eq. 7:

$$D_e = f(K_i, v_i, v_c, D_i, D_c) \qquad \text{(Eq. 7)}$$

where v_i and v_c are constants for a given system. Even when D_i and D_c are constant, D_e is a function of K_i, which may be concentration dependent.

The Partition Coefficient—K_i may change with drug concentration in various ways such as dissociation of the drug, dimerization, complex formation, *etc.* Let us now examine a situation where dissociation of the drug is involved.

It is assumed that (*a*) the drug molecule dissociates in the internal phase, (*b*) only the nonionized species dissolves in the continuous phase, and (*c*) equilibrium of nonionized species always exists between the internal and the continuous phases. The water-in-oil emulsion of pyridine hydrochloride is an example of this case. The concentration relationships among the various species for this system are: (*a*) in the internal phase:

$$K_a = \frac{(A)\,(H^+)}{(AH^+)}$$

$$C_i = (AH^+) + (A)$$

$$K_w = (H^+)\,(OH^-)$$

Received April 14, 1967, from the College of Pharmacy, University of Michigan, Ann Arbor, MI 48104

Accepted for publication August 9, 1967.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This investigation was supported by a grant from Smith Kline & French Laboratories, Philadelphia, Pa., and by grant GM13368 from the National Institute of General Medical Sciences, U. S. Public Health Service, Bethesda, Md.

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$$(H^+) + (AH^+) = CA + (OH^-)$$

(*b*) in the continuous phase:

$$C_c = (A)_c$$

(*c*) at the interface:

$$\frac{(A)_i}{(A)_c} = \frac{1}{P_0}$$

where K_a is the acid dissociation constant of drug, and C_1 and C_i are the drug concentrations in the internal phase and the continuous phase, respectively. CA is the hydrochloric acid concentration in internal phase, P_0 is the true partition coefficient of the drug (partition coefficient of the nonionized species), and K_w is the ion product for water.

Solving these equations simultaneously, one can get Eq. 8.

$$K_i = \frac{C_i}{C_c} = \frac{1}{P_0} + \frac{CA + \sqrt{CA^2 + \frac{4K_w}{P_0K_a}(C_c + P_0K_a)}}{2(C_c + P_0K_a)} \quad \text{(Eq. 8)}$$

The total drug concentration is given by Eq. 9:

$$C_e = v_c \cdot C_c + v_i \cdot C_i = (v_c + v_iK_i) \cdot C_c \quad \text{(Eq. 9)}$$

From Eqs. 8 and 9, it is clear that the partition coefficient, K_i, is a function of concentration, C_e.

The Time Dependence of the Amount of Drug Released—When the diffusion coefficient, D, is a function of concentration, C (the subscript e is dropped from D_e and C_e), the equation for one-dimensional diffusion is given by Eq. 10, instead of Eq. 1.

$$\frac{\partial C}{\partial t} = \frac{\partial}{\partial x}\left(D\frac{\partial C}{\partial x}\right) \qquad \begin{matrix} t = 0 \text{ and } 0 < x, C = C_0 \\ t > 0 \text{ and } x = 0, C = 0 \end{matrix} \quad \text{(Eq. 10)}$$

Applying Boltzman's method (3), one can obtain Eq. 11:

$$C = a\int_0^\lambda \frac{1}{D}\exp.\left(-\int_0^\lambda \frac{\lambda}{2D}\,d\lambda\right)d\lambda \quad \text{(Eq. 11)}$$

$$a = \frac{C_0}{\int_0^\infty \frac{1}{D}\exp.\left(-\int_0^\lambda \frac{\lambda}{2D}\,d\lambda\right)d\lambda} \quad \text{(Eq. 12)}$$

where $\lambda = x/\sqrt{t}$.

Then the nonsteady state drug release rate per unit area, G, is given by:

$$G = \left(D\frac{\partial C}{\partial x}\right)_{x=0} \quad \text{(Eq. 13)}$$

Integration of Eq. 13, using Eqs. 11 and 14, gives:

$$\left(D\frac{\partial C}{\partial x}\right)_{x=0} = \frac{1}{\sqrt{t}}\left(D\frac{\partial C}{\partial \lambda}\right)_{\lambda=0} \quad \text{(Eq. 14)}$$

$$q = 2a\sqrt{t} \quad \text{(Eq. 15)}$$

where q is the amount of drug released per unit area, and a is the same constant defined by Eq. 12.

These results show that, even if D is concentration dependent, the release pattern is exactly the same as the constant D case. *"Square root" relationship still holds.*

Computation of a—It is quite difficult to solve Eq. 12 and evaluate a, analytically, even though D is a known function of C. Therefore, a numerical calculation method is expedient.

If the region ($0 \sim C_0$) is divided into N equal parts, the following relationships hold:

$$C_j = a\int_0^{\lambda_j}\frac{1}{D}\exp.\left(-\int_0^\lambda \frac{\lambda}{2D}\,d\lambda\right)d\lambda \quad (j = 1, 2, \ldots N) \quad \text{(Eq. 16)}$$

$$C_N = C_0,\ \lambda_N = \infty$$

$$C_j - C_{j-1} = \frac{C_0}{N} = a\int_0^{\lambda_j}\frac{1}{D}\exp.\left(-\int_0^\lambda \frac{\lambda}{2D}\,d\lambda\right)d\lambda - a\int_0^{\lambda_{j-1}}\frac{1}{D}\exp.\left(-\int_0^\lambda \frac{\lambda}{2D}\,d\lambda\right)d\lambda \quad \text{(Eq. 17)}$$

$$(j = 1, 2, \ldots N)$$

therefore

$$\int_{\lambda_{j-1}}^{\lambda_j}\frac{1}{D}\exp.\left(-\int_0^\lambda \frac{\lambda}{2D}\,d\lambda\right)d\lambda = \frac{C_0}{aN} = \text{constant} \quad \text{(Eq. 18)}$$

$$(j = 1, 2, \ldots N)$$

The constant a is computable from these relationships. The procedure is as follows.

(*a*) Divide C_0 into N equal parts, C_j,

$$C_j = C_0 \times \frac{j}{N} \quad (j = 1, 2, \ldots N) \quad \text{(Eq. 19)}$$

(*b*) Calculate D_j, which is a known function of C_j, from C_j obtained by the previous step.

(*c*) Choose arbitrary value for λ_1, and evaluate M_1, given by Eq. 20, applying the proper numerical integration method:

$$M_1 = \int_0^{\lambda_1}\frac{1}{D}\exp.\left(-\int_0^\lambda \frac{\lambda}{2D}\,d\lambda\right)d\lambda \quad \text{(Eq. 20)}$$

With the trapezoidal rule, M_1 is given by:

$$M_1 \simeq \frac{\lambda_1}{2}\left[\frac{1}{D_0} + \frac{1}{D_1}\exp.\left(-\frac{\lambda_1^2}{4D_1}\right)\right] \quad \text{(Eq. 21)}$$

(*d*) Look for the value of λ_2 which satisfies:

$$\int_{\lambda_1}^{\lambda_2}\frac{1}{D}\exp.\left(-\int_0^\lambda \frac{\lambda}{2D}\,d\lambda\right)d\lambda = M_1 \quad \text{(Eq. 22)}$$

(*e*) Repeat the similar procedure and calculate $\lambda_3, \lambda_4 \ldots \lambda_{N-1}$ which satisfies:

$$\int_{\lambda_{j-1}}^{\lambda_j}\frac{1}{D}\exp.\left(-\int_0^\lambda \frac{\lambda}{2D}\,d\lambda\right)d\lambda = M_1 \quad \text{(Eq. 23)}$$

Applying the trapezoidal rule, integration of Eq. 23 becomes calculable:

$$\int_{\lambda_{j-1}}^{\lambda_j}\frac{1}{D}\exp.\left(-\int_0^\lambda \frac{\lambda}{2D}\,d\lambda\right)d\lambda \simeq \left(\frac{\lambda_j - \lambda_{j-1}}{2}\right)(T_j + T_{j-1})$$

$$T_j = \frac{1}{D_j}\exp.\left\{-\frac{\lambda_j^2}{4D_j} - \sum_{k=2}^{j}\frac{\lambda_{k-1}\cdot\lambda_k\cdot(D_k - D_{k-1})}{4\cdot D_k\cdot D_{k-1}}\right\} \quad \text{(Eq. 24)}$$

(f) Calculate the integration from λ_{N-1} to infinity. Assuming D is constant, D_{N-1}, between λ_{N-1} and infinity, and by applying the infinite series for the error function, one obtains Eq. 25:

$$\int_{\lambda_{N-1}}^{\infty} \frac{1}{D} \exp.\left(-\int_0^{\lambda} \frac{\lambda}{2D}\, d\lambda\right) d\lambda \simeq \frac{\sqrt{\pi}}{\sqrt{D_{N-1}}}\left(1 - \sum_{n=0}^{\infty} \frac{(-1)^n X^{2n-1}}{(2n+1)n!}\right) \exp.\left(-\sum_{n=2}^{N-1} \frac{\lambda_{n-1}\lambda_n(D_n - D_{n-1})}{4 \cdot D_{n-1} \cdot D_n}\right) \quad \text{(Eq. 25)}$$

where $X = \lambda_{N-1}/2\sqrt{D_{N-1}}$

(g) If the value obtained at (f) is equal to M_1 within the allowable accuracy, a is calculated by:

$$a = \frac{C_0}{NM_1} \quad \text{(Eq. 26)}$$

(h) If (g) is not true, modify λ_1 according to the result of the comparison, *i.e.*, when the value of (f) is greater than M_1, increase λ_1. Repeat the whole procedure from (c) to (f) until (g) becomes true.

EXPERIMENTS

In order to test the validity of the theory, the release rates of pyridine from w/o emulsions were determined.

Apparatus—The apparatus used to determine drug release from emulsions is shown in Fig. 1. Compartments A and B are made of glass. Compartment B contains 345 ml. of water (or hydrochloric acid) and behaves as a sink for releasing drug. Solution samples are taken through C with a pipet. The emulsion is placed in compartment A at zero time. M is a cellophane membrane[1] separating the emulsion from the aqueous sink. The whole set-up is immersed in a constant-temperature water bath at 30°.

Emulsion—Water-in-oil emulsions of pyridine hydrochloride were made using sorbitan sesquioleate, HLB 3.7,[2] as emulsifier and hexadecane as the oil phase. Fifteen milliliters of a 2% solution of pyridine in water or in hydrochloric acid (0.063 N, 0.084 N, 0.126 N, 0.189 N, and 0.252 N) was added to a 50-ml. test tube containing 5 ml. of hexadecane and the weighed amount of sorbitan sesquioleate (0.64 Gm.). Stoppered tightly, the test tube was shaken vigorously for 20 min. This procedure gave a thick, stable emulsion, and the type of the emulsion was determined to be w/o by electric conductivity.

Ten milliliters of the emulsion was put into compartment A of the cell, and the release of pyridine was determined as a function of time. To make the sink more effective, the same concentrations of hydrochloric acid as the internal phase of emulsions were used in the B compartment of the cell. Samples of the solution were taken at predetermined periods. Drug content was determined by UV absorbance measurement (at 255 mμ). Amount of drug released, Q, was plotted against the square root of time. Results are shown in Fig. 2.

Diffusion Coefficient of Pyridine in Homogeneous System—Diffusion coefficients of pyridine in hexadecane, D_c, and in water, D_i, were determined with the apparatus (above) applying Eq. 2 for calculation. A small amount of glass wool was used in compartment A in order to prevent convection currents setting up (when the emulsions were used, the viscosities were high enough to prevent convection and such precautions were unnecessary). The amount of drug released, Q, was plotted against square root of time. Results are shown in Fig. 3. Diffusion coefficients were calculated from the slope of the straight line of Fig. 3 by Eq. 27:

$$D = \left(\frac{\text{slope}}{2 \cdot C_0 \cdot A}\right)^2 \pi \quad \text{(Eq. 27)}$$

[1] Visking Co., Chicago, Ill.
[2] Arlacel C, Atlas Chemical Co., Wilmington, Del.

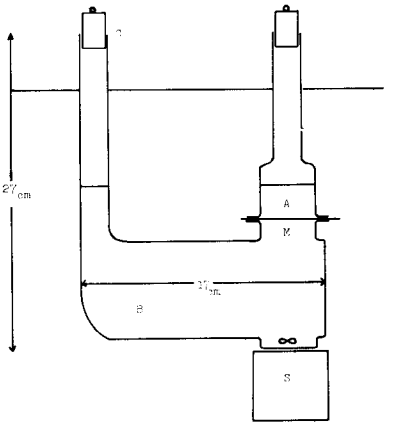

Fig. 1—Apparatus for drug release from emulsions. See text for discussion.

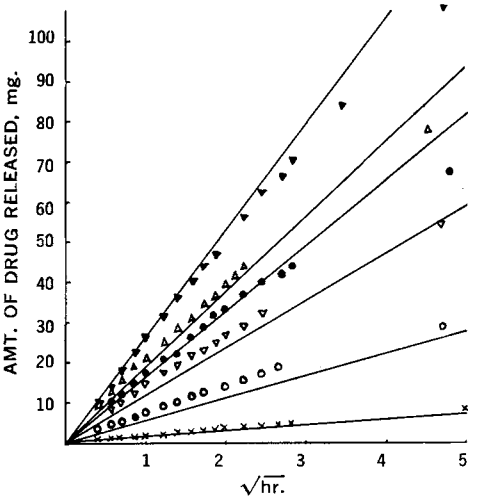

Fig. 2—Comparison of experimental data with theory. Amount of pyridine released from w/o emulsion vs. *square root of time. Key:* ▼, △, ●, ▽, ○, *and* × *represent* CA = *0, 0.063, 0.084, 0.126, 0.189, and 0.252 mole/L., respectively. Straight lines are theoretical values computed using Brug_eman's equation.* V_c = *0.25;* V_i = *0.75;* P_0 = *0.570;* $D_c = 1.42 \times 10^{-5}$ *cm.²/sec.;* $D_i = 1.19 \times 10^{-5}$ *cm.²/sec.;* C_0 = *0.189 mole/L.;* $K_a = 5.89 \times 10^{-6}$ *mole/L.*

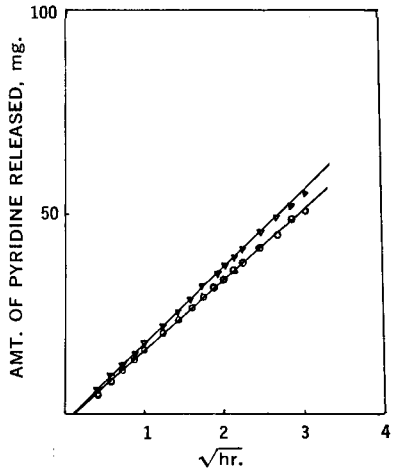

Fig. 3—Amount of pyridine released from homogeneous solutions. Key: ▽, hexadecane; ○, water. Cross-sectional area of cell, A, is 7.54 cm.².

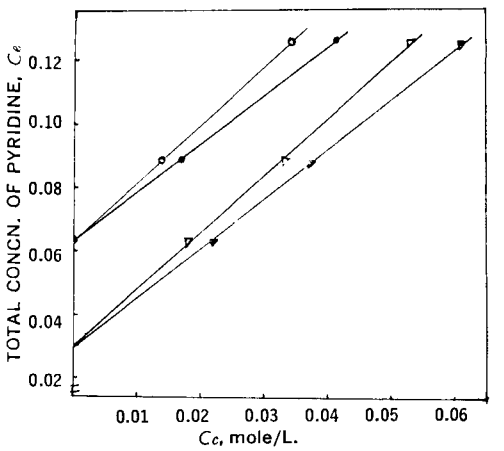

Fig. 4—Plots of C_e vs. C_c. *See text. Key:* ○, ●, CA = *0.126 mole/L.;* △,▲, CA = *0.063 mole/L.;* ○,△, *sorbitan sesquioleate 0.16 Gm./10 ml. hexadecane;* ●,▲, *sorbitan sesquioleate 0.64 Gm./10 ml. hexadecane;* $V_i = V_c = 0.5$.

where A is the cross-sectional area (cm.²) of the diffusion compartment and a constant for a given cell. A was determined from the release rate of 0.05 N hydrochloric acid for which the diffusion coefficient is known to be 2.93×10^{-5} cm.²/sec. at 25° and 3.61×10^{-5} cm.²/sec. at 35° (4). A value of 1.42×10^{-5} cm.²/sec. was obtained for the diffusion coefficient of pyridine in hexadecane, D_c, at 30°, and 1.19×10^{-5} cm.²/sec. was obtained for pyridine in water or in 0.252 N HCl (1% pyridine), D_i, at 30°.

Partition Coefficient—*Absence of Surfactant*—Ten milliliters of the aqueous solution of pyridine was added to a 50-ml. centrifuge tube, containing 10 ml. of hexadecane, which was stoppered tightly and shaken vigorously until equilibrium was reached. The mixture was centrifuged and the aqueous phase was separated from the oil phase. The pyridine contents of both phases were determined as well as the pH of the water phase. The concentration of the nonionized species in the water phase was calculated from the pH and the acid dissociation constant. The partition coefficient of the nonionized species, P_0, was calculated from the results. The data are shown in Table I.

Presence of Surfactant (5)—When a surfactant which is necessary for making the emulsion is present, its effects have to be taken into account. However, it is difficult to define K_i exactly in this situation.

For the practical purpose, however, it is convenient and reasonable to assume that the surfactant changes P_0, but nothing else, by solubilizing more pyridine into the hexadecane phase. Therefore:

$$P_0^* = P_0 + K(S) \qquad \text{(Eq. 28)}$$

where P_0^* is the partition coefficient of nonionized species in the presence of a surfactant. Here (S) is the surfactant concentration and K is constant. Because of the low solubility of sorbitan sesquioleate in water, all of the sorbitan sesquioleate used to make the emulsion is assumed to exist in the hexadecane phase.

Ten milliliters of the aqueous solution of pyridine was added to a 50-ml. centrifuge tube containing 10 ml. of hexadecane and the weighed amount (0.64 Gm.) of sorbitan sesquioleate. Stoppered tightly, the tube was shaken until equilibrium was reached. When the surfactant was used, centrifugation (7,000 r.p.m., 5 hr.) separated the oil phase but did not clear the water phase. Consequently, the determination of pyridine in the water phase was difficult.

In Eq. 8 as $4K_w/P_0K_a$ $(C_c + P_0K_a)$ is less than 10^{-7} mole/L. and negligible compared to C_A, which is greater than 6.3×10^{-2} mole/L., Eq. 29 may be used instead of Eq. 8.

$$K_i = \frac{C_i}{C_c} = \frac{1}{P_0} + \frac{C_A}{C_c + P_0K_a} \qquad \text{(Eq. 29)}$$

Table I—Partition Coefficient, P_0, of Pyridine Between Hexadecane and Water in the Absence of Surfactant

	Pyridine Concn. in Water Phase, mole/L.	Pyridine Concn. in Hexadecane Phase, mole/L.	pH of Water Phase	Nonionized[b] Pyridine Concn. in Water Phase, mole/L.	P_0 Partition Coefficient of Nonionized Species
1[a]	0.1095	0.0141	5.10	0.0418	0.337
2[a]	0.1106	0.0142	5.10	0.0422	0.336
3	0.0188	0.0058	6.20	0.0163	0.356
4	0.0895	0.0287	6.66	0.0820	0.350
5	0.1799	0.0587	6.87	0.1765	0.333

[a] Water phase is 0.063 N HCl. [b] Nonionized concentration = total concn. in water × $[K_a/K_a + (H^+)]$, $K_a = 5.89 \times 10^{-6}$ mole/L.

TABLE II—PARTITION COEFFICIENT, P_0^*, OF PYRIDINE BETWEEN HEXADECANE AND WATER IN THE PRESENCE OF SURFACTANT

Sorbitan Sesquioleate Concn. (S) Gm./10 ml. Hexadecane	V_i	V_c	HCl Concn. in Water Phase CA mole/L.	Slope	$P_0^* V_i/$ Slope $- V_c$	$P_0^* - P_0$[a]	K
0.16	0.500	0.500	0.063	1.84	0.37	0.03	0.19
0.16	0.500	0.500	0.126	1.86	0.37	0.03	0.19
0.64	0.500	0.500	0	1.64	0.44	0.10	0.16
0.64	0.500	0.500	0.063	1.61	0.45	0.11	0.17
0.64	0.500	0.500	0.126	1.55	0.48	0.14	0.22
1.28	0.667	0.333	0.063	1.53	0.56	0.22	0.17

[a] 0.34 was used as the value of P_0 (*cf.* Table I).

Therefore:

$$C_e = \left(v_c + \frac{v_i}{P_0}\right) C_c + \frac{v_i C_c C_A}{C_c + P_0 K_a} \qquad \text{(Eq. 30)}$$

Equation 30 shows that when C_c is large enough compared to P_0K_a, the relationship between C_c and C_e is linear. The slope is $v_c + v_i/P_0$, and the intercept is v_iC_A.

The pyridine concentration in the hexadecane phase, C_c, was determined and plotted against the total concentration, C_e. Figure 4 shows the results. P_0^* was calculated from the slope of the straight line, namely:

$$P_0^* = \frac{v_i}{\text{slope} - v_c} \qquad \text{(Eq. 31)}$$

The P_0^* values at various surfactant concentrations are shown in Table II. K is fairly constant at the different concentrations of surfactant, and therefore Eq. 28 holds reasonably well in this situation. The average value of K, 0.18, was used for the theoretical computations of pyridine release rate from emulsion.

Computation of the Theoretical Slopes—The computations were executed by the IBM 7090 digital computer. The flow chart is shown in Fig. 5. Results are in Table III and are shown also in Fig. 2.

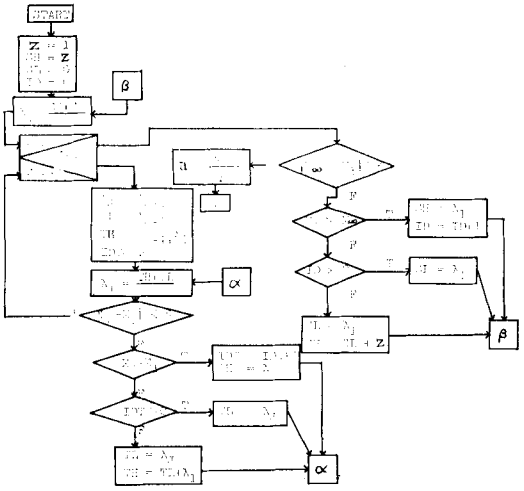

Fig. 5—Flow diagram showing the procedure for the computation of a. ϵ = *accuracy allowance (allowable error);* M_1 = *calculated value of Eq. 21;* M_J = *calculated value of Eq. 24;* M_∞ = *calculated value of Eq. 25.*

Discussion—The relatively good linearity of the data shown in Fig. 2 supports the validity of "square root" relationship for the variable diffusion coefficient case. Leveling off of the curve at large t is due to the finite thickness of emulsion in the diffusion compartment.

In Fig. 3, which shows the release of pyridine from homogeneous solution, the curve did not start from the origin but gave a short lag time. It is believed that this time lag of 0.6 min. was caused by the initial diffusion of pyridine in the membrane as well as by the initial absorption of pyridine by the membrane. Details of the lag time phenomenon are discussed under *Appendix*.

The Bruggeman equation (Eq. 4) appears to be better than the Wagner-Wiener equation (Eq. 3) for estimating effective diffusion coefficient in the present situation.

Considering the fact that the theoretical slope is computed from independently determined parameters, agreement of theoretical and experimental results is extremely satisfactory.

From these results, it is obvious that quantitative theory is quite useful and should have extensive applications in the future.

APPENDIX

One of the time-lag phenomena that is involved in the experimental situations of this report is the well-known lag time of Barrer (6) which is a measure of the period required for the absorption of the drug by the membrane.

TABLE III—THEORETICAL VALUES OF a[a]

CA mole/L.	N	60	80	100
0	B	0.022318	0.022321	0.022323
	W	0.022311	0.022313	0.022315
0.063	B	0.015728	0.015752	0.015764
	W	0.015106	0.015132	0.015146
0.084	B	0.013720	0.013749	0.013760
	W	0.012978	0.013003	0.013014
0.126	B	0.0099014	0.0099215	0.0099319
	W	0.0089659	0.0089859	0.0089960
0.189	B	0.0046754	0.0046931	0.0047020
	W	0.0036538	0.0036719	0.0036809
0.252	B	0.0012180	0.0012220	0.0012244
	W	0.00048505	0.00048687	0.00048798

[a] The parameters are: $D_c = 1.42 \times 10^{-5}$ cm.²/sec., $C_0 = 0.189$ mole/L., $D_i = 1.19 \times 10^{-5}$ cm.²/sec., $K_a = 5.89 \times 10^{-6}$ mole/L., $P_0^* = 0.570$, B = Bruggeman equation, W = Wagner-Wiener equation.

Barrer's lag time is given by:

$$\tau_B = \frac{h^2}{6D} \qquad \text{(Eq. 32)}$$

where h is the thickness of the membrane and D is diffusion coefficient.

Another lag time is present because the diffusion coefficient of the drug in the membrane is smaller than that in the bulk.

Let D_1 be the diffusion coefficient in the membrane, the thickness of which is h, and D_2 that in the bulk. Also, let C_1 be the concentration in the membrane and C_2 in the bulk. Then assume that the diffusion is expressed by the following equations:

$$\text{at } t = 0 \qquad C_1 = C_0 \qquad -h \leq x \leq 0$$
$$C_2 = C_0 \qquad 0 \leq x$$
$$\text{at } t > 0 \qquad \frac{\partial C_1}{\partial t} = D_1 \frac{\partial^2 C_1}{\partial x^2} \qquad -h < x < 0$$
$$\frac{\partial C_2}{\partial t} = D_2 \frac{\partial^2 C_2}{\partial x^2} \qquad 0 < x$$
$$D_1 \frac{\partial C_1}{\partial x} = D_2 \frac{\partial C_2}{\partial x} \text{ and } C_1 = C_2$$
$$\text{at } x = 0 \text{ and } C_1 = 0 \text{ at } x = -h$$

Solutions to these equations with the indicated boundary conditions are given by Carslaw (7).

The amount of drug released from this system, q, is given by Eq. 33:

$$q = \int_0^t \left(D_1 \frac{\partial C_1}{\partial x}\right)_{x=-h} dt = \int_0^t \times \frac{C_0\sqrt{D_1}}{\sqrt{\pi t}} \left\{1 + 2 \sum_{n=1}^{\infty} \alpha^n \exp. -n^2h^2/D_1t\right\} dt \qquad \text{(Eq. 33)}$$

where $\alpha = \dfrac{\sqrt{D_2} - \sqrt{D_1}}{\sqrt{D_2} + \sqrt{D_1}}$

At large t, Eq. 34 is obtained:

$$q = \frac{2C_0\sqrt{D_2}}{\sqrt{\pi}} \left\{\sqrt{t} - \frac{h\sqrt{\pi}}{2\sqrt{D_2}} \left(\frac{D_2}{D_1} - 1\right)\right\} \qquad \text{(Eq. 34)}$$

Therefore the lag time due to slow membrane diffusion, τ_M, is expressed by:

$$\tau_M = \frac{h^2\pi}{4D_2} \left(\frac{D_2}{D_1} - 1\right)^2 \qquad \text{(Eq. 35)}$$

From steady-state diffusion experiments of pyridine through a cellophane membrane (other than those reported in this paper), a value of 3.43×10^{-4} cm./sec. was obtained for the value of D_1/h. If the thickness of the cellophane membrane is 0.015 cm., τ_B is calculated to be 3 sec., and τ_M to be 39 sec.

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Keyphrases

Pyridine release from emulsions
pH effect on pyridine release—w/o emulsions
Diffusion coefficient—homogeneous system
Partition coefficient—pyridine
Diffusion coefficient equations
Computer flow chart—theoretical slopes for pyridine release

Analysis of Data on Drug Release from Emulsions III

Boundary Effect on Pyridine Release from Water-in-Oil Emulsions as a Function of pH

By TAMOTSU KOIZUMI* and W. I. HIGUCHI

The influence of a membrane separating an unstirred emulsion from a well-stirred sink upon the drug release rate has been studied experimentally and theoretically. Experimental data have been compared to theory. Numerical methods were employed in the analysis with the help of the IBM 7090 computer.

IN THE PREVIOUS paper (1) the release of drug from an amine–amine hydrochloride solute mixture in a water-in-oil emulsion into an aqueous sink was studied experimentally and theoretically. In the treatment of the theory it was assumed that the sink is perfect and that the drug concentration at the emulsion–sink interface is always maintained at zero. This model is shown in Fig. 1. While this assumption was reasonable for the experimental conditions of that study, it may not necessarily be always true when a membrane is used to separate an emulsion from an aqueous sink as is frequently done in studies of this kind.

Another model is proposed in this report. This is illustrated in Fig. 2. The region, $x < -h$, is the aqueous sink which is not perfect for the emulsion because of the membrane of thickness h, which acts as a barrier and separates the emulsion from the sink. Steady-state diffusion may be assumed in the region $-h < x < 0$ characterized by an effective diffusion coefficient, D_m. The region $0 \leq x \leq 1$ is the emulsion for which the effective diffusion coefficient may be calculated by either the Bruggeman or the Wagner-Wiener equation as was done previously (1).

The following appropriate partial differential equations for one-dimensional diffusion may be written:

at $x = 1$ and for all t,

$$\frac{\partial C_e}{\partial x} = 0 \qquad \text{(Eq. 1)}$$

At $0 \leq x \leq 1$ and for $t = 0$,

$$C_e = C_0 \qquad \text{(Eq. 2)}$$

and for $t > 0$,

$$\frac{\partial C_e}{\partial t} = \frac{\partial}{\partial x}\left(D_e \frac{\partial C_e}{\partial x}\right) \qquad \text{(Eq. 3)}$$

At $x = 0$ and for all t,

$$C_m = K_m \cdot C_e \qquad \text{(Eq. 4)}$$

and for $t > 0$,

$$D_e \frac{\partial C_e}{\partial x} = D_m \left\{\frac{(C_m)_{x=0} - (C_m)_{x=-h}}{h}\right\} \qquad \text{(Eq. 5)}$$

At $-h \leq x \leq 0$ and for all t,

$$\frac{\partial C_m}{\partial x} = \frac{(C_m)_{x=0} - (C_m)_{x=-h}}{h} \qquad \text{(Eq. 6)}$$

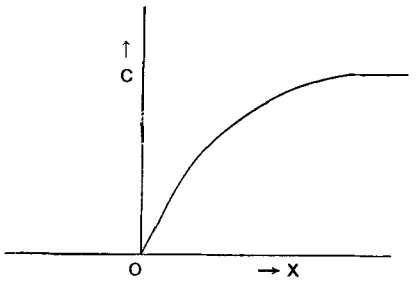

Fig. 1—Boundary conditions used in the previous report.

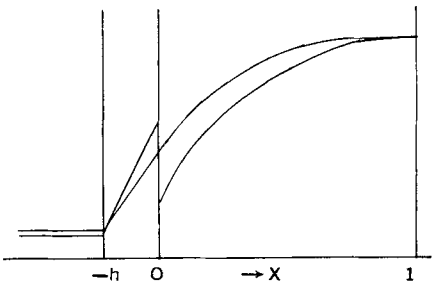

Fig. 2—Boundary conditions used in this report. Key: continuous curve represents the case where the partition coefficient between emulsion and membrane is unity; discontinuous curve is the case where the partition coefficient is not unity.

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This investigation was supported by a grant from Smith Kline & French Laboratories, Philadelphia, Pa., and by grant GM13368 from the National Institute of General Medical Sciences, U. S. Public Health Service, Bethesda, Md.

* Leave of absence from: Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan.

At $x = -h$ and for $t = 0$,

$$C_m = 0 \qquad \text{(Eq. 7)}$$

and for $t > 0$,

$$C_m = f(t) \qquad \text{(Eq. 8)}$$

Here C_e is the drug concentration in the emulsion, C_m is the drug concentration in the membrane, D_e is the effective diffusion coefficient which is a known function of C_e, and K_m is the apparent partition coefficient between the emulsion and the membrane. When the sink has a finite volume V_0, then $f(t)$ is given by:

$$f(t) = \frac{Q}{V_0} \qquad \text{(Eq. 9)}$$

where Q is the amount of drug released to the sink in time, t.

For obtaining numerical solutions, Crank (2) recommends the introduction of a new variable, S, when the diffusion coefficient is concentration dependent. This S is defined by Eq. 10:

$$S = \frac{\int_0^{C_e} D_e dC_e}{\int_0^{C_0} D_e dC_e} \qquad \text{(Eq. 10)}$$

With this new variable Eqs. 1 to 4 become Eqs. 11 to 14:

At $x = 1$, for all t,

$$\frac{\partial S}{\partial x} = 0 \qquad \text{(Eq. 11)}$$

At $o \leq x \leq 1$, for $t = 0$,

$$S = 1 \qquad \text{(Eq. 12)}$$

and for $t > 0$,

$$\frac{\partial S}{\partial t} = D_e \frac{\partial^2 S}{\partial x^2} \qquad \text{(Eq. 13)}$$

At $x = 0$, for $t > 0$,

$$\frac{\partial S}{\partial x} = \frac{D_m\{(C_m)_{x=0} - (C_m)_{x=-h}\}}{h \int_0^{C_0} D_e dC_e} \qquad \text{(Eq. 14)}$$

Applying the finite-difference method (3) to Eqs. 11 to 14, one can obtain the following equations:

At $x = 1$,

$$S_{n-1}{}^{+} = (S_n{}^{+} - S_n) \cdot \frac{2}{(D_n{}^{+} + D_n)\frac{\Delta t}{(\Delta x)^2}} + (S_n{}^{+} + S_n) - S_{n-1} \qquad \text{(Eq. 15)}$$

At $0 < x < 1$,

$$S_{i-1}{}^{+} = (S_i{}^{+} - S_i)\frac{2(\Delta x)^2}{(D_i{}^{+} + D_i)\Delta t} + 2(S_i{}^{+} + S_i) - (S_{i+1}{}^{+} + S_{i+1}) - S_{i-1} \qquad \text{(Eq. 16)}$$

At $x = 0$,

$$\frac{-S_2 + 4S_1 - 3S_0}{2(\Delta x)} = \frac{D_m\{(C_m)_{x=0} - (C_m)_{x=-h}\}}{h \int_0^{C_0} D_e dC_e} \qquad \text{(Eq. 17)}$$

Here the range 0 to 1 was divided into a number of equal intervals, Δx, and the time into intervals of Δt. S_{i-1}, S_i, S_{i+1} are the S values at $(i-1)\Delta x$, $i\Delta x$, $(i+1)\Delta x$, respectively, at $t = m\Delta t$, and $S_{i-1}{}^{+}$, $S_i{}^{+}$, $S_{i+1}{}^{+}$ are the corresponding values at $x = (i-1)\Delta x$, $i\Delta x$, $(i+1)\Delta x$ at time $(m+1)\Delta t$.

Similarly, $D_i{}^{+}$ and D_i are effective diffusion coefficients corresponding to the concentration at the point $i\Delta x$ at $t = (m+1)\Delta t$ and $t = m\Delta t$, respectively.

THE COMPUTATION PROCEDURE

The procedure for computation is as follows.

(*a*) Divide the emulsion thickness, 1, into n equal lengths of Δx and divide time into equal intervals of Δt.

(*b*) Set $S_i = 1$ (i = o to n)—initial condition.

(*c*) Choose an arbitrary value (between 0 and 1) of $S_n{}^{+}$.

(*d*) Calculate C_e corresponding to the value of $S_n{}^{+}$ chosen at (*c*). Equation 10 may be used for this calculation.

(*e*) Calculate the effective diffusion coefficient corresponding to the concentration, C_e, just calculated at (*d*). This is $D_n{}^{+}$.

(*f*) Calculate $S_{n-1}{}^{+}$ by Eq. 15.

(*g*) Calculate C_e corresponding to $S_{n-1}{}^{+}$, and then $D_{n-1}{}^{+}$.

(*h*) Calculate $S_{n-2}{}^{+}$ by Eq. 16.

(*i*) Repeat (*g*) and (*h*) until S_0 is calculated.

(*j*) Calculate C_e corresponding to S_0, *i.e.*, $(C_e)_{x=0}$. Calculate $(C_m)_{x=0}$ using Eq. (4), and also calculate $(C_m)_{x=h}$ using Eqs. 8 and 9, where:

$$Q = k \int_0^1 (C_0 - C_e)dx \qquad \text{(Eq. 18)}$$

k = cross-sectional area of the emulsion layer.

(*k*) Calculate separately the lefthand side and the righthand side of Eq. 17 and compare to see whether Eq. 17 holds.

(*l*) If the calculated values of the left and right sides of the equation are consistent within the range of error allowed, the value of Q obtained at (*j*) is the amount of drug released at $t = \Delta t$.

(*m*) If (*l*) is not the case, modify the value of $S_n{}^{+}$ depending on the results of (*k*). Then repeat steps from (*d*) to (*k*), until Eq. 17 holds. When Eq. 17 is obeyed, the most recent value of (Q) is the amount of drug released by $t - \Delta t$.

(*n*) Replace all values of S_i by the new values of $S_i{}^{+}$ obtained, and repeated procedure (*c*) to (*m*) until the desired time ($t = m\Delta t$) is reached. Here m is the number of times step (*n*) is repeated.

THE DETERMINATION OF D_m/h

Figure 3 is the schematic model of the apparatus which was used to determine the D_m/h value.

Compartments 1 and 2 were stirred, and diffusion took place inside the membrane only. At $t = 0$, $C_1 = C_0$ and $C_2 = 0$.

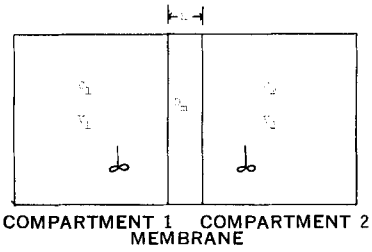

Fig. 3—Schematic model of apparatus for determination of D_m/h.

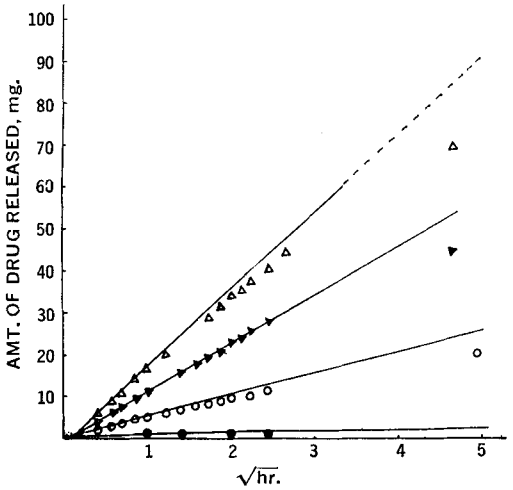

Fig. 4—Comparison of experimental data with theory. Amount of pyridine released from w/o emulsion vs square root of time. Sink is plain water, △, ▼, ○, and ● represent $C_A = 0.063, 0.126, 0.189,$ *and* 0.252 *mole/L., respectively. Solid lines are theoretical value computed by Bruggeman equation.* $V_c = 0.25$; $V_i = 0.75$; $P_o = 0.570$; $D_c = 1.42 \times 10^{-5}$ *cm.²/sec.*; $D_i = 1.19 \times 10^{-5}$ *cm.²/sec.*; $C_o = 0.189$ *mole/L.*; $K_a = 5.89 \times 10^{-6}$ *mole/L.*; $\Delta x = 0.1327$ *cm.*; $\Delta t = 360$ *sec.*; $D_m/h = 3.43 \times 10^{-4}$ *cm./sec.*

Conservation of mass gives Eq. 19 for this situation:

$$V_1C_1 + V_2C_2 = V_1C_0 = (V_1 + V_2)C_0' \quad \text{(Eq. 19)}$$

Assuming steady-state diffusion, Fick's first law gives:

$$\frac{d(V_2C_2)}{dt} = K\left(\frac{D_m}{h}\right)(C_1 - C_2) \quad \text{(Eq. 20)}$$

From Eqs. 19 and 20, Eq. 21 is obtained:

$$\log\left(\frac{C_0^1 - C_2}{C_0^1}\right) = -\frac{K(V_1 + V_2)}{2.303\ V_1 \cdot V_2}\left(\frac{D_m}{h}\right)t \quad \text{(Eq. 21)}$$

where C_1 and C_2 are the drug concentrations in compartments 1 and 2, respectively. V_1 and V_2 are the volumes of compartments 1 and 2, and K is area of the membrane.

When the logarithm of $(C_0^1 - C_2)/C_0^1$ is plotted against time, a straight line will be obtained. The value of D_m/h is then calculated from the slope of the line.

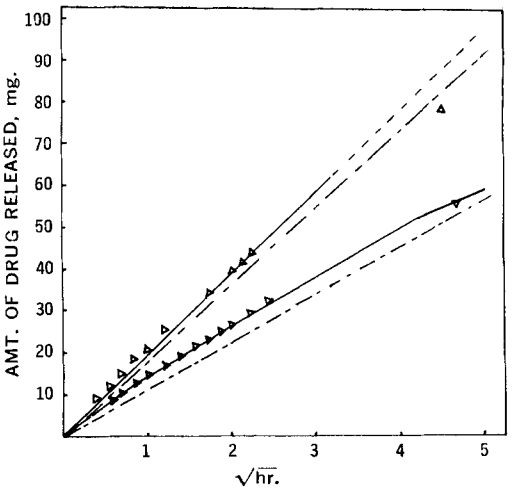

Fig. 5—Comparison of experimental data with theory. Amount of pyridine released from w/o emulsion vs. square root of time. Sink is hydrochloric acid, the concentration of which is equal to C_A. △ *and* ▽ *represent* $C_A = 0.063$ *and* 0.126 *mole/L., respectively. Key: ——, theoretical values computed by this method; - - -, theoretical values computed by the method of the previous report.* $V_c = 0.25$; $V_i = 0.75$; $P_o = 0.570$; $D_c = 1.42 \times 10^{-5}$ *cm.²/sec.*; $D_i = 1.19 \times 10^{-5}$ *cm.²/sec.*; $C_o = 0.189$ *mole/L.*; $K_a = 5.89 \times 10^{-6}$ *mole/L.*; $\Delta x = 0.1327$ *cm.*; $\Delta t = 360$ *sec.*; $D_m/h = 3.43 \times 10^{-4}$ *cm./sec.*

TABLE I—DATA FROM STEADY-STATE DIFFUSION EXPERIMENTS[a]

Time, min.	C_2 (10^{-3} mole/L.)	$(C_0^1 - C_2)/C_0^1$
0	0	1.0000
10	0.078	0.9696
20	0.141	0.9450
30	0.195	0.9240
45	0.287	0.8881
60	0.360	0.8596
90	0.515	0.7992
120	0.661	0.7423
150	0.795	0.6901
180	0.927	0.6386

[a] $C_0 = 5.643 \times 10^{-3}$ mole/L., $V_1 = 100$ ml., $C_0^1 = 2.565 \times 10^{-3}$ mole/L., $V_2 = 120$ ml.

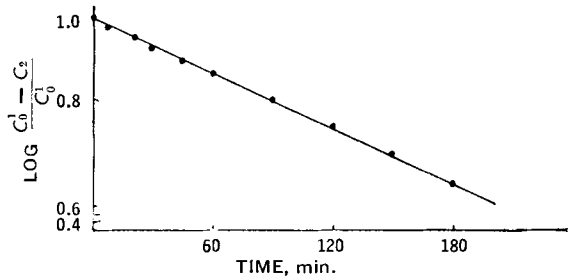

Fig. 6—Plots of log $\left(\frac{C_0^1 - C_2}{C_0^1}\right)$ vs. t. *See text.*

EXPERIMENTAL

The apparatus, the diffusion coefficients in homogeneous solution, and the partition coefficients were the same as reported previously (1). In order to

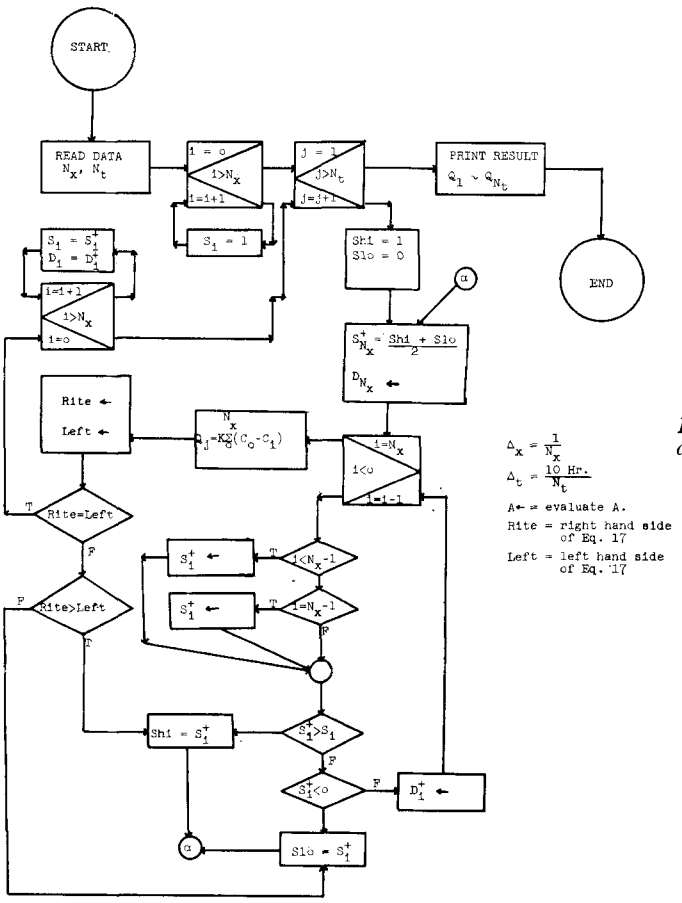

Fig. 7—Flow diagram showing the procedure for the computation of theoretical values.

TABLE II—THEORETICAL VALUES OF THE AMOUNT OF PYRIDINE RELEASED[a]

Time, hr.	C_A = 0.063 mole/L.	C_A = 0.126 mole/L.	C_A = 0.189 mole/L.	C_A = 0.252 mole/L.
0	0	0	0	0
1.0	0.1134591	0.07203754	0.03303765	0.01795251
2.0	0.1650710	0.1043185	0.04752852	0.02280451
3.0	0.2046400	0.1290364	0.05863856	0.02663112
4.0	0.2379512	0.1498451	0.06798362	0.02996549
5.0	0.2672553	0.1681557	0.07620181	0.03368697
6.0	0.2936981	0.1846839	0.08362651	0.03694266
7.0	0.3179419	0.1998395	0.09043241	0.03987066
8.0	0.3403976	0.2138898	0.09677280	0.04255156
9.0	0.3613336	0.2270172	0.1027032	0.04503749
10.0	0.3809442	0.2393349	0.1082879	0.04736435

[a] Expressed as the fraction of total amount in the system. Sink is plain water.

TABLE III—THEORETICAL VALUES OF THE AMOUNT OF PYRIDINE RELEASED[a]

Time, hr.	C_A = 0.063 mole/L.	C_A = 0.126 mole/L.
0	0	0
1.0	0.1305398	0.09348550
2.0	0.1835966	0.1267081
3.0	0.2239798	0.1520191
4.0	0.2579157	0.1732450
5.0	0.2877549	0.1919180
6.0	0.3146762	0.2087835
7.0	0.3393585	0.2242616
8.0	0.3622198	0.2386266
9.0	0.3835348	0.2520417
10.0	0.4034949	0.2646448

[a] Expressed as the fraction of total amount in the system. Sink is hydrochloric acid with concentration equal to C_A.

make the sink more ineffective, plain water was used instead of hydrochloric acid in the compartment B of the cell.

The amount of drug released, Q, was plotted against the square root of time. Results are shown in Fig. 4.

Determination of D_{mh}—The same apparatus as that used in the release experiments was used except both the inside of compartment B and compartment A were stirred, the latter by a screw-type stirrer attached to a motor driven glass rod. The following conditions were used: $C_0 = 5.643 \times 10^{-3}$ M, $V_1 = 100$ ml., $V_2 = 120$ ml., $K = 6.16$ cm.^{2}, and $C_0^1 = 2.565 \times 10^{-3}$ M.

Samples were taken from compartment 2 at predetermined intervals. Concentrations were

determined by UV absorbance. Results are shown in Table I. The logarithm of $(C_0^1 - C_2)/C_0^1$ was plotted against time, and Fig. 6 was obtained. The slope of the straight line was calculated to be 0.0605 hr.$^{-1}$, and D_m/h to be 1.234 cm./hr. or 3.43 $\times$ 10^{-4} cm./sec. This same value was also used under *Appendix* of the previous report (1).

The Computation of Theoretical Values—The computations were executed by the help of the IBM 7090 digital computer. The simplified flow chart is shown in Fig. 7. Results are in Tables II and III and shown in Figs. 4 and 5.

Discussion—The excellent agreement of computed values and experimental results can be seen in Figs. 4 and 5.

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Diffusion equations, one-dimensional
Computer flow chart—theoretical values of pyridine release

Chemically Defined Medium for the Production of *Fusarium graminearum*

By ROBERT D. IMHOLTE and L. C. SCHRAMM*

A chemically defined medium for production of *Fusarium graminearum* has been developed and modified. A series of carbon and nitrogen sources was studied, resulting in the selection of glucose and ammonium succinate as primary substrates. Using the economic coefficient as a criterion for efficiency, the optimum concentrations for glucose and succinic acid were 3 and 0.5 percent, respectively. Growth weight and pH changes as well as glucose, nitrogen, and succinic acid utilization were determined during a typical fermentation period.

FUSARIUM GRAMINEARUM is the imperfect stage of *Gibberella zea* [*G. saubinetti* (Mont.) Sacc.], a plant pathogen causing stalk, root, and ear rot of corn and scab disease on wheat and barley. This organism has been classified in the order Hypocreales of the class ascomycetes. Ingestion of scabbed grains by domestic animals having simple stomachs elicits digestive system disturbances, vomiting, and in extreme cases, death (1–3). Bread made from scabbed grains has been called "intoxicating bread." Recent work on the fungus has resulted in the isolation of an anabolic uterotrophic compound (4, 5). Most investigators concerned with *F. graminearum* metabolites have utilized natural or incompletely defined substances as growth media. These have included potato infusion enriched with dextrose, scabbed barley, and cracked corn (1–5). The present report is concerned with the development of a completely defined chemical medium for the production of the fungus and a study of the growth habits on this medium. With such a medium it is possible to control the total environment of the fungus in order to facilitate extraction, metabolic, and animal toxicity studies.

Received April 14, 1967, from the College of Pharmacy, University of Minnesota, Minneapolis, MN 55455

Accepted for publication September 11, 1967.

Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

The authors acknowledge support of this work by grant UI-00133-04 from the National Center for Urban and Industrial Health and a Mead-Johnson undergraduate award (1965–1966).

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EXPERIMENTAL MATERIALS AND METHODS

A culture of *F. graminearum* was maintained by serial transfer on potato dextrose agar (PDA) slants. After initial growth for 1 week in an incubator at 28°, the cultures were stored at 4°. The liquid medium

determined by UV absorbance. Results are shown in Table I. The logarithm of $(C_0^1 - C_2)/C_0^1$ was plotted against time, and Fig. 6 was obtained. The slope of the straight line was calculated to be 0.0605 hr.$^{-1}$, and D_m/h to be 1.234 cm./hr. or 3.43 $\times$ 10^{-4} cm./sec. This same value was also used under *Appendix* of the previous report (1).

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EXPERIMENTAL MATERIALS AND METHODS

A culture of *F. graminearum* was maintained by serial transfer on potato dextrose agar (PDA) slants. After initial growth for 1 week in an incubator at 28°, the cultures were stored at 4°. The liquid medium

used at the beginning of these experiments was derived from the PDA slant culture medium. It consisted of 20% potato infusion and 3% glucose, and was designated potato–dextrose broth (PDB).

Analytical determinations were accomplished in the following manner. A sufficient quantity of 500-ml. conical flasks, each containing 100 ml. of media, were plugged with disposable foam enclosures and autoclaved for 15 min. Inoculum was prepared by transferring a small portion of mycelium from a stock PDA slant to a flask of the same medium to be used for each experiment. This inoculum flask was incubated on a rotary shaker (1 in. diameter rotation, 300 r.p.m.) at 28° for 5–7 days. At the end of the incubation period, the contents of the flask were homogenized in a sterile Waring blender for 30 sec. The experimental flasks were inoculated by aseptically transferring a small loop of the well-stirred homogenized inoculum to each flask. The flasks were then incubated on the rotary shaker for a sufficient period of time. At arbitrarily specified intervals two flasks were harvested and treated as follows. The contents of each flask were filtered through a previously tared filter paper, the mycelium and filter paper frozen, then freeze-dried and weighed. The weight of the freeze-dried mycelium represented growth weight. The pH of the filtrate from the filtration described above was measured; a 15-ml. sample of the filtrate was preserved frozen. The quantity of glucose remaining in the filtrate was measured by the method of Shaffer and Somogyi (6). Nitrogen was measured by the method of Kala (7) and also by the Kjeldahl method.

RESULTS AND DISCUSSION

In an early investigation of the toxic metabolite of *F. graminearum* the PDB medium was devised to permit rapid production of large quantities of mycelium. When cultured in the PDB medium either in 500-ml. conical flasks on a shaker or in a 5-L. fermentor, the organism produced abundant growth and profuse quantities of red colored microconidia. Growth weight, pH, glucose, and nitrogen changes were determined during a typical incubation period of *F. graminearum* on this medium. The results are summarized in Fig. 1.

From this figure it can be ascertained that the maximum dry weight of mycelium obtained was

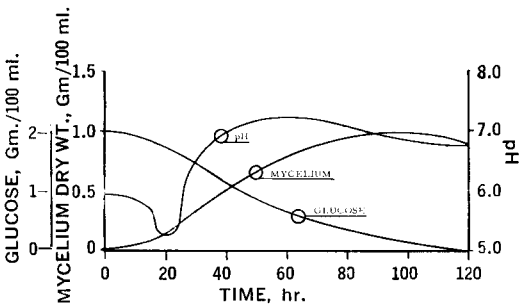

Fig. 1—Patterns of growth, pH changes, and glucose utilization of Fusarium graminearum *in potato–dextrose broth.*

0.94 Gm./100 ml., occurring approximately 100 hr. after inoculation. The pH of the medium varied from 5.25 to 7.25. The pH at the termination of the experiment (140 hr.) was 6.9. After an initial 15-hr. lag period, glucose was depleted in a logarithmic fashion during the logarithmic growth phase of the organism. (It is interesting to note that the minimum pH was recorded at the beginning of this logarithmic growth phase.) Glucose was apparently completely utilized at 110 hr. Maximum growth weight was reached before the glucose was depleted, indicating that glucose was not limiting growth. Nitrogen did not appear to be utilized since the slope of the nitrogen analysis regression line was −0.001. Because of the abundant production of the colored microconidia (about 8% of total dry weight) which hindered extraction studies, and because the mycelium produced in this medium was not acceptable to experimental animals as a food substitute in toxicity studies, it was necessary to investigate other media in anticipation that a completely defined synthetic medium might result from such further experimentation.

Semisynthetic media contain in part natural products and in part discrete chemical compounds. The absolute quantities of the chemical compounds may be varied between zero and the upper limits of solubility. Natural product constituents used in growth media are in many cases unknown. In addition, the reproducibility of the composition of a natural product is difficult due to the inherent variability of most biological systems. A synthetic medium would therefore be desirable if for no other reason than absolute control of all ingredients in the environment of the organism.

In order to evaluate and establish the limits of certain medium constituents capable of supporting growth, a number of semisynthetic media were constructed before attempting growth on a synthetic defined medium.

Glucose, mannitol, sucrose, and starch were screened singly and in combination, using neopeptone, yeast extract, and casein hydrolysate also singly and in combination as nitrogen sources. In these early screening experiments dry weight of mycelium after a 120-hr. incubation period was used as the criterion for efficiency. Although none of the media were as efficient as the original PDB medium, growth was supported by all media containing a nitrogen source; no growth was noted in media lacking nitrogen. Nitrogen was thus established as a requirement for growth.

Since *F. graminearum* did not appear to be a fastidious fungus, a chemically defined synthetic medium developed by Pacifici *et al.* (8) for the production of *Claviceps purpurea* was employed in the search for suitable substrates. This medium (see under *appendix*) not only supported growth, but was superior to PDB using terminal growth weight as criterion for efficiency.

The carbon and nitrogen modifications of Pacifici's medium are illustrated in Tables I and II.

Table I indicates that although Pacifici's original medium containing mannitol is capable of supporting growth of *G. zea*, either glucose or sucrose is more efficient in the combination with succinic acid. From the modifications made, it is apparent that a medium containing 3% glucose or sucrose and 0.1% to 1% succinic acid is most efficient. The criterion

TABLE I—CARBON SOURCE VARIATIONS—PACIFICI MEDIUM

Carbon Source, %	Succinic Acid, %	Mycelium Dry Wt.,[a] Gm.	Econ. Coeff.[b]
Mannitol, 3	3	1.59	63
Sucrose, 5	0.5	1.95	83.5
Sucrose, 3	3	1.23	48
Sucrose, 3	0.5	1.38	93
Sucrose, 1	0.5	0.56	87.5
Glucose, 5	3	2.03	61
Glucose, 3	3	1.72	68
Glucose, 3	1	1.65	101
Glucose, 3	0.5	1.50	105
Glucose, 3	0.1	1.29	104
Glucose, 3	0.05	0.84	69
Glucose, 1	3	0.85	49.5
Glucose, 0.5	3	0.47	31
...	3	0.45	34

[a] Average of three replicate flasks; 168 hr. fermentation. [b] Economic coefficient = mycelium dry wt./Gm. carbon consumed × 100.

TABLE II—NITROGEN SOURCE VARIATION—MODIFIED PACIFICI MEDIUM[a]

Nitrogen Source[b]	Mycelium Dry Wt.,[c] Gm.
Ammonium succinate[d]	1.72
Ammonium nitrate	0.46
Ammonium chloride	0
Ammonium formate	0
Ammonium bromide	0.18
Ammonium carbonate	0.32
Ammonium dihydrogen phosphate	0
Ammonium sulfate	0
Potassium nitrate	0.38
Calcium nitrate	0.78
Sodium nitrate	0.23
Neopeptone	0.20

[a] The basic medium consisted of 3% glucose, 3% succinic acid, and salts as listed in the Pacifici medium under *Appendix*. [b] Nitrogen sources were supplied in an amount equivalent to the nitrogen content of 3% ammonium succinate. [c] Average of three replicate flasks; 168 hr. fermentation. [d] Succinic acid was deleted from this modification.

for efficiency in this experiment was the economic coefficient; the greater numbers indicate greater utilization of carbon substrate. Succinic acid was considered as part of the carbon source in calculating the economic coefficient. For purposes of calculation, it was assumed that all sugar and succinic acid added to the medium was consumed.

The quantity of succinic acid does not appear to be critical between 0.1 and 1%. The economic coefficient drops sharply below 0.1% and above 1%. Using optimum succinic acid concentration, glucose appears to be the growth limiting factor at 3%; no further modifications of glucose concentration have been attempted.

Table II indicates that succinic acid, partially neutralized with ammonium hydroxide, is the most efficient nitrogen source. Although the nitrogen variations were accomplished before the optimum concentration of ammonium succinate was known, it is probable that similar results would be obtained using a nitrogen content equivalent to 0.1 to 1% ammonium succinate. Starting pH of the media was adjusted to 5.1–5.5 in all cases.

Variations in concentration of the major salts (magnesium sulfate and potassium hydrogen phosphate) indicated that the original quantities were in fact optimum.

No modifications of the micro elements have been attempted.

During the course of medium modification, a second analysis was accomplished. The medium used consisted of glucose 3%, succinic acid 3%, and salts as in Pacifici's original medium. Growth weight and pH changes as well as glucose utilization were determined. The results are summarized as Fig. 2.

Glucose was utilized in a logarithmic fashion, being depleted 80 hr. after inoculation. Growth weight did not reach a maximum until after glucose had been completely used, indicating utilization of succinic acid after glucose depletion. After an initial lag phase, the pH rose throughout the incubation period. The beginning of the rise coincided with the end of glucose utilization. The increase in pH could be explained on the basis of nonutilization of nitrogen (or only slight utilization) and metabolism of succinic acid after glucose had been almost completely depleted. This would account for the observed predominant ammonia odor in the medium, and the high ending pH (8.3). Maximum growth weight was 1.4 Gm./100 ml.

Decreasing the succinic acid to 5 Gm./L. (see under *Appendix*) resulted in still a different picture of metabolism. A fermentation similar to the previous ones was accomplished using the low succinic

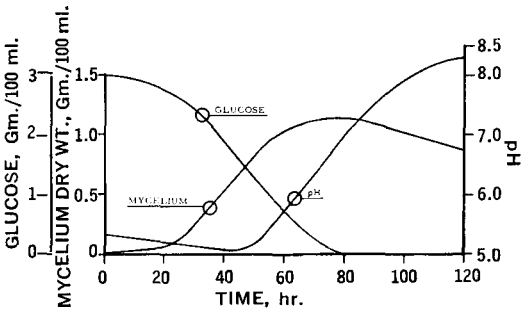

Fig. 2—Patterns of growth, pH changes, and glucose utilization of Fusarium graminearum *in the defined medium containing 3% succinic acid.*

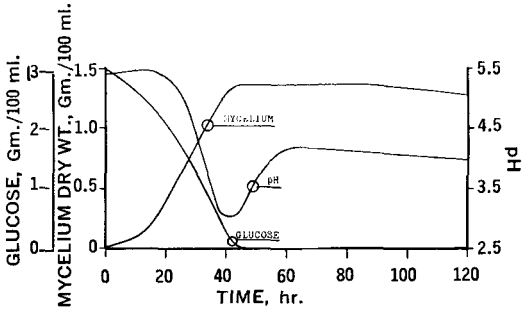

Fig. 3—Patterns of growth, pH changes, and glucose utilization of Fusarium graminearum *in the defined medium containing 0.5% succinic acid.*

acid concentration. The results are summarized in Fig. 3.

As in the previous experiment, glucose was utilized in a logarithmic fashion, being depleted in this case 45 hr. after inoculation. Growth weight, reached a maximum at the same time, indicating glucose as the growth weight limiting factor. After dropping from an initial pH of 5.4, the pH of the medium began to increase at the time glucose was depleted from the medium. It is probable that the succinic acid again was metabolized after glucose was depleted. In this fermentation, because of the low concentration of succinic acid, ammonia was also present in a much lower concentration. The final pH (4.0) was much lower than that encountered in other media. Maximum growth weight was 1.4 Gm.

SUMMARY

The experiments indicated above have resulted in the formulation of a completely defined medium for the production of *F. graminearum*. This medium was derived from one used for the production of another hypocrealic ascomycete, *Claviceps purpurea*. It appears that the optimal carbon sources are glucose (3%) and succinic acid (0.5%). Nitrogen is supplied in the form of ammonium hydroxide to adjust the pH of the medium to 5.1–5.5.

APPENDIX

Potato-Dextrose Medium (PDB)—Freshly diced potato, 400 Gm.; glucose, 30.0 Gm.; tap water *q. s. ad.* 1000 ml.

Pacifici Medium—Mannitol, 30 Gm.; succinic acid, 30 Gm.; KH_2PO_4, 1.0 Gm.; $MgSO_4 \cdot 7H_2O$, 0.3 Gm.; $FeSO_4 \cdot 7H_2O$, 14 mg.; $MnSO_4 \cdot 4H_2O$, 6.7 mg.; $ZnSO_4 \cdot 7H_2O$, 3.7 mg.; H_3BO_3, 2.5 mg.; KI, 0.76 mg.; $AlCl_3 \cdot 6H_2O$, 0.054 mg.; $CuSO_4 \cdot 5H_2O$, 0.03 mg.; NH_4OH *q.s.* to pH 5.5; distilled water *q. s. ad.* 1000 ml.

Defined Medium—Glucose, 30 Gm.; succinic acid, 5.0 Gm.; KH_2PO_4, 1.0 Gm.; $MgSO_4 \cdot 7H_2O$, 0.3 Gm.; $FeSO_4 \cdot 7H_2O$, 14 mg.; $MnSO_4 \cdot 7H_2O$, 6.7 mg.; $ZnSO_4 \cdot 7H_2O$, 3.7 mg.; H_3BO_3, 2.5 mg.; KI, 0.76 mg.; $AlCl_3 \cdot 6H_2O$, 0.054 mg.; $CuSO_4 \cdot 5H_2O$, 0.03 mg.; NH_4OH *q.s.* to pH 5.4; distilled water *q. s. ad.* 1000 ml.

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Keyphrases

Chemical media for *Fusarium graminearum*
Potato-dextrose broth
Glucose—succinic acid—ammonium hydroxide media
Glucose consumption—analysis
Nitrogen consumption—analysis

Effect of Complex Formation on Drug Absorption V

Studies on the Mechanism of the Secobarbital Absorption-Enhancing Effect of Polysorbate 80 in Goldfish

By GERHARD LEVY and JUDITH A. ANELLO

Polysorbate 80 in concentrations of 0.0005 to 0.01 percent increased significantly the absorption rate of secobarbital by goldfish. This absorption-enhancing effect was obtained also if the fish were immersed in polysorbate 80 solution, washed, and then placed in a solution of secobarbital without the surfactant. The ratio of rate constants for absorption of nonionized and ionized secobarbital, respectively, was not affected by polysorbate 80. The results of this study indicate that polysorbate 80 enhances the absorption of secobarbital by increasing the permeability of the biologic membrane to the drug, rather than by forming a more rapidly absorbed nonmicellar polysorbate–secobarbital complex in the bulk phase of the drug solution.

THE EFFECTS of surfactants on drug absorption are of considerable interest and have been investigated extensively (1, 2). Of particular pharmaceutical importance are the possible effects of surfactants at low concentrations, such as might be encountered when dosage forms containing surfactants are dissolved in or diluted with various body fluids following oral or parenteral admistration, or following application to mucous membranes. Unfortunately, appreciable technical difficulties may be encountered in studies of the effect of low concentrations of surfactants on drug absorption (3). The use of goldfish, as described in previous reports from this laboratory (4–6), obviates many of these problems (3). Good correlation has been noted between the results of drug absorption studies with goldfish and rats (5, 7), and similarly between results obtained in rats and in man (8).[1]

A recent study in this laboratory has shown that the absorption rate of secobarbital by goldfish was increased significantly in the presence of 0.01% polysorbate 80, and decreased significantly by high concentrations (1–2%) of the surfactant (3). On the other hand, polysorbate 80 had no effect on the absorption rate of ethanol[2] and certain other alcohols (3). It was noted by appropriate physicochemical studies and from a kinetic analysis of the biologic data that the modification of secobarbital absorption by high concentrations of polysorbate 80 represents the net effect of an enhanced intrinsic absorption rate constant in the presence of the surfactant and a decreased thermodynamic activity of secobarbital resulting from micellar complexation (3). The absorption-enhancing effect of polysorbate 80 was believed to be due either to a direct effect of the surfactant on the permeability of the biologic membrane, or to the formation of a nonmicellar drug–surfactant complex which is more rapidly absorbed than the free drug (3). The available data did not make it possible to distinguish between these possibilities. The purpose of this investigation to be described here was to study the mechanism of the absorption-enhancing effect of polysorbate 80 and to determine the basis for the selectivity of this effect.

EXPERIMENTAL

Goldfish, *Carassius auratus*, common variety, weighing 8–10 Gm., were used. All fish used in a given experiment (*i.e.*, listed in any one table) were from the same lot. The composition of drug solutions, methods for determination of absorption rate, and the method for the determination of surface tension were the same as described previously (3) except as noted in the next paragraph.

All absorption studies were carried out on groups of 5 fish immersed simultaneously in 1 L. of drug solution contained in a 2-L. capacity glass beaker placed in a water bath at 20 ± 0.5°. Pretreatment consisted of placing the 5 fish for the indicated time in 1 L. of 0.01% polysorbate 80 in 0.05 *M* THAM buffer (pH 5.9) or in THAM buffer without surfactant. The temperature of these solutions was maintained at 20 ± 0.5° by means of a water bath. After pretreatment, the fish were transferred with a fish net to a pan containing 3 L. distilled water at

Received June 21, 1967, from the Biopharmaceutics Laboratory, Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication August 28, 1967.

This investigation was supported in part by grant R01 AM 08753 03 PET from the U. S. Public Health Service, Bethesda, Md.

Previous paper: Reuning, R. H., and Levy, G., *J. Pharm. Sci.*, **56**, 843(1967).

[1] This statement refers to the relative absorption rates of a series of passively absorbed drugs in solution.

[2] Results of recent studies (12) raise the possibility that the mechanism of the pharmacologic effect of ethanol on goldfish differs at low and high concentrations. However, additional experiments conducted in this laboratory have shown that polysorbate 80 has no apparent effect on ethanol absorption even at a relatively low concentration (3.5%) of the latter.

TABLE I—EFFECT OF POLYSORBATE 80 ON SECOBARBITAL ABSORPTION BY GOLDFISH[a]

Group	Polysorbate 80 Concn., % w/v	Time of Death,[b] min.	Significantly Different ($p < 0.01$) from Group	Surface Tension,[c] dyne/cm. Before	After
A	None	27.9 (9.4)[d]	*B*, *C*, *D*	63.6	64.0
B	0.0005	17.8 (4.2)	*A*, *D*	51.0	52.7
C	0.001	16.0 (3.6)	*A*	48.1	49.1
D	0.01	14.2 (1.3)	*A*, *B*	43.0	42.6

[a] Fish were immersed in 0.020% sodium secobarbital at pH 5.9 and 20 ± 0.5°. [b] Mean of 10 fish. [c] Mean of 4 determinations. [d] Standard deviation in parentheses.

TABLE II—EFFECT OF PRETREATMENT WITH POLYSORBATE 80 ON SECOBARBITAL ABSORPTION BY GOLDFISH AND ON SURFACE TENSION OF SECOBARBITAL SOLUTIONS

Pretreatment Conditions		Time of Death in Secobarbital Soln.,[a] min.	Surface Tension of Secobarbital Soln. at End of Expt.,[b] dyne/cm.
Soln.	Immersion Time, min.		
0.05 *M* Tris, pH 5.9	30	27.0 (5.8)[c]	63.7
0.01% polysorbate 80 in 0.05 *M* Tris, pH 5.9	30	21.4[d] (4.1)	63.7

[a] Mean of 20 fish immersed in 0.020% sodium secobarbital at pH 5.9 and 20 ± 0.5°. [b] Mean of 4 determinations. [c] Standard deviation in parentheses. [d] Statistically significantly different ($p < 0.01$) from group not pretreated with polysorbate 80.

TABLE III—EFFECT OF DURATION OF PRETREATMENT WITH POLYSORBATE 80 ON SECOBARBITAL ABSORPTION BY GOLDFISH

Pretreatment Conditions		Time of Death in Secobarbital Soln.,[a] min.	Significance of Difference
Soln.	Immersion Time, min.		
0.05 *M* Tris, pH 5.9	1	19.1 (4.1)[b]	$p < .05$
0.01% polysorbate 80 in 0.05 *M* Tris, pH 5.9	1	16.6 (3.1)	N.S.
0.05 *M* Tris, pH 5.9	60	20.4 (3.2)	N.S
0.01% polysorbate 80 in 0.05 *M* Tris, pH 5.9	60	16.8 (3.3)	$p < .01$

[a] Mean of 20 fish in 0.020% sodium secobarbital at pH 5.9 and 20 ± 0.5°. [b] Standard deviation in parentheses.

room temperature where they remained for 4 min. The latter procedure was repeated twice for a total of 3 washes of 4 min. each. The fish were then placed in secobarbital solution as described above.

RESULTS AND DISCUSSION

The principal investigational approach used in this study was to determine the absorption rate of secobarbital in fish previously exposed to polysorbate 80 and in fish exposed only to buffer before being immersed in secobarbital solution. An initial concern was the possibility that sufficient surfactant may be transferred mechanically together with the polysorbate 80 treated fish to the secobarbital solution so that the latter would not really be free of surfactant. It was desirable therefore to determine the concentration range in which polysorbate 80, in the presence of secobarbital, will enhance significantly the rate of absorption of the latter. The data listed in Table I show that polysorbate 80 concentrations as low as 0.0005% have a pronounced absorption-enhancing effect. Polysorbate 80 as such, in the concentrations employed in this study, has no obvious deleterious effect on goldfish (3). It was noted that surface tension measurements provide a sensitive semiquantitative indication of surfactant concentration in the range employed in this study. There was only a small and possibly insignificant increase in the surface tension of the solutions at the end of the experiment; this suggests that only a small amount of the surfactant could have been adsorbed on the fish membranes.

Exposure of goldfish to 0.01% polysorbate 80 for 30 min., followed by three washings in water, resulted in a significantly enhanced absorption of secobarbital when the fish were placed in secobarbital solutions which did not contain polysorbate 80 (Table II). The magnitude of this effect was independent of the duration of exposure of the fish to surfactant; fish placed in polysorbate 80 solution for 1 min. subsequently absorbed secobarbital as rapidly as did fish maintained in polysorbate 80 solution for 60 min. before being placed in secobarbital solution (Table III). There was no detectable mechanical carry-over of polysorbate 80 to the secobarbital solution; the effectiveness of the three washings is evidenced by the fact that the secobarbital solutions used for surfactant-treated and control fish, respectively, had the same surface tension at the end of the experiment (Table II). Results obtained in control fish were not affected by duration of immersion in buffer solution (1 *versus* 60 min.) preceding their placement in secobarbital solution. Preliminary experiments had shown also that the response to secobarbital of fish

TABLE IV—EFFECT OF POLYSORBATE 80 ON ABSORPTION OF NONIONIZED AND IONIZED FORMS OF SECOBARBITAL BY GOLDFISH

Compn. of Soln.					
Polysorbate 80, %	Sod. Secobarbital, %	pH[a]	Time of Death,[b] min.	Ratio of Time of Death With:Without Polysorbate	k_n/k_i[c]
None	0.020	6.9	41.4 (8.5)[d]	0.52	16.6
0.01	0.020	6.9	21.6 (3.8)		
None	0.125	8.9	40.7 (13.3)	0.52	16.5
0.01	0.125	8.9	21.2 (3.4)		

[a] 0.05 *M* Tris buffer at 20 ± 0.5°. [b] Mean of 10 fish. [c] Ratio of absorption rate constants for nonionized and ionized secobarbital. [d] Standard deviation in parentheses.

subjected to the several transfers required for pretreatment and subsequent washings did not differ from the response of fish placed directly in secobarbital solution. The times of death of control fish and pretreated fish in the experiments listed in Table II were somewhat longer than those in the experiments listed in Table III, but the relative magnitude of the surfactant effect was similar in each set of experiments. The absolute difference in death times of the control fish in the two sets of experiments is due to the use of different lots of fish. This lot to lot difference has been found previously and necessitates the use of appropriate controls in each set of experiments.

The experimental results listed in Tables II and III show that polysorbate 80 can increase the absorption rate of secobarbital in goldfish even if the two substances are not present together in the bulk phase of the solution. This leads to the conclusion that the absorption-enhancing effect of polysorbate 80 is due to a direct effect of the surfactant on the permeability characteristics of the fish membranes. The experimental results do not support an assumption that the enhanced absorption of secobarbital is due to the formation of a more rapidly absorbed nonmicellar drug–surfactant complex (9). In addition, no physicochemical evidence for such a complex in aqueous solutions containing up to 0.01% polysorbate 80 has been found in this laboratory (3).

The experimental data suggest that a small amount of polysorbate 80 is adsorbed rapidly on the absorbing membranes of fish, and that this in turn causes a more rapid absorption of secobarbital and certain other drugs. The fact that exposure of the fish to polysorbate 80 for 1 min. elicits the same effect as exposure for 60 min. shows not only that the surfactant acts rapidly, but also that the effect is relatively time independent. This is in agreement with the result of the previously reported kinetic analysis of the effect of polysorbate 80 on secobarbital absorption (3); the surfactant effect could be expressed in terms of a rate constant for the enhanced absorption and thus appeared to be essentially time independent. This would not be expected to occur if the surfactant acts by slowly removing lipoid constituents from the membrane or by causing the leaching out of some other membrane component. The rapid effect of polysorbate 80 is more consistent with a mechanism involving adsorption of surfactant molecules on the biologic membrane, thereby making the latter more permeable to certain drugs. Such a mechanism *could* involve interaction of adsorbed surfactant with the drug, thereby "augmenting the amount of drug on the surface of the biologic membranes" (9). Thus, a distinction between a membrane effect and a complexing effect as the basis for the absorption-enhancing mechanism of polysorbate 80 is a matter of semantics; the present study distinguishes between a membrane effect (including a possible complex formation between *membrane-bound* surfactant and drug) and an effect due to the formation of nonmicellar drug–surfactant complex in the *bulk phase* of the solution. It is apparent from the tabulated data that polysorbate 80 exerts a more pronounced effect on secobarbital absorption in the presence of the drug than when used as a pretreatment. This is probably due to partial desorption of surfactant from the fish membranes during the washings.

The failure of polysorbate 80 to enhance the absorption of low molecular weight alcohols and its effectiveness in increasing the absorption of two lipoid-soluble barbiturates of considerably higher molecular weight suggested that the surfactant might selectively increase absorption across the lipoid portion of biologic membranes without affecting the rate of drug permeation through membrane pores (3). These considerations (and a provisional assumption that secobarbital anion is absorbed solely or mainly by the pore route) led to an experiment to assess the relative effect of polysorbate 80 on the absorption rate of the nonionized and ionized forms of secobarbital, respectively. This involved determination of secobarbital absorption at pH 6.9 and 8.9 with and without surfactant (Table IV). Secobarbital has a pKa of 7.9 at 20° (10) and is about 90% nonionized at the low pH and 90% ionized at the higher pH. A more pronounced surfactant effect on the absorption of the nonionized form of secobarbital would be reflected by a greater overall effect at low pH than at higher pH where ionized secobarbital contributes appreciably to the total absorption. In fact, the surfactant affected the absorption of both species to an equal degree (Table IV).[3] This is evident not only from the respective ratios of times of death with and without surfactant, but also from the ratios of the rate constants for the absorption of nonionized and ionized secobarbital in the absence and presence of polysorbate 80. The ratios of these rate constants were calculated from two simultaneous equations of the form:

[3] Note also that the *relative* effect of 0.01% polysorbate 80 in the experiments listed in Table IV is exactly the same (50% decrease in time of death) as in the experiments listed in Table I.

$$L = k_n C_n T_L + k_i C_i T_L \qquad \text{(Eq. 1)}$$

where L is the lethal dose of secobarbital, k_n and k_i are the rate constants for the absorption of nonionized and ionized secobarbital, respectively, C_n is the concentration of nonionized secobarbital, C_i is the concentration of ionized secobarbital, and T_L is the time of death (4, 5). The experimental data obtained at pH 6.9 and 8.9 were used for each pair of equations, one for drug absorption in the absence of polysorbate 80, the other for absorption in the presence of the surfactant. The $k_n:k_i$ ratio was 16.5 in each case, showing that the absorption of ionized and nonionized secobarbital was enhanced equally by polysorbate 80. The experiments listed in Table IV were designed to yield a relatively constant T_L irrespective of pH by using an appropriately higher over-all concentration of secobarbital at pH 8.9 as determined from preliminary experiments. The $k_n:k_i$ ratio of 16.5 obtained in this manner is in relatively good agreement with the ratio of about 10 determined several years ago in this laboratory by an entirely different experimental approach where the over-all concentration of secobarbital was kept constant and T_L was variable (4).

The results of this study provide a better understanding of the nature of the absorption-enhancing effect of polysorbate 80 although the exact mechanism, and the basis for its specificity, must still remain speculative. One possibility is that surfactant molecules adsorbed to the surface of the biologic membrane interact with barbiturates [but not with the low molecular weight alcohols studied previously (3)] and thereby enhance to an equal degree the penetration of nonionized barbiturates across the lipoid portion of the membrane and the diffusion of barbiturate anions through pores in the membrane. This mechanism is difficult to accept on a rigorous basis, particularly since there is no evidence for nonmicellar complexation of secobarbital with polysorbate 80 at concentrations up to 0.01% of the latter. In addition, significant micellar complexation of secobarbital by polysorbate 80 occurs only with the nonionic form of secobarbital (11). It seems much more likely that secobarbital anion (mol. wt. >200) is absorbed solely or primarily across the lipoid portion of biologic membranes rather than through pores in the membrane, *i.e.*, by the same route as nonionized secobarbital. The similarity in the absorption-enhancing effect of polysorbate 80 on the ionized and nonionized forms of secobarbital, and the lack of effect on the absorption of low molecular weight alcohols, can then be explained by assuming that the surfactant affects drug absorption across the lipoid barrier portion of the biologic membrane but does not affect diffusion of drug through membrane pores.

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Keyphrases

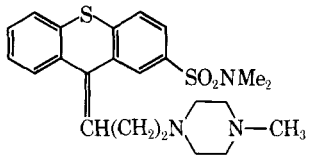
Complex formation effect on drug absorption
Secobarbital—polysorbate 80 system
Absorption-enhancing effect in goldfish
Polysorbate 80 pretreatment effect on secobarbital absorption
Polysorbate 80 effect on lipoid barrier
Absorption rate equations
Membrane permeability

Metabolism of Thiothixene

By D. C. HOBBS

The metabolism of thiothixene, as shown by studies with rats and dogs employing ^{35}S-labeled drug, reflects the fundamental structural similarity of this thioxanthene derivative to other tricyclic psychotherapeutic agents. Like other members of this large group of compounds, it appears to be well absorbed orally, rapidly distributed, and metabolized to a variety of related compounds which are secreted mainly into the bile. The chemical similarity also suggests that it might bind to melanin. Although *in vivo* studies with hooded rats do indeed indicate a preference of thiothixene and/or its metabolites for pigmented over nonpigmented tissues, *in vitro* studies indicate that it is actually one of the least strongly bound of these drugs. In contrast to excreta and other tissues which contain a variety of metabolites, the brain contains only unchanged thiothixene.

THIOTHIXENE (I),[1] the *cis* isomer of *N,N*-dimethyl-9- [3 - (4 - methyl - 1 - piperazinyl)propylidene]-thioxanthene-2-sulfonamide, is a new potent tricyclic psychotherapeutic agent with antipsychotic activity comparable to that of several widely used phenothiazine drugs (1–5).



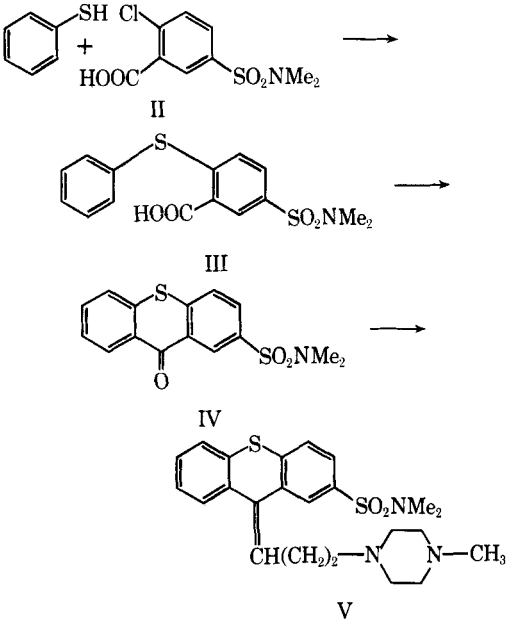
I

The studies to be reported here were undertaken to place the metabolism of thiothixene in perspective with that of existing tricyclic psychotherapeutic agents. In these initial studies using thiothixene labeled with ^{35}S in the nucleus, most of the doses employed (about 8 mg./Kg.) were those which elicit pharmacological effects in acute experiments. It should be noted, however, that these doses are at least ten to twenty-fold higher (on a mg./Kg. basis) than the usual daily human dose.

Since previous reports have indicated that certain tricyclic psychotherapeutic drugs chemically related to thiothixene interact with melanin *in vitro* and *in vivo* (6) and may be responsible for some clinical side effects in melanin-containing tissues (7), hooded rats, which have pigmented eyes and areas of black skin and hair, were included in some of the studies.

METHODS

Labeled Thiothixene—Thiophenol-^{35}S was condensed with 2-chloro-5-dimethylsulfamoylbenzoic acid (II) in alkaline dimethylformamide at 130–140°. The resulting acid (III) underwent ring closure with polyphosphoric acid at 70°. The ketone (IV), in a Wittig reaction with triphenyl-3-(4-methylpiperazinyl)propylphosphonium bromide hydrobromide, yielded V, a mixture of thiothixene and the undesired *trans* isomer. After removal of the latter as the crystalline phosphate, thiothixene-^{35}S was obtained by crystallization of the base from the mother liquor. The initial specific activity of the product was 13.4 mc./mmole. A second lot of material (1.7 mc./mmole) was obtained by dilution of the mother liquors with unlabeled thiothixene, followed by recrystallization. (Scheme I.)



Scheme I

Chromatographic examination of both lots of labeled thiothixene in paper and thin-layer systems established the identity and radiopurity (>98%) of the material.

Drug Administration—Thiothixene base, dissolved in pH 4, 0.25 *M* acetate buffer, was administered to rats and dogs by stomach tube or by intraperitoneal injection. Most of the studies were carried out at a dose level of 8 mg./Kg. However,

Received June 26, 1967, from the Medical Research Laboratories, Chas. Pfizer & Co., Inc., Groton, CT 06340
Accepted for publication September 27, 1967.
The assistance of Dr. J. F. Muren with a portion of the synthesis and Dr. L. D. Sharp with the dog bile duct cannulation is acknowledged. Skilled technical assistance was rendered by Mrs. D. DeMots, Miss A. G. Connolly, and Mr. P. E. Chernowsky.

[1] Navane is the registered trademark of Chas. Pfizer & Co., Inc.

TABLE I—EXCRETION OF RADIOACTIVITY[a] BY RATS (AV. WT. 140 GM.) FOLLOWING SINGLE INTRAPERITONEAL OR ORAL DOSES OF 8 mg./Kg. LABELED THIOTHIXENE

	Intraperitoneal		Oral	
	Conditioned[b] (2 Rats)	Unconditioned (4 Rats)	Conditioned[b] (2 Rats)	Unconditioned (4 Rats)
Urine, hr.				
0–24	3.8 (2.6– 4.9)	4.4 (3.1– 5.6)	2.6 (2.6– 2.6)	3.6 (2.8– 5.1)
24–48	0.7 (0.5– 0.8)	3.8 (1.0– 6.4)	0.3 (0.3– 0.3)	1.1 (0.5– 1.5)
48–96	0.3 (0.3– 0.3)	0.5 (0 – 1.3)	0.3 (0.3– 0.3)	0.3 (0.2– 0.3)
Total	4.8 (3.4– 6.0)	8.7 (4.6–11.8)	3.2 (3.2– 3.2)	5.0 (3.5– 6.4)
Feces, hr.				
0–24	43.1 (41.3–44.9)	10.0 (0 –38.7)	73.2 (64.7– 81.7)	45.6 (25.7– 70.4)
24–48	10.9 (8.7–13.1)	21.2 (0.1–72.1)	25.9 (10.8– 41.1)	57.1 (28.3–107.7)
48–96	3.5 (2.3– 4.6)	0.7 (0 – 1.8)	1.7 (0.8– 2.6)	2.8 (0 – 5.1)
Total	57.5 (55.9–59.1)	31.9 (0.5–72.9)	100.8 (78.1–123.6)	105.5 (75.8–133.4)
Total	62.3	40.6[c]	104.0	110.5

[a] Expressed as average (range) percent of dose administered. [b] These rats had previously received 1 mg. unlabeled thiothixene/day for several weeks. [c] Two of the animals in this group were greatly depressed and excreted very little feces, thus decreasing the apparent excretion of radioactivity.

the effects of chronic administration on levels of radioactivity in the liver, eyes, and dark skin of hooded rats were investigated in a group of animals given daily oral doses of 1 mg./Kg. Two animals were sacrificed each second day and the tissues assayed for radioactivity. After 13 days, drug administration was terminated in these animals and in another group of hooded rats which had been dosed simultaneously with 10 mg./Kg. thiothixene. Animals from both groups were then sacrificed at intervals to assess the rate of removal of drug from these tissues.

Sample Collection and Treatment—Animals were maintained in metabolism cages and excreta collected separately. Feces were homogenized with water, lyophilized, and assayed for radioactivity by scintillation spectrometry after digestion with a quaternary ammonium bactericide.[2] Tissue samples were homogenized with the bactericide and aliquots assayed; skin samples did not include the hair, and stomach contents were washed out prior to homogenization. Urine samples were assayed directly for radioactivity.

Chromatographic Procedures—Urine samples were accumulated from rats receiving daily intraperitoneal doses of unlabeled thiothixene. After treatment with a glucuronidase–sulfatase mixture,[3] an alkaline chloroform extract was prepared and concentrated for thin-layer chromatography. A similar extract was prepared from urine not treated with enzyme. Samples of feces from rats receiving labeled thiothixene by the oral and intraperitoneal routes were extracted before and after glucuronidase treatment at neutral, acid, and alkaline pH and concentrated extracts similarly chromatographed. Bile samples obtained 1–1.5 hr. after administration by these two routes were also included for comparison, as well as a neutral extract of liver. Thin-layer system EDW consisted of ethyl acetate-diethylamine–water (90:15:5) on Silica Gel GF. Areas on the thin-layer strips containing radioactive materials were delineated with a Vanguard model 880 Autoscanner and by autoradiography with Kodak type F X-ray film.

[2] Hyamine, Packard Instrument Co.
[3] Glusulase, Endo Products, Inc.

In Vitro Studies—Melanin was isolated from beef eyeballs by differential centrifugation, according to Potts (6). It was found that a concentration of 5 mg. melanin granules/ml. bound chlorpromazine to the same extent as that reported by Potts for 10 mg./ml. The former concentration was therefore used in the comparative study with 2.5 μmoles compound/ml.

Isolation of Sulfur-Containing Nondrug Related Materials—Twelve rats each received subcutaneous injections of 50 mg. of β-naphthylamine in oil (an amount found sufficient, in preliminary experiments, for the facile recovery of its metabolite) and 1 mg. labeled thiothixene (intraperitoneal) on each of 2 successive days. Urine was collected for a total of 5 days and 2-amino-1-naphthylsulfuric acid isolated by the method of Laidlaw and Young (8).

The method of Dziewiatkowski (9) was used to sample body cysteine in the form of an excreted mercapturic acid. Accordingly, 2 rats each received oral doses of 140 mg. of bromobenzene and intraperitoneal doses of 2 mg. of labeled thiothixene. Crystalline *p*-bromo-phenylmercapturic acid was isolated from the 24-hr. urines as described (9).

Cysteine was isolated (10) from the total carcass protein (except gastrointestinal tract contents) of rats 1 day (bromobenzene animals) and 5 days (β-naphthylamine animals) after administration of labeled thiothixene.

RESULTS

Excretion—Following single intraperitoneal or oral doses of labeled thiothixene, urine and feces were collected and assayed after various intervals (Table I). The route of drug administration did not appear to have an appreciable effect on the route or rate of excretion; the differences in fecal radioactivity were probably artifactual, resulting from the small amount of feces excreted by the depressed animals receiving intraperitoneal thiothixene. Some animals in each group were pretreated with several daily doses of unlabeled drug; these animals excreted the radioactive material earlier than those not pretreated. Whether this was due to saturation of tissue binding sites by the unlabeled material or to induction of drug metabolism cannot be answered by these data.

Most of the radioactivity was excreted in the feces, indicating extensive biliary secretion of thiothixene and/or its metabolites by the rat. That this actually occurred was established by direct measurement of radioactivity in the bile from two rats (Table II) and a dog (Table III) receiving labeled thiothixene. The high levels of radioactivity observed indicate the importance of this route of excretion and the high efficiency of drug absorption following administration by the oral and intraperitoneal routes. The dog excreted a greater fraction of the administered radioactivity *via* the urine than did the rat.

Distribution—Following single doses of labeled thiothixene in the rat, various tissues were removed after 4 or 24 hr. for estimation of tissue radioactivity (Table IV). Four hours after dosing, all tissues examined contained higher levels of radioactivity after the intraperitoneal dose than after the oral dose. However, this may not be a valid index of the relative efficiencies of absorption by these two routes; material absorbed from the intestinal tract goes first to the liver where it appears to be rapidly metabolized and excreted into the bile, whereas drug absorbed from the peritoneal cavity goes first to the general circulation.

At the earlier interval, the highest concentration was seen in the stomach, even after intraperitoneal administration. After 24 hr., however, the liver was the only tissue with appreciable amounts of label. To further investigate levels in the liver, a group of rats receiving single intraperitoneal doses of labeled thiothixene were sacrificed at intervals of up to 28 days; the decline in radioactivity occurred with an early half-life of 3 days and a later half-life of approximately 4.5 days (Table V). Plasma levels of radioactivity in these animals declined more rapidly.

The distribution of radioactivity within the brain of the rat 1 hr. after thiothixene administration was determined, as indicated in Table VI. Small differences were observed, but the ratio of the extreme values was only 1.7. Autoradiography of

TABLE II—BILIARY SECRETION[a] OF RADIOACTIVITY BY THE RAT FOLLOWING ORAL AND INTRAVENOUS ADMINISTRATION OF 8 mg./Kg. LABELED THIOTHIXENE

hr.	Intravenous	Oral
0–1/2	0.3	2.5
1/2–1	2.1	7.4
1–1 1/2	4.3	10.3
1 1/2–2	5.8	10.7[b]
2–3	10.0	15.2
3–4	10.5[b]	18.4
4–5	13.3	22.7
5–6	14.9	25.7
6–7	16.2	...
7–22	22.7[c]	30.5
22–30	28.8	43.5
30–48	...	65.0

[a] Cumulative percent of dose administered. [b] A small amount of bile lost from these samples, but there should be little effect on cumulative values. [c] A considerable amount of bile lost from this sample; extrapolation to expected bile flow rate means increasing total 0–30 hr. excretion from 28.8 to 37.1% of the dose administered.

TABLE III—BILIARY AND URINARY EXCRETION[a] OF RADIOACTIVITY BY THE DOG FOLLOWING ORAL ADMINISTRATION OF 14 mg./Kg. LABELED THIOTHIXENE

hr.	Urine	Bile
0–2	...	9.5
2–5	...	23.3
5–22	16.9	46.5
22–29	18.0	48.9
29–46	19.2	51.8

[a] Cumulative percent of dose administered.

TABLE V—LIVER AND PLASMA RADIOACTIVITY LEVELS (mcg. THIOTHIXENE EQUIVALENT/Gm.) IN RATS AFTER SINGLE INTRAPERITONEAL DOSES (8 mg./Kg.) OF LABELED THIOTHIXENE

Time	Liver	Plasma
2 hr.	14.86 (2)[a]	0.93 (2)
6 hr.	9.37 (2)	0.25 (2)
1 day	6.84 (2)	0.06 (2)
2 days	5.88 (2)	0.02 (2)
3 days	3.13 (2)	...
5 days	2.64 (4)	0.01 (2)
7 days	1.84 (2)	...
14 days	0.32 (2)	...
21 days	0.21 (2)	...
28 days	0.06 (2)	...

[a] Number of animals.

TABLE VI—DISTRIBUTION OF RADIOACTIVITY WITHIN THE RAT BRAIN 1 hr. AFTER A SINGLE INTRAPERITONEAL DOSE (10 mg./Kg.) OF LABELED THIOTHIXENE

	mcg. Thiothixene Equivalent/Gm.
Cerebral hemispheres	0.72
Olfactory bulb	0.66
Cerebellum	0.54
Paraflocculus	0.43
Thalamus	0.60
Medulla	0.59
Corpus callosum	0.73

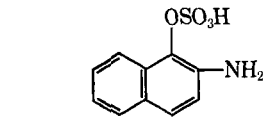
TABLE IV—^{35}S TISSUE LEVELS (mcg. THIOTHIXENE EQUIVALENT/Gm.) IN RATS FOLLOWING A SINGLE DOSE (8 mg./Kg.) OF THIOTHIXENE-^{35}S

	Intraperitoneal		Oral	
	4 hr.	24 hr.	4 hr.	24 hr.
Heart	1.12, 1.50	0.14, 0.09	0.21, 0.13	0.14, 0.05
Lung	4.75, 2.05	0.70, 0.80	0.85, 0.90	0.09, 0.10
Liver	11.43, 7.91	4.42, 5.91	4.96, 7.17	6.60, 5.71
Kidney	1.50, 0.46	0.61, 0.38	0.47, 0.68	0.42, 0.19
Stomach	23.50, 10.69	0.23, 0.46	9.12, 14.35	0.04, 0.05
Skin	1.65, 1.31	0.23, 0.32	0.04, 0.05	0.28, <0.01
Muscle	1.20, 1.25	0.09, 0.14	0.04, 0.09	0.03, 0.02
Brain	0.23, 0.09	0.02, 0.04	0.04, 0.02	0.01, 0.01

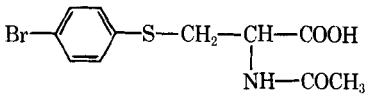
thin slices of brain indicated that more activity was associated with the gray matter than with the white matter.

Since thiothixene is to be administered chronically, tissue levels after multiple doses were investigated (Table VII). Under these conditions, higher and longer-lasting levels were noted in all of the tissues examined, the liver again showing the highest concentrations. Upon chronic administration, as after a single dose, the brain contained the lowest level of radioactivity of these tissues. An additional study was made of levels of radioactivity in liver, eyes, and skin of hooded rats receiving thiothixene in daily oral doses of 1 mg./Kg. [close to that found satisfactory for human clinical use (4)] and 10 mg./Kg. (Fig. 1). Amounts of label in these tissues of the low-dose animals had reached equilibrium by approximately the fifth dose. Following the cessation of drug administration, levels in liver and skin of both high-dose and low-dose animals declined logarithmically with a half-life of approximately 5 days. Levels in the eye remained stationary for several days, then declined at a rate considerably lower than that observed in the liver.

With the exception of the *N*-methyl group(s), fragments from the tricyclic psychotherapeutic drugs do not appear to be incorporated into normal tissue constituents. Some direct evidence on this point for thiothixene was obtained by the isolation of constituents which might contain labeled sulfur derived from thiothixene. Accordingly, 2-amino-1-naphthylsulfuric acid (VI), excreted as a result of the hydroxylation and etherification with sulfate of administered β-naphthylamine, and *p*-bromophenylmercapturic acid (VII), excreted upon condensation



VI



VII

of administered bromobenzene with *N*-acetylcysteine, were isolated. Cysteine, derived from total body protein, was also obtained. The absence of radioactivity in these substances indicates that the sulfur of tissue sulfate and cysteine cannot arise from the ring sulfur of thiothixene.

Interaction of Thiothixene with Melanin—To further assess the relative affinity of thiothixene for melanin-containing tissues as compared to the same tissues without melanin, the tissue levels in the eyes and skin of hooded (pigmented) rats were compared with those in albino animals (Table VIII). The dark skin of the hooded rats contained more label than the white skin of the same animals or the skin of albino rats. Statistical analysis indicated that the difference was significant at the 5% level. Drug concentrations in the pigmented eyes, although comparable to those of the liver at the same interval (Table IV), were nevertheless much higher than those observed in the eyes of the albino rats.

In vitro, the affinity of thiothixene for melanin isolated from beef eyeballs was compared with that of several other tricyclic psychotherapeutic drugs and the *trans* isomer of thiothixene (Table IX). Values for triflupromazine and amitriptylene were quantitatively different from those obtained by Potts (6) in a similar study, although the relative ranking of these two compounds and chlorpromazine was the same. This may have been due to a difference in the potency of the melanin preparations. The extent of binding noted with thiothixene (as well as its *trans* isomer) was similar to that observed with amitriptyline, the least bound of a much larger group of psychotherapeutic drugs investigated by Potts (6).

Metabolites of Thiothixene—Alkaline chloroform extracts of rat urine, before and after treatment with glucuronidase, upon thin-layer chromatography in system EDW, revealed the presence of several UV absorbing spots. There were only traces of material moving in the thiothixene region, but one

TABLE VII—TISSUE LEVELS (mcg. THIOTHIXENE EQUIVALENT/Gm.) IN THE RAT AFTER EIGHT DOSES[a] OF THIOTHIXENE ^{35}-S

	Days After Last Dose		
	1	*2*	*3*
Heart	1.07	0.61	0.81
Lung	2.08	1.67	1.87
Liver	21.35	15.62	12.32
Kidney	3.58	1.77	2.96
Stomach	3.37	3.05	2.84
Muscle	0.98	0.23	0.66
Brain	0.06	0.03	0.21

[a] Three rats (av. wt. 120 Gm.) each received 8 mg./Kg. intraperitoneal thiothixene on days 1, 2, 3, 4, 5, 8, 9, 10.

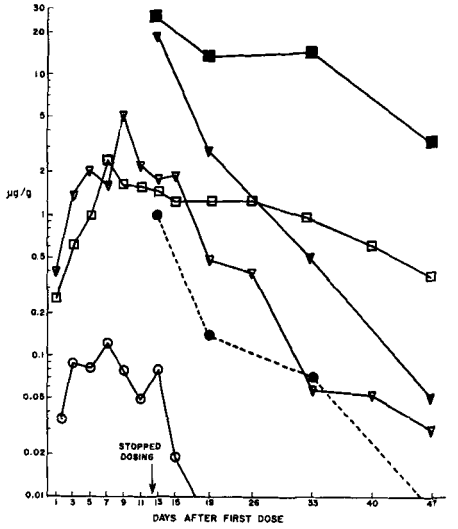


Fig. 1—Levels of radioactivity (expressed as mcg./Gm. thiothixene) in selected tissues of hooded rats during and subsequent to 13 daily oral doses of labeled thiothixene. Key: ■, eyes, 10 mg./Kg.; □, eyes, 1 mg./Kg.; ▼, liver, 10 mg./Kg.; ▽, liver, 1 mg./Kg.; ●, dark skin, 10 mg./Kg.; ○, dark skin, 1 mg./Kg.

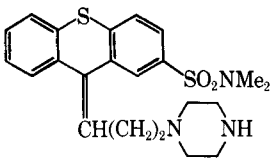
TABLE VIII—TISSUE LEVELS OF ^{35}S (mcg. THIOTHIXENE EQUIVALENT/Gm.) IN SKIN AND EYEBALLS OF HOODED AND ALBINO RATS AFTER A SINGLE INTRAPERITONEAL DOSE OF 8 mg./Kg. THIOTHIXENE-S^{35}

Time →	24 hr.					7 Days	
	Albino		Hooded			Hooded	
Rat No. →	*1*	*2*	*3*	*4*	*5*		*6*
Eyeballs	0.5		12.45		6.69		5.12
Dark skin	...	...	2.36	0.19	0.14		1.01
White skin	0.18	0.10	0.13	0.04	0.06		0.03

TABLE IX—BINDING OF VARIOUS PSYCHOACTIVE DRUGS TO MELANIN GRANULES

	% Bound	
	This Study	Data of Potts
Chlorpromazine	50.4	52
Triflupromazine	44.0	33
Chlorprothixene	52.1	..
Amitriptyline	37	10
Thiothixene	33.5, 33.5	..
P-4657A (*trans* isomer)	36.4	..

of the major metabolites was chromatographically similar to the demethyl derivative (VIII).



VIII

Extracts of feces, bile, and a neutral extract of liver from animals receiving labeled thiothixene were also examined in system EDW (Fig. 2). A large number of metabolites was again observed, including the demethyl derivative, with little or no unchanged thiothixene. All of the bile samples of Table II were chromatographed in the same system with no discernible changes in the pattern of metabolites during the course of the excretion.

Extracts of various portions of the rat brain 1 hr. after thiothixene administration, upon thin-layer chromatography, revealed a single radioactive substance, chromatographically identical to thiothixene. An extract of liver from the same animal showed the usual multiplicity of metabolites.

Extracts of feces (before and after glucuronidase treatment), prepared at neutral, acid, and alkaline pH levels and after heating with strong alkali gave qualitatively similar patterns of metabolites, the data of Fig. 2 being representative.

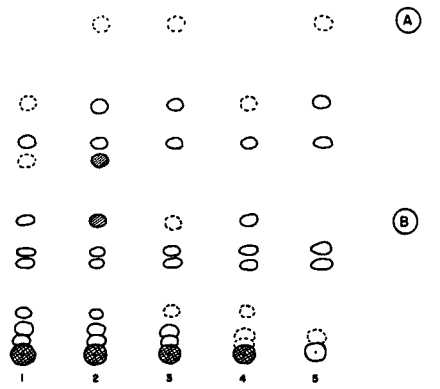


Fig. 2—Thin-layer chromatography of thiothixene metabolites. Key: 1, *bile, i.p. dose;* 2, *bile, oral dose;* 3, *extract of feces, i.p. dose;* 4, *extract of feces, oral dose;* 5, *extract of liver, i.p. dose;* A, *thiothixene;* B, N,N-*dimethyl-9-(3-piperazinylpropylidene)-thioxanthene-2-sulfonamide* (N-*demethyl-thiothixene*).

DISCUSSION

The experimental results reported here indicate that the absorption, distribution, and excretion patterns observed with thiothixene in the rat are generally similar to those of other tricyclic psychotherapeutic agents, as would be expected from the structural relationships involved.

As with other tricyclic psychotherapeutic agents (11–13), thiothixene was rapidly and extensively absorbed following oral administration, very little unchanged drug being recovered in the feces. Further evidence for its prompt assimilation is afforded by the high recovery of radioactivity in the bile of the rat. The low and rapidly diminishing blood levels observed with thiothixene, as with other related drugs (11, 13–16), in the presence of facile absorption indicate rapid excretion and/or distribution into various tissue compartments. The ratio of liver to plasma radioactivity increased with time indicating that the liver is a major depot for storage of thiothixene metabolites. Once absorbed, thiothixene was rapidly metabolized, since bile obtained 0.5 hr. after drug administration apparently contained all of the metabolites noted in later samples.

The relatively large amount of radioactivity excreted *via* the bile is in accord with the structure of thiothixene: comparative studies in rats (17) and dogs (13) with various phenothiazines indicate that biliary secretion is highest for those compounds which contain the 4-methyl-1-piperazinyl side chain. Reabsorption from the intestine (enterohepatic circulation) occurs with many phenothiazines (13, 18–20) and may also be a factor in the disposition of thiothixene. The 5:2 ratio of biliary to urinary excretion after thiothixene administration in the dog is considerably lower than that previously observed with prochlorperazine and trifluoperazine in the dog (13). Nevertheless, the feces still account for more than half of the material excreted. In both dogs and rats, therefore, the liver must play a dominant role in the excretion of thiothixene.

There appear to be no appreciable differences be-

tween the amounts of label excreted in the urine and feces from conditioned animals as compared to those not previously receiving unlabeled thiothixene. The amounts excreted in the feces of the orally dosed animals were greater than those derived from the intraperitoneally dosed animals, but this was probably due to the depressed state of some members of the latter group from which a smaller amount of feces was obtained and therefore does not represent depressed absorption of thiothixene. As discussed above, the biliary secretion was similar by both routes.

Data on tissue levels indicate that thiothixene and its metabolites are spread widely throughout the body, as is the case for the tricyclic psychotherapeutic drugs in general (12, 14, 18). Tissue concentrations in the rat 4 hr. after thiothixene administration were generally higher following intraperitoneal as compared to oral administration; however, at the 24-hr. interval, there were no appreciable differences between levels of radioactivity in the two groups of animals. Concentrations in the lung were lower than those previously noted with chlorpromazine and trifluoperazine (11), prochlorperazine (21), thioridazine (22), and perphenazine (16). The high initial drug levels in the stomach of rats after thiothixene parallel results obtained with chlorpromazine in mice (23) and were presumably a function of the basicity of the two compounds (and their metabolites) coupled with acidity of the gastric mucosa. This phenomenon is not associated with retention of drug within the stomach after oral dosing, since it also occurs upon intraperitoneal administration of thiothixene. Amounts of label in the liver were relatively high and declined at a slower rate than that of the other tissues examined (except the eye of pigmented rats). This long half-life, however, is similar to that observed with several other related drugs (11). Although multiple daily doses of thiothixene resulted in higher and longer-lasting levels in the liver (as well as other tissues), the ^{35}S-concentrations seen in this study were much lower than those achieved with comparable doses of chlorpromazine in cats (24) and prochlorperazine in rats (14, 21).

The low thiothixene levels found in the brain are typical for this type of drug, being noted also with promazine (25), thioridazine (11), trifluoperazine (11), and chlorpromazine (11, 20). The high brain levels of chlorpromazine relative to other tissues, noted by some investigators (24, 26–27) may have been due to methods of extraction and assay which are primarily responsive to unchanged drug. With thiothixene, as with perphenazine (28), chlorpromazine (29), and prochlorperazine (29), only the unchanged drug could be detected upon chromatographic examination of extracts of brain tissue 1 hr. after drug administration. Whether metabolites of thiothixene begin to appear at later intervals was not assessed. The uniform distribution of thiothixene throughout the rat brain at this morphological level has counterparts in studies with thioridazine (11), but differential accumulation has been observed with chlorpromazine (24, 29) and prochlorperazine (29). The higher levels of thiothixene in the gray matter as compared to white matter have also been seen with chlorpromazine (23).

The melanin-containing areas of the rat skin are definite sites of drug retention, having significantly more ^{35}S than unpigmented skin from the same animals or skin from albino rats. However, the pigmented structures of the eye appear to be the areas of highest retention. In the eye, levels approximating those of the liver were rapidly achieved; since the choroid, where the drug presumably accumulates (30), is only 8% of the total wet weight of the eyeball, levels in this structure corrected for more direct comparison with the data of Potts (6) are therefore much higher than those of the liver. Similarly, chlorpromazine and prochlorperazine result in concentrations in pigmented structures up to 100-fold higher than the corresponding liver concentrations (6). *In vitro* studies with thiothixene indicate that its increased psychotherapeutic potency is not reflected in an increased affinity for melanin. In fact, the extent of melanin binding was similar to that of amitriptyline, another nonphenothiazine and the least bound of a larger series of tricyclic psychotherapeutic drugs studied by Potts (6). Since chlorprothixene, another thioxanthene derivative, is highly bound *in vitro* to melanin, the relatively low binding observed with thiothixene may be associated with the dimethylsulfamoyl substituent rather than with a change in the nucleus from phenothiazine to thioxanthene. Nevertheless, the results here indicate that drugs bearing the thioxanthene nucleus behave qualitatively as do their phenothiazine counterparts.

Rapid equilibration of drug levels within the eye and liver was indicated in experiments involving daily oral administration of thiothixene. Concentrations of radioactivity stopped rising after only 5 days. The eye and liver tissue levels attained with doses of 10 mg./Kg. were approximately 10 times those achieved after 1 mg./Kg., indicating no impairment of absorption, distribution, or metabolism within this range. Following withdrawal, levels began to decline immediately in the liver and skin, but there was a lag phase in the eye following which label was removed at a rate slower than that in the liver. The absence of a similar lag in the removal of label from skin (where it is presumably also bound to melanin) may indicate a difference in the nature of the complex. This may be due to differences in the chemical nature of the thiothixene metabolite interacting with the melanin, or in further transformations in the complex once formed. It has been suggested (31) that 7-hydroxychlorpromazine or a further metabolite thereof is the active species involved in chlorpromazine pigmentation; a similar thiothixene derivative may conceivably be involved.

As with the related tricyclic drugs, thiothixene was rapidly metabolized to a wide variety of derivatives with little of the unchanged drug being recovered. The liver appears to be the major site of metabolism, since bile samples obtained within 0.5 hr. of drug administration contained as great a diversity of metabolites as urine and bile samples taken at later intervals. The only metabolite of thiothixene yet tentatively identified is the *N*-demethyl derivative; such demethylation on the side chain is a common feature in the metabolism of drugs of this type.

Although inorganic sulfate has been implicated as a metabolite of ^{35}S-labeled chlorpromazine (26), indicating fission of the ring system, later studies (32) with chlorpromazine and promazine revealed no

evidence for such a reaction. Ten percent of the sulfur of phenothiazine, but an insignificant amount from promazine, was excreted as inorganic sulfate by dogs (25). There have been no subsequent reports concerning the derivation of sulfate from the tricyclic psychotherapeutic agents. Since the thioxanthene nucleus may behave in a manner unlike that of the related phenothiazine drugs, direct evidence on the ultimate fate of the nuclear sulfur was sought. Cysteine has been shown to be derived, in part, from inorganic sulfate (33) and was therefore felt to be a reliable indicator of sulfur metabolism. Since a mercapturic acid derived from administered bromobenzene and cysteine, an ethereal sulfate resulting from detoxication of administered β-naphthylamine, and tissue protein cysteine were all devoid of measurable radioactivity following the administration of a single high dose of thiothixene-^{35}S, it can therefore be reasonably concluded that the nuclear sulfur of thiothixene cannot enter the body sulfur pool. This would also tend to indicate that the nucleus remains intact, as has been concluded for the phenothiazine drugs.

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Keyphrases

Thiothixene metabolism
^{35}S-Labeled thiothixene—synthesis
Tissue distribution thiothixene
Thiothixene melanin interaction
Thiothixene excretion
Scintillation spectrometry—analysis
TLC analysis of urine
Thiothixene metabolites

Acid Dye Method for the Analysis of Thiamine

By V. DAS GUPTA* and DONALD E. CADWALLADER

An accurate, precise, and simple method for the analysis of thiamine has been developed. The method is based on the salt formation of thiamine with an acid dye, bromothymol blue. There is no interference from other vitamins, hormones (ethinyl estradiol and progesterone), and excipients. The nature of reaction between thiamine and bromothymol blue appears to be a simple acid–base reaction. The stoichiometric balance has been established to be one part of dye to one part of thiamine.

THIAMINE is an essential nutritional or therapeutic component of a great many oral and injectable pharmaceutical preparations. Many biological and chemical methods for the quantitative determination of thiamine have been proposed. Chemical assay methods are usually preferred to biological methods, since analysis can be performed rapidly and economically, and they are usually more reliable for routine determinations.

Although a number of chemical assay methods of thiamine are known, only the coupling reaction with diazotized 6-aminothymol (1) and thiochrome reaction (2) have been developed as precise methods of assay. In recent years the thiochrome method has been used almost exclusively, but close attention to details makes this method unsuitable for rapid control work. The development of an accurate colorimetric method of analysis for thiamine is the object of this investigation. Schill and Marsh (3) and Schill (4–6) have studied the photometric determination of amines and quaternary ammonium compounds with bromothymol blue. They reported that thiamine reacts with bromothymol blue to form a salt which can be determined photometrically. The present investigation includes studies pertinent to the nature of the salt, factors influencing its formation, and the stoichiometric balance.

EXPERIMENTAL

Reagents—All the chemicals and reagents used were either USP, NF, or A.C.S. grade. Bromothymol blue was purchased from Fisher Scientific Co. and was used without further purification.

Preparation of Solutions—A chloroform solution of the dye (4×10^{-4} *M*) was prepared by dissolving 62.44 mg. of bromothymol blue in enough chloroform to make 250 ml. of solution. This solution was diluted with chloroform to obtain various other strengths as needed. These solutions were stable for at least 1 month.

Received June 21, 1967, from the School of Pharmacy, University of Georgia, Athens, GA 30601

Accepted for publication August 30, 1967.

Abstracted in part from a thesis submitted by V. Das Gupta to the Graduate School, University of Georgia, in partial fulfillment of Doctor of Philosophy degree requirements.

Adapted from the manuscript submitted by V. Das Gupta, which received first prize in the Southern Section in the 1967 Lunsford Richardson Pharmacy Awards Competition.

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An aqueous buffer solution of bromothymol blue (2×10^{-4} *M*) was prepared by dissolving 62.44 mg. of the dye in approximately 0.5 ml. of 0.2 *M* sodium hydroxide solution and then adding enough pH 6.0 buffer solution to make 500 ml. The pH of the solution was determined with a Beckman Zeromatic pH meter. This solution was stable for at least 2 weeks.

Aqueous solutions of thiamine were prepared by dissolving 40.0 mg. of either thiamine hydrochloride or thiamine mononitrate in enough buffer solution to make 100 ml. These stock solutions could be used to prepare solutions of lower thiamine concentrations by diluting with appropriate buffer solutions. Solutions with pH 6.6 or lower were prepared fresh daily while those with values higher than pH 6.6 were prepared immediately before use, since thiamine is unstable at higher pH values.

Buffer solutions were prepared according to directions in the USP (7). Buffer solutions between pH 4.0 and 8.0 were prepared by taking 50 ml. of 0.2 *M* potassium dihydrogen phosphate solution, adding an appropriate amount of 0.2 *M* sodium hydroxide solution or 0.2 *M* hydrochloric acid solution, and then adding enough distilled water to make 200 ml. Solutions between pH 1.0 and 4.0 were prepared by adding an appropriate amount of 0.2 *M* hydrochloric acid to distilled water, and solutions between pH 9.0 and 11.5 were prepared by adding 0.2 *M* sodium hydroxide to distilled water. The pH of each solution was determined with a Beckman Zeromatic pH meter.

Determination of Thiamine Dye Salt in Organic Phase—*Procedure A*—A 10.0-ml. quantity of buffer solution containing an appropriate amount of thiamine alone or with other substances was placed in a 125-ml. separator. A 10.0-ml. quantity of dye solution in chloroform (1×10^{-4} *M* unless otherwise indicated) was added to 10.0 ml. of the aqueous solution, and the separator was shaken vigorously by hand for 1 min. The nonpolar and polar phases were allowed to separate and the chloroform layer was collected. The aqueous phase was extracted with 2 additional 10.0-ml. quantities of chloroform and the 3 chloroform extracts were combined. The chloroform solution was evaporated in a 50-ml. beaker using low heat (60°) to a volume less than 10 ml. After the solution had cooled to room temperature, chloroform was added to make 10.0 ml. of solution. A portion of this solution was centrifuged for 5 min. at 2500 r.p.m., and the absorbance of the clear solution was read at 420 mμ (slit width 0.03 mm.) using a Beckman DU spectrophotometer. A blank was prepared by substituting 10.0 ml. of plain buffer solution for thiamine solution in the above procedure.

Procedure B—This procedure is the same as above

except that only one 10.0-ml. quantity of chloroform was used for extraction instead of three.

Procedure C—A 5.0-ml. quantity of pH 6.0 buffer solution containing 10.0 mcg./ml. of thiamine hydrochloride was combined with 5.0 ml. of buffered dye solution in a 125-ml. separator. A 10.0-ml. quantity of organic solvent was added and the separator was shaken vigorously by hand for 1 min. The polar and the nonpolar phases were allowed to separate, and the organic phase was collected and centrifuged at 2500 r.p.m. for 5 min. The absorbance of the clear solution was read at 420 mμ. A blank was prepared by substituting plain buffer solution (5.0 ml.) for thiamine solution in the above procedure.

Determination of Dye in Aqueous and Organic Phases—To determine the amount of dye in the aqueous phase of an experimental run, the aqueous layer was collected and centrifuged for 5 min. at 2500 r.p.m., and the absorbance was measured at 503 mμ (slit width 0.015 mm.), the isosbestic point of bromothymol blue, with a Beckman DU spectrophotometer, and compared with a standard.

To determine the amount of dye in the organic phase of an experimental run, the organic layer was collected and a portion added to an equal volume of alkaline aqueous solution (pH 11.5) in a 125-ml. separator. The funnel was vigorously shaken by hand for 1 min., the aqueous phase was collected, and the absorbance of the solution measured at 503 mμ as described above.

The isosbestic point is that wavelength where the absorption of a dye in solution is dependent only on the concentration of dye and not the pH. The isosbestic point of bromothymol blue was determined according to the procedure of Reilley and Swayer (8). A stock solution of bromothymol blue was prepared by dissolving 100 mg. of the dye in enough 20% v/v ethyl alcohol to make 100 ml. Two milliliters of the stock solution was placed in a 50-ml. volumetric flask, 20 ml. of distilled water and 8 drops of concentrated hydrochloric acid were added, and the volume was brought to 50 ml. with distilled water. The pH of this solution was approximately 1. A pH 6.9 solution was prepared by placing 2.0 ml. of the stock solution in a volumetric flask and adding sufficient quantity of pH 6.9 phosphate buffer to make 50 ml. A solution having pH 13 (approximately) was prepared by placing 2.0 ml. of the stock solution in a 50-ml. volumetric flask, adding 28 drops of 3.5 *M* sodium hydroxide solution, and diluting to 50 ml. with distilled water. An absorption spectrum of each of the 3 solutions between 365 to 600 mμ was recorded on the same graph paper using a Perkin-Elmer 202 spectrophotometer. The point of intersection where the 3 curves have the same absorbance is the isosbestic point for bromothymol blue (Fig. 1).

Determination of Thiamine in Aqueous and Organic Phases—The determination of thiamine in aqueous phase was carried out according to the USP assay method (2). The fluorescence of each solution was measured using a Coleman model 12 C fluorophotometer and thiamine content was determined by comparison with standard readings.

The determination of thiamine in an organic solvent was carried out by first mixing the organic phase with an equal volume of 0.2 *M* hydrochloric acid in a 100-ml. beaker and evaporating the organic

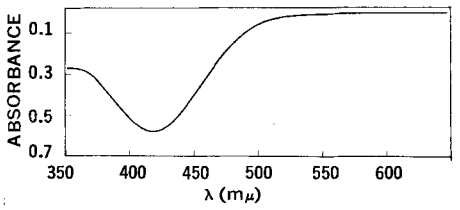

Fig. 1—Absorption spectrum of bromothymol blue.

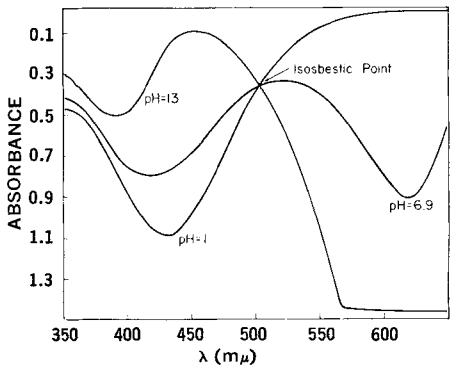

Fig. 2—Absorption spectra of thiamine dye salt.

phase by heating at 80°. After the solution was cooled to room temperature, 0.2 *M* hydrochloric acid was added to give a thiamine salt concentration of approximately 0.2 mcg./ml. The thiamine assay was then carried out as described above.

Determination of the Wavelength of Maximum Absorption—A 10.0-ml. quantity of pH 6.6 buffer solution containing approximately 8.5 mcg./ml. of thiamine hydrochloride was placed in a 125-ml. separator. A 10.0-ml. quantity of bromothymol blue solution (1×10^{-4} *M*) in chloroform was added to the aqueous solution, and the separator was shaken vigorously for 1 min. The two phases were allowed to separate, the chloroform phase was collected, and the absorption spectrum of the thiamine dye salt in solution was obtained by scanning on a Perkin-Elmer 202 spectrophotometer. The absorption spectrum is shown in Fig. 2.

Determination of Maximum Dye Concentration—Ten-milliliter quantities of pH 6.0 buffer solution containing 4.0 mcg./ml. of thiamine hydrochloride were placed in 125-ml. separators. Ten-milliliter quantities of dye in chloroform solutions (various concentrations of dye) were added to the separators, and each mixture was treated in the manner described under procedure *B* for the determination of thiamine dye salt. The results are presented in Table I.

Preparation of Calibration Curves—Using procedure *A*, pH 6.6 buffer solutions containing various concentrations of thiamine hydrochloride or thiamine mononitrate were assayed for thiamine dye salt concentrations. The absorbance readings were used to prepare calibration curves and to test for conformity to Beer's law. The results are shown in Fig. 3.

Determination of Best Organic Solvent—Using procedure *C*, various organic solvents were tested to determine the best solvent for extracting the

TABLE I—ABSORBANCE READINGS WITH INCREASING DYE CONCENTRATION[a]

Dye Concn., moles/L.	Absorbance at 420 mμ
2.5×10^{-5}	0.244
5.0×10^{-5}	0.272
1.0×10^{-4}	0.292
2.0×10^{-4}	0.325[b]

[a] Each absorbance value is an average of 3 experiments. Concentration of thiamine HCl was always 4.0 mcg./ml. All individual readings were within ±3% of the mean reading. [b] Reading is high probably due to emulsion formation. This limits the maximum concentration to 1×10^{-4} moles/L.

TABLE II—PERCENTAGE OF THIAMINE DYE SALT EXTRACTED BY VARIOUS SOLVENTS[a]

Solvent	% Extracted
Benzene	6.5
Carbon tetrachloride	1.3
Chloroform	95.3
Methylene chloride	80.4
Petroleum ether	2.6
Toluene	5.2
Xylene	5.2

[a] Each value is an average of 2 experiments. All individual readings were within ±3% of the mean reading.

TABLE III—EFFECT OF pH ON THE EXTRACTION OF THIAMINE DYE SALT[a]

pH	Absorbance at 420 mμ, Procedure B[b]	Absorbance at 420 mμ, Procedure A[c]
2.0	0.000	0.000
3.0	0.000	0.000
3.9	0.020	0.020
4.3	0.205	0.285
5.2	0.274	0.304
5.4	0.285	0.305
5.6	0.290	0.305
5.8	0.290	0.305
6.0	0.290	0.305
6.2	0.290	0.305
6.4	0.280	0.304
6.6	0.270	0.304
6.8	0.250	0.305
7.0	0.224	0.281
7.2	0.188	0.282
7.4	0.130	0.235
7.6	0.130	0.235
8.0	0.052	0 092
10.0	0.000	0.000

[a] Each absorbance value is an average of three experiments. All individual readings were within ±3% of the mean reading. [b] One 10.0-ml. portion of chloroform was used to extract the thiamine salt. [c] Three 10.0-ml. portions of chloroform were used to extract the thiamine dye salt.

thiamine salt. The results are presented in Table II.

Determination of the Effect of pH—Various solutions (pH range 2 to 10) containing 4.0 mcg./ml. of thiamine hydrochloride were prepared and assayed for thiamine dye salt according to procedures *A* and *B*. The results are shown in Table III.

Determination of Effect of Time and Temperature on Stability of Thiamine Dye Salt—A pH 6.6 buffer solution containing 4.0 mcg./ml. of thiamine hydrochloride was used in these studies.

Procedure *A* was used to determine the thiamine dye salt; however, after the absorbance of the chloroform layer was determined initially, it was allowed to stand for 1, 2, and 3 hr. at ambient temperature (25° ± 2°). The absorbance of the chloroform solution was measured at hourly intervals and the results are given in Table IV.

In other experiments the absorbance of the chloroform solution was determined initially, and then the solution was diluted to 3 times its initial volume with chloroform. This solution was heated in a beaker at 60° for approximately 10 to 15 min. until the volume was less than 10 ml. After cooling to room temperature, the solution was adjusted to its original volume and the absorbance of this solution measured. The result is shown in Table IV.

Tolerance of Assay Method to Diverse Substances—Buffer solutions (pH 6.0 and 6.6) containing various amounts of diverse substances were prepared. The concentration of each test substance in solution was approximately 4 times that usually present in commercial products. The interference of each substance was individually determined by substituting 10.0 ml. of the test solution for 10.0 ml. of thiamine solution and carrying out the assay according to procedure *A*. Any absorbance in the final chloroform solution was indicative of interference. The results are shown in Table V.

TABLE IV—EFFECT OF TIME AND TEMPERATURE ON THE STABILITY OF THE THIAMINE DYE SALT[a]

Time, hr.	Temp., °C.	Absorbance at 420 mμ
0	25	0.307
1	25	0.306
2	25	0.306
3	25	0.306
0.25	60	0.306

[a] Each absorbance value is an average of two experiments. All individual readings were within ±3% of the mean reading.

TABLE V—EFFECT OF DIVERSE SUBSTANCES[a]

Substance	Concn., mcg./ml.	Absorbance Values at 420 mμ, pH 6.0	Absorbance Values at 420 mμ, pH 6.6
Amaranth	1.0	0.000	0.000
Butyl-*p*-hydroxybenzoate	0.1	0.000	0.000
DL-Calcium pantothenate	100.0	0.000	0.000
Copper sulfate	10.0	0.000	0.000
Ethinyl estradiol	1.0	0.000	0.000
Ethyl alcohol[b]	0.05[b]	0.060[c]	0.056[c]
Ferrous sulfate	500.0	0.000	0.000
Folic acid	5.0	0.000	0.000
Glycerin[b]	0.05[b]	0.000	0.000
Lactose	30.0	0.000	0.000
Magnesium sulfate	15.0	0.000	0.000
Manganese sulfate	10.0	0.000	0.000
Molybdenum sulfate	10.0	0.000	0.000
Nicotinamide	50.0	0.045	0.000
Progesterone	5.0	0.000	0.000
Pyridoxine HCl	10.0	0.000	0.000
Riboflavin	10.0	0.000	0.000
Starch	30.0	0.000	0.000
Sugar	30.0	0.000	0.000
Vitamin A acetate	50.0	0.000	0.000
Vitamin B_{12}	0.02	0.000	0.000
Vitamin C	200.0	0.000	0.000
Vitamin D_2	5.0	0.000	0.000
Zinc sulfate	10.0	0.000	0.000

[a] Each absorbance value is an average of two experiments. [b] Concentration expressed in ml./ml. for these liquids. [c] There is no interference if the alcohol present is evaporated using gentle heat before assaying.

Experiments were carried out in which pH 6.6 solutions were prepared to contain 0.0, 4.0, 6.0, and 8.0 mcg./ml. of thiamine hydrochloride or mononitrate and all of the substances listed in Table V. (Each substance was present in the concentration listed in Table V.) These solutions were tested for interference in the same manner as described above. The results are presented in Table VI.

Effect of decomposition products on the acid dye method was studied by preparing an aqueous solution containing 20 mcg./ml. of thiamine mononitrate and all the substances listed in Table V (the concentration of each substance in solution was 5 times the concentration listed in Table V). The pH of this solution was adjusted to 6.4 with sodium hydroxide (0.2 *M*). Initial assays for thiamine were run both by the USP method (2) and by the acid dye method (procedure *A* using pH 6.6 buffer). After storing this solution at 60° for 85 days, the thiamine content was again determined using the methods described above. Results are presented in Table VII.

Reproducibility of Results—To test the reproducibility of results, 12 assays were run by the USP method (2) and 12 by the acid dye method using procedure *A* and thiamine mononitrate (4 mcg./ml.) in a pH 6.6 buffer solution. Results are presented in Table VIII.

Determination of Stoichiometric Balance—Buffer solutions (pH 7.7) containing 10.0, 20.0, or 30.0 mcg./ml. of thiamine hydrochloride or thiamine mononitrate were prepared for these studies. The high pH was used to prevent the passage of any free dye into the chloroform layer. Each of the thiamine solutions was mixed with 2×10^{-4} *M* bromothymol blue in chloroform solution according to the directions in procedure *B*. After the nonpolar and polar layers were separated and collected, the chloroform and aqueous solutions were assayed for thiamine and dye according to the methods described under *Determination of Thiamine and Dye in Aqueous and Organic Phases*. The results are presented in Table IX. The stoichiometric balance was calculated by using the following equation:

$$\frac{\text{concn. of thiamine in } CHCl_3}{\text{mol. wt. of thiamine}} : \frac{\text{concn. of dye in } CHCl_3}{\text{mol. wt. of dye}}$$

Several experiments were carried out to confirm the stoichiometric balance of 1:1. Twenty milligrams of thiamine mononitrate was added to 100 ml. of bromothymol blue in chloroform solution (12.48 mcg./ml.) in a 250-ml. separator, and the mixture was shaken intermittently over a period of 3 hr. After 3 hr. the chloroform layer exhibited a lower absorbance value (at 420 mμ) than was expected for complete salt formation. A 5-ml. quantity of distilled water was then added and the mixture was shaken vigorously for several minutes, after which time the equilibrium was established. After the two phases had separated, the aqueous solution was colorless and the chloroform solution gave an expected absorbance reading. Subsequent experiments were carried out in which 5 ml. of water was added immediately and the 3-hr. shaking period was eliminated. Similar experiments were carried out using a higher dye concentration (24.96 mcg./ml.). The results are presented in Table X.

TABLE VI—COMBINED EFFECT OF DIVERSE SUBSTANCES[a]

Concn., Thiamine, mcg./ml.		Absorbance at 420 mμ	
HCl	Mononitrate	Thiamine Alone	Thiamine + Added Substances
0.00	0.00	0.000	0.000
4.00	0.00	0.305	0.306
6.00	0.00	0.456	0.457
8.00	0.00	0.608	0.609
0.00	4.00	0.312	0.313
0.00	8.00	0.622	0.624

[a] Each absorbance value is an average of 3 experiments. All individual readings were within ±3% of the mean reading. Each substance was present in the concentration listed in Table V. Procedure *A* and buffer solution of pH 6.6 were used in all the assays.

TABLE VII—EFFECT OF DECOMPOSITION PRODUCTS ON THE ACID DYE METHOD AS COMPARED WITH THE USP METHOD[a]

	Percentage Thiamine Mononitrate Remaining After	
	Initial	85 Days
USP method	100.3	10.1
Dye method	100.4	9.8

[a] Each percentage is an average of 3 assays. All individual readings were within ±3% of the mean reading.

TABLE VIII—REPRODUCIBILITY OF RESULTS (DYE METHOD *Versus* USP METHOD) ON A SAMPLE (4.0 mcg./ml.) OF THIAMINE MONONITRATE

Sample	Found, mcg./ml. Dye Method	Found, mcg./ml. USP Method	Deviation, mcg. Dye Method	Deviation, mcg. USP Method
1	4.08	4.04	0.08	0.04
2	4.02	4.00	0.02	0.00
3	4.04	4.08	0.04	0.08
4	4.02	3.94	0.02	0.06
5	3.94	3.92	0.06	0.08
6	4.00	4.04	0.00	0.04
7	3.98	4.08	0.02	0.08
8	4.03	4.00	0.03	0.00
9	4.00	4.06	0.00	0.06
10	3.95	3.92	0.05	0.08
11	3.93	3.94	0.07	0.06
12	4.03	3.98	0.03	0.02
Av. deviations	4.00 + 0.04	4.00 + 0.05	...	. .

Physical Properties of the Salts—A 300-ml. quantity of pH 6.6 buffer solution containing 100 mcg./ml. of thiamine hydrochloride or mononitrate was vigorously skaken with 300 ml. of dye in chloroform solution (1×10^{-4} *M*) in a 1,000-ml. separator for 1 min. The phases were allowed to separate and the chloroform solution containing the thiamine dye salt was collected in a 600-ml. beaker, and evaporated to a small volume (3 to 5 ml.) on a hot plate (60°). The remaining chloroform was allowed to evaporate at ambient temperature and the salt was dried in a desiccator. The melting points of the salts were determined using a Thomas-Hoover capillary melting point apparatus.

DISCUSSION AND CONCLUSIONS

Optimum Conditions for Assay—The results show that a method based on the reaction between bromothymol blue and thiamine (procedure *A*) in a pH 6.6 buffer solution can be used for the analysis

TABLE IX—DISTRIBUTION OF THIAMINE AND DYE BETWEEN CHLOROFORM AND WATER (pH 7.7) FOR THE DETERMINATION OF STOICHIOMETRIC BALANCE[a]

Total Thiamine Salt Concn., mcg./ml.	Concn., mcg./ml. of Thiamine Salt: In Water	Thiamine Salt: In Chloroform	Bromothymol Blue: In Water	Bromothymol Blue: In Chloroform	*M* Ratio of Thiamine to Dye
			Hydrochloride		
10.0	4.71	5.29	114.70	10.10	1.00:1.03
20.0	9.43	10.57	104.58	20.22	1.00:1.03
30.0	14.15	15.85	94.48	30.32	1.00:1.03
			Mononitrate		
10.0	4.50	5.50	114.35	10.45[b]	1.00:1.00
20.0	9.25	10.75	104.25	20.55	1.00:1.00
30.0	13.95	16.05	94.28	30.52	1.00:1.00

[a] Each value is an average of 3 experiments. All individual readings were within ±3% of the mean reading. [b] Absorbance value for this concentration of thiamine dye salt in chloroform was 0.398.

TABLE X—CONFIRMATION OF STOICHIOMETRIC BALANCE[a]

Dye Concn., mcg./ml.	Absorbance at 420 mμ: Expected[b]	Found
12.48	0.475	0.472
24.96	0.950	0.944

[a] Each absorbance value is an average of 3 experiments All individual readings were within ±3% of the mean reading. [b] From Table IX (*Footnote b*) we find that 10.45 mcg./ml. of dye in chloroform gives an absorbance value of 0.398. Assuming a stoichiometric balance of 1:1, a dye concentration of 12.48 mcg./ml. in chloroform will be expected to give an absorbance value of 0.475.

of thiamine in pharmaceutical preparations (Tables III and V) and the best dye concentration is 1×10^{-4} moles/L. (Table I). The optimum pH value of 6.6 is not in agreement with a 7.4 value reported by Schill (6).

Beer's law is followed within the range of 4 to 10 mcg./ml. of thiamine (Fig. 3) at 420 mμ (Fig. 2). The best solvent for extraction is chloroform (Table II) and the thiamine dye salt is very stable in this solvent (Table IV). The acid dye method is at least as accurate and precise as the USP method. The reproducibility of results based on 12 samples (4 mcg./ml.) was 4.00 ± 0.04 *versus* 4.00 ± 0.05 by the method (Table VIII).

Nature of Thiamine Dye Salt—The nature of the reaction between bromothymol blue and thiamine seems to be a simple acid–base reaction. The stoichiometric balance of the reaction is 1:1 (Tables IX and X). The thiamine salts formed with bromothymol blue have high molecular weights (925.2 for thiamine monochloride dye salt and 915.28 for thiamine mononitrate dye salt) as compared with thiamine hydrochloride (337.28) and thiamine mononitrate (327.36); hence they are more soluble in organic solvents. The melting points of thiamine dye salts were 255–258° (with decomposition). These high melting points indicate that ion–ion pairs are salts and not loose complexes. Additional work is being carried out to establish the composition of these salts.

Effect of Diverse Substances—No interference in the analytical procedure was observed from other vitamins, minerals, hormones (ethinyl estradiol and progesterone), and excipients except for a slight interference from ethyl alcohol and nicotinamide (Tables V and VI). Lack of interference was sxpected, since none of these substances is as etrong a base as is thiamine.

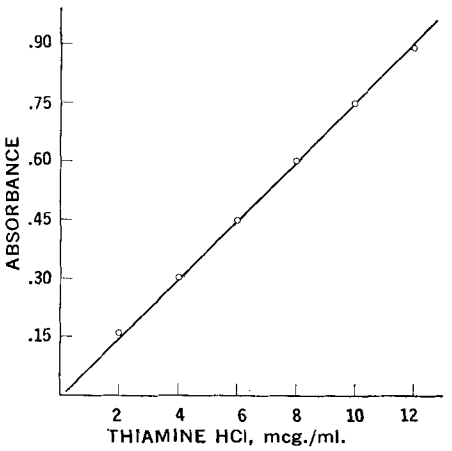

Fig. 3—Standard curve for thiamine hydrochloride. A similar curve was obtained with thiamine mononitrate.

The interference from ethyl alcohol may be due to increase in the polarity of the nonaqueous phase which results in the occurrence of more dissociated dye in this phase. A slight interference from nicotinamide at pH 6.0 and no interference at pH 6.6 was not studied further.

Interference from the decomposition products of thiamine and other vitamins is also negligible (Table VII). Apparently none of the products of decompositon behaves as a strong base. This being the case, it may be possible to ascertain the degradation products. If, for example, it is assumed that thiamine was oxidized on storage at 60°, then thiochrome, the decomposition product, is formed, which means the amino group of the pyrimidine ring, which is probably responsible for the formation of thiamine dye salt, is lost.

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Keyphrases

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Thiamine–bromothymol blue salt formation
Thiamine–bromothymol blue salt physical properties
Stoichiometric balance
Bromothymol blue isosbestic point

Shortcomings in Pharmacokinetic Analysis by Conceiving the Body to Exhibit Properties of a Single Compartment

By S. RIEGELMAN, J. C. K. LOO, and M. ROWLAND

In the past, pharmacokinetic assessment of drug absorption, metabolism, and excretion usually have been based on the concept of the body behaving as a single compartment. After rapid i.v. injection, provided that blood samples are taken sufficiently soon after injection, at least a bi-exponential curve is obtained. The initial portion of this curve, the so-called rapid distribution phase, has been ignored without proof, and it has been assumed that the single-compartment concept does not introduce large errors into the subsequent calculations. On the basis of first principles, at least one additional peripheral compartment must exist for virtually any compound introduced into the body. Such a model is physiologically compatible with distribution of the drug throughout the body under perfusion and diffusion forces. The two-compartmental open-system model is discussed in respect to the error introduced into the usual absorption rate and elimination rate calculations and on the estimation of the volume of distribution of various drugs.

PHARMACOKINETIC STUDIES are usually undertaken to attempt to define, as accurately as possible, the rates of absorption, metabolism, and excretion of a drug and its metabolites. The analyses of data, obtained after administration of a drug to man or animals, have most commonly been based on the presumption that it is adequate to consider the body as exhibiting the properties of a single compartment. Since a model is only conceived to serve the purposes of the scientist, it need not be any more complex (nor should it be any less simplified) than required to serve this function. However, it is scientifically unsound to continue to accept a model because it is often used; instead it should be questioned until there is sufficient evidence to support its adequacy for the purposes for which it was designed. Surprisingly, within the knowledge of the authors, there appears to have been no critical tests of the validity of the single-compartmental model. This paper is the first of a series which will attempt to examine this model and point to some of the limitations and incorrect reasoning that it imposes on these analyses.

It is the purpose of this paper to emphasize that a central and at least one peripheral compartment appear essential to describe adequately the distribution of the drug in the body. Not only is such a model sufficiently realistic when viewed on a physiological basis, but it is mathematically more acceptable than the one-compartmental model.

DISCUSSION

Physiological Aspects of the Mammillary Model—Compounds are distributed throughout the body by the blood and vascular network acting as a carrier system. One conceives of a model which represents drug distribution and elimination to be made up of a central compartment with interchanging connections with one or more peripheral compartments. Such a model has been called a mammillary model (1). While blood is a major

Received April 14, 1967, from the Departments of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94122
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essential component of the central compartment, it will be made clear later that the compartment is larger than the plasma volume. When a drug is injected rapidly into a vein, it initially mixes with a small volume of plasma forming a bolus. However, as it passes through various capillary beds, filtration and diffusion forces cause some of the molecules to transfer into the surrounding tissues. These events take place extremely rapidly (2), so that even during the first few moments while the drug solution becomes physically mixed with the plasma, the solute has already penetrated into a much larger volume.

The rate of uptake of a compound into the tissues is controlled by several forces, including the rate of flow of blood through the tissue, the mass (volume) of the tissue, and the partition characteristics of the compound between the plasma and the tissue. The interrelationship of these factors is defined by Fick's law of perfusion and has been discussed in detail by Kety (3) and others (4, 5). The rate of flow of blood through the tissues varies in each tissue from highs of 500 ml. to less than 2 ml. of blood/100 ml. of tissue/min. The partition coefficient of the compound into the various tissues also is affected by many factors, such as its relative lipoid solubility, pH, and complexation with proteins, nucleic acids, *etc.* It is clear from the above that drugs will distribute into tissues in a highly complex manner and in a real sense will exhibit different concentrations down to the subcellular level. This almost infinite degree of complexity brings confusion into the issue of multiple-compartmental analysis merely because we are so anxious to place physiological meanings to the compartments defined by a particular experiment.

The distribution of a drug into various tissues as described above is spoken of in anesthesia and physiological literature (5, 6) as the perfusion-limited, multiple-compartmental model. If the body must be divided into N-compartments, there should be $N - 1$ exponential terms in the resultant equation.[1] The fact that it is rarely possible to determine more than two or three exponential terms in these studies indicates that many of the tissue compartments exhibit sufficient similar properties to pool into large groups or are too small to be detected. The anesthesiologist–physiologist conceives of the body as dividing into four groups according to their perfusion and partition characteristics.

A highly perfused lean tissue group, consisting of the heart, lung, hepatoportal system, kidneys, endocrine glands, and under certain instances the brain and spinal system.

A poorly perfused lean tissue group, consisting of the large mass of muscle and skin tissue.

A fat group, consisting of the adipose tissue, including marrow.

A negligible perfusion tissue group, consisting of bone (not marrow), teeth, ligaments, tendons, cartilage, contents of the alimentary canal, and hair. This final group can usually be ignored in pharmacokinetic analysis except in those cases when considering drugs (tetracyclines) or elements (calcium, phosphate) which concentrate in this compartment.

Recently, Perl and co-workers have shown (7) that the multiple-compartmental model based on distribution of the compound solely by a perfusion-limited process is unrealistic, at least as it applies to fat-soluble compounds (8). Even though fat is estimated to have approximately the same perfusion rate as muscle tissue, many compounds are much more soluble in the fat than in other tissue masses. Therefore, direct diffusion between the fat pads and the adjacent tissues becomes a significant pathway due to the gradient in concentration which develops as the neighboring tissues "fill up" at unequal rates from the common source, the arterial blood. Perl and co-workers propose that the adipose tissue should be considered to be stored into two types of depots. In one (subcutaneous, interstitial, *etc.*), the adipose tissue has a boundary surface in contact with the more slowly perfused lean tissues, such as the resting muscle and skin. In the other, the adipose tissue (omental, perirenal, *etc.*) has a boundary surface in contact with the highly perfused lean tissue. Strong evidence to support this contention was presented from studies on the simultaneous uptake of nitrous oxide and cyclopropane in man at subanesthetic levels. These compounds have identical partition characteristics into body fluids and tissues, except that cyclopropane is eight times more soluble in fat than is nitrous oxide. An approximately constant 5% difference in the alveolar concentration of the two gases was obtained when they were simultaneously administered. This was larger and more rapidly evolved than could be explained on any physiologically acceptable modification of the simple perfusion-limited, multicompartmental model. The magnitude and rate of onset of the concentration difference could only be adequately explained on the basis of diffusional forces modifying the perfusion-limited distribution and causing the cyclopropane to enter more rapidly the two separate fat compartments *via* the neighboring tissues.

From the above discussions it seems likely that one would not have to consider the body to be so highly compartmentalized as was originally thought. In effect, the two fat compartments surrounded by tissues of different perfusion characteristics simply modify the effective volume of these two tissue groups and their rate of equilibration at least insofar as the pharmacokinetic calculations are concerned. Therefore, since the uptake of the drug by the highly perfused tissue group is usually so rapid, it can be conceived mathematically to be part of the central compartment. Also, expanding the volume of the central compartment to include some extracellular fluids and the highly perfused tissue mass as well appears to be physiologically acceptable and mathematically logical.

Simultaneous to distributing throughout the body, virtually all drugs undergo metabolism. The effect of metabolism is superimposed on the distribution from the very instant the drug enters the tissues and the magnitude of its influence on the observed rate constants for the loss of the compound from the body varies from compound to compound. This effect will be discussed in detail below. The major site for metabolism, liver, as well as the minor sites, the kidneys and gastrointestinal tissues, are all included in the central compartment in the presently conceived model. This will simplify many mathematical considerations (9). From the viewpoint of what can be detected from the blood,

[1] The so-called two-compartmental open-system model in an exact sense has at least one additional compartment, the urine, feces, or metabolite.

it is, therefore, physiologically acceptable to conceive of a bi- or tri-exponential function representing body distribution of the drug from a central into one or two peripheral compartments, with metabolism and excretion taking place from the central compartment. This viewpoint in itself does not prove that a two- or three-compartmental model is better than the one-compartmental model, or that a more complex model may not be needed to meet the known physiological or pharmacological facts. The intent of the above discussion is to emphasize that a two-compartmental model may be all that is necessary to achieve the declared goals of accurate assessment of the rates of absorption, metabolism, and excretion. A possible method of establishing valid experimental support for a given model can be made through studies of absorption which will be presented later.

Mathematical Basis for Reducing N-Compartmental System into a Two-Compartmental Model—From a purely pragmatic point of view, the above simplification of complex physiological phenomena needs no further justification. Yet Sharney, Wasserman, and co-workers (10) have shown that under certain precise conditions a system consisting of an arbitrarily large number of interchanging pools can be represented exactly by a two-pool or three-pool model. These authors made no *a priori* assumptions about the relative sizes of the peripheral pools and, therefore, such mammillary systems may include very small as well as very large peripheral compartments. They emphasized that such a representation of complex mammillary systems by a simple two- or three-pool model is a perfectly valid and rigorous procedure. Because no individual compartment is detected, the corresponding rate constants out of the peripheral compartments are defined by Sharney to be equal since they are not specific in any sense nor do they apply to any real tissue compartment (10). They are, however, essential to the authors' model in that they define the disposition of the drug into the rest of the body. Without their definition, the absorption and probably the metabolism and excretion characteristics of the drug are not accurately definable.

Estimation of Tissue Compartment on Basis of Material Balance—One might suggest that a peripheral compartment is detectable only in the early phases of the time course of the drug in the body and that the body takes on the characteristics of a single-compartmental model when the blood data appear to indicate monoexponential decay. The loss of the drug from the body is then specified to be the elimination rate constant. However, without presuming any specific model for distribution of a drug after an intravenous injection, it can be shown that at least one peripheral (tissue) compartment exists *throughout* the time course of the drug in the body. Material balance requires that the following equation holds true at any time:

$$\text{dose} = V_pC_p + M + E + T \quad \text{(Eq. 1)}$$

where

V_pC_p = mass of the original dose, D, in the central compartment at any time, t, as indicated by the concentration of the drug in the plasma, C_p.

V_p = volume of the undefined central compartment.

$C_p^\circ = D/V_p$ = concentration of the drug at zero time as defined by extrapolation of the curve back to $t = 0$.

M = mass of the original drug metabolized (from central compartment) by *all* processes.

E = mass of original drug excreted (from central compartment) by all routes.

T = mass of the original dose distributed to the peripheral or so-called tissue compartment at any time.

It is further presumed that metabolism and excretion take place by first-order processes with the rate constants, k_m and k_e, respectively, *i.e.*,

$$dM/dt = k_mV_pC_p \quad \text{(Eq. 2)}$$

$$dE/dt = k_eV_pC_p \quad \text{(Eq. 3)}$$

Also, defining the sum of these two processes as the elimination rate constant, $k_{el} = k_m + k_e$, then the amount of the original dose lost by all processes of metabolism and excretion up to time, t, is obtained by integrating Eqs. 2 and 3:

$$M + E \Big|_0^t = k_{el}\, V_p \int_0^t C_p dt \quad \text{(Eq. 4)}$$

where the integral term $\int_0^t C_p dt$ is the area under the plasma concentration time curve from the time of the injection to time t. Substitution into Eq. 1 and rearranging gives:

$$T = D - k_{el}\, V_p \int_0^t C_p dt - V_pC_p \quad \text{(Eq. 5)}$$

At a sufficient time, arbitrarily defined as infinite time, for all the drug to be eliminated from the body, Eq. 4 becomes:

$$\text{dose} = M + E \Big|_0^\infty = k_{el}V_p \int_0^\infty C_p dt \quad \text{(Eq. 6)}$$

from which we can substitute for $k_{el}V_p$ in Eq. 5.

$$T = D - D\frac{\int_0^t C_p dt}{\int_0^\infty C_p dt} - V_pC_p \quad \text{(Eq. 7)}$$

Equation 7 is, therefore, a method of estimating the mass of the drug which is in the tissue compartment at any time. Dividing through by dose, results:

$$T/D = 1 - \frac{\int_0^t C_p dt}{\int_0^\infty C_p dt} - C_p/C_p^\circ \quad \text{(Eq. 8)}$$

Equation 8 is useful to describe the fate of the compound from data obtained from intravenous administration of that compound. The term C_p/C_p° is the fraction of the original dose remaining in the central compartment at any time. The term T/D is the fraction of the dose which has entered and remains in the peripheral compartment at any time. Finally, the term

$$\int_0^t C_p dt \Big/ \int_0^\infty C_p dt$$

is the fractional area term and represents the fraction of the original dose which has been lost from the

central compartment by metabolism and excretion. While both metabolism and excretion can take place from the peripheral compartments, on the basis of the present knowledge, both of these processes predominantly take place in tissues included within the central compartment. Figures 1–5 are based on calculations using Eq. 8 on selected data from compounds of interest. It is clear from these data that the size of the tissue compartment varies markedly from compound to compound, as does the instant at which the central and tissue compartments reach equilibrium ($dT/dt = 0$). However, in each of the examples illustrated the data indicate the continued existence of the tissue compartment throughout the test period.

The parallelism of the slope of the two lines is a point that creates a great deal of confusion. The fraction of the drug lost from each compartment is identical when the two compartments are in apparent distribution equilibrium. However, the two compartments are not in steady state of equilibrium throughout this period, since drug mass is exchanging between the compartments per unit time. Neither can they be lumped into a single compartment without loss of certain mathematical accuracy in the calculation of the absorption and the elimination rate constants.

Many drugs cannot be easily studied from blood specimens due to the small dose administered or at times when only a small fraction of the dose remains in the central compartment. This is particularly true of amine drugs. The value of the study of urinary excretion data was shown by the farsighted and basic studies of Nelson (16–18) and others (19, 20). It should be noted that Eq. 3 listed above intrinsically presumes constant urinary clearance. Therefore, if urinary excretion samples were taken at sufficient frequency, a similar analysis of the data could be made. This is seen in Fig. 6 where blood and urinary data on mannitol (21) are represented. Unfortunately, it is not easy to obtain sufficient urine samples for such an analysis in each case. However, analysis of urinary data for the absorption and elimination rate constants are no more valid than their counterparts calculated from the blood data; indeed, it is likely that they may be less accurate due to variations in urinary pH, flow rate, and when a small fraction of the drug is excreted intact.

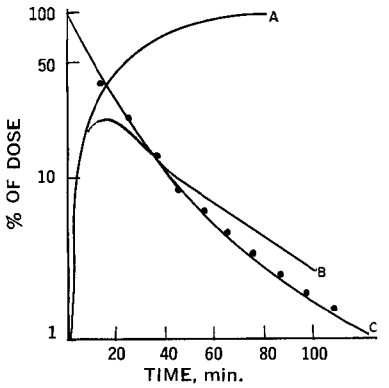


Fig. 1—Percent of dose estimated to be in the central compartment, tissue compartment, and eliminated after a 100-mg. i.v. injection of penicillin G into a male (12). Key: A, *amount eliminated;* B, *tissue compartment;* C, *central compartment.*

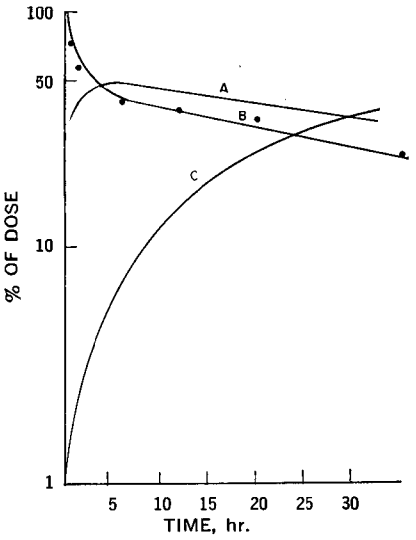
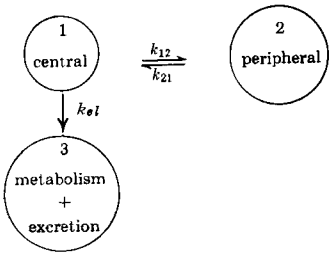


Fig. 2—Percent of dose estimated to be in the central compartment, tissue compartment, and eliminated after a 1.0-Gm. i.v. injection of pentobarbital into a male (13). Key: A, *tissue compartment;* B, *central compartment;* C, *amount eliminated.*

Two-Compartmental Open-System Model—The above discussions all lead to the inevitable conclusion that the body behaves more like a two-compartmental open system, such as is indicated in the diagram shown in Scheme I.



Scheme I

It is believed that this model was first related to drug kinetics by Teorell in 1937 (22). Since then it has been discussed in various degrees of sophistication by Dominguez (23), Sheppard (24), Solomon (25), Shore (26), Riggs (27), Mathews *et al.* (28), Nelson (29), Resigno and Segre (30), Sharney *et al.* (31), and others. Solution of the differential equations resulting from such a model yields the following integrated solution:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 9)}$$

$$C_p^\circ = A + B \qquad \text{(Eq. 10)}$$

or

$$1 = A/C_p^\circ + B/C_p^\circ = A' + B'$$

$$A' = (k_{21} - \alpha)/\beta - \alpha;\ B' = (k_{21} - \beta)/\alpha - \beta \qquad \text{(Eq. 11)}$$

$$\alpha \cdot \beta = k_{21} \cdot k_{el} \quad \text{(Eq. 12)}$$

$$\alpha + \beta = k_{12} + k_{21} + k_{el} \quad \text{(Eq. 13)}$$

$$\int_0^\infty C_p dt = A/\alpha + B/\beta = C_p^\circ/k_{el} = D/V_p k_{el} \quad \text{(Eq. 14)}$$

Therefore

$$k_{el} = 1/(A'/\alpha + B'/\beta) \quad \text{(Eq. 15)}$$

$$k_{21} = A'\beta + B'\alpha \quad \text{(Eq. 16)}$$

$$k_{12} = A'B' (\beta - \alpha)^2/k_{21} \quad \text{(Eq. 17)}$$

The best method of estimating the coefficients, A and B, and the hybrid rate constants, α and β, of Eq. 9 are not the subject of this paper except to point out that no mathematical method will substitute for sufficient blood samples taken at the critical times and accurately assayed. Several authors have discussed the analysis of these curves (32–34) and present numerical and computer solutions of the equation. Wagner and Northam recently discussed the relationship between the volume of distribution and the half-life of a compound after an i.v. injection (35) wherein they present several equations equivalent to those listed above.

Influence of Two-Compartmental Open-System Model on Estimation of Absorption Rate Constant—It should be clear from examination of Figs. 1–6 that a variable fraction of the drug molecules leave the central compartment during the distributive phase. Therefore, when the drug is not injected but is absorbed from a site, such as the gastrointestinal tract, a fraction of the absorbed molecules is distributing into the tissue compartment. This phenomenon cannot be ignored while calculating the true absorption rate.

It will be shown in a later paper (36) that calculation of the absorption rate ignoring the existence of the peripheral compartment is subject to significant errors and that an absorption rate equation including a peripheral tissue compartment term allows recovery of known rates of drug infusion into the body, while the former methods lead to incorrect estimates. These facts lend significant support to the presently conceived model. Since such calculations are essential to the basic understanding of drug absorption, their correct interpretation is not unimportant, particularly since *in vivo–in vitro* relationships have been drawn up on the basis of these types of analyses (37, 38).

Influence of the Two-Compartmental Open-System Model on Calculations of Volume of Distribution—A detailed discussion of the calculations of the volume of distribution (V_d) as it is affected by the concept of a two-compartmental open-system model has been presented by Riggs (39). The volume of distribution of a compound can only be defined when the tissue compartment is in equilibrium with the central compartment (*i.e.*, when $dT/dt = 0$) at least insofar as this model is concerned.[2] The volume of distribution is merely one of the kinetic constants of the model being tested. Within the experimental error, the same value should be obtained from the various methods of estimation. The V_d's calculated by the several biased methods

[2] This is not mathematically the unambiguous definition for the steady state of equilibrium (27), but applies in the model since it is assumed that no drug loss takes place from the tissue compartment.

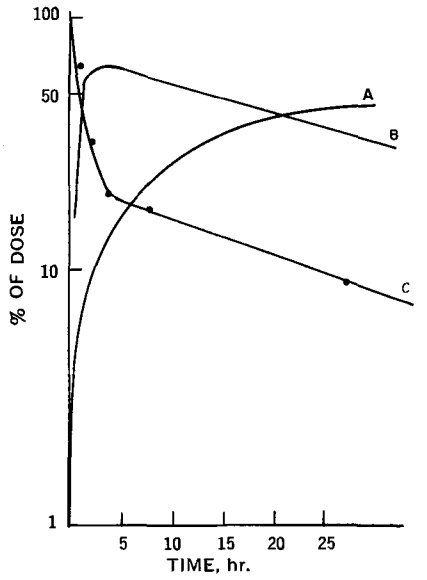


Fig. 3—Percent of dose estimated to be in the central compartment, tissue compartment, and eliminated after a 750-mg. i.v. injection of thiopentobarbital into a male (13). Key: A, amount eliminated; B, tissue compartment; C, central compartment.

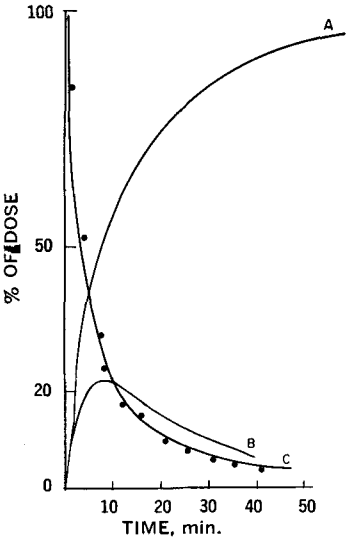


Fig. 4—Percent of dose estimated to be in the central compartment, tissue compartment, and eliminated after a 650-mg. i.v. injection of acetylsalicylic acid into a male (14). Key: A, amount eliminated; B, tissue compartment; C, central compartment.

vary considerably, when sufficient data points are taken, which they should not do if the single-compartmental model held. However, calculation of the volume constant defined by the two-compartmental open-system model presented above appears to yield a constant value independent of the method of calculation (40).

Influence of Two-Compartmental Open-System Model on Estimation of Metabolism and Excretion Rate Constants—It is clear from Eqs. 12 and 13

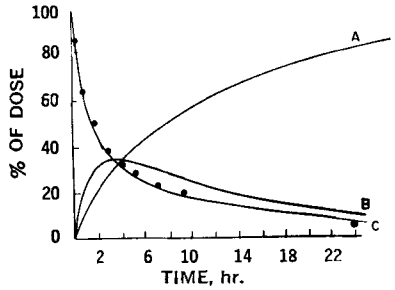


Fig. 5—Percent of dose estimated to be in the central compartment, tissue compartment, and eliminated after a 142-mg. i.v. injection of griseofulvin into a male (15). Key: A, *amount eliminated;* B, *tissue compartment;* C, *central compartment.*

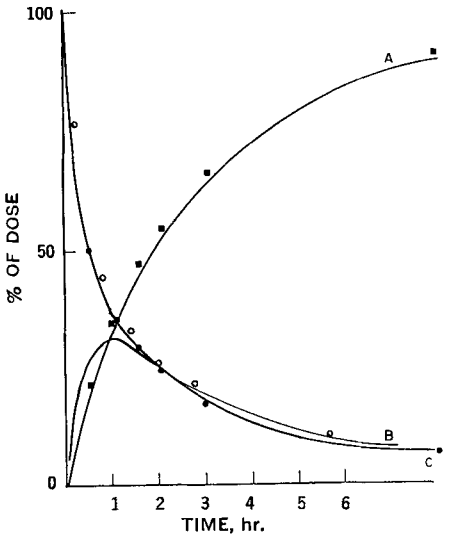


Fig. 6—Percent of dose estimated to be in the central compartment, tissue compartment, and eliminated after a 25.8-Gm. i.v. injection of mannitol into a male. Also included is a curve representing the normalized urinary excretion data (21). Data from subject: R = 3. *Curves drawn with two-compartment analog computer program. Points represent appropriate blood and urine data. Key:* A, *amount eliminated;* B, *tissue compartment;* C, *central compartment.*

that α and β are hybrid rate constants, each influenced by all of the rate constants of the system. One must be warned, therefore, that with such a multiple exponential equation, one has no right to identify a particular term with a particular process. The elimination rate constant for the process is denoted as k_{el} in the two-compartmental open-system model given above. It is incorrect to designate β, the slower rate constant of the bi-exponential, as the elimination rate constant as is the usual practice in pharmacokinetics. However, some descriptive term should be available to refer to this hybrid constant. Therefore, the term "*disposition* rate constant[3]" is proposed, attempting thereby to imply that it includes both distribution and elimination.

In order to illustrate the relation between the true elimination rate constant, k_{el} and β, Eq. 14 may be rearranged as follows:

$$k_{el} = C_p^\circ/(A/\alpha + B/\beta) \qquad \text{(Eq. 18)}$$

However, A/α is often negligible relative to the magnitude of B/β. Equation 18 may be reduced to the following approximation:

$$k_{el} \approx \left(\frac{C_p^\circ}{B}\right)\beta \qquad \text{(Eq. 19)}$$

The ratio C_p°/B varies from one drug to another, but often ranges from 1.5–2.5. However, compounds which distribute out of the central compartment into the peripheral compartment(s) to a large degree will show a much higher proportionality constant. For example, from the published data on intravenous injection of radioactive digitoxin (41), it can be estimated that k_{el} is at least 15 to 40 times larger than the slowest disposition rate constant.[4] Most amine drugs are known to distribute to a large extent into the tissues. This will result in a high ratio of C_p°/B, and therefore, the true elimination constant may be many times larger than the value of the slowest disposition rate constant. In some pharmacokinetic evaluations, this error may play a minor role, such as when one is attempting to evaluate dosage form effects. However, when urinary data are being analyzed, it is common to presume that the slowest disposition rate constant is identical with the true elimination rate constant and that the metabolic rate constant is equal to the disposition rate constant times the fraction of the drug metabolized. Such assumptions result in a large error.

When a pharmacologically related and structurally similar series of compounds are being studied, it is exceedingly important to attempt to evaluate the true elimination rate constant. Introduction of one organic substituent into a molecule may cause a significant change in the tissue distribution, as well as in the metabolism and excretion processes. It is important to discriminate among these factors to a greater extent than we have done to date if we are to better understand how substituents are influencing distribution, metabolism, and excretion characteristics.

Not only does the two-compartmental open-system model become important in the analysis of absorption, metabolism, and excretion of drug, it also markedly influences the analysis of turnover rate of normal body constituents, such as ascorbic acid (42), glucose (43), iron (44), and other compounds. The turnover rate cannot be defined by injecting a known dose and estimating the decline to the homeostatic level, presuming the drug is distributing into a single compartment. It is clear that such an analysis is based on a questionable postulate, since most of these drugs have been shown (45, 46) to require multicompartmental models to define their disposition and elimination from the body.

Relationships of Two-Compartmental Model to Consideration of Drug Interaction in Man—In recent years, it has become apparent that drug

[3] The term *dispose* is taken from the French, meaning to place apart.

[4] In this instance, a tri-exponential was observed by the author (41).

administered during or immediately before asecond compound is taken may influence the pharmacological activity of the second drug. Several different mechanisms have been proposed for these drug interactions. In one instance, the first drug is said to induce changes in the level of metabolizing enzymes. When the second drug is administered, it undergoes a more rapid rate of metabolism. A second form of drug interaction has been postulated for some drugs when given concurrently where the first drug partially displaces the second drug from protein binding sites, thereby allowing for a change in drug distribution. In other instances, the second drug may cause an increase in the excretion rate or may reduce the rate of absorption. It should be apparent that the two (or multiple) compartmental open-system model is a much more meaningful model to examine the relative effect of drug *A* on drug *B*.

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Keyphrases

Pharmacokinetics of drug absorption
Mammillary model
Compartments, consideration of body as one and two
Absorption rates—two compartmental
Drug distribution volume—two compartmental
Tissue compartment—estimation
Metabolism-excretion rates, two compartmental

In Vivo Determination of Certain Aralkylamines

By WINTHROP E. LANGE, JOSEPH M. THEODORE*, and FREDERICK J. PRUYN†

A micro-method has been developed by which blood concentrations of certain aralkylamines can be quantitatively determined. The method makes use of thin-layer chromatography and fluorometry. Quantitative determinations are made by measuring the fluorescence exhibited by the rose bengal complex of the drug. The method utilizes 0.1-ml. samples of capillary blood from which 0.05 mcg. of drug has been detected.

IN RECENT YEARS a pharmaceutical need has arisen for newer and more sophisticated analytical methods. This need has been brought about by the continuous development of potent therapeutic agents, the *in vivo* distribution and the metabolic fate of which have not been completely determined. Chlorpheniramine maleate, dextromethorphan hydrobromide, phenylephrine hydrochloride, and phenylpropanolamine hydrochloride are representative of the aralkylamines that fall into the above category.

Many *in vitro* analysis methods have been reported for the quantitation of aralkylamines contained in pharmaceutical preparations. Gas chromatography has been successfully employed whereby various polyethylene glycols and silicone gum rubber have served as column coating material (1–4). The sensitivities attained, however, were not sufficient to permit the *in vivo* quantitation of the drugs.

Several paper and thin-layer chromatographic separation procedures have been recently reported to effect resolution of some aralkylamines. Cox *et al.* (5) studied 12 antihistamines. Schriftman and Shultz (6) reported a two-dimensional paper chromatographic separation procedure for a mixture of chlorpheniramine maleate, dextromethorphan hydrobromide, and phenylephrine hydrochloride. The developing media were butanol–acetic acid–water (5:1:3), and chloroform–ether (1:1).

With the advent of fluorometric techniques it was possible to quantitate lower concentrations of many compounds. It was shown by Duggan *et al.* (7) that phenylephrine fluoresces maximally at pH 1.

Brodie, Udenfriend *et al.* (8, 9) reported general methods for the estimation of basic compounds. The methods generally included extraction procedures for the isolation of the compounds from their metabolic products and from naturally occurring substances. They concluded that fluorometry was the method of choice whenever possible.

Cohen (10) found that nonfluorescent compounds, or those that were weakly fluorescent, could be converted to a highly fluorescent complex by treatment with rose bengal.

Little has been reported about the *in vivo* distribution and the excretion rate of many of the common aralkylamines. Brossi *et al.* (11) could not determine dextromethorphan blood levels. They did report, however, that from 2 to 16% of the drug was excreted in the urine.

Woods *et al.* reported that blood levels had been determined for the *levo* isomer of the drug, which had been administered to dogs and to monkeys. The isotopic technique showed the maximum concentration of the drug to be present in the plasma after 1 hr. (12).

Willner reported on the excretion of the metabolic products of dextromethorphan (13). He found the drug to be excreted both *per se* and as demethylated derivatives. These findings were made when a paper chromatographic separation procedure was used.

The excretion rate for phenylpropanolamine hydrochloride was studied by Heimlich *et al.* (14). They reported that about 90% of an orally administered 50-mg. dose of the drug was excreted, according to first-order kinetics, within 24 hr.

Excretion rates for chlorpheniramine and phenylephrine were studied by Cavallito *et al.* (15). An isotopic procedure, which required 15-ml. samples of blood or of urine, showed the excretion rates to vary widely among individuals. No apparent relationship was found to exist between the blood level and the excretion rate of tritiated phenylephrine.

Although the information cited above is extremely valuable, it is clear that there has been no general method for the quantitative, *in vivo* determination of the aralkylamines. This paper

Received August 7, 1967, from the Department of Pharmacy, Massachusetts College of Pharmacy, Boston, MA 02115

Accepted for publication September 26, 1967.

Abstracted from theses submitted by Joseph M. Theodore and Frederick J. Pruyn to the Massachusetts College of Pharmacy in partial fulfillment of Doctor of Philosophy degree requirements.

This investigation was supported by a research grant from Chesebrough-Pond's, Inc., Clinton, Conn.

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provides a practical means for performing such analyses both rapidly and accurately.

EXPERIMENTAL

Reagents and Chemicals

The reagents and chemicals used were: acetone, spectroanalyzed, Fisher certified reagent; chloroform, Fisher certified reagent; ethyl alcohol USP; ethyl ether USP; methanol, Fisher certified reagent; ammonium hydroxide solution USP; and ethyl acetate, Mallinckrodt. Dragendorff's reagent and 0.2% aqueous ninhydrin solution were used as indicators. Rose bengal, Fisher certified reagent, was used and was purified by extraction with ethyl acetate and chloroform by the method described by Cohen (10). Chlorpheniramine maleate, chlorpheniramine free base, dextromethorphan hydrobromide, dextromethorphan free base, phenylephrine hydrochloride, and phenylpropanolamine hydrochloride were supplied by Chesebrough-Pond's, Inc.

Apparatus

The thin-layer chromatographic plates used were prepared by coating 20 cm. square glass plates with 0.5 mm. of a Silica Gel G slurry, which was prepared by mixing 1 part by weight of Silica Gel G, Brinkmann Instruments, Inc., with 2 parts by volume of distilled water. The developing chambers and the appropriate micropipets were purchased from the Fisher Scientific Co. The instrument used was a Turner fluorometer, model 110. The instrument was equipped with a 520–560 mμ green lamp, Turner No. 110-854; primary excitation filters No. 1-60 (546 mμ) and No. 58 (525 mμ); and a No. 23-A (570 mμ) secondary emission filter. The 12 × 75 mm. cells used were carefully matched and were selected for low fluorescence.

Methods

Thin-Layer Chromatographic Separation of the Aralkylamines—A thin-layer chromatographic plate, which had been stored in a desiccator, was activated for 20 min. at 110°. A 25-μl. volume of a standard ethanolic solution, containing 25 mcg., 25 mcg., and 75 mcg. of chlorpheniramine maleate, dextromethorphan hydrobromide, and phenylephrine hydrochloride, respectively, was applied to the plate 3 cm. from the lower right corner. The solvent was allowed to evaporate spontaneously at room temperature.

The chromatogram was then developed in an ammoniacal methanol system, which was attained by placing a 50-ml. beaker, containing ammonium hydroxide solution, in a corner of the developing chamber 0.5 hr. before the insertion of the plate. The chromatogram was developed for 1.5 hr., during which time the front moved a distance of 15 cm. The chromatogram was air-dried for 1 hr. at room temperature, rotated clockwise through 90°, and developed in an ammoniacal chloroform–ether (1:1) system for 1.25 hr. The front moved a distance of 15 cm.

The two-dimensional chromatogram was air-dried at room temperature, sprayed with 0.2% aqueous ninhydrin solution, and heated for 15 min. at 110°. The appearance of a purple spot indicated the presence and the location of phenylephrine hydrochloride. After cooling to room temperature, the chromatogram was sprayed with Dragendorff's reagent. The immediate appearance of 2 reddish orange spots indicated the presence and the location of chlorpheniramine maleate and of dextromethorphan hydrobromide.

The resolution of a mixture of chlorpheniramine maleate, phenylephrine hydrochloride, and phenylpropanolamine hydrochloride was effected by the above procedure also. The phenylpropanolamine hydrochloride was detected by the ninhydrin reagent.

Quantitative In Vivo Determination of Chlorpheniramine Maleate—Two 0.1-ml. samples of whole capillary blood were collected from the fingertips of the subjects. The samples were collected with a sterile microlance and a serological pipet after the fingertips had been cleansed with alcohol. One sample was used as a blank and the other as a control, which was prepared by adding 0.05 mcg. of chlorpheniramine maleate to the 0.1-ml. blood sample. One 4-mg. chlorpheniramine maleate tablet[1] was then administered orally to each subject. Capillary blood samples were collected 1, 2, 3, 4, and 5 hr. after the administration of the drug.

All samples, including those to be used as blanks and as controls, were precipitated with 50 μl. of 10% aqueous trichloroacetic acid solution, mixed well with a small glass rod, and centrifuged until the supernatant liquid was clear. The supernatant liquids were applied to 0.5-mm. Silica Gel G chromatographic plates with a 100-μl. micropipet. The plates had previously been activated as has been described above. The chromatograms were developed in ammoniacal chloroform–ether (1:1) for 1 hr. and air dried at room temperature. The outermost columns of the chromatograms, to which had previously been applied 25 mcg. of chlorpheniramine maleate, were sprayed with Dragendorff's reagent to determine the exact R_f value of the drug. The areas of the chromatogram containing the drug were scraped from the plate, care being taken to insure the removal of equal areas. The samples thus removed were transferred to 18 × 150 mm. Pyrex test tubes. To each tube was added 2 ml. of ethanol. The tubes were shaken for 15 min. on a Burrell wrist action shaker. The contents of each tube were filtered through Whatman No. 1 filter paper, the filtrates being collected in 2-in. evaporating dishes. The tubes were rinsed with 2 ml. of ethanol and the rinsings passed through the appropriate filters, which were rinsed with 1 ml. of ethanol. To each filtrate was added 50 μl. of ammonium hydroxide solution.

The filtrates were evaporated to dryness on a steam bath. To each dish was added 1 ml. of an acetone–chloroform (1:1) solution containing 0.05 mcg. of rose bengal. Each sample was diluted with 3 ml. of acetone–chloroform (1:1). The contents of each dish were mixed by gentle swirling and transferred to fluorometric cells. Fluorometric analyses of the samples were carried out using the previously described filter system.

Tenth milliliter urine samples were also analyzed by the same procedure except that the urine samples were chromatographed directly.

Quantitative In Vivo Determination of Dextro-

[1] Chlortrimeton, Schering Corp.

TABLE I—R_f VALUES DETERMINED FOR CERTAIN ARALKYLAMINES[a]

Drug	Solvent A[b]	Solvent B[c]
Chlorpheniramine maleate	0.68	0.43
Dextromethorphan hydrobromide	0.51	0.53
Phenylephrine hydrochloride	0.43	0.00
Phenylpropanolamine hydrochloride	0.64	0.10

[a] All plates were coated with 0.5 mm. of Silica Gel G. [b] Ammoniacal methanol. [c] Ammoniacal chloroform–ether (1:1).

methorphan Hydrobromide—The preceding procedure for the determination of blood and of urine concentrations of chlorpheniramine maleate was used with the following exceptions. A 10-ml. dose of a dextromethorphan hydrobromide syrup,[2] containing 15 mg. of the drug in each 5 ml., was administered orally. Acetone was used to extract the drug from the Silica Gel G. Each sample was treated with 0.75 mcg. of rose bengal, and a 1% transmission filter was incorporated into the system.

In Vivo Determination of Phenylephrine Hydrochloride—The preceding procedure for the determination of blood and of urine concentrations of chlorpheniramine maleate was used with the following exceptions. A 5-ml. dose of a phenylephrine hydrochloride elixir,[3] containing 5 mg. of the drug in each 5 ml., was administered orally. Each sample was treated with 0.15 mcg. of rose bengal and 25 µl. of ethanol was added to each sample cell. The chromatogram was developed in ammoniacal methanol and 0.2% aqueous ninhydrin solution was used to indicate the R_f value of the drug.

RESULTS AND DISCUSSION

The two-dimensional thin-layer chromatographic separation procedure effected excellent resolution of the drugs. Table I shows the R_f values that were determined for each drug. It was noted that the ammoniacal chloroform–ether (1:1) solvent medium should be prepared freshly and that no more than two chromatograms be developed in such a system. The ammoniacal methanol solvent medium gave satisfactory results for up to five chromatograms.

The samples for fluorometric analysis were prepared in an acetone–chloroform (1:1) solvent medium. It was found that the acetone gave rise to an approximate tenfold fluorescence potentiation, which attained a maximum after about 20 min. Figure 1 shows the fluorescence exhibited by various concentrations of chlorpheniramine free base when complexed with rose bengal. The graph shows that accurate measurements could be made in the submicrogram concentration range.

The *in vivo* determinations were carried out using six normal males, from 23 to 34 years of age. All subjects were in a semifasting condition as only liquid nourishment was allowed during the 8 hr. preceding, and throughout the duration of, the test. All subjects had been deprived of drugs of any kind for the 72 hr. preceding the test.

[2] Romilar Syrup, Sauter Laboratories, Inc.
[3] Neo-Synephrine Elixir, Winthrop Laboratories.

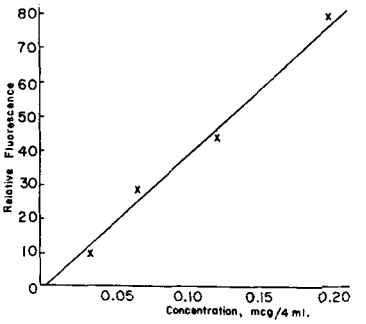

Fig. 1—Fluorescence-concentration relationship of chlorpheniramine free base when complexed with rose bengal.

TABLE II—CHLORPHENIRAMINE MALEATE BLOOD LEVELS OBTAINED FROM ORAL ADMINISTRATION OF THE DRUG[a]

Subject	(mg. % × 10²) 1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
A	0.97	1.47	3.30	2.30	1.10
B	1.87	1.77	2.80	2.20	1.40
C	1.30	1.60	1.10	0.94	...
D	2.30	2.30	2.60	2.30	1.80
E	2.50	2.90	2.50	1.70	1.40
F	1.50	1.50	2.10	...	1.30

[a] Chlortrimeton, 4 mg., Schering Corp.

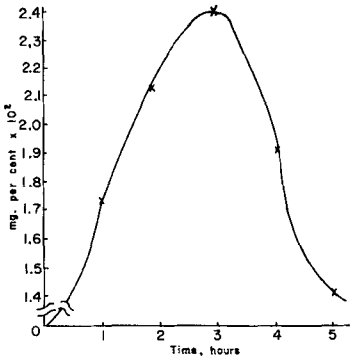

Fig. 2—Mean blood level distribution of chlorpheniramine maleate after oral ingestion of a 4-mg. dose.

The results of the analyses showed chlorpheniramine maleate to exhibit a definite absorption pattern. The maximum blood level appeared within 3 hr. after the oral administration of the drug and was followed by a rapid decline thereafter. Table II shows the chlorpheniramine maleate blood concentrations, in milligrams percent, that were determined from the subjects that were studied. Figure 2 shows the mean blood level distribution of chlorpheniramine maleate. The analysis of the urine showed that approximately 17% of the orally administered drug had been excreted unchanged within 6 hr.

A comparison of the blood concentrations that were produced from a 15-mg. and from a 30-mg. oral dose of dextromethorphan hydrobromide was made. Analyses were carried out 1, 2.5, 4.5, and 6 hr. after the ingestion of the drug. It was found that approximately 10, 25, and 16% of the orally admin-

istered 30-mg. dose was in the blood stream after 1, 2.5, and 6 hr., respectively. The 1 and the 2.5 hr. blood concentrations produced by the 15-mg. oral dose were insignificantly different from the respective concentrations produced by the 30-mg. dose, but the 4.5 and the 6 hr. concentrations were substantially lower. Approximately 8% of the orally administered drug was excreted unchanged within 6 hr.

The presence of phenylephrine hydrochloride was detected in human blood but statistical quantitative determinations have not as yet been carried out.

The *in vitro* applications of the fluorometric analysis procedure to other aralkylamines has been studied. Phenindamine required 3 times its weight of rose bengal to give the optimum degree of fluorescence. The dye–drug weight ratios were 25:1, 10:1, 1:1, and 0.5:1, respectively, for dextroamphetamine, phenylpropanolamine, phenyltoloxamine, and thonzylamine.

It should be noted that only the amine free bases respond to the fluorometric analysis procedure, thus it is necessary that the salts be converted to the free bases before analyses are carried out.

It was determined that a 99% recovery of the drugs from Silica Gel G had been effected. The recovery from blood and from urine was also nearly quantitative. The use of blanks and of controls, which were prepared from the subject's blood or urine, increased the accuracy of the procedure in that any inherent fluorescence would become nulled.

It is felt that this method can be applied to other aralkylamines and, since it has been shown that resolution of drug mixtures can be effected, that simultaneous determinations can possibly be carried out.

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Keyphrases

Aralkylamines
In vivo determination of aralkylamines
Chlorpheniramine maleate–dextromethorphan
HBr–phenylephrine HCl mixture
TLC separation
Rose bengal–aralkylamine complex
Fluorometry–analysis

Concept of a Volume of Distribution and Possible Errors in Evaluation of This Parameter

By S. RIEGELMAN, J. LOO, and M. ROWLAND

To be pharmacokinetically correct, an estimate of the volume of distribution must establish a volume of a fictitious single compartment at a steady state of equilibrium and therefore independent of the role of metabolism and excretion. It has been shown previously that it is mathematically and physiologically more correct to conceive the body as exhibiting the properties of at least a two-compartmental rather than a single-compartmental model, which necessitates careful definition of the instant in time when the steady state (ss) of equilibrium exists. A mathematical relationship between the volume of distribution at steady state and those estimated by often used but incorrect methods is presented. Experimental data on acetylsalicylate, salicylate, griseofulvin, and several barbiturates are presented. The pharmacokinetic significance of these data is discussed.

THE VOLUME of distribution is not only an essential parameter in pharmacokinetic calculations, but is also a useful concept in physiology. However, examination of the literature in both fields clearly indicates a misunderstanding of the essential criteria which must be met when calculating this constant. Since the volume of distribution is a parameter of a particular model used to describe the distribution of a drug in the body, its physiological meaning is limited by the model. Nonetheless, it is a constant so that if the model is to be consistent its value must be independent of the method of measurement. In an earlier paper of this series (1), a detailed discussion was presented of the mathematical, physiological, and pharmacokinetic basis for representing the body as a mammillary model consisting of a central compartment with at least one peripheral compartment. Most of the presently published pharmacokinetic evaluations of drug absorption and elimination rates have been based on the supposition that the body need only be considered as a single compartment in assessing these constants. Little or no effort has been expended to evaluate the error involved in these measurements based on this presumption of a single-compartment model.

The purpose of this paper is to present some mathematical interrelationships of the various methods of calculation of the volume of distribution. A two-compartmental open-system model will be used to calculate the volume of distribution, Vd_{ss}, which exists at a steady state of equilibrium between these two compartments. The relationships between this Vd_{ss} and that obtained by various biased[1] methods of measurement will be derived. Data will be presented which allow one to predict the apparent Vd[2] obtained by one of the biased methods of calculating this constant and to assess its relative error.

Received April 14, 1967, from the Departments of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94122

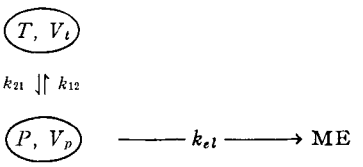
Accepted for publication August 29, 1967.

This research was supported in part by a research grant from the Research Committee, San Francisco Academic Senate, and from Glenbrook Laboratories.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

DISCUSSION

Definition of the Two-Compartmental Open-System Model—The two-compartmental open system model may be represented as follows:



where T = the amount of drug in the peripheral (tissue) compartment at any time, t,

P = the amount of the drug in the central compartment at time, t,

ME = the amount of the drug eliminated by all processes of metabolism and excretion, assumed to take place exclusively in the central compartment, up to time, t.

V_p, V_t = the volumes of central and tissue compartments, respectively.

Where

$$Vd_{ss} = V_t + V_p$$

and

k_{12}, k_{21} = first-order rate constants of distribution,

k_{ei} = the sum of the simultaneous processes of metabolism and excretion all assumed to be first order.

Widmark and Tandberg (2), Teorell (3), and Dominguez (4) recognized that from the kinetic point of view the so-called central compart-

[1] It will be shown subsequently that these estimates are biased in that they are dependent upon the elimination rate constant and distribution constants.

[2] We shall arbitrarily define Vd as the so-called constant value arrived at when estimated assuming the single-compartmental model applies. All other volumes of distribution, analyzed on the basis of the two-compartmental model, will be specified with a subscript referring to the method of their calculation.

ment includes, in addition to the plasma volume, that portion of the intercellular fluids and tissues which appear to spontaneously come into equilibrium with the blood. Riegelman *et al.* (1) pointed out that the peripheral compartment may well represent the muscle, skin, and a portion of the fat depot. However, both of these volumes are kinetic parameters of the model, varying with the partition and binding characteristics of the compounds, so that it serves no useful purpose in attempting to attach any physiological meaning to the values obtained. Such a two-compartmental open-system model, as defined in the distribution and elimination scheme illustrated above, results in the following bi-exponential equation:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 1)}$$

where C_p = concentration of drug in plasma
α and β = hybrid rate constants dependent on all three specific rate constants of the model (see under *Appendix*).
$C_p^0 = A + B$
V_p = volume of central compartment

and

$$V_p = \text{dose}/C_p^0 \qquad \text{(Eq. 2)}$$

When sufficient time has passed such that the first term in the bi-exponential $Ae^{-\alpha}$ becomes vanishingly small, Eq. 1 reduces to a mono-exponential, which according to the single-compartmental model[3] becomes:

$$C_p = Be^{-\beta t} \qquad \text{(Eq. 3)}$$

Upon integration between zero and infinity, we arrive at the area under the curve, whereupon Eq. 1 becomes:

$$\text{area} = \int_0^\infty C_p dt = A/\alpha + B/\beta \qquad \text{(Eq. 4)}$$

and Eq. 3 becomes:

$$\text{area} = B/\beta \qquad \text{(Eq. 5)}$$

Essential Criteria in the Definition of a Volume of Distribution—Riggs (5) has presented an excellent analysis of the volume of distribution concept and some of the biased methods of calculating this parameter. He emphasizes that essential to its basic definition is the concept of an equilibrium state. The volume of distribution of a given compound in the whole body, with reference to its equilibrium concentration, $(C_p)_{eq.}$, in the plasma, is given by

$$Vd_{ss} = (Q_{tot.})_{eq.}/(C_p)_{eq.} \qquad \text{(Eq. 6)}$$

where $(Q_{tot.})_{eq.}$ = the quantity distributed throughout the entire system at equilibrium.

In the two-compartmental open-system model several difficulties in the evaluation of Vd_{ss} are immediately apparent. Simultaneous to the distribution of the drug into the second compartment is the consecutive loss by metabolism and excretion. This results in a steady state of equilibrium of the drug between the two compartments, central and peripheral, occurring at only one instant following an i.v. injection, *i.e.*, when the rate of change of drug in the peripheral compartment is zero ($dT/dt = 0$).

Riggs (5) has shown that the volume of distribution, Vd_{ss} can be estimated from the rate constants into and out of the central and peripheral compartments,

$$Vd_{ss} = \frac{k_{12} + k_{21}}{k_{21}} V_p \qquad \text{(Eq. 7)}$$

The necessity for this definition lies in the fact that it is impossible to sample the (fictitious) peripheral compartment and, therefore, the drug is conceived to be distributed in a volume referred to the concentration in the plasma at equilibrium (see Eq. 6).

Extrapolation Method—When the single-compartment concept is applied to the calculation of Vd, the mono-exponential portion of Eq. 1 is extrapolated to the y-intercept, and the result is:

$$B = \text{dose}/Vd \qquad \text{(Eq. 8)}$$

or

$$Vd = \text{dose}/B \qquad \text{(Eq. 9)}$$

However, by the two-compartmental model, the extrapolated value B is given by:

$$B = \frac{\text{dose}\ (k_{21} - \beta)}{V_p(\alpha - \beta)} \qquad \text{(Eq. 10)}$$

so that

$$Vd_{\text{extrap.}} = \text{dose}/B = V_p(\alpha - \beta)/(k_{21} - \beta) \qquad \text{(Eq. 11)}$$

It will be shown under the *Appendix* that Eq. 11 can be expressed in terms of the specific rate constants as follows:

$$Vd_{\text{extrap.}} = \frac{2\ V_p \cdot Z}{k_{21} - k_{12} - k_{el} + Z} \qquad \text{(Eq. 12)}$$

where $Z = [(k_{12} + k_{el} - k_{21})^2 + 4k_{12} \cdot k_{21}]^{1/2}$.

Area or Clearance Method—According to the single-compartment model, a second method of calculating Vd is by application of the area equation (Eq. 5) which upon substitution into Eq. 8 results in:

$$Vd = \text{dose}/\beta \cdot \text{area} \qquad \text{(Eq. 13)}$$

The same volume is obtained from urinary clearance studies as the following will show. If the processes of excretion and metabolism are presumed to obey first-order kinetics with constants k_e and k_m, respectively, and:

$$k_m + k_e = \beta \qquad \text{(Eq. 14)}$$

or

$$f \cdot \beta = k_e \qquad \text{(Eq. 15)}$$

where $f = k_e/(k_e + k_m)$.

The amount of the dose excreted intact from zero time until the drug is entirely eliminated is:

$$f \cdot \text{dose} = k_e \cdot Vd\ C_p dt \qquad \text{(Eq. 16)}$$

Substituting for k_e from Eq. 15, rearranging, and cancelling we arrive at Eq. 13.

With the two-compartmental model, the Vd_{area} can be estimated by the following equation, which is derived under *Appendix:*

$$Vd_{\text{area}} = Vd_{ss} + \frac{k_{el} - \beta}{k_{21}} V_p \qquad \text{(Eq. 17)}$$

[3] Many authors use the symbol K instead of β for the first-order rate constant of Eq. 3.

Zero-Order Infusion Method—Occasionally a drug is administered by constant infusion. If this is continued for a sufficient length of time, the drug becomes equilibrated in the body fluids.

Correcting for the drug eliminated during the infusion process, an estimate of the volume of distribution can be obtained. Since drug is lost by metabolism and excretion during the infusion process, the amount of drug remaining in the body ($D_{corr.}$) can be estimated by the ratio of the area under the blood curve from zero to the end of the infusion, divided by the total area, or:

$$Vd = \text{dose}/(C_p)_{eq.}\left[1 - \frac{\int_0^t C_p dt}{\int_0^\infty C_p dt}\right] = \text{dose}_{(corr.)}/C_{p_{eq.}} \quad \text{(Eq. 18)}$$

where $C_{p_{eq.}}$ = the plasma concentration at equilibrium.

This volume of distribution can be estimated from the two-compartmental model, resulting in

$$Vd_{inf.}^0 = Vd_{ss} + k_{el}/k_{21}\ (V_p) \quad \text{(Eq. 19)}$$

A comparison can now be made of the methods of estimation using the different proposed compartmental models. One cannot be satisfied simply by obtaining a constant value for the volume of distribution, since it is obvious that by insufficient sampling in the early period, the experimenter would miss the distributive phase and only obtain a monoexponential. Under this situation all the methods of estimation would arrive at the same incorrect value. What is important, however, is to attempt to describe the error which would be involved in these estimates, using data which result in well-defined bi-exponential equations.

EXPERIMENTAL PROCEDURE

The *N*-methylglucamine salt of acetylsalicylic acid (ASA) and of salicylic acid (SA) was prepared by mixing equimolar amounts of the base and acid. The solution was bacteriologically filtered and injected into the antecubital vein by rapid i.v. injection (over 5–10 sec.) or by constant infusion over a 1-hr. period with a Harvard infusion pump. Griseofulvin was formulated into a parenteral solution by dissolving the drug in sterilized polyethylene glycol 300. The solution was not injected directly into the test subject, but by diluting it into a rapidly flowing normal saline solution, which was being administered intravenously over a 3–6-min. period. The griseofulvin was also infused at a constant rate over a 2-hr. period of time. The exact details of preparation will be given in a subsequent paper where each system will be discussed at length.

RESULTS AND DISCUSSION

Table I lists the calculated and predicted values of the various volumes of distribution after an i.v. injection of ASA to three males. All three subjects showed large differences between the various estimates. However, the resultant predicted values of $Vd_{extrap.}$, Vd_{area}, and $Vd_{inf.}^0$ are in excellent agreement with the observed biased estimates. Stated another way, these biased estimates would result in a constant value for Vd_{ss}. It is immediately seen that the $Vd_{extrap.}$ always produces the largest error and in this instance has a value which is approximately twice that of Vd_{ss}. If the single-compartmental model is assumed, these estimates of Vd would be identical and equal to $Vd_{extrap.}$, *i.e.*,

$$Vd = D/B = D/\beta \text{ area} = D_{corr.}/C_{p_{eq.}} = Vd_{extrap.} \quad \text{(Eq. 20)}$$

and had insufficient data points been taken so as to miss the distribution phase, it therefore follows that the error will be as great as that of the $Vd_{extrap.}$ estimate.

Table II includes the data obtained after injection of various doses of SA into the same three test subjects. In this instance, the values obtained from the biased methods are almost identical with the Vd_{ss}. One might presume, therefore, that the single-compartmental model is adequate to represent the fate of this drug in the body. However, when ASA is administered intravenously as indicated in Table I, the rapid metabolic transformation of ASA to SA results in the accumulation of SA in the body. Analog computer simulation of these data was studied using the two-compartmental system for the

TABLE I—VOLUME OF DISTRIBUTION ESTIMATES AFTER i.v. INJECTION OF ASA IN MAN

Subject[a]	Biased Vd's: D/B	$D/\beta \cdot$ Area	$D_{(corr.)}/C_{p_{eq.}}$	V_p
1	19,700	13,800	...	6500
2	17,600	14,100	...	6900
3	21,000	13,900	15,700	5650
	Predicted Values: $Vd_{extrap.}$	Vd_{area}	$Vd_{inf.}^0$	Vd_{ss}
1	19,800	13,800	...	10,400
2	17,300	14,000	...	11,700
3	21,000	13,800	16,400	10,100

[a] The appropriate constants from which the calculations were made are listed below in running sequence for subjects *1–3*, respectively. Dose = 650, 650, 325, 1240 mg. (inf.); A = 67, 56, 42 mcg./ml.; α = 0.23, 0.314, 0.257 min.$^{-1}$; B = 33, 37, 15.5 mcg./ml.; β = 0.0495, 0.0506, 0.0478 min.$^{-1}$; area = 956, 920, 487 mcg./ml. × min.; k_{el} = 0.105, 0.110, 0.117 min.$^{-1}$; k_{12} = 0.067, 0.105, 0.0825 min.$^{-1}$; k_{21} = 0.109, 0.157, 0.104 min.$^{-1}$.

TABLE II—VOLUME OF DISTRIBUTION ESTIMATES[a] AFTER i.v. INJECTION OF SA IN MAN

Subject[b]	Biased Vd's: D/B	$D/\beta \cdot$ Area	V_p
1	10,400	10,200	5700
2	9,680	9,650	5900
3	8,800	8,700	5300
	Predicted Values: $Vd_{extrap.}$	Vd_{area}	Vd_{ss}
1	10,400	10,350	10,200
2	9,680	9,650	9,620
3	8,750	8,590	8,500

[a] These volume estimates are valid for the doses used since it is known that the metabolism and excretion characteristic of SA varies with dose. [b] The appropriate constants from which calculations were made are listed in a running sequence for subjects *1–3*, respectively. Dose = 508, 484, 508 mg.; A = 40, 32, 38 mcg./ml.; α = 0.140, 0.216, 0.173 min.$^{-1}$; B = 49, 50, 58 mcg./ml.; β = 0.0032, 0.0026, 0.0027 min.$^{-1}$; area = 15,500, 19,400, 21,600 mcg./ml. × min.; k_{el} = 0.0058, 0.0040, 0.0044 min.$^{-1}$; k_{12} = 0.061, 0.085, 0.064 min.$^{-1}$; k_{21} = 0.078, 0.133, 0.1055 min.$^{-1}$.

TABLE III—VOLUME OF DISTRIBUTION ESTIMATES AFTER i.v. INJECTION OF GRISEOFULVIN IN MAN

Subject[a]	Biased Vd's		
	D/B	$D/\beta \cdot$Area	V_p
1	129,000	120,000	62,000
2	138,000	117,800	56,000
3	145,000	118,000	60,000
	Predicted Values		
	$Vd_{extrap.}$	Vd_{area}	Vd_{ss}
1	129,000	120,100	112,000
2	142,000	118,600	104,000
3	146,000	121,700	103,000

[a] The appropriate constants from which the calculations were made are listed below in a running sequence for subjects *1–3*, respectively. Dose = 142.128, 90 mg.; A = 1.2, 1.35, 0.90 mcg./ml.; α = 0.60, 0.63, 0.41 hr.$^{-1}$; B = 1.1, 0.93, 0.62 mcg./ml.; β = 0.0445, 0.075, 0.063 hr.$^{-1}$; area = 26.0, 14.5, 12.1 mcg./ml. × hr.; k_{el} = 0.0850, 0.157, 0.126 hr.$^{-1}$; k_{12} = 0.25, 0.284, 0.145 hr.$^{-1}$; k_{21} = 0.31, 0.243, 0.200 hr.$^{-1}$.

ASA disposition and both the one-compartmental and the two-compartmental constants for the SA disposition. It was immediately apparent that for each subject, the single-compartmental disposition of SA did not fit the data and underestimated the values of the SA in the earlier time periods (6). In contrast, when both the ASA and SA were conceived to be distributing into two compartments, an excellent fit was obtained. This is once again a strong indication that the two-compartmental model is essential to the treatment of the disposition of SA as well as ASA, particularly if one wishes to estimate the absorption rate constants.

It might appear from the data on salicylates that the results obtained with ASA were unique to this compound due to its very fast metabolism and that drugs with slower elimination rates may be adequately analyzed for their volume of distribution by one of the biased methods. However, the data given in Table III for the i.v. studies on griseofulvin, in which the values of $0.693/\beta$ were from 9.5 to 17 hr., do not support this postulate. The biased estimates result in significant variation with the various methods of calculation on all three subjects. Further, the relatively large error in the biased estimates when compared with Vd_{ss} indicate that all three rate constants defining the distribution and elimination processes interact in affecting the biased estimates. A 2-hr. zero-order infusion was performed on one subject; however, this was not long enough to result in equilibration between the compartments (which would have required continuous infusion for at least 4 hr.).

In reviewing the four equations by which the volume of distribution can be estimated, it can be seen that the Vd_{ss} defined by Eq. 7 is the only one which is not influenced by the elimination processes. The $Vd_{inf.}{}^0$ is defined by Eq. 19 and includes k_{el}. This is equally true of $Vd_{extrap.}$ defined by Eq. 12, and Vd_{area} as defined by Eq. 17. Since the drug itself or a second drug may induce changes in the metabolic rate, it seems illogical to accept a definition of a volume of distribution which is dependent on metabolic changes. Indeed, the same can be said for the excretion rate, which can be affected by concomitant administration of other drugs.

The foregoing discussion leads to a number of important points. All the biased estimates $Vd_{extrap.}$, Vd_{area}, and $Vd_{inf.}{}^0$ will result in overestimation of the value of Vd_{ss} and will only approach this latter value when the elimination rate constant, k_{el}, becomes small in respect to k_{12} and k_{21}. This is readily seen by making k_{el} zero in each relevant equation. However, the $Vd_{extrap.}$ will always result in the biggest error. This can produce erroneous results and therefore misleading conclusions. An example is seen in the case of ASA and SA. From the $Vd_{extrap.}$ values, one might deduce that the volume of distribution of ASA was approximately twice that of SA. Comparison of their Vd_{ss} values, however, show them to be quite similar, which would seem more plausible as they have similar physical chemical properties.

In 1953 Brodie and co-workers administered pentobarbital and thiopentobarbital by rapid intravenous injection into test subjects (7). The data were taken from the excellent figures found in the reference and the constants of the bi-exponential equations were estimated by the usual procedures. The following were the results: thiopentobarbital (dose = 750 mg.)—$C_p = 10e^{-1.15t} + 2.6e^{-0.03t}$; V_p = 60,500 ml.; Vd_{ss} = 270,000 ml. Pentobarbital (dose = 1000 mg.)—$C_p = 8.5e^{-1.4t} + 7.6e^{-0.017t}$; V_p = 62,000 ml.; Vd_{ss} = 130,000 ml.

Many studies of thiopentobarbital have indicated that a more complex distribution model is required for an understanding of period of sleep induced by the drug relative to its distribution and metabolism (8, 9). It appears from the data of Brodie *et al.*, however, that not more than a two-compartmental model is necessary to fit the available data, and if one were attempting to estimate the true absorption rate of the drug or assess its true elimination rate constant, that this model may be adequate. It is interesting to note the similarity of the estimated volume of the central compartment for the two drugs, approximately 61,000 ml., and the large difference in the estimated volume of the tissue compartment(s)—68,000 ml. with pentobarbital and over 210,000 ml. with thiopentobarbital. These estimates are compatible with the suggestion that the latter drug enters the muscle, skin, and fat depots of the body to a higher degree than does pentobarbital. While these data have been obtained for only two compounds within a test series, these results appear to indicate that much more definitive information could be obtained from careful application of the two-compartmental model.

Most of the earlier pharmacokinetic studies have utilized one of the biased methods of analyzing for the volume of distribution. Dost and co-workers (10), Krueger-Thiemer (11), Gladtke (12), and other European workers (13) have utilized the extrapolation procedure to estimate the volume of distribution and in effect have assumed a single-compartmental model. The area or clearance method has been used by Wagner and co-workers (14), by Levy (15), and by many other American workers in our field. In many of these studies, the authors also estimated the metabolism and excretion rate constants, by assuming that the apparent elimination rate constant was equal to k_{el}, the true elimination rate constant. However, one can assess the error involved in making this assumption. If one presumes that sufficient blood samples are taken after an i.v. dose to define the so-called distribution phase, the area under the concentration-time curve, can be expressed in two alternative fashions, namely:

$$\text{area} = \int_0^\infty C_p dt = \text{dose}/Vd_{ss}\cdot\beta = \text{dose}/V_p\cdot k_{el} \qquad \text{(Eq. 21)}$$

Cancelling common terms, we arrive at the following:

$$\beta = V_p/Vd_{\text{area}}\cdot k_{el} \qquad \text{(Eq. 22)}$$

Therefore estimates of the metabolism and excretion rates, based on the fraction excreted intact, and presuming Eq. 14 applies, will be seriously in error. From examination of the volume ratios for the drug discussed above, it can be seen that the true elimination rate constants will be from twofold to fivefold larger than the apparent elimination rate constant, β. These discrepancies are due to the fact that β is a hybrid rate constant, for which we propose the alternative designation, *disposition* rate constant to indicate that it is dependent on the distribution rate constants k_{12} and k_{21}, as well as the elimination rate constant, k_{el}. It seems essential, therefore, that due caution be taken in making these estimates of metabolism and excretion rate constants, for mathematically according to the multicompartmental models, β can never exactly equal k_{el}.

There are some pharmacokinetic situations in which it is not important to define the volume of distribution in accordance with the two-compartmental model in order to correctly make the desired mathematical analysis of the data. It is to be noted that the denominators in Eq. 21—$Vd_{\text{area}}\cdot\beta$ or $V_p\cdot k_{el}$—must be equal and both represent the total clearance value for the drug from the body[4] by all processes of metabolism and excretion. The units of clearance are volume per unit time (ml./min.). Wagner proposed a method of estimating the mean equilibrium concentration, $\bar{C}_{p_{\text{eq.}}}$, of a drug in the blood given without interruption over a sufficient time period with a constant dosing interval of $\Delta t = t_2 - t_1$. This method in effect utilizes the relationship defined in Eq. 21, which includes these equivalent clearance terms. He assumed that a constant fraction of the administered dose (F) would be absorbed during each administration, and that at equilibrium the area under the concentration–time curve for the dosing interval would become equal to the total area under the curve from a single dose:

$$\text{area}\Big|_0^\infty = \text{area}\Big|_{t_1}^{t_2} = \frac{F\cdot D}{\text{clearance}} = C_{p_{\text{eq.}}}\cdot\Delta t \qquad \text{(Eq. 23)}$$

Since clearance is merely the ratio of the dose divided by the area (expressed in proper units), the calculation of $C_{p_{\text{eq.}}}$ becomes independent of the model. A valid estimate of the mean equilibrium concentration can be made from an analysis of a single dose as long as the other assumptions apply.

SUMMARY

A calculated volume of distribution is only one parameter of a model describing the fate of a drug in the body. However, calculations of that volume can lead to some evidence of the deficiencies of the single-compartmental model for the drug at hand. Nevertheless, even if the calculations of the V_d for the single-compartmental model result in a constant value, this in itself is not adequate information as to the validity of the model and additional tests must be met.

[4] The term body clearance is used in the analogous context to urinary clearance and represents the ml. of plasma cleared from the drug by all metabolic and excretion processes per unit time.

APPENDIX

Definition of $Vd_{\text{extrap.}}$ from the Two-Compartmental Model—On the basis of the two-compartmental open-system model as defined in this paper, the constants of the resultant bi-exponential equation can be defined as follows:

$$C_p = \frac{D(k_{21}-\alpha)}{V_p(\beta-\alpha)}e^{-\alpha t} + \frac{D}{V_p}\frac{(k_{21}-\beta)}{(\alpha-\beta)}e^{-\beta t} \qquad \text{(Eq. 1}a\text{)}$$

where

$$\alpha = (b + \sqrt{b^2 - 4c})/2$$

$$\beta = (b + \sqrt{b^2 - 4c})/2$$

and

$$b = k_{12} + k_{21} + k_{el}$$

$$c = k_{21}\cdot k_{el}$$

After sufficient time the first exponential term vanishes and back extrapolation of the linear portion of the semilog plot to the y-intercept yields:

$$B = \frac{D}{V_p}\frac{(k_{21}-\beta)}{(\alpha-\beta)} \qquad \text{(Eq. 2}a\text{)}$$

On rearrangement one has:

$$Vd_{\text{extrap.}} = \frac{D}{B} = \frac{V_p(\alpha-\beta)}{k_{21}-\beta} \qquad \text{(Eq. 3}a\text{)}$$

which is identical to Eq. 11 in the text. However, it can be shown that:

$$\alpha - \beta = (b^2 - 4c)^{1/2} \qquad \text{(Eq. 4}a\text{)}$$

$$= [(k_{12} + k_{el} - k_{21})^2 + 4k_{21}\cdot k_{12}]^{1/2} = Z$$

and

$$k_{21} - \beta = {}^1/_2[k_{21} - k_{12} - k_{el} + (Z)] \qquad \text{(Eq. 5}a\text{)}$$

substitution from Eqs. 4a and 5a into Eq. 3a results in:

$$Vd_{\text{extrap.}} = \frac{2V_p(Z)}{k_{21} - k_{12} - k_{el} + (Z)} \qquad \text{(Eq. 6}a\text{)}$$

which is represented as Eq. 12 in the text.

Definition of Vd_{area} from the Two-Compartmental Model—After sufficient time after the i.v. dose, the bi-exponential curve reduces to a mono-exponential. Expressed in terms of mass of the drug in accordance with the single-compartmental model we have:

$$dP/dt = -\beta P \qquad \text{(Eq. 7}a\text{)}$$

However, according to the two-compartmental model throughout the total curve the following equation applies:

$$dP/dt = k_{21}T - (k_{12} + k_{el})P \qquad \text{(Eq. 8}a\text{)}$$

Substituting for the differential, dP/dt for Eq. 7a and dividing by P we have:

$$\beta = k_{12} + k_{el} - k_{21}\frac{T}{P} \qquad \text{(Eq. 9}a\text{)}$$

or on rearrangement:

$$\frac{T}{P} = \frac{(k_{12} + k_{el} - \beta)}{k_{21}} \quad \text{(Eq. 10a)}$$

The volume of distribution, Vd_{area} can be expressed as:

$$Vd_{area} = \frac{T + P}{P/V_p} = (1 + T/P)\ V_p \quad \text{(Eq. 11a)}$$

Substituting for T/P from Eq. 10a, we have:

$$Vd_{area} = 1 + \left(\frac{k_{12} + k_{el} - \beta}{k_{21}}\right) V_p \quad \text{(Eq. 12a)}$$

$$= \left(\frac{k_{21} + k_{12}}{k_{21}}\right) V_p + \left(\frac{k_{el} - \beta}{k_{21}}\right) V_p$$

and finally

$$Vd_{area} = Vd_{ss} + \left(\frac{k_{el} - \beta}{k_{21}}\right) V_p \quad \text{(Eq. 13a)}$$

which is Eq. 17 in the text.

Definition of $Vd_{inf.}^0$ from the Two-Compartmental Model—After a constant zero-order infusion is maintained for a sufficient length of time, an equilibrium is established. Then the rate in and the rate out are such that a constant concentration of drug is achieved in the central compartment and a $Vd_{inf.}^0$ can be calculated as follows:

$$dP/dt = k_{21}T - k_{el})P = 0 \quad \text{(Eq. 14a)}$$

$$k_{21}T = (k_{12} + k_{el})\ P \quad \text{(Eq. 15a)}$$

$$T/P = \frac{k_{12} + k_{el}}{k_{21}} \quad \text{(Eq. 16a)}$$

From Eq. 7 in the text, we have:

$$Vd_{inf.}^0 = \frac{(Q_{tot.})}{(C_p)_{eq.}} = \frac{P + T}{P/V_p} \quad \text{(Eq. 17a)}$$

$$Vd_{inf.}^0 = (1 + T/P)\ V_p \quad \text{(Eq. 18a)}$$

Substituting for T/P from Eq. 16a, we have:

$$Vd_{inf.}^0 = 1 + \left(\frac{k_{12} + k_{el}}{k_{21}}\right) V_p \quad \text{(Eq. 19a)}$$

which can be simplified according to the definition of Vd_{ss}:

$$Vd_{inf.}^0 = Vd_{ss} + \left(\frac{k_{el}}{k_{21}}\right) V_p \quad \text{(Eq. 20a)}$$

The latter is represented as Eq. 19 in the text.

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Keyphrases

Volume of distribution
Acetylsalicylicacid–*n*-methylglucamine salt distribution
Salicylic acid–*n*-methylglucamine salt distribution
Griseofulvin distribution
Open-model system–two compartmental
Mathematical interrelation of calculation methods
Distribution volume—calculation errors
Distribution rate constant equations
Zero-order infusion method

Gas Chromatographic Determination of Ethanol in Pharmaceuticals

Comparison of Polyethylene Glycol 400 and Divinylbenzene Polymer Columns

By JOSEPH T. PIECHOCKI

Gas chromatography with a flame ionization detector was used with an official NF XII and a divinylbenzene polymer (DVBP) column to determine ethanol in pharmaceuticals. The DVBP column separated ethanol from methanol and gave results comparable to the polyethylene glycol 400 (PEG 400) column for various types of pharmaceuticals.

GAS CHROMATOGRAPHY has been used for the quantitative determination of alcohol in pharmaceuticals and other products for several years (1–5). Because of its numerous advantages, this method was investigated by the Pharmaceutical Manufacturers Association (6) and was adopted by the National Formulary (7). Elefant *et al.* (8) showed the advantages of the flame ionization detector over the thermal conductivity cell for the determination of ethanol.

Most earlier GLC methods for separating alcohols have utilized a polyethylene glycol column. Although this column was the most satisfactory of those available, ethanol could not be separated from methanol under the conditions suggested in NF XII (9). Another disadvantage is that the solvent peak elutes after the sample and internal standard peaks, unnecessarily prolonging the analysis time (8). The presence of water will affect the over-all sensitivity of the flame detector, although it does not give a response itself (10).

Recently a divinylbenzene polymer (DVBP) column[1] has been used for the separation of ethanol and chloroform in pharmaceuticals (11).

Hollis (12) introduced the use of porous polymer beads in gas chromatography. DVBP beads[2] more easily solved the problem of separating low molecular weight alcohols, acids, and glycols. Porous polymer beads have many advantages; they need no liquid phase, are hard and do not break during packing, require no pretreatment, have a large number of theoretical plates per unit volume, and condition in a relatively short time (approximately 1 hr.).

DVBP, unlike polyethylene glycol 400 (PEG 400),[3] does not retain water. When an aqueous solution is used, water will be eluted in the solvent peak or will be the first compound eluted from the DVBP column.

EXPERIMENTAL

Instrument—A Barber-Colman 5000 gas chromatograph equipped with a model 5062 oven, a flame ionization detector, and a 1-mv. recorder was used throughout this study.

PEG 400 Column—A 183 cm., 3-mm., U-shaped glass column was packed with 20% PEG 400 on 80/-100 mesh Anakrom ABS prepared by the slurry procedure (13), and conditioned at 125° for approximately 48 hr.

DVBP Column—A 120 cm., 3-mm., U-shaped glass column was packed with 80/100 mesh DVBP and conditioned at 235° for approximately 1 hr.

Operating Conditions—The column temperatures were 100° for PEG and 150° for DVBP. The temperatures of the detector and injector were 210° and 225°, respectively. The flow rates were nitrogen, 50 ml./min.; oxygen, 400 ml./min.; hydrogen, 50 ml./min. The voltage was 300 v., the sensitivity 100, the attenuation 5, and the AFS 1.0×10^{-9}.

Solutions—A standard alcohol solution was prepared according to the NF XII procedure (9), then diluted to approximately 2% (v/v). When necessary, samples were diluted with distilled water to approximately 2% alcohol (v/v). A 2% (v/v) solution of acetone in distilled water was used as an internal standard.

The final solution for injection was prepared by transferring 10.0 ml. of the diluted sample or stand-

Received July 17, 1967, from the Baltimore District Laboratories, Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Baltimore, MD 21201

Accepted for publication August 30, 1967.

[1] Polypak-2, available from F and M Scientific Division, Hewlett-Packard Corp., Avondale, Pa.

[2] Available commercially as Porapak Q from Waters Associates, Inc., Framingham, Mass.

[3] Carbowax 400.

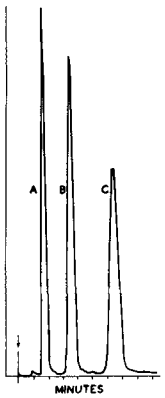

Fig. 1—Separation of alcohol standards by gas chromatography on DVBP column. Key: A, methanol; B, ethanol; C, isopropyl alcohol. DVBP column, 122 cm., 3 mm. i.d.; column temperature, 150°; injector temperature, 225°; flow rate, 50 ml. nitrogen 1 min.; flame ionization detector; detector temperature, 210°; sensitivity 1 × 10^{-9} AFS.

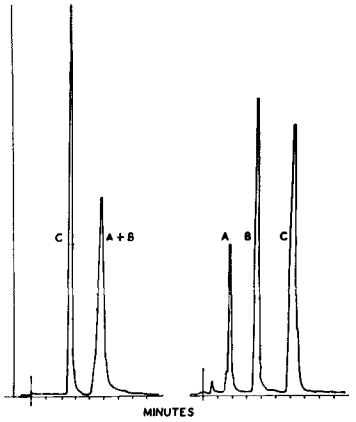

Fig. 2—Comparison of columns for gas chromatographic separation of alcohols. Key: A, methanol; B, ethanol; C, acetone. Left: PEG 400 column, 183 cm. ,3 mm. i.d.; column temperature, 100°. Right: DVBP column, 4 ft., 3 mm. i.d.; column temperature 150°. All other conditions identical: injector temperature, 225°; flow rate, 50 ml. nitrogen/min.; flame ionization detector; detector temperature, 210°; sensitivity 1 × 10^{-9} AFS.

ard alcohol solution and 10.0 ml. of the diluted acetone standard to a 100-ml. volumetric flask, diluting to volume with distilled water, and mixing.

If the sample contains acetone, two identical dilutions can be made, eliminating the internal standard from one solution and making the appropriate corrections, or another internal standard can be used.

Calculations—All calculations were made by using net integrator counts and the following formula:

$$\% \text{ alcohol} = \frac{\text{net sample counts}}{\text{net std. counts}} \times \frac{\text{net std. acetone counts}}{\text{net sample acetone counts}} \times \text{dilution factor} \times \text{concn. of std. (\%)}$$

RESULTS AND DISCUSSION

DVBP was used for about 6 months in this laboratory; no major difficulties were incurred and results are reproducible. However, solutions containing borates should not be injected into either column (14). Figure 1 shows that the three commonly used alcohols are completely separated on DVBP columns.

Retention times for ethanol and acetone were 4.0 and 6.0 min., respectively, on DVBP under the conditions stated in the experimental section (see Fig. 1).

If peak height is used as the method of quantitation, raising the column temperature to 200° will further reduce the retention time, with little loss in resolution. A complete chromatogram can then be obtained in approximately 4 min.

A comparison of the PEG 400 and DVBP is shown in Fig. 2. Methanol and ethanol are not separated on the PEG column with the conditions described in NF XII (9).

Varying amounts of the standard alcohol solution were injected onto the columns to determine the effect of injection size on response. Figure 3 shows a plot of the ratio of net alcohol counts to net acetone counts *versus* sample size. The DVBP column gave a higher net number of integrator counts/μl. of injected acetone than the PEG column.

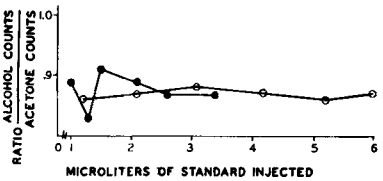

Fig. 3—Response ratio as a function of μl. injected: comparison of the two columns. Key: ○, DVBP; ●, PEG.

TABLE I—COMPARISON OF ETHANOL DETERMINATIONS ON PEG 400 AND DVBP COLUMNS

Product Type	—% Ethanol— PEG	DVBP	% Declared
Brown mixture NF	11.5	11.5	9–11
Mercurochrome	56.7	56.7	52
Antihistamine expectorant	3.0	3.0	3
Barbiturate expectorant	15.2	15.2	15
Paregoric USP	47.5	50.0	45
	47.0	46.4	...
	47.4	...	...
Terpin hydrate with codeine elixir	40.0	42.5	40
	41.2	39.6	...
	39.6	...	...
Elixir phenobarbital (synthetic)	12.7	13.8	12–15
Mouthwash	19.2	18.9	18.5
	18.1	17.4	17.0

Table I compares the analyses of ethanol content of various products on both the PEG and DVBP columns. Results from the two columns were similar. Table II lists the analyses of ethanol from

TABLE II—RESULTS OF ETHANOL DETERMINATION ON VARIOUS COMMERCIAL PRODUCTS USING THE DVBP COLUMN

Product Type	% Ethanol Declared	% Ethanol Found	% of Amt. Decl.
Antihistamine elixir	2.3	2.4	103
Antihistamine expectorant	3.5	3.8	109
Antihistamine syrup	15	14.6	97
Cough syrup	10	11.0	110
Cough syrup	3.0	3.0	100
Cough syrup	14	14.7	105
Three bromides elixir NF	3–5	4.3	107
Three bromides elixir NF	3–5	4.0	100
Phenobarbital with bromides	20	21.5	107
Corn remedy	42.5	42.0	99
Tincture merthiolate	50	51.4	103
Alcohol soln. of coal tar	83	93.8	113

TABLE III—RESULTS OF ANALYSIS OF SYNTHETIC PHENOBARBITAL ELIXIR BY VARIOUS OFFICIAL METHODS

Method	% Ethanol	Found	Av.
Pycnometer (25°)	13.9	14.2	14.0
Immersion refractometer	13.2	13.5	13.4
GLC direct on DVBP	13.8	13.7	13.7
Distillate on GLC	13.1	13.4	13.3

different types of pharmaceuticals on the DVBP column.

A synthetic mixture of elixir of phenobarbital USP was prepared and analyzed in duplicate on the DVBP column, by the distillation procedure (7), and by the immersion refractometer method (15). The results are shown in Table III. The amount of alcohol determined by refractometry on the distillate was in good agreement with the amount determined when the distillate was injected into the gas chromatograph.

CONCLUSIONS

Columns of DVBP (porous polymer beads) offer advantages for alcohol determinations, and give results as good as or better than the commonly used PEG 400 columns.

With a 150° column temperature, a sample can be injected every 7 min., resulting in 8 analyses/hr. By raising the column temperature to 200°, the time can be reduced to approximately 4 min. with little loss in resolution.

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Keyphrases

Ethanol in pharmaceuticals
GLC analysis
Divinylbenzene polymer column
Acetone–ethanol separation

Quantitative Determination of Some Synthetic Antiparkinsonism Agents

By L. G. CHATTEN and W. J. RACZ

Nonaqueous titrimetry was employed to estimate quantitatively some of the compounds which are useful in treating Parkinsonism. The method was applied to both the crystalline material and their dosage forms. This technique was found to yield results which agreed well with those obtained by either the official procedures or by methods submitted by the manufacturer. Several solvent systems were employed for the analysis of the various compounds, but either chloroform alone or a phenol–chloroform mixture was found to be almost universally applicable. Indicators were found for all the medicinal agents investigated in both of the aforementioned major solvent systems. As an additional means of quantitative assay, the amine salts were precipitated as their tetraphenylborates and the resulting derivative was then determined quantitatively by nonaqueous titrimetry.

IN THE past decade, several synthetic anticholinergic compounds have been found to be useful in controlling the symptoms of Parkinsonism. These compounds are potent systemic pharmacological agents and, therefore, accurate methods of estimating both the crystalline materials and their dosage forms must be available.

A survey of the literature revealed a complete lack of methods for quantitatively determining some of these compounds. Furthermore, although procedures have been published for some of these organic medicinals, in general, they are long and tedious.

For biperiden hydrochloride, no method of quantitative estimation could be found in the literature. On the other hand, the "National Formulary" (1) describes a procedure for cycrimine hydrochloride tablets which is based upon the infrared absorbance of a chloroform solution. The major limitation of this procedure is the large number of extractions which must be performed to ensure complete removal of the drug. The pure drug is assayed by visual nonaqueous titrimetry in glacial acetic acid.

Chafetz (2) devised a method of quantitative estimation for phenyramidol hydrochloride which was based on periodate oxidation followed by ultraviolet spectrophotometric measurement of the resulting araldehyde.

The "British Pharmacopoeia" (3) presents a method for both pure procyclidine hydrochloride and the pharmaceutical dosage forms. It is based on extraction of the liberated base and its estimation by residual titrimetry, but the technique suffers from the same limitations as the NF method for cycrimine HCl tablets. Rogers (4) described an ultraviolet absorption method for procyclidine HCl which required special slit width adjustments, whereas Pellerin and coworkers (5) employed a technique which involved sodium lauryl sulfate as titrant. *Drug Standards* (6) contains a monograph which outlines a nonaqueous titrimetric procedure for the closely related compound, tricyclamol chloride. A mixed solvent system of chloroform and glacial acetic acid is utilized and the end point determined either potentiometrically or visually. The analysis of the capsules was carried out directly on the powdered substance. However, for tricyclamol chloride tablets, the active ingredient was extracted with methanol prior to estimation.

The "United States Pharmacopeia" (7) utilizes a nonaqueous titration in chloroform to assay pure trihexyphenidyl hydrochloride whereas the same compendium describes a colorimetric extraction procedure for the dosage forms. The BP (3) employs methods similar to those described in the USP (7). Kráčmar, Blazek, and Stejskal (8) devised a polarographic procedure for the quantitative estimation of trihexiphenidyl, and Bozsai and Vastogh utilized a similar technique (9). However, since this substance does not contain a functional group which can be readily oxidized or reduced under an applied potential, a suitable group must be introduced. This process may be lengthy and the reactions involved must be quantitative.

It is the purpose of this investigation to develop rapid and accurate analytical procedures which can be employed in routine quality control of these medicinal agents both as raw materials and in pharmaceutical dosage forms.

Received March 13, 1967, from the Faculty of Pharmacy, University of Alberta, Edmonton, Alberta, Canada.

Accepted for publication August 29, 1967.

W. J. Racz is indebted to the Canadian Foundation for the Advancement of Pharmacy and the National Research Council of Canada for financial assistance during the course of this investigation.

The organic medicinals and their dosage forms were generously supplied by the following pharmaceutical companies: Burroughs Wellcome & Co. (Canada) Ltd., procyclidine HCl and Kemadrin 5 mg. tablets); Eli Lilly and Co. (Canada) Ltd., cycrimine HCl, Pagitane 1.25 and 2.50 mg. tablets, tricyclamol chloride and Elorine 50 mg. capsules; Knoll Pharmaceutical Co., diperiden HCl and Akineton 2 mg. tablets; Mead Johnson Laboratories, Analexin 400 mg. capsules; Neisler Laboratories Ltd., phenyramidol HCl; Cyanamid of Canada Ltd., trihexyphenidyl HCl, Artane 2 and 5 mg. tablets.

EXPERIMENTAL

Apparatus

Metrohm Potentiograph model E.336 equipped with a glass-calomel electrode pair, Beckman Zeromatic II pH meter fitted with a glass-calomel electrode system, magnetic stirring apparatus, conventional laboratory glassware, sintered-glass funnels of medium porosity, sintered-glass crucibles of fine porosity, microburet graduated to 0.01 ml.

Reagents and Solutions

Acetone; chloroform; *N,N*-dimethylformamide; glacial acetic acid; phenol; sodium acetate; sodium tetraphenylboron; acetate buffer solution, pH 4.6; crystal violet T.S. USP; 0.1 *N* HCl; 0.05 *N* HCl; 2 *N* HCl; mercuric acetate T.S. USP; 0.5% methyl red in methanol; methyl red solution BP; 0.05 *N* perchloric acid in dioxane (standardized against primary standard potassium acid phthalate); 5% sodium tetraphenylboron solution in water (filtered through a sintered-glass funnel of medium porosity); thymol blue in *N,N*-dimethylformamide USP; 0.25% tropaeolin 00 in methanol.

All chemicals employed in this investigation were either A.C.S. or reagent grade quality.

The antiparkinsonism compounds and their commercial products studied in this investigation are as follows: biperiden HCl (2 mg.),[1] cycrimine HCl (2.5 and 1.25 mg.),[2] phenyramidol HCl (400 mg.),[3] procyclidine HCl (5 mg.),[4] tricyclamol chloride (50 mg.),[5] trihexyphenidyl HCl (5 and 2 mg.).[6]

Procedures for Crystalline Materials

The purity of trihexyphenidyl HCl was determined by the procedure of the USP (7). Procyclidine HCl was estimated by the BP (3) method and cycrimine HCl by the NF (1) procedure.

General Nonaqueous Procedure

Approximately 50 mg. of the material under investigation was accurately weighed into a 150-ml. beaker and dissolved in 50 ml. of the solvent of choice with the aid of magnetic stirring. Mercuric acetate T.S. (1.5 ml.) was then added and the titration followed potentiometrically using 0.05 *N* perchloric acid in dioxane as titrant.

Since official methods were not available for biperiden HCl, tricyclamol chloride, and phenyramidol HCl, the purity of these compounds was determined by the foregoing nonaqueous technique when glacial acetic acid was utilized as solvent system.

Modification 1—Chloroform was employed as the solvent for cycrimine HCl, procyclidine HCl, trihexyphendyl HCl, and tricyclamol chloride. In addition to the potentiometric determinations, visual titrations were also carried out using 3 drops of a saturated solution of methyl red in methanol as indicator. The end point was found to be purplish red in color.

Modification 2—A solvent system comprising equal parts of chloroform and *N,N*-dimethylformamide was employed for the same 4 substances mentioned under *Modification 1*. The end point was detected potentiometrically.

Modification 3—In this instance, the solvent system was similar to that employed by Chatten (10) for the titration of various nitrate and sulfate salts of organic bases. Methyl red was found to be the indicator of choice for biperiden HCl, whereas tropaeolin 00 in anhydrous methanol was employed for phenyramidol HCl. The end point for the latter was orange-brown in color.

For the general procedure as well as all modifications, a blank was performed on the solvent system. Where applicable, the appropriate indicator was included. Corrections were made of all titrant volumes.

Sodium Tetraphenylborate Procedure

This technique was a combination and a modification of the methods of Smith, Worrell, and Sinsheimer (11) and Chatten, Pernarowski, and Levi (12).

A sample (25 to 50 mg.) of the drug was accurately weighed into a 150-ml. beaker and 50 ml. of acetate buffer (pH 4.6) was added. The mixture was stirred magnetically until the drug had dissolved. An excess of the 5% solution of sodium tetraphenylboron was added and the mixture stirred for an additional 20 min. The precipitate was removed with the aid of suction filtration through a sintered-glass crucible of fine porosity. The beaker and the tetraphenylborate were washed with 200 ml. of water. Then the crucible and tetraphenylborate were dried for a period of not less than 18 hr. in a vacuum desiccator over phosphorus pentoxide. The magnetic stirring bar was washed with a small quantity of acetone, the acetone retained in the beaker, and the beaker also dried in a vacuum desiccator over calcium chloride.

Ten milliliters of acetone was added to the crucible containing the tetraphenylborate and the mixture was stirred to dissolve the compound. The solution was then filtered into the dried beaker in which the compound had been prepared. The crucible was washed with an additional 10 ml. of acetone and the filtrate collected in the beaker. Positive pressure was used to facilitate the filtration process. To the filtrate, 30 ml. of glacial acetic acid, 1.5 ml. of mercuric acetate T.S., and 1 drop of crystal violet T.S. were added and the mixture titrated to a blue end point (13) with perchloric acid in dioxane as titrant. A titration light was used to facilitate detection of the end point. A blank titration was performed on the solvent system.

All six antiparkinsonism agents were assayed by this method.

The tetraphenylborate of tricyclamol was prepared, purified, and the equivalent weight determined by the method of Chatten and Doan (13). The adduct was submitted for elemental analysis and the results were as follows: tricyclamol tetraphenylborate, m.p. 197–199°; purity by nonaqueous titrimetry, 99.5%.

Anal.—Calcd. for $C_{44}H_{52}BNO$: C, 84.93; H, 8.43; N, 2.25. Found: C, 84.78; H, 8.26; N, 2.11.

Procedures for Pharmaceutical Dosage Forms

All dosage forms were analyzed by pharmacopeial

[1] Trademarked as Akineton.
[2] Trademarked as Pagitane.
[3] Trademarked as Analexin.
[4] Trademarked as Kemadrin.
[5] Trademarked as Elorine.
[6] Trademarked as Artane.

procedures or by methods supplied by manufacturers, wherever this was possible.

Trihexyphenidyl HCl tablets, cycrimine HCl tablets, and procyclidine HCl tablets were assayed by the procedures of the USP (7), NF (1), and the BP (3), respectively.

Capsules of tricyclamol chloride and phenyramidol HCl were analyzed by methods provided by the respective manufacturers.

A published procedure was not available for the analysis of biperiden HCl tablets.

General Nonaqueous Procedure

A sample of the powdered tablet or capsule material was accurately weighed into a 150-ml. beaker and 25 ml. of chloroform was added. The mixture was stirred magnetically for 40 min. and the solution then filtered through a sintered-glass funnel of medium porosity. The beaker and funnel were washed with an additional 25 ml. of chloroform. A slight excess of mercuric acetate solution was added and the titration followed potentiometrically using perchloric acid in dioxane as titrant. The end point was also detected visually by employing methyl red as indicator. A blank titration was performed on the solvent system and the appropriate correction made.

This method was employed to analyze the dosage forms of the following compounds: cycrimine HCl, procyclidine HCl, and trihexyphenidyl HCl. Tricyclamol chloride capsules were also determined by the above procedure but the samples were not filtered prior to titration.

Tricyclamol chloride capsules were also titrated by a modification in which a 1:1 mixture of dimethylformamide–chloroform was used as solvent. The end point was detected potentiometrically.

Biperiden HCl tablets were assayed by another modification in which the solvent system was 2.5% phenol in chloroform. The same solvent system with a stirring time of 30 min. was employed for phenyramidol capsules. The end point was determined either potentiometrically or visually using tropaeolin 00 as indicator. The end point was found to be orange-brown in color. A blank was determined on the solvent system and capsule excipients prior to the addition of mercuric acetate.

Sodium Tetraphenylborate Method

The pharmaceutical dosage forms were also assayed by the tetraphenylborate method, with the introduction of certain modifications. The finely ground tablet or capsule material was accurately weighed into a 150-ml. beaker and about 2 ml. of acetate buffer was added. The powder was triturated with buffer solution until a smooth paste resulted. The remainder of the 50 ml. of buffer was added and the mixture stirred magnetically for 40 min., and the general procedure was followed beginning with the words "An excess of a 5% solution of sodium tetraphenylboron ...."

The commercial products of biperiden HCl, phenyramidol HCl, procyclidine HCl, tricyclamol chloride, and trihexyphenidyl HCl were determined by this method.

RESULTS AND DISCUSSION

Glacial acetic acid was chosen as the solvent system for determining the purity of those compounds for which no published or accepted procedures existed. It has been extensively used for the analysis of crystalline pharmaceutical substances (3, 7) because of its excellent solvent properties for organic medicinal agents. The potentiometric break obtained in glacial acetic acid was well-defined and thus the end point could be determined accurately. However, this solvent is not suitable for the nonaqueous titration of pharmaceutical dosage forms, because a large number of interfering excipients are soluble in it (14). As noted from Table I, the results obtained in this solvent are in excellent agreement with the theoretical values.

In order to select a suitable titration medium, the solubility of each compound was determined in a variety of solvents which could also be applied to the dosage forms. Trihexyphenidyl HCl, procyclidine HCl, cycrimine HCl, and tricyclamol chloride were found to be soluble in chloroform and 2-propanol. While compounds with the exception of tricyclamol chloride were readily soluble in chloroform, dissolution was slow in 2-propanol.

The potentiometric titration of trihexyphenidyl HCl was attempted in 2-propanol but the inflection point was not well-defined and hence the end point was difficult to determine. On the other hand, in chloroform, the potentiometric break was well-defined and the end point easily detected. Clair and Chatten (14) reported that very few tablet excipients interfere with nonaqueous titrations which are performed in chloroform and hence it was the solvent of choice for the aforementioned materials as it possessed many desirable attributes.

The four antiparkinsonism agents were also titrated in chloroform and the end point was detected with methyl red in anhydrous methanol. Thymol blue can also be utilized as an indicator for the titration of these substances and, in fact, is recommended by the USP (7) for the assay of trihexyphenidyl HCl. However, the former indicator was selected because its color change appeared to be more distinct than that of thymol blue.

In Fig. 1, curve A depicts the potentiometric curve for the titration of procyclidine HCl in chloroform, whereas curve B illustrates the titration of tricyclamol chloride in chloroform. The color changes of methyl red are illustrated on the same figure. Curve C shows the titration of procyclidine HCl in chloroform with thymol blue as indicator.

Tricyclamol chloride was found to be only slowly soluble in chloroform. Therefore, a more suitable solvent, *N,N*-dimethylformamide, was selected. In addition, this solvent was observed to possess excellent solubilizing properties for the hydrochlorides of trihexyphenidyl, cycrimine, procyclidine, and phenyramidol. However, titration of tricyclamol chloride in *N,N*-dimethylformamide resulted in a depressed and poorly defined end point. Investigation of mixed solvent systems resulted in a mixture of chloroform and *N,N*-dimethylformamide (1:1) which gave sharp end points and yet retained excellent solvent powers for the compounds previously mentioned. Unfortunately, a suitable indicator could not be found for the chloroform–*N,N*-dimethylformamide solvent system.

Phenyramidol HCl could not be titrated in *N,N*-dimethylformamide–chloroform as the potentio-

TABLE I—SUMMARY OF DATA FOR ANALYSIS OF CRYSTALLINE COMPOUNDS EXPRESSED AS PERCENT RECOVERY ± STANDARD DEVIATION

Compd.	Official Method	Potentiometric Titration in Glacial Acetic Acid	Potentiometric Titration in Chloroform	Visual Titration in Chloroform	Potentiometric Titration in DMF–$CHCl_3$	Potentiometric Titration in Phenol–$CHCl_3$	Visual Titration in Phenol–$CHCl_3$	Tetraphenylborate
Trihexyphenidyl hydrochloride	100.0 ±0.54	...	...	99.6 ±0.25	100.1 ±0.36	...	...	97.9 ±1.12
Procyclidine hydrochloride	98.7 ±1.96	...	99.9 ±0.45	99.9 ±0.42	100.1 ±0.62	...	...	97.5 ±1.58
Cycrimine hydrochloride	99.5 ±0.58	...	100.0 ±0.43	99.3 ±0.32	100.1 ±0.64	...	...	98.6 ±1.49
Biperiden hydrochloride		99.9 ±0.89	...	...	...	100.2 ±0.71	99.9 ±0.41	98.5 ±.94
Phenyramidol hydrochloride		100.1 ±0.38	...	...	...	100.2 ±0.37	99.9 ±0.40	98.8 ±1.91
Tricyclamol chloride		99.8 ±0.53	99.4 ±0.25	99.5 ±0.32	99.9 ±0.49	...	...	95.1 ±0.66

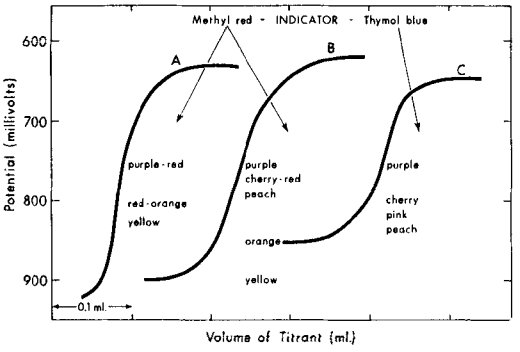

Fig. 1—Titrations in chloroform. Key: A, *procyclidine HCl;* B, *tricyclamol chloride;* C, *procyclidine HCl.*

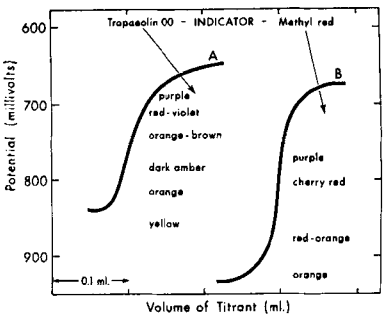

Fig. 2—Titrations in phenol–chloroform. Key: A, *phenyramidol HCl;* B, *biperiden HCl.*

metric break was very poorly defined. In addition, biperiden HCl was found to possess a low solubility in chloroform and hence a combination of the two factors created the need for another solvent system for these two compounds. The present investigation showed that biperiden HCl and phenyramidol HCl were soluble in a system containing 2.5% phenol in chloroform and these substances were readily titratable in this solvent system. The weak acidity of the solvent system may enhance the weak basicity of phenyramidol HCl whereas *N,N*-dimethylformamide would have the opposite effect upon the antiparkinsonism agent.

Tropaeolin 00 in methanol was the indicator of choice for phenyramidol HCl in the phenol–chloroform system and Fig. 2, curve A depicts the titration curve together with the indicator changes. Methyl red can also be used and was, in fact, selected for biperiden HCl. Curve B illustrates the titration curve and indicator changes for that substance.

Tetraphenylborate Method

Sodium tetraphenylboron has been used for the characterization and determination of organic medicinal agents containing basic nitrogen functional groups. Smith, Worrell, and Sinsheimer (11) have titrated some sympathomimetic amines with sodium tetraphenylboron and detected the end point amperometrically.

In a previous report, Chatten and Doan (13) prepared in excellent yields the tetraphenylborates of the compounds under study in this project. It was of interest, therefore, to attempt to modify their procedure in order to obtain quantitative results.

An acidic pH in the range of 4 to 6 has been reported to be suitable for the preparation of the tetraphenylborates of organic bases (11). It was found that buffered solutions in the pH range of 4 to 5.6 gave reasonable analytical results, while in a more acid system, the tetraphenylborates of the substances in this investigation exhibited increased solubility. Hence, the acetate–acetic acid buffer pair (pH 4.6) was selected for solvent power and convenience

One disadvantage of the tetraphenylborate method is the difficulty encountered in removing the precipitate from the vessel in which it was prepared. To overcome this problem, the beaker was allowed to dry and the acetone solution of the tetraphenylborate filtered into it.

The end point of the titration was found to appear very slowly and false end points occurred prior to the stoichiometric end point. The addition of mercuric acetate T.S. to the tetraphenylborate solution allowed the stoichiometric end point to be reached in a shorter space of time because the premature end points were few and very short-lived.

Examination of Table I reveals the various nonaqueous methods which were developed in this

investigation for the hydrochlorides of trihexyphenidyl, procyclidine, and cycrimine gave excellent agreement with the official procedures. No official methods were available for biperiden HCl, phenyramidol HCl, and tricyclamol HCl. Because of their widespread utility, however, potentiometric titrations in glacial acetic acid were accepted as standards and all other techniques were compared to them. For reasons of solubility, which have been already explained, the hydrochlorides of biperiden and phenyramidol were titrated in a phenol–chloroform solvent system. The results obtained are in excellent agreement with those in glacial acetic acid. For tricyclamol chloride, all nonaqueous techniques gave virtually identical results. Although the results by the tetraphenylborate method were slightly lower in almost every instance than those obtained with all other techniques, the values were believed to be within experimental error between two methods. In general, the recoveries were considered to be acceptable.

The results obtained from the analysis of the pharmaceutical dosage forms are listed in Table II. No official or manufacturers' procedure was available for biperiden HCl tablets. In addition, quantitative recoveries could not be obtained for this preparation by the tetraphenylborate method.

The analysis of phenyramidol HCl capsules was carried out by four methods: (*a*) and (*b*) potentiometric and visual nonaqueous titration in the phenol–chloroform solvent system, (*c*) preparation and titration of the tetraphenylborate, and (*d*) the manufacturers' procedure. Excellent agreement was obtained by the four methods of assay applied to this dosage form.

For trihexyphenidyl HCl, the four methods of analysis gave very good agreement, as shown by Table II, for the 5-mg. tablet. Small but not significant anomalies occurred with the 2-mg. product. For both nonaqueous procedures, it was necessary to remove interfering excipients by filtration, prior to titration.

The results of the nonaqueous assays for tricyclamol chloride in chloroform were below those obtained by the manufacturers' method. This may be caused by the slow solubility of the drug in chloroform. The higher recoveries in a mixed solvent system of *N,N*-dimethylformamide–chloroform are probably due to the enhanced solubility of tricyclamol chloride in that system. The low recoveries obtained by the tetraphenylborate procedure may be attributed to the suggestion that the quaternary ammonium compound does not form tetraphenylborates as readily as the other substances do.

As noted from Table II, the results obtained by the nonaqueous methods for procyclidine HCl are in good agreement with the official procedure of the BP (3). Filtration of the chloroform solutions prior to carrying out the titrations was found to be necessary as undissolved excipients interfered with the end points. It is noted that the tetraphenylborate method yielded values which appear to be higher than those obtained by the official procedure. This discrepancy is probably due to excipient interference.

For cycrimine HCl the results with the nonaqueous procedures agreed well with the NF (1) method for the 2.5-mg. tablet. However, for the 1.25-mg. product, the results by the nonaqueous techniques were much too low. No satisfactory explanation can be offered for this anomaly, since increasing the extraction time did not increase the percent recovery.

The tetraphenylborate method could not be applied to cycrimine HCl tablets as the recoveries obtained were much above the theoretical values. It would appear that these tablets contain an excipient which is capable of reacting with sodium tetraphenylboron and this results in overestimation.

It is worthy of mention that analysis of procyclidine HCl 5 mg., trihexyphenidyl HCl 5 mg., and cycrimine HCl 2.5-mg. tablets was attempted in the *N,N*-dimethylformamide–chloroform mixture but gross overestimation occurred in all three instances. This would seem to indicate that a titratable excipient which is soluble in dimethylformamide but not in chloroform must be present in the tablet formulations.

TABLE II—SUMMARY OF DATA FOR ANALYSIS OF DOSAGE FORMS BY VARIOUS PROCEDURES EXPRESSED AS PERCENT POTENCY ± STANDARD DEVIATION

Compd.	Official or Manufacturers' Procedure	Potentiometric Titration in Chloroform	Visual Titration in Chloroform	Potentiometric Titration in DMF–$CHCl_3$	Potentiometric Titration in Phenol–$CHCl_3$	Visual Titration in Phenol–$CHCl_3$	Tetraphenylborate
Biperiden HCl, 2 mg.	...	...	...	...	94.9	95.8	No result
	...	...	...	...	±0.72	±0.90	
Phenyramidol HCl, 400 mg.	93.9	...	...	...	92.2	91.7	92.7
	±0.29	...	...	...	±0.98	±0.18	±0.67
Trihexyphenidyl HCl, 5 mg.	97.3	96.6	96.2	...	...	...	97.1
	±1.65	±1.58	±0.27	...	...	...	±2.32
Trihexyphenidyl HCl, 2 mg.	106.9	104.6	106.9	...	...	...	104.9
	±0.89	±1.39	±0.40	...	...	...	±2.20
Tricyclamol chloride, 50 mg.	101.0	98.5	98.6	99.4	...	...	94.7
	±0.56	±1.15	±0.91	±0.70	...	...	±2.63
Procyclidine HCl, 5 mg.	94.5	94.6	94.2	...	...	...	97.7
	±3.08	±0.71	±0.53	...	...	...	±1.62
Cycrimine HCl, 2.5 mg.	94.5	93.8	92.2	...	...	...	No result
	±5.69	±1.23	±0.23	...	...	...	
Cycrimine HCl, 1.25 mg.	95.2	80.7	80.4	...	...	...	No result
	±1.53	±1.20	±1.39	...	...	...	

SUMMARY

A nonaqueous method of analysis utilizing chloroform as solvent has been developed for four compounds and their dosage forms. The results obtained are in excellent agreement with those obtained by the official or manufacturers' methods, but the nonaqueous procedure is much more rapid.

Phenol–chloroform mixture was employed with good results as solvent for the analysis of two of the organic medicinal agents investigated.

N,N-Dimethylformamide–chloroform mixture was successfully utilized as a solvent system for the nonaqueous titration of four of the crystalline salts. However, the solvent could be applied only to one dosage form.

A method based on precipitation of the amine as the tetraphenylborate and then estimating the tetraphenylborate by nonaqueous titrimetry was developed. This procedure does not have as wide an application as does direct nonaqueous titrimetry, but good analytical data were obtained for five of the dosage forms. This method would appear to be susceptible to excipient interference.

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Keyphrases

Antiparkinsonism agents, synthetic
Titrimetry, nonaqueous-quantitative analysis
Tetraphenylborate salt formation
Perchloric acid in dioxane titrant
Mercuric acetate T.S. and mercuric acetate plus crystal violet T.S. indicators
Methyl red and methyl red plus crystal violet T.S. indicators
Tropaeolin 00—indicator
Colorimetric end point determination
Potentiometric end point determination

Gas Chromatographic Determination of Trisulfapyrimidines in Tablets

By JOHN W. TURCZAN

A procedure for the quantitative determination of individual sulfapyrimidines in mixtures has been developed. Analyses of known mixtures show an accuracy of 1–2 percent. The method is based on acid hydrolysis of the trisulfapyrimidines to sulfanilic acid and their respective heterocyclic amines, followed by the separation and determination of the amines by gas chromatography.

THE SEPARATION and quantitative determination of the three sulfapyrimidines, which are frequently dispensed together, is a difficult problem. In the usual methods of analysis the aromatic primary amino group or the acidic hydrogen (1) is determined. These procedures are therefore not specific and cannot determine individual sulfapyrimidines in a multi-sulfapyrimidine mixture. A paper chromatographic procedure (2) has been developed which identifies and quantitates mixtures of sulfapyrimidines and other sulfonamides.

An alternative method proposed here is based on gas chromatography after an acid hydrolysis (3, 4) of the sulfapyrimidines to sulfanilic acid and their respective heterocyclic amines. A study was made to determine optimum conditions for the hydrolysis. The following procedure was then used to analyze known mixtures and commercial tablets.

Received June 21, 1967, from the New York District Laboratories, U. S. Department of Health, Education, and Welfare, Food and Drug Administration, Brooklyn, NY 11232

Accepted for publication August 21, 1967.

The author acknowledges the assistance of Evelyn Sarnoff, Dr. R. Ginell, and Gertrude Lowell, in the preparation of this report, and Anthony Romano, Jr., Minneapolis District, Anthony C. Celeste, Kansas City District, and Martin Goldstein, New Orleans District.

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By JOHN W. TURCZAN

A procedure for the quantitative determination of individual sulfapyrimidines in mixtures has been developed. Analyses of known mixtures show an accuracy of 1–2 percent. The method is based on acid hydrolysis of the trisulfapyrimidines to sulfanilic acid and their respective heterocyclic amines, followed by the separation and determination of the amines by gas chromatography.

THE SEPARATION and quantitative determination of the three sulfapyrimidines, which are frequently dispensed together, is a difficult problem. In the usual methods of analysis the aromatic primary amino group or the acidic hydrogen (1) is determined. These procedures are therefore not specific and cannot determine individual sulfapyrimidines in a multi-sulfapyrimidine mixture. A paper chromatographic procedure (2) has been developed which identifies and quantitates mixtures of sulfapyrimidines and other sulfonamides.

An alternative method proposed here is based on gas chromatography after an acid hydrolysis (3, 4) of the sulfapyrimidines to sulfanilic acid and their respective heterocyclic amines. A study was made to determine optimum conditions for the hydrolysis. The following procedure was then used to analyze known mixtures and commercial tablets.

Received June 21, 1967, from the New York District Laboratories, U. S. Department of Health, Education, and Welfare, Food and Drug Administration, Brooklyn, NY 11232

Accepted for publication August 21, 1967.

The author acknowledges the assistance of Evelyn Sarnoff, Dr. R. Ginell, and Gertrude Lowell, in the preparation of this report, and Anthony Romano, Jr., Minneapolis District, Anthony C. Celeste, Kansas City District, and Martin Goldstein, New Orleans District.

EXPERIMENTAL

Apparatus—A Barber-Colman 5000 gas chromatograph with a hydrogen flame detector was used. The column, 6 ft. by 4 mm., was packed with 5% SE-30 and 5% Carbowax 20M on Chromosorb W (60–80 mesh; hexamethyldisilazane-treated). The column was conditioned overnight at 180° with a nitrogen flow of 140 ml./min. The operating conditions were: column temperature, 150°; flash heater temperature, 260°; cell temperature, 260°; carrier gas, prepurified nitrogen; flow rate of carrier gas (outlet), 120 ml./min.; pressure of hydrogen, 16 p.s.i.; air, 40 p.s.i.

The instrument was equipped with a 1-mv. recorder and all determinations were made with an electrometer setting of 300 and an attenuation of 2×; at this setting 1.2 mcg. of 2-amino pyrimidines will give a recorder response of about 70% of full-scale deflection.

Standard Solutions—*Internal Standard*—Four milligrams per milliliter, 2-amino-4,6-dimethylpyridine or 2-amino-4-methylpyridine in water. If procedure given below is to be used, take 5-ml. aliquot (yielding concentration of 0.2 mg./ml. when diluted) for standard and sample solution.

Standard Solution—A mixture containing 0.2 mg./ml. of 2-aminopyrimidine, 0.2 mg./ml. of 2-amino-4-methylpyrimidine, 0.2 mg./ml. of 2-amino-4,6-dimethylpyrimidine, and 0.2 mg./ml. of internal standard in water.

Procedure—*Preparation of Sample*—Weigh and finely powder not less than 20 trisulfapyrimidine tablets. Weigh accurately a portion of the powder equivalent to about 150 mg. of total sulfapyrimidines into a glass-stoppered 50-ml. centrifuge tube, add 4 ml. of concentrated sulfuric acid, stopper, mix, and place in an oven at 130° for 1.5 hr. Shake every 15 min. to insure complete contact of powder with hot acid. Cool by holding under running water and transfer quantitatively, using approximately 50 ml. of water, into a 100-ml. volumetric flask. Make alkaline to litmus by slowly adding approximately 8 ml. of 50% sodium hydroxide, maintaining solution at room temperature. Add 5.0 ml. (equivalent to 20 mg.) of internal standard and dilute to volume.

Chromatography and Calculations—Inject enough standard solution to insure 30–80% deflection (about 4–6 μl. if above procedure is followed). Measure the retention time (R.T.) of the standards from the solvent front to the apex of the peaks and calculate recorder response for each by multiplying retention time by the peak height (5–7). Using the recorder response for each standard and for the internal standard in the standard mixture, and their respective concentrations, determine the R value by Eq. 1. To obtain consistent results, try 3–4 preliminary injections. The R value should be constant within 3%; if it is not constant, make additional injections. When consistent responses are obtained, inject a sample to give approximately the same peak heights as the standard solution. Calculate the concentration of each sulfapyrimidine (using recorder responses) in its final solution by Eq. 2.

$$R = \frac{A_0 C_1}{A_1 C_0} \qquad \text{(Eq. 1)}$$

$$C_3 = \frac{A_3 C_2 A_1 C_0}{A_0 C_1 A_2} \qquad \text{(Eq. 2)}$$

where

A_0 = (R.T. × h) = recorder response for standard,
A_1 = recorder response of internal standard in standard mixture,
A_2 = recorder response of internal standard in sample mixture,
A_3 = recorder response of sample,
C_0 = concentration of standard,
C_1 = concentration of internal standard in standard mixture,
C_2 = concentration of internal standard in sample mixture,
C_3 = concentration of sulfapyrimidine in sample mixture.

Note: if the concentration of the internal standard in standard and sample is the same ($C_1 = C_2$), then Eq. 2 becomes:

TABLE I—KNOWN USP MIXTURE OF TRISULFAPYRIMIDINES

	Sulfadiazine			Sulfamerazine			Sulfamethazine		
Mixture	Added, mg.	Found, mg.	Recovery, %	Added, mg.	Found, mg.	Recovery, %	Added, mg.	Found, mg.	Recovery, %
1	50.1	49.47	98.7	49.9	49.65	99.5	51.5	52.02	101.0
2	48.8	47.73	97.8	50.6	50.04	98.9	51.8	51.54	99.5
3	48.9	49.19	100.6	52.6	51.76	98.4	49.4	48.86	98.9
4	55.3	54.69	98.9	50.2	49.55	98.7	49.7	50.35	101.9
5	52.7	51.86	98.4	49.1	48.51	98.8	48.6	48.21	99.2

TABLE II—COMMERCIAL TABLET PREPARATION

	Sulfadiazine			Sulfamerazine			Sulfamethazine		
Sample	Declared, mg.	Found, mg.	Declared, %	Declared, mg.	Found, mg.	Declared, %	Declared, mg.	Found, mg.	Declared, %
1	167	165.0	98.8	167	166.5	99.7	167	165.3	99.0
2	161	159.1	98.8	161	164.1	101.9	161	163.4	101.5
3	167	168.8	101.1	167	170.1	102.2	Not present or declared		
4	163	159.9	98.1	163	165.1	101.3	163	165.4	101.5
5	167	163.3	97.8	167	165.2	98.2	167	165.5	99.1
6	161	158.3	98.3	161	161.8	100.5	161	158.6	98.5
7	163	158.4	97.2	163	160.7	98.6	Not present or declared		
8	167	161.8	96.9	167	165.0	98.8	167	166.3	99.6

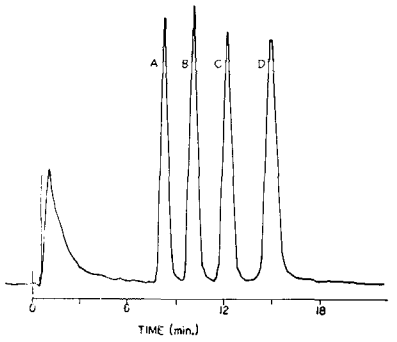

Fig. 1—Chromatogram of 3 sulfapyrimidines and internal standard mixtures. Key: A, 2-aminopyrimidine; B, 2-amino-4-methylpyrimidine; C, 2-amino-4,6-dimethylpyrimidine; and D, 2-amino-4,6-dimethylpyridine (internal standard); 1.2 mcg. of each injected.

$$C_3 = \frac{A_3 C_0 A_1}{A_0 A_2} \qquad \text{(Eq. 3)}$$

All calculations are made in terms of the final dilution. To calculate the concentration in the original sample, the sample size, the molecular weights, and the average weight per tablet must be taken into account; then Eq. 3 becomes:

$$C_3 = \frac{A_3 C_0 A_1}{A_0 A_2} \times \frac{\text{av. wt./tab.} \times \text{mol. wt. of sulfa drug}}{\text{wt. of spl.} \times \text{mol. wt. of hydrolysis product}}$$

$$= \text{mg. of sulfa drug/tablet} \qquad \text{(Eq. 4)}$$

DISCUSSION AND RESULTS

To determine the optimum conditions for hydrolysis, the experimental conditions were varied and completeness of hydrolysis was determined by UV spectrophotometry. The time of hydrolysis was kept at 1 hr. and the amount of sulfapyrimidine at 100 mg., while the concentration of hydrolyzing acid was varied from 20% to concentrated. Concentrated sulfuric acid (reagent grade) was found to be necessary for complete hydrolysis.

The time of hydrolysis was varied from 10 min.–180 min., while the amount of acid was kept constant at 2 ml. of concentrated sulfuric acid and the amount of sulfapyrimidine at 100 mg. The minimum time necessary for complete hydrolysis was found to be 1 hr.

An experiment in which the amount of sulfapyrimidine was varied from 50–220 mg., while the amount of concentrated acid was kept constant at 2 ml. and the time at 1 hr., demonstrated that no more than 190 mg. should be treated with 2 ml. of acid.

Individual 150-mg. portions of sulfadiazine, sulfamerazine, and sulfamethazine were hydrolyzed under the following conditions: 4 ml. of concentrated sulfuric acid, 90 min., at 130°. The resulting solutions were analyzed by both UV spectrophotometry and gas chromatography. Recoveries by gas chromatography correlate well with those by UV spectrophotometry. They ranged from 98.9–101.1% by UV and from 95.6–99.5% by GC for sulfadiazine; 98.1–101.5% by UV, and 97.8–99.1% by GC for sulfamerazine; 98.0–102.0% by UV, and 97.4–99.2% by GC for sulfamethazine.

Five synthetic mixtures containing equal parts of sulfadiazine, sulfamerazine, and sulfamethazine were analyzed by the proposed procedure (GLC) (Table I) with very good results. Analysis of eight commercial preparations (Table II) showed good agreement with declared values.

If the relative retention time (R.R.T.) of sulfadiazine, which yields 2 aminopyrimidine, is taken as 1.00, then the R.R.T. of sulfamerazine, which yields 2-amino-4-methylpyrimidine, is 1.24, that of sulfamethazine, which yields 2-amino-4,6-dimethylpyrimidine, is 1.52, and that of the internal standard, 2-amino-4,6-dimethylpyridine, is 1.89 (Fig. 1).

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Keyphrases

Trisulfapyrimidine tablets analysis
Sulfadiazine, sulfamerazine, and sulfamethazine combination
Acid hydrolysis
UV determination of hydrolysis
GLC analysis

Determination of Terpin Hydrate in Elixirs Containing Codeine or Dextromethorphan Hydrobromide by Gas–Liquid Chromatography

By F. P. MAHN, V. VISWANATHAN, and B. Z. SENKOWSKI

A gas–liquid chromatographic procedure for the quantitative analysis of terpin hydrate was investigated. A Pyrex column packed with 10 percent Carbowax 20M terephthalic acid on Chromosorb W, hexamethyldisilane (HMDS) treated, was found to give satisfactory elution characteristics. Pharmaceutical preparations containing terpin hydrate, such as elixirs with dextromethorphan hydrobromide or codeine, were analyzed by the proposed procedure with recoveries of 100 ± 3 percent. The internal standard used was biphenyl. The effect of temperature on retention volume is reported.

THE CHEMICAL STRUCTURE of terpin hydrate indicated that the compound could lend itself to rapid and precise gas chromatography. A survey of the literature failed to disclose any previous studies utilizing this technique.[1] Procedures were reported by Platt and James (1) for the quantitative analysis of terpin hydrate using colorimetry. These authors treated phosphomolybdic acid with terpin hydrate under controlled conditions and measured the resulting color. Milos (2) reported on collaborative results which supported the adoption of the colorimetric determination. Vadodaria, Parikh, and Mukheyi (3) modified the colorimetric method of Platt and James and reported results with a precision of ±3%. These methods are time-consuming and involve the isolation of terpin hydrate in comparatively pure form by solvent extraction prior to actual analysis. Microscopic methods (4, 5) and refractometric methods (6) also were reported for the analysis or identification of terpin hydrate. However, the use of these techniques was limited primarily to qualitative identification.

Vapor-phase chromatography was investigated, and it was found that elution of terpin hydrate under isothermal conditions resulted in a symmetrical peak. The column selected for best results was 10% Carbowax 20M terephthalic acid on Chromosorb W, HMDS treated. Since analysis could be carried out directly on alcoholic dilutions of terpin hydrate preparations, errors due to excessive handling were minimized. The precision of the method therefore is dependent primarily upon the instrumental conditions and response. In order to compensate for column characteristics, instrumental variations, and sample introduction technique, an internal standard, biphenyl, was employed. Biphenyl is well suited for the experimental conditions in terms of detector linearity and retention time. Analyses were carried out on elixir terpin hydrate and elixir terpin hydrate with codeine or dextromethorphan hydrobromide NF.

Linearity of detector response with concentration, detection levels, interferences, and recovery data are presented.

EXPERIMENTAL

Operational Parameters—The instrument used for this work was a Varian Aerograph model 600 gas chromatograph equipped with a hydrogen flame ionization detector. The column used was a Pyrex coil, 5 ft. long and 3 mm. i.d., packed with 10% Carbowax 20M terephthalic acid on Chromosorb W, HMDS treated, 60/80 mesh. The temperatures were: column, 160°; injector port, 250° with a Pyrex insert; and detector, 160°. The flow rates were: carrier gas, nitrogen, 40 ml./min.; detector gas, hydrogen, 20 ml./min.; and air, 300 ml./min. All injections were made using a 10-μl. Hamilton syringe with the injection volume being approximately 2 μl. The instrument was operated at a range of 10 and attenuation of 64×, or equivalent, to result in at least a 50% response of the recorder scale. The recorder used was a 0–1 mv. Texas Instrument with a pen response of 0.4 sec. and a chart speed of 0.75 in./min. All peak areas were measured using a disk integrator. Under the conditions stated the relative retention time of terpin hydrate was 1.38 with respect to the internal standard, biphenyl, which has a specific retention time of approximately 4 min. (Fig. 1).

Standard Preparation—*Internal Standard*—Accurately weigh 50 mg. of biphenyl into a 25-ml. volumetric flask. Dissolve and dilute to volume with absolute ethanol. Mix well.

Reference Standard—Accurately weigh 85 mg. of terpin hydrate into a 25-ml. volumetric flask. Dissolve and dilute to volume with absolute ethanol. Mix well.

Working Standard (Prepare in Duplicate)—Into a small glass-stoppered flask, pipet 1 ml. of the bi-

Received May 31, 1967, from the Analytical Research Laboratory, Hoffmann-LaRoche, Inc., Nutley, NJ 07110
Accepted for publication August 21, 1967.

[1] Subsequent to the submission of this paper, a similar method was published: Kurlansik, L., Damon, D., Klein, H., and Salim, E. F., *J. Pharm. Sci.*, **56**, 1160(1967).

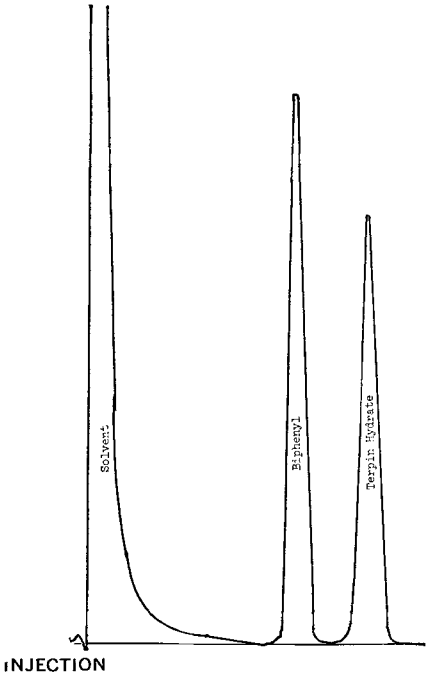

Fig. 1—Standard chromatogram.

phenyl standard solution and 1 ml. of the terpin hydrate standard solution. Stopper and mix thoroughly. Chromatograms were run and all peak areas were measured from the integrator pen trace.

Sample Preparation—Accurately pipet a 5-ml. aliquot of elixir terpin hydrate sample, equivalent to 85 mg. of terpin hydrate, into a 25-ml. volumetric flask. Dilute to volume with absolute ethanol and mix thoroughly.

Into a small glass-stoppered flask, pipet 1 ml. of the above prepared sample solution and 1 ml. of the biphenyl solution (internal standard solution). Stopper and mix the solutions thoroughly (working sample solution).

Chromatograph this mixture in duplicate as described in the above procedure. After the elution of the terpin hydrate, the chromatogram is allowed to run for an additional 10 min. to allow for the elution of glycerol, if present, which has an approximate retention time of 12 min. When the glycerol peak has been completely eluted, the column is ready for another run.

Calculations—Assay for terpin hydrate in the elixir.

mg. of terpin hydrate per 5 ml. of elixir =

$$\frac{Ths}{Bps} \times \frac{Bp}{Th} \times 85 \text{ mg.}$$

Ths = peak area of terpin hydrate in sample,
Bps = peak area of biphenyl in sample,
Bp = peak area of biphenyl in standard,
Th = peak area of terpin hydrate in standard,
85 = mg. of terpin hydrate.

RESULTS AND DISCUSSION

Although daily conditioning of the column was found to be unnecessary, it is not advisable to use the first run for quantitative information. This initial run should be used to determine instrument sensitivity and retention data.

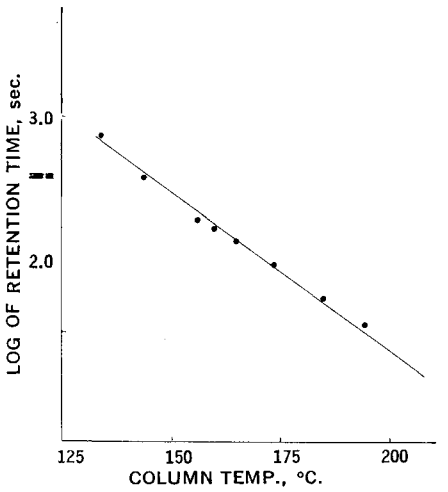

Fig. 2—Column temperature versus *retention time for terpin hydrate.*

As is well known, insufficiently loaded columns may cause tailing due to adsorptive effects which result from incomplete coverage of the support by the liquid phase. If adsorption occurs, quantitative work is considerably hampered. The column used in this study contained a high ratio of liquid phase to inert support in order to minimize any adsorptive effects.

An attempt was made to utilize standard copper tubing for the column, however, the erratic results obtained were indicative of possible decomposition due to catalytic effects. A Pyrex column therefore was used in all experiments.

The detector response for approximately 3.4 mcg. of terpin hydrate and 2.2 mcg. of biphenyl with the change in hydrogen flow rate, at constant air and carrier gas was obtained. The optimum flow ratio of hydrogen to carrier gas was 1:2.

TABLE I—ANALYSIS OF PRODUCTS CONTAINING TERPIN HYDRATE AND RECOVERY DATA

	Terpin Hydrate, mg./5 ml.			
	Theoret.	Added	Found	
Sample 1				
Elixir terpin hydrate placebo	...	...	Av. of 2 detn.	91.2
	...	90.7		101%
Sample 2				
Elixir terpin hydrate	85.0	...	Av. of 2 detn.	82.4
				97%
	85.0	18.8	Av. of 2 detn.	103.3
				100%
Sample 3				
Elixir terpin hydrate with dextromethorphan hydrobromide	85.0	...	Av. of 2 detn.	85.3
				100%
	85.0	15.8	Av. of 2 detn.	98.2
				97%
Sample 4				
Elixir terpin hydrate with codeine	85.0	...	Av. of 4 detn.	82.0
				97%
	85.0	13.5	Av. of 10 detn.	97.7
				99%
Sample 5				
Elixir terpin hydrate with dextromethorphan hydrobromide	50.0	...	Av. of 6 detn.	50.0
				100%
	50.0	11.2	Av. of 4 detn.	59.6
				97%

The detector response with varying concentrations of terpin hydrate and biphenyl followed a linear relationship and the minimum measurable quantity was 5 nanograms.

The effect of column temperature on retention time was investigated and a linear relationship obtained (Fig. 2). The equation calculated by the method of least squares is $\log t_r = -0.014\ T_c + 4.7$, where t_r = retention time in seconds and T_c is the column temperature in °C. A plot of the log of the retention volume *versus* $1/T$, where T = absolute temperature, also yielded a linear relationship. The apparent ΔH_s was calculated from the equation $\log V_R = -\Delta H_s/2.3RT + C$ and found to be −12.4 Kcal./mole, where the slope of the resulting straight line was determined by the method of least squares.

A number of products containing terpin hydrate was assayed. The results and recovery data are presented in Table I. The precision is ±3%.

Under the conditions described, no interference in the analysis of the samples was encountered. Apparently dextromethorphan hydrobromide and codeine were not eluted, or were in too low a concentration to result in a measurable detector response. However, it should be pointed out that measurable interference due to glycerol will occur unless complete elution of this compound from the column is carried out prior to subsequent injections. The retention time of glycerol is approximately 12 min. Continued use of the column will result in the build-up of sugar which tends to skew the elution peaks and decrease the precision of the method. When this occurs, the column should be replaced.

SUMMARY

A gas–liquid chromatographic method for the determination of terpin hydrate in elixirs has been developed. The procedure involves diluting the sample with ethanol, adding an internal standard, biphenyl, and chromatography.

The precision of the developed method was ±3% and duplicate analyses can be completed within 1 hr.

Detection levels, interferences, and recovery data are presented.

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Keyphrases

Terpin hydrate elixirs
Codeine terpin hydrate elixir—analysis
Dextromethorphan terpin hydrate hydrobromide elixir—analysis
GLC analysis

Technical Articles

Unit Tablet Assay of 17α-Ethynylestradiol-3-methyl Ether (Mestranol) by a Direct Colorimetric and an Automated Fluorometric Method

By J. P. COMER, P. HARTSAW, and C. E. STEVENSON

Unit tablet assays of 17α-ethynylestradiol-3-methyl ether (mestranol) were obtained with optimum precision and accuracy by direct dissolution of sample tablets and standardized reference tablets in sulfuric acid–methanol (70:30) reagent. An automated method which utilized basic automatic analyzer (AutoAnalyzer) components and a Turner fluorometer was more rapid but less precise than the direct method.

THE ASSAY of low dosage combination steroid tablets has been a challenge to several analysts. Gänshirt and Polderman (1) separated ethinyl estradiol from tablet excipients and decomposition products by thin-layer chromatography (TLC), and measured the color formed with sulfuric acid–water (80:20) with a relative standard deviation (RSD) of ±3.2%. Comer (2) mentioned the utility of the sulfuric acid–methanol (70:30) reagent for the determination

Received April 14, 1967, from the Analytical Development Department, Eli Lilly and Co., Indianapolis, IN 46206
Accepted for publication August 30, 1967.
Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

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of 17α-ethynylestradiol-3-methyl ether (mestranol) in combination with 6-chloro-6-dehydro-17-α-acetoxy progesterone (chlormadinone acetate). The reagent had been used for several years for routine control assays of mestranol.

Schultz (3) compared a gas chromatography (GC) method and a TLC method for mestranol in the presence of chlormadinone acetate. Recoveries of 96.4 and 98.2% with a 1.5-hr. assay time by GC and 95.5% with a 3–4 hr. assay time for TLC were reported on tablets containing 80 mcg. of mestranol. The mestranol, separated by TLC, was measured at 278 mμ. Boughton *et al.* (4) formed the trimethylsilyl ethers of ethinyl estradiol and 17-β-hydroxy-6-α-methyl-17-(1-propynyl]-androst-4-en-3-one and separated them by GC. An accuracy of 98% with RSD of ±8.6% was reported for 100-mcg. ethinyl estradiol tablets by the GC method.

Heusser (5) separated mestranol and chlormadinone acetate on Silica Gel G using ether–cyclohexane (8:2) as the solvent. After extraction of the silica gel the mestranol was determined colorimetrically with 1.5% titanium chloride in sulfuric acid solution. Using composite samples of 20 tablets containing 100 mcg. of mestranol, Heusser obtained an accuracy of 97.9% and a RSD of ±4.2% from 20 determinations. Tsilifonis and Chafetz (6) reported on a similar method for ethinyl estradiol. Cali and Khoury (7) then presented their studies on an automated method for ethinyl estradiol and mestranol. This paper presents a direct method and an automated method which offered advantages in economy, precision, and accuracy over previous methods for unit tablet assays.

EXPERIMENTAL

Direct Method—Many problems normally involved in the quantitative measurement of microgram amounts of tablet ingredients were simplified by the utilization of the properties of a simple tablet formulation of the two steroids with sodium bicarbonate. During the assay development, it was found that when unit tablets were ground prior to sampling, adsorption of mestranol to the grinding vessel became a significant error. Dissolution of the whole tablet in an aqueous solution and subsequent addition of the color reagent resulted in considerable retardation of color formation. The dissolution of the whole tablet directly in the color reagent was achieved. However, quantitation by the addition of mestranol standards dissolved in methanol to the color reagent proved unsuccessful due to a dilution of the color reagent and the change in the sulfuric acid–methanol concentrations. Color development of dry residue mestranol standards gave erratic results. Quantitation was obtained when a tablet of known concentration was dissolved under the same conditions as sample tablets.

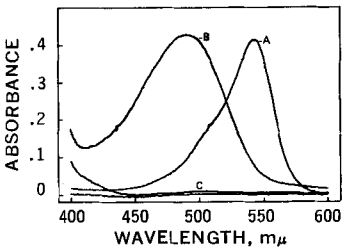

Fig. 1—Spectra in sulfuric acid–methanol (70:30) reagent. Key: A, *mestranol tablet at 5° for 2 hr.;* B, *chlormadinone acetate tablet at 25° for 2 hr.;* C, *chlormadinone acetate tablet at 5° for 2 hr.*

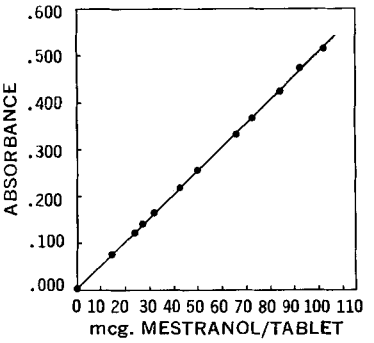

Fig. 2—Absorbance versus *concentration.*

The method thus evolved in which a weighed single tablet is placed into a 25-ml. conical flask containing 20 ml. of the sulfuric acid–methanol (70:30) color reagent which is situated in an ice bath equipped with a magnetic stirring assembly. The solution is stirred with a magnetic stirring bar, and the mestranol color developed simultaneously as the tablet dissolves. The absorbance of the clear solution containing the completely reacted tablet is then measured at 545 mμ *versus* a reagent blank in 5-mm. cells. The spectrum is shown in curve A of Fig. 1. When the color reaction is carried out in the presence of chlormadinone acetate, the mestranol color development process must be carried out at a reduced temperature or a positive interference occurs due to a reaction of chlormadinone acetate with the color reagent. Spectra B and C show the interference of chlormadinone acetate at 25° and 5°.

The linearity of the color reaction was determined by placing weighed portions of reference tablets into the color reagent. The tablets had previously been standardized in mcg. steroid/Gm. by a manual multiple chloroform extraction technique performed by several analysts on several days. The data in Fig. 2 illustrate the conformance to Beer's law. The RSD of the dozen replicates listed in Table I was 1.05% including tablet heterogeneity.

For routine control purposes, nine sample tablets and three reference tablets are run simultaneously in a special stirrer—ice bath apparatus illustrated in Fig. 3. The stirrers are a series of 12 small clock

TABLE I—PRECISION DATA ON UNIT TABLET ASSAYS

Colorimetric Method Abs./Gm. Tablet	Automated Method Peak Ht./Gm. Tablet
3.45	3.93
3.46	3.85
3.49	3.84
3.49	3.88
3.46	4.00
3.52	3.96
3.52	3.86
3.49	3.95
3.42	3.88
3.49	3.85
3.40	3.90
3.46	3.91
Av. 3.47	Av. 3.90
RSD 1.05%	RSD 1.30%

Fig. 3—Magnetic stirrer–ice bath apparatus.

motors with attached magnets. The motors are mounted on the bottom of an inverted aluminum cake pan. Nonmagnetic metal grids were placed in an upright pan to separate the flasks (8). The individual tablet weights and absorbance values are submitted to a computer and various parameters printed out. The data in Table II are a condensation of computer information from several of these nine tablet replicates on various lots of tablets fron export areas. These are expressed in terms of the RSD of mcg. of mestranol/Gm. tablet to remove variance of tablet weight. The comparison statistic is the assay value of a conventional extraction assay on a composite of 10 tablets. The direct method described yielded about six samples/hour/analyst counting reagent preparation and clean-up time and produced the optimum accuracy and precision over many other methods evaluated for unit tablet assay for control purposes.

Automated Method—An automated procedure was desired that would minimize variation and permit a greater number of unit tablet assays. Cali and Khoury (7) outlined an automated fluorometric assay using 90% sulfuric acid. Jakovljevic (9) had previously utilized the author's color reagent (70:30) for the fluorometric assay of mestranol in urine.

The excitation spectrum at an emission of 556 mμ and the emission spectrum at an excitation of 526 mμ for the automated sulfuric acid reagent for mestranol are shown in Fig. 4. This method utilizes basic automatic analyzer[1] components and a Turner model 111 fluorometer with a flow cell door conversion kit.[2]

The description below follows the flow diagram in Fig. 5. A weighed tablet is dropped into the blender of the Solid prep unit,[2] and 120 ml. diluent [H_2O–denatured alcohol (100 parts alcohol + 5 parts methanol) 85:15] are added. Dissolution of the tablet in the blender occurs, and an air segmented sample is withdrawn. The air is then debubbled at the A_2 glass fitting. An unsegmented portion of the sample stream is pumped to a 0.038 in. (0.965 mm.) i.d. Teflon mixing coil where it is segmented into a stream of ethylene dichloride. This coil was described by Kuzel (10). It consists of 36 loops of Teflon tubing wound around an empty chart paper roller. After leaving the first Teflon extraction coil, the aqueous portion is desegmented from the ethylene dichloride stream by pumping all of the aqueous phase along with a portion of the ethylene dichloride to waste from a C_2 glass fitting. The remainder of the ethylene dichloride is forced by difference into a 36 loop 0.053 in. (1.35 mm.) i.d. Teflon mixing coil where it is segmented into a stream of H_2SO_4. Upon leaving the second Teflon extraction coil, the ethylene dichloride is desegmented from the H_2SO_4 stream in a modified fluorometric flow cell and the fluorescence of the H_2SO_4 is measured in the Turner instrument. The shape of this flow cell is indicated in the flow diagram. The system operates at a rate of 20 determinations/hour.

Interferences—An interesting quality control problem was solved by a study of interference on the color reaction. A lot of tablets from a foreign production facility assayed low by the direct colorimetric method but assayed correctly by the manual extraction procedure. The actual raw material excipients used in the lot, or water up to 5%, in the tablets did not retard the color reaction. Sulfite, thiosulfate, phosphate, chloride, bicarbonate, and sodium ions alone in small amounts caused no problems. Exhaustive analyses of the tablets for many components showed no significant difference over normal tablets except for nitrate content. Normal tablet values were less than 1 p.p.m. nitrate, and the troublesome lot 6 p.p.m. Experiments were made to evaluate the effect of nitrate. Aliquots of $NaNO_3$ were evaporated along with solutions containing 80 mcg. of mestranol or ethinyl estradiol. Bicarbonate and color reagents were added and the color developed for 1 hr. The effect of nitrate on fluorescence on 1 mcg. of mestranol was studied in a similar manner excluding the bicarbonate. Fluorescence measurements were made on a model 110 Turner fluorophotometer using No. 58 Kodak Wratten and 1-60 Corning primary filter and a No. 22 Kodak Wratten secondary filter. Since the retardation of the reactions by nitrate was confirmed, the search for its source in the tablet was continued.

Nitrate was not observed in any of the tablet raw materials. The source of nitrate was found to be the glassine paper used in manufacturing at the foreign facility. Typical assays for nitrate for foreign glassine paper were 18–21 p.p.m. and for domestic glassine paper 0.1 p.p.m. The colorimetric nitrate assays (11) were confirmed by a

[1] AutoAnalyzer, Technicon Corp., Chauncey, N. Y.
[2] Technicon Corp., Chauncey, N. Y.

TABLE II—DATA ON ROUTINE CONTROL SAMPLES

Lot	Tablets, Mestranol: Direct Method RSD[c]	Tablets, Mestranol: Av. mcg./Gm.	Tablets, Mestranol: Manual Method,[b] mcg./Gm.	Tablets Mestranol Plus Chlormadinone OAc: Direct Method[a] RSD[c]	Tablets Mestranol Plus Chlormadinone OAc: Av. mcg./Gm.	Tablets Mestranol Plus Chlormadinone OAc: Manual Method,[b] mcg./Gm.
1	.628%	750	758	.301	781	778
2	.527	773	776	.631	772	768
3	.780	798	802	.366	772	765
4	.795	786	798	.400	763	780
5	.219	805	801	.212	768	768

[a] Data from 9 tablet replicates using 3 standardized tablets as reference. [b] Data from a single assay on a 10-tablet composit sample. [c] Relative standard deviation.

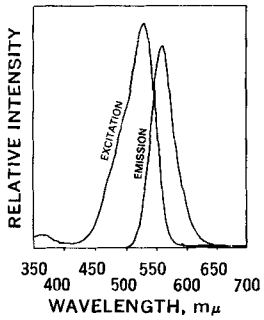

Fig. 4—Excitation and emission spectra.

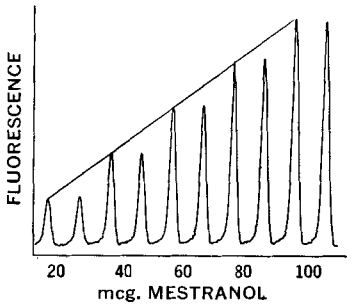

Fig. 6—Peak height versus *concentration for automated assay.*

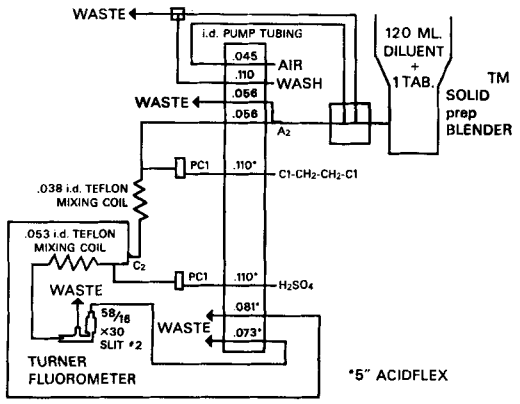

Fig. 5—Flow chart for automated assay.

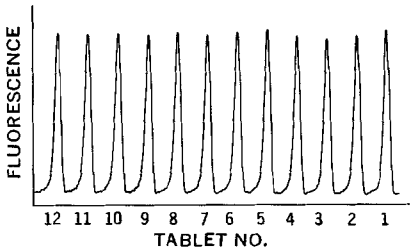

Fig. 7—Precision of automated assay.

polarographic procedure (12). When the source of nitrate was removed, the assay results returned to normal values. Hydrogen peroxide and nitrites also inhibited color formation but these were not found in tablets. Related steroids such as estradiol methyl ether, estrone methyl ether, and estrone produced insignificant positive interference when added in amounts up to 500 mcg. per tablet.

RESULTS AND DISCUSSION

The direct colorimetric method for mestranol using a reference standard tablet has proved to be a very precise and accurate method for routine control (Table II) with a relative standard deviation often less than 1% including tablet heterogeneity but excluding tablet weight variance. The interference of chlormadinone acetate is insignificant if the temperature is held below 5°. The use of the special stirrer shown in Fig. 3 allowed a multiple assay at a rate of about six samples/hour/analyst.

The fluorescence of mestranol in sulfuric acid which is more sensitive than the color reaction was used as the means of measurement for automation. The interferences caused by nitrate by direct dissolution of the tablet led to the solvent extraction scheme as outlined in the flow diagram of the automated method. The relative fluorescence intensity was found to have a linear relationship to mestranol concentration as illustrated by the straight line connecting the peak heights in Fig. 6. The mestranol was added in solution in varying amounts to the sample cups. A run of 12 reference standard tablets, shown in Fig. 7, produced a RSD of 1.3% including tablet heterogeneity. These values are listed in Table I.

The retardation of the color and fluorescence reaction if nitrate is present is significant in con-

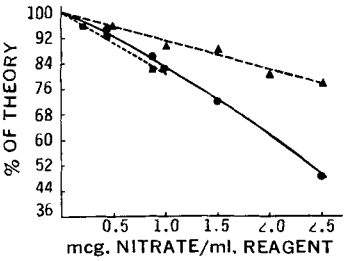

Fig. 8—Effect of nitrate on reactions. Key: ▲, mestranol fluorometric assay; ●, mestranol colorimetric assay; ■, ethinyl estradiol colorimetric.

centrations above 0.1 mcg./ml. of reagent as shown in Fig. 8.

SUMMARY

The utility of H_2SO_4 to produce color or fluorescence of α-ethinyl estradiol and derivatives has been established by several authors (1, 2, 5–7). When used in conjunction with a standardized reference tablet, the method yields optimum precision and accuracy for mestranol. The fluorescence measurement is more sensitive and more suitable for automation but less precise than the color measurement. Nitrate, nitrite, and hydrogen peroxide retard the reaction with sulfuric acid.

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(12) Frischmann, J., Analytical Development Department, Eli Lilly and Co., Indianapolis, Ind., unpublished data.

Keyphrases

17α-Ethynylestradiol-3-methyl ether (mestranol) tablets
Colorimetric analysis
Sulfuric acid–methanol color reagent
Automated fluorometric analysis
Fluorescence produced by sulfuric acid
Nitrate effect on analysis accuracy

Notes

Preparation of Some Phenyl Pyridyl Ethers with Antifungal and Antibacterial Properties

By RICHARD O. MUHLHAUSER* and EUGENE C. JORGENSEN†

2-Methyl-4-chlorophenyl-4′-pyridyl ether (II) and 2-chlorophenyl-4′-pyridyl ether (III) were prepared by condensation of *N*-pyridyl-4-pyridinium chloride hydrochloride (I) with appropriate phenols. These compounds were found to be effective as antifungal agents but were less effective as antibacterial agents. Compound II had the greatest antifungal activity and the least toxicity.

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Nuclear magnetic resonance and infrared spectral studies supported the structures assigned to com-

Received May 11, 1967, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94122
Accepted for publication, August 30, 1967.
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The authors thank Drs. Nancy C. Hall and Joseph Z. Krezanoski, Barnes-Hind Laboratories, Sunnyvale, Calif., for the assistance in obtaining the antifungal results shown in Table III.
This investigation was supported by grants AM 04223 and PHS 5 TI GM 0728 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
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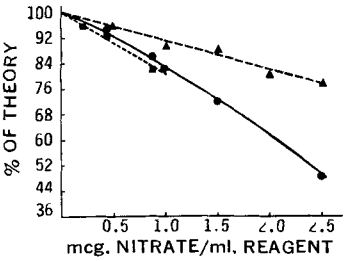

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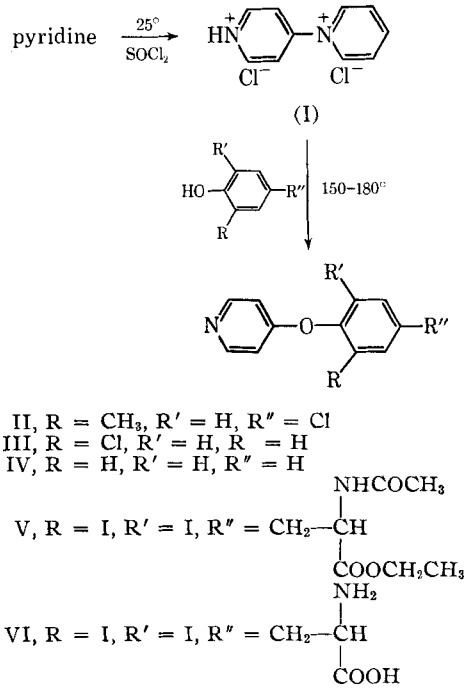


II, R = CH_3, R′ = H, R″ = Cl
III, R = Cl, R′ = H, R = H
IV, R = H, R′ = H, R″ = H

V, R = I, R′ = I, R″ = CH_2—CH(NHCOCH$_3$)—COOCH$_2$CH$_3$

VI, R = I, R′ = I, R″ = CH_2—CH(NH_2)—COOH

Scheme I

pounds II-VI. In the case of compound V, the NMR spectrum was correlated with spectra obtained for substituted thyronines by Lehman and Jorgensen (3). A broad peak at approximately 8.5 p.p.m. was assigned to the α protons adjacent to the pyridyl nitrogen of V (4–5).

Antimicrobial and antifungal evaluation of compounds II and III was performed by the small-tube screening procedures of Catalfomo and Schultz (8) and by the paper disk diffusion method (9).

The data in Tables II and III illustrate the activity of these compounds against representative fungi and bacteria.

These results agree with those obtained by Hata *et al.* (10) and Tomita *et al.* (11), and suggest that both the pyridyl ring and the phenolic ether are operative in producing the antifungal activity.

Results from the screening tests showed that compound II completely inhibited the growth of *T. mentagrophytes* at a concentration of 0.142 mmoles/L. (31.2 mcg./ml.). Partial inhibition was obtained at a concentration of 0.045 mmoles/L. (10 mcg./ml.). Undecylenic acid at a concentration of 0.054 mmoles/L. (10 mcg./ml.) proved less effective.

Evaluation of antifungal activity by the paper disk method (9) showed that a concentration of 2 mg./ml. of II completely inhibited the growth of several other fungal species. Results obtained by this method are shown in Table III.

Compound II had no apparent effect on the mean survival time of mice infected with *Plasmodium berghei*, whereas compound III significantly decreased the life span of these mice. This indicates that compound II, the more effective antifungal agent, is the less toxic of the pair of compounds tested.

Compounds V and VI were synthesized for testing as possible thyromimetic compounds and were not tested for antifungal activity. They were found to be inactive as thyromimetics by the rat antigoiter assay method (12).

EXPERIMENTAL

All melting points were taken on a Thomas-Hoover melting point apparatus and are uncorrected. The infrared spectra were determined with a Beckmann IR5 spectrophotometer by suspending the solid materials in potassium bromide disks. Nuclear magnetic resonance spectra were determined in deuterochloroform with tetramethylsilane as an internal standard. Microanalyses were performed by the Microanalytical Laboratory, University of California, Berkely, Calif.

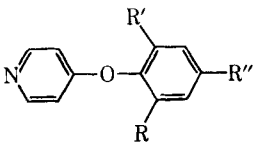
Substituted Phenyl Pyridyl Ethers (Table I; II–IV) General Procedure—A mixture of *N*-pyridyl-4-pyridinium chloride hydrochloride (1) (I, 15.0 Gm., 0.0645 mole) and the appropriate phenol (0.071 mole) was heated at 160–165° in an oil bath, forming a clear melt within a few minutes. On cooling, the residue was treated with 10% sodium carbonate solution and the dark-colored upper layer was removed and taken up in chloroform. The chloroform solution was extracted twice with 2 *N* sodium hydroxide solution (400 ml. total) and with water (800 ml.) and dried over sodium sulfate. After filtration, the chloroform was removed *in vacuo*, and the oil was purified by washing it several times with water (to remove pyridine) followed by distillation *in vacuo*. The clear distillates obtained crystallized after standing at room temperature for a few days. Pertinent data are listed in Table I.

N-Acetyl-3,5-diiodo-4-(4′-pyridinoxy) Phenyl-L-alanine (V)—Prepared from I (5 Gm., 0.0215 mole) and *N*-acetyl-3,5-diiodo-L-tyrosine ethyl ester (13) (11.92 Gm., 0.0237 mole). The mixture was fused at 165–180° as described above. The cooled, brittle, black solid was stirred with a hot solution of sodium carbonate (250 ml.) and the suspension was allowed to stand overnight. The next day, the polymeric-like suspension was treated again with hot sodium carbonate solution and insoluble material was collected by filtration. The gummy solid was dissolved in acetone, treated three times with decolorizing carbon,[1] and filtered. The solvent was concentrated and water added. A buff-white solid, m.p. 169-170°, was obtained. The product was recrystallized from acetone–water to give a white solid, m.p. 169-171°. A mixed melting point determination with *N*-acetyl-3,5-diiodo-L-tyrosine ethyl ester gave a depression in melting point. The solid was chromatographically homogeneous on Silica Gel H with an R_f = 0.75 (ethyl acetate); $\bar{\nu}_{max.}$ in cm.$^{-1}$, 1257 (s), 1269 (s) (ether), 1669 (s) (amide), 1751 (vs) (ester), 3349 (m); NMR peaks at δ = 8.5 p.p.m. (broad) (α protons), δ = 7.65 p.p.m. (2,6 protons), δ = 6.7 p.p.m. (β protons).

3,5-Diiodo-4-(4′-pyridinoxy) Phenyl-L-alanine (VI)—Prepared from compound V by acid hydrolysis. A mixture of V (0.736 Gm., 0.00127 mole), glacial acetic acid (24.5 ml.), and concentrated hydrochloric acid (16.36 ml.) was heated at reflux temperature for 3.5 hr. according to the method of Jorgensen and Slade (12). Additional

[1] Norit.

TABLE I—COMPOUNDS SYNTHESIZED



Compd.	R	R′	R″	M.p., °C.	Formula	Anal., % Calcd.	Found
II	CH_3	H	Cl	53–55°	$C_{12}H_{10}ClNO$	C, 65.61 H, 4.59 N, 6.38	65.42 4.51 6.56
III[a]	Cl	H	H	54–55	$C_{11}H_8ClNO$		
IV[b,c]	H	H	H	48	$C_{11}H_9NO$		
V	I	I	CH_2—CH(NHCOCH$_3$)—COOCH$_2$CH$_3$	169–171	$C_{18}H_{18}I_2N_2O_4$	C, 37.26 H, 3.13 N, 4.83	37.33 3.33 4.80
VI	I	I	CH_2—CH(NH_2)—COOH	216–217.5	$C_{14}H_{12}I_2N_2O_3$	C, 32.97 H, 2.37 I, 49.76	32.73 2.35 49.98

[a] Lit. (2) m.p. 54–55°. [b] Lit. (6) m.p. 44°. [c] Lit. (7) m.p. 48°.

TABLE II—ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF PHENYL PYRIDYL ETHERS

Compd.	Concn., mmoles/L.	*T. mentagrophytes* 7 Day	*T. mentagrophytes* 14 Day	*Botrytis cinerea* 7 Day	*Botrytis cinerea* 14 Day	*Staphylococcus aureus* 48 hr.	*Escherichia coli* 48 hr.
II[a]	1.14[b]	—[c]	—	—	—	—	—
	0.57	—	—	—	—	4+	4+
	0.285	—	—	1+	2+	4+	4+
	0.228	—	—				
	0.142	+—	—	3+	4+		
	0.071	1+	2+				
	0.045	1+	4+				
III[d]	2.42	—	—	—	—	—	—
	1.21	—	—	—	—	4+	4+
	0.605	—	—	2+	4+		
	0.302	+—	+—	3+	4+		
	0.150	1+	2+	4+	4+		
Undecylenic acid	0.54	—	—				
	0.27	—	—				
	0.054	3+	4+				
	0.027	3+	4+				
	0.00 (Alcohol control)	3+	4+				
Griseofulvin (in dimethylformamide plus buffer)[e]	0.142	—	—				
	0.0284	+—	4+				
	0.0142	1+	4+				
	0.000 (DMF-buffer control)	3+	4+				

[a] 2-Methyl-4-chlorophenyl-4′-pyridyl ether. [b] Alcohol 5% or less plus organism. [c] +, growth; —, no growth; +—, uncertain growth. [d] 2-Chlorophenyl-4′-pyridyl ether. [e] Dimethylformamide plus buffer 5% or less, plus a DMF control plus sterility control.

hydrochloric acid (6 ml.) was added at the end of 1 hr. and again at the end of 2.5 hr. Additional glacial acetic acid (6 ml.) was added at the end of 2.5 hr. The cooled solution was filtered and the solvents removed *in vacuo*. The residue was dissolved in hot ethanol (50 ml.) and water (50 ml.) was added. After filtration, the pH was adjusted to 2.0; on cooling, crystals deposited. The crystals were redissolved by heating, and a hot 10% sodium acetate solution was added to bring the pH to 5.0. The solution was cooled and the precipitated white solid collected by filtration and dried; m.p. 229–231° dec. Two more isoelectric precipitations gave 0.38 Gm. (58% yield) of a white solid, m.p. 216–217.5° dec.; $\bar{\nu}_{max.}$ 820 (m), 860 (m), 1200 (ms), 1260 (m), 1400 (s), 1590 (s), 2800–3100 (b) (amino acid). Thin-layer chromatography on Silica Gel H using a solvent system of butanol–acetic acid–water

TABLE III—ANTIFUNGAL ACTIVITY OF 2-METHYL-4-CHLOROPHENYL-4′-PYRIDYL ETHER (II) AGAINST TEST ORGANISMS

	Observations: 7 Days	Control	Ethyl Alcohol	1/100	1/500
109	*Epidermophyton floccosum*	++[a]	++	++	++
112	*Microsporum audouinii*	++	+++	0	0
113	*Microsporum canis*	++	++	0	0
114	*Microsporum gypseum*	+++	++++	0	++
116	*Trichophyton gallinae*	++++	++++	+	++++
119	*Microsporum tonsurans*	++	++	0	0
120	*Trichophyton violaceum*	++	++	0	0
121	*Trichophyton mentagrophytes*	+++	+++	+	+++
	13 Days				
109	*Epidermophyton floccosum*	++	+++	+++	++
112	*Microsporum audouinii*	++	+++	0	0?
113	*Microsporum canis*	+++	+++	0	+?
114	*Microsporum gypseum*	+++	+++	0	++
116	*Trichophyton gallinae*	++++	++++	+	++++
119	*Microsporum tonsurans*	+++	+++	0	0
120	*Trichophyton violaceum*	+++	++	0	0
121	*Trichophyton mentagrophytes*	++++	++++	+	+++

[a] +, growth; 0, no growth; 0?, uncertain growth.

(100:30:10) showed a single spot (ninhydrin positive) for the white solid. The starting material (V) was negative to ninhydrin.

Biological Materials and Methods—The fungal species used were *Trichophyton mentagrophytes*, ATCC 9972, and *Botrytis cinerea*. The bacterial species employed were *Escherichia coli* and *Staphylococcus aureus* from the collections maintained at the department of Pharmacognosy, School of Pharmacy, University of California.[2]

The fungal organisms were maintained on Sabouraud dextrose medium[3] (liquid and agar), and the bacteria were maintained on nutrient medium[4] (broth and agar). The liquid cultures also served as a source of inocula.

For studies using *T. mentagrophytes*, the mycelial growth from 5–7-day old stationary cultures was homogenized in a sterile semimicro Waring blender for 30 sec. and added as 0.5 ml. inocula to the surface of an agar slant containing 9.5 ml. of Sabouraud's agar containing the test compounds. The tubes were incubated for 2 weeks at room temperature and growth was recorded at 7-day and 14-day intervals.

For antibacterial studies, actively growing 2–4-day old liquid cultures of *S. aureus* No. 209 P and *E. coli* No. 6522 were used as inocula. A bacterial suspension of 0.5 ml. was added to 9.5 ml. of nutrient broth No. 4A containing the dilutions of the compounds to be tested. The broth cultures were incubated for 48 hr. at 37°, and turbidity was then read to determine growth.

Results of the preliminary antifungal and antibacterial screening tests are shown in Table II.

In all cases, sterility controls were run for each sample of nutrient culture medium and for alcohol controls plus organism. Solutions of the agents to be tested were prepared in 95% ethanol and then diluted. In the case of griseofulvin, the compound was dissolved in dimethylformamide at various dilutions, and these dilutions were again diluted with phosphate buffer.

The second antifungal screening test on compound II was carried out in another laboratory.

Four sterile S and S absorbent disks (12.5 mm.) were placed in four Petri dishes containing Sabouraud's medium. The dishes were labeled as control, ethyl alcohol, $^1/_{100}$, and $^1/_{500}$. Compound II was dissolved in ethanol and dilutions of $^1/_{100}$ and $^1/_{500}$ were prepared. To the disks were added 2 drops of ethanol to one dish, 2 drops of $^1/_{100}$ dilution, 2 drops of $^1/_{500}$, and control was untouched. The disks were allowed to set 30 min. Then each set of disks was inoculated with a single culture of the respective organism and the plate was sealed with 3M Parafilm. The plates were then incubated at room temperature. Observations were taken at the end of 7 and 13 days. Results are shown in Table III.

Antimalarial Test[5]—Compounds II and III were administered subcutaneously in oil to mice at dose levels of 40, 160, and 640 mg./Kg. of body weight. The mice were infected with a lethal dose of *Plasmodium berghei* 3 days prior to administration of test compounds. With compound III, toxic deaths of all test animals were observed at all dose levels. No toxic deaths were observed with compound II, but the mean survival time of 6.2 days was not significantly prolonged.

[2] Obtained through the courtesy of Mrs. Evelyn Johnson and Dr. Robertson Pratt, Department of Pharmacognosy, School of Pharmacy, University of California Medical Center, San Francisco, Calif.

[3] Difco.

[4] Difco No. 4A.

[5] Antimalarial tests were performed at the Walter Reed Army Institute of Research, Washington, D. C.

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Keyphrases

Phenyl ethers—synthesis
Antibacterial activity of phenyl pyridyl ethers
Antifungal activity of phenyl pyridyl ethers
Antimalarial activity of phenyl pyridyl ethers
NMR spectrometry
IR spectrometry–structure
Microanalysis

Analog Computer Simulation of Rheological Systems I Pseudoplastic Flow

By GERALD J. YAKATAN and OSCAR E. ARAUJO

A method for the quantitative characterization of pseudoplastic systems using the analog computer was reported. Three parameters were obtained (one of which can be maintained constant for a given system) which completely describe the shape of rheograms for various CMC mucilages. The method developed can be carried out rapidly, the computer program is relatively simple, and the results should provide an easy means of communication among rheologists desiring to accurately compare experimental data on pseudoplastic systems.

THE IMPORTANCE of pseudoplastic flow in pharmaceutical systems is apparent when one considers that most hydrocolloids and dilute suspensions exhibit this rheological pattern. A large number of pharmaceutical emulsions and semisolid preparations also behave as pseudoplastic systems.

The quantitative characterization of pseudoplasticity has been a difficult problem for the rheologist. The application of an empirical exponential equation involving the use of a log-log plot has been commonly accepted (1–3). However, the use of this equation has been questioned by several workers (4).

Other investigators have proposed various equations with a theoretical basis, but the involved calculations necessary to obtain the constant described considerably reduced their practical value (5–7).

In 1961 Shangraw *et al.* (8) proposed another equation in an attempt to provide a much better representation of non-Newtonian systems. The equation is:

$$F = f + \eta_\alpha S - b_\gamma e^{-aS} \qquad \text{(Eq. 1)}$$

where F is the shearing stress, S is the rate of shear, and f, η_α, a, and b_γ are constants characteristic of a particular system.

The problems involved in adequately characterizing pseudoplastic flow systems seemed to offer a challenging area of research. The concept of developing an equation with a minimum number of parameters which would completely characterize all pseudoplastic systems was an interesting one. It was also felt that the analog computer would be of great assistance in solving these problems.

A typical rheogram of a pseudoplastic substance appeared to be composed of a "first-order" and a "zero-order" segment. Consequently, a general equation combining the integrated expressions for a zero-order and a first-order process would be (9):

$$y = ax + b(1 - e^{-cx}) \qquad \text{(Eq. 2)}$$

where y and x are variables for the particular system and a, b, and c are constants for the system.

For a specific rheological system, Eq. 2 becomes:

$$F = aS + b(1 - e^{-cS}) \qquad \text{(Eq. 3)}$$

where F is the shearing stress, S is the rate of shear, and a, b, and c are constants. For purposes of computer programming, F was made directly proportional to voltage and S directly proportional to time, and Eq. 3 becomes:

$$V = at + b(1 - e^{-ct}) \qquad \text{(Eq. 4)}$$

EXPERIMENTAL

The analog computer is rapidly becoming an extremely important tool in aiding the solution of complex scientific and engineering problems. In most modern computers, the continuous variables are d.c. voltages. The electronic analog computer makes possible the building of an electrical system in which d.c. voltages will vary with time in a manner similar to the variable of interest in the actual system under study.

Received May 1, 1967, from the Department of Pharmacy, College of Pharmacy, University of Florida, Gainesville, FL 32601
Accepted for publication September 12, 1967.
The authors are indebted to D. J. Weber for his suggestions and encouragement.
This investigation was supported in part by predoctoral fellowship 5-FI-GM-29,944-02 awarded to G. J. Yakatan from the National Institute of General Medical Sciences, U. S. Public Health Service, Bethesda, Md.

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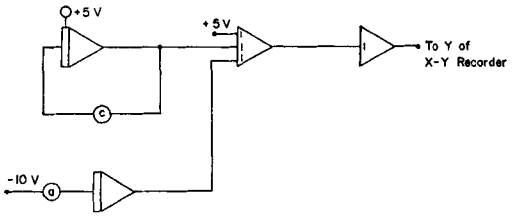

Fig. 1—Analog computer program for simulation of pseudoplastic flow systems.

The analog computer offers a number of distinct advantages for its utilization in the simulation of physical systems. The simple turn of a knob feeds in design parameters. The computer provides an instant-by-instant dynamic picture of each change in a parameter, and each change can be visually compared with any other. Above all, the computer offers simplicity, rapidity, and accuracy in operation.

The present study made use of the Pace TR-10 analog computer[1] and the output from the computer was fed into a Moseley Autograf model 2D-2 X-Y recorder[2] on which the curves were recorded.

The program used to simulate Eq. 4 is shown in Fig. 1. Variable potentiometer settings were used for the parameters a and c. The value of b was maintained constant at +5 v. throughout the study. Plots of shearing stress against rate of shear for pseudoplastic systems pass through the origin. The corresponding computer plot represented by Eq. 4 will also pass through the origin since at $t = 0, v = 0$. Equation 4 was now used to simulate pseudoplastic flow systems by varying two parameters, a and c.

Assuming that pseudoplastic rheograms, obtained by plotting shearing stress *versus* rate of shear, are composed of first-order and zero-order segments, it was deemed advisable to vary one of the two available parameters while holding the other constant. This would show the separate effect of each parameter on the shape of the pseudoplastic curves. The results of this study are shown in Figs. 2 and 3. As expected, the effect of each parameter cannot be separated entirely over the range of values chosen. Nevertheless, an extremely good indication of the effect of varying each parameters was obtained. From these data it seemed probable that by judiciously varying the parameters a and c, rheograms could be drawn which simulate pseudoplastic curves obtained from real systems. To test this theory it was decided to obtain actual experimental data on CMC mucilages known to exhibit pseudoplastic flow.

The rheological measurements were carried out on a Stormer viscosimeter. A weight hanger with various slotted weights provided the shearing stress in Gm. The rate of shear was reported in r.p.m. The CMC mucilages were allowed to equilibrate for 6 hr. after preparation, before any measurements were made. The rheological data, obtained at 20 ± 0.1°, are given in Table I.

Figure 4 shows the plots of the experimental points for the various mucilages and the computer curves which were drawn to fit these points. The values of a and c used to draw the simulated computer curves are listed in Table II, along with the values of a and c for the real systems corrected for scaling factors used by the computer. The values of these parameters were determined in the following manner.

A—The experimentally obtained curves, in Gm. *versus* r.p.m., were plotted on graph paper of the same size as that used to draw Figs. 2 and 3.

B—The plot of the experimentally determined points was superimposed on Fig. 2 to roughly estimate the value of c. This procedure was repeated using Fig. 3 to approximate the value of a.

C—These approximate values of a and c were then fed into the computer by adjusting the appropriate potentiometers, and the resulting curve was drawn by the computer on the graph paper containing the experimental points. The recorder was set so that 1 v. = 1 in. and 1 sec. = 1 in. of chart paper.

D—The first curve drawn by the computer never exactly fits the data. However, from this first approximation, changes were made in the values of a and c until a good fit was obtained. This adjustment was always accomplished within 10 min.

E—Now values of a, b, and c are simply read off the potentiometer settings. These values are the parameters of Eq. 4 and consequently must be corrected to the parameters of Eq. 3, which describes the experimental system.

To correct c, the following equation should be used:

$$c_{corr.} = P \times \frac{M}{R} \qquad \text{(Eq. 5)}$$

where P is the potentiometer setting c in Fig. 1, M is actual machine time at R r.p.m., *i.e.*, if it takes the computer 1 sec. to move 50 r.p.m. on the abscissa then $M/R = 1/50$. To correct a, the following equation should be used:

$$a_{corr.} = P' \times \frac{k}{k'} \qquad \text{(Eq. 6)}$$

where P' is the potentiometer setting a in Fig. 1, k is the proportionality constant relating F to V, and k' is the proportionality constant relating S to t. To correct b, the following equation should be used:

$$b_{corr.} = P'' \times k \qquad \text{(Eq. 7)}$$

where P'' is the potentiometer setting represented by +5 V in Fig. 1, and k is the proportionality constant relating F to V. Thus for the systems investigated $b_{corr.} = 5\ V \times 100 = 500$. The corrected values for a, b, and c may now be substituted into Eq. 3 to give an equation which completely describes the pseudoplastic rheogram.

Since the Stormer viscosimeter was used in the present study, it should be pointed out that the constants obtained by computer fit of these data will be different from those obtained from data accumulated from another viscosimeter. However, the general equation and the computer program are applicable regardless of the instrument employed.

DISCUSSION

The empirical log-log plot frequently used to describe pseudoplastic flow very often does not truly characterize the experimental curve. It is not unusual for this type of plot to produce a great deal of scatter in the points or even another curved line.

[1] Manufactured by Electronics Associates, Inc.
[2] Manufactured by Hewlett-Packard, Inc.

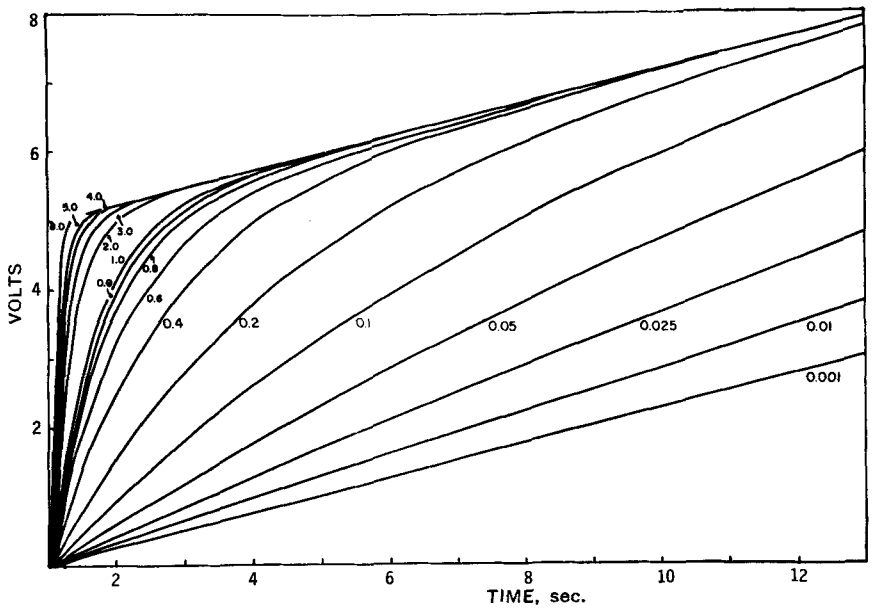

Fig. 2—Effect of variation of c (a = 0.3) *on the shape of pseudoplastic flow curves.*

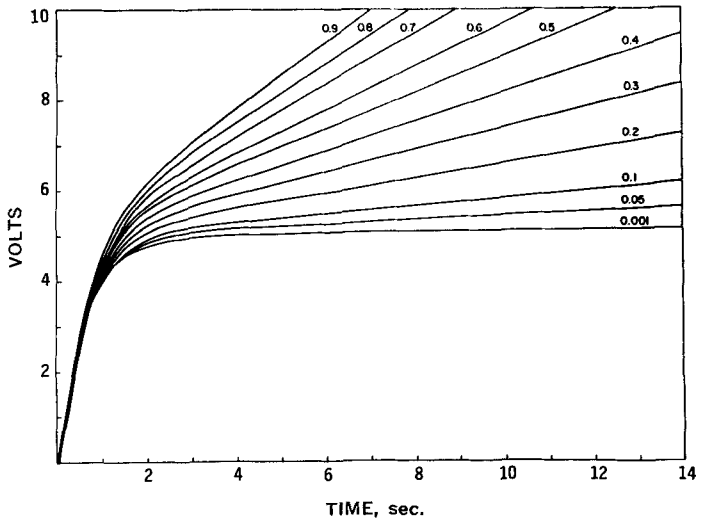

Fig. 3—Effect of variation of a (c = 1.0) *on the shape of pseudoplastic flow curves.*

TABLE I—RHEOLOGICAL DATA FOR VARIOUS CMC MUCILAGES

2.8% w/w		2.2% w/w		1.6% w/w	
Gm.	r.p.m.	Gm.	r.p.m.	Gm.	r.p.m.
250	45.5	100	38.9	40	40.9
300	58.6	120	49.1	50	53.2
350	72.3	150	65.0	60	66.0
400	91.4	175	80.8	80	92.2
500	127	200	94.4	100	120
650	198	250	128	150	195
800	286	300	163	200	286
900	351	400	238	250	373
		500	328		

The slope of this "line" yields a so-called index of pseudoplasticity.

The method proposed in this study offers three parameters to describe the experimental data and characterize the entire flow curve quantitatively. While the approach is still admittedly empirical, no difficulty has been encountered in successfully characterizing any of the experimental data employed. Figure 4 clearly demonstrates that a wide variety of pseudoplastic curves can be accurately simulated.

It is realized that not all rheologists can count an analog computer as one of the research tools commonly at his disposal in the laboratory. Nevertheless, almost every research investigator in rheology, whether in industrial or academic circles, has an analog computer within reasonably easy reach. The computer program presented here is a model of simplicity, mastery of which would require no

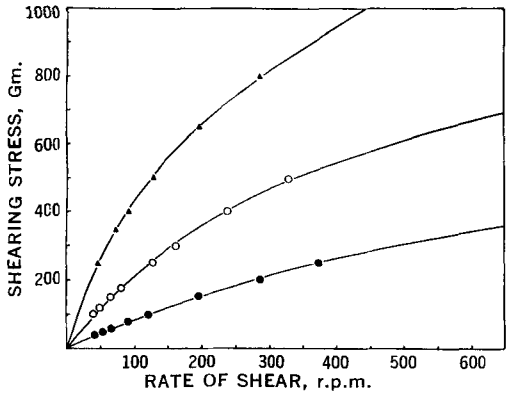

Fig. 4—Computer fits of actual data for CMC flow curves. Computer scale: 1 v. = 100 Gm. and 1 sec. = 50 r.p.m Lines represent computer fit and points represent the actual rheological data. Key: ▲, 2.8%; ○, 2.2%; ●, 1.6%.

TABLE II—VALUES OF *a* AND *c* OBTAINED FROM COMPUTER DATA TO FIT VARIOUS CMC RHEOGRAMS

% w/w CMC	*a*	*c*	$a_{corr.}$	$c_{corr.}$
2.8	0.55	0.55	1.10	1.10×10^{-2}
2.2	0.165	0.225	0.33	4.5×10^{-3}
1.6	0.01	0.09	0.02	1.8×10^{-3}

more than 30 min. instruction even to those completely alien to computer programming.

In summary, this paper has presented a method for the quantitative determination of three parameters which completely characterize the shape of pseudoplastic flow curves. Evidence has been presented regarding the general applicability of the method by making use of actual experimental data. The method is rapid and avoids any extensive calculations. It is hoped that the use of this procedure will lead to a common means of communication among rheologists, working with pseudoplastic systems, who wish to compare experimental data. It is also possible that with the aid of the analog computer some new light may be thrown on the mechanism of pseudoplastic flow. There is a distinct possibility that the same type of treatment presented here may be applied to other rheological systems. Work in these areas is being pursued at the present time.

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Keyphrases

Rheology
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FISH ARE widely used for experimental purposes and for biological assays and have been used for a variety of biological studies including outstanding works in experimental embryology, endocrinology, and nerve physiology (1). The gross anatomy and physiology of fish are comparable to that of mammals, and fish contract many of the same diseases as do mammals. Fish have become popular as experimental tools because they are relatively inexpensive and easily kept in the laboratory.

The use of fish in drug absorption studies has led to the development of a theory by Levy and Gucinski (2) describing the uptake of drugs from a bathing solution by goldfish. The theory describes the uptake of drugs by fish under appropriate conditions as a simple diffusion process dependent upon the concentration of the drug in the external bathing solution.

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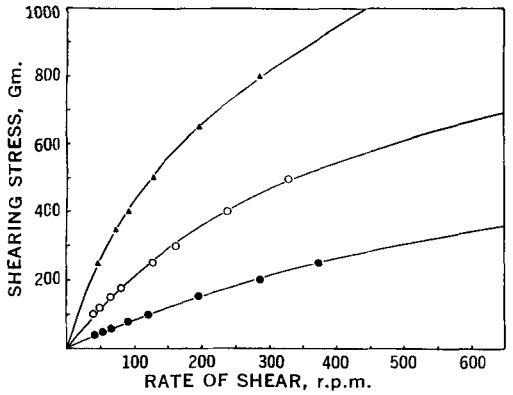

Fig. 4—Computer fits of actual data for CMC flow curves. Computer scale: 1 v. = 100 Gm. and 1 sec. = 50 r.p.m Lines represent computer fit and points represent the actual rheological data. Key: ▲, 2.8%; ○, 2.2%; ●, 1.6%.

TABLE II—VALUES OF *a* AND *c* OBTAINED FROM COMPUTER DATA TO FIT VARIOUS CMC RHEOGRAMS

% w/w CMC	*a*	*c*	$a_{corr.}$	$c_{corr.}$
2.8	0.55	0.55	1.10	1.10×10^{-2}
2.2	0.165	0.225	0.33	4.5×10^{-3}
1.6	0.01	0.09	0.02	1.8×10^{-3}

more than 30 min. instruction even to those completely alien to computer programming.

In summary, this paper has presented a method for the quantitative determination of three parameters which completely characterize the shape of pseudoplastic flow curves. Evidence has been presented regarding the general applicability of the method by making use of actual experimental data. The method is rapid and avoids any extensive calculations. It is hoped that the use of this procedure will lead to a common means of communication among rheologists, working with pseudoplastic systems, who wish to compare experimental data. It is also possible that with the aid of the analog computer some new light may be thrown on the mechanism of pseudoplastic flow. There is a distinct possibility that the same type of treatment presented here may be applied to other rheological systems. Work in these areas is being pursued at the present time.

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The uptake of a weak acid over a pH range encompassing the pKa of the drug has been studied in goldfish and was shown to be consistent with the pH- partition theory. In their studies Levy and Gucinski have shown that the uptake of a nonelectrolyte, ethanol, by goldfish does not depend upon the pH of the external medium.

Levy's theory for goldfish has been applied to the common guppy (*Lebistes reticulatus*) (3). The uptake of ethanol was found to conform to the theory within certain concentration limits. The guppy appeared to show promise as a suitable animal for drug absorption studies. Guppies previously have been used as experimental animals in many types of toxicity studies (4).

The effect of pH on the rate of absorption of nonelectrolytes has not been examined in detail. A variable pH should have no profound effect on the physical characteristics of a nonelectrolyte, but absorbing membranes might be sensitive to these changes. Membranes generally are believed to possess pores, allowing for the diffusion of small molecules through the membrane without entering the lipid phase. Kavanau (5) has proposed a mechanism whereby the diameter of the pores changes between open and closed configurations. In his theory the transformation is mediated by proton activity. It is possible then that varying pH could significantly alter the rate of absorption of a neutral molecule by causing a structural change in the absorbing membrane. The study reported here was undertaken to find what effect pH variability might have on the rate of absorption of a neutral molecule in guppies and goldfish.

EXPERIMENTAL

To investigate the effect of varying pH upon the absorption of ethanol through intact membranes both the guppy and the goldfish were used. The time for a pharmacological effect to occur has been shown previously to be a function of the rate of absorption (2, 3). A change in the time required for the effect to occur at constant ethanol concentration was considered to reflect a change in the absorption rate.

Two different pharmacological effects were used as end points—overturn (loss of equilibrium) and death (cessation of gill and mouth movement). The times required to attain the end point from the immersion were termed "overturn time" and "survival time," respectively. The overturn end point was used with the guppies because cessation of gill and mouth movements was difficult to detect due to their size. The death end point was used on goldfish because they did not give a "sharp" overturn end point but appeared to lose their equilibrium by degrees. Both end points used on the same lot of fish gave parallel log-log plots of time for effect *versus* concentration, indicating that both end points were the same function of concentration.

Materials—The ethanol solutions were prepared from absolute ethanol of reagent quality (U. S. Industrial Chemical Co.). The solutions were prepared by accurately measuring the ethanol into a volumetric flask. A quantity of buffer and sodium chloride necessary to give 0.05 *M* buffer concentration and an ionic strength of 0.05 was added with distilled water; the pH was adjusted to the desired value with dilute NaOH or HCl, and the solution was made to volume. The ionic strength adjustment was based upon calculation of the fraction of the buffer in its ionic form at each pH. All solutions contained 0.05 *M* buffer adjusted to 0.05 in ionic strength.

Three buffers were used in preparing the solution: 2-(*N*-morpholino)-ethane-sulfonic acid.H_2O(MES, Calbiochem), *N,N* - bis(2 - hydroxyethyl)glycine (bicine, Calbiochem), and tris-(hydroxymethyl)-aminomethane (tris or THAM, Fisher Scientific Co.). The buffers supplied by Calbiochem are relatively new in biological studies. The pKa of MES is 6.09, of bicine 8.26, and of tris 8.14 at 25° (6). The buffers were found by preliminary experiments to be innocuous to the fish.

Fish—Common guppies (*Lesbistes reticulatus*) were obtained from a local supplier of tropical fish. They were maintained at 25° and sized as described previously (3). Only male fish were used.

Goldfish (*Carassius auratus*), approximately 4 cm. in length, were purchased in a single lot from a local pet shop.

Method—A 4% ethanol solution was used with the guppies and a 6% ethanol solution with the goldfish. Nine solutions were used in the guppy study and 10 in the goldfish study. The solutions in each study were identical except for pH and buffer. The solutions were hypotonic to fish blood so the osmotic pressure vector across the absorbing membranes was in the same direction as in natural environmental conditions, although not quite so large (7). Prior to use, the solutions were placed in a water bath at 25° and allowed to attain temperature equilibrium.

The fish were exposed to the drug solutions by removing them from the tank with a plastic net and dropping them into the ethanol solution. Thirty milliliters of solution in a 100-ml. beaker was used for the guppies, and 100 ml. of solution in a 250-ml. beaker was used for the goldfish. A stopwatch was started when the fish made contact with the solution. The 100- or 250-ml. beaker was then placed inside a 600-ml. beaker that was suspended in a constant-temperature bath at 25°. The temperature of the bathing solution was thus maintained at 24–25°. Each fish was observed until the end point was reached. The stopwatch was stopped, the fish removed from the solution, and the time recorded. A fresh solution was used for each fish, and the fish were used only once. Twelve guppies and six goldfish were used for each solution.

To minimize bias in determining the end point, the solutions were coded by letter, the code being unknown to the person determining the end point. The solutions were administered in a random order established from a table of random numbers. These procedures were designed to eliminate the effects of the order in which the fish were removed from the tank and the bias of the observer in end point determination and fish selection.

RESULTS

Table I lists the results of the studies with guppies. At each pH the means from all buffers are averaged to give a collected mean. A *t* test of differences among the means in the guppy experiments showed that at the 95% confidence level there are significant differences between the collected

TABLE I—OVERTURN TIMES IN MINUTES FOR GUPPIES IN 4% ETHANOL[a]

	pH				
	5.0	6.0	7.0	8.0	9.0
		MES			
	17.25(3.93)	15.30(2.22)	12.20(2.59)	...	...
		Tris			
	...	14.88(2.59)	16.01(1.68)	14.81(1.80)	...
		Bicine			
	...	...	14.65(2.76)	12.65(1.74)	11.34(1.76)
Collected mean	17.25	15.09	14.28	13.73	11.34

[a] Each reported time represents the mean of 12 fish. Standard deviation in parentheses.

TABLE II—SURVIVAL TIMES IN MINUTES FOR GOLDFISH IN 6% ETHANOL[a]

	pH				
	5.0	6.0	7.0	8.0	9.0
		MES			
	14.30(1.25)	12.30(1.17)	17.05(1.51)	...	...
		Tris			
	...	15.28(0.98)	13.91(1.98)	14.23(0.79)	14.91(1.54)
		Bicine			
	...	...	15.61(2.72)	13.56(0.40)	14.08(0.96)
Collected mean	14.30	13.79	15.52	13.89	14.49

[a] Each reported time represents the mean of six fish. Standard deviation in parentheses.

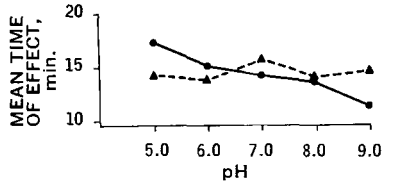

Fig. 1—Variation of mean time of the effect of ethanol in buffered solutions at different pH levels. Points represent collected means for all solutions buffered with MES, tris, and bicine. Key: ▲, survival times (min.) for goldfish in 6% ethanol; ●, overturn times (min.) for guppies in 4% ethanol.

means at pH 5 and 6, pH 6 and 8, pH 7 and 9, pH 8 and 9, as well as between pH values farther apart than these. With the individual buffers there is a trend toward shorter overturn times with increasing pH, except for the tris-buffered series. The differences among the means for the tris series are not statistically significant.

Table II lists the results of the studies with goldfish. There are no statistically significant differences at the 95% confidence level among any of the collected means, and there appears to be no trend paralleling the changes in pH with individual buffers.

Figure 1 depicts the comparison between goldfish and guppies of the effect of ethanol at various pH levels.

DISCUSSION

Several factors may have an appreciable effect on the absorption rate of a molecule. The more important factors are concentration, temperature, pH, osmotic pressure, ionic strength, degree of ionization of the molecule, size of the molecule, and its lipid solubility. If a buffer is present, as in this study, the degree of ionization of the buffer may be important.

In these experiments, the concentration of the ethanol (4% for guppies and 6% for goldfish) and the buffer (0.05 *M*) was held constant. The osmotic pressure of all the solutions was nearly equal although the exact osmotic pressure exerted by the buffers is not known. The ionic strength of the solutions was constant at 0.05. Because ethanol is nonionized in aqueous solutions, the ionization of the molecule was not a variable. The size of the molecule and its lipid solubility also remained constant in all of the solutions.

The degree of ionization of the buffer could possibly have an effect upon the absorbing membranes of the fish due to ion-membrane interactions. In their buffer ranges MES and bicine are anionic as their molecules have fully dissociated carboxyl groups and partially ionized amino groups depending on the pH of the solution. As the pH is increased in the buffering range of the molecule the amino group becomes less ionized, resulting in an increased net negative charge on the molecule from the fully ionized carboxyl group. Tris, however, is not zwitterionic but is a substituted amine, and as the pH is increased, the number of positively charged buffer ions decreases in the buffering range of the molecule. It is unlikely that charges upon the buffer molecules would influence the absorption rate of ethanol in this study as all solutions were of equal ionic strength, and there is no qualitative difference among the charges on the buffer ions and the sodium and chloride ions present. To maintain the pH of the solutions it is necessary that buffers be present.

The parameter which this study concerns is pH and its effect upon the absorbing membranes of guppies. From the data collected in this study it appears that the pH of the solution has a significant effect upon the rate of absorption of ethanol by guppies. The absorbing membranes of the fish are

apparently altered, causing an increase in the rate of absorption as the hydrogen-ion activity becomes less. The nature of this alteration could be a change in the membrane pore size or some other structural change in the membrane.

The extremes of the pH range used in this study are not harmful to either of the fish which may live for extended periods at either pH 5.0 or 9.0. Further, it is doubtful that the changes in pH have interfered with the integrity of the membrane. The possibility of a synergistic toxicity of the hydrogen ion–ethanol combination, independent of absorption rate, could not be ruled out. Preliminary experiments on guppies with strychnine, however, showed a similar increase in absorption rate at high pH levels. The observed absorption rate increase was considerably greater than that accounted for by calculation of the amount of strychnine present as the free base at elevated pH levels.

Results from this study indicate that the absorbing membranes of the guppies are pH sensitive. The use of this fish as an experimental model in studying drug absorption probably should be limited to those studies in which the pH of the solutions used is constant. Absorption studies that are run with pH as a variable factor probably will be influenced by the changing absorption rates due to varying pH, and any change in absorption rate in such a study may not be meaningful. Goldfish, on the other hand, appear to have absorbing membranes which are not appreciably altered by pH changes *per se*.

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Keyphrases

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Membrane sensitivity to pH

Mixed Glycol Acetal Dimethylcarbamates *via* Transacetalation of Certain Di- and Trihydric Alcohols

By CLAUDE PIANTADOSI, FRED T. SEMENIUK, and ROBERT K. RAUCH

Mixed acetals of a new type were synthesized by an acetal exchange reaction between the mono dimethylcarbamate of ethylene, propylene, and trimethylene glycols and the diethyl acetals of butyraldehyde, heptaldehyde, and benzaldehyde. The glycerol carbonate (followed by dimethylaminolysis) also underwent transacetalation and dimethylcarbamate esters of the dioxolane-type were synthesized.

A NUMBER of acyclic acetals (1), cyclic acetals (2), and their derivatives (3–9) have been reported in the literature. Their potentialities as hypnotics, anticonvulsants, and skeletal muscle relaxants (3–9) have been noted. In view of the fact that mixed acetals are similar in structure to the pharmacologically active glycerol ethers, it was of interest to synthesize some of the mixed acetal carbamates in order to determine the feasibility of the transacetalation reaction for preparing such compounds. Exchange reactions between alcohols in acetal linkage and alcohols have long been known. They have been reported to be applicable for primary alcohols (10, 11), for secondary and tertiary alcohols and phenols (11), for ethylene and trimethylene glycol (12), and for glycerol (13–15). The term "transacetalation" has been coined and may be applied generally to cover all such acetal exchange reactions (13). The experimental procedures presented in this paper describe the use of this reaction with the glycol monocarbamates of ethylene, propylene, trimethylene, and glycerol with the diethyl acetals of benzaldehyde, butyraldehyde, and heptaldehyde. Attempts to prepare the unsubstituted carbamate derivatives by this method were not successful.

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Accepted for publication August 3, 1967.
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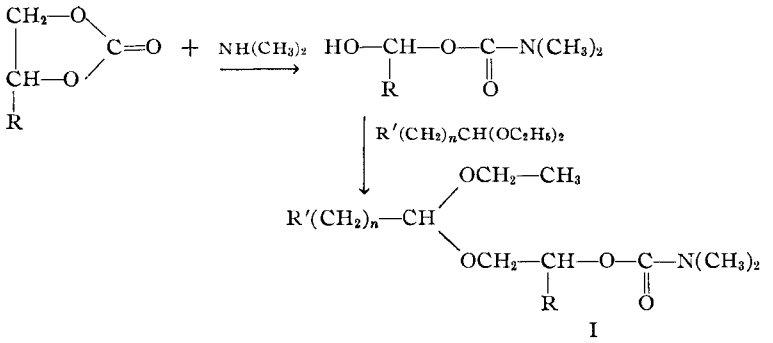
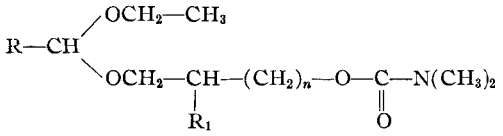


R = H or CH_3, R′ = aliphatic or aromatic. n = 0, 1, 2, 5

Scheme I

TABLE I—MIXED ACETAL DIMETHYLCARBAMATES



R	R_1	n	Formula	Yield, %	B.p., °C. (mm.)	Anal., % C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
C_3H_7	H	0	$C_{11}H_{23}NO_4$	60	83 (0.3)	56.63	56.87	9.94	10.04	6.01	5.89
C_6H_5	H	0	$C_{14}H_{21}NO_4$	66	122 (0.3)	62.90	63.19	7.92	7.88	5.24	5.25
C_6H_{13}	H	0	$C_{14}H_{29}NO_4$[a]	54	108 (0.3)	61.06	61.58	10.61	10.32	5.09	5.30
C_6H_5	CH_3	0	$C_{15}H_{23}NO_4$	57	122 (0.4)	64.03	64.22	8.24	8.39	4.98	5.10
C_3H_7	CH_3	0	$C_{12}H_{25}NO_4$	49	80 (0.4)	58.27	58.49	10.19	10.42	5.66	5.76
C_6H_{13}	CH_3	0	$C_{15}H_{31}NO_4$	60	110 (0.4)	62.25	62.04	10.80	10.84	4.84	4.88
C_3H_7	H	1	$C_{12}H_{25}NO_4$	57	93 (0.3)	58.27	58.78	10.19	10.00	5.66	5.56
C_6H_5	H	1	$C_{15}H_{23}NO_4$	43	124 (0.2)	64.03	64.66	8.24	8.36	4.98	4.85
C_6H_{13}	H	1	$C_{15}H_{31}NO_4$[b]	50	108 (0.3)	62.25	62.24	10.80	10.99	4.84	4.95

[a] 2-(1-Ethoxyheptoxy)ethyl benzoate was prepared in the usual manner, b.p. 39° (0.3). Calcd. for $C_{18}H_{28}O_4$: C, 70.08; H, 9.15. Found: C, 70.29; H, 9.50. [b] 3-(1-Ethoxyheptoxy)propyl benzoate was prepared, b.p. 52° (0.4). Calcd. for $C_{19}H_{30}O_4$: C, 70.77; H, 9.38. Found: C, 70.34; H, 9.61.

cm.$^{-1}$ and the C—O—C bands at 1050–1275 cm.$^{-1}$. Scheme I for the synthesis of 3-(1-ethoxybutoxy) propyl dimethylcarbamate(I) illustrates the reaction sequence for the preparation of mixed acetal dimethylcarbamates. Data with respect to these derivatives are listed in Table I. Scheme II depicts the route used in the synthesis of dioxolane carbamates (*Va*) and mixed glycerol carbamate acetals (IV*b*).

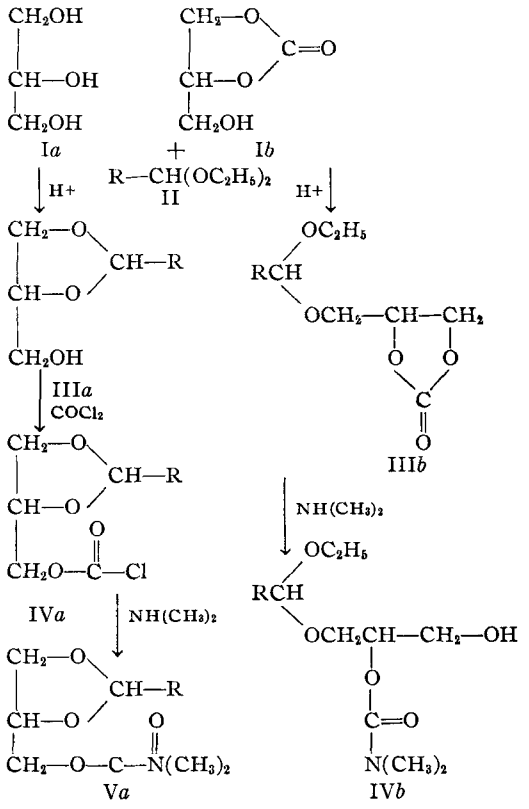
EXPERIMENTAL

All boiling points and melting points are uncorrected. Analyses were determined by Weiler and Strauss Microanalytical Laboratory, Oxford, England, Spang Microanalytical Laboratory, Ann Arbor, Mich., and M-H-W Laboratories, Garden City, Mich. Infrared spectra were recorded with a Perkin-Elmer Infracord.

3-(1-Ethoxybutoxy)propyl Dimethylcarbamate (I)—The following procedure is based on a modification of the method of Alquier (20) and subsequently of others (15). This method was used for all the products obtained from ethylene, propylene, and trimethylene glycols. Table I indicates pertinent data with respect to the mixed acetal dimethylcarbamates synthesized.

In a three-necked flask equipped with a stirrer, a thermometer, and a distilling head for the collection of the alcohol evolved, were placed 13.3 Gm. (0.1 mole) of ethylene glycol monodimethylcarbamate and 58.4 Gm. (0.4 mole) of butyraldehyde diethyl acetal. The reaction mixture was heated with vigorous stirring on an oil bath. When the temperature of the reaction mixture reached 115°, a drop of concentrated HCl was added, and ethanol began to evolve. After the theoretical amount of ethanol was obtained, the reaction mixture was cooled, and 50 ml. of ether was added. The ethereal solution was washed once with 30 ml. of saturated $NaHCO_3$, and three times with 30-ml. portions of cold water. The ether extract was dried over anhydrous sodium sulfate and the ether was removed under reduced pressure. The resulting product was purified by distillation under reduced pressure. Compounds listed in Table I were prepared in an analogous manner.

4 - Hydroxymethyl - 2 - n - hexyl - 1,3 - dioxolane Dimethylcarbamate (Va)—To 37.6 Gm. (0.2 mole) of 4-hydroxymethyl-2-*n*-hexyl-1,3-dioxolane dissolved in 80 ml. of benzene was added dropwise 0.2 mole of phosgene. The mixture was stirred for 3 hr. at which time 0.2 mole of dimethylaniline was added. Stirring was maintained for another 30 min. and the mixture was washed with ice water. A separation of benzene and water layers occurred, and the benzene layer was poured into 150 ml. of a 25% aqueous solu-



Scheme II

tion of dimethylamine. The reaction mixture was placed in an ice bath and stirred for 3 hr. The benzene layer was separated, dried over anhydrous potassium carbonate, and removed by evaporation. The residue was purified by distillation under reduced pressure, b.p. 106–107° (0.14 mm.); n_D^{25} 1.4517.

Anal.—Calcd. for $C_{13}H_{25}NO_4$: C, 60.20; H, 9.72; N, 5.40. Found: C, 60.76; H, 9.54; N, 5.46.

In a similar manner the benzaldehyde analog was prepared. After evaporation of the benzene, distillation of the residue under vacuum afforded 12.8 Gm. of a yellow colored liquid, b.p. 140° (0.14 mm.); n_D^{25} 1.5180. Upon standing in the cold (5°), approximately 1 Gm. of white crystals was collected from this liquid fraction, m.p. 115–116°. Both liquid and solid were submitted for analyses.

Anal.—Calcd. for $C_{13}H_{17}NO_4$: C, 63.13; H, 6.82; N, 5.58. Found: (solid) C, 63.23; H, 6.88; N, 5.26. (Liquid): C, 63.26; H, 6.66; N, 5.36.

Mixed Glycerol Carbamate Acetals (IVb)—To 11.8 Gm. (0.1 mole) of freshly distilled glycerol carbonate was added 75.2 Gm. (0.4 mole) of heptaldehyde diethyl acetal. The reaction mixture was heated to 150° and two drops of concentrated HCl was added. After cooling, ether was added, followed by neutralization with a weak base. The mixture was then washed with water, and the organic layer was dried over anhydrous potassium carbonate and subsequently removed by evaporation. Distillation of the residue gave a product (III*b*) with b.p. 147° (0.23 mm.); n_D^{27} 1.4444.

Anal.—Calcd. for $C_{13}H_{24}O_5$: C, 59.98; H, 9.29. Found: C, 59.78; H, 9.17.

Similarly, the corresponding benzaldehyde derivative was obtained, b.p. 155–157° (0.14mm.); n_D^{27} 1.5079.

Anal.—Calcd. for $C_{13}H_{15}O_5$: C, 61.89; H, 6.39. Found: C, 62.03; H, 6.35.

To obtain the carbamate (IV*b*) 0.1 mole of III*b* was placed in 100 ml. of a 25% aqueous solution of dimethylamine. The mixture was set aside for 4 days, and the layers separated and distilled, b.p. 143° (0.15 mm.); n_D^{25} 1.4488.

Anal.—Calcd. for $C_{15}H_{31}NO_5$: C, 58.99; H, 10.23; N, 4.59. Found: C, 59.07; H, 10.06; N, 4.50.

The corresponding benzaldehyde derivative was obtained as a viscous yellowish-brown liquid. This oil was purified in a molecular still, b.p. 160° (0.13 mm.); n_D^{25} 1.5020.

Anal.—Calcd. for $C_{15}H_{23}NO_5$: C, 60.59; H, 7.80; N, 4.71. Found: C, 61.00; H, 8.03; N, 4.59.

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Keyphrases

Glycol acetal dimethylcarbamates
Glycol acetal dimethylcarbamates—synthesis
Transacetalation of di- and trihydric alcohols
IR spectrophotometry—structure

Utilization of Hydrophilic Gums for the Control of Drug Substance Release from Tablet Formulations II. Influence of Tablet Hardness and Density on Dissolution Behavior

By H. E. HUBER and G. L. CHRISTENSON

The influence of tablet hardness and density variation on *in vitro* dissolution of dye from controlled release tablets made with different types and viscosity grades of hydrophilic polymers was investigated. Tablet volumes were maintained constant and the weight varied to obtain varying degrees of hardness and density. Tablets made at varying hardness did not show markedly different release characteristics as evaluated by an *in vitro* method. Different release patterns were, however, observed with tablets prepared from different gums.

A PREVIOUS REPORT (1) from these laboratories presented data to illustrate the utilization of hydrophilic gums as a means of controlling rate of drug release from tablet formulations. It was shown that the type and concentration of gum in a formulation could markedly influence disintegration and dissolution behavior. Recently, Lapidus and Lordi (2) published the results of their related investigations on factors affecting the release of water-soluble drugs from a compressed hydrophilic matrix. Their studies were concerned with theoretical aspects of the problem and focused attention on the kinetics of drug release in the initial stages of the process and from a single face of a tablet. They were able to show that under such conditions the release pattern appeared to follow the same relationships as were proposed by Higuchi (3, 4) for solid drugs dispersed in solid matrices.

The present investigation was conducted to determine, in part, the influence of formulation and processing variables on the release rate characteristics of tablets formulated with hydrophilic gums as the delaying matrix. In this study, the relationships between tablet hardness and release rate characteristics have been investigated. It was felt that a comparison of hardness measurements would reflect, quantitatively, differences in density and porosity of tablets made at different compressional forces.

Differences in density and porosity could possibly influence the rate of tablet dissolution by, for example, affecting the initial rate of penetration of water at the tablet surface and thus the rate of formation of the gel barrier at the tablet periphery. Such considerations are of practical importance since complete uniformity of compressional force is difficult to maintain in production operations and variations of release rate as a function of processing variables must be known to maintain reasonable tablet-to-tablet uniformity with regard to release rate characteristics.

Tartrazine (FD&C Yellow No. 5) was used in this study as a tracer to evaluate release characteristics. It was chosen because of analytical considerations. The release of the dye from a tablet could be easily followed spectrophotometrically and the spectral characteristics of the dye are pH independent over the pH range investigated. In addition, the dye served to visually indicate uniformity of distribution throughout the tablet. The gums selected for this study were carboxypolymethylene (CPM), sodium carboxymethylcellulose (NaCMC), and hydroxypropyl methylcellulose (HPMC). Three different viscosity grades of the latter gum were investigated (HPMC 100 cps., 4,000 cps., and 15,000 cps.).

EXPERIMENTAL

Tablet Granulations—Five different gums were investigated, CPM, NaCMC, HPMC 100 cps., HPMC 4,000 cps., and HPMC 15,000 cps. In each formulation equal parts of lactose and gum were mixed with 0.4% micropulverized tartrazine. The mix was slugged and screened, and 2% magnesium stearate was added for lubricant.

Preparation of Tablets—Two approaches can be used to vary the hardness characteristics of tablets. A constant weight of granulation can be used and the compression pressure exerted by the machine varied through adjustment of the die fill volume. As a result, tablets of different hardness would be obtained but the tablets would also have different volumes. Alternatively, tablets with different hardness values and constant volume can be prepared by using different weights of granulation and maintaining a constant fill volume. The second approach was used, because if weight is held constant and volume varied, differences in dissolution rate could be expected as a result of differences in surface area, and therefore the data would have little value in answering the basic question regarding influence of hardness on release rate characteristics.

Granulation was weighed out on a class A prescription balance and compressed on a Stokes model F tablet machine adjusted to provide a $^3/_8$-in. flat-faced tablet 4.1 to 4.2 mm. thick and with Pfizer hardnesses of approximately 6, 10, or 15 Kg. Once the weight required to obtain a desired hardness was found, additional tablets were made and hardness checked.

Six tablets were then made by weighing out granulation and compressing it at that particular machine setting. These 6 tablets were then weighed on a Mettler balance to the nearest 0.1 mg. and their thickness measured with an Ames micrometer to the nearest 0.01 mm. Volume was calculated using $\pi r^2 h$. Apparent density was calculated from average tablet weight/volume. Three of the 6 tablets, with apparent density values on the high and low side, were used to determine an average hardness. The remaining tablets were subjected to *in vitro* dissolution.

Received April 14, 1967, from the Pharmaceutical Research and Development Department, Scientific Laboratories, The Wm. S. Merrell Co., Division of Richardson-Merrell, Inc., Cincinnati, OH 45215

Accepted for publication August 16, 1967.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

Dissolution Study—Each tablet was placed in the basket rack assembly of a USP Stoll-Gershberg apparatus, lowered into an 800-ml. beaker containing 600 ml. of simulated gastric fluid (no enzymes) maintained at 37 ± 0.5° and operated for 1 hr. At the end of 1 hr., the basket rack, with tablet, was transferred to another 800-ml. beaker containing 600 ml. of simulated intestinal fluid (no enzymes) and the operation continued. A sample of the simulated gastric fluid was filtered through Whatman No. 42 paper and its absorbance measured at 420 mμ on a Spectronic 20 colorimeter. At hourly intervals the apparatus was stopped, the volume of simulated intestinal fluid adjusted to 600 ml., operated again for a short interval to assure mixing, and a 10-ml. sample was withdrawn. After filtration and reading the absorbance, the sample was returned to the beaker. Hourly assays were made until the tablet was dissolved or 7 hr. of data were obtained. Those tablets that remained undissolved were stirred on a magnetic stirrer until dissolved and an end point assay run. Three determinations were made for each hardness value.

Percent dye released each hour was calculated on the basis of total dye released for each tablet. By treating the data in this manner, differences in tablet weight and therefore total tablet dye content were eliminated.

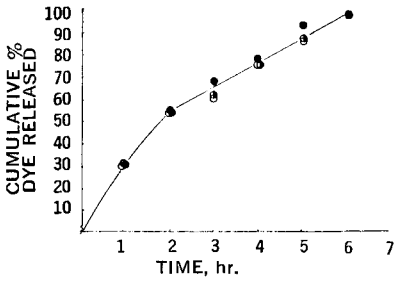

Fig. 1—Influence of tablet hardness on in vitro *dissolution of dye from CPM tablets. Key: ○, 6.1 Kg. average hardness; ◑, 12.1 Kg. average hardness; ●, 14.7 Kg. average hardness.*

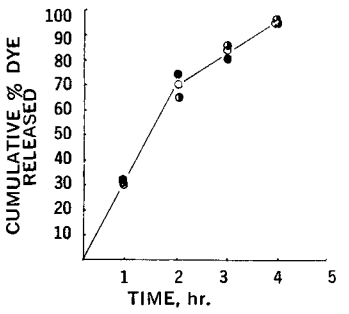

Fig. 2—Influence of tablet hardness on the in vitro *dissolution of dye from HPMC 100 cps. tablets. Key: ○, 6.3 Kg. average hardness; ◑, 8.8 Kg. average hardness; ●, 13.5 Kg. average hardness.*

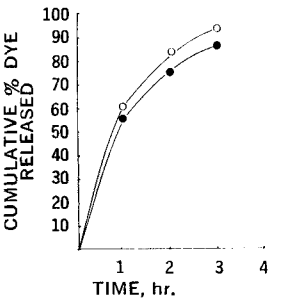

Fig. 3—Influence of tablet hardness on in vitro *dissolution of dye from NaCMC tablets. Key: ○, 5.7 Kg. average hardness; ●, 11.7 Kg. average hardness.*

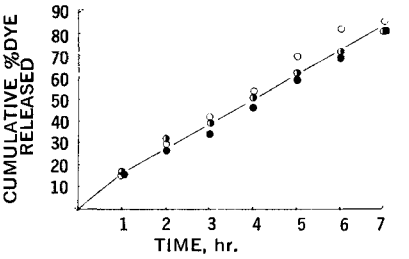

Fig. 4—Influence of tablet hardness on in vitro *dissolution of dye from HPMC 4,000 cps. tablets. Key: ○, 5.3 Kg. average hardness; ◑, 8.4 Kg. average hardness; ●, 11.8 Kg. average hardness.*

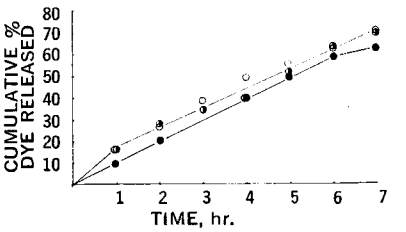

Fig. 5—Influence of tablet hardness on in vitro *dissolution of dye from HPMC 15,000 cps. tablets. Key: ○, 5.7 Kg. average hardness; ◑, 10.4 Kg. average hardness; ●, 13.7 Kg. average hardness.*

TABLE I—TABLET HARDNESS AND DENSITY

Gum	Hardness, Kg.	Apparent Density, Gm./ml.
CPM	6.1	1.03
	12.1	1.11
	14.7	1.18
HPMC, 100 cps.	6.3	1.19
	8.8	1.23
	16.6	1.30
HPMC, 4,000 cps.	5.3	1.23
	8.4	1.25
	11.8	1.29
HPMC, 15,000 cps.	5.7	1.19
	10.4	1.24
	13.7	1.28
NaCMC	5.7	1.33
	11.7	1.41

RESULTS

No significant differences in release rate of dye could be seen for tablets made with CPM, HPMC

100 cps., and HPMC 4,000 cps. at approximately 6, 10, and 15 Kg. hardness (Figs. 1, 2, and 4). Average apparent tablet densities and corresponding hardness values are shown in Table I.

HPMC 15,000 cps. and NaCMC tablets demonstrated a slightly reduced rate of dye release during the first hour of *in vitro* dissolution as a result of increased hardness and density. The rates of dye release for the remaining dissolution period remained approximately the same, regardless of tablet hardness (Figs. 3 and 5 and Table I).

DISCUSSION

The data indicate that one can expect little or no change in dissolution rate pattern as a result of alteration in tablet density and porosity for some hydrophilic gum formulations. If changes occur, they probably will appear during the initial phase of the dissolution period and the shape of the dissolution profile will not be markedly altered. Apparently, the affinity of some gums for water will overcome any deterring influence which an increased density or decreased porosity may tend to exert on the initial rate of water penetration into the tablet surface. In those cases where the gum and/or formulation exhibit a decreased affinity for water, an increase in density will deter water penetration and reduce the rate of drug substance dissolution until a gel barrier is formed. After the gel barrier is formed, the rate of drug substance release is further reduced and becomes dependent on the rate at which drug substance diffuses through the gel and the rate at which the gel barrier is mechanically removed by agitation.

SUMMARY AND CONCLUSIONS

In an investigation of *in vitro* dissolution behavior for tablets compressed to different degrees of hardness, no changes in dissolution rate pattern were observed for three formulations. In two formulations, a slightly reduced rate of dissolution was noted during the first hour; however, no marked change in the total dissolution profile was observed. Different release rate profiles were observed with tablets prepared from different gums.

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Keyphrases

Tablet formulation dissolution
Hydrophylic resin tablet formulations
Tablet hardness
Tablet density
Simulated gastric fluid
Simulated intestinal fluid
Colorimetric analysis

Tetracyclic Phenothiazines I. Reinvestigation of the Brominations of Some Pyrido[3,2,1-*kl*]phenothiazines

By ARNOLD R. MARTIN, GERALD G. BRIGGS, and TIMOTHY J. YALE

Brominations of both 1,2-dihydro-3-keto-3*H*-pyrido[3,2,1-*kl*]phenothiazine (I) and its sulfoxide (VII) gave 3-hydroxypyrido[3,2,1-*kl*]phenothiazinium bromide (VI). Treatment of 3-keto-3*H*-pyrido[3,2,1-*kl*]phenothiazine (II) with bromine gave 2-bromo-3-hydroxypyrido[3,2,1-*kl*]phenothiazinium bromide (XII). Structural assignments of VI and XII have been made on the basis of their spectral properties, correcting those previously reported.

As a result of an interest in tetracyclic phenothiazine derivatives as intermediates for the synthesis of pharmacologically interesting agents, the reactions of 1,2-dihydro-3-keto-3*H*-pyrido-[3,2,1-*kl*] phenothiazine (I) and 3-keto-3*H*-pyrido-[3,2,1-*kl*]phenothiazine (II) with bromine (Scheme I) were reinvestigated. Harfenist (1) reported the bromination of I to give a "bromo derivative," which he assumed to be the bromoketone (III), on the basis of its elemental analysis. Heating of the "bromo derivative" in aqueous methanol caused its conversion to the unsaturated ketone (II). The reaction of II with bromine gave an unstable "dibromide," assumed to be the addition product (IV). Attempts to recrystallize IV reportedly gave the unsaturated bromoketone (V). The interpretation of the infrared and ultraviolet spectra of the two bromination products originally prepared by Harfenist requires reassignment of their structures.

RESULTS AND DISCUSSION

The infrared spectrum of the "bromo derivative" obtained from I lacks a carbonyl band, but shows the characteristics of an ionized amine salt, *i.e.*, a nearly continuous series of bands in the region of about 2300–2700 cm.$^{-1}$(2). The high melting point of the "bromo derivative" and its solubility behavior (it is insoluble in nonpolar solvents) further militate against the bromoketone structure III. The aromatized salt structure VI, on the other hand, is consistent with the analytical, spectral, and

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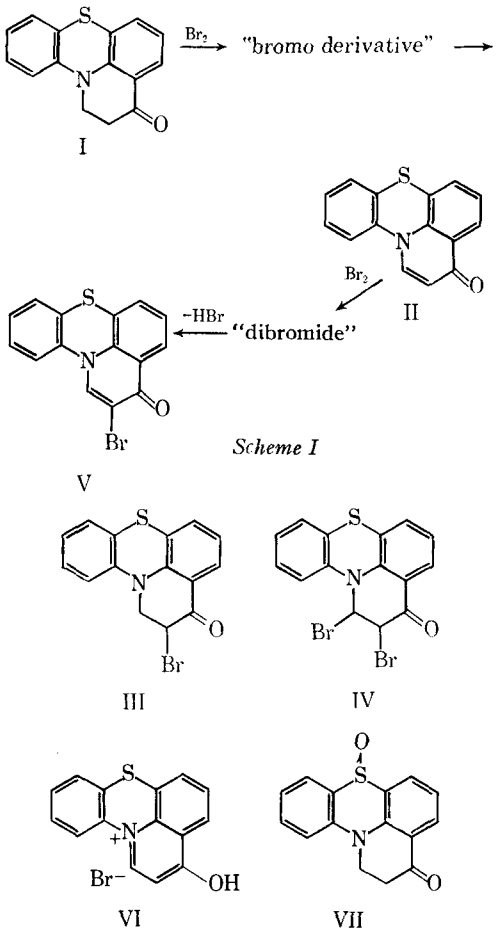


Scheme I

physical data. The salt would be expected to lose a proton in polar solvents readily to give the weak conjugate base II.

The ultraviolet spectra of I, II, and VI are shown in Fig. 1. The spectra of I and II in acetonitrile have been reported previously (3). Comparison of the spectra of I and VI reveal differences which appear unreconcilable on the basis of anticipated relationships between the spectra of an aromatic ketone and its corresponding α-bromo derivative. The relationship of VI as the conjugate acid of II was confirmed by investigating the spectral behavior of II in 50% ethanol containing varying concentrations of hydrochloric acid. The behavior of the long wavelength band is shown in Fig. 2. An isosbestic point is evident at 413 mμ. The absorption maxima of II in the presence of ethanolic hydrochloric acid are essentially the same as those of VI in acetonitrile, except for minor wavelength shifts due to differences in solvent and differences in absorptivity, probably resulting from differences in the amount of dissociation in the two solvents.

Hromatka *et al.* (4), in an effort to obtain a derivative of I chlorinated in one or both of the benzene rings, reacted the sulfoxide VII in hot aqueous ethanolic hydrochloric acid. A good yield of the unsaturated ketone II was obtained. This result prompted us to react VII with bromine, giving an excellent yield of VI. It would seem likely that the synthesis of VI (or II, depending on the workup) by bromination of I or VII, or from the action of hydrochloric acid on VII, might proceed through the common intermediate VIII. This intermediate was originally suggested by Harfenist (1) for Hromatka's preparation of II. A doubly charged intermediate like VIII has been proposed by Schmalz and Burger (5) to explain the reductive nuclear chlorination and nitration of *N*-alkylphenothiazine sulfoxides. Loss of a proton from VIII, followed by enolization, would give VI (Scheme II). The intermediate VIII could possibly rise from either of the perbromides, IX or X, or the protonated form of the sulfoxide XI, depending on the reactants.

It is noteworthy that, if the bromination of I is carried out at ice bath temperature, a brown solid containing roughly two equivalents of bromine and exhibiting a carbonyl band in the infrared can be isolated. Heating the solid causes evolution of hydrogen bromide, giving VI. Efforts to obtain a

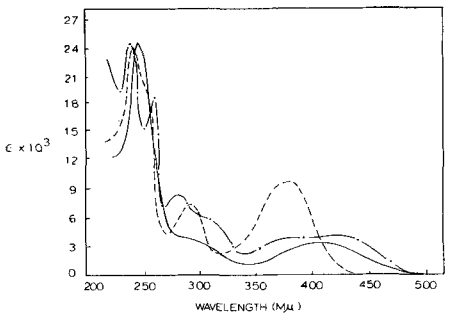


Fig. 1—1,2-Dihydro-3-keto-3H-pyrido[3,2,1-kl]-phenothiazine (I) (——) in 95% ethanol; 3-keto-3H-pyrido[3,2,1-kl]phenothiazine (II) (- - -) in 95% ethanol; 3-hydroxypyrido[3,2,1-kl]phenothiazinium bromide (VI) (— · —) in acetonitrile.

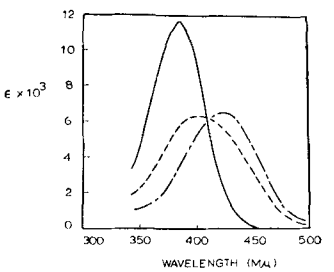
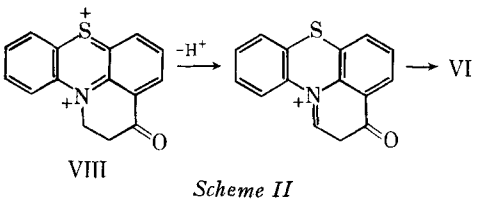
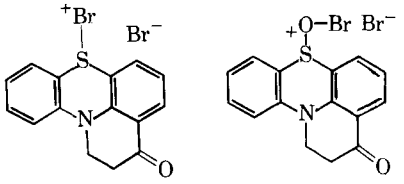


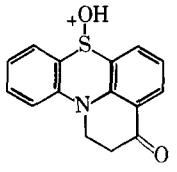
Fig. 2—3-Keto-3H-pyrido[3,2,1-kl]phenothiazine (II) (——); II in 1.6 N HCl (- - -); II in 3.8 N HCl (— - —) (all in 50% ethanol).



Scheme II



IX X

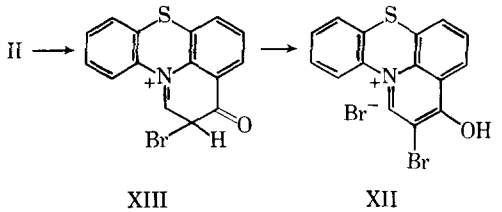


XI

satisfactory analysis of the intermediate, thought to be the S-perbromide (IX), have not been successful. The possibility that the brown solid is the bromoketone (III), which eliminates hydrogen bromide when heated, cannot be entirely ruled out.

It occurred to the authors that Harfenist's "dibromide," obtained from the reaction of II with bromine, is probably the hydrobromide salt XII. The infrared spectrum of this substance, consistent with expectations, lacks a carbonyl band and shows a continuous series of absorptions in the 2300–2700 cm.$^{-1}$ region. Efforts to study the ultraviolet spectrum of XII were frustrated by its lack of solubility in nonpolar solvents and by its conversion to the unsaturated bromoketone (V) in polar solvents. A study of the spectral behavior of V in the presence of acid demonstrated that it is a much weaker base than II. Concentrations of hydrochloric acid causing significant wavelength shifts in the spectrum of II did not effect comparable changes in the spectrum of V. Evidently, the base weakening inductive effect of the bromine atom is more important than possible stabilization of the conjugate acid of II by intramolecular hydrogen bonding of the proton with the nonbonding electrons of the bromine atom. The aromatized salt XII can be visualized as being formed *via* enolization of an intermediate ion XIII as shown in Scheme III.

The fact that II undergoes electrophilic substitution rather than addition is a further indication of its aromatic character. Infrared data also suggest a high degree of aromaticity exists in this and related systems (3). Comparable substitution reactions of *N*-substituted-2- and 4-pyridones appear in the literature (6–8). For example, bromination of 1-methyl-2-pyridone is reported to give 3,5-dibromo-2-pyridone (6). The reaction of 3,5-dicarboxy-1,2,6-trimethyl-4-pyridone with iodine in acetic acid reportedly gave the corresponding 3,5-diiodo derivative in which loss of the carboxy groups occurred (8). The behavior of II toward other electrophilic reagents is under investigation in this laboratory.



XIII XII

Scheme III

EXPERIMENTAL

Melting points were taken in a Thomas-Hoover capillary apparatus and those below 240° are corrected. Infrared spectra were determined in KBr pellets with a Beckman IR-5 spectrophotometer. Ultraviolet spectra were determined with a Cary model 15 spectrophotometer.

3-Hydroxypyrido[3,2,1-kl]phenothiazinium Bromide (VI)—The procedure employed by Harfenist (1) for the bromination of 1,2-dihydro-3-keto-3*H*-pyrido[3,2,1-*kl*]phenothiazine (I) was employed. Concentration of the mother liquid improved the yield to 85–90%. $\nu_{max.}$ 3058, 3012 (aromatic C—H); about 2300–2700 continuous band series (ionized OH); 1616 (aromatic C=C); no ketone band in the region 1680–1690 or aliphatic C—H band near 2900. When the addition of bromine was carried out in an ice bath, a brown solid was formed almost immediately. It was collected on a filter, washed with carbon tetrachloride, and allowed to dry at 25°. When heated on a melting block the substance evolved hydrogen bromide. The infrared spectrum of the brown solid showed it to contain some VI. A ketone band at 1682 cm.$^{-1}$ was also evident in the spectrum.

Bromination of 1,2-Dihydro-3-keto-3H-pyrido-[3,2,1-kl]phenothiazine-7-oxide (VII)—A solution of 3.25 Gm. (12 mmoles) of VII in hot anhydrous dioxane was cooled to 50° and 2.1 Gm. (13.2 mmoles) of bromine in 25 ml. of dioxane was added with stirring over a 3-min. period. The mixture was then heated for 10 min. on a steam bath causing copious evolution of hydrogen bromide and the precipitation of an orange solid, m.p. 255–266°, following recrystallizations from nitromethane. Concentration of the reaction mixture gave additional material, totaling 3.4 Gm. (85%).

3-Keto-3H-pyrido[3,2,1-kl]phenothiazine (II)—A mixture of 3.32 Gm. (10 mmoles) of VI and 10 ml. of triethylamine was allowed to stand 15 min. The triethylamine was removed and the resulting solid was collected on a filter and washed with water. A nearly quantitative yield of II was obtained, m.p. 206°·[Lit. (1) 207°]. $\nu_{max.}$ 3064, 3014 (aromatic C—H); 1621 (C=O).

2 - Bromo - 3 - hydroxypyrido[3,2,1 - kl]phenothiazinium Bromide (XII)—Harfenist's procedure for the reaction of II with bromine was employed (1). Recrystallization of the material thus obtained from anhydrous ethyl acetate gave orange needles, m.p. 149–155°. [Lit. (1) 146–about 159°.] $\nu_{max.}$ 3060, 3015 (aromatic C—H); about 2300–2700 continuous band series (ionized O—H); 1628 (aromatic C=C); no keto band in the region of 1680–1690 or aliphatic C—H band near 2900. Attempts to determine the ultraviolet spectrum of XII in dioxane or acetonitrile gave a spectrum identical with that of V (see below).

2 - Bromo - 3 - keto - 3H - pyrido[3,2,1 - kl]phenothiazine (V)—A mixture of 4.11 Gm. (10 mmoles) of XII and 15 ml. of triethylamine was allowed to stand 15 min. The triethylamine was removed; the residue was collected on a filter and washed with water. A nearly quantitative yield of V was

obtained, m.p. 146–147° from 95% ethanol. [Lit. (1) 145–147.3°.] $\nu_{max.}$ 3048, 3022 (aromatic C—H); 1620 (C=O). $\lambda_{max.}$ 248 (ϵ 32,000); 301 (ϵ 6,800); 387 (ϵ 11,700). The ultraviolet absorption maxima did not undergo appreciable wavelength shifts in 4 *N* hydrochloric acid in 50% ethanol.

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Keyphrases

Tetracyclic phenothiazines
Bromination of pyrido[3,2,1-*kl*] phenothiazines
IR Spectrophotometry–structure
UV Spectrophotometry–structure

Quantitative Separation and Estimation of Steroid Mixtures by Thin-Layer Chromatography II. Determination of Progesterone and Estradiol Benzoate and of Progesterone, Testosterone Propionate, and Estradiol Benzoate in Mixtures

By TATJANA BIĆAN-FIŠTER

Rapid and simple procedures were developed for the TLC separation and subsequent quantitative determination of progesterone and estradiol benzoate as well as progesterone, testosterone propionate, and estradiol benzoate in vegetable oil solutions and microcrystalline water suspensions. The solvent system proposed permits not only a rapid quantitative separation of the steroids from one another but at the same time enables the separation of the steroids from the vegetable oil. No special extraction of steroids from the oil is thus necessary. The separated steroids after extraction from the adsorbent are each determined by the colorimetric and fluorimetric method, respectively.

In a recent communication (1) concerning the quantification on a microscale of steroid mixtures after thin-layer chromatographic (TLC) separation a procedure was proposed for the determination of progesterone and testosterone propionate in oil solution.

Because of the lack of rapid methods for the routine control of preparations containing steroid mixtures, the present communication describes rapid and simple procedures for the determination of mixtures of progesterone and estradiol benzoate as well as progesterone, testosterone propionate or butyrate, and estradiol benzoate.

The procedures described have been adapted to the quantification of steroid mixtures in vegetable oil solutions and crystalline water suspensions commercially available in Yugoslovia.

EXPERIMENTAL

Materials and Apparatus

Reagents—Dissolve 0.4 Gm. of isonicotinhydrazide in methanol, add 0.5 ml. hydrochloric acid (37%), and fill up to 100 ml. with methanol. Set aside the reagent for 24 hr. before use.

Received March 20, 1967, from the Institute for the Control of Drugs, Zagreb, Yugoslavia.
Accepted for publication August 15, 1967.
The author thanks Mrs. A. Harapin for technical assistance.

Adsorbent—Fluorescent Kieselgel HF_{254} (E. Merck, Darmstadt).

Reference Standards—Progesterone, testosterone propionate, estradiol benzoate [Organon, Oss (Holland)].

Apparatus—Thin-layer chromatography outfit with regulation thickness spreader (Desaga, Heidelberg), Agla micrometer syringe (Burroughs Wellcome & Co., London), ultraviolet lamp, 254 mμ (Hanau), fluorimeter [Kipp (Delft)].

Solvent System—Cyclohexane–ether–ammonia (8:2:0.5 v/v).

Preparation of Plates

Chromatoplates were prepared following the technique described by Stahl. Plates 20 × 20 cm. were coated (0.5 mm. layer thickness) with a slurry prepared by mixing with a pestle 35 Gm. Kieselgel HF_{254} with 85 ml. of water in a mortar. A batch suffices for six plates. The plates were air-dried for 10 min. at room temperature and thereafter activated by heating at 130° for 4 hr. and stored in a desiccator until used.

Procedures

A—TLC Separation of Progesterone and Estradiol Benzoate in Oil Injection Solutions—A 3.0-ml.

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Procedures

A—TLC Separation of Progesterone and Estradiol Benzoate in Oil Injection Solutions—A 3.0-ml.

quantity of the olive oil solution (about 60 mg. progesterone and about 9.0 mg. estradiol benzoate) was diluted in a 10-ml. volumetric flask with chloroform up to the mark. Twice, 20 µl. of this sample solution (samples *1* and *2*) and 20 µl. of each of the corresponding standard solutions were applied with a microsyringe on a Kieselgel HF $_{254}$ coated plate along the starting line (2 cm. from the lower edge) as 2 cm. horizontal lines. The chromatogram was developed in a chamber previously saturated for 24 hr. with the solvent. When the solvent reached about 1 cm. from the upper edge of the plate (about 60 min.), the plate was removed from the chamber, air-dried for a few minutes, and the separated steroids located under low wavelength light (Fig. 1). The UV absorbing zones of the sample and the standards were marked with an ample margin around each zone and quantitatively scraped off the plate, each into a separate 50-ml. glass-stoppered flask. Progesterone was thus extracted from the adsorbent with 5 ml. methanol while estradiol benzoate was extracted with 5 ml. chloroform. The flasks were stirred well for 30 min. and centrifuged.

Determination of Progesterone—Three-milliliter aliquots of each supernatant solution of sample and standard progesterone were pipeted into 50-ml. flasks, 4 ml. of the INH reagent was added, and the mixtures were set aside for 1 hr. The absorbances were measured at 380 mµ against a reagent blank.

$$\text{concn.}_x = \frac{\text{concn.}_{st.} \cdot A_x}{A_{st.} \cdot 3}$$

concn.$_x$ = concentration of progesterone per ml.,
concn.$_{st.}$ = concentration of progesterone in 10 ml. standard solution,
A_x = mean value of absorptivities of samples *1* and *2*,
A_s = absorptivity of standard solution,
3 = dilution factor.

Determination of Estradiol Benzoate—Three-milliliter portions of each supernatant solution of sample and standard estradiol benzoate were evaporated to dryness in 10-ml. volumetric flasks by heating on a water bath. The residues were dissolved in 88% sulfuric acid filled to the mark with the same acid. After 1 hr. the developed fluorescences were determined relative to the standard fluorescent solution of estradiol benzoate using filter B_2.

$$\text{concn.}_x = \frac{\text{concn.}_{st.} \cdot A_x}{A_{st.} \cdot 3}$$

concn.$_x$ = concentration of estradiol benzoate per ml.,
concn.$_{st.}$ = concentration of estradiol benzoate in 10 ml. standard solution,
A_x = mean value of absorptivities of samples *1* and *2*,
A_s = absorptivity of standard solution,
3 = dilution factor.

Standard Solution of Progesterone—Dissolve 60.0 mg. progesterone in a 10-ml. volumetric flask in chloroform and fill to the mark with the same solvent.

Standard Solution of Estradiol Benzoate—Dissolve 9.0 mg. estradiol benzoate in a 10-ml. volumetric flask in chloroform and fill to the mark with chloroform.

Standard Fluorescent Solution of Estradiol Benzoate—Dilute 10 µl. of the standard solution of estradiol benzoate in a 10-ml. volumetric flask with 88% sulfuric acid to the mark.

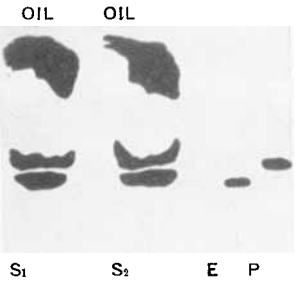

Fig. 1—Chromatogram for olive oil solution of progesterone and estradiol benzoate solution. Key: S_1, S_2 = duplicate test samples; E = estradiol benzoate; P = progesterone.

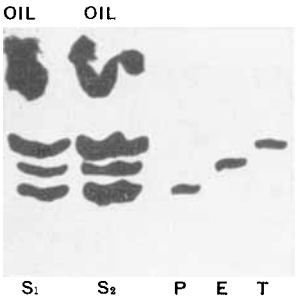

Fig. 2—Chromatogram for olive oil solution of progesterone, estradiol benzoate, and testosterone propionate. Key: S_1, S_2 = duplicate test samples; P = progesterone; E = estradiol benzoate; T = testosterone propionate.

B—TLC Separation of Progesterone, Testosterone Propionate, and Estradiol Benzoate in Oil Injection Solution—A 3.0-ml. quantity of the olive oil solution (about 60 mg. progesterone, 75 mg. testosterone propionate, and 9 mg. estradiol benzoate) was diluted in a 10-ml. volumetric flask filled to the mark with chloroform. Proceed as described under *TLC Separation of Progesterone and Estradiol Benzoate* in procedure *A* using the additional standard solution of testosterone propionate (Fig. 2).

Determination of Progesterone and Testosterone Propionate—Three-milliliter aliquots of each supernatant solution of sample and standard progesterone and testosterone propionate were pipeted into 50-ml. volumetric flasks and the determination of both steroids proceeded as described under *Determination of Progesterone* in procedure *A*.

Determination of Estradiol Benzoate—A 3.0-ml. quantity of each standard and sample supernatant chloroform solution of estradiol benzoate was treated in the same manner as described under *Determination of Estradiol Benzoate* in procedure *A*.

Standard Solution of Testosterone Propionate—Dissolve 75.0 mg. testosterone propionate in a 10-ml. volumetric flask in chloroform and fill to the mark with chloroform.

C—Testosterone Butyrate and Estradiol Benzoate in Microcrystalline Water Suspension—The contents of 1 ampul (about 7.5 mg. estradiol benzoate and 150 mg. testosterone propionate) is extracted in a separator with three times 10 ml. chloroform. The combined chloroform extracts are collected in a 50-ml. volumetric flask which is then filled to the mark with chloroform. Twenty microliters of this solution and of the corresponding standard solutions are applied to a Kieselgel HF_{254} plate along the start as 2-cm. horizontal lines, and the separation and determination of testosterone butyrate is carried out as

directed for progesterone, and that of estradiol benzoate as directed for the same steroid in procedure *A*.

Standard Solution for Testosterone Butyrate—Dissolve 150.0 mg. testosterone butyrate in a 10-ml. volumetric flask in chloroform and fill to the mark with chloroform.

Standard Solution of Estradiol Benzoate—Dissolve 10.0 mg. estradiol benzoate in a 10-ml. volumetric flask in chloroform and fill to the mark with the solvent.

D—Progesterone and Estradiol Benzoate in Microcrystalline Suspension—The contents of 1 ampul (about 200 mg. progesterone and 10 mg. estradiol benzoate) are extracted in the same manner as described under procedure *C*. Twice, 20 μl. of the chloroform extract and 20 μl. each of the corresponding standard solutions are applied to the plate, and the separation and determination of steroids are carried out as directed under procedure *A*.

Standard Solution of Progesterone—Dissolve 40.0 mg. progesterone in a 10-ml. volumetric flask in chloroform and fill up to the mark with chloroform.

Standard Solution of Estradiol Benzoate—Dissolve 10.0 mg. estradiol benzoate in a 10-ml. volumetric flask in chloroform and fill up to the mark with chloroform.

RESULTS AND DISCUSSION

The separation of progesterone from estradiol benzoate and progesterone, testosterone propionate, and estradiol benzoate achieved best resolution when 0.5 ml. of ammonia was added to the nonpolar cyclohexane–ether system to make it more polar. [The nonpolar cyclohexane–ether system was previously used (1) for the separation of progesterone and testosterone propionate.] It is known that the R_f values of steroids mainly depend on the number of C and O atoms, *i.e.*, the C/O index, which is largely determined by the polarity of the molecule. The migration of steroids on the plate can thus be explained by the fact that the less polar ester, testosterone propionate, with a longer side chain and a C/O index of 7.3, migrated faster than the more polar diketone, progesterone, with a C/O index of 10.3, and the phenolic ester, estradiol benzoate, with a C/O index of 8.3 (Fig. 2).

The methods used for the quantification of steroids extracted from the adsorbent permit the determination of about 50 mcg. of testosterone propionate and progesterone and about 6 mcg. of estradiol benzoate. This is important since steroids are generally compounded in pharmaceutical formulations in low concentrations. For the determination of the Δ^4-3 ketosteroids, progesterone and testosterone propionate, the colorimetric method by Umberger (2) was adapted. The visual fluorescence of estradiol benzoate, in sulfuric acid solution as an approach to determining steroid substances present in preparations in submicrogram amounts was found to be very useful. Data given by Szalikowsky (3) on the sulfuric acid fluorescence of various steroids proved valuable as a basis for the development of a quantitative procedure. It was found that steroid levels of 3 mg./ml. in 88% sulfuric acid could be measured satisfactorily. The Beer-Lambert law was obeyed over a suitable range of 20 to 140 mcg. of the steroid.

Table I—Analysis of Standard Steroid Mixtures

	Progesterone	Estradiol Benz.	Progesterone	Testosterone Prop.	Estradiol Benz.
	Added, mcg.		Added, mcg.		
	72.00	7.20	72.00	9.00	10.00
No.	Recovered, mcg.		Recovered, mcg.		
1	71.00	7.34	70.71	9.00	10.28
2	71.62	6.81	71.79	8.77	9.82
3	71.79	7.38	70.80	8.92	10.19
4	72.37	7.28	72.92	8.65	9.69
5	72.52	7.01	72.19	8.98	11.09
6	71.44	7.35	71.67	9.08	9.98
7	72.00	7.27			
8	71.12	7.12			
9	71.12	6.91			
Mean	71.67	7.18	71.68	8.90	10.17
Limits of error (P = 0.95)	99.40–100.60	96.37–103.63	96.60–103.40	97.10–102.90	98.04–101.96

Table II—Analysis of Commercial Multiple Pharmaceutical Preparations

Prepn.	Label Claim	Found, mg.		% of Label Claim	
Oil Injection Soln.					
Progesterone	20.0	19.2	20.1	96.1	100.5
Estradiol benz.	2.0	1.8	2.0	94.4	100.0
Progesterone	20.0	20.4	20.8	102.0	104.0
Testosterone prop.	25.0	23.6	25.8	94.3	103.2
Estradiol benz.	3.0	2.8	3.2	93.6	106.6
Microcrystalline Water Suspension					
Testosterone butyr.	50.0	47.9	49.8	95.8	98.0
Estradiol benz.	2.5	2.5	2.6	100.0	104.0
Progesterone	200.0	193.0	195.8	96.5	97.9
Estradiol benz.	10.0	9.9	10.5	99.6	105.2

The effect of time on the formation of fluorescence was determined by treatment of 20 mcg. of estradiol benzoate with 10 ml. of 88% sulfuric acid. The fluorescence was stabilized at room temperature after 1 hr. and was found to be practically stable for an additional hour.

To determine the precision of the procedures, analyses were carried out with standard steroid vegetable oil solutions prepared in this laboratory and containing exactly known quantities of steroids in approximately the same concentrations present in commercial preparations.

It is evident from the data in Table I that good reproducibility with a satisfactory standard deviation is obtained. The determination of steroids in commercial dosage forms showed that the results obtained are in good agreement with the labeled amount of the respective steroids. Results of these determinations are given in Table II.

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Keyphrases

Steroid mixtures—analysis
Progesterone and estradiol benzoate in oil
Progesterone, testosterone propionate, and estradiol benzoate in oil
Testosterone butyrate and estradiol benzoate aqueous suspension
Progesterone and estradiol benzoate aqueous suspension
TLC separation
Fluorometric analysis
UV analysis

Miotic Activity Produced by Inhibitors of Acetylcholinesterase

By A. Z. BOOTH, J. P. LONG, and K. R. LONG

Various biological activities of bis-phenacyl derivatives were investigated. The picoline derivatives demonstrated potent activity as inhibitors of human red blood cell cholinesterase. The compounds were effective miotic agents in mice and rabbits. Marked differences in toxicity of the positional isomers were noted. The 3-picoline derivative is a very toxic agent in mice, however it is equal to 2-picoline in activity as an inhibitor of acetylcholinesterase. Since the 2-picoline derivative is much less toxic, some mechanism other than inhibition of acetylcholinesterase must be involved to explain the high toxicity of the 3-picoline derivative.

SEVERAL SERIES of bis-quaternary compounds have been prepared that demonstrate potent inhibition of acetylcholinesterase. In general these compounds are much more selective for acetylcholinesterase than they are for pseudo cholinesterase (1). Since the innervation of the circular muscles of the iris is cholinergic and thus the degree of muscle tone is indirectly controlled by functional acetylcholinesterase, the biological activity was evaluated for the potent inhibitors of acetylcholinesterase described in this report. Quaternary compounds that are inhibitors of cholinesterase (*e.g.*, neostigmine) have not been particularly effective as miotic agents, probably because of difficulty of penetration across the cornea. In this series of agents this difficulty apparently was overcome by obtaining compounds that are more active biologically than neostigmine and incorporating into the structure components that would be expected to increase the lipid solubility.

METHODS

The ability to inhibit human red blood cell cholinesterase was measured using a Radiometer titrator type TTTlc titrograph type SBR 2C with Alga syringe buret. The reagents used were as follows: NaCl, 21.6%; NaCl, 22.5%; NaCl, 0.9%; acetylcholine iodide, 0.250 *M*; acetyl-β-methylcholine bromide, 0.5 *M*; Potassium hydrogen phthalate, 0.7351 Gm./200 ml. (2.0 ml. = 36 μmoles NaOH); 0.1 *N* NaOH (carbonate free). Two milliliters of potassium hydrogen phthalate is added to 10.0-ml. distilled water and this solution is titrated under N_2 with 0.1 *N* NaOH. From this titration curve the ordinate of the titrigraph chart is calibrated directly in μmoles NaOH used, which is equivalent to μmoles of liberated acid (cholinesterase activity). The abscissa of the chart gives time in minutes. Values are read as μmoles/ml./min. Red cells are prepared by centrifuging the blood sample at a fixed RFC and then washing twice with normal saline. The washed cells are suspended in an equal volume of normal saline (*i.e.*, 50% solution). To 22.0 ml. of distilled water is added 1.0-ml. whole blood, washed red cells, or plasma. The mixture is allowed to stand for 10 min. at 37–38° to allow for hemolysis and temperature equilibration. A 1.0-ml. amount of 21.6% NaCl is added and the mixture is again allowed to stand for a few minutes so that the temperature will equilibrate. The reaction mixture is then placed on the titrator and N_2 is blown over the top. After adjusting to pH 7.4 (preliminary titration), 1.0 ml. of acetylcholine iodide (0.250 *M*) is added and the reaction rate followed for about 8 min. If the true cholinesterase only is to be measured, 1.0 ml. of acetyl-β-methylcholine (0.5 *M*) is added in place of the acetylcholine iodide. Some samples were found to have too much

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Supported in part by grants NB-1396 and NB-4431 from the U. S. Public Health Service, Bethesda, Md.

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activity to measure even when 0.5 ml. of sample was used. In these instances 0.25 ml. of sample diluted with 0.25 ml. of normal saline was used. Inhibition studies were performed by allowing the enzyme reaction to proceed 3–5 min. to attain maximum activity and let the rate become linear. The inhibitors were added to 0.1-ml. amounts and the concentration in the reaction varied from 10^{-6} to 10^{-9} *M* depending upon the particular inhibitor employed.

The LD_{50} was calculated from data obtained with mice weighing 17–22 Gm. The compounds were administered intraperitoneally to three groups of 10 mice/group. Each compound was dissolved in distilled water and concentrations varied by 0.3 log intervals. The LD_{50}, 95% fiducial limits, and their relative activities were calculated by the methods of probits (2).

The miotic activities of the compounds were evaluated in mice and rabbits. The mice weighed 17–25 Gm. and 10 mice were used for each concentration of each drug. The compounds were prepared in distilled water and applied to the right cornea. The concentrations were varied by 0.48 log intervals. The solution was allowed to remain in contact with the cornea for 10 sec. and then the drug was washed off with distilled water (3). The amount of miotic activity was recorded at 10-min. intervals using a calibrated eyepiece and a dissecting scope (3). The relative activities of the compounds were calculated using a 2 × 2 parallel line assay (2).

Similar experiments were performed with Dutch rabbits weighing from 2–2.5 Kg. Seven rabbits were used for each dose of each compound. The pupil diameters were measured by a karatometer before and at 0.5-hr. intervals after the application of the compounds, which were dissolved in saline, with concentrations varied by 0.48 log intervals. The animals were observed for systemic effects such as diarrhea and salivation. The data were treated statistically as described above for mice.

A series of rabbits were anesthetized with sodium phenobarbital (200 mg./Kg.) administered intravenously and the influence of compounds *A* and *B* on pupil size and any alteration of arterial blood pressure responses to acetylcholine administered intravenously was evaluated.

RESULTS

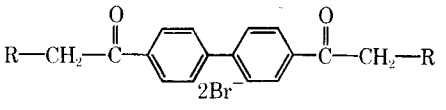
A summary of the structures and corresponding biological activity is shown in Table I.

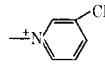
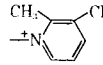
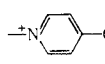
Toxicity—A wide variation in the LD_{50} following intraperitoneal administration was demonstrated by the compounds. One of the compounds, *B*, exhibited very high toxicity and it appeared to produce death by a mechanism other than inhibition of cholinesterase. The onset of observable symptoms and time for death with this compound in mice was slow, usually requiring 10 to 20 min. In contrast, the time for death for the remaining compounds was usually less than 8 min. The toxicity of the other compounds was less than that observed for neostigmine.

Inhibition of Acetylcholinesterase—The α- and β-picolines (compounds *A* and *B*) were approximately 1000 times more active than neostigmine bromide. Compound *C*, α,β-picoline, was somewhat less active. The γ-picoline derivative as well as the piperidine derivative (compounds *D* and *E*) were less active. Also the picoline compounds indicated a more rapid inhibition of the acetylcholinesterase than did neostigmine.

Miotic Activity—These quaternary compounds demonstrated miotic activity in both mice and rabbits. The duration of action was more than 2

TABLE I—BIOLOGICAL ACTIVITIES OF VARIOUS BIS-QUATERNARY INHIBITORS OF ACETYLCHOLINESTERASE



Compd.	R	LD_{50}[a] mg./Kg. (95% CI)	Acetylcholinesterase MI_{60}	Relative Miotic Activity (95% Fiducial Limits) Mice	Rabbits
Neostigmine	...	0.61 (0.46–0.80)	7.8×10^{-6}	...	...
A		6.7 (5.9–7.5)	8.2×10^{-9}	1[b]	1[c]
B		0.067 (0.047–0.101)	8.2×10^{-9}	0.65 (0.35–0.95)	0.55 (0.30–0.92)
C		0.88 (0.78–1.11)	3.0×10^{-8}	0.96 (0.76–1.22)	1.22 (0.87–1.75)
D		8.2 (6.6–12.4)	1.5×10^{-7}	0.11 (0.07–0.45)	0.43 (0.24–0.70)
E		8.4 (7.1–10.5)	4.7×10^{-6}	0.04 (0.03–0.06)	0.15 (0.08–0.36)

[a] The compounds were administered intraperitoneally. [b] The minimal concentration used for assay in mice was 0.011%. [c] The minimal concentration used for assay in rabbits was 0.033%.

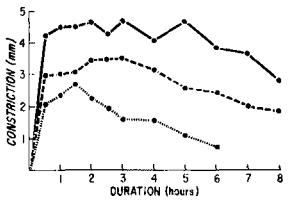


Fig. 1—Duration response curves in unanesthetized Dutch rabbits following topical application of compound A. Key: top, 0.44%; middle, 0.15%; bottom, 0.033%.

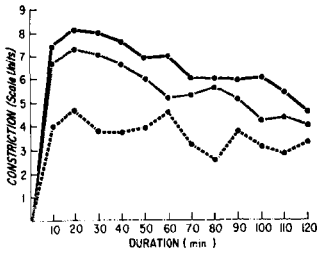


Fig. 2—Duration response curves for miosis in mice for compound A following topical application. Key: top, 0.1%; middle, 0.033%; bottom, 0.011%.

hr. in mice and 8 hr. in rabbits. The time to reach maximal inhibition in mice was 20 min. and approximately 0.5 hr. in rabbits. The dose-effect curves for compound *A* in rabbits and mice are shown in Figs. 1 and 2. Five rabbits were anesthetized and the right carotid artery was cannulated for blood pressure measurements. Control responses to acetylcholine, epinephrine, and norepinephrine were obtained. Compound *A* (0.6%) was applied to the cornea. During the following hour no alteration in blood pressure occurred and no potentiation or antagonism of the acetylcholine depressor response was observed. Also there was no alteration in the pressor responses to epinephrine or norepinephrine. With compound *B*, topical application of 0.5% solution to the anesthetized rabbits produced respiratory failure after a period of about 1 hr.

DISCUSSION

If heterocyclic substitution is placed on the bisphenacyl moiety, potent inhibitors of acetylcholinesterase are obtained. Compound *B* is of interest because of the high toxicity that apparently cannot be explained by its inhibition of cholinesterase. Since these are analogs of hemicholinium (4) it may be that this compound is exhibiting hemicholinium-type action.

Apparently these compounds cross the "corneal barrier" and reach acetylcholinesterase in concentrations sufficient to produce miosis of considerable duration. With the biphenyl nucleus as well as the substituted pyridine derivatives, the compounds may have components that contribute considerably to lipid solubility.

With the exception of compound *B* there was no evidence of systemic absorption of the compounds. Therefore it may be that the compounds can be localized effectively to the site of application, namely the cornea and anterior chamber of the eye.

SUMMARY

It has been demonstrated that a series of bis-quaternary phenacyl derivatives are potent inhibitors of acetylcholinesterase and are active miotic agents when applied topically to the cornea of both mice and rabbits. There is a wide variation in the LD_{50} of the compounds and the mechanism of toxicity is unknown for compound *B*. The high toxicity of this compound does not appear to be related to its ability to inhibit cholinesterase. The compounds, with the exception of compound *B*, do not appear to be absorbed systemically in quantities sufficient to potentiate the depressor response of acetylcholine.

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Keyphrases

Acetylcholinesterase inhibitors
Bis-quaternary ammonium compounds
Miotic activity
LD_{50} values

Crystal Structure of Thiolphthalide

By ELI SHEFTER

The crystal and molecular structure of thiolphthalide has been determined by X-ray crystallographic analysis. The crystals of this compound are orthorhombic (space group P $2_12_12_1$) with cell dimensions of a = 4.104 Å., b = 11.835 Å., and c = 14.602 Å. The structure was determined by Patterson and Fourier methods and refined by least squares to a final R value of 0.070. The two C—S bond lengths are 1.83 Å. and 1.77 Å., with the latter bond adjacent to the carbonyl group. The angle subtended by the C—S bonds is 94°.

THOUGH THE importance of thiolesters in biological systems has been well established (1), there appears to be a lack of structural data on such compounds in the literature. Significant structural variations have been observed between acetylcholine (2) and acetylthiolcholine (3), but the accuracy of these structures does not permit a detailed comparison of the valence states about the respective ester linkages. In an attempt to obtain more definitive information about the resonance in thiolesters the crystal structure of thiolphthalide has been completed.

EXPERIMENTAL

Elongated colorless prisms obtained by sublimation were used, and the unit cell dimensions for these crystals were measured on a General Electric XRD-6 diffractometer with Ni filtered Cu $K\alpha$ radiation. They were found to be a = 4.104 ± 0.001 Å., b = 11.835 ± 0.003 Å., c = 14.602 ± 0.007 Å., and $\alpha = \beta = \gamma = 90.0°$. The systematic absences of the $h\,0\,0$ ($h = 2n + 1$), $0\,k\,0$ ($k = 2n + 1$), and $0\,0\,e$ ($e = 2n + 1$) reflections indicated the space group P $2_12_12_1$. The density calculated from the X-ray data is 1.42 Gm./cm.^{3} (assuming 4 molecules in the unit cell). Attempts were made to measure the density of the material in potassium iodide solutions, but an accurate value was not obtained.

The intensity data were collected by the stationary counter-stationary crystal technique with balanced Ni-Co filters. The mounted crystal had to be coated with petroleum jelly in order to retard its sublimation. Even though it was so covered, a 20% decrease in intensity was observed over the whole data collection period. A correction for this effect was applied to the intensities. Lorentz and polarization factors were applied to the data as well as an adjustment for the anisotropy of transmission about the ϕ axis. A correction for the α_1-α_2 splitting was also made using a correction similiar to that of Tulinsky *et al.* (4). In the 2θ range of the data collection (0–120°), 640 reflections out of a total possible number of 660 had intensities significantly greater than their background counts.

The Patterson function revealed the sulfur position and from subsequent Fourier syntheses the locations of the other atoms were obtained. The positional and thermal parameters were refined by least squares using a block diagonal matrix approximation. A weighting of $1/f_c$, where f_c is the scattering factor for carbon, was utilized in the least squares analysis. This weighting is approximately equivalent to differential synthesis weighting. The 20 unobserved reflections were given zero weight. In the later stages of refinement the hydrogen atoms were located from an electron density difference Fourier synthesis (see Fig. 1)[1] and included in the final cycles of least squares. The final R value was 0.070 (including the 20 unobserved reflections which were given half the minimum observable intensity). The atomic form factors used in all the above calculations were those tabulated by Cromer and Waber (5).

The atomic positional and thermal parameters for the atoms together with their estimated standard deviations are given in Table I. A list of the observed and calculated structure factors amplitudes will be supplied upon request.

RESULTS AND DISCUSSION

The intramolecular bond distances and angles for the molecule are shown in Fig. 2. These values were not corrected for the thermal libration of the molecule. The mean standard deviation of the intramolecular bond lengths and angles for the nonhydrogen atoms are 0.01 Å. and 0.6°. The average error for the C—H bonds is 0.10 Å.

The two C—S bonds differ significantly in their bond lengths. The C(8)—S distance of 1.83 Å. agrees well with the "commonly" accepted C—S single bond length of 1.82 Å. (6), while the C(7)—S bond is significantly shorter than this value. The latter bond appears to have approximately 35% double bond character (estimated from the data presented in *Reference 6*). The C—O— bonds in various lactones (7–10) also show an analogous difference in their molecular structures. It is worthy to note that the bond length of the carbonyl group is quite similar to the average value found for the same group in the two γ-lactone moieties of anemonin (7), where the value is 1.19 Å. In comparing the thiol-lactone portion of this molecule to anemonin, a most striking difference is observed between the C—S—C and C—O—C angles, which 94.5 and 110° (mean value), respectively, this

Received June 22, 1967, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication August 18, 1967.

This research was supported by grant CA-10104-01 from the National Cancer Institute, U.S. Public Health Service, Bethesda, Md.

The author is indebted to Professor David Harker, Roswell Park Memorial Institute, for the use of his facilities, to the Computing Center of this University for a generous gift of computing time, and especially to Professor Henry G. Mautner, School of Medicine, Yale University, for his interest, and for the elongated colorless prisms which he so kindly furnished.

The *Chemical Abstracts* nomenclature for this compound is benzo(*c*)-thiophen-1-(3*H*)-one.

Note Added in Proof—Recent calculations by G. Edwin Wilson, Jr. [*Tetrahedron Letters*, **21**, 2007(1967), and personal communication] are consistent with the findings in this paper. He also suggested the possibility that the smaller C—S—C angle may also be due to the larger 3 p orbital of sulfur.

[1] Ellipses were drawn using a program written by C. K. Johnson, Oak Ridge Laboratory, Oak Ridge, Tenn.

TABLE I—ATOMIC POSITIONAL AND THERMAL PARAMETERS[a]

Atom	x/a	y/b	z/c	B_{11}/B_{iso}	B_{22}	B_{33}	B_{12}	B_{13}	B_{23}
C(1)	0.4191 (21)	0.0867 (5)	0.3776 (4)	0.0618 (53)	0.0047 (4)	0.0038 (3)	−0.0019 (30)	0.0015 (27)	0.0002 (6)
C(2)	0.3572 (22)	−0.0180 (5)	0.3390 (5)	0.0641 (51)	0.0051 (5)	0.0043 (3)	−0.0025 (33)	0.0056 (29)	−0.0004 (7)
C(3)	0.4543 (24)	−0.1145 (6)	0.3867 (6)	0.0926 (73)	0.0050 (5)	0.0061 (4)	0.0038 (38)	0.0119 (33)	0.0001 (8)
C(4)	0.6030 (29)	−0.1022 (7)	0.4696 (6)	0.0944 (73)	0.0088 (7)	0.0057 (4)	−0.0165 (50)	0.0097 (37)	−0.0053 (9)
C(5)	0.6678 (25)	0.0028 (9)	0.5068 (5)	0.0799 (71)	0.0123 (8)	0.0044 (4)	0.0075 (56)	0.0035 (33)	0.0012 (10)
C(6)	0.5692 (24)	0.0988 (6)	0.4619 (5)	0.0766 (66)	0.0075 (6)	0.0044 (3)	0.0093 (38)	−0.0022 (29)	0.0008 (8)
C(7)	0.2987 (22)	0.1834 (6)	0.3204 (5)	0.0761 (64)	0.0062 (5)	0.0045 (4)	0.0029 (36)	0.0023 (29)	0.0009 (8)
C(8)	0.1931 (24)	−0.0180 (6)	0.2479 (5)	0.0857 (70)	0.0062 (6)	0.0046 (4)	−0.0058 (35)	−0.0049 (30)	−0.0007 (8)
O	0.3192 (21)	0.2817 (4)	0.3384 (4)	0.1265 (66)	0.0042 (3)	0.0072 (4)	0.0066 (30)	0.0005 (31)	−0.0001 (6)
S	0.1263 (6)	0.1302 (2)	0.2179 (1)	0.0744 (14)	0.0082 (1)	0.0047 (1)	0.0026 (11)	−0.0062 (8)	0.0017 (2)
C(3)H	0.454 (267)	−0.177 (76)	0.359 (62)	4.1					
C(4)H	0.712 (272)	−0.152 (85)	0.506 (66)	4.7					
C(5)H	0.780 (206)	0.012 (64)	0.552 (49)	1.4					
C(6)H	0.719 (217)	0.156 (67)	0.485 (53)	2.4					
C(8)H$_1$	−0.062 (312)	−0.056 (88)	0.244 (72)	6.5					
C(8)H$_2$	0.390 (278)	−0.078 (81)	0.207 (65)	4.7					

[a] The estimated standard deviations are given in parentheses × 10^4 except those for the isotropic temperature factors of the hydrogens, which have an average error of ±2.0 Å.^{2}. The anisotropic temperature factor is of the form: $T = \exp. -(B_{11}h^2 + B_{22}k^2 + B_{33}\ell^2 + B_{12}hk + B_{13}h\ell + B_{23}k\ell)$.

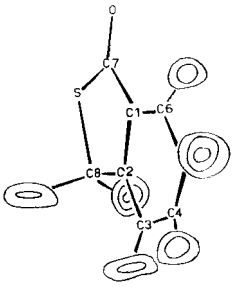

Fig. 1—Hydrogen atom electron density contours in a difference Fourier synthesis computed at the conclusion of the refinement. Contour levels at 0.1 eÅ.$^{-3}$, starting at 0.1 eÅ.$^{-3}$.

difference may be indicative of the ability of the sulfur atom to use its *d* orbitals in this bonding situation.

Within experimental error the other bond lengths and angles of the molecule agree favorably with their expected values.

The 10 nonhydrogen atoms comprising the molecule are essentially planar. The least squares equation of the plane through these atoms is:

$$-0.8897\ X - 0.0358\ Y + 0.4552\ Z = -0.9511 \text{ Å}.$$

where X, Y, and Z are the coordinates in Å. measured along a, b, and c, respectively. The root mean square deviation of the 10 atoms from this plane is 0.001 Å.

The packing arrangement of the molecules in the crystal lattice is shown in Fig. 3. The shortest intermolecular contacts are represented as dashed lines. With the exception of the C(8)—H2...O intermolecular contact, all the distances appear to be normal van der Waals contacts. Even though the estimated standard deviation for the C(8)H2...O distance is 0.12 Å., the contact is substantially smaller than the sum of the van der Waals radii between the respective atoms [1.2Å.(H) + 1.4 Å.(O)]. C—H...O hydrogen bonds have been found in a number of heterocyclic structures (11).

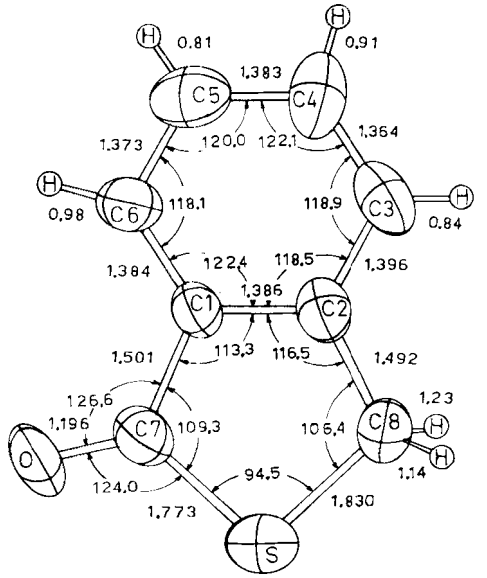

Fig. 2—The intramolecular bond distances and angles in thiolphthalide. The thermal ellipsoids are also shown.

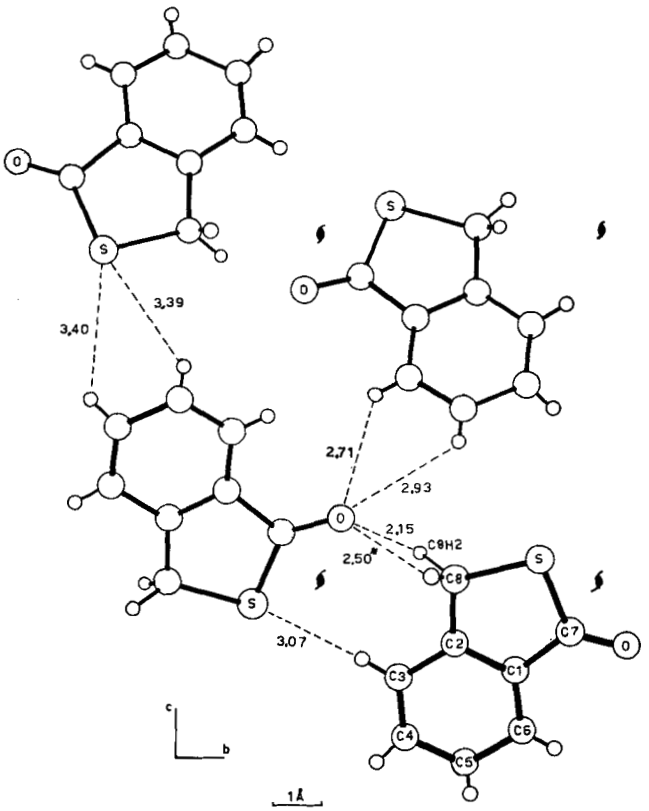

Fig. 3—The arrangement of the molecules as viewed down the a-axis. Dashed lines show shortest intermolecular contacts.

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Keyphrases

Thiolphthalide
Crystal structure of thiolphthalide
X-Ray analysis–crystal structure
Atomic positional and thermal parameters

Interaction of a Photosensitizer with Dipalmitoyl Lecithin

By R. SCHAUBMAN and A. FELMEISTER*

Monomolecular films of lecithin spread on aqueous subphases containing chlorpromazine hydrochloride were studied. The effects of ultraviolet irradiation and pH were determined. The degree of penetration of the chlorpromazine into the lecithin film was found to be dependent on surface pressure, pH, and ultraviolet irradiation. The biological significance of the data is discussed in relationship to a mechanism for photosensitization.

OVER 70 years ago Raab (1) reported the first photosensitized reaction in a biological system, noting that the killing power of acridine on paramecium was markedly increased in the presence of light. Since that time numerous other compounds have been implicated as photosensitizers, including such modern medicinal agents as the sulfonamides, phenothiazines, tetracyclines, and salicylanilides (2). While a number of theories have been advanced to explain this phenomenon (1–3), neither the exact mechanism of photosensitization nor the cellular constituents involved have been clearly established (4). The following fundamental steps, though, are generally assumed to occur: absorption of light by the photosensitizing agent; a photochemical reaction involving the photosensitizing agent, oxygen, and some cellular component; and development of increased cell permeability, probably as a result of the interaction of the photo-produced species with the constituents necessary for maintaining cellular integrity. The resultant clinical developments *i.e.*, erythema, wealing, edema, and in more drastic situations, hemorrhage and irreversible cell damage, are apparently then a consequence of increased cell permeability.

It is reasonable to postulate that this increased cell permeability involves interactions of the photo-produced species with the phospholipids or sterols of the membrane. These components are the principal structural elements which make up the membranes of the epidermal and dermal cells (5), and it would be expected that changes in their structure or orientation might lead to increased cell permeability or lysis.

A number of *in vivo* and *in vitro* models have been employed to study the photosensitization process, including erythrocytes, protozoa, and the skin of test animals (4, 6, 7). While all of these models are useful in assessing the damage produced by the photosensitization process, none of them serve to establish the particular cellular components involved in the reaction. The work of Allison *et al.* (4), however, did indicate that disruption of the membranes of lysosomes and mast cells might be the primary event in photosensitization.

It is the purpose of this work to investigate the usefulness of monomolecular films as models for studying photosensitized reactions with cell membrane constituents. For this initial phase, a spread film of L-α-dipalmitoyl phosphatidylcholine (lecithin) was selected, since it has been reported to be the principal phospholipid of epidermal cells (5). Chlorpromazine hydrochloride (CPZ) was selected as the photosensitizer because of the large number of cases of photosensitization reported implicating this drug (8), and because there is extensive information available concerning the photo-properties of CPZ (8–11).

EXPERIMENTAL

Materials—The L-α-dipalmitoyl phosphatidylcholine (lecithin) was obtained, chromatographically pure, from Mann Chemical Co., New York, N. Y. Chlorpromazine hydrochloride (CPZ), obtained from Smith Kline & French Laboratories, Philadelphia, Pa., was used without further purification. The water used was first deionized with a mixed resin bed, and then double distilled from alkaline permanganate using all glass equipment. All other chemicals were reagent grade.

Equipment and General Methods—Surface tension was measured by means of a Rosano tensiometer (12) using a thin roughened platinum plate. Films were spread on a 900-ml. capacity Langmuir-type trough equipped with a movable Teflon barrier. The trough temperature was maintained constant at 25° ± 0.1 by circulating water from a constant-temperature water bath. The edges of the trough were lightly coated with paraffin to prevent wetting. Ultraviolet irradiation of the films was accomplished by means of a Mineralite model V-41 UV lamp[1] fitted with a filter to screen out radiation below 270 mμ. The lamp was positioned about 50 mm. above the trough. The lecithin was dissolved in hexane–ethanol (95%:5% v/v) and spread on the aqueous subphase using an Agla micrometer syringe.[2] Measurements of pH and absorbance were made with a Beckman Zeromatic pH meter and a Beckman DB spectrophotometer, respectively.

Studies of Nonirradiated Films—The surface pressure (surface tension of the subphase minus the surface tension of the subphase in the presence of the film) of the lecithin film was determined at different areas per molecule on a subphase containing CPZ ($1 \times 10^{-4} M$) at pH values of 3.0 and 6.0. The subphase was adjusted to pH 3.0 and 6.0 with 0.001 *M* HCl and sodium acetate–acetic acid buffer (0.1 *M*), respectively. The ionic strength of the HCl solution was adjusted to 0.1 with sodium chloride. Surface pressure was also determined in the absence of CPZ for comparison.

Studies of Irradiated Films—Films were spread under the same conditions as used for the nonirradiated studies. Irradiation was initiated with the films at zero surface pressure and continued for 2 min. The surface pressure–surface area (π-A) curves were determined either immediately or 5 min. after irradiation.

Received April 14, 1967, from the College of Pharmaceutical Sciences, Columbia University, New York, NY 10023
Accepted for publication August 30, 1967.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
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[1] Ultra-violet Products, Inc., San Gabriel, Calif.
[2] Burroughs Wellcome Corp., Tuckahoe, N. Y.

Pure lecithin films, *i.e.*, in the absence of CPZ, were irradiated for 30 min. to determine their ultraviolet stability.

To determine the effect of initial pressure, films were irradiated for extended periods of time at two different initial film pressures.

RESULTS AND DISCUSSION

Irradiation of pure lecithin film at pH 3.0 and 6.0 for 30 min. showed no change in surface pressure, demonstrating the stability of these films to the ultraviolet radiation. Irradiation of the pH 3.0 subphase containing 1×10^{-4} *M* CPZ in the absence of the film also produced no change in surface pressure, though some decomposition did occur as evidenced by formation of color and changes in absorbance spectrum. However, at pH 6.0 an increase in surface pressure was observed even in the absence of the lecithin film. This increase in surface pressure apparently was due to the formation of an insoluble film, whose π-*A* characteristics were markedly dependent on rate of compression.

The π-*A* curves for the lecithin on a 1×10^{-4} *M* CPZ subphase at pH 3.0 and 6.0 were the same (Fig. 1). A π-*A* curve for pure lecithin (*i.e.*, in the absence of CPZ) is included for comparison. It is clear that film pressure develops at much larger lecithin areas when CPZ is included in the subphase. This apparently is the result of penetration of the CPZ molecule into the lecithin film leading to the formation of a mixed film. On continued compression of the nonirradiated film it can be seen that the π-*A* curves for both the lecithin and lecithin–CPZ monolayers become identical, due to the ejection of CPZ from the film at about 31 dynes/cm.

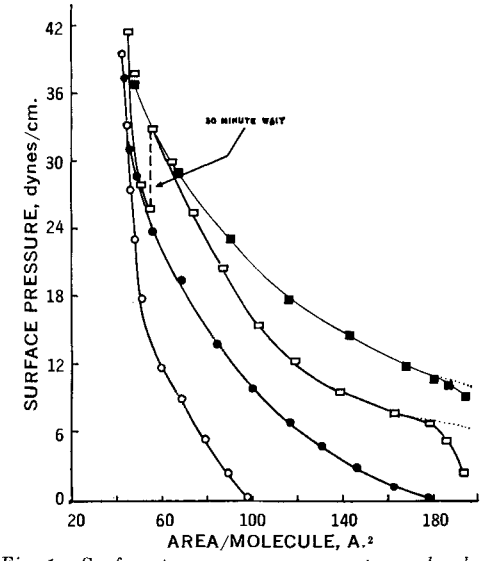

Fig. 1—Surface pressure versus *area per molecule for L-α-dipalmitoyl phosphatidylcholine at pH 3.0 and 6.0, 25°, and ionic strength 0.1. Key:* ○, *zero chlorpromazine HCl at pH 3.0 and 6.0;* ●, 1×10^{-4} M *chlorpromazine HCl at pH 3.0 and 6.0;* □, 1×10^{-4} M *chlorpromazine HCl at pH 3.0 irradiated for 2 min. at zero initial pressure;* ■, 1×10^{-4} M *chlorpromazine HCl at pH 6.0 irradiated for 2 min. at zero initial pressure; dotted lines indicate the curve that results if the readings are initiated 5 min. after the termination of the irradiation rather than immediately after.*

pressure. Similar results have been reported by Zografi *et al.* (13).

At both pH values irradiation results in significantly higher pressure at all areas as compared to the corresponding nonirradiated films. The rapid rise in surface pressure seen in the initial part of the π-*A* curves was observed consistently when the surface tension readings were begun immediately after the end of the irradiation. If, instead, the readings were begun 5 min. after the end of the irradiation, this sharp rise was not observed. The initial part of the curves then started at a higher pressure as indicated by the dotted lines (Fig. 1). The remainder of the curves were the same under both conditions. Thus it appears that changes occur, possibly in the composition of the photospecies, its orientation, or its interaction with the film or other components of the system, for a short period after the irradiation has ceased. However, apparently, after this initial period no further changes occur in the photoproduced species over the time of observation. At very high pressures it appears that the π-*A* curves of the irradiated films approach that of the pure lecithin. This suggests that the irradiated component as well as the nonirradiated CPZ is ejected from the film at very high pressures. Because of the uncertainty of the data at pressures very near the collapse pressure, it was not established whether the curves do eventually coincide and whether all of the irradiated components are ejected.

If the compression of the irradiated film on the pH 3.0 subphase was stopped on reaching an area of about 60Å² (33 dynes/cm. pressure) and maintained at this area for 30 min., the pressure gradually decreased to the value previously recorded for the nonirradiated film. Further compression then showed the same π-A characteristics as did the nonirradiated film (Fig. 1). When the compression was stopped at an area of 100Å.² or greater (*i.e.*, an area at which the pressure is about 17 dynes/cm. or less) and maintained at that area for 30 min., no change in surface pressure with time was observed. Thus it appears that the decrease in surface pressure with time, observed at the smaller area/molecule, was the result of a gradual ejection of the photo-produced species from the film rather than any change in the properties of the species itself.

At pH 6.0 the π-*A* curve after irradiation was shifted considerably to the right of that obtained at pH 3.0 (Fig. 1). However, if the surface pressure at each area developed from the irradiation of CPZ at pH 6.0 in the absence of the lecithin was subtracted from this curve, the resultant curve appeared to qualitatively follow that obtained at pH 3.0. Thus it appears that the differences observed at the two pH values are at least in part due to the increase in surface activity which results from irradiation of CPZ alone at pH 6.0. In addition, at pH 6.0 the change in π that resulted after a 2 min. irradiation decreased with increasing film pressure beyond 4 dynes/cm. The opposite effect was noted above an initial pressure of 8 dynes/cm. when the film was irradiated at pH 3.0 (Fig. 2). The reason for these differences was not determined, but they might be due to a number of factors, including the formation of different photo species at each pH value as well as the influence of pH on the rate of the photo reaction and on the photo species–film interaction.

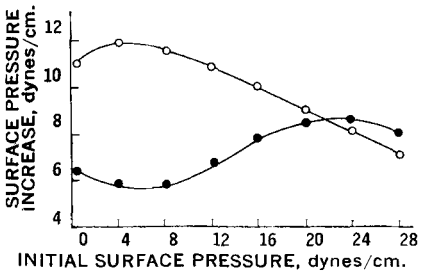

Fig. 2—The increase in surface pressure at various initial surface pressures for L-α-dipalmitoyl phosphatidylcholine at 25°, ionic strength 0.1, and 1 × 10^{-4} M chlorpromazine HCl following ultraviolet irradiation of the film at zero surface pressure for 2 min. Key: ●, pH 3.0; ○, pH 6.0.

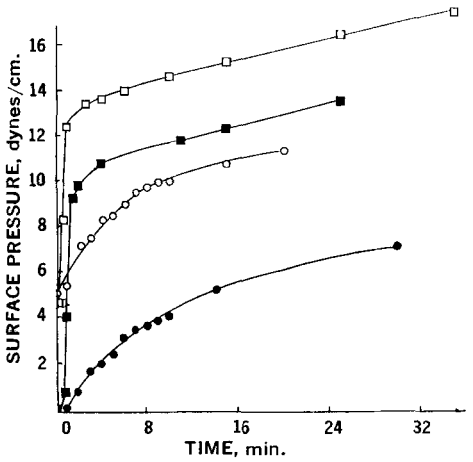

Fig. 3—Surface pressure versus *time of ultraviolet irradiation for L-α-dipalmitoyl phosphatidylcholine at 25°, ionic strength 0.1, and 1 × 10^{-4} M chlorpromazine HCl. At pH 3.0 irradiation started at zero surface pressure (●), and at 5.1 dynes/cm. surface pressure (○); at pH 6.0 irradiation started at zero pressure (■), and at 4.9 dynes/cm. surface pressure (□).*

When irradiation at pH 3.0 was extended beyond 2 min., it was noted that the rate of increase in surface pressure was fairly rapid during the first 7 to 8 min., after which the rate tailed off (Fig. 3). To determine whether this change in rate was a direct result of the increase in pressure that accompanied irradiation, and thus possibly a change in concentration and/or orientation of CPZ in the film, the effect of compression prior to irradiation was studied. The lecithin film, spread on a subphase containing 1 × 10^{-4} *M* CPZ at pH 3.0 was compressed to 5.1 dynes/cm. prior to the start of irradiation. The same rapid increase in pressure was noted, followed by tailing off after about 7–8 min. (Fig. 3). At pH 6.0 the increase in pressure developed much more rapidly and reached a higher pressure before leveling off (Fig 3). However, at both pH values the initial pressure (within the limited range studied) seemed to have no significant influence on either the rate of increase or total increase of surface pressure which resulted from irradiation. Thus it appears that the surface pressure which develops during the course of irradiation does not influence the photo reaction.

To determine whether the irradiated interaction was dependent on the presence of the film, the subphase at pH 3.0 was irradiated for 2 min. prior to spreading of the lecithin. The π-*A* curve of the lecithin film spread on this pre irradiated subphase was not significantly different from that obtained in previous experiments when the film was spread first and then irradiated for 2 min. at zero initial pressure.

CONCLUSION

Seeman *et al.* (14) have shown that CPZ penetrates the membrane of erythrocytes *in vitro*, producing membrane stabilization at concentrations of 10^{-4} *M* or lower, and lysis at concentration above 10^{-4} *M*. These effects have been related to the surface activity and the ability of this drug to penetrate spread monolayers (14). In view of this, and the results of this preliminary study, it appears that in CPZ mediated photosensitized reactions this drug may first penetrate the membranes of the cells of the dermis or basal layers of the epidermis at levels sufficient to stabilize these cell membranes. This is quite reasonable since plasma and tissue levels of CPZ are normally well below 10^{-4} *M*. Subsequent irradiation would tend to increase the pressure within the cell membranes until it reaches a level equivalent to that produced by lytic concentrations of CPZ. The result would be increased cell permeability, with its subsequent clinical effects.

It appears that a monomolecular lecithin film is a useful model to study the interactions of photosensitizing agents at cell membranes. Additional work is currently in progress to determine the effects of other photosensitizing agents on this model as well as the influence of antioxidants and ultraviolet absorbers.

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Keyphrases

Dipalmitoyl lecithin monomolecular films
Chlorpromazine HCl-lecithin film interaction
UV irradiation of film
Surface pressure-effect on film
Photosensitization-relation to chlorpromazine HCl penetration

Some Effects of Humidity and Heat on the Tableting Properties of Microcrystalline Cellulose Formulations I

By MUHAMMAD A. SHAH and ROBERT G. WILSON

Microcrystalline cellulose was exposed to the atmosphere of the tableting room. It was found that this exposed material did not tablet as well as material taken from the original, sealed container. Heating of the exposed material improved its tableting characteristics as was shown by increases in the weight and hardness of the tablets produced. Lubrication of the exposed material served to increase tablet weight, but this was accompanied by a decrease in the hardness of the tablets. The combination of heat and lubrication also gave heavier tablets, but again at a sacrifice in hardness.

MICROCRYSTALLINE CELLULOSE is a comparatively new material for use in the manufacture of pharmaceutical tablets. Its significance to the pharmaceutical industry is indicated by its recent inclusion in the "National Formulary" (1) as a pharmaceutic aid (tablet diluent). Several groups of investigators have reported on research work involving this new material. Among them, Lee *et al.* (2) studied the effect of water vapor pressure on the moisture sorption and stability characteristics of microcrystalline cellulose based tablets. Reier and Shangraw (3) found that microcrystalline cellulose-containing tablets swelled, gained in weight, and lost in hardness when exposed to humid conditions. The effect was shown to be a reversible phenomenon.

During preliminary work, it was observed that microcrystalline cellulose which had been exposed to the normal atmospheric conditions of the tableting room did not perform as well in tableting as did material of the same batch which was drawn from the original, closed container. The problem seemed to lie in the flow of material from the feed hopper of the tableting press. Several methods were suggested for improving the performance of the exposed material and experiments were carried out to test the effects of heat and/or lubricant upon the exposed microcrystalline cellulose. The results of two of these experiments are reported here.

MATERIALS AND EQUIPMENT

The microcrystalline cellulose,[1] as supplied was packed in a polyethylene bag contained in a standard cylindrical fiber drum. The magnesium stearate[2] was of USP quality.

All tablets were compressed on a single-punch Stokes model B tablet machine, using $^3/_8$ in. standard concave punches and die. The die fill adjustment and upper punch movement were set so they would remain essentially constant throughout any one part of the investigation. The tablets were compressed at the rate of 70 tablets per minute. Tablet weights were measured with an analytical balance. Tablet hardness was determined using a Pfizer hardness tester. The USP disintegration test, using distilled water, and the Roche Friabilator were used to test disintegration and friability, respectively. Where it was necessary to heat the microcrystalline cellulose, a bacteriological incubator was used in which the temperature could be held to $\pm 2°$.

Received June 9, 1967, from the College of Pharmacy, University of Houston, Houston, TX 77004

Accepted for publication August 10, 1967.

Abstracted from a thesis submitted by Muhammad A. Shah to the University of Houston, Houston, Tex., in partial fulfillment of Master of Science degree requirements.

[1] Marketed as Avicel by FMC Corp., New York, N. Y.

[2] Distributed by R. F. Reyson Chemicals Co., New York, N. Y.

EXPERIMENTAL

A—Nonlubricated Microcrystalline Cellulose—In one part of the experimental work, microcrystalline cellulose was removed from the original container and exposed to the uncontrolled atmosphere of the tableting room for a 3-week period. During this time the temperature ranged from 10–28°, at a relative humidity of 50–80%. An amount of exposed material sufficient for a 60-min. tableting run was divided into three portions. One of these was placed in an oven at 37° for 16 hr., while the other two were held under ambient room conditions. The tableting press was adjusted, and all three batches were then run consecutively, starting with a room temperature batch, continuing with the heated batch, and then finishing the run with the second batch of material at room temperature. This design served two purposes: (*a*) by running the room temperature microcrystalline cellulose before and after the heated batch, it could be shown that the press settings would remain essentially constant throughout the entire run; and (*b*) it was possible to compare the tableting properties of the heated and unheated portions of the exposed material. The tablets were collected in a predetermined pattern so as to avoid any variation due to the start-up of the press or shifts from one material to another in the course of the tableting run. The results obtained are shown in Table I.

There was but a slight variation in tablet weight or in tablet hardness between tablets made from the same portion and no significant difference between the two batches of room temperature material compressed before and after the heated batch. This indicates that the press settings remained constant, and the powder flowed at uniform rates during these two parts of the experiment. There was, however, a marked and statistically significant difference between the exposed unheated, and the exposed heated, portions of the material. Heating the exposed microcrystalline cellulose caused it to fill the die cavity to a greater extent and thus to produce a heavier, harder tablet. This was accomplished without a sacrifice in the disintegration characteristics of the tablets and with what appeared to be a slight improvement in tablet friability.

B—Lubricated Microcrystalline Cellulose—In another part of the work, microcrystalline cellulose was exposed for a 3-week period and again divided

TABLE I—EFFECT OF MICROCRYSTALLINE CELLULOSE ON TABLETS

Property	Microcrystalline Cellulose—Room Temp., Compressed Before Heated Portion	Microcrystalline Cellulose—Heated 16 hr. at 37°
Av. tablet wt. (100 tablets)	220.8 mg.	240.8 mg.
S. D. in av. tablet wt.	1.7 mg.	1.3 mg.
Av. tablet hardness (20 tablets)	10.6 lb.	14.5 lb.
S. D. in av. tablet hardness	0.6 lb.	0.6 lb.
Disintegration time (USP test)	Practically instantaneous	Practically instantaneous
Friability (Roche Friabilator)	0.15% loss in wt.	0.11% loss in wt.

into three parts. One part was held as an untreated control. The other two parts were lubricated with magnesium stearate at a concentration of 1% by weight. One of these lubricated portions was then heated in an oven at 37° for 16 hr., while the other was held under room conditions. The press was set, and the three portions were processed in sequence through the tableting machine. The results obtained are shown in Table II. The data on the tablets produced from the plain, unlubricated microcrystalline cellulose show that the tablets of part *B* were lighter than those of part *A*, *i.e.*, a different press setting, as well as having been treated at a different temperature level. The results of the two parts of the experiment are not to be compared against each other. Considering part *B*, alone, it is evident that the addition of magnesium stearate gave a marked and significant improvement in average tablet weight, and that an even further and again statistically significant improvement was obtained by heating the lubricated material for 16 hr. at 37° prior to tableting. However, these significant increases in the average weights of the tablets produced were accompanied by important decreases in tablet hardness and friability. In addition it was observed that the lubricated microcrystalline cellulose seemed to give tablets with imperfect surfaces, and ones which would presumably be too soft for most commercial purposes. Further studies would be required before a definite decision could be reached on this point.

TABLE II—EFFECT OF MAGNESIUM STEARATE ON MICROCRYSTALLINE CELLULOSE TABLETS

Property	Microcrystalline Cellulose—Plain (No Lubricant), Held at Room Temp.	Microcrystalline Cellulose—1% Magnesium Stearate, Held at Room Temp.	Microcrystalline Cellulose—1% Magnesium Stearate, Heated at 37°
Av. tablet wt. (100 tablets)	196.6 mg.	249.8 mg.	257.3 mg.
S. D. in av. tablet wt.	1.7 mg.	1.7 mg.	1.3 mg.
Av. tablet hardness (20 tablets)	9.6 lb.	6.1 lb.	6.2 lb.
S. D. in av. tablet hardness	0.7 lb.	1.2 lb.	1.4 lb.
Disintegration time (USP test method)	Practically instantaneous	Practically instantaneous	Practically instantaneous
Friability (Roche Friabilator)	0.18% loss in wt.	0.92% loss in wt.	0.87% loss in wt.

SUMMARY

Microcrystalline cellulose was used in direct compression tableting studies. It was observed that the tableting characteristics of the material changed with time during exposure to the atmosphere of the tableting room. Three procedures were suggested as possible ways to improve the performance of the exposed material. These were: (*a*) to heat the exposed material, (*b*) to add magnesium stearate as a lubricant, and (*c*) to combine the effects of heat and lubrication.

An analysis of variance, using the F ratio, was applied to the data from each of the experimental runs. It was found that heating the exposed microcrystalline cellulose prior to its direct compression gave a statistically significant ($P < 0.01$) increase in both the weight and hardness of the finished tablets. The use of magnesium stearate as a lubricant also increased the tablet weight significantly ($P < 0.01$), but there was an undesirable decrease in the hardness of the finished tablets. The combined use of heat and lubricant gave an increase in average tablet weight over that obtained with lubricant alone, but the hardness of the tablets was still at a low and unsatisfactory level. Futher studies are being conducted on the effects of exposure upon the tableting characteristics of microcrystalline cellulose. More should be known about the underlying reasons for the changes that have been observed.

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Keyphrases

Microcrystalline cellulose tablets
Moisture effect
Heat effect
Magnesium stearate lubricant effect
Tablet hardness
Tablet friability

Effect of Molecular Conformation on Interaction of Organic Species in Water. Benzamides and Anisamides with Theophylline and Riboflavin

By MASAHIRO NAKANO* and TAKERU HIGUCHI†

Complexing tendencies of *N,N*-dimethylbenzamide and *N,N*-dimethylanisamide toward theophylline and riboflavin in water were found to be significantly less than those of *N*-substituted and *N*-monomethyl analogs. It is suggested that rotation of *N,N*-dimethylamide group about the benzene ring-carbonyl carbon bond may inhibit formation of such complexes. *N,N*-Dimethylcinnamamide whose *N,N*-dimethylamide group can lie on the same plane as the styrene group was shown to be as good an interactant as the unsubstituted cinnamamide.

HIGUCHI AND PISANO (1) and Nakano with Higuchi (2) have observed that some *ortho*-substituted benzoate ions other than hydroxy were generally much weaker binding agents than the unsubstituted and the corresponding *meta*- or *para*-substituted benzoate ions. In these systems the observed effect was attributed to steric interferences by groups in the *ortho* position.

The present report is concerned with a similar observation resulting from a substitution in a side chain. *N,N*-Dimethylbenzamide was found to be a significantly weaker interactant toward theophylline and riboflavin than unsubstituted and *N*-monosubstituted benzamide. Measurements were also carried out on anisamides and cinnamamides.

EXPERIMENTAL

Materials—Benzamide (Eastman), anisamide (Aldrich), and cinnamamide (Aldrich) were recrystallized from water, m.p. 128°, 166°, 147°, respectively. *N*-Methylbenzamide, *N*-methylanisamide, *N,N*-dimethylbenzamide, *N,N*-dimethylanisamide, and *N,N*-dimethylcinnamamide were synthesized from their acid chlorides (Aldrich) and gaseous monomethylamine or dimethylamine in ether and purified by vacuum distillation or by recrystallization from water, m.p. 80°, m.p. 116°, b.p. 132° at 15 mm., m.p. 42°, and m.p. 103°, respectively. The PMR spectrum of each compound was also checked on a Varian A-60A spectrometer. Theophylline (m.p. 272°) and riboflavin were of NF and USP grade, respectively, and were used without further purification.

Solubility Studies—For benzamide–theophylline systems, increasing amounts of benzamide solution ($0–3 \times 10^{-1}$ *M*) were pipeted into vials and the solutions were brought to a constant final volume (10 ml.) in presence of fixed, excess amount of solid theophylline. For cinnamamide–theophylline systems, increasing amounts of theophylline solution ($0–3 \times 10^{-2}$ *M*) were pipeted into vials and excess amounts of cinnamamides were added. Equilibration was effected by rotation of the vials in a water bath thermostated at 25.0 ± 0.1° for 48 hr.

After equilibration, an aliquot of the supernatant liquid was withdrawn and analyzed spectrophotometrically after dilution with methanol. Wavelengths chosen were 282 mμ for benzamide–theophylline systems and 290 mμ for cinnamamide–theophylline systems. Small absorption due to the known amount of benzamides for the former systems and that of theophylline for the latter systems were substracted from the total observed absorbance. Apparent 1:1 stability constants were computed according to the phase-solubility technique (3).

Spectrophotometric Studies—Stability constants for riboflavin complexes were evaluated from spectroscopic measurements. Volumetric flasks, each containing 4×10^{-5} *M* riboflavin and varying amount of benzamides or anisamides ($0–5 \times 10^{-2}$ *M*), were equilibrated in a 25° water bath. These solutions were brought to final equilibrium in a Cary 15 spectrophotometer cell compartment kept at 25°. The absorbance was measured at 490 mμ and the Foster *et al.* method (4) was employed for the computation of stability constants.

PMR Studies—Proton magnetic resonance (PMR) spectrum of *N,N*-dimethylbenzamide (5×10^{-2} *M*) in water at 25° was recorded on a Varian A-60A spectrometer with a V-6057 variable temperature accessory. PMR spectra of anisamides (3×10^{-2} *M*) in deuterium oxide were recorded on the same spectrometer at temperature of the probe (38°). DSS was used as an internal reference.

RESULTS AND DISCUSSION

Experimentally observed apparent stability constants of benzamide and substituted benzamides interacting with theophylline as determined by the solubility method are shown in Table I. It is probable that these values reflect, to a limited degree, contributions from species other than 1:1;

TABLE I—APPARENT 1:1 STABILITY CONSTANTS (M^{-1}) WITH THEOPHYLLINE IN WATER AT 25° DETERMINED BY THE SOLUBILITY METHOD

Compd.	Stability Constant (M^{-1})
Benzamide	12
N-Methylbenzamide	13
N,N-Dimethylbenzamide	2

Received July 10, 1967, from the School of Pharmacy, University of Wisconsin, Madison, WI 53706

Accepted for publication August 9, 1967.

M. Nakano expresses his appreciation to Professors K. Kakemi and H. Sezaki, Kyoto University, for their encouragement and interest.

This work was supported in part by grant GM-05830 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

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but these are not expected to affect significantly the relative values. Corresponding values obtained spectrophotometrically for a series of amides interacting with riboflavin are shown in Table II. For

TABLE II—APPARENT 1:1 STABILITY CONSTANTS (M^{-1}) WITH RIBOFLAVIN IN WATER AT 25° DETERMINED BY THE SPECTROPHOTOMETRIC METHOD

Compd.	Stability Constant (M^{-1})
Benzamide	8
N-Methylbenzamide	10
N,N-Dimethylbenzamide	2
Anisamide	21
N-Methylanisamide	33
N,N-Dimethylanisamide	4

both systems it would appear that a single *N* substitution produced a small but a definite increase in binding tendencies. *N,N*-Dimethyl series, however, exhibit in every case a dramatic drop-off in their associative ability. It is suggested that this situation may arise as a result of the difference in the steric conformation of the disubstituted amides as compared to the others. Benzamides and *N*-methylbenzamides can have completely planar structures which would permit maximum conjugation between the amide groupings and the benzene rings. These have, as expected, closely similar spectra (5). With *N,N*-dimethylbenzamides, on the other hand, a completely planar structure is impossible because of the interference between the methyl groups and the *ortho* hydrogen atoms (5). The interference can be relieved by rotation about the Ph—CO bond or the CO—NMe_2 bond. It is more likely that the amide group remains planar because of its high resonance energy (6), and that rotation takes place about the Ph—CO bond. The ultraviolet spectrum of *N,N*-dimethylbenzamide in water, furthermore, has no peak above 220 mμ, a situation characteristic of nonplanar molecules (7). The PMR spectrum of *N,N*-dimethylbenzamide at 25° in water, moreover, was observed to have 2 peaks, separated by 7 c.p.s. from each other, corresponding to 2 different methyl groups with respect to carbonyl group. This effect is most likely due to hindered rotation about the CO—NMe_2 bond. The relative PMR chemical shifts of the *ortho* and *meta* hydrogens in anisamides shown in Fig. 1 also appear to reflect the effects of *N* methylation.

The complexing tendency toward theophylline of *N, N*-dimethylcinnamamide, whose amide group is sufficiently removed from the benzene ring for steric hindrance to planarity to be absent, was also examined and the results are shown in Table III.

Unlike the benzamide *N, N*-dimethylcinnamamide

TABLE III—APPARENT 1:1 STABILITY CONSTANTS (M^{-1}) WITH THEOPHYLLINE IN WATER AT 25° DETERMINED BY THE SOLUBILITY METHOD

Compd.	Stability Constant (M^{-1})
Cinnamamide	32
N,N-Dimethylcinnamamide	30

appears to be almost as good an interactant as the unsubstituted cinnamamide and has essentially the same absorption as the latter when account is taken of the bathochromic effect of the methyl groups (5).

It is believed that the interactions of aromatic molecules in water are likely plane to plane stacking (2). If this is true, the observed poor complexing tendencies of *N, N*-dimethylbenzamides are possibly due to prevention of approaching molecules from coming closer to the aromatic ring. The decreased interactive tendencies of the dialkyl derivatives, on the other hand, may also arise in part from the reduction in resonance caused by the steric effect.

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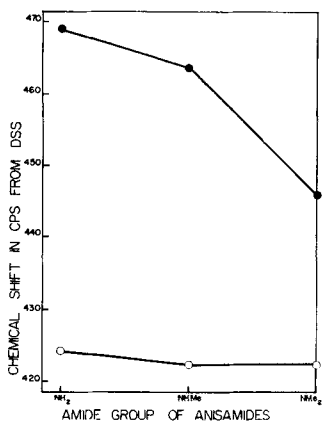

Fig. 1—The plots showing the chemical shifts of ortho (●) *and* meta (○) *protons to amide group in anisamides* (3×10^{-2} M) *in* D_2O.

Keyphrases

- Molecular conformation effect on complex formation
- Benzamide and substituted benzamide–theophylline systems
- Cinnamamide and substituted cinnamamide–theophylline systems
- Benzamide and substituted benzamide–riboflavin systems
- Anisamide and substituted anisamide–riboflavin systems
- PMR spectrometry
- UV spectrophotometry–complex stability
- Stability constants

Method for Determining Ethchlorvynol in Urine and Serum by Gas Chromatography

By DON W. ROBINSON

A new gas chromatographic technique for the identification and quantitative determination of ethchlorvynol in urine and serum is described. The method was developed because of the need for a rapid analysis of the drug in cases of suspected overdosage. The procedure was tested at concentrations of 3 to 100 mcg./ml.

PREVIOUS METHODS (1, 2) for the determination of ethchlorvynol,[1] a nonbarbiturate hypnotic, in biological fluids have involved distillations and chemical reactions. The procedure described is specific and does not require altering the ethchlorvynol molecule.

In developing a gas chromatographic method, it was noted that the presence of alcohol in urine would interfere with the ethchlorvynol peak. The syringe and injector become clogged when serum was injected directly. These problems were solved by the addition of an extraction step.

Two drugs of similar structure, ethinamate[2] and methylparafynol,[3] were found not to interfere. Kazyak and Knoblock have also shown different retention times for these compounds on a silicone column (3).

Recovery of therapeutic and overdoses of ethchlorvynol using chemical methods have been reported in the literature (1, 2).

EXPERIMENTAL

Method and Materials—*Instrument*—A gas chromatograph equipped with a flame ionization detector. A 5 ft. by $^1/_8$ in. outside diameter stainless steel column packed with Silicone L-46[4] 20% on acid washed Chromosorb W 80 to 100 mesh.[5] The liquid phase was applied by the evaporation technique. The column should be conditioned at 225° for 24 hr. prior to use. A Hamilton microliter syringe equipped with a Chaney adapter set to deliver 5 μl.

Instrument Conditions—Column temperature, 125°; injector temperature, 150°; nitrogen carrier gas, 22 ml./min.; electrometer, maximum sensitivity; and attenuator, 1.

Reagents and Solutions—Carbon disulfide analytical reagent.[6] Urine and serum from a subject who has not recently ingested ethchlorvynol and which does not produce an ethchlorvynol response after extraction and injection into the chromatograph.

Procedure—Weigh accurately 0.1 Gm. of ethchlorvynol into a 100-ml. volumetric flask. Bring to volume with urine or serum which does not contain the drug. This solution contains 1000 mcg./ml. and is used for making further dilutions. The solution may be frozen and used at a later date.

Urine Analysis—Using a separator, extract 50 ml. of urine with two portions of carbon disulfide, 30 and 20 ml. Inject 5 μl. of the carbon disulfide layer and measure the area of the ethchlorvynol peak. Extract a standard of ethchlorvynol in urine at the suspected concentration in exactly the same manner. Inject 5 μl. of the standard extract and measure the peak area.

Serum Analysis—The extraction procedure for serum must be modified because of the usually small volume available. Pipet 5 ml. of serum along with 5 ml. of carbon disulfide into a glass-stoppered centrifuge tube. Shake gently for 2 min. Care must be taken to prevent formation of an emulsion which cannot be broken. Centrifuge at 2000 r.p.m. for 15 min. Inject 5 μl. of the carbon disulfide layer and measure the peak area. Treat a standard of ethychlorvynol in serum at the suspected concentration in exactly the same manner.

Under the conditions described, the ethchlorvynol should be eluted in 3 to 4 min. A chromatogram is shown in Fig. 1. Using toluene as the reference standard, the relative retention time is 3.0:

Calculation—

$$\frac{\text{area sample peak}}{\text{area std. peak}} \times \text{concn. of std., mcg./ml.} = \text{sample concn., mcg./ml.}$$

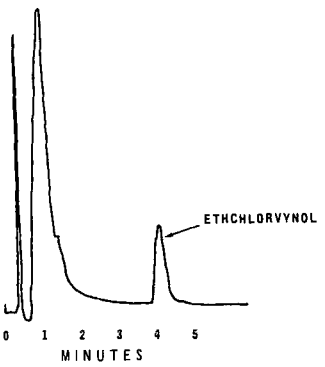

Fig. 1—Chromatogram of carbon disulfide extract from human serum containing 18 mcg./ml. ethchlorvynol. Five foot × $^1/_8$ in. stainless steel column packed with 20% Silicon L-46 on Chromosorb W 80 to 100 mesh. Temperature 125°; nitrogen at 22 ml./min.

DISCUSSION

Carbon disulfide was chosen as the extracting solvent because of its good solvent properties and because of the low response it produces on the flame ionization detector (4).

Received May 15, 1967, from the Analytical Research Department, Abbott Laboratories, North Chicago, IL 60064
Accepted for publication August 8, 1967.

[1] Placidyl. Abbott Laboratories, North Chicago, Ill.
[2] Marketed as Valmid by Eli Lilly & Co., Indianapolis, Ind.
[3] Marketed as Dormison by Schering Corp., Union, N. J.
[4] Silicone Division, Union Carbide Corp.
[5] Johns-Manville Corp.
[6] Mallinckrodt No. 4352. Purification is not necessary.

The standard extracts are stable and may be tightly sealed and stored at low temperature for use at a later date.

To further confirm the presence of ethchlorvynol, the extract can be chromatographed on a column of different polarity. A 5 ft. by $^1/_8$ in. outside diameter stainless steel column packed with diethylene glycol succinate 20% on Gas-Pack W[7] 60/80 gives good separation. When operated under the same conditions employed with the Silicone L-46 and using benzyl alcohol as the reference standard, the relative retention time for ethchlorvynol is 0.54.

To establish the linearity of the method, standard curves were prepared for both urine and serum. Concentrations of 5, 10, 25, 50, and 100 mcg./ml. were prepared and extracted. The curves are shown in Figs. 2 and 3. The ability of the procedure to recover the drug quantitatively from urine and serum was tested. The results were obtained by comparing the sample to a standard of similar concentration. Two of the urine samples also contained 0.2% ethanol to check for possible interferences. The results are in Tables I and II.

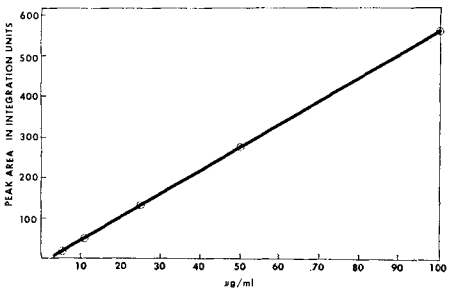

Fig. 2—Standards of 5, 10, 25, 50, and 100 mcg./ml. ethchlorvynol in urine extracted with CS_2.

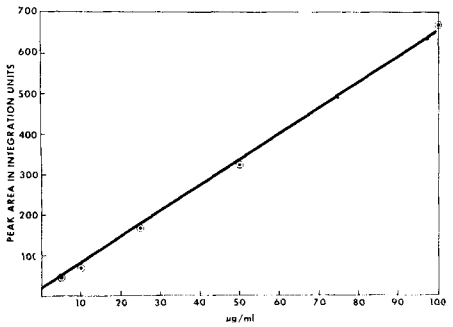

Fig. 3—Standards of 5, 10, 25, 50, and 100 mcg./ml. ethchlorvynol in serum extracted with CS_2.

The method can be performed in approximately 1 hr. and used to confirm suspected misuse of the drug.

[7] Chemical Research Services.

TABLE I—RECOVERY OF ETHCHLORVYNOL FROM HUMAN URINE

Added to Urine, mcg./ml.	Found, mcg./ml.
3[a]	2
3[a]	2
3[a]	2
18	18
18	17
18	19
33	32
33	31
33	32
65[a]	70
65[a]	61
65[a]	63
82	81
82	83
82	87

[a] Also contained 0.2% ethanol.

TABLE II—RECOVERY OF ETHCHLORVYNOL FROM HUMAN SERUM

Added to Serum, mcg./ml.	Found, mcg./ml.
3	3
3	4
3	3
18	19
18	18
18	18
33	33
33	34
33	33
65	65
65	67
65	66
82	82
82	79
82	80

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Keyphrases

Ethchlorvynol—analysis
Urine—extraction of ethchlorvynol from
Plasma—extraction of ethchlorvynol from
Carbon disulfide reagent
Gas chromatographic analysis

Mechanism of Action of Starch as a Tablet Disintegrant II Effect of Pepsin and Surfactants on Swelling of Starch Grains at 37°

By JAMES T. INGRAM and WERNER LOWENTHAL

The study of the effects of the individual components of simulated gastric fluid USP on starch grain swelling is completed. There is no statistically significant swelling demonstrated by pepsin in the submersion medium when compared to simulated gastric fluid and distilled water. Surfactants demonstrated no significant effect on starch grain swelling over that of distilled water. Apparent effectiveness of surfactants in improving tablet disintegration in some cases is postulated as being due to an increase in the rate of wetting. In the same experiment, the significant differences observed between corn and amioca starches and the moisture contents of these two starches are postulated as being due to the initial size differences of the grains being maintained during the experiment.

It does not seem probable that untreated or unmodified starch will swell sufficiently at 37° to cause tablet disintegration. If swelling is the primary mechanism whereby starch exerts its disintegrant effect, then this property must be imparted to the starch by the environmental conditions. The minimal effects of acid and salts in the submersion medium were demonstrated previously (1). Changes in pH had little effect on swelling; however, evidence was obtained to show that starch grains may swell more at a pH of 5.3 than in lower pH media. Salts did effect increased swelling, and the results indicated that salts of polyvalent cations produced more swelling than the salts of monovalent cations. Ionic concentration did not show an effect on swelling.

In order to complete the study of the effects of the individual components of simulated gastric fluid USP (SGF) on starch swelling, pepsin is included as a submersion medium in the present study.

Since surfactants have been shown to be effective in certain cases in improving tablet disintegration (2, 3), an experiment is included to determine their effect on starch swelling. Surfactants, when sprayed onto granulations containing starch, were shown by Cooper and Brecht (2) to be generally effective in improving tablet disintegration. A total of 21 surfactants, representing anionic, cationic, and nonionic classifications as well as a wide range of chemical and physical properties, were incorporated into calcium lactate tablets containing starch. Disintegration tests on these tablets revealed that the four surfactants that were most efficient in improving disintegration time were anionic in ionization, waxy solids, and structurally related to sulfosuccinic acid.

Aradi (3) also studied the influence of surfactants on disintegration and found a positive relationship between surfactants and improvement of disintegration time.

Gray and Schoch (4) studied the effect of various surfactants on the swelling behavior of several typical starches. Viscosity measurements on aqueous solutions of the starches heated above the pasting range were used as a measure of swelling. It was reasoned that if the surfactant complexes strongly with the linear fraction of the starch, it should restrict swelling. If no such complex is formed or if a waxy starch is used, the surfactant should act as a wetting agent and assist hydration of the starch molecules with subsequent swelling of the starch grains. Results of the study show that polar surfactants which complex strongly with the linear fraction restricted the swelling and solubilization of corn, potato, and even waxy sorghum starches. Cationic quaternary ammonium surfactants anomalously increased the hydration of corn and waxy sorghum starches, but drastically reduced the swelling and solubilization of potato starch.

EXPERIMENTAL

The study was planned to determine the effect of pepsin and surfactants on starch grain swelling. The swelling of starch grains was determined microscopically by measurement of the individual grain diameters using the procedure described previously (1). Two full factorial experimental designs were used and analyses of variance were calculated to determine the effects of pepsin and surfactants with various starches. Two types of starches at two moisture contents were used to determine if the type of starch and moisture content would have any effect on swelling in the submersion media used and to give more sensitive experimental design.

Commercial grades of corn and amioca (waxy corn) starches were chosen for the investigation. For the high moisture content, the commercially available starches[1] were used. For the low moisture content cornstarch, a commercial redried cornstarch[2] (P825) containing 1.5% maximum moisture was used. For the low moisture content amioca starch, the commercially available amioca starch was dried at 93° to a constant moisture content of 2.88% w/w.

RESULTS AND DISCUSSION

To investigate the effect of pepsin on swelling of starch grains, a $2 \times 2 \times 3$ full factorial experimental design was used. A 0.32% w/w aqueous solution of pepsin, distilled water, and SGF were used as submersion media. It was previously shown that there is no significant difference between distilled

Received June 8, 1967, from the School of Pharmacy, Medical College of Virginia, Richmond, VA 23219
Accepted for publication August 15, 1967.
Abstracted in part from the thesis presented by James T. Ingram to the Graduate School, Medical College of Virginia, in partial fulfillment of Master of Science degree requirements.
Previous paper: Ingram, J. T., and Lowenthal, W., *J. Pharm. Sci.*, **55**, 614(1966).

[1] Melojel (MJ) and amioca starch marketed by National Starch and Chemical Corp.
[2] Marketed as Purity 825 by National Starch and Chemical Corp.

TABLE I—COMPARISON OF MEAN GRAIN SIZES OF STARCHES AFTER SUBMERSION FOR 5 min. IN PEPSIN SOLUTION, SGF, AND DISTILLED WATER

Moisture Content	Medium: Distilled Water	SGF	Pepsin
	Corn		
High (MJ)	6.129[a]	6.408	6.133
Low (P825)	5.919	5.805	6.665
	Amioca		
High	6.157	6.388	6.571
Low	5.672	6.320	6.197

[a] Mean grain sizes in scale divisions; one scale division = 1.8 μ.

TABLE II—EFFECT OF SURFACTANTS IN SUBMERSION MEDIUM ON MEAN GRAIN SIZES OF STARCHES

Moisture	Medium	Time, min.: 0[a]	5
	Corn		
High (MJ)	Distilled water	6.133[b]	6.129
	P80	6.133	4.846
	BzC	6.133	5.867
	NaLS	6.133	6.181
Low (P825)	Distilled water	5.676	5.919
	P80	5.676	5.664
	BzC	5.676	5.422
	NaLS	5.676	5.052
	Amioca		
High	Distilled water	5.482	6.157
	P80	5.482	5.740
	BzC	5.482	5.728
	NaLS	5.482	5.319
Low	Distilled water	5.063	5.672
	P80	5.063	4.949
	BzC	5.063	4.897
	NaLS	5.063	5.796

[a] Unsubmerged. [b] Mean grain sizes in scale divisions; one scale division = 1.8 μ.

water and SGF as submersion media, and that the significance of the time effect was due to the difference in grain diameters between unsubmerged starch (0 time) and submerged starch (1). For these reasons, the time effect was not investigated. Distilled water and SGF were included as a basis of comparison.

The starches were slurried in the submersion media at 37° and the mean grain diameters were determined after submersion for 5 min. Table I shows the mean grain sizes and the variables investigated. In the analysis of variance, interactions were not significant and were combined with the error term. There was no significant difference demonstrated among any of the variables investigated.

The effectiveness of surfactants in improving tablet disintegration may be due to the fact that surfactants increase the rate of starch grain wetting. Nogami, Nagai, and Uchida (5) state that immersional wetting might be a controlling factor in disintegration of tablets made under low compressional force. In order to investigate the possibility that surfactants improve tablet disintegration by increasing the swelling capability of the starch grains, a 4 × 2 × 2 × 2 factorial experimental design was used.

Table II shows the variables studied and the mean grain sizes. Polysorbate 80 (P80), benzalkonium chloride (BzC), and sodium lauryl sulfate (NaLS) were selected as the surfactants because they represent each of the ionic classifications—nonionic, cationic, and anionic, respectively. Aqueous solutions containing 1% surfactant were used as submersion media and distilled water was included as a control. Due to the high mobility of the starch grains in the surfactant solutions, measurement of the grain diameters was almost impossible when the slides were prepared directly from the surfactant slurry. To overcome this difficulty, the sample removed from each slurry was mixed with glycerin, which does not affect the swelling of starch grains (6). This increased the viscosity sufficiently to fix the grains and allow measurement of the diameters. In the analysis of variance, the three-factor interactions were not significant and were combined with the error term. The type of starch × time interaction was significant. Type of starch and moisture content were significant at the 0.5% level. These differences are probably due to maintenance of the initial grains size difference for the duration of the experiment. Significant differences among the surfactants and distilled water were not demonstrated.

SUMMARY AND CONCLUSIONS

Previous work on the effect of environmental conditions on starch grain swelling is reviewed and determination of the effects of the individual components of simulated gastric fluid USP was completed. There was no statistically significant swelling demonstrated by pepsin in the submersion medium when compared to simulated gastric fluid and distilled water.

Surfactants did not significantly affect starch swelling when compared to distilled water. Surfactant effectiveness in occasionally improving tablet disintegration is apparently not due to any effect on starch grain swelling, but is more likely due to an increase in the wetting rate of the tablet resulting from the decrease in surface tension.

The study of the effects of tableting procedure on starch grain swelling and tablet disintegration is being completed.

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Keyphrases

Cornstarch grains
Starch in simulated gastric fluid
Pepsin effect on starch
Surfactant effect on starch
Starch grain diameter determination

Phosphorus–Nitrogen Compounds VII. Urethan Derivatives

By L. A. CATES and J. A. NELSON

A series of phosphorylated urethans was synthesized for antitumor screening. Results indicate that the P(O)NHC(O)OEt moiety does not contribute directly to an oncolytic effect against lymphoid leukemia.

BIS(1-AZIRIDINYL)PHOSPHINYL CARBAMATES have been extensively investigated as potential anticancer drugs (1–4); these derivatives being synthesized specifically to incorporate two biologically active groups into a single molecule, *i.e.*, the so-called "dual antagonist." Papanastassiou and Bardos (2) prepared a phosphorylated urethan lacking an aziridine group to support their premise that for a dual antagonist "each synergistic component should be present in the molecule in such form (chemical linkage) that it can exert its biological activity." This "open-chain" analog, *N,N,N′,N′*-tetraethyl-*N″*-carbethoxyphosphoramide, was inactive in Walker carcinosarcoma 256 but equal to urethan against adenocarcinoma 755 in mice. The difficulty these investigators encountered in the synthesis of this compound involved ammonolysis of the labile ester group and separation of the desired carbamate from the corresponding carbamide.

In view of the antileukemic activity of urethan it was considered advisable to prepare further *N*-phosphorylated derivatives (Table I) for testing against this tumor system. None of the phosphorylated urethans (compounds I–V, VII, and IX, Table II) exhibited significant oncolytic properties. These results indicate that previously reported (1) superior activities of bis(1-aziridinyl)phosphinyl carbamates over nitrogen mustards against leukemia may be due to the effect of the carbamate portion on some other factor, such as distribution, absorption, or elimination.[1]

It has been reported that urethan is *N*-hydroxylated *in vivo* and the resulting *N*-hydroxyurethan may be the active metabolite which is responsible for its radio-mimetic effects (5). Also, Skipper *et al.* (6, 7) observed reduced antileukemic activity in *N*-alkyl derivatives of urethan. It is possible, therefore, that *N*-substitution, such as phosphorylation, may interfere with hydroxylation, and thus with the oncolytic activity, of urethan.

Previous studies (8, 9) have indicated that direct phosphorylation of physiologically active amines results in a reduction of activity and toxicity, whereas separation of phosphorus and nitrogen atoms by a carbamoyl group has afforded derivatives with some degree of antitumor activity (10). VIII represents a ureido separation of these atoms and a comparison of its activity with that of VII may provide a basis for the study of related derivatives. A future paper in this series describes the synthesis of phosphoric triamides incorporating urethan and methoxyurea moieties.

The phosphinylcarbamates herein reported were prepared *via* the reaction between dichlorophosphinylurethan and various amines or ammonia. Ammonolysis of carbamate to carbamide was shown, by means of elemental microanalyses and infrared absorption in the 1660 cm.$^{-1}$ (ureas) instead of the 1700–1735 cm.$^{-1}$ range (urethanes) (11), to occur in only one case (IX). The low yield of VI may be accounted for on this basis although no corresponding carbamide was isolated. A high yield of III was obtained despite the use of a strong base which would expectedly result in ammonolysis. VII and VIII were prepared in good yield by condensing phosphinylidynetris isocyanate with ethanol or ethyl carbazate, respectively.

Compounds II–V and IX were incidentally screened for antimalarial activity.[2] None of these derivatives extended survival time of mice infected with lethal doses of *Plasmodium berghei*.

EXPERIMENTAL

Ethyl Bis(diaminophosphinyl) Carbamates[3] (I–VI)—Dichlorophosphinylurethan (2) (0.1 mole) in toluene or benzene (100 ml.) was added to aniline, *p*-toluidine, cyclohexylamine, 2-aminopyridine, or phenylhydrazine (0.4 mole) in toluene or benzene (100 ml.) with stirring and <50°. The reaction mixtures were allowed to remain overnight and filtered, and the residues were washed with water and recrystallized once or twice from the appropriate solvent. In the case of II, the reaction mixture filtrate was spin-evaporated to yield a dark oil which was dissolved in dilute NaOH, treated with charcoal, filtered, and acidified with concentrated HCl. Recrystallization of the resulting white precipitate from ether gave the product. I and IV were similarly dissolved in alkali and precipitated with acid as a purification step. VI was prepared by passing dry ammonia into an ethereal solution of dichlorophosphinylurethan (0.2 mole) for 5 min. with stirring and isolating the product from the precipitate.

Triethyl Phosphenylidynetris Carbamate (VII) and Triethyl 3, 3′, 3″-[phosphinylidynetris (iminocarbonyl)] tricarbazate (VIII)—Ethanol or ethyl carbazate (0.1 mole) in acetonitrile (50 ml.) was added to phosphinylidynetris isocyanate (0.03 mole) (phosphoryl isocyanate, Alfa Inorganics, Inc.) in ether or acetonitrile (200 ml.) with stirring and 5–10°. After remaining overnight, the precipitates were collected, washed with ether, and dried *in vacuo*. Neither product showed absorption in the 2250–2275 cm.$^{-1}$ region (isocyanates).

Received June 30, 1967, from the College of Pharmacy, University of Houston, Houston, TX 77004

Accepted for publication September 19, 1967.

This investigation was supported in part by research grant CA-08711 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.

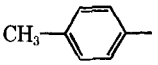
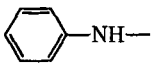
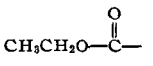
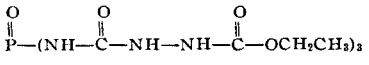
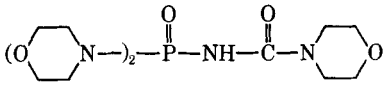
Previous paper: Cates, L. A., *J. Med. Chem.*, **10**, 924 (1967).

[1] Since the submission of this paper, it has been reported that the replacement of a urethan moiety in bis(2,2-dimethylaziridinyl) phosphinyl carbamate by an ethoxy group gives a much more effective antitumor agent. See Chmielewicz, Z. F., Bardos, T. J., Munson, A., Babbitt, H. I., and Ambrus, J. L., *J. Pharm Sci.*, **56**, 1179(1967).

[2] Testing results furnished by Walter Reed Army Medical Center, Washington, D. C.

[3] Also termed *N*-ethoxycarbonyl-*N′,N″*-disubstituted phosphoric triamides or phosphoramides.

TABLE I—PHOSPHORYLATED URETHANS

Compd.	R	M.p.,[a] °C.	Recrystn.[b] Solvent	Yield, %	ν cm.⁻¹ (C=O)[c]	Formula	Anal., %[d] Calcd.	Found
I		193–196	Et	47	1700	$C_{15}H_{18}N_3O_3P$	C, 56.5 H, 5.7 N, 13.2	C, 56.1 H, 5.8 N, 13.0
II		174–176	E	40	1700	$C_{17}H_{22}N_3O_3P$	C, 58.8 H, 6.4 N, 12.1	C, 58.3 H, 6.4 N, 12.0
III		161–163	Et	81	1700	$C_{15}H_{30}N_3O_3P$	C, 54.4 H, 9.1 N 12.7	C, 54.4[e] H, 9.2[e] N, 12.6[e]
IV		170–175	Et	20	1710	$C_{13}H_{16}N_5O_3P$	C, 48.6 H, 5.0 N, 21.8	C, 48.7 H, 5.2 N, 22.0
V		184–186	Et	70	1700	$C_{15}H_{20}N_5O_3P$	C, 51.6 H, 5.8 N, 20.0	C, 51.5 H, 5.7 N, 19.8
VI	H—	156–158	A	7	1710	$C_8H_{10}N_3O_3P$	C, 21.6 H, 6.0 N, 25.1	C, 21.6 H, 5.9 N, 24.7
VII		145	...	75	1700	$C_9H_{18}N_3O_7P$	C, 34.7 H, 5.8 N, 13.5	C, 34.7 H, 5.9 N, 13.4
Misc.								
VIII		175–177	...	72	1670–1750	$C_{12}H_{24}N_9O_{10}P$	C, 29.7 H, 5.0 N, 26.0	C, 29.6 H, 5.0 N, 25.7
IX		192–195	Et	30	1640	$C_{13}H_{25}N_4O_5P$	C, 44.8 H, 7.2 N, 16.1	C, 44.8 H, 7.4 N, 16.0

[a] Determined on a Fisher-Johns apparatus and are uncorrected. [b] Et, ethanol; E, ether; A, acetone. [c] Obtained with a Beckman IR-8 spectrophotometer using a Nujol mull. All compounds had infrared spectra in agreement with their assigned structures. [d] Determined by Coleman C—H and N analyzers. [e] Performed by Schwarzkopf Analytical Laboratories, Woodside, N. Y.

TABLE II—ANTICANCER SCREENING[a]

Compd.	Test System	Dose, mg./Kg.[d]	T/C × 100
I	LE[b]	400	104
II	LE	400	98
III	LE	200	90
	WM[c]	150[e]	72
IV	LE	400	101
V	LE	400	89
	WM	200	72
VII	LE	400	93
IX	LE	400	107

[a] Data from CCNSC. [b] Lymphoid leukemia L-1210. [c] Walker 256 (intramuscular). [d] Highest dose permitting all test animals to survive. [e] Only dose used; permitted 4/6 survivors.

N-(Dimorpholinophosphinyl)-4-morpholinecarboxamide (IX)—This compound was isolated during an attempted synthesis of the corresponding carbamate using the method described for I–V.

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Keyphrases

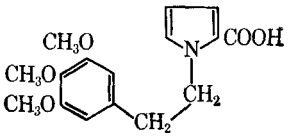
Phosphorus–nitrogen compounds
Urethan derivatives–phosphorylated
IR spectrophotometry–structure
Anticancer activity

Peyote Alkaloids IV. Structure of Peyonine, Novel β-Phenethylpyrrole from *Lophophora williamsii*

Sir:

In a recent study on the peyote alkaloids, we reported (1, 2) isolation of five crystalline quaternary bases, four of which were identified. A sixth quaternary base was obtained in a purified form and a seventh crystalline compound was a nonalkaloidal substance. Further work on the peyote constituents resulted in an isolation of an eighth new compound designated peyonine.[1] Structural study on peyonine is the subject of this communication and has revealed that this is a novel β-phenethylpyrrole.

Peyonine, m.p. 131–133.5°, isolated from purified methanolic extracts of *Lophophora williamsii* (Lem.) Coult., shows a positive Ehrlich reaction, indicating the presence of an indole, a pyrrole, or a phenolic compound (3, 4). The mass spectrum (Fig. 1) is characterized by peaks at *m/e* 305 (parent), 261 (loss of a carbon dioxide), and 181 (base, evidently the trimethoxybenzyl ion, see below). The IR spectrum revealed a broad hydroxyl peak at 3600–2500 cm.$^{-1}$, a carbonyl peak at 1670, and aromatic peaks at 1595, 820, and 750 cm.$^{-1}$ The ultraviolet absorption spectrum in methanol showed a maximum at 261 mμ (ε10,000) which base shifted to 257 (9700), and acid shifted to 266 (10,700). The NMR spectrum ($CDCl_3$, TMS = 0) revealed a broad peak at 10.70 p.p.m. (1 H, COOH), 6.32 (2 H singlet, polyoxygenated aromatic), 4.35 and 2.98 (2 H each, triplets, A_2X_2, $ArCH_2CH_2$), and 3.85 (9 H, singlet, aromatic OCH_3); in benzene this last peak appeared as two singlets, 3.63 (6 H) and 3.85 [3 H, *o,o'* disubstituted aromatic methoxyl (6)]. Multiplets at 7.15, 6.70, and 6.12 (1 H each) are assigned to the protons of an *N*-substituted pyrrole-2-carboxylic acid. The spectral properties of pyrrole-2-carboxylic acid substantiated this assignment. IR (KBr) carbonyl: 1660; aromatic 1550; 750 cm.$^{-1}$; UV, $\lambda_{max.}^{MeOH}$ 261 mμ(ε 13,200) (8), shifted by base to 254 (12,000) and by acid to 263 (14,700). The spectral data and the biogenetic considerations suggested that peyonine was most likely 1-(β-3′, 4′, 5′-trimethoxyphenylethyl)-pyrrole-2-carboxylic acid (I).



I

The assigned structure I was confirmed by the synthesis of peyonine. Treatment of mescaline with methyl 2,5-dimethoxytetrahydro-2-furoate (9) in refluxing glacial acetic acid produced peyonine methyl ester. Saponification furnished crystalline peyonine identical (TLC, GLC, UV, IR, NMR, and mixed m.p. undepressed) with the isolated compound.

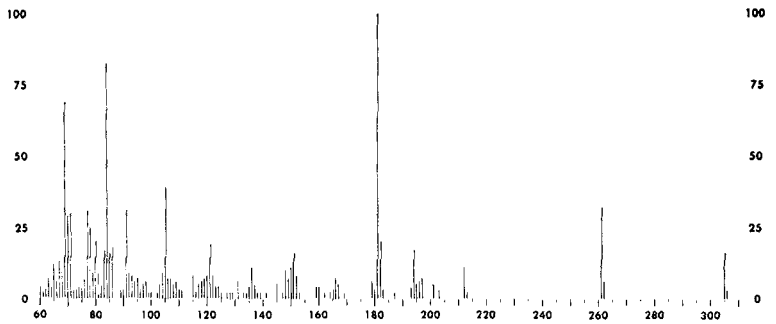


Fig. 1—Mass spectrum of peyonine.

Although proline (2-pyrrolidinecarboxylic acid) is an amino acid of wide natural occurrence, peyonine appears to be the first simple pyrrole-2-carboxylic acid derivative isolated from natural source, its structure elucidated, and the assigned structure proven by a synthesis.

It seems reasonable to speculate that the pyrrole ring of peyonine is produced in the plant by condensation of a 1,4-dicarbonyl compound such as an α-ketoglutaric acid derivative or its equivalent with mescaline or its precursors. The biosynthesis of peyonine, the preparation of com-

[1] Isolation and synthesis of peyonine have been separately reported. (See *Reference 7* for the published abstract.)

pounds related to the isolated β-phenylethylpyrrole, and pharmacological studies of peyonine and related compounds are currently being investigated.

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Received July 5, 1967.
Accepted for publication November 1, 1967.
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Studies at Howard University were generously supported by grant MH11119-02 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
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Keyphrases

Peyote alkaloids
Peyonine structure determination
Mass spectrometry
IR spectrophotometry—structure
UV spectrophotometry—structure
NMR spectrometry

Remarks on Synthesis of Benzofurans

Sir:

A recent note in this Journal by P. K. Sharma *et al.* (1) reported the synthesis of several substituted benzofurans. It appears, however, that there are some discrepancies in this report worthy of further mention.

The authors describe the reaction of benzyl bromide with hydroxymethyl propiophenone,[1] and *p*-nitrobenzyl bromide with 2-hydroxypropiophenone and 2-hydroxy-3-aceto-6-methylpropiophenone to yield, respectively, 4-methyl-6-phenylbenzofuran, 2-(*p*-nitrophenyl)benzofuran, and 2-aceto-5-methyl-7-nitrophenylbenzofuran. The products to be expected (2) in the first two cases, respectively, are 2-phenyl-3-ethyl-5-methylbenzofuran and 2-(*p*-nitrophenyl)-3-ethylbenzofuran, while in the third case the product might be expected to be 2-(*p*-nitrophenyl)-3-ethyl-4-methyl-7-acetylbenzofuran and/or 2-(*p*-nitrophenyl)-3,6-dimethyl-7-propionylbenzofuran. On first consideration the discrepancies appear to be one of nomenclature (2, 3), but additional contemplation reveals the problem to be an error on the part of the authors. Utilizing the reactants and conditions stated it is impossible to obtain the products alleged. Since the authors offer no analytical data or spectra to substantiate their claims, it is doubtful as to the true nature of the products. For example, the authors report the melting point of their alleged 2-(*p*-nitrophenyl)benzofuran to be 193°, while the literature value (4), substantiated by a good carbon–hydrogen analysis, is 182°. Indeed, in view of the same literature report (4), the reaction between benzyl bromide and hydroxymethyl propiophenone might *not* be expected to proceed under the *mild* conditions described, but might terminate at the benzyloxy stage.[2] Finally, the structure depicted for their alleged compound 2-aceto-5-methyl-7-nitrophenylbenzofuran is actually 1-methyl-4-acetyl-8-nitro*di*benzofuran (2).

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REVIEWS

Design of Active-Site-Directed Irreversible Enzyme Inhibitors. By B. R. BAKER. John Wiley & Sons, Inc., 605 Third Ave., New York, NY 10016, 1967. xiii + 325 pp. 15.5 × 23.5 cm. Price $13.50.

Professor Baker has written an interesting book about the work he has been doing for the last six years on the design, synthesis, and evaluation of what he calls "active-site-directed irreversible inhibitors." The book itself deals primarily with design of these inhibitors.

Dr. Baker's ideas initially were based largely on the established mechanism of action of azaserine, which is known to combine, because of its similarity to glutamine, reversibly at the active site of phosphoribosylformylgycineamidine synthetase using the glutamine binding points, and then irreversibly inactivate the enzyme by reaction of its diazomethyl group with a thiol group in the active site. The design of such inhibitors, which have been termed endo-alkylating agents, is severely limited because of the steric requirements of the active site. On the other hand, Professor Baker has found that an inhibitor can be designed to fit an enzyme active site and at the same time bear a group capable of reacting with a functional group on the enzyme surface but outside the active site. This concept of an exo-alkylating agent has two advantages. First, the *position* of the change made in a metabolite to give an antimetabolite becomes more important than the nature or size of the change. Second, and more important ultimately, such an inhibitor has an extra dimension of specificity, since even closely related isozymes differ in their secondary and tertiary structure.

Professor Baker uses the "6-mercaptopurine story" to illustrate the parameters involved in the use of enzyme inhibitors as chemotherapeutic agents. He then discusses the development of his exo-mechanism concept. Chapter 2 is devoted to a general discussion of the forces involved in the reversible binding of substrates to enzymes. Chapters 3–5 deal with the mode of binding to specific enzymes. Chapter 6 is concerned with the types of changes in the structure of a metabolite that can be expected to produce inhibitors and the types of changes that may lead to modified substrates, while Chapter 7 discusses bulk tolerance in enzyme–inhibitor complexes. Chapter 8 details the kinetic parameters important to active-site-directed irreversible inhibition and studies, other than the author's, on this type of inhibition. Chapters 9–12 are devoted to the details of the *modus operandi* developed by Professor Baker in his studies of lactic and glutamic dehydrogenases, dihydrofolic reductase, and other folate cofactor area enzymes, while Chapter 12 describes the work of other investigators on adenosine deaminase. Unexpectedly, the last chapter is devoted to enzyme-specific columns, but the relationship of this subject to the author's interests is obvious.

This book should be studied by any serious student of medicinal chemistry. I would agree with the author's statement "Although these active-site-directed irreversible inhibitors have not yet led to useful chemotherapeutic agents, the future for such discoveries appears based on scientifically sound premises." That is a lot more than can be said for much of the work being carried out today on the design and synthesis of potentially useful medicinals.

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JOURNAL OF Pharmaceutical Sciences

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Review Article

Spectroscopy of Amines of Pharmaceutical Interest

By I. B. EISDORFER, R. J. WARREN, and J. E. ZAREMBO

ALTHOUGH THE USE of alkaloidal amines of plant origin as therapeutic agents extends well back into the history of mankind, it was not until the advent of synthetic organic chemistry in the 19th century that the first successful attempts were made to simulate and improve upon nature. Early in this century it was discovered that many body substances either are amines, are metabolized to amines, or can be simulated by amines. Certainly by its brilliant work elucidating the structure and function of epinephrine (1), norepinephrine (2), ephedrine (3), and the other sympathomimetic amines now so widely used in medicine, pharmaceutical chemistry has to a great extent been amine chemistry.

Amines are, of course, basic nitrogen-containing groups derived from ammonia. Amino groups can be found or placed on aromatic as well as aliphatic skeletons; they can be fused into alicyclic rings, be attached to atoms other than carbon, be quaternized to increase their basicity, or be located adjacent to other functional groups that make them neutral or even acidic. It is not the purpose of this review to cover all possible amino group combinations. For the purpose of this review we will restrict our discussion to basic amino-containing compounds in which the amino group is attached directly to an aliphatic carbon with no other substitution on the nitrogen other than alkyl groups or hydrogens. There may of course, be aromatic, or heteroatom-containing groups elsewhere in the molecule.

Received from the Research and Development Division, Smith Kline & French Laboratories, Philadelphia, PA 19130

The various forms of spectroscopy, UV, IR, NMR, Raman, mass, *etc.*, are all well-established techniques for elucidating gross and fine structure of organic compounds, for evaluating compound interactions, and under appropriate circumstances for quantitatively determining compounds in mixtures. It is the purpose of this paper to review specific applications of the various forms of spectroscopy for amine-containing compounds that have been or are likely to be of interest to people working in the field of pharmaceutical chemistry. Our primary purpose is to describe how spectroscopy can help elucidate the amine function but secondarily also to attempt to relate spectroscopy to the whole molecule.

Of the various spectroscopic techniques covered here, ultraviolet and infrared absorption are best known and most widely applied in structure identification and quantitative analysis. Nuclear magnetic resonance is now perhaps the most useful and most rapidly growing branch of spectroscopy for gross and fine structure determination. Mass spectroscopy shows great promise for rapidly unraveling difficult structures particularly when only small sample sizes are available, as for example, in the effluent from a gas chromatograph. Raman spectroscopy has much promise for structure assignment work, but it still awaits development of simple inexpensive equipment before its potential can be adequately realized. The most promising of all developments in organic structure identification is the combined use of all spectroscopic techniques to attack a single problem (4, 5). This very powerful pro-

cedure can become even more so when applied in conjunction with an interpretive computer. This certainly is the direction of future development in this field.

There are a number of good review articles and books that have been published on the use of the various spectroscopic techniques in analyzing amine-containing organic structures (6–8). Previous reviews in this journal have covered certain aspects of the subject quite adequately and should be referred to for further information on the subject.

NUCLEAR MAGNETIC RESONANCE

To attempt to review all of the literature for every report of nuclear magnetic resonance data on amines would be an impossible task. The number of scientific papers including NMR data has increased at an almost unbelieveable rate. Five years ago 12% of the reports in the *Journal of Organic Chemistry* contained NMR data either as the major topic of discussion or as supporting data for structure assignment. Today, it would be safe to say that no more than 12% do not contain NMR data. It has been reported (9) that in 1964 NMR was mentioned in every sixth paper appearing in the chemical literature of the United States. In other words, every journal reporting synthesis or structure elucidation of organic compounds serves as a repository for NMR data. Realizing the difficulties inherent in undertaking a search of such dimensions and the practical limits of time and space allotted here, this reviewer has chosen to present a broad review of the techniques developed specifically for amines. These data will be supplemented with references to pharmaceutical and biologically important amines.

The use of nuclear magnetic resonance in the elucidation of the structure of amines has found wide application. The identification of *N*-alkyl groups by NMR has been described by several workers (10–13), and the technique has largely replaced other methods for identifying *N*-alkyl groups. Ma and Warnhoff (14) and others (15–18) have discussed the application of NMR for detection of *N*-methyl groups in particular. The NMR method for *N*-methyl analysis is described as being dependent on a pronounced downfield shift of the resonance peak of basic *N*-methyl groups in solvents of increasing acidity. The effect is due to the decreased shielding of the methyl protons of the positively charged ion relative to those of the free amine. This has also been mentioned by earlier investigators (17–19). Ma and Warnhoff have presented the NMR spectral data on a series of 116 compounds containing *N*-methyl and/or other methyl groups and have recorded the spectra in deuterochloroform, perdeuteroacetic acid, and trifluoroacetic acid. *N*-Methyl protons in secondary amines (aliphatic) have been found to be decidedly less deshielded in strong acid than *N*-methyl groups in tertiary amines. The explanation offered for this behavior is that the charge on the protonated secondary amine is more dispersed (20), and therefore, the smaller charge has a smaller deshielding effect on the *N*-methyl protons. A large number of chemical shift values and spin-spin splitting constants was given.

An extension of the method to the identification of primary, secondary, and tertiary aliphatic amines has also been reported (16, 21). Once again it is found that the effects of strongly acidic solvents on the chemical shift and spin-spin splitting constants of the protons α to the amine ion are characteristic for the particular functional group. The protons adjacent to the primary amine ion, NH_3^+, are split into a quartet; those adjacent to secondary ion, NH_2^+, into a triplet, and those adjacent to tertiary ion, NH^+, into a doublet. This phenomenon is also reported by Loewenstein and Meiboom (22) and Jackman (23). The nuclear magnetic resonance spectra of amino acids have been reported. Among the more interesting studies that have been reported are those on the correlation of NMR spectra with the L- and *meso* structures of cystine (24) and of the effect of optical activity of amino acids on their NMR spectra (25). There have been comprehensive analyses of many specific amino acid spectra using NMR (26–29); much detail on the spectra of the individual acids is given. In addition, Thompson *et al.* (21) have shown that spectra of *N*-substituted amino acids examined in alkaline deuterium oxide and in trifluoroacetic acid show chemical shifts and first-order spin-spin splitting patterns that are useful for identification of *N*-methyl, *N*-methylene, *N*-phenyl, and other groups attached to the nitrogen of an amino acid.

Data on aromatic amines are not as plentiful as those on aliphatic amines unless one wishes to consider the spectra of individual amines reported in the literature. The spectrum and chemical shift of the aromatic protons in aniline have been reported (30), and Ma *et al.* (14) have included data on 13 aromatic and *N*-heterocyclic amines in their work. Identification of *N*-phenyl amines by acidic solvent effects on the chemical shift and spin-spin splitting constants of the phenyl protons were reported (31) to provide the basis for the identification of the *N*-phenyl groups

in primary, secondary, and tertiary amines.

In addition to these rather general techniques for identification of amines, there have been collections and compilations of NMR data on nitrogen-containing compounds. One of the most recent and extensive papers is presented by Slomp and Lindberg (32), in which the structural and chemical shift data for 2306 hydrogens influenced by nitrogen in a variety of ways have been correlated and charted. There are also publications on specific classes of nitrogen-containing compounds such as the alkaloids (33). Other heterocyclic systems which have been recorded and analyzed are the pteridines (34), pyridines (35), piperidines (36), purines (37), pyrrolines (38). A general treatment of heterocyclics by NMR is also available (39). Reports on pharmaceutical applications of NMR have been presented (40), and Jardetzky and Jardetzky (41) give many examples from the biological field.

For those who have found that trying to keep abreast of all the current NMR literature is impossible, but who would like to stay up to date, there remains the abstract services (42, 43).

Techniques

Spin-Decoupling—Equipment is commercially available for heteronuclear spin-decoupling and has been used with success. Anderson and Silverstein (16) have described the decoupling of the NH^+ protons from the nitrogen in amine salts. The actual performance of a spin-decoupling experiment is straightforward, and an experienced operator can accomplish it in a relatively short time. For a complete and, excellent review of spin-decoupling see Baldeschwieler and Randall (44). Heteronuclear spin-decoupling equipment can be purchased either from the various instrument manufacturers or from Nuclear Magnetic Resonance Specialties, Inc., New Kensington, Pa.

Micro Sampling—Most instrumental techniques are taxed to the limit of sensitivity in the pharmaceutical and biological areas where a constant problem is paucity of sample. In the study and identification of metabolites, for instance, frequently there is an insufficient amount of sample to obtain spectral data by routine methods. This is especially true in NMR work, but the use of a CAT (computer of average transients) has eliminated much of the problem of small sample sizes. A spectrum, is scanned repeatedly a preselected number of times. The signal output from the spectrometer is stored and accumulated by the computer which presents a final spectrum with a total signal proportional to the number of passes through the spectrum and with the noise accumulated as the square root of the number of passes. This gain in signal to noise ratio and the buildup of sample signal allows one to obtain an NMR spectrum on microgram quantities of sample. Applications of the technique to biological systems have been reported (45).

INFRARED SPECTROSCOPY

An earlier review in this Journal by Carol (46) presented the fundamental theoretical concepts and techniques of infrared spectrophotometry as applied to pharmaceutical analyses. In the present report we shall confine our interest to the relatively narrow range of amines. The areas of interest in the infrared spectra of amines concern themselves primarily with the stretching and bending or deformation vibrations of the amine function. These regions have been assigned (47–49) as follows: N—H stretching region for primary and secondary amines from 3500–3300 cm.$^{-1}$; NH_2 bending or deformation approximately 1650 cm.$^{-1}$. Primary amines are identified by two absorption bands in the 3500–3300 cm.$^{-1}$ region (47–52). A third band is often found at slightly lower frequency due to hydrogen bonding. This third band is generally broader than the two bands at higher frequency (53). The 1650 cm.$^{-1}$ region is also useful in identifying primary amines in that it contains the NH_2 deformation band. This absorption band is usually broad and of medium intensity (47, 54) and shifts to higher frequency on hydrogen bonding in a similar manner as the NH_2 stretching vibration (53). Because of its location, the absorption band can often be assigned only with difficulty when the complexity of the molecule increases, and aromatic and other functional groups such as secondary amides are present. In such cases a variety of techniques may be brought to bear on the problem. The two techniques most commonly used are deuteration of the NH_2 groups (55, 56) and/or formation of an amine salt (57). Deuteration causes a shift of the NH_2 absorption to lower frequency, and salt formation involves a shift to lower frequencies as well as other changes in the absorption bands. Although for secondary amines one might expect only one absorption band in the 3500–3300 cm.$^{-1}$ range, there are often two bands observed due to association (53). The band from the associated NH is broader than that of the free NH, just as in the case of the associated NH_2 band. However, the integrated absorption intensity of the absorption bands of primary amines is approximately double that for secondary amines.

Tertiary amines, lacking the NH function, offer no absorption bands in the areas 3500–3300 or 1650 cm.$^{-1}$. They are difficult if not impossible to identify as free bases by infrared spectroscopy. There exists the possibility of identifying the group attached to the nitrogen atom, *i.e.*, CH_3N, C_2H_5N, *etc.*, but in the final analysis it would appear that the best method of identification by infrared would be to form a derivative of the amine.

From what has been said thus far, one might get the impression that differentiation of primary, secondary, and tertiary amines could be made quite simply. However, such is not always the case. Duval (58) describes how various factors such as molecular association, interactions with solvents, and poor dispersion in some instruments make identification and differentiation of amine classes an extremely difficult proposition.

A great amount of effort has been exerted in the study of inter- and intramolecular interactions of amines (53, 59). The conclusion reached almost unanimously is that spectra, to be compared qualitatively or quantitatively, should be measured in the same solvent under the same conditions.

For positive identification of amines it has been found useful in many cases to form an amine salt. This is particularly true of pharmaceutical amines which are generally administered in the form of a salt. The formation of the NH_3^+, NH_2^+, and NH^+ ions results in a shift of the amine absorption band to lower frequency. The wavenumbers at which the resulting absorption bands occur are specific for each class of amine. Primary amine salts have been reported to show a series of weak peaks with a broad absorption band of medium to weak intensity located between 1960 and 2080 cm.$^{-1}$. Secondary amine salts have been found to have a series of three bands between 2860 and 2040 cm.$^{-1}$; splitting of one or more of these bands has been observed in some cases. Tertiary amine salts, which are by far the most common class of amines used pharmacologically, have the simplest absorption spectra since they contain only one hydrogen on the nitrogen. The distinguishing feature of NH^+ spectra is a broad and intense peak located in the range of 2770–2380 cm.$^{-1}$. The exact location of the absorption band depends on whether the molecule contains water of hydration (64). There is of course strong hydrogen bonding in all of the crystalline amine salts and very little absorption found at the normal N—H frequency range 3500–3300 cm.$^{-1}$.

One thing to be remembered in dealing with amine salts is to avoid preparation of alkali halide disks whenever possible. Changes in spectra arising from hydrogen bonding and interaction with halide disks have been reported (65).

Near IR—Near infrared spectrophotometry in this discussion refers to the area of the spectrum between 1.0 and 2.5 μ. We are eliminating the fundamental N—H region because it has been adequately covered in the preceding section. The area deals largely with stretching vibrations involving hydrogen bound to a variety of other atoms. The absorption bands in this region are overtones or combination bands of vibrations originating in the fundamental region. The principal use of the area is in quantitative analysis; however, it is possible to differentiate primary, secondary, and tertiary amines on the basis of their absorption here. In addition to other absorptions, primary amines have a peak at about 2.0 μ, which is due to a combination band resulting from N—H bending and stretching vibrations in the fundamental region (66). Secondary and tertiary amines lack the 2.0 μ band, but secondary amines have an absorption in the first overtone region which is located at about 1.5 μ. Tertiary amines have no absorption due to NH and contribute nothing to this area of the spectrum as free bases.

The area of the combination band (1.95–2.0 μ) has great quantitative value in determining primary amines alone or in combination with secondary and tertiary amines (67, 68). The chief disadvantages to more extensive use of this region of the spectrum is the increased sample size required for significant absorption in the overtone region and the lack of suitable solvents. Carbon tetrachloride is the only solvent transparent in the region from 1.0 to 3.0 μ. Carbon disulfide has only one peak in the area of 2.2 μ, which makes this a useful solvent also. However, because of solubility problems on one hand and possible solvent interaction on the other, it is sometimes difficult to operate under the most desirable conditions. Whetsel *et al.* (53), in describing solvent and concentration effects on the near infrared NH bands of primary amines state that the absorption bands tend to broaden and shift to longer wavelength with increasing concentrations. Intensities and band widths vary markedly from solvent to solvent and great care should be exercised in comparing qualitative as well as quantitative data on the same material unless the data have been collected under similar conditions.

The spectroscopy of amine salts in the near infrared region has recently been described (69). A technique using samples in the solid state is described by which qualitative and quantitative

data on the salts were obtained. Primary amine salts were distinguished from secondary and tertiary amine salts on the basis of the presence of a band at 2.18 μ and the absence of a band at 2.05 μ.

For a review of near infrared spectrophotometry in general, the reader is referred to the chapter by Goddu in "Advances in Analytical Chemistry" (75).

Far IR—During the last few years instrumentation extending beyond the 650 cm.$^{-1}$ region has become commercially available, and many laboratories have begun investigating the range of 700–200 cm.$^{-1}$ for potential application. The techniques and methods of analysis used in the 4000–7500 cm.$^{-1}$ region are generally applicable to the far infrared. Window materials and solvents are not used interchangeably from one region to another, but there is an adequate supply of these for both regions. For example, the range to 400 cm.$^{-1}$ can be covered with potassium bromide cell windows and disks, while cesium bromide and cesium iodide transmit reasonably well to about 300 cm.$^{-1}$. Polyethylene has been shown (76) to be a useful window material for far infrared work. Good solvents for the far infrared are available although not as common as those available for the infrared region. Wyss *et al.* (77) have published a collection of 12 organic solvents for possible use in the far infrared.

For solid state samples the common alkali halide disks have been found to be most useful. Cesium iodide has also been used to press disks. Polyethylene and hydrocarbon wax have also been suggested as possible disk matrices (78). The information obtained in this range of the spectrum is related to the skeletal vibrations in the amines. Although the amount of information available on amines in this range is small relative to the 4000–7500 cm.$^{-1}$ range, some work has been reported on NH_2 vibrations (70). Tsuboi *et al.* (71) and Fukushima (72) have investigated amino acids in some detail in this region and have reported on NH_2, ND_2, and NH_3^+ vibrations. While the potential of the far infrared for providing a wealth of information for routine analysis is low, the area will undoubtedly provide more supplementary information that will aid in identifying molecular vibrations.

Attenuated Total Reflectance—The technique of attenuated total reflectance should be mentioned in this review of pharmaceutical amines because of its ability to obtain spectra with relative ease on samples which would ordinarily be difficult or impossible to handle. While the technique of ATR is known principally for its application to surfaces, it is also extremely useful in obtaining spectra on small amounts of sample. Microgram quantities of sample spread out as a thin film on an ATR sample plate or crystal yield spectra of excellent quality. The practical applications of the technique include analysis of metabolites isolated from biological systems and fractions from thin-layer and gas–liquid chromatograms, in amounts that heretofore would have precluded any attempt at identification by infrared spectra.

The principles of attenuated total reflectance have been set forth by Fahrenfort (73) and Harrick (74). The equipment in the form of attachments for present infrared spectrometers is available commercially from several manufacturers, including Wilks Scientific, the Perkin-Elmer Corp., and Barnes Engineering Co.

ULTRAVIOLET SPECTROPHOTOMETRY

Ultraviolet spectrophotometry has become an established, fundamental technique in pharmaceutical research and analysis as well as in the broad area of organic and inorganic analysis. Its widespread use and acceptance is such that for a general review of theory and techniques the reader is referred to several recent texts and not necessarily to papers appearing in current journals. The possible exception to this statement is the review of ultraviolet spectrometry appearing biannually in *Analytical Chemistry*. The theory and applications of ultraviolet spectrometry have been treated in detail in such recent books as "Absorption Spectroscopy," by Bauman (79), "Theory and Applications of Ultraviolet Spectroscopy," by Jaffe and Orchin (80), "Molecular Spectroscopy: Methods and Applications in Chemistry" (81), "Ultraviolet and Visible Spectroscopy: Chemical Applications" (82), "Advances in Analytical Chemistry and Instrumentation" (83). Special topics have been covered in other volumes such as "Interpretation of the Ultraviolet Spectra of Natural Products" (84), "Spectrophotometric Analysis of Drugs" (85), and a review and tabulation of the ultraviolet spectra of alkaloids (86). The latter review is especially useful since it contains all the available ultraviolet data on alkaloids up to and including 1964. The data are arranged according to structural type for more convenient study.

There are several collections of spectra and spectral data available which provide quantitative as well as qualitative data on amines as well as other organic classes. Perhaps the most useful of these is the four-volume work "Organic Electronic Spectral Data" (87). This collection

provides qualitative and quantitative data, often in several solvents, for each compound. The data are derived from a literature search involving some 69 journals. It is probably the most comprehensive work of its kind.

Ultraviolet spectrometry has found application in the study of aromatic amines in many areas of pharmaceutical analysis. The technique has been used in organic analysis and structure elucidation as well as to study stability, kinetics, ionization constants, mixtures, drug levels, and metabolites.

Organic Analysis—It has been mentioned previously that ultraviolet spectrophotometry is an established, fundamental technique in pharmaceutical analysis. Because of its general use in all phases of analysis it is often shunted aside and neglected in one of its most basic applications—structure elucidation. Many people bypass ultraviolet spectra completely in favor of the latest or what they consider to be more sophisticated techniques in the solution of organic structure problems. While other, newer techniques undoubtedly make important contributions in structural analyses, ultraviolet spectra can give much in the way of complementary or supplementary evidence for a specific structure proof. As a matter of fact, the determination of small amounts of organic material is very often done more conveniently by ultraviolet spectrometry than by any other method. Recent examples of the use of ultraviolet spectra in structural analysis are described in the work of Kamlet, Adolph, and Hofsommer (88) who showed that comparison spectra of 2,4-dinitroaniline and its *N*-alkyl and *N,N*-dialkyl derivatives permit discernment of the spectral effects of steric enhancement and inhibition of resonance. Baker *et al.* (89) have shown that it is possible to identify positional isomers in *N*-substituted uracils containing additional substituents in the 5- and 6-positions. Ultraviolet spectra taken in neutral and 0.1 *N* alkaline solvents show that substitution in the N-1 position results in a shift of less than 2 mμ. Substitution in the N-3 position results in a shift of 24–36 mμ.

In the quantitative analysis of aromatic amines, ultraviolet spectrometry plays a major role. Standard methods of analyses of aromatic amines invariably include UV as one of the methods of choice. Recent reports of the use of the technique include a spectrophotometric method for the determination of poldine methylsulfate (90), phenethanolamine after oxidation with periodate to benzaldehyde (91), and ephedrine, phenylpropanolamine, phenyramidol, and phenylephrine (92). In addition, Brandstaetter-Kuhnert *et al.* (93) have described a combination thermomicroscopic and ultraviolet spectrophotometric method for the qualitative determination of a series of antihistamines. Determination of thiobarbiturates by simultaneous recording of two spectra of the same material, differentiated by a spectrum shift due to regulated acidification of an alkaline solution of the thiobarbiturate, has been reported by Williams *et al.* (94). This use of differential UV spectrophotometry allows determination without interference from regular barbiturates. Needless to say it would be impossible to include here all of the articles and papers relating to the UV spectra of amines. For methods and applications for specific amines, the reader may consult the applications review articles in *Analytical Chemistry* or *Chemical Abstracts.*

Kinetics—Reaction rates and mechanisms of reactions in solution are conveniently followed by ultraviolet spectroscopy. An added advantage of following reactions this way is the detection and identification of intermediates and/or other unexpected products. Repetitive scans of the spectra of reaction mixtures at fixed time intervals permit observation and calculation of the amount of product as it is formed. The method, of course, is dependent on either formation of a new absorption band, which increases with time, or the decrease in intensity of the band of the starting material as it reacts to form a product. This method has been used by Eisenthal and Katritsky (95) to measure the rate of hydrolysis of 1-methoxypyridinium salts. A study involving oxidation and bromination of phenothiazine showed that the phenothiazine sulfoxide was the first stable product formed (96).

Szulczewski, Shearer, and Aguiar (97) studied the kinetics and mechanism of isomerization and hydrolysis of 4,6-diamino-1-(3,5)-dichlorophenyl-1,2-dihydro-2,2-dimethyl-1,3,5-triazine in dilute aqueous solution. In this study, which may be considered a typical kinetic study involving UV spectroscopy, a standard solution was prepared and diluted into various buffers held at the temperature of the run. Samples of the thermostated solutions were withdrawn periodically, and the ultraviolet spectrum of the intact starting material was obtained. The absorbance of the solution was read and the amount of unchanged material was calculated from a standard plot of the starting material.

Stability—The method for stability studies is the same as that used for kinetic work. The method provides stability data in a relatively short space of time, and the stability of the

material in question can be checked under a variety of conditions, *e.g.*, heat, light, time, *etc.* Recent applications of this method are reported by Garrett and Schraeder (98) and Simpson and Zappala (99). Riegelman and Fisher (100) have used the method to measure the stability of epinephrine. UV may also be used in stability studies in conjunction with thin-layer chromatography where decomposition products are isolated and determined by UV.

Amine Complexes—The formation of amine complexes showing absorption in the UV range has been used in the determination of amines. A number of articles describing formation of these UV-absorbing complexes have appeared recently. Ferric chloride has been found to complex with pyridine (101). The complexes of mercury and aniline have been studied (102), and the interaction of mercuric acetate with indole derivatives has been reported (103). Iodine has been found to complex with aniline (104), tertiary amine *N*-oxides (105), and 2,2-bipyridines (106). In a report by Lim *et al.* (107), a study of the solubilization and stabilization of phenobarbital by aminoalcohols is described. The increased solubility of phenobarbital by aminoalcohols is attributed to the formation of a "complex" or salt. An ultraviolet method for quantitative analysis of phenobarbital was used throughout the study.

Ionization Constants—The changes in the spectra of aromatic amines brought about by change of the pH of the solution reflect the changes in electronic state in going from free base to ion. These spectral changes are useful in studying tautomeric equilibria and for determining ionization constants of amines. The procedure generally followed in determining pKa values is to obtain the absorption spectra of the amine in basic solution, the amine ion in acidic solutions, and mixtures of the two forms in suitable buffer solution. The pH of the buffer solution is measured, and this in addition to the absorbance values at a given wavelength permits calculation of the pKa value from the following relationship:

$$\mathrm{pKa} = \mathrm{pH} + \log \frac{A_B - A_{OH^-}}{A_{H^+} - A_B} + \log \gamma^+$$

where A_B, A_{OH^-}, and A_{H^+} are the absorbance in the buffer, basic, and acidic solutions, respectively, and γ^+ is the activity coefficient of the ion in the buffer solution. This procedure or variations and modifications of it have been used to calculate the pKa values of many aromatic amines. For ultraviolet or pKa data on individual amines, the reader is referred to *Chemical Abstracts.* Recently pKa values of 2-(2′-thienyl)-pyridine and of methyl-2-picolyl sulfide were determined by Kahmann, Sigel, and Erlenmeyer (108) and of the dichloroanilines by Robinson (109). Apparent dissociation constants of some phenolic amines reported also (110), and there are reports of special techniques for individual amines and/or circumstances. For instance, determination of ionization constants on submilligram amounts of sample has been reported by Rinehart and Stafford (111).

Mixtures—Ultraviolet spectrophotometric analysis of mixtures is carried out routinely on two- and three-component systems. This is made possible, of course, by the fact that absorbance is linear with concentration and that the absorbances of various components in a mixture are additive. This concept is treated extensively in the text by Bauman (79) and others. Examples of its practical applications are found in the recent literature. Endriz (112) has described an ultraviolet spectrophotometric determination of heroin hydrochloride, methapyrilene hydrochloride, and quinine hydrochloride mixtures. The spectra for heroin, methapyrilene, and quinine show points of maximum absorption at 280 mμ, 313 mμ, and 348 mμ, respectively. Methapyrilene and quinine do not seriously interfere with the heroin maximum, and methapyrilene in dilute acid solution absorbs only slightly at the quinine maximum of 348 mμ. The procedure is fast and accurate enough for forensic purposes.

Analyses of chloramphenicol and tetracycline hydrochloride mixtures (113) and phenobarbital and pentobarbital in pharmaceutical mixtures (114) are further examples of the utility of UV spectrophotometry in this area.

Metabolite and Drug Levels—Because of the sensitivity of the ultraviolet method, the amount of sample required for spectral identification is very small; drug levels and metabolites have been determined in biological tissues and fluids after separation and extraction. The method is used extensively and routinely. Since most materials are not photosensitive, the technique is nondestructive, and the metabolite or drug can be recovered for further testing. Reports have been published for most pharmaceutical amines whose metabolites have been investigated discussing the UV spectra of starting material and metabolites. For UV data on specific metabolites, once again the reader is referred to *Chemical Abstracts* data on the specific parent amine.

RAMAN SPECTROSCOPY

Raman spectroscopy is presently undergoing a revitalization after years of relative inactivity. After the discovery of Raman spectroscopy in 1928, a wealth of information regarding molecular structure of organic and inorganic compounds was obtained. A large number of scientific papers was presented and published in the 1930's dealing with the analysis of molecular vibration by Raman techniques. Subsequently, however, infrared spectroscopy eclipsed Raman and took the lead in widespread use and application in industrial laboratories. The principal difficulty with Raman technique was the great care and time-consuming aspects associated with obtaining good data. In spite of the fact that good, reliable, and revealing information could be obtained with Raman, the experimental difficulties were too great for most people. Industrial chemists, especially, were reluctant to contend with the weak lines produced by Raman scatter, which took hours to produce a photographic spectrum of sufficient intensity, when they could prepare and obtain an infrared spectrum in a few minutes. Cost of equipment was also a factor which limited its use in most laboratories. The result of these experimental and cost disadvantages was the decline and overshadowing of Raman by infrared.

The development of the continuous laser as a source for Raman spectroscopy has revived and stimulated new interest in this technique. The laser, by increasing the intensity of the Raman scatter lines, has once more made the technique attractive. The additional development of electronic devices has made possible photoelectric readout and in a few minutes a complete vibrational spectrum from 4000–50 cm.$^{-1}$ can be recorded. The spectrum is recorded as intensity *versus* wavelength, and since the intensity is directly proportional to the concentration, the technique lends itself very well to quantitative analysis. Other advantages of laser-Raman are that the scatter lines produced by a laser source are sharper and easier to read than those from other sources. Focusing and collimating are simplified. Ultraviolet radiations from conventional sources which cause photodecomposition of some molecules is eliminated. Use of the ionized noble gas laser allows different laser lines of excitation to be used, and in some cases this eliminates the problem of fluorescence which often prevents obtaining a spectrum when a conventional Hg lamp is used. A sample containing water, which would obscure a good part of the infrared spectrum, can be run successfully by the Raman technique because the spectrum of water is weak in the Raman. The whole range from 4000–50 cm.$^{-1}$ can be seen without instrumental changeover (prisms, gratings, *etc.*). Selection rules for the Raman effect permit observations of some vibrations which do not make themselves known in infrared spectroscopy. For example, rotational transitions of molecules are observed for molecules which do not possess a permanent dipole moment. Vibrations involving groups such as $R_1R_2C{=}CR_1R_2$ which have very weak infrared absorptions at best can be seen clearly with Raman. The advent of the laser source has not only stimulated interest in academic and industrial scientists but has stirred the commercial world as well. With low-cost equipment available and more research on low-cost instrumentation promised, the outlook for increased use of Raman spectroscopy is very favorable.

For the best comprehensive summary of Raman spectroscopy up to 1960, see Brandmuller and Moser (115). Other review articles on the subject are those by R. N. Jones and M. K. Jones (116), A. C. Jones (117), and Green (118). A number of books devoted in whole or in part to Raman have appeared recently (119–122). The recent impetus given to Raman is indicated not only in the increased number of publications covering the subject but also in the beginnings of a shift of Raman from the academic laboratories exclusively to more industrial laboratories. The use of Raman to provide information on the C—C, C=C, and C—H skeletal bands of complex molecules will provide more information leading to the elucidation of structures. If the recent interest in Raman can be sustained and nurtured by more organic and analytical chemists, the amount of information involving group frequency-structure correlations in Raman will assume an importance on a par with infrared correlations used routinely in nearly every organic and analytical laboratory.

The principal applications of Raman in the area of organic chemistry have been in the field of hydrocarbons and amino acids. Studies have been made on glycine (123, 124), diglycine barium chloride monohydrate (125), and triglycine selenate (123). Stretching frequencies of N—H hydrogen bonding of these compounds have been investigated and reported by the same authors. Raman spectra of glycine have also been reported by Ghazanfar (126). Methyl, ethyl, and propylamine have been studied by Wolff and Staschewski (127). A number of other molecules have been investigated singly or in conjunction with infrared studies on the same molecule. It is often difficult to locate the

Raman spectra of the material in such cases because the spectra are often listed under infrared headings with little or no attention given to the Raman data in the journal index. In addition to this more recent data, there are, of course, data on individual amines listed in collections such as the Landolt–Bornstein (128) tables which include assignments for spectra. There are many amines and nitrogen-containing heterocyclics which are included in this collection. While the collection and accumulation of this data were slow and painstaking over the years, the data and assignments are of good quality and can be of great service not only in Raman work but in infrared as well.

MASS SPECTROMETRY

Introduction—The history of structural organic chemistry is filled with methods used to resolve complex molecules by breaking them into small pieces, which can be easily and unambiguously identified, and then assembling these pieces in a logical sequence to determine the original compound. Obviously as molecules become larger and more complex the task becomes more formidable. This differs in principle from infrared spectroscopy where functional groups present in the molecule can be identified and the positions of the groupings in respect to one another obtained. Isolated nuclear magnetic resonance data also provide fragmentary data and, if unsupported, does not permit unambiguous identification of many chemical species.

Just as NMR has become a valuable tool to provide unique structural information about unknown molecules and to complement infrared spectroscopy, mass spectrometry has also become a valuable complementary technique to provide additional unique information about molecules, and to facilitate the assignment and verification of chemical structures. Unlike ultraviolet, nuclear magnetic resonance, and infrared spectroscopy where compounds are recovered after analysis, mass spectrometry is a destructive technique; however, one might be consoled in knowing that fractional parts of a microgram of sample, which otherwise might be insufficient for other instrumental methods, are sufficiently large to obtain a perfectly good mass spectrum. Another important feature of mass spectrometry, in addition to its sensitivity is the fact that, even though a sample is impure, considerable structural information can be obtained, and although the sample is destroyed, a permanent record of the data can be obtained in an extremely short period of time. Scans of masses up to approximately 500 atomic mass units can be made in a matter of seconds.

There has been an aura of mystery associated with mass spectrometry because of the complex nature of a mass spectrum which can comprise a hundred or more peaks, and the lack of a simplified theoretical approach for interpreting these data. In spite of the immensity of data provided by a mass spectrometer, this technique has come of age and has become an equally important partner of the other spectroscopic techniques. This rise in importance and popularity is due, for the most part, to the efforts of such men as Professor F. McLafferty, Purdue, Dr. Klaus Biemann, M.I.T., Dr. R. I. Reed, Glasgow University in Scotland, Professor Carl Djerassi and his co-workers, Stanford, Professor Einar Stenhagen, Dr. Raynor Ryhage, Dr. J. H. Beynon, Dr. J. S. Shannon, and several others who have contributed significant data to the literature of this subject.

Mass spectra of compounds containing the amine function have been studied extensively (7, 129–136, 138). In the work of Gohlke and McLafferty (132), 67 aliphatic amines were studied, fragmentation patterns were presented, and methods for identifying amines were given. Collin (129) reported on the mass spectra of aliphatic amines including appearance potential data, likely structure, and additional mass spectral correlation data. Judson and Struck (153) have also correlated mass spectral data on aliphatic amines. The theory of unimolecular decomposition as applied to mass spectra electron bombardment of aliphatic amines has been discussed by Hurzeler, Ingraham, and Morrison (154). Budzikiewicz and co-workers (7) have discussed the mass spectra of aliphatic, cycloalkyl, aromatic, and steroidal amines as well as other nitrogen functions in their book and publications. A correlation of the mass spectral data of alicyclic amines has been reported by Saunders and Williams (155). This class of amines has been studied by several other investigators (155–159). The fragmentation process of some cyclic amines (158) and the location of the rearranging atom in some alicyclic amines (156) was established by deuterium labeling. The usefulness of dimethylamine derivatives in the study of steroidal fragmentation was presented by Pelah and co-workers (160). Geminal amine mass spectra (134), derivatives of amines (161, 162), and the mass spectra of amino acids (163–167) and derivatives (164) of amino acids have been studied extensively. Aromatic amines (140–143) and heteroaromatic *N*-containing compounds (168) have been studied extensively because of their significance in the pharmaceutical industry.

A discussion of all the above aspects of amine mass spectrometry is a formidable and quite impractical plan in this type of report; however, this is an attempt to present a practical coverage of pharmaceutically important amine-type functions, the factors that have provided the basis of mass spectral study of these compounds, and an understanding of the factors involved in using mass spectrometry for further study and future research in these areas.

In its most simplified version, mass spectrometry of organic molecules involves the bombardment of a hypothetical molecule, ABCD, in the vapor state with a high energy electron beam. If the ionization potential of the molecule is less than the energy of the electron bombardment, a single electron will be expelled from the molecule and a molecular ion ($ABCD^{+\cdot}$) is produced (*A*):

$$ABCD + e^- \longrightarrow ABCD^{+\cdot} + 2\,e^- \quad (A)$$

If this ion ($ABCD^{+\cdot}$) is accelerated without further breakdown and deflected in the magnetic field of the mass spectrometer, it will be collected at a radius that corresponds to the molecular weight. This, in fact, is the basis of molecular weight determinations by mass spectrometry. Further and more intense electron bombardment will break bonds and produce additional ions and smaller fragments of the original molecule. As more bonds are broken a multiple stepwise process will occur, producing one positively charged ion and additional neutral species:

Fragmentation:
$$ABCD^{+\cdot} \longrightarrow ABC^+ + D\cdot \quad (B)$$
$$ABC^+ \longrightarrow BC^+ + A\cdot \quad (C)$$
$$BC^+ \longrightarrow B^+ + C\cdot \quad (D)$$
$$BC^+ \longrightarrow C^+ + B\cdot \quad (E)$$

Rearrangement:
$$ABCD^{+\cdot} \longrightarrow AD^+ + BC\cdot$$
$$ABCD^{+\cdot} \longrightarrow AC^+ + BD\cdot$$

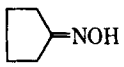
Ionization:
$$ABCD \longrightarrow ABCD^+ + 2\,e^-$$
$$ABCD \longrightarrow ABCD^{2+} + 4\,e^- \quad \text{(multiple ionization)}$$

The ions and fragments which form will depend upon the bond broken, its dissociation energy, and the stability of the charged and neutral species formed. This formation of a charged particle is analogous to carbonium ion and multiple bond formation, with the consequent stabilization of the ion by formation of positively charged particles having considerable stability, such as tropylium, azatropylium, acylium, oxonium, and ammonium ions.

$R'-CH=\overset{+}{O}H$ (oxonium)

$R-C\equiv O^+$ (acylium)

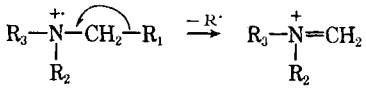
(tropylium)


(cyclic oxonium)

$$R'-CR''-NH_2^{+\cdot} \longrightarrow R'-CR=NH_2^+$$

(ammonium)

In general, the stability of a molecular ion will be enhanced by any structural feature which permits spreading of the odd electron charge over the molecular ion rather than concentrating it at any one atom. Thus charge delocalization leads to greater stability. In aromatic compounds, conjugated unsaturated systems, and functional groups having unsaturated groups, stable bonds are produced. In alicyclic systems where two bonds have to be broken to produce a fragment, additional stability is encountered. In amine compounds with which we are concerned here, the most easily removed electron in the ionization process is from the lone pair (nonbonding) electrons of the nitrogen atom. The process may be represented as in Scheme I.



Scheme I

[Homolytic processes will be indicated by a fishook (↷) which implies a one-electron shift, whereas an arrow (↷) represents a heterolytic (two-electron shift) (7).]

In dealing with the mass spectra of amine compounds we are dealing with a molecule having a nitrogen atom which provides more information than a nonnitrogen-containing molecule. In compounds having an odd number of nitrogen atoms, the molecular weight is odd, and in those having an even number of nitrogens, the molecular weight is even. In organic molecules containing carbon, hydrogen, and oxygen, fragments arising from the cleavage of *one bond* without hydrogen, transfer will always appear at odd mass numbers. The reverse of this occurs in compounds with an odd number of nitrogens, *e.g.*, one-bond fission of an odd nitrogen-containing compound will produce an even fragment. Elimination of neutral stable molecules is a frequent fragmentation mechanism. Nitrogen forms such stable species as HCN and NH_3 which are removed from amine-containing compounds. Amines frequently constitute a more volatile group than other nitrogen species, *e.g.*, NO_2^-, amido, *etc.*, and conversion to an amine permits easy handling and specific fragmentation,

which permits facile identification of unknown species. Deuteration of amino nitrogens also permits convenient study of reaction pathways and rearrangement processes. These points will be elaborated in the context of this paper.

The principles of mass spectrometry are dealt with in great detail in several textbooks (7, 169–174) and will be summarized here only briefly. To record the mass spectrum of a compound the sample has to be admitted into the ionization chamber of a mass spectrometer at pressures of 10^{-6} to 10^{-8} torr. For easily volatile materials this is done by placing the sample in a reservoir of the inlet system either as a gas or vapor at pressures of about 10^{-2} torr. and allowing it to "leak" through a very small orifice into the ionization chamber where it is bombarded with a high energy electron stream having a potential of about 10 to 100 e.v. (usually 70). The pressure in the chamber is kept low by a series of mercury diffusion pumps. Both positively and negatively charged particles and neutral fragments are produced. Only the positively charged particles are accelerated in the chamber, where the ion beam is focused magnetically and electrostatically (only in double focusing instruments) into a circular path. The radius of this path depends upon the ratio of the mass to charge of the cations (m/e). This radius is related to the accelerating voltage, V, and the magnetic field by the expression: $R = (2mV/eH^2)^{1/2}$. The mass spectrometer is designed so that only particles which describe a radius R will pass through the exit slit and be recorded by the ion collector. The current of the particles is of the order of 10^{-15} to 10^{-8} amps. This current is amplified and recorded *via* an electrometer and recording galvanometer which produces signals according to the relative abundance of the ions. The m/e value of the collected ions will be $R^2H^2/2V$ and will depend upon instrument design and applied electrical and magnetic field. The complete m/e spectrum is obtained by changing either the magnetic field, H, or the accelerating voltage, V, during the recording of a spectrum. It should be noted that mass spectra are recordings of discontinuous signals, unlike the continuous recordings in the infrared or ultraviolet region, since atomic masses vary in integral units. The units of m/e of atomic mass to charge in units of electric charge are termed "mass number" or atomic mass units (a.m.u.).

Instrumentation—The recent availability of moderately priced mass spectrometers (about $25,000) has permitted many smaller pharmaceutical laboratories and universities with limited budgets to enter the mass spectrometric field. As a result of this interest and activity, current literature on this subject has increased logarithmically in the number of published articles over the last few years and has significantly advanced the science. The lower priced instruments have resolutions of 500–850 with a 10% valley over the 1–300 mass range (Perkin-Elmer model 270) and up to 2500–3000 (AEI Model 15 and the Hitachi Model RMU6E).

High resolution instruments with a capability of 1 part in 30,000 or better are much more expensive ($80,000+). These instruments permit the analysis of fragments of about 5 p.p.m. or better. To be considered as high resolution, an instrument must have the capability of making mass measurements with a resolving power ($m/\Delta m$) of 10,000 or better with a 10% valley definition and at the same time have a precision of better than ± 10 p.p.m. measuring capability.

Much of our present mass spectrometric instrumentation is designed for use in qualitative organic structural analysis, and differs from earlier instrumentation which was used for the determination of isotope masses and, later, for relative abundances of these masses. These very early instruments were developed in the 1930's into precision equipment for mass/charge determinations which led to exhaustive studies using stable isotope tracers. About 1940 commercial instrumentation was first placed on the market.

There are two major types of commercially available high resolution mass spectrometers, the Mattauch-Herzog and the Nier-Johnson type. In both of these instruments there is an electrostatic field used as an energy selector, which controls the energy spread in the ion beam produced by the initial kinetic energy of the ions. The differences are in the methods of focusing the ions. In the Mattauch-Herzog system all the ions are focused simultaneously in one plane, and a collector slit can, therefore, be placed along any point in the plane. By scanning the magnetic field a complete mass spectrum of the ions can be obtained. This can be displayed either on a photographic plate or a high speed recorder. These, of course are permanent high resolution mass spectrum recordings. For very high precision mass determination of peaks, a peak matching procedure is used incorporating an oscilloscopic screen. In the Nier-Johnson type system the ion beam is refocused at a single point, and it is at this point where the collector slit is placed. In either system, of course, a complete mass spectrum can be recorded within a fraction of a second to a few minutes, depending upon the resolution desired and also on the particular spectrum being

obtained. A third type of instrument, the "time of flight" mass spectrometer, works on a different ion separation principle. Here a pulse of ions which has been produced by the ion bombardment is accelerated down a straight evacuated tube. In the course of their flight, the ions are separated according to the momentum they have received during their acceleration. These ions arrive at the ion collecter plate in order of increasing mass, the lightest arriving first and the higher ones follow in increasing order of mass. An instrument with capabilities of unit mass resolution of 700 ($m/\Delta m$) can be used for general organic structural analysis and identification. It has the advantage of attaining high scan speeds of a few milliseconds and is useful for thermodynamic, combustion, and fast reaction studies. It does not, however, meet the requirements for high resolution work.

The newest entry into the mass spectrometer field is the double focusing cycloidal medium resolution mass spectrometer (Varian Associates) which produces a cycloidal ion trajectory in which focusing depends only on the m/e of the ion and not on the direction or velocity of the charged particle.

Gas chromatograph attachment to the mass spectrometer permits mixtures to be separated and analyzed while the chromatograph is being run without the need for collecting individual peaks. This aspect will be discussed more fully later. Many instruments are equipped with detection systems which allow detection of negative ions produced in the fragmentation process. All the instruments mentioned here are suitable for use with pharmaceutical amines.

Sample Requirements and Manipulation—Boiling points and volatilities of organic amines range from the low boiling liquid to solid. Many of the amines are conveniently isolated and recrystallized as salts. Introduction of these materials into a mass spectrometer involves injection *via* a gas chromatograph, a gas inlet system, or a solid direct insertion probe. Modification by derivatization as silyl derivatives, trifluoroacetyl, or acetyl derivatives also provides convenient procedures for volatilizing the more intractable or heat-sensitive compounds. Probably the most elegant feature of the mass spectrometric method is the sensitivity of the instrument; submicrogram quantities of sample are sufficient for a mass spectrum (176). Biemann (175) has used 0.25 mcg. (1.5×10^{-9} moles) of phenylalanine and was able to get unambiguous identification of the compound. Elliot and co-workers (176) have obtained excellent accuracy of mass measurements with as little as 0.005 mcg. (5×10^{-9} Gm.) of ibogaine.

It is obvious, of course, but important that pure uncontaminated samples be used for the most valuable retrievable information. This presumes the use of high purity and sufficiently volatile solvents, which can be pumped out of the instrument before volatilization of the sample, when a direct sample insertion technique is used or when samples are being isolated from mixtures for insertion as liquids. This precaution is usually not necessary when good separation is possible *via* a gas chromatographic sample separation and insertion technique. Occasionally when an amine sample does not give a good molecular ion, introduction of a salt will help. If this is a hydrochloride it presents other problems by corroding equipment and being difficult to pump out completely. Nonvolatile compounds may sometimes be safely placed directly in the ion beam where, under the effect of high vacuum and temperature, small amounts are flaked off into the ion beam to produce a good spectrum. When a smoke or aerosol can be created in the ion source, highly intractable compounds inert to chemical modification are frequently analyzed successfully.

Molecular Weight—The molecular ion of amine compounds can be obtained if the compounds do not decompose under the influence of heat and electron bombardment at the low pressures (about 10^{-6}–10^{-8} torr.) that they will experience in the sample chamber of the mass spectrometer. In cases where decomposition does take place, more stable derivatives may be prepared by silylation, acetylation, trifluoroacetylation, *etc.* (discussed later). In most cases with amines, as with other compounds the "parent" or "molecular" ion may be found in the normal fragmentation pattern. It usually appears as the largest peak of the highest molecular weight multiplet of peaks in the mass spectrum, and represents a single electron removed by electron bombardment of the original compound: $M \rightarrow M^+ + e$.

McLafferty (132) examined the spectra of 67 aliphatic amines and found that the molecular ion was present, although in small relative abundance, in amines containing up to 16 carbon atoms. With primary amines the molecular ion intensity reaches a minimum (about 0.2–0.4%) in the region of nine to 12 carbon atoms. When the α-carbon atom is substituted, the molecular ion intensity is greatly diminished. McLafferty points out that in α-substituted primary amines containing four or more carbon atoms, the molecular ion is useless for analytical purposes; whereas, with α-substituted secondary amines, the molec-

ular ion is indistinguishable when there is a total of approximately eight carbon atoms. In cases where the longest chain of an α-substituted amine has more than four carbon atoms, the molecular ion cannot be used for molecular weight determinations.

In compounds where the amine function is attached or is part of an aromatic or alicyclic system, there is greater ion stability (177) and the molecular ion abundance increases significantly.

As the intensity of the parent peak of higher molecular weight amine compounds becomes indistinguishable precluding its use for determining molecular weight, there is increased possibility of observing the ion occurring at a molecular weight + 1, $(M + 1)^+$. This, of course, is now an even mass ion for compounds containing an odd number of nitrogen atoms, and as such is a diagnostic device, which helps to pick out this ion. This anamolous type of ion can also be found in alcohols, ethers, sulfides, glycols, and nitriles and is due to a bimolecular reaction which occurs in the ion stream. A striking feature of this ion which makes it useful for analytical purposes is the fact that it is pressure sensitive. The relative abundance of this $(M+1)^+$ peak increases with increased sample pressure and with decreased ion repeller potential. This peak is especially valuable with high molecular weight amines, and its presence can be demonstrated by rescanning the spectrum of these compounds at the higher mass range after reducing the repeller potential, thereby allowing longer ion residence time in the ion stream for reaction to occur. Increasing the sample pressure by adding another organic compound will also increase the relative amount of this peak. Munson and Field (178) have used chemical ionization mass spectrometry for certain amines and indicate that the ions in the ionization chamber of the mass spectrometer will produce a spectrum, which is more useful for determining the structure and the molecular ion for certain amines, and they indicate that the ions in the mass spectrum are predominantly high molecular weight fragments; whereas, the converse is true for electron impact spectra. Since the ionization potentials of primary aliphatic amines are greater than 8.5 v. and between 7 and 8 v. for tertiary aliphatic amines, the authors point out the differences indicated for the parent ion of these types of compounds are sufficiently different to allow isomers to be distinguished by this technique.

The $M+1$ ion indicated above was first reported by Wertzeler and Kinder (135) and later work (129) by Collin indicates that the $M+1$ ions arise from a reaction such as the following:

$$RNH_2 + RNH_2 \longrightarrow RNH_3^+ + RNH$$

To recognize the molecular ion in amines, certain tests may be applied to the highest mass peak that can help to distinguish whether this ion is not the molecular ion. These tests cannot determine if the converse is true.

Where the possibility exists that impurities are present, contributions from background or other artifacts, suitable steps such as partial ionization, examination of the compounds by thin-layer chromatography or gas chromatography should be used to complement these data. A combination of gas chromatography and mass spectrometry (discussed later) may provide a suitable system for obtaining a meaningful mass spectrum of the compound.

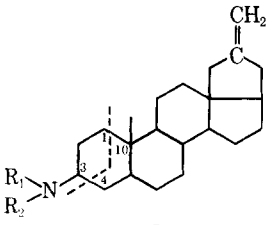
Since the molecular ion is produced by electron impact and the loss of one electron from the molecule, the ion produced must be an odd-electron ion, M^+, and appear at an odd mass. An even electron ion, M^+, appearing at high mass at an even mass number is usually the molecular ion. For an even mass ion to be a molecular ion it must contain an odd number of nitrogen atoms. In cases where major ions appear at positions separated from the highest mass ion by anomolous mass values or elemental composition by high resolution, mass measurement is illogical; the molecular ion is probably not the molecular ion or else there probably is an impurity in the sample. Mass losses of 3 to 14, 21 to 25, 33, 37, and 38 are highly improbable, and an examination of the masses compiled by McLafferty (179) in his mass spectral correlations will help to establish whether such an ion is probable. To prevent possible mistakes in considering the molecular weight of compounds, which have atoms where isotopes are naturally present, such as with bromine or chlorine, the mass should be calculated on the basis of the lower mass of the two isotopes. Low energy bombardment may also help to establish the identity of the molecular ion. Since the molecular ion is the precursor ion, it requires less energy for its formation, and as the electron energy is lowered, it should be the last one to disappear. In the case of some amines this would be difficult because of the low abundance of the molecular ion, but for derivatives, this should be suitable. An ion with the lowest appearance potential is not necessarily the molecular ion; only the converse would be true. Empirical formulas can frequently be determined from the two small peaks at $(M+1)^+$ and $(M+2)^+$, which are due to the isotopes of C, H, O, N, Cl, Br, S, *etc.*, present in the compound. If an illogical mass is obtained,

further investigation of other peaks, sample purification, *etc.*, are in order. Molecular weight determinations may also be field ionization (180) and rate of effusion techniques. Once the molecular ion has been identified, interpretation of the remainder of the spectrum can start.

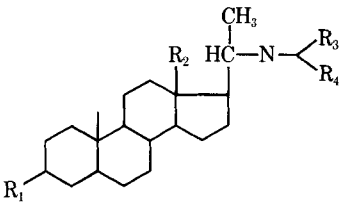
Use of Isotopes—Radioactive and stable isotope use in chemical, biochemical, and physiological fields has increased over the years very markedly. The availability of ^{18}O, deuterium, ^{13}C, and ^{15}N has provided the stimulus for research in biological and chemical fields where only small amounts of isolated compounds are available for study, and where incorporation of these materials into a molecule provides an elegant way to determine the mode of action, as well as to provide a means of following the compound in a living system. It is, therefore, not unusual to incorporate a stable isotope in a drug and use the mass spectrometer to assist in identifying and following the metabolic fate of the drug in an animal or microbial system. Because many pharmaceutical compounds have an amine function associated with them, it is not uncommon to exchange the active hydrogen atoms attached to a nitrogen with deuterium or to synthesize an amine or other nitrogen-containing pharmaceutical with ^{15}N.

Mechanisms (7, 158) of mass spectral fragmentation have been studied *via* deuterium exchange in many phases. The replacement of —N—H with N—D or NH_2 with —ND_2 leads to fragment ions of one and two atomic mass units greater than the parent; however, in the case of tertiary amines there is no exchange, and hence no effect.

Deuteration is achieved readily by equilibration of the sample with deuterium oxide. Deuteration has been useful to study fragmentation patterns, to determine structure, to examine ionization potentials. After deuteration of an amine, comparison of the fragments and the molecular ion permits identification of the fragments which have the nitrogen containing the deuterium. In cases where there are two nitrogens present in the molecule, the procedure becomes particularly useful. Systematic studies have been made by Pelah *et al.* (147) upon the migration mechanisms of hydrogen migration in amides and amines, and for the selective replacement of certain hydrogen atoms in *N*-acetylcyclohexylamine. Fragmentation of steroidal amines was also studied after deuteration. Incorporation of deuterium in the amine nitrogen of *Holarrhea* alkaloids of types I and II by Dolejs *et al.* (181) permitted these workers to ascertain the number of methyl groups bound to the individual nitrogen atoms.

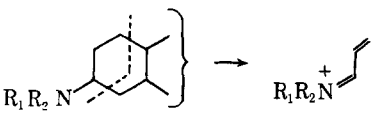


I



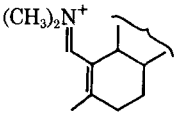
II

By the fragmentation process in Scheme II, cleavage in these molecules was shown to occur by rupturing of the C_3—C_4 bond (β-bond to the nitrogen atom) and the C_{1-10} to give fragments as those in the scheme. The nitrogen ion which was formed was stabilized by splitting off or transfer of hydrogen atoms. Adequate metastable peaks in sufficient abundance were also found to verify the fragmentation. Where R_1 and R_2 are methyl the inductive effect of the methyl group stabilizes the positive charge on the nitrogen atom (*Reference 175*, p. 88) and produces high abundance fragments. Additional studies on the structure and stereochemical relationships of steroidal dimethylamines (152) done by Pelah and co-workers supported these studies by deuterium labeling experiments. This work showed the most abundant ion in the fragmentation of steroidal dimethylamines corresponds to the immonium ion (III) and they predicted this



m/e = 56
70
84

Scheme II



III

species in terms of charge localization on the nitrogen of the molecular ion and subsequent fragmentation by rational bond homolysis and hydrogen transfer. They also showed in their studies that dimethylamino compounds were desirable derivatives for mass spectral purposes.

The exchange of active hydrogen for deuterium in amines can very conveniently be carried out by injection of D_2O into a gas chromatograph, and then immediate injection of the amine with the replaceable hydrogen. Alternatively, this can be done with the sample in contact with D_2O in the heated inlet system of the mass spectrometer.

Ambiguities arising from the splitting out, for example, of an M-28 peak, which could be due to the loss of H_2CN, CO, C_2H_4, or the appearance of a M-27 peak due to the loss of C_2H_3 or HCN, can be resolved in many cases with deuterium labeling.

Aside from the labeling aspect of isotope mass spectral data, the isotope peaks $M+1$ and $M+2$ can be used to calcuate the molecular formula of a compound. Details may be found in the textbooks mentioned above (170–175).

Elemental Analysis and High Resolution Mass Spectrometry—In addition to the information obtained from the molecular ion determination, information on molecular structure is obtained from an elemental analysis of secondary ions which provides information on the arrangements of the atoms in the molecule. Compounds which contain several isotopic elements (polyisotopic) provide unique peak abundances in proportion to the abundance of the isotopes normally associated with an element. These data are useful both qualitatively as well as quantitatively. They not only indicate the presence of these elements but often tell how many of the atoms are present in the molecule. The monoisotopic elements found with organic compounds include fluorine, phosphorus, and iodine. These are also useful in studying fragmentation mechanisms and compound structures. Perfluorokerosene, for example, is one of the most widely used internal standards for peak identification and peak matching to obtain very accurate mass values.

Microcombustion analysis for the elements, because of its inherent errors, falls short of the capabilities of the mass spectrometer; however, the data provided by such determinations very often complements mass spectrometric data and also provides information on the compound such as inorganic ash content, compound stability, nature and ease of compound decomposition, volatility, ease of sublimation, *etc.* Many researchers use combustion data to verify or support their mass spectral findings. One must, however, recognize that unit mass measurements with low as well as high resolution instruments provide accurate means for establishing exact molecular formulas. Because of this high degree of accuracy, high resolution mass spectral determination has removed the ambiguities that arise from combustion analysis data and has simplified this aspect of structural elucidation problems. This has been extremely useful in pharmaceutical applications for high molecular weight amine compounds, alkaloids, polypeptides, *etc.* (151), which differ from other possible structures by mass units less than one. Microelemental analysis would have been completely inadequate to assign exact empirical formulas for such compounds. Another advantage with mass data of this type is for determining molecular formulas of mixtures of compounds, if there is supporting additional information available on a sample. When molecular species present or suspected as being pressed in a sample have sufficiently different masses, they can often be recognized and determined because of unusual fragment ions which are not present in normal fragmentations. By changing parameters on the instrument such as ionization voltages to below ionization potentials of some of the compounds present in a mixture, distinction can be made between several compounds; by changing temperature, a fractionation is often possible; or by coupling a gas chromatograph, separate compounds in a mixture can be determined as they elute from a column. These are discussed later.

High resolution mass spectrometers which use both electrostatic and magnetic focusing systems are capable of determining and distinguishing between molecular formulas of monoisotopic molecular weights to values of less than a few parts per million of the actual mass on as little as 0.2 mcg. of sample. The modern approach to determining elemental formulas for all the ions in a mass spectrum with high resolution mass spectrometers was introduced by Biemann and co-workers (182). High resolution mass spectra may be recorded on magnetic tape, photographic plates, recorder charts, or obtained by peak matching techniques using an oscilloscope (171), or by combinations of all these techniques. These data may be fed into a computer (184), and the information may be displayed as an "elemental map" containing all the ion species arranged in columns according to mass, elemental composition, intensity of peaks, and elemental combinations (183). Element mapping makes use of all the peaks in a mass spectrum, unlike

that in normal spectra where many of the low abundance peaks are disregarded. These lesser abundance peaks very often provide much of the information, which allows a complete structure determination, that otherwise would either be impossible or extremely time consuming to obtain. Biemann (185, 186) has provided several examples of this "elemental map" technique for the analysis of high resolution mass spectra of alkaloids, steroids, amines, and many other compounds. Powers and Wolstenholme (183) used element mapping to determine the structure of linear peptides, cyclic peptides, and cyclodepsipeptides. They report that computer interpretation of the high resolution mass spectra of peptides completely removes the necessity for quantitative amino acid analysis. For linear peptides, acylation provides sufficient information for the computer to identify all the peaks by their atomic composition. Spectra at a resolution of 10,000 can be run in as little as 10-sec. intervals thereby permitting this technique to be used simultaneously with gas chromatography. The result is that unambiguous assignments of atomic composition of molecular and fragment ions are possible even in complex mixtures.

The accuracy of measurement is limited to the capability of the instrument to resolve very close peaks, and this determines, to a large extent, how unique a differentiation can be made to select two or more possible elemental combinations to fit an empirical formula. In cases where it is necessary to determine the differences in mass of a compound containing a CH_4 (m/e = 16.031300) or an oxygen (15.994915) where the difference in mass is (0.036385) or 36.4 micro mass units (m.m.u.) it would require an instrument with a resolving power ($m/\Delta m$) = 16/0.036385 = 440. If this difference existed in a molecule with a mass of 160, an instrument with a resolution of 4400 would be required (*e.g.*, 160/0.036385 = 4400), and, as the case may very well be with pharmaceutical compounds, a mass twice this amount, *e.g.*, 320, would not be unusual. This, of course, would require a resolution of 8,800 or very nearly 10,000 which approaches the resolution limits for many "high resolution" instruments. For a compound containing C, H, N where a distinction between CH_2 *versus* N is required (Δm = 12.6 m.m.u.) an instrument with a resolving capability of ±5 m.m.u. would be necessary. Other more demanding resolution capability (±1 m.m.u.) would not be unusual.

Biemann (186) has reported many of the techniques where high resolution mass spectrometry can be used. The voluminous data accumulated from mass measurements have made element mapping a very desirable technique and have made interpretation of spectral data an attractive technique. Suitable computer programs (183, 184) have been developed to handle such mass spectrometric data, and frequently when such data are presented, interpretation may be made by use of a few significant peaks which show the parent ion, the important fragments, and the fragmentation process with reasonable certainty. Many aliphatic amines (187) are determined this way. Several approaches to the interpretation of high resolution spectra have been reported and should be consulted for more details and the rationale used. The usefulness of high resolution mass spectrometery used in conjunction with element mapping or exact fragment determination has been reported in several reviews (188–194).

Sample Modification—*Derivatives*—Incorporation of stable and radioactive isotopes in compounds for mass spectral analysis was described earlier. Amine compounds very often can be successfully analyzed by means of their more stable derivatives. These compounds may be acetylated with acetic anhydride or other suitable anhydride, trifluoroacetylated, or converted into silyl derivatives (195).

Many easily obtainable and efficient silylating reagents such as bis-trimethylsilyl, acetamide (BSA), hexamethyldisilizane, trimethylchorosilane, bromomethyldimethylchlorosilane (197), and chloromethyldimethylchlorosilane may be used. The halogenated compounds are useful for work at low concentrations of sample, using gas chromatographs incorporating electron capture detectors at low or only moderate temperatures. Amino acids (198,199) are readily converted into volatile, stable derivatives with BSA, *N*-trimethylsilydiethylamine, *N*-trimethylsilydiethylamine.

Hill (170) and Reed (169) give detailed instructions on the utilization of chemical reactions to convert many compounds to amines to obtain characteristic fragmentation patterns for structural elucidation. They give examples for converting amides and nitro compounds to amines, nitriles to primary amines, isonitriles to secondary amines, *etc.*, to assist in characterizing them. Thus treatment of primary aromatic amines with nitrous acid and heat converts them to phenols, secondary amines to N-nitroso and tertiary amines to C-nitroso derivatives. The mass spectra of these compounds are usually quite easily distinguished because of characteristic peaks. In the case of C-nitroso *versus* *N*-nitroso compounds,

the *N*-nitroso compound should lose NO (M-30) easily. Reed (169) has treated the subject of distinguishing compounds containing one, two, or more nitrogens adequately. Budzikiewicz, Djerassi, and Williams (7) have illustrated the usefulness of the information in compound identification and reaction mechanisms of fragmentation patterns. They have also shown the utility of using stable isotopes in such studies. Several reviews on this subject are also available (131, 188).

Gas Chromatography–Mass Spectrometry—Resolution of many of the difficult problems associated with isolation of components in a mixture, especially when they are present in trace amounts, has been greatly facilitated when the advantages of a gas chromatograph have been combined with those of a mass spectrometer. The capability of rapid scanning of peaks emerging from a gas chromatograph, even those small trace peaks or peaks which have shoulders or tails, has provided information which previously has been extremely difficult and time consuming to obtain. Because of the speed at which a spectrum can be obtained on a mass spectrometer, a peak appearing in the gas chromatographic recording can frequently be scanned several times to determine if it is, indeed, a single component, or whether it is an unresolved mixture. It is easy, of course, to see the potentialities of this technique for studying the reaction products of an organic synthesis, microbiological transformation, metabolic transformation, or identification of the components in a simple mixture. Reaction mechanisms and much of the physical chemistry of compounds may be deduced by the procedure. Because mass spectrometers handle small samples (as little as 0.2 mcg.) with relative ease, the high plate efficiency of capillary gas chromatographic columns can be exploited. Thus, materials obtained from human, animal, or agricultural isolations can be examined easily. Research in the study of drug metabolites of chlorpromazine found in human blood (200) have been monitored by Holmstedt, Hammar, and Ryhage, Karolinska Institute. Their studies have indicated that chlorpromazine disappears from the blood in about 4 hr., but the drug metabolites continue to circulate for an indefinite time, and rather significant to medical and pharmaceutical scientists is the fact that breakdown products differ from person to person.

In addition to the advantages described above, the gas chromatograph provides a convenient way to introduce volatile gases, liquids, and volatile solids into the mass spectrometer. Rapid volatilization and hence low residence times at high temperatures often permit heat-sensitive compounds, such as steroids and their derivatives, to be examined without thermal decomposition.

In the past few years mass spectrometer–gas chromatographic combination instruments have become available commercially. These instruments couple gas chromatographs to a mass spectrometer by three popular separators (201), the Biemann-Watson, the Becher-Ryhage (202), and the Llewelyn types (203). The first two are based on the concept of sample enrichment by gas diffusion, whereas the Llewelyn, a two-stage separator, is based on the use of a permeable barrier separation where an elastomer barrier sets up a condition somewhat similar to the liquid phase of a gas chromatographic column. The gas not being very soluble does not permeate the barrier greatly, whereas the organic liquid, on the other hand, does. The result is an enriched gas on the opposite side of the barrier to the one in which the gas passes. For additional enrichment a two-stage separator is used. These elastomeric membranes are made of silicone rubber and permit very rapid organic liquid permeability while enriching the gas at the same time. Since permeation of different organic materials varies considerably, the gas chromatograph–separator cannot be used strictly as a quantitative tool; however, the gas chromatograph itself should be considered the quantitative tool (see above), and as such, does not affect the utility of the system. The operating parameters in using fast scanning, high resolution mass spectrometry in tandem with gas chromatography have been reported by McMurray, Greene, and Lipsky (204), by Watson and Biemann (205), and many others (206-208). A direct application for the separation and identification of optically resolved amino acids is reported in the work by Halpern and coworkers (209).

Quantitative Analysis—Quantitative mass spectral analysis was pioneered and certainly exploited by the petroleum industry. While many of the applications of mass spectrometery are for qualitative analysis or structural elucidation, quantitative mass spectrometric analysis of a 30-component mixture can be done rapidly and accurately ($\pm 1\%$) (210). The method used in quantitative mass spectrometric analysis is similar to that in infrared or ultraviolet spectrometry. Peaks are chosen from the spectrum of pure compounds and used for calibration. Ideal peaks are those which have high intensity and freedom from interference from other components in the mixture being analyzed. After choosing these peaks, measure-

ments are made for all the compounds present in the mixture and a calibration curve is prepared. Simultaneous linear equations are set up, one for each compound. Thus, a 30-component mixture would require 30 × 30 sets of equations (211). These are best handled using an inverse matrix and a computer program (213). A desk calculator is quite satisfactory for small systems once a matrix has been set up (175). Where occasional analyses, say a few a week, are required, mass spectrometry would probably be impractical; however, in cases where hundreds of analyses on multicomponent systems are needed, the mass spectrometer is a very efficient technique. Calculations for quantitative analysis are detailed in texts on mass spectrometry (169, 212, 213).

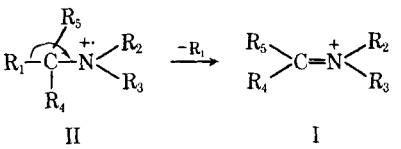
When mass spectrometry is combined with gas chromatography, the mass spectrometer sees only fractional parts of the sample because of the variances due to separator efficiencies and varying efficiencies of ion collection. This is due to the fact that ions pass through a variety of electrical and magnetic fields before reaching the collector. Exact conditions, careful control of temperature, and careful calibration are necessary for good mass spectral quantitative data. It is much more reasonable to use the gas chromatographic data for quantitative analysis and the mass spectrometer for the qualitative identification of the components present in the mixture.

Interpretation of Mass Spectra of Amines—After the molecular ion of a compound has been located and an empirical formula established from the mass spectral evidence, identification of structure begins. This involves the assemblage of the spectral fragments, a rational picture of the elements, functional groups, and other structural features into a chemical structure which will stand the tests of all or any other evidence that is presented, *e.g.*, spectral or chemical. The whole sequence of events involved in breaking down a molecule into smaller, more simple fragments must, in effect, be reversed. An understanding of these fragmentation processes has been the subject of extensive investigation by many serious individuals (145, 148, 154, 196, 214–218), but their work, for the most part, has been limited to practical, mechanistic, and semiempirical approaches. Their efforts, on the other hand, have permitted a logical interpretation of compounds based on spectral evidence. There has, however, been only limited effort made on the development of the theoretical aspects of mass spectrometry.

Many of the relationships in the fragmentation of simple amine structures may be extended to more complex pharmaceutical amine compounds, and an understanding of these will help to identify the structure of the more complex pharmaceutical amine structures. These structural features which appear in the mass spectra of amines are considered below on an individual basis. It should be remembered that identification and interpretation of mass spectral data is much easier and more meaningful if the data are plotted under high resolution conditions, and the formula of each peak determined from accurate mass measurement or an element map is prepared. Lacking such capabilities, much structural evidence may be gleaned from low (unit) or medium resolution instruments (231).

Aliphatic Amines—In considering the spectra of aliphatic amines, considerable information about the fragmentation processes may be deduced by a comparison with the spectra of known ethers and alcohol compounds (133). It might well be imagined that the spectra of amines would be more complex because of the nature of a nitrogen-containing molecule where there exists the possibility of three rather than two side chains. Primary aliphatic amines (129–133, 214) fragment by a C—C cleavage at the bond β to the nitrogen, by alpha cleavage, and by rearrangement and cleavage. Many examples of this type of fragmentation are found in the references given earlier (129–134). Rules regarding the fragmentation of aliphatic amines are included which were derived from a large number of amine compounds, many of which had been tagged with deuterium.

Important in the primary fragmentation process of an aliphatic amine (132) is the removal of the lone pair of electrons of the heteroatom, followed by cleavage of the carbon-carbon bond adjacent to the nitrogen atom (α-cleavage) to give a positive ion (I) and an alkyl fragment (R_1) (Scheme III).



II I

Scheme III

Ion I is the most intense peak in the spectra of primary, secondary, and tertiary alkyl amines (II, $R_4 = R_3 = H$) and α-mono- and α-disubstituted primary amines (II, $R_2 = R_3 = H$, R_4 = alkyl or H). When R_3 and/or $R_4 = H$, an M-1 peak is usually visible. The largest alkyl group is lost preferentially and hydrogen is eliminated only when no other alternative exists. Since the

amino group usually directs the fragmentation process (215) in preference to other functional groups because of the ease with which it loses an electron and because of the stronger resonance stabilization of the ion fragment, the only structural information available is that from the low mass region.

Examples of the fragmentation which occur in several amines of biological importance were reported by Beckett and co-workers (136) and by others (137) and are shown in Scheme IV. This information has been proposed to aid in the identification of metabolites as well as parent compounds in the urine of dosed individuals.

One mass unit differences in possible fragments differentiate between these two isomers (137).

Amino Acid Esters—Fragmentation of α-amino acid esters (217–223) occurs according to the general Scheme V, where β-cleavage is the preferred mode of fragmentation. The charge is carried on the amine fragment as expected. If the ester is an ethyl, the ester fragment will have a mass of 102 and can be used to identify the NH_2 position in other amino acid esters. From the molecular weight, the amine fragment, as well as the ester fragment, any substitution in the α-carbon position readily can be deduced.

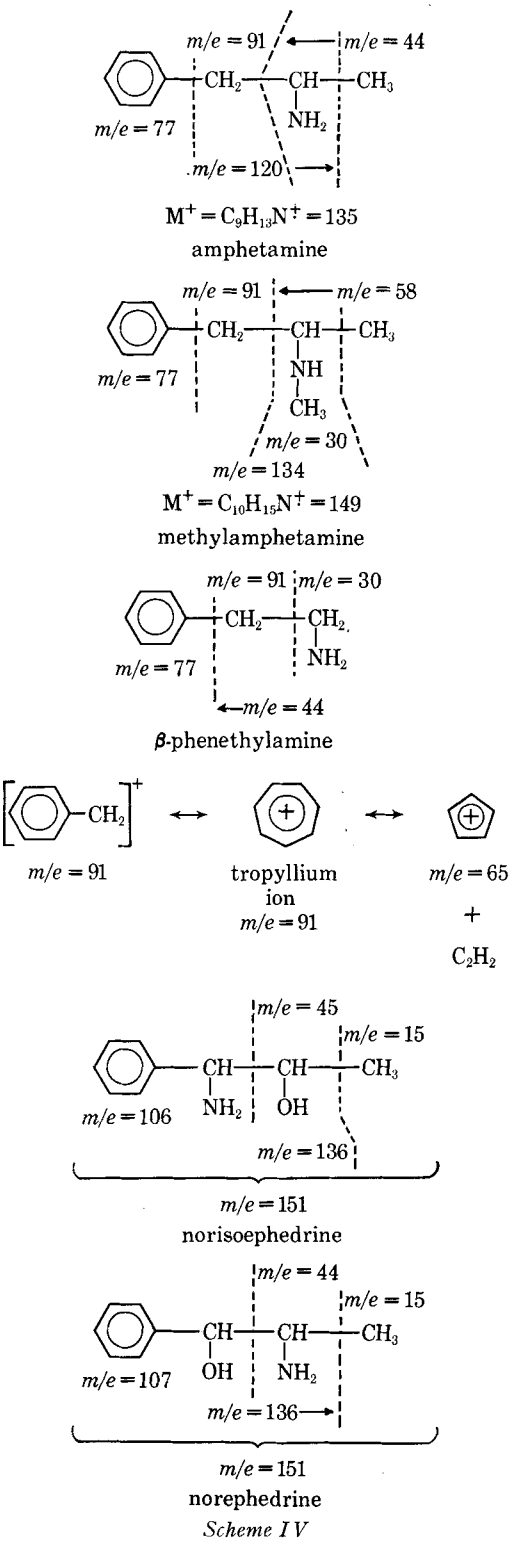
Just as in the aliphatic amine compounds, many amino acid esters have weak molecular ions but do have a characteristic M+1 peak detected by changes in pressure (*vide supra*).

Using the information obtained by studying known amino acid structures, it is possible to assign structures to new amino acids. Thus, Biemann *et al.* (224, 225) established the structure of lysopine, the lysine analog of octapine, which contains an arginine moiety. The processes leading up to the identification of new amino acids by mass spectrometry are elegantly described by Biemann (139).

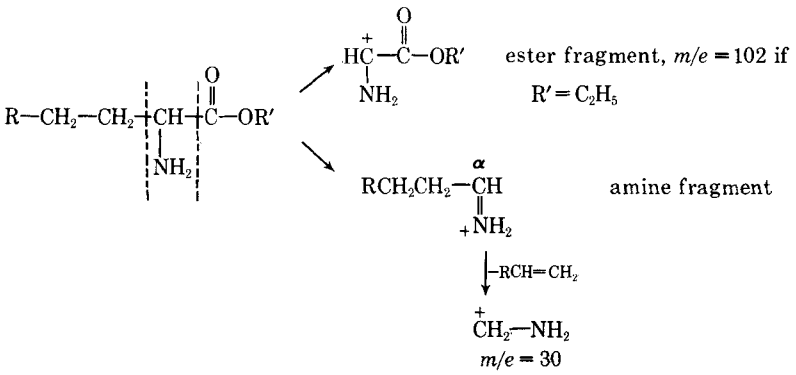
Aromatic Amines—The mass spectra of primary aromatic amines show that there are no bonds which are easily broken, therefore aniline loses one of the amine hydrogen atoms to give a moderately strong peak at M-1, also a peak in which HCN is lost. *N*-Alkyl anilines decompose by cleavage of a C—C bond next to a nitrogen to give an ion fragment of *m/e* = 106 (Scheme VI).

The mass spectra of aromatic amines have been reported by several authors (226–229).

Other Amine Structures—Reports on the mass spectra of cyclic amines include the work of Duffield and co-workers (230) and Gallegos and Kiser (231). Geminal amines (134) are described by Kostyanovskii and Pan'shin. Mass spectrometry has been successfully used to determine the amino acid sequence in oligopeptide methyl esters (146) and has proved ex-



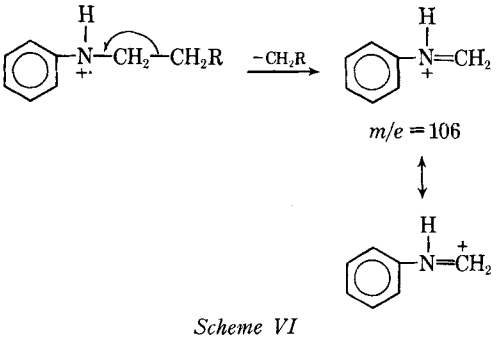
Scheme IV



Scheme V

tremely useful in structural and stereochemical problems of steroidal dimethylamines (147). Nitrogen in a steroid very strongly and consistently directs the mass spectral fragmentation, so it is very desirable to use mass spectrometry for the study of many steroidal amines to complement other spectral techniques. The fragments have diagnostic value for determining both the size and number of alkyl groups attached to nitrogen and also the nature of carbons in other functional groups in related molecules. Smith and Grostic (230) have described a procedure for mass spectrometric identification of the metabolites of an orally active diuretic agent, methyl *N*-(*o*-aminophenyl)-*N*-(3-dimethylaminopropyl) anthranilate. The mass spectra of enamines (149), and acetylene-amine adducts (150) have been studied. The recent review by Spiteller (216) on the mass spectrometry of heterocyclic compounds includes information on many amine-containing alkaloids, heterocyclic, and other compounds and should be consulted for an excellent description of the uses to which mass spectrometry may be put in the study of many biologically related compounds. Rearrangements (133) and hydrogen transfer reactions (132) in amides and amines are also reported.

In the field of pharmaceutical chemistry mass spectrometry has made a significant and important impact in the last 10 years and it promises



Scheme VI

to pioneer even greater pathways in the future. It should contribute much understanding of the processes involved in microbial as well as animal metabolism, and potentially supply valuable information for the development and advancement of new drug designs by providing the means for identification and elucidation of new structures of matter.

SUMMARY

The various branches of spectroscopy hold much promise for organic amine structure elucidation and analysis. Procedures and techniques for deducing information about these compounds are now quite well established, although new approaches are being developed daily. Future developments in this field are likely to include development of new sophisticated techniques and equipment, simplification of known techniques and equipment, and, most of all, wider use of computers to allow the research worker to learn more with less effort.

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Keyphrases

Amines–spectroscopy review
NMR spectrometry–structure
IR spectrophotometry–structure
UV spectrophotometry–analysis
Raman spectroscopy
Mass spectrometry

Research Articles

Role of Wetting on the Rate of Drug Release from Inert Matrices

By PARVINDER SINGH*, SAURABH J. DESAI*, ANTHONY P. SIMONELLI, and WILLIAM I. HIGUCHI

This investigation has shown that matrix permeability and rates of permeation of the matrix by the solvent can individually limit drug release rates. This was found to be a function of the pore size distribution of the matrix and the permeation pressure of the release media defined by its surface tension and contact angle. Methods useful in separating the various roles of the above were developed and are presented. They include the correlation and use of external pressure, vacuum techniques, surface tension and contact angle measurements, and porosimeter data. The results have been used to develop models to illustrate the possible systems that can be encountered. Concepts such as rates of pore permeation, varying solubility dependence, and tortuosity are developed and applied to these models.

PREVIOUS release studies have indicated that the polyethylene matrix gave excellent results in the presence of surfactant (1). The experiments in water, however, gave comparatively poor results as release rates were very slow and apparent tortuosities very high. It was speculated at that time that polyethylene was hydrophobic in nature and that wetting was not fully achieved.

Studies involving the effect of increasing concentrations of dibasic potassium phosphate on salicylic acid release from polyethylene matrices surprisingly showed no rate increase due to potassium phosphate even though the solubility increased by a factor of 45. There was, however, a dramatic rate increase in the presence of surfactant, even though the solubility remained constant.

These studies prompted this investigation in order to further clarify the possible mechanisms which can be operative under various conditions.

EXPERIMENTAL

Release Rate Determinations—The general procedure used for investigating the release rates has been previously described (2). It was necessary, however, to redesign the apparatus to allow the pressure to be kept constant at different pressures, as illustrated in Fig. 1.

A water-jacketed (C) glass conical flask with 2

Received April 14, 1967, from the College of Pharmacy, University of Michigan, Ann Arbor, MI 48104
Accepted for publication September 11, 1967.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
Supported in part by a grant from Parke, Davis and Co., Detroit, Mich., and in part by a grant from Ciba Pharmaceutical Co., Summit, N. J.
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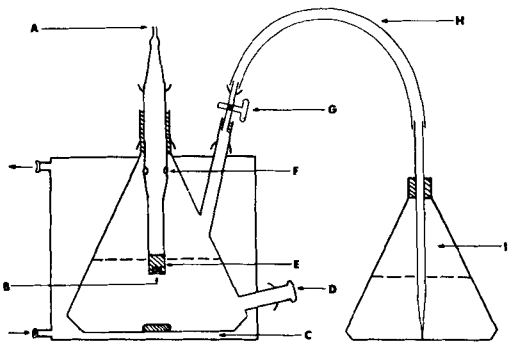

Fig. 1—Schematic diagram of the apparatus used to study release rates under positive net pressures.

side arms was used for these experiments. The tablet was mounted on a holder with a ground-glass joint to fit into the ground-glass necked conical flask. The lower part of the tube (E) was sealed to prevent hydrodynamic flow during the pressure experiments. The holder (A) was designed so that vacuum or pressure could be applied to its upper end and transmitted to the surface of the solvent by means of the lower openings (F).

Pressure was applied and maintained at a constant level by utilizing a tank of compressed nitrogen. To maintain a closed system when it was charged with solvent under vacuum conditions, the side arm (G) was connected by means of a tube (H) to a flask containing the solvent. Before vacuum was applied, the connecting tube (H) and stopcock bore (G) were completely flushed with the solvent to remove any entrapped air. A vacuum pressure of less than 0.1 mm. Hg was obtained to make certain all air was removed from the matrix before the solvent from the flask (I) was allowed to flow into the apparatus. The length of the tablet holder was sufficient to ensure that the tablet was not exposed to air, but was completely immersed in solvent prior to the release of the vacuum.

It should be emphasized that once the matrix is immersed in solvent, air can no longer enter the matrix, and a pressure differential of 1 Atm. remains on the system even though the vacuum is eliminated. Since this net solvent pressure of penetration is given by $P_{net} = P_{vacuum} + P_{external}$ it can be readily seen that each specific pressure can be achieved by many combinations of the above. For obvious technical reasons, however, the vacuum contribution was restricted to 1 Atm. pressure.

Samples were withdrawn using a 10-ml. syringe which was injected through a self-sealing multidose vial stopper (D). The sample outlet was placed so that it was always below the solvent level to prevent a change in pressure due to loss or addition of air to the system whenever fluid was removed or added.

Contact Angle Measurements—Contact angle measurements were made for all experiments reported in this study. The solutions used were made by dissolving enough drug in the solvent to provide a concentration equivalent to approximately 95% of its solubility in order to approximate the conditions existing in the matrix at the solvent front boundary.

The tablet was placed on an adjustable platform kept perfectly horizontal by means of a leveling device. A small drop of the test solution was placed on the tablet surface, and the contact angle was measured using a Gaertner[1] telemicroscope fitted with a protractor eyepiece and an objective lens (magnification 5×). The entire unit was mounted on an adjustable stand. The hairline of the protractor eyepiece was adjusted to coincide with the flat surface of the tablet, and the intersection of the two hairlines was fixed at the solid–liquid–air triple interface. With this baseline adjustment made, the reading on the protractor eyepiece was recorded. The eyepiece was then rotated until the horizontal hairline was made tangent to the liquid–air interface at the table surface. The angle difference between these two readings was equal to the contact angle used in these studies. These contact angles are not the contact angles in the classical sense because of the presence of capillaries and other heterogeneities on the surface. It should therefore be noted that the term contact angle, as referred to in the text, is in reality only an apparent value. However, it should also be emphasized that these measurements, and not the classical ones, are valid and provide a useful method of comparing the wetting tendencies of the different systems studied.

Other Parameters—The solubilities and diffusion coefficients were measured using methods reported earlier (3). The surface tension measurements were carried out using the Wilhelmy plate method (4). The solutions used were again made by dissolving enough drug in the solvent to provide a concentration equivalent to approximately 95% of its solubility.

RESULTS AND DISCUSSION

Water and Surfactant Data—As mentioned in the introduction, the release profile of salicylic acid from the polyethylene matrix was studied utilizing a wide range of dibasic potassium phosphate concentrations, thereby providing increasing solubilities. The results of these studies, along with the apparent solution–tablet contact angles, are presented in Table I. The expected slopes listed in the last column of Table I have been calculated using the 0.01 *M* K_2HPO_4 slope as the reference, and then calculating the release rates for the dibasic potassium phosphate systems based on the predicted square root of solubility dependence (2). It is clear that this dependence is not observed, as the experimental rates for the various systems studied are relatively constant and do not show the predicted five- to six-fold increase. This strongly indicates that the diffusion model is not applicable under the above conditions.

Examination of Table I further shows that for all these dibasic potassium phosphate solutions, the contact angles are extremely high (>90°), and reflect a nonwetting solid–liquid interface. This suggests that drug release may possibly be controlled by the rate of solvent penetration.

If this is true, the release rates should be considerably increased by reducing the contact angle to below 90°. Studies in water and 0.01% dioctyl

[1] Gaertner Scientific Corp., Chicago, Ill.

TABLE I—PARAMETERS CHARACTERIZING THE SALICYLIC ACID–POLYETHYLENE SYSTEM IN VARYING CONCENTRATIONS OF DIBASIC POTASSIUM PHOSPHATE IN WATER

Solvent, moles K_2HPO_4 in H_2O	θ, °	cos θ	γ, dyne/cm.	γ cos θ	$C_s \times 10^2$, Gm./ml.	$Q/t^{1/2} \times 10^5$ Exptl. Gm./sec.$^{1/2}$	$Q/t^{1/2} \times 10^5$ Expected[a] Gm./sec.$^{1/2}$
0.01	95	−0.105	60.0	−6.30	0.43	1.22	1.22
0.05	95	−0.105	59.3	−6.20	1.00	1.28	1.81
0.10	98	−0.156	59.0	−9.20	1.60	1.32	2.45
0.25	99	−0.174	58.3	−10.14	3.72	1.22	3.61
0.50	99	−0.174	57.0	−9.92	6.70	1.10	4.83
1.00	97	−0.139	56.7	−7.88	11.5	1.06	6.31

[a] The experimental slope with 0.01 *M* K_2HPO_4 used as reference.

TABLE II—PARAMETERS CHARACTERIZING THE SALICYLIC ACID–POLYETHYLENE SYSTEM IN VARYING CONCENTRATIONS OF DIBASIC POTASSIUM PHOSPHATE IN AOT SOLUTIONS

Solvent moles K_2HPO_4	% AOT	θ, °	cos θ	γ, dyne/cm.	γ cos θ	$C_s \times 10^2$, Gm./ml.	$Q/t^{1/2} \times 10^5$ Exptl. Gm./sec.$^{1/2}$	$Q/t^{1/2} \times 10^5$ Expected Gm./sec.$^{1/2}$	τ, Exptl.
...	...[a]	92	−0.052	60.4	−3.14	0.26	0.53	2.20	107
...	0.01	56	0.559	38.1	21.30	0.26	1.08	2.20	25
...	0.05	0	1.00	30.4	30.40	0.27	3.00	2.20	3.3
0.01	0.01	31	0.857	31.3	26.82	0.43	3.49	2.81	4.1
0.01	0.05	0	1.00	26.3	26.30	0.44	3.40	2.81	4.2
0.05	0.01	29	0.875	27.5	24.06	1.00	4.40	4.28	5.9
0.05	0.05	0	1.00	26.4	26.40	1.04	4.42	4.28	5.9
0.10	0.01	32	0.848	27.6	23.41	1.75	5.42	5.63	6.6
0.25	0.01	32	0.848	26.6	22.56	3.78	8.30	8.30[b]	6.0

[a] Data in this row refer to water as the solvent. [b] The experimental slope with 0.25 *M* K_2HPO_4 and 0.01% AOT used as reference.

sodium sulfosuccinate[2] (AOT) confirmed this. Release rate studies of 20% salicylic acid from a polyethylene matrix were then carried out in varying concentrations of dibasic potassium phosphate solutions containing 0.01% AOT.

Examination of the 0.01% AOT results shown in Table II demonstrate the desired increase in rates with increasing solubility. The rate shown by the solvent not containing any dibasic potassium phosphate, however, was considerably lower than expected. At the same time there was a seemingly concomitant higher contact angle and surface tension.

To investigate this effect further, the above studies were repeated using 0.05% AOT, and these results are also tabulated in Table II. Interestingly, the rates in dibasic potassium phosphate solutions did not change, but the water data show a dramatic increase. A comparison of the rates and their corresponding contact angles and surface tensions clearly shows a strong relationship. The rate was greatly increased when the contact angle was lowered from a value greater than 90° to either 56° or 31°. The surface tensions were also correspondingly lowered. The slope was not changed, however, when the angle was lowered from 31° to essentially 0°, but dramatically increased when the angle was lowered from 56°. At the same time the surface tension also remained constant when the contact angle was lowered from 31°, but decreased when the angle was lowered from 56° to 0°. This implies that the rate is either controlled by the surface tension or that an effective maximum wetting effect is achieved at a contact angle between 31° and 56°. This observation is also implied by the fact that only the solutions of contact angles equal to or greater than 56° significantly show slower rates. Lastly, the apparent tortuosity values also are reasonable for all solutions but those showing contact angles of 56° or greater, as seen by the water data showing values of 107, 25, and 3.3.

The nonsurfactant solutions of Table I, on the other hand, had high contact angles and surface tensions, indicating that in those systems solvent penetration into the matrix may have been the reason for nonconformity to the diffusion model.

It would be highly desirable at this point to determine the individual roles that surface tension and contact angles play in this regard. A study in which contact angle can be continuously varied while a relatively constant surface tension is maintained could shed light on these roles, and in this way the two effects could be separated. Polysorbate 80[3] provided such a system. A series of varying concentrations of polysorbate 80 was selected so that the contact angle decreased from 97° to 46° with little change in surface tension, with the hope of obtaining a varying rate of solvent penetration. Table III shows that the release rates of salicylic acid considerably increase with increasing concentration of polysorbate 80. Figure 2 and Table III indicate that the experimental rates show a strong relationship with the corresponding contact angle, but are relatively independent of the surface tension. A large effect is also demonstrated regarding the apparent tortuosities which decrease exponentially.

This strongly suggests that the contact angle or degree of wetting is more important than surface

[2] Marketed as Aerosol OT by the American Cyanamid Co., Wayne, N. J.

[3] Marketed as Tween 80 by Atlas Chemical Industries, Wilmington, Del.

TABLE III—PARAMETERS CHARACTERIZING THE SALICYLIC ACID–POLYETHYLENE SYSTEM AS A FUNCTION OF POLYSORBATE 80 CONCENTRATION

Solvent, 0.1 *M* K_2HPO_4 in % Polysorbate	$C_s \times 10^2$, Gm/ml.	θ, °	cos θ	γ, dyne/cm.	$\gamma \cos \theta$	$Q/t^{1/2} \times 10^5$ Gm./sec.$^{1/2}$	τ
0	1.69	98	−0.156	59.0	−9.20	1.32	111
0.001	1.62	97	−0.139	42.6	−5.92	1.60	75.8
0.005	1.62	93	−0.070	44.0	−3.08	1.94	51.6
0.01	1.62	87	+0.052	38.4	2.00	2.22	39.4
0.05	1.63	59	+0.515	38.2	19.67	4.26	10.7
0.10	1.62	46	+0.695	40.0	27.80	5.20	7.2
0.01% AOT	1.75	32	+0.848	27.6	23.41	5.42	6.6

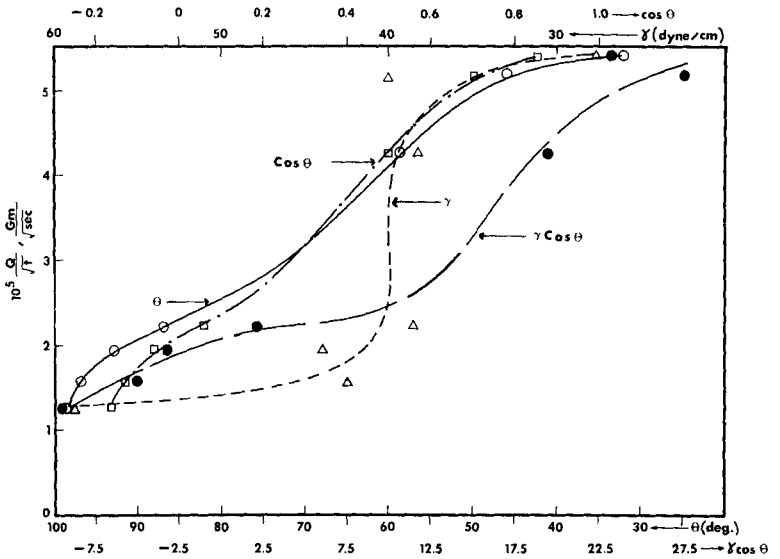

Fig. 2—Relationship between the release rates and the variables controlling penetration of the matrix.

tension in determining solvent penetration. In these studies, however, the surface tension was already considerably lower (40 dynes/cm.). The studies in water, listed in Table I, on the other hand, are involved with a high surface tension in addition to a high contact angle, and in this situation the contact angle may not be the more important factor. The contact angle effect, independent of the surface tension effect, can be studied by using a matrix which shows a moderate contact angle in water. It was found that a matrix made of 70% polyvinyl chloride (PVC) and 30% polyethylene satisfied these requirements, as it exhibited a wetting contact angle (~70°) in the presence of nonsurfactant solutions. The study of Table I, *i.e.*, by varying the solubility using dibasic sodium phosphate, was then repeated using this matrix. The data are tabulated in Table IV and show that despite the presence of high surface tension of a nonsurfactant solution, the rate is increased with increasing solubility, and low tortuosity values are obtained. This shows that the degree of wetting is the important factor controlling matrix permeability and is the reason that the solubility effect was not observed in the experiments of Table I.

TABLE IV—PARAMETERS CHARACTERIZING THE SALICYLIC ACID–POLYVINYL CHLORIDE–POLYETHYLENE SYSTEM IN VARYING CONCENTRATIONS OF DIBASIC SODIUM PHOSPHATE IN WATER

Solvent, moles Na_2HPO_4 in H_2O	$C_s \times 10^2$, Gm./ml.	$Q/t^{1/2} \times 10^5$ Exptl., Gm./sec.$^{1/2}$	$Q/t^{1/2} \times 10^5$ Expected., Gm./sec.$^{1/2}$	τ Exptl.
0	0.26	2.57	2.57[a]	6.0
0.10	1.00	6.00	6.37	6.8
0.25	3.70	10.6	9.70	5.0
0.50	6.60	12.6	13.0	5.8
0.75	9.10	13.0	15.2	7.2
1.00	11.5	13.5	17.1	8.1

[a] Reference.

Pressure Studies as a Function of Solubility—The question of whether the apparent wetting dependence is an all-or-nothing effect or whether it is a rate effect, however, has yet to be answered.

These roles of solvent penetration can be further investigated by applying external pressure to the system as its permeation rate will increase with an increase of external pressure on the solvent. In this way the role of the rate of solvent penetration can be illustrated.

With the above in mind, experiments utilizing water as the release medium were carried out. The results tabulated in Table V show the feasibility of this method as the rates indeed are significantly increased and the apparent tortuosities decreased by an increase in pressure, despite a constant solubility, surface tension, and contact angle. As a matter of fact, the release rates and tortuosities obtained at higher pressures were comparable to those obtained from the same system (no K_2HPO_4) in the presence of surfactant (see Table II), and

TABLE V—EFFECT OF PRESSURE ON THE RELEASE RATES AND CORRESPONDING TORTUOSITIES OF THE SALICYLIC ACID–POLYETHYLENE SYSTEM IN WATER

Net Pressure, p.s.i.	$Q/t^{1/2} \times 10^5$ Gm./sec.$^{1/2}$	τ
0	0.53	107
10	0.90	37.1
15	0.98	31.3
20	1.36	16.2
25	1.90	8.3
30	2.10	6.8
40	2.50	4.8

TABLE VI—COMPARISON OF THE EFFECTS OF PRESSURE ON THE RELEASE RATES OF THE SALICYLIC ACID–POLYETHYLENE SYSTEM AS A FUNCTION OF SOLUBILITY IN WATER

Net Pressure, p.s.i.	Exptl. Slope $\times 10^5$ Gm./sec.$^{1/2}$ 0 M 0.26[a]	0.05 M 1.00[a]	0.10 M 1.60[a]	0.25 M 3.72[a]
0	0.53	1.28	1.32	1.22
10	0.90	...	1.38	...
15	0.98	1.18	1.30	1.36
20	1.36	1.69	2.14	2.80
25	1.90	2.75	3.50	4.15
30	2.10	2.90	4.30	5.92
40	2.50	3.43	4.05	6.70

[a] Solubility in Gm./ml. $\times 10^2$.

suggest that at higher pressures a solubility effect should be observed.

This was substantiated by obtaining the release rates of salicylic acid in a series of release media at 40 p.s.i. external pressure. Data presented in the lowest row of Table VI show that the rate is increased with an increasing concentration of dibasic potassium phosphate, and thereby show that the solubility dependence is observed when 40 p.s.i. is applied to the aqueous system but is not observed at atmospheric pressure (Table I).

At this point it may be worthwhile to try to delineate the role played by solvent penetration in eliminating the solubility effect in terms of a hypothetical model utilizing an increasing net permeation pressure. This net permeation pressure, P_n, is defined as

$$P_n = P_p - P_i \qquad \text{(Eq. 1)}$$

where P_i is the internal pressure resisting solvent penetration, P_p is the permeability pressure $= P_w + P_e$; P_w is the pressure due to wetting, and P_e is the external pressure applied to the system.

In this model, the net permeation pressure is initially negative. This can be done by applying vacuum to a system previously loaded with solvent under atmospheric pressure. The entrapped air then causes an internal pressure which pushes solvent out of the matrix. This prevents solvent penetration and yields no drug release (see Fig. 3, top left).

This condition will persist until the net pressure is positive, at which point solvent will begin to penetrate (Fig. 3, bottom left). At low positive net pressures, however, the solvent rate of contact with solid drug is slower than the rate of drug diffusion, resulting in a subsaturated solution at the moving solvent boundary. As long as this condition persists, the release rate of the drug will

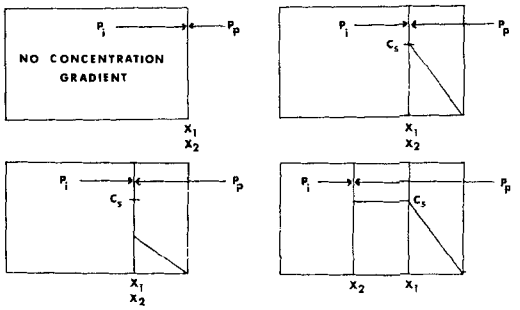

Fig. 3—Hypothetical model describing the conditions existing under a varying net pressure on the system.

be independent of the solubility. As the net positive pressure is further increased, the condition will be reached when the solvent rate of contact with solid drug becomes equal to the rate of drug diffusion (Fig. 3, top right). At this point no further increase in the rate of drug release will occur with further increase in net pressure. The solvent front, however, must continue to penetrate the matrix at a proportionally higher rate (Fig. 3, bottom right). This means that two boundaries must exist at net pressures above the minimum critical value—a solvent boundary, and a solid drug boundary, enclosing a region containing a saturated solution in equilibrium with its solid drug.

This model can be further clarified by exploiting the expression for the steady state at the solid drug–liquid interface, as the rate of drug solution must be equivalent to the rate of drug diffusion. The rate of solution is given by the amount of drug per unit volume of tablet, A, times the rate of the solid drug–liquid boundary movement, dx_1/dt. The rate of diffusion is given by the effective diffusion coefficient, $D\epsilon/\tau$, times the concentration gradient, dC/dx or Cx_1/x_1, where Cx_1 is the concentration of drug at the solid drug boundary. Then:

$$A \frac{dx_1}{dt} = \frac{D\epsilon}{\tau} \frac{Cx_1}{x_1}$$

(See Fig. 3, bottom right.) For the diffusion model to be operative the rate of permeation of the solvent must be equal to or greater than the rate of solid drug boundary movement, and permit a buildup of Cx_1 to the solubility level. The above equation can be rearranged to yield:

$$\frac{dx_1}{dt} = \frac{D\epsilon}{A\tau} \cdot \frac{Cs}{x_1} = \text{min. solvent permeation rate} \qquad \text{(Eq. 2)}$$

This shows that the minimum rate of penetration must increase as the porosity, the diffusion coefficient, or solubility of drug is increased, but it may permeate more slowly with a higher tortuosity, higher percentage of drug embedded in the matrix, and as penetration proceeds.

In an effort to verify the requirements of an increasing permeation rate with increasing solubility, the experiment conducted at 40 p.s.i. was repeated as a function of lower pressures, and the results are tabulated in Table VI and plotted in Fig. 4. Each curve seems to support the previous discussion as the rates of drug release appear to increase and

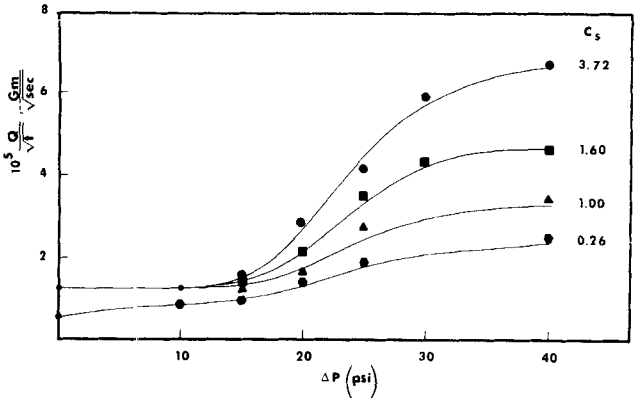

Fig. 4—Comparison of the effect of pressure on the release rates of the salicylic acid–polyethylene system as a function of solubility in water. Solubility is expressed in Gm./ml. × 10^2. (See Table VI.)

then plateau with an increased rate of solvent penetration. Furthermore, the plateau appears to require a higher rate of solvent penetration as drug solubility increases.

The family of curves in Fig. 4, however, also displays characteristics not predicted by the previous discussion. The rates of drug release do not increase with increasing solvent penetration rates until a net pressure of about 15 p.s.i. is reached, although the previous discussion indicated that this should begin at 0 p.s.i. This implies that an all-or-nothing effect is predominant below 15 p.s.i., but that above 15 p.s.i. the rate of penetration is controlling.

Pore Size Distribution—The initial plateau region of Fig. 4 displaying the all-or-nothing pressure effect can be explained if one includes the pore size distribution in the proposed model. This can be done by using the Washburn equation (5) which relates pore diameter to the solvent pressure required to initiate its penetration. Then,

$$P_i = -\left(\frac{4}{d_i}\right)\gamma \cos\theta \qquad \text{(Eq. 3)}$$

where P_i is the minimum net pressure required to achieve penetration, γ is the surface tension of the solvent, θ is the contact angle of the solid–solvent interface, and d_i is the minimum pore diameter penetrated by solvent at pressure P_i.

The above equation is valid only for contact angles greater than 90° (which is true of the system under consideration) and indicates that the all-or-nothing effect may be due to solvent impenetrability of certain pores present in the matrix which are below minimum diameter. This equation indicates that the pressure opposing solvent penetration is proportional to the product of the surface tension and the cosine of the wetting angle. Examination of this product, listed in Table I, indicates that all solutions are under the influence of a force opposing solvent penetration. Examination of Table II shows, however, that there is a positive permeation force manifested. More enlightening, on the other hand, is a re-examination of Table III, which shows that the above product ranges from an opposing penetration force to one of strong permeation. The plot in Fig. 2 strongly suggests the possibility that the presence of a restrictive pore distribution is operative in Fig. 4.

Figure 4 shows that the range of critical pressures begins at 20 p.s.i. and continues to a point past 40 p.s.i., indicating that there is a range of pore size which are not penetrated at a lower pressure. The distribution of pore sizes limiting penetration corresponding to the above range of critical pressures was calculated using the Washburn equation and found to range from 0.32 μ down to a value less than 0.12 μ. The average pore size of the above distribution was estimated to be 0.24 μ, using 25 p.s.i. as its critical pressure, which corresponds to the average point of inflection on the rising portions of all Fig. 4 curves. Porosimeter (6) data were obtained and indicated that the pore size distribution ranged from 40 μ down to below 0.06 μ. There was, however, a sharp peak beginning at 2 μ extending to 0.3 μ with its maximum at 0.8 μ. It was felt that this was in very good agreement with the hypothesis, if one considers that first, the porosimeter yields essentially data at equilibrium, whereas the pressure studies are rate data. One would therefore expect that the critical pressure would be increased, and therefore its apparent pore size distribution would be exhibited at lower pore sizes. Second, there may be distortion of the pores due to compression of preceding empty lumen occurring with the porosimeter at the higher pressures used. Last, the effective diameter (7) of a tortuous pipe will be less than the true diameter when considering hydraulic flow.

To further investigate the phenomenon, 0.005% polysorbate was added to the release media and release studies were again made as a function of the pressure. The contact angle for this system decreased from 98° to 93°, and the surface tension from 59 dynes/cm. to 44 dynes/cm. This means that the critical pressure should be lowered by a large factor of 0.6 (from the Washburn equation). The results of these studies are presented in Table VII and Fig. 5, and a large shift is seen supporting

Table VII—Comparison of the Effects of Pressure on the Release Rates of the Salicylic Acid–Polyethylene System as a Function of Solubility in 0.005% Polysorbate 80 Solution

Net Pressure, p.s.i.	Exptl. Slope × 10^5 Gm./sec.$^{1/2}$ 0 M 0.26[a]	0.05 M 1.00[a]	0.10 M 1.62[a]	0.25 M 3.74[a]
0	1.64	2.05	1.94	2.32
5	2.50	3.52	4.10	4.92
10	2.76	4.10	5.20	6.40
15	2.70	4.10	5.05	7.60

[a] Solubility in Gm./ml. × 10^2.

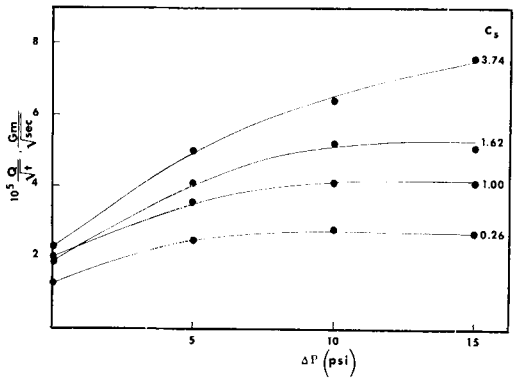

Fig. 5—Comparison of the effects of pressure on the release rates of the salicylic acid–polyethylene system as a function of solubility in 0.005% polysorbate solutions. Solubility is expressed in Gm./ml. × 10^2. (See Table VII.)

the previous discussion. Comparable release rates are seen in Figs. 4 and 5, although for the system containing polysorbate they occur at about 15 p.s.i. lower than in the absence of polysorbate. This implies that the addition of 0.005% polysorbate is equivalent to an application of 15 p.s.i.

This above observation can be quantitated by using the Washburn equation (Eq. 3) to determine the external pressure required for penetration under both water and surfactant conditions. Obviously, the difference will yield the contribution due to the addition of 0.005% polysorbate to the solution. Then,

$$P_{\text{polysorbate contribution}} = P_{\text{polysorbate}} - P_{\text{water}} = -\frac{4}{d}(\gamma_{PSO}\cos\theta_{PSO} - \gamma_{H_2O}\cos\theta_{H_2O})$$

and this yields a value of 10 p.s.i., which agrees very well with the above value.

The degree of the solubility dependence as a function of the pressure exhibited in Figs. 4 and 5 prior to the maximum plateau can be determined from a plot of the rate of drug release *versus* the square root of solubility. For better comparison, however, the ordinate and abscissa can be normalized by using the release rates and the corresponding solubility value in 0.25 *M* K_2HPO_4 solution for each pressure. Ideally, all curves should be superimposable with a slope of one, as the effect of all other factors (such as porosity, tortuosity, drug percent, and diffusion coefficient) are eliminated by taking a ratio, yet Figs. 6 and 7 show that the slopes are less than one and increase with an increasing rate of solvent penetration. Drug unavailability cannot be used to explain the results as the total amount of drug released showed that there was enough drug present to produce a saturated solution in the matrix. This indicates that there are complicating kinetic factors involved.

The Effect of Pressure Variation Under Wetting Conditions—If these factors are not dynamic in nature, there should not be pressure dependence of the release rates exhibited under wetting conditions. To test this point, two series of experiments were performed, one at a relatively high constant solubility level, 1.62 Gm./100 ml. (0.1 *M* K_2HPO_4), using a varying concentration of polysorbate solutions; and another at a relatively low constant solubility level, 0.26 Gm./100 ml. (H_2O), using a varying concentration of AOT solutions. The results of these experiments are tabulated in Tables VIII and IX, and are plotted in Figs. 8 and 9, respectively.

Both curves show that the release rates are not independent of pressure under these conditions. In addition, the application of external pressure and the permeation pressure due to wetting are evidently complementary, since with an increase in the permeation pressure (by increasing the wetting power of the release medium), less external pressure must be applied to achieve the maximum rate. In fact, the release rates no longer are dependent on the external pressure if the wetting power is sufficiently increased. The persistent pressure effect, even under wetting conditions, strongly reinforces the possibility that the partial solublity. dependence is due to complicating kinetic factiors These kinetic factors can be brought about by the presence of a complex distribution of pore sizes of which porosimeter data only showed two distri-

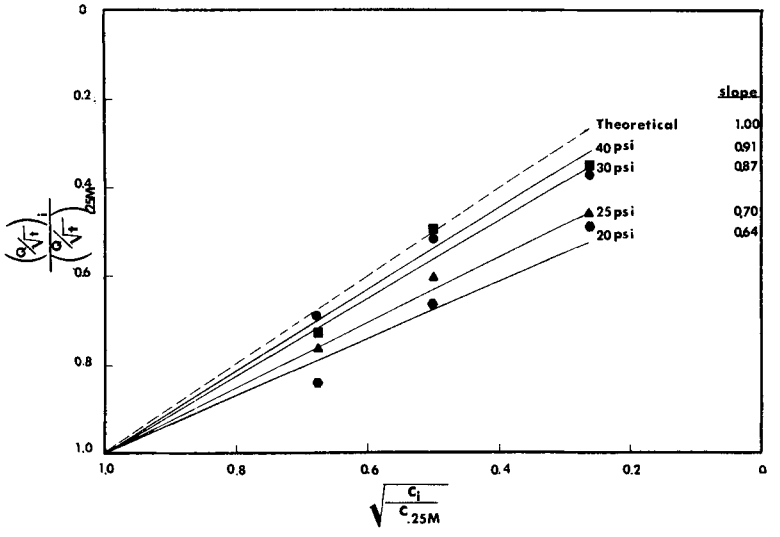

Fig. 6—Effect of increasing pressure on the observed solubility dependence for the same set of experiments as in Fig. 4, using 0.25 M as reference.

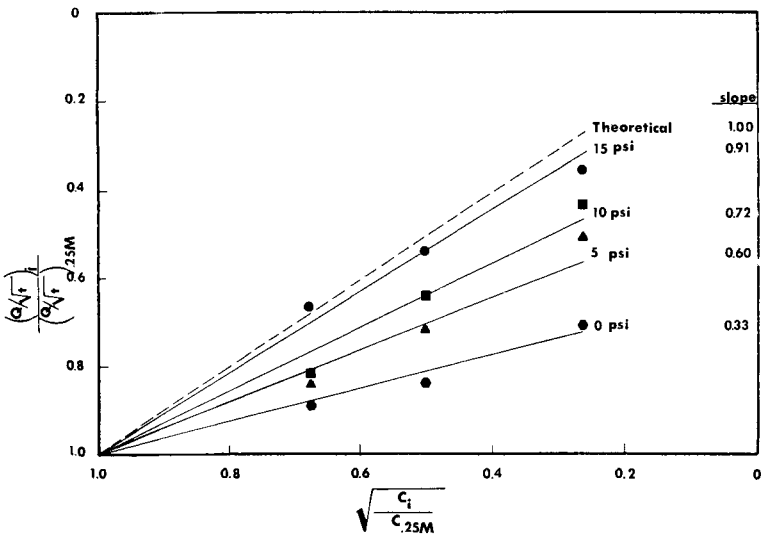

Fig. 7—Effect of increasing pressure on the observed solubility dependence for the same set of experiments as in Fig. 5, using 0.25 M *as reference.*

TABLE VIII—COMPARISON OF THE EFFECTS OF PRESSURE ON THE RELEASE RATES OF THE SALICYLIC ACID–POLYETHYLENE SYSTEM AS A FUNCTION OF THE CONTACT ANGLE IN POLYSORBATE SOLUTIONS

Net Pressure, p.s.i.	Exptl. Slope[a] × 10^5 Gm./sec.$^{1/2}$				
	0%[b]	0.005%[b]	0.01%[b]	0.05%[b]	0.10%[b]
0	1.32	1.90	2.22	3.80	5.20
5	1.36	4.10	4.54	5.20	6.00
10	1.38	5.20	5.50	5.95	5.90
15	1.30	5.05	5.50	5.95	5.95
Contact angle, $\theta°$	98	93	87	58	46
$\cos\theta$	−0.156	−0.070	0.052	0.530	0.695
γ, dynes/cm.	59.0	44.0	38.4	38.2	40.0
$\gamma\cos\theta$	−9.204	−3.080	1.997	20.246	27.800

[a] The solubility was maintained at 1.62×10^{-2} Gm./ml. [b] Percent polysorbate.

TABLE IX—COMPARISON OF THE EFFECTS OF PRESSURE ON THE RELEASE RATES OF THE SALICYLIC ACID–POLYETHYLENE SYSTEM AS A FUNCTION OF THE CONTACT ANGLE IN AOT SOLUTIONS

Net Pressure, p.s.i.	Exptl. Slope[a] × 10^5 Gm./sec.$^{1/2}$			
	0%[b]	0.005%[b]	0.01%[b]	0.05%[b]
0	0.53	1.00	1.08	3.00
15	0.98	1.63	2.30	2.92
30	2.10	2.40	2.60	...
40	2.50	2.67	2.76	...
Contact angle, $\theta°$	92	76	56	0
$\cos\theta$	−0.052	0.242	0.559	1.00
γ, dynes/cm.	60.4	42.0	38.1	30.4
$\gamma\cos\theta$	−3.141	10.164	21.298	30.400

[a] The solubility was maintained at 2.6×10^{-3} Gm./ml. [b] Percent AOT.

butions. Since all of the porosity was not accounted for, it is very likely that the unaccounted for porosity is composed of pores whose size distribution is so small it would require prohibitive pressures to detect. The higher resistance of these pores to flow, however, can greatly affect the rates of permeation and diffusion. These rates can also be complicated by other factors such as microenvironmental heterogeneities and contact angles which can exist throughout the matrix porosity.

Model Defining the Role of Solvent Penetration—It would be useful at this time to review the techniques and concepts that have been developed in this study, and show how they may be used to characterize various matrix systems.

It would appear, first of all, that one must consider kinetic as well as static factors. These include the degree of possible penetration which can range from matrix impermeability to one of complete permeability. The rate of permeation, on the other hand, can also be very important, and can be rate determining if too slow.

If one wishes to apply the results of this investigation to a particular inert matrix, however, one must be able to characterize each system, so that the variables can be identified which must be altered to obtain a desired drug release profile. The following discussion considers the different systems that can exist with the above in mind.

If incomplete penetration of the matrix occurs, the porosities used in the theoretical equations will obviously be too large, and consequently will yield

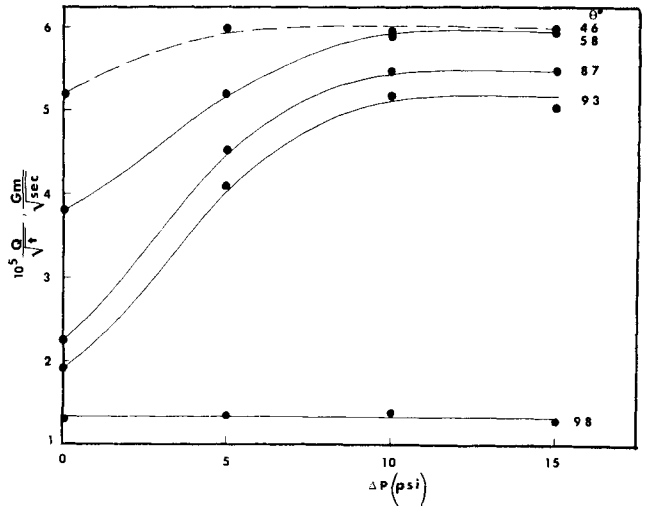

Fig. 8—Comparison of the effects of pressure on the release rates of the salicylic acid–polyethylene system as a function of the contact angle in polysorbate solutions. Solubility maintained constant at 1.6 × 10^{-2} Gm./ml. (See Table VIII.)

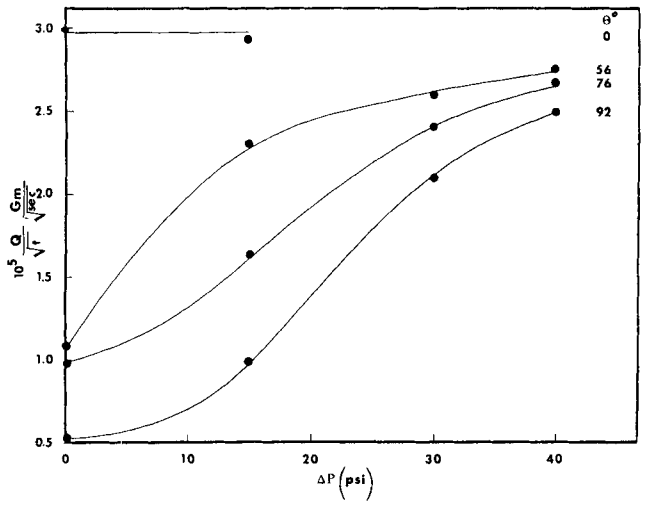

Fig. 9—Comparison of the effects of pressure in the release rates of the salicylic acid–polyethylene system as a function of the contact angle in AOT solutions. Solubility maintained constant at 2.6 × 10^{-3} Gm./ml. (See Table IX.)

abnormally high tortuosities. The rate of solvent penetration will strongly influence the characteristics exhibited by this system. For example, if the permeation rate is too slow, the release rate will be increased by either an increase in pressure or on addition of surfactant. The effect of pressure, however, may not be evident until a critical pressure is applied. This system is also characterized by an absence of a solubility dependence effect. These characteristics describe the salicylic acid release in water as shown by the effects of pressure and surfactant, tabulated in Tables II, III, V, and VI, and the solubility effect shown in Table I.

If the permeation rates, on the other hand, are sufficiently rapid, a solubility dependence will be exhibited. There also will be a concomitant decrease in the apparent tortuosity to an intermediate value. The pressure and surfactant studies will reveal the same behavior as the first case. These characteristics describe the salicylic acid release in 0.005% polysorbate solution as shown by the effects of pressure and surfactant in Table VIII and the solubility effect in Table VII. The appplication of a critical pressure was not necessary in this system.

If complete penetration of the matrix is accomplished, needless to say, only the rate of permeation of the solvent can be limiting. If it is too slow, no solubility dependence is found, both pressure and surfactant effects can be observed, and high apparent tortuosities will be present. This condition can be achieved if one utilizes a highly soluble drug in too low a percent in the tablet.

The rate of permeation, however, can be sufficiently rapid to manifest a solubility dependence, but not rapid enough to demonstrate it to the maximum. This system will be characterized by pressure and surfactant effects with intermediate values of apparent tortuosities. This description would appear to describe the salicylic acid release in 0.005% polysorbate solution, but this possibility is easily eliminated due to the high nonwetting contact angle. These characteristics, however, can be used to describe the release of salicylic acid in 0.005% AOT. The pressure and surfactant effects are shown in Table IX. The solubility dependence was not determined for this system, but examination of Fig. 9 leaves little doubt that a partial solubility dependence would be observed.

The ideal situation of complete penetration and

sufficiently rapid permeation rates, needless to say, will exhibit no pressure or surfactant effects, and yield a maximum solubility dependence with low tortuosities. These characteristics apply very well to salicylic acid release in 0.05% AOT as shown by Table II and Fig. 9.

It appears that the techniques developed and presented here lend themselves very well to a definitive description and characterization of the particular matrix system one is dealing with, and in particular, permit the selection of the proper variables to alter in order to remedy nonideal matrix systems to provide the desired release profiles.

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Keyphrases

Drug release rates
Matrices–inert
Wetting effect–drug release
Surfactant effect–wetting
Contact angle measurements
Pressure effect–drug release
Diffusion coefficients
Permeation rates–solvent

Use of the Overturn End Point for the Estimation of Absorption and Elimination Kinetics in Goldfish

By MILO GIBALDI and CHARLES H. NIGHTINGALE

The advantages of overturn time as a pharmacologic end point for studies of biologic membrane permeation in goldfish are presented. Studies with both pentobarbital and ethanol showed good reproducibility. Redeterminations of overturn time after 18 hr. provided excellent agreement with initial determinations with both drugs. Evidence is presented to suggest a complex mechanism of action of ethanol in goldfish. A kinetic model is derived which relates overturn time and recovery time and permits the estimation of an elimination rate constant from pharmacologic response data alone.

Fish, particularly goldfish, have been used since 1867 as test animals for determining the toxicity of various substances, and a number of techniques have been developed for this purpose (1). A useful approach has been measurement of the time required to produce a well-marked stage of toxicity such as death.

Powers (2), after an extensive series of experiments with goldfish using a variety of drugs and chemicals, concluded that for every toxic substance, over a certain concentration range, the survival time is inversely related to the concentration. For many years the idea prevailed that the magnitude of the slope of a reciprocal time of death-concentration plot was indicative of intrinsic toxicity (1). Recently, Levy and Gucinski (3) have demonstrated that the slope of such a plot is actually a complex function of both the intrinsic toxicity and the absorption rate of the drug. Furthermore, these workers proposed that the reciprocal time of occurrence of any suitable pharmacologic response for a given drug will be directly proportional to the absorption rate of the drug under certain specified conditions (*viz.*, absorption occurs by passive diffusion, drug concentration gradient across the absorbing membranes remains essentially constant, drug metabolism in the fish is negligible, and the pharmacologic end point occurs without significant delay after a given amount of drug is absorbed). Knowledge of the amount of drug in the goldfish body required to induce a pharmacologic response permits the calculation of the absorption rate constant from reciprocal time of response-concentration plots. Even in the absence of such knowledge, relative absorption rates of a single drug under different experimental conditions may be assessed from the slopes of reciprocal time-concentration plots (3).

Another apparently well-marked pharmaco-

Received July 17, 1967, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication September 28, 1967.
This investigation was supported in part by institutional grant GU-1864 from the National Science Foundation.

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logic response that has been used in the goldfish is the so-called overturn time (1). The overturn end point marks the time, after exposure to a given substance, at which the fish appear to lose their sense of balance and float on their side or upside-down. This end point seems somewhat analogous to the loss of righting reflex in the rat.

Use of the overturn time rather than time of death as a pharmacologic end point offers certain advantages in that the fish survive the study and may, after a suitable period of time, be used again in other experiments. In well-designed experiments a fish or a group of fish may serve as its own control, thereby affording a decided statistical advantage. Furthermore, the ability to reuse fish in a series of experiments allows conservation of test animals which minimizes the problems of lot-to-lot variation (3). In certain toxicity studies the overturn time end point has been claimed to be better defined than the time of death end point since with some drugs the fish enter a deep anesthetic stage which may be mistaken as death (1).

A further advantage of the use of overturn time as a pharmacologic criterion of drug absorption is that the duration of pharmacologic response may be determined. The duration of response should be related to the body drug levels in the fish as well as the rate of drug elimination from the fish.

It is the purpose of the present report to demonstrate the utility of the overturn time end point for the determination of drug absorption and exsorption rates in the goldfish.

EXPERIMENTAL

Goldfish, *Carassius auratus*, common variety, weighing from 5 to 10 Gm. were used. All fish utilized in a given experiment were from the same lot. Three different lots were used during the study. Solutions were prepared daily, as required, from reagent grade or USP materials. The test drugs, ethanol and sodium pentobarbital, were dissolved in 0.05 *M* phosphate buffer, adjusted to pH 6.1.

Evaluation of Pharmacologic Response—All measurements of overturn time (T_0) and duration of effect (T_R) were made at 24–26°. The overturn time was taken as the time required for the fish to lose the ability to maintain itself upright after immersion in drug solution. When the first signs of overturn were observed the end point was tested by attempting to turn the fish on its side with a stirring rod. Unaffected fish will either not allow themselves to be placed on their side or will immediately right themselves. The end point was judged as the time when a fish placed on its side did not right itself immediately. The parameter T_R was defined as the time required (from $t = T_0$) for a fish to regain the ability to right itself immediately after being placed on its side.

The determination of T_0 was carried out with individual fish in 200 ml. of drug solution in 250-ml. beakers. At $t = T_0$ the fish was immediately (or after a timed interval of 2 or 4 min.) placed in approximately 5 gal. of distilled water. The time required from immersion of the fish in the reservoir to recovery of righting reflex is T_R.

At one concentration with each drug the fish were removed from the drug solution after determination of T_0 and placed in a holding tank containing 10 gal. of distilled water. These fish were restudied under identical conditions after 18 hr.

RESULTS AND DISCUSSION

It was found in preliminary experiments that immersion of goldfish in the buffer solution for 24 hr. had no apparent effect on the fish. In addition the overturn time found in 3% ethanol solutions with and without buffer was virtually identical. Hence, it was concluded that the buffer system had no direct effects on pharmacologic response.

According to the model proposed by Levy and Gucinski (3), the time required for a pharmacologic response (T) is related to drug concentration (C) in the following manner:

$$1/T = KC \qquad \text{(Eq. 1)}$$

where $K = ka/A_B$. The term ka is the apparent absorption rate constant (which includes the absorptive surface area) and the term A_B is the amount of drug required in the fish to elicit the pharmacologic response. The requisite assumptions for Eq. 1 have been detailed (3).

Levy and Miller (4) have found that the relationship between time of death and concentration of various barbiturates including pentobarbital and various alcohols is in agreement with Eq. 1. The results of the present study with pentobarbital (shown in Fig. 1) using overturn time as the pharmacologic end point rather than time of death are also in good agreement with the biologic model. A plot of $1/T_0$ as a function of concentration demonstrated linearity over a concentration range from 10 to 50 mg.%. The significance of a least-squares treatment on a reciprocal plot, without appropriate weighting factors, is questionable. Accordingly, the

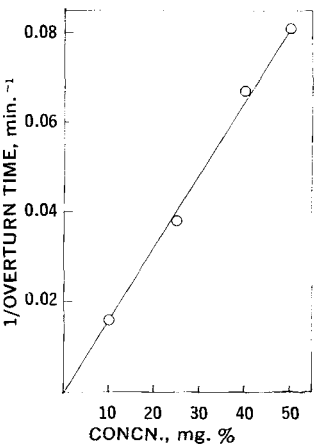

Fig. 1—Reciprocal time of overturn of goldfish as a function of pentobarbital concentration in pH 6.1 buffer.

TABLE I—OVERTURN TIMES OF GOLDFISH IN VARIOUS CONCENTRATIONS OF PENTOBARBITAL

Concn., mg. %	No. of Animals	Av. Overturn Time, min.	S.E.
10	5	62.8	4.18
25	4	26.4	1.28
40	5	15.0	1.25
50	10	12.3	0.69

TABLE II—OVERTURN TIMES (MIN.) OF INDIVIDUAL GOLDFISH IN SODIUM PENTOBARBITAL AND ETHANOL, INITIALLY (*A*) AND AFTER 18 HR. (*B*)

	Na Pentobarbital 50 mg. %		Ethanol 3% (v/v)	
	A	*B*	*A*	*B*
	7.5	8.0	4.2	4.0
	10.5	11.5	4.6	4.2
	11.0	12.0	5.2	5.7
	13.0	13.5	6.7	6.0
	14.0	14.5	6.8	7.0
Mean	11.2	11.9	5.5	5.4
S.E.	1.12	1.11	0.53	0.56

TABLE III—OVERTURN TIMES OF GOLDFISH IN VARIOUS CONCENTRATIONS OF ETHANOL

Concn., % v/v	No. of Animals	Av. Overturn Time, min.	S.E.
1.5	4[a]	58.1	4.78
2.0	5	28.0	4.02
3.0	5	13.6	1.87
4.0	5	8.4	0.32

[a] A total of 5 fish was studied but one fish showed no response over a 2-hr. period and was not included in the calculation.

data in Fig. 1 were fitted by eye and the resulting line appears to approximate the origin. The mean data obtained at each concentration of pentobarbital along with standard error calculations are summarized in Table I.

The results of retesting a group of fish after exposure to 3% ethanol or 50 mg.% pentobarbital are shown in Table II. The excellent agreement of the mean values, range, and standard errors between the initial study and the second study, conducted 18 hr. later, indicates clearly the statistical advantages to be gained by the use of a pharmacologic end point other than time of death.

The results of the overturn time–ethanol concentration studies are shown in Table III. The reciprocal time-concentration plot shown in Fig. 2 clearly indicates that the data do not adhere to the theoretical model. Although a linear relationship exists between $1/T_0$ and ethanol concentrations between 1.5 and 4.0% (v/v), a clearly defined intercept is observed at a concentration of about 1%. These findings are similar to those reported recently by Hall and Hayton (5) using overturn time of goldfish and guppies in ethanol solutions.

The model proposed by Levy and Gucinski (3) requires conditions where the fish body functions as an infinite sink, *i.e.*, drug concentration in the fish never exceeds 10 to 20% of drug concentration in the bathing solution. Accordingly, if the concentration required to elicit a pharmacologic response in the fish is $1/5$ to $1/10$ the concentration of the drug

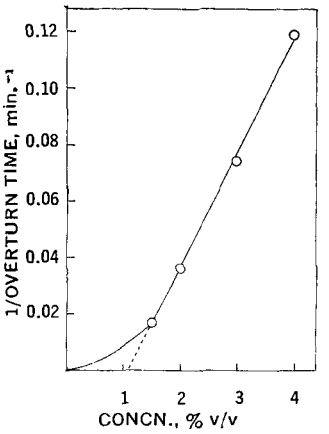

Fig. 2—Reciprocal time of overturn of goldfish as a function of ethanol concentration. Data suggest a concentration intercept of 1% (v/v).

solution, one would anticipate a change in the order of absorption kinetics and a lack of agreement with Eq. 1 at these low concentrations.

On the basis of the present findings it would appear that the minimum body level of ethanol required to elicit overturn in the goldfish is in the order of 150 to 300 mg. %. Interestingly, this estimate approximates the toxicity level of ethanol in man. Sollmann (6) reports that an ethanol blood level of 300 mg. % is required to induce a stuporous condition or sedation in man.

An alternative possibility to explain the intercept is that the fish is capable of metabolizing ethanol. If this were the case, the minimum body level of ethanol required to induce overturn may be significantly lower than 150 mg. % and one would still observe a concentration intercept of approximately 1%.

The ethanol studies also indicated lack of agreement with the biologic model at concentrations above 4% (v/v). Experiments conducted at 5% and 7.5% ethanol gave reciprocal times of overturn that were significantly higher than those predicted by extrapolating the results in Fig. 2. These tentative findings suggested a change in the mechanism of penetration or pharmacologic effect of ethanol in the goldfish as a function of concentration.

On the basis of these findings, it was decided to restudy a second lot of fish and to determine overturn times over a 2 to 8% concentration range at 1% intervals.[1] The results of this second study are shown in Table IV and Fig. 3. Once again a concentration intercept was noted at about 1%. In this particular lot of fish a linear relationship between $1/T_0$ and concentration was found from 2 to 5% ethanol.

The data in Fig. 3 clearly indicate a discontinuous increase in $1/T_0$ above 5% ethanol, *i.e.*, time of overturn is significantly shorter than can be predicted from the low concentration data. The present results suggest the absence of a concentration intercept if high ethanol concentration data are extrapolated to zero concentration. This finding is in agreement with that of Levy and Miller (4).

[1] Concentrations above 8% ethanol gave rise to T_0 values of less than 1 min. and corresponding $1/T_0$ values are subject to large errors.

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extracted with portions of methanol. The methanolic extracts (2 L.) were concentrated to about 50 ml. and passed through 20 Gm. Amberlite IRA 400 (OH) resin column. The resin was washed with 150 ml. 50% aqueous methanol. The effluents were evaporated to yield 123 mg. of choline. The base was identified by converting it to picrate and crystallizing the product from absolute ethanol. The melting point of the picrate, 242–244°, was not depressed by mixing the compound with reference choline picrate and IR spectra of the two samples were identical.

The unidentified polar base, which had zero R_f value on the preparative paper chromatograms, was isolated by extracting the corresponding band with 50% aqueous methanol acidified with formic acid. The product obtained after evaporation was saved.

DISCUSSION

An investigation of *Lophophora williamsii* constituents resulted in isolation and identification of four crystalline compounds. Three bases, which were new peyote alkaloids, were designated—anhalotine, lophotine, and peyotine—and were identified as metho derivatives corresponding to anhalidine, lophophorine, and pellotine, respectively. A fourth base which was obtained as a picrate was identified as choline.

Of the three quaternary tetrahydroisoquinolines isolated and identified, lophotine and peyotine are expected to be optically active. Since only small amounts of the two compounds were available, the optical rotations of the compounds could not be determined. However, the melting points of lophotine iodide isolated and that of the methiodide prepared from the natural lophophorine were the same and the mixed melting point of the two showed no depression. This suggested that the isolated compound was identical with the prepared.

The melting point of crystalline peyotine iodide was lower than the melting point of the methiodide prepared from *dl*-pellotine. The isolated compound softened at 114° and thereafter solidified and then melted at 185–186°. It is possible that the isolated compound is an optically active substance or that the compound in part may have racemized during the isolation. According to Spaeth and Kesztler (18) pellotine base itself undergoes racemization and this had led the authors to suggest that the optically active base is present in the plant; the compound is racemized during extraction or aging of the drug. As indicated earlier, small amounts of peyotine iodide isolated did not permit any further work. The R_f values, IR, and UV spectra of the isolated and prepared compounds were, however, identical.

With the isolation and identification of anhalotine, lophotine, and peyotine the expected occurrence of the quaternary bases corresponding to the known peyote secondary and tertiary bases—namely, anhalamine, anhalidine, anhalonine, lophophorine, anhalonidine, and pellotine—is realized. However, the isolated quaternary bases—namely, the three tetrahydroisoquinolines, choline, and the other two unidentified basic compounds—occur only as minor constituents of peyote.

The quaternary compound corresponding to mescaline, the major and important peyote base, appears not to be present in a detectable amount in the plant. In connection with this, it may be indicated that *N*-methylmescaline has been isolated as a minor alkaloid of peyote (19) and *N*,*N*-dimethylmescaline (≡ tricocereine) which though known to be present in the cactus plant, *Trichocereus terscheckii* (Perm.) (20), has not yet been isolated from the mescal buttons.

As indicated earlier in a recent study thin-layer chromatographic identification of candicine has been reported (5) from peyote quaternary base fraction. In connection with the identification of this compound, the following may be noted.

In Table I are listed the R_f values of anhalotine, lophotine, and peyotine iodides and choline chloride. For comparison R_f values of candicine iodide have also been given. The results recorded in the table indicate that the R_f values of candicine iodide are either too close to or the same as that of the quaternary alkaloids which have been isolated in our studies. Even on alumina G plate and the solvent system carbon tetrachloride-methanol-glacial acetic acid (12:28:1) used (5), candicine iodide has the same R_f value as peyotine iodide. Wagner's reagent a nonspecific reagent, was used for location of the quaternary bases on the chromatogram by these authors. Therefore, distinction of the compound(s) present on the spot having an R_f value same as candicine iodide, into phenolic and nonphenolic quaternary bases, could not be made. In this study tetrazotized di-*o*-anisidine was used for the location of the phenolic bases. The nonphenolic and the phenolic quaternary bases were further detected and distinguished by the use of the modified Dragendorff's reagent. The former agent yielded distinct violet colored spots with the phenolic tetrahydroisoquinoline bases anhalotine, peyotine, and also other nonquaternary phenolic bases anhalamine, anhalidine, anhalonidine, and pellotine. On the other hand candicine, a phenolic quaternary β-phenylethylamine and other related compounds namely, tyramine, *N*-methyltyramine, and hordenine, gave yellow colored spots. In the chromatographic examination of the quaternary base fraction of peyote, no yellow colored spot at the candicine R_f location was observed. This observation and the fact that the R_f values of candicine iodide are the same as, or too close to, other quaternary bases, suggest that the candicine cannot be identified by the method reported and its occurrence in peyote has not been proven.

In the present studies, in addition to the quaternary bases, the known peyote alkaloids namely, lophophorine, pellotine, and anhalonine were obtained by a method differing from that reported previously (8–11). The modified method is based on the fact that from an aqueous extract acidified with hydrochloric acid, the three known bases can be extracted as hydrochlorides with chloroform. Inasmuch as the isolation of the three compounds is accomplished in earlier stages of extraction, the modified method reported herein can be considered as a convenient procedure for the isolation of at least lophophorine and pellotine.

One noteworthy point regarding isolation of pellotine that needs to be pointed out is that, even though this is a phenolic alkaloid, it can be extracted from an aqueous potassium hydroxide

solution with chloroform and ether. Spaeth and Becke (8) also have reported such a behavior for the peyote phenolic alkaloids. According to them, these compounds were at least in part extracted with ether from an aqueous potassium hydroxide solution even though they used concentrated solution (50%) of aqueous potassium hydroxide.

In conclusion, it may be indicated that although the β-phenylethylamine quaternary alkaloids, candicine and coryneine (≡ 3-hydroxycandicine), have been isolated from some cacti (1,20), *Lophophora williamsii* appears to be the first cactus plant from which simple quaternary tetrahydroisoquinolines have been isolated and identified. Occurrence of such compounds in other cacti could be expected, and as in peyote, these compounds may be present at least as minor basic constituents.

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Keyphrases

Peyote alkaloids
Alkaloids, quaternary–*Lophophora williamsii*
Anhalotine, lophotine, and peyotine–isolation
IR spectrophotometry–structure
UV spectrophotometry–structure
NMR spectrometry

Thalictrum Alkaloids V

Isolation

By MAURICE SHAMMA and BERNARD S. DUDOCK

The experimental procedure which led to the isolation of the new alkaloids thalifendlerine (I), thalifendine (II), thalidastine (III), thaliporphine (IV), preocoteine (V), and thalidezine (VI), from *Thalictrum fendleri* is described in detail. Known alkaloids also found in *T. fendleri* are berberine (VII), jatrorrhizine (VIII), glaucine (IX), magnoflorine (X), ocoteine (XI), hernandezine (XII), and thalicarpine (XIII). A completely new dimeric aporphinebenzylisoquinoline alkaloid of only partially elucidated structure is thaldimerine (XIV). A novel procedure for the separation of tertiary alkaloids consists of partition chromatography of the mixture of hydrochloride salts using a cellulose column in the system methyl ethyl ketone–water.

THE STRUCTURAL ELUCIDATION of a variety of new isoquinoline-type alkaloids from *Thalictrum fendleri* has previously been reported. The alkaloids involved were thalifendlerine (I), thalifendine (II), thalidastine (III), thaliporphine (IV), preocoteine (V), and thalidezine (VI) (1–4). In addition, the presence of the following known alkaloids in *T. fendleri* was indicated: berberine (VII), jatrorrhizine (VIII), glaucine (IX), magnoflorine (X), ocoteine (XI), hernandezine (XII) (3), and thalicarpine (XIII). The purpose of the present paper is to describe the isolation procedure employed to obtain these 13 compounds. Additionally, the isolation of the completely new alkaloid thaldimerine (XIV) will also be discussed, although lack of sufficient material has so far precluded the complete structural elucidation.

Coarsely ground *T. fendleri* was extracted with methanol and the solvent was evaporated. The oily residue obtained was taken up in dilute acetic acid and filtered. The filtrate was extracted with chloroform and ether to remove acidic and neutral components. The aqueous acidic layer was then made just basic with concentrated ammonium

Received August 18, 1967, from the Department of Chemistry, Pennsylvania State University, Univerity Park, PA 16802
Accepted for publication October 2, 1967.
This investigation was supported by grant GM-10608 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
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solution with chloroform and ether. Spaeth and Becke (8) also have reported such a behavior for the peyote phenolic alkaloids. According to them, these compounds were at least in part extracted with ether from an aqueous potassium hydroxide solution even though they used concentrated solution (50%) of aqueous potassium hydroxide.

In conclusion, it may be indicated that although the β-phenylethylamine quaternary alkaloids, candicine and coryneine (≡ 3-hydroxycandicine), have been isolated from some cacti (1,20), *Lophophora williamsii* appears to be the first cactus plant from which simple quaternary tetrahydroisoquinolines have been isolated and identified. Occurrence of such compounds in other cacti could be expected, and as in peyote, these compounds may be present at least as minor basic constituents.

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Keyphrases

Peyote alkaloids
Alkaloids, quaternary–*Lophophora williamsii*
Anhalotine, lophotine, and peyotine–isolation
IR spectrophotometry–structure
UV spectrophotometry–structure
NMR spectrometry

Thalictrum Alkaloids V

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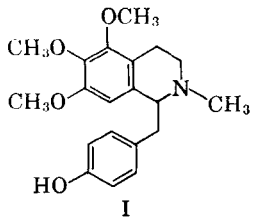
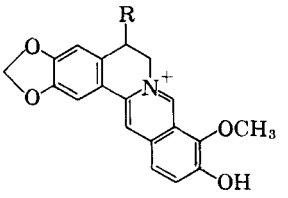
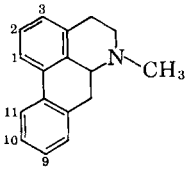
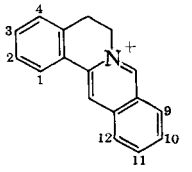


I

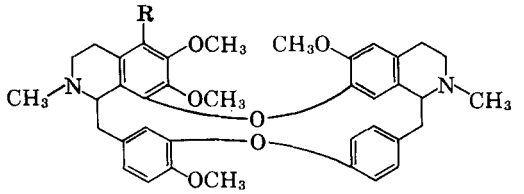


II, R = H
III, R = OH

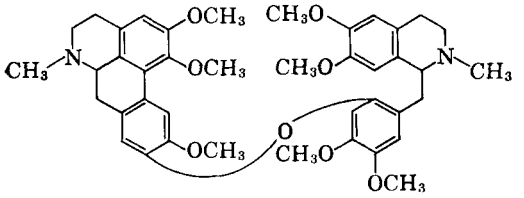




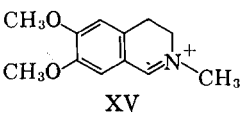
IV, 1-Hydroxy-2,9,10-trimethoxy
V, 1-Hydroxy-2,3,9,10-tetramethoxy
VII, 2,3-Methylenedioxy-9,10-dimethoxy
IX, 1,2,9,10-Tetramethoxy
VIII, 2,9,10-Trimethoxy-3-hydroxy
X, 1,11-Dihydroxy-2,10-dimethoxy-*N*-methyl salt
XI, 1,2-Methylenedioxy-3,9,10-trimethoxy



VI, R = OH
XII, R = OCH_3



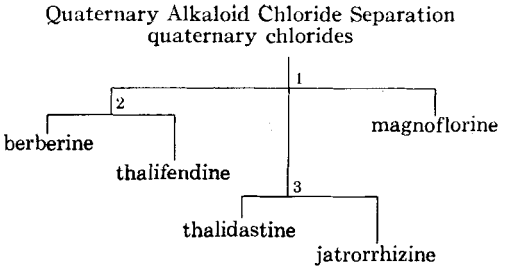
XIII



XV

hydroxide, and reextracted with chloroform and ether to remove the tertiary alkaloids. The aqueous solution was acidified and the quaternary alkaloids were precipitated as their reineckate salts, which were then converted to the alkaloid chloride salts by ion exchange.

Quaternary Alkaloid Chloride Separation



1, partition chromatography
2, alumina chromatography
3, preparative TLC

Scheme I

Preliminary attempts to fractionate the quaternary alkaloid chloride mixture, employing fractional crystallization and adsorption chromatography on columns of alumina, silica gel, and magnesium silicate,[1] gave relatively poor separations. Partition chromatography, previously shown to be successful in fractionating highly complex quaternary alkaloid mixtures (5), was therefore employed. Methyl ethyl ketone saturated with water was chosen as the partition system for the large-scale column chromatography on cellulose. This solvent combination gave a good separation, and had the advantage of being fast running, reasonably stable, and odorless, and low boiling so that it could easily be removed without alkaloid decomposition.

The partition column used cellulose powder as the inert support. The cellulose was first saturated with the stationary phase (water saturated with methyl ethyl ketone) then "dry-packed" (6) into the column and finally equilibrated with the mobile phase. The quaternary chloride mixture, dissolved in the stationary phase bound to the cellulose powder, was then added to the top of the equilibrated column and the separation was begun by allowing the mobile phase to slowly flow down the column. Efforts were made to control the temperature, and the packing and drip-rate fluctuations. The complete procedure is described in detail under *Experimental.*

The quaternary alkaloid mixture was thus separated into distinct fractions as summarized in Scheme I. Fractions were combined on the basis of their paper and thin-layer chromatographic pattern, and usually further purified by adsorption chromatography on alumina or preparative thin-layer chromatography (TLC).

Due to the success of partition chromatography in fractionating the quaternary alkaloids, the same procedure was employed to separate the

[1] Florisil. Floridin Co., Pittsburgh, Pa.

TABLE I—FRACTIONATION OF TERTIARY ALKALOIDS BY PARTITION CHROMATOGRAPHY[a]

Fractions	Flasks[b]	Alkaloids isolated
1	1–6	No well-defined alkaloids isolated
2	7–15	Thalifendlerine (I), 3 Gm.[c]
3	16–20	Thalifendlerine (I), 200 mg. Ocoteine (XI), 200 mg. Glaucine (IX), 5 mg. Preocoteine (V), 60 mg.
4	21–28	Thaliporphine (IV), 70 mg.
5	29–35	Thaliporphine (IV) 30 mg. and other minor alkaloids
6	36–55	Not investigated to date[d]
7	56–61	
8	62–89	
9	90–105	
10	106–115	
11	Methanol washing	Hernandezine (XII), 50 mg. Thalicarpine (XIII), 20 mg. Thaldimerine (XIV), 20 mg Thalidezine (VI), 300 mg.

[a] The column was 180 cm. in height, 10 cm. wide, and contained 1,500 Gm. of cellulose powder. [b] Each flask contained about 100 ml. of solution. [c] The weight refers to the alkaloid free base obtained from 15 Kg. of dried plant. [d] TLC revealed the presence of hernandezine and thalicarpine.

tertiary bases. The total tertiary alkaloid mixture, which was only half as prevalent as the quaternary alkaloid fraction, was converted to hydrochloride salts and fractionated on large cellulose columns with methyl ethyl ketone saturated with water, in the same manner as previously described. To our knowledge, this is the first time such an isolation procedure has been employed for the fractionation of tertiary alkaloids. Upon completion of the partition chromatography, the column was washed thoroughly with methanol to insure complete removal of the alkaloids. Similar fractions were combined on the basis of their TLC patterns. This procedure gave 11 fractions which are indicated in Table I.

The general procedure for separating the components of each fraction was to convert the tertiary alkaloid salts back to the free bases, and then to employ adsorption chromatography on neutral alumina with chloroform, containing increasing amounts of methanol, as solvent. The fractions from this chromatographic separation were further purified by preparative TLC.

It was repeatedly found in the course of the present investigation that the true complexity of the crude alkaloid mixtures could not be determined prior to the large-scale partition chromatography on cellulose. It is therefore felt that the use of large partition columns as the first step in alkaloid fractionation is decidedly advantageous, and facilitates the isolation of minor alkaloids.

The new tertiary alkaloid thaldimerine (XIV) which has not previously been described was obtained as a transparent light brown oil which was homogeneous by TLC. The UV spectrum with $\lambda_{max.}^{EtOH}$ 303 and 283 mμ showed a marked bathochromic shift upon the addition of base, so that the alkaloid must be phenolic. The NMR spectrum of a deuteriochloroform solution exhibited two *N*-methyl groups superimposed at 2.48δ (six protons), and six *O*-methyls at 3.57 (three protons), 3.79 (nine protons), and 3.92δ (six protons), underscoring the fact that the alkaloid must be dimeric. The mass spectrum with a molecular ion at *m/e* 699 pointed to the formula $C_{40}H_{46}O_9N_2$, indicating the alkaloid to be an aporphine-benzylisoquinoline dimer. The base peak at *m/e* 206 represents the moiety XV. Other intense peaks were situated at *m/e* 491, 411, 397, 340, 222, and 192. It is quite tempting to present a complete structural expression for thaldimerine on the basis of these data. The authors shall refrain, however, from doing so, pending the isolation of additional thaldimerine for further study.

EXPERIMENTAL

All melting points were taken on a block manufactured by the Nalge Co. and are uncorrected. Infrared spectra were taken on a Beckman IR-5 spectrophotometer, and the ultraviolet spectra were measured in distilled 95% EtOH on a Cary model 14 spectrophotometer. The nuclear magnetic resonance spectra were taken on a Varian Associates A-60 instrument unless indicated otherwise, in deuteriochloroform or trifluoroacetic acid with tetramethylsilane as internal standard.

Mass spectra were obtained with a Nuclide 12-90-G1.1 mass spectrometer using the following procedure. The sample was placed into the heated inlet and the temperature was gradually raised until a sufficient ion current was obtained. The probe was then removed, thoroughly cleaned, and a fresh alkaloid sample added. It was then placed into the heated inlet at the previously determined temperature and the spectrum was taken immediately after a sufficient ion current was reached. In no case was a spectrum used after the sample had been in the heated inlet for more than 2 min. because of rapid alkaloid decomposition at the extreme temperatures employed.

Thin-layer chromatography was performed on a purified silica gel.[2] Preparative TLC was run on plates 8 × 8 in., (20 × 20 cm.) coated with a 1-mm.-thick adsorbent layer. Paper chromatography was performed by the descending procedure on Whatman No. 1 paper. Cellulose powder refers to Whatman ashless grade.[3] The ion-exchange resin[4] was chromatographic grade, 100–200 mesh, chloride form. Methyl ethyl ketone (MEK) was purchased from Eastman Organic Chemicals and purified by refluxing in Zn dust for 8 hr., followed by distillation from Zn dust. Ammonium reineckate was purchased from Matheson, Coleman and Bell and dissolved in water only prior to immediate use.

The following procedures were strictly adhered to:

[2] Adsorbosil I. Applied Science Laboratories, State College, Pa.
[3] Chromedia CF 11.
[4] Amberlite CG 400. Matheson, Coleman and Bell.

(*a*) solvent evaporations were performed below 60°, either at reduced pressure or under a nitrogen stream, (*b*) samples were stored under nitrogen in the dark, (*c*) compounds were removed from the preparative TLC adsorbent as soon as the solvent had evaporated, (*d*) quaternary alkaloid reineckate salts were converted to their chloride form within 4 hr. of their preparation, (*e*) after use, all chromatographic columns were washed with methanol to insure complete alkaloid removal.

Plant Extraction and Preliminary Separation—The *T. fendleri* samples used in this research were harvested during three consecutive summers in the area surrounding Tabiona, Utah. They were air dried and shipped to State College, Pa. The plant material (a total of 40 Kg. was used for this investigation) was ground to a fine powder and extracted in a Soxhlet extractor with MeOH, until the MeOH was clear and gave a negative Mayer's test (7) (usually 3–5 days). The extracts, upon evaporation of the MeOH, gave a thick, viscous residue which was dissolved as much as possible in dilute (3–5%) HOAc and filtered with the aid of Filtercel. Approximately 1 L. of acid solution was used to dissolve the extract from each 1 Kg. of dried plant. The solution was then extracted with an equal volume of $CHCl_3$ to remove nonalkaloidal components. The aqueous layer was basified (pH 8) with concentrated NH_4OH, and reextracted with an equal amount of $CHCl_3$, followed by an equal amount of Et_2O. The $CHCl_3$ and Et_2O layers were combined and the solvent was removed to give a black oil containing the total tertiary alkaloid mixture. It was found preferable not to keep alkaloids in $CHCl_3$ solution due to slow decomposition.

The aqueous layer was now freed of organic solvent at reduced pressure, and acidified (pH 2) with concentrated HCl. Freshly prepared, warm (50–60°), aqueous ammonium reineckate solution was added until the alkaloids were completely precipitated. After cooling to 0°, the precipitate was removed by suction filtration. It was immediately dissolved in acetone giving a deep red solution. This solution was diluted with an equal volume of MeOH and the alkaloids were converted to the chloride form by ion exchange as described below. Slight differences in relative alkaloid concentrations were noted in plant batches of different years.

Ion-Exchange Conversion of Alkaloid Reineckates to Chlorides—The yellow ion-exchange resin was completely converted to the chloride form by soaking in 1 *N* aqueous HCl for 10 min. and drying by suction filtration. It was then washed once in distilled water and redried. The resin was now washed three times with acetone–MeOH (1:1) and finally suspended in acetone–MeOH (1:1) for 30 min., with occasional stirring. This mixture was stirred to form a slurry which was poured slowly into a chromatographic column containing a glass wool plug. After the resin had settled (30 min.) the stopcock was opened, and the resin was washed repeatedly with acetone–MeOH (1:1) until the eluate was clean and gave no residue upon evaporation of 25 ml. of eluant. The deep red alkaloid reineckate solution in acetone–MeOH (1:1) was now placed on the column. A successful conversion was indicated by a yellow effluent of alkaloidal chlorides if concentrations are low, or more generally by a deep red band at the top of the column. The completeness of the conversion could also be checked by the disappearance of the 310 mμ reineckate anion peak (8) in the effluent. The longer alkaloid reineckate solutions were permitted to stand, the poorer was the subsequent conversion to the chloride form.

Chromatography—*Adsorption Chromatography*—All adsorption chromatography columns were prepared as a slurry. Alumina, which was found to be superior to other adsorbents, was Baker neutral alumina, 100–200 mesh, slurry pH 6–8. It was stored tightly sealed and was not activated before use. Silica gel and magnesium silicate were found to give poorer results due to the difficulty of desorbing some of the alkaloids from their surface.

Partition Chromatography—The large partition columns of cellulose, which were the major means of fractionation employed in this study, were carefully prepared and equilibrated prior to use. They were packed dry using a procedure modified from Kupchan (6) as follows.

Dry cellulose powder (500 Gm.) was placed in a 5-L. round-bottom flask equipped with a tight-fitting stopper covered with aluminum foil. Water saturated with MEK (250 ml.) (the lower phase of the MEK–H_2O system, *i.e.*, the stationary phase) was added, and the mixture was shaken vigorously to break up all lumps. The cellulose powder, appearing dry at this stage, had now been impregnated with the stationary phase. Approximately 10 Gm. of this cellulose powder was placed into the chromatography column and tamped down lightly. This procedure was repeated usually 20–30 times, until the proper size column was built. The cellulose powder was now topped with 3 or 4 filter paper disks cut to fit. Care was taken so that at no stage was the cellulose powder exposed to the air for more than a few minutes because the stationary phase impregnated on it could easily evaporate.

After the column had been built, a large amount of mobile phase, MEK–H_2O (MEK–H_2O = methyl ethyl ketone saturated with water), was added and allowed to run through. This washing and equilibration was continued for at least 8–48 hr., whereby the pressure ridges became less pronounced. A successfully equilibrated column gives a clear eluate, a cloudy eluate indicating incomplete equilibration. The equilibration of the column can be destroyed by even moderate temperature fluctuations, in which case it has to be reequilibrated prior to use.

The placing of the sample on the column was usually the most critical step in the chromatographic separation. Since alkaloid salts are generally only slightly soluble in the mobile phase (MEK–H_2O), the following procedure was used to place them on the column.

The alkaloid chlorides (5 Gm.) were dissolved in 10 ml. of stationary phase (water saturated with methyl ethyl ketone) and the solution was added to 20 Gm. of dry cellulose powder and shaken vigorously to break up all lumps. This cellulose powder was then carefully placed on the top of the previously prepared and equilibrated column, and tamped down lightly. This was topped with 3 or 4 filter paper disks, and the chromatography was started by the addition of the mobile phase.

In general, 1 Gm. of crude alkaloid chloride per 100 Gm. of dry cellulose powder was used to insure an optimum separation. The large cellulose columns employed contained 1,500 Gm. of cellulose and

TABLE II—INITIAL QUATERNARY ALKALOID SEPARATION

Fraction	Alkaloids Isolated[a]
I	Berberine chloride (VII), 1 Gm.
	Thalifendine chloride (II), 200 mg.
II	Jatrorrhizine chloride (VIII), 150 mg.
	Thalidastine chloride (III), 700 mg.
III	Magnoflorine chloride (X) 10 Gm.[b]

[a] The weight refers to the amount of alkaloid, in the chloride form, that was obtained from 15 Kg. of dried plant. [b] This weight is approximate since only a small fraction of the crude magnoflorine chloride was purified.

TABLE III—ALUMINA COLUMN CHROMATOGRAPHY OF FRACTION I (TABLE II)

Solvent	Alkaloids Present
Acetone–methanol (9:1)	Berberine
Acetone–methanol (1:1)	Uncharacterized alkaloid
Methanol	Thalifendine

were 180 cm. in height and 10 cm. wide, and a maximum of 50 Gm. of alkaloid chloride was added at one time. No column was used twice to insure against cross-contamination of alkaloid fractions.

Occasionally another procedure of sample application was employed for the separation of protoberberine alkaloids. In this case the column was prepared and equilibrated as previously described. Solid crude quaternary chlorides were now added to a 250-ml. conical flask containing 100 ml. of the mobile phase (MEK–H_2O). The flask was stoppered tightly and shaken vigorously. After 2 hr. the mobile phase was decanted off and 100 ml. of fresh mobile phase was added and the procedure was repeated until the desired amount of quaternary chloride was dissolved. This mobile phase saturated with alkaloids, sometimes amounting to 2 L. in volume, was now added to the column and the separation was begun. Despite the large volume used in the sample application, an excellent separation of protoberberine alkaloids could be achieved. This procedure was not tried with other complex quaternary mixtures.

Fractionation of Quaternary Alkaloids—The quaternary alkaloid chloride mixture was first separated on a large partition column in MEK–H_2O as previously described. The eluate was separated into three fractions, the first two of which were bright yellow and were shown by TLC in MeOH to contain three alkaloids each. The final fraction was colorless, exhibited a brilliant blue-white fluorescence under UV light, and consisted of primarily one component. Table II summarizes the alkaloids isolated from these three fractions.

Fraction I (see Table II) upon evaporation of the MEK–H_2O gave a yellow oil showing a number of spots on thin-layer chromatography. A neutral alumina column was prepared as a slurry in the solvent acetone–methanol (9:1). The yellow oil was dissolved in acetone–methanol (1:2), carefully placed on the alumina column, and elution was begun with acetone–methanol (9:1), which brought down the first yellow band. Elution was continued with acetone–methanol (1:1), which brought down another yellow band, and was completed with pure methanol, which brought down the final, yellow-orange band. The results of this alumina column are summarized in Table III.

Berberine (VII)—Upon evaporation of the acetone–methanol (9:1), the residue crystallized readily from methanol and was shown to be identical to authentic berberine chloride in terms of melting point; found 205°, reported 205° (9), mixed m.p. 205°, superimposable IR spectra (KBr pellet and Nujol mull), NMR spectra in trifluoroacetic acid and UV spectra. TLC and paper chromatography confirmed the identity of the isolated berberine chloride with authentic material.

Thalifendine (II)—Upon elution of the alumina column containing fraction I with methanol, an orange and very diffuse band emerged. Since this band had no sharply defined boundaries, a large volume of methanol was used to insure complete elution. After evaporation of the solvent, an orange solid remained. This solid showed only one spot on TLC and was converted to a yellow solid by addition of dilute hydrochloric acid. The yellow solid crystallized readily from methanol, and was recrystallized to give fine yellow needles sintering above 230°. The structural elucidation of thalifendine has been reported (1).

Thalidastine (III)—The second fraction obtained from the large cellulose column, fraction II (see Table II), gave a yellow viscous oil upon evaporation of the solvent. This oil gave three spots on TLC, all of which appeared to be protoberberine-like from their yellow-orange color. The oil was separated by preparative TLC in methanol into two easily distinguishable bands as follows: top band, R_f 0.4–0.5 thalidastine; bottom band R_f 0.0–0.1 jatrorrhizine and uncharacterized yellow alkaloid.

Both bands were eluted from the TLC plate with methanol which also dissolved some of the adsorbent. The alkaloids were freed of this impurity by alumina column chromatography with absolute ethanol. On evaporation of the ethanol, thalidastine appeared as an orange oil and jatrorrhizine and the uncharacterized yellow alkaloid as a yellow-orange oil.

The orange oil from the top band mentioned above was dissolved in methanol, aqueous HCl was added until the solution turned yellow, and upon evaporation of the solvent yellow crystalline thalidastine chloride was obtained. The structural study of this alkaloid has been described (2).

Jatrorrhizine (VIII)—Fraction II (see Table II), in addition to containing the previously mentioned thalidastine, also contained two other protoberberine alkaloids, the known alkaloid jatrorrhizine and a yellow uncharacterized salt. These two alkaloids were easily separated from thalidastine by preparative TLC in methanol, but it was found very difficult to separate them from each other. After repeated failures it was decided to employ a magnesium silicate column from which jatrorrhizine could be eluted, but not the uncharacterized salt. Using this column with acetone–methanol (1:1) as solvent, pure jatrorrhizine (one spot on TLC) was obtained as a red-brown oil. This oil was dissolved in methanol, hydrochloric acid was added until the color turned yellow, and upon evaporation jatrorrhizine chloride crystallized as fine needles.

The isolated material was shown to be identical with authentic jatrorrhizine chloride by comparison

of NMR spectra in trifluoroacetic acid and UV spectra, both of which were superimposable. Additionally TLC and paper chromatography confirmed the identity of the two samples. The isolated material was reduced in a microhydrogenator with Adams catalyst in methanol for 24 hr. A white crystalline reduction product was obtained in quantitative yield. This was shown to be identical to authentic tetrahydrojatrorrhizine by melting point comparison; found 210°, reported 216° (10) mixed m.p. 210°, practically superimposable IR ($CHCl_3$) and mass spectra, and TLC R_f values in several solvent systems.

Magnoflorine (X)—Fraction III (see Table II) was the last and largest fraction eluted from the large cellulose column. Upon evaporation of the solvent a thick brown oil was obtained. This oil gave one spot on TLC and paper chromatography, with very few minor spots also present. A small amount of this oil was rechromatographed on a long thin cellulose column with MEK–H_2O as solvent. Part of the eluate from this column crystallized upon evaporation of solvent.

The isolated material and magnoflorine chloride were shown to be identical through IR (Nujol mull) and UV spectral comparisons. Additional TLC and paper chromatography confirmed the spectral results. Magnoflorine is the most prevalent alkaloid in *T. fendleri*.

Preliminary Separation of the Crude Tertiary Alkaloids—The solid crude tertiary alkaloids (50 Gm.) were dissolved in 50 ml. of 2 *N* methanolic HCl, the pH being kept highly acidic by dropwise addition of concentrated HCl when needed. The MeOH and excess acid were removed very thoroughly under a nitrogen steam and *in vacuo*. The tertiary hydrochloride salts were then subjected to partition chromatography (1,500 Gm. cellulose powder) in the same manner as described above for the quaternary chloride salts. Upon elution from the column and combination of like fractions the solvent was removed and the tertiary hydrochloride salts were converted back to their free bases. This was done by dissolving the salts in 10–20 ml. of water followed by dropwise addition of concentrated NH_4OH until the solution was just basic and then repeated extraction with $CHCl_3$. The $CHCl_3$ layer was removed, dried with Na_2SO_4, filtered, and evaporated to dryness. All adsorption chromatography and structural elucidation was done on the tertiary free bases. The large partition separation of the tertiary hydrochloride mixture is summarized in Table I.

Thalifendlerine (I)—Fraction 2 (see Table I) gave a light brown oil upon evaporation of the solvent. This oil, showing one spot on TLC, crystallized upon the addition of methanol. After several recrystallizations from the same solvent and then from acetonitrile, large white rods were obtained. The chemistry of this interesting benzylisoquinoline alkaloid has been described (1).

Separation of Tertiary Alkaloid Fractions 3 and 4—Fraction 3 (see Table I) gave a brown oil upon evaporation of the solvent. TLC showed it to be a highly complex mixture composed of at least 10 alkaloids, although some were present to only a minor extent. TLC also indicated that many components of this fraction were present in the succeeding fraction, designated fraction 4. Fraction 4 was further purified in the same manner as will be described for fraction 3, and will therefore not be discussed in detail.

TABLE IV—SEPARATION OF FRACTION 3

Flasks	Solvents	Alkaloids
1–9	$CHCl_3$	Ocoteine (XI), glaucine (IX)
10–20	$CHCl_3$	None
21–27	1% MeOH	None
28–35	1% MeOH	Preocoteine (V)
36–39	3% MeOH	Preocoteine (V), thaliporphine (IV)
40–44	3% MeOH	Thaliporphine (IV), thalifendlerine (I)
44–53	5% MeOH	Thalifendlerine (I), plus other alkaloids
54–64	10% MeOH	Several alkaloids present

The brown oily fraction 3 (1.2 Gm.) was dissolved in chloroform, filtered, and chromatographed on a neutral alumina column (90 Gm.) prepared as a slurry in chloroform. After the addition of 400 ml. of chloroform, methanol was added in increasing increments (gradient elution chromatography) until a 10% methanol solution in chloroform was reached. The results of this adsorption chromatography are given in Table IV.

Flasks 1–27 contained 25 ml. each and 28–64 ml. each of solvent.

Flasks 1–9 (see Table IV) were combined and evaporated to dryness, giving a light yellow oil which failed to crystallize. TLC showed this oil to be composed of at least three components which were easily separated by preparative TLC in acetone. The top band was not characterized, the middle band was identified as ocoteine (XI), and the lower band corresponded to glaucine (VII).

Ocoteine (XI)—The middle band from the preparative TLC, gave a UV spectrum with $\lambda_{max.}^{EtOH}$ 314, 302, 283, and 232 mμ (log ε 4.1, 4.2, 4.2, and 4.4). The NMR spectrum showed one *N*-methyl at 2.54 δ, three *O*-methyl groups at 4.01 (three protons) and 3.92 δ (six protons), one methylenedioxy as two doublets at 6.05 and 5.92 δ (J = 1.5 c.p.s.), and two aromatic protons at 6.78 and 7.62 δ. The UV and NMR spectra were practically superimposable with those of an authentic sample of known ocoteine. TLC confirmed the identity of the two samples. In addition, the mass spectrum of the isolated alkaloid showed a molecular ion (M^+) at m/e 369 ($C_{21}H_{23}NO_5$) and other large peaks at 354 (M-15), 338 (M-31), and 326 (M-43), all characteristic of aporphine alkaloids, and in agreement with the reported structural assignment (11).

Glaucine (IX)—The lower band from the preparative TLC gave a colorless oil, λ_{max}^{EtOH} 300, 280, and 218 mμ, showing no shift on addition of base. This UV spectrum indicated the isolated alkaloid to be a 1, 2, 9,10- substituted, nonphenolic aporphine, and strongly suggested the alkaloid was glaucine (12). This assumption was strengthened by the mass spectrum, which showed a large molecular ion (M^+) at m/e 355 ($C_{21}H_{25}NO_4$), and the other expected peaks at 340 (M-15), 324 (M-31), and 312 (M-43) (11). The isolated alkaloid was then shown to be the known base glaucine by means of a direct comparison with an authentic sample in terms of UV and mass spectral comparisons and TLC R_f values. The structural assignment was performed on less than 5 mg. of isolated material.

Preocoteine (V)—Flasks 28–35 (Table IV) were combined and upon evaporation of the solvent gave a light green oil. This oil was shown by TLC to contain some nonalkaloidal material, and this impurity was separated by preparative TLC in methanol–acetone (5:3). The alkaloid moved to the middle of the plate and the impurity was located at the solvent front. Upon removal from the plate with chloroform–methanol (9:1), preocoteine exhibited $\lambda_{max.}^{EtOH}$ 314, 304, 279, and 222 mμ, with a marked bathochromic shift upon addition of base. The NMR spectrum in deuteriochloroform at 100 Mc. showed the presence of one *N*-methyl at 2.52 δ, four *O*-methyl groups at 3.92 δ (six protons), and 3.88 δ (six protons), and two aromatic protons, one downfield at 7.99 δ characteristic of a C-11 proton, and the other at 6.74 δ (12). The absence of a relatively upfield methoxyl indicates the phenolic function is at C-1 (12). The UV spectrum of preocoteine strongly resembles that of ocoteine (XI). The structural elucidation of preocoteine has been recorded (4).

Thaliporphine (IV)—Flasks 36–39 (see Table IV) were combined and upon solvent evaporation gave an oil showing two spots on TLC. These two components were easily separated by preparative TLC in chloroform–methanol (19:2). The top band proved to be preocoteine (V). The lower band was eluted from the purified silica gel with chloroform–methanol (9:1), and crystallized with difficulty from methanol. The chemistry of this new aporphine alkaloid, designated thaliporphine, has been discussed (4).

Flasks 40–44 (see Table IV) were combined and the solvent removed. TLC showed it to contain thaliporphine (IV) and thalifendlerine (I). These two alkaloids were separated by preparative TLC in methanol–chloroform (2:19), the top band being thaliporphine, and the bottom band thalifendlerine.

Flasks 44–53 and 54–64 (see Table IV) after combination and solvent evaporation were shown to be complex mixtures of at least eight components each by TLC in methanol–chloroform (1:1). Preparative TLC in this system allowed the isolation of some thalifendlerine from flasks 44–53. The other components of both fractions were present in too small amounts to be isolated.

Fractions 5 and 6 were chromatographed on a column of neutral alumina, using chloroform with increasing amounts of methanol as the eluent. Additional quantities of thaliporphine were isolated from this procedure together with other minor alkaloids which were not characterized.

Hernandezine (XII) and Thalicarpine (XIII)—The methanol washing from the large cellulose column (see Table I) proved to be rich in alkaloids. After solvent evaporation the oily residue was dissolved in chloroform and chromatographed on a neutral alumina column. The solvent polarity was gradually increased by the addition of methanol, until pure methanol was used. Fractions were combined on the basis of their TLC patterns in methanol–acetone (5:3). This chromatographic procedure gave two distinct fractions: an early fraction eluted with pure chloroform, and a late fraction eluted with chloroform–methanol. The results of this chromatographic procedure are summarized in Table V.

Table V—Separation of Methanol Washing

Early fraction (eluted with chloroform)	Hernandezine (VIII) Thalicarpine (XIII)
Late fraction (eluted with chloroform containing methanol)	Thaldimerine (XIV) Thalidezine (II)

The early fraction showed three spots on TLC either in methanol–acetone (5:3) or in methanol–chloroform (1:1). Preparative TLC in these solvents was used to separate these three components. The top band was identified as the known alkaloid hernandezine (VIII), the middle band was shown, by comparative TLC to be thalicarpine (XIII), and the bottom band was present in too small amounts to be investigated.

Hernandezine, m.p. 158° (MeOH), was obtained upon washing the purified silica gel with methanol–chloroform (1:9). A direct comparison of the isolated material with an authentic sample showed the two materials to be identical in terms of IR (chloroform), UV, and mass spectral comparisons, and TLC R_f values. A structural reevaluation of hernandezine has been reported (3).

Thalicarpine (XIII) obtained by washing the purified silica gel with methanol–chloroform (1:9) was identified by spectral comparison with an authentic sample, and comparative TLC.

Thaldimerine (XIV)—The late fraction (see Table V) showed two spots on TLC in methanol–acetone (5:3). After many trials it was decided that methanol–ether (7:3) was a suitable solvent combination for preparative TLC. A complete separation of the two alkaloids was achieved only after repeated use of preparative TLC. The upper band proved to be a new alkaloid, designated thalidezine (VI). The lower band, which was oily and turned brown on exposure to iodine vapor, was also a new alkaloid and was named thaldimerine (XIV).

Thalidezine (VI)—The new bisbenzylisoquinoline alkaloid thalidezine (Table V) was obtained in crystalline form by repeated preparative TLC separations from the crude thaldimerine fraction above. Recrystallization from acetone gave 300 mg. of white needles, m.p. 158–159°. The structural elucidation of thalidezine has been reported (4).

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Keyphrases

Thalictrum alkaloids
Alkaloid isolation–*Thalictrum fendleri*
Chromatography, partition and adsorption–separation
TLC and paper chromatography–separation
NMR spectrometry
IR spectrophotometry–structure
UV spectrophotometry–structure

Inhibitors of Monoamine Oxidase

Influence of Methyl Substitution on the Inhibitory Activity of β-Carbolines

By BENG T. HO, WILLIAM M. MCISAAC, KENNETH E. WALKER, and VICENTE ESTEVEZ

A number of tetrahydro and aromatic β-carbolines, mostly with a methyl substituent at various positions, were synthesized and their *in vitro* inhibitory activities on monoamine oxidase evaluated. Substitution of a methyl group at the N-9 nitrogen of tetrahydro-β-carboline gave a potent competitive inhibitor of the enzyme. Methyl groups at C-1 of both tetrahydro and aromatic β-carbolines generally reduced the potency, whereas introduction of a methyl group at the N-2 nitrogen of tetrahydro-β-carboline gave a compound of equal activity.

FOR MANY YEARS, since the discovery of iproniazid, inhibitors of the enzyme monoamine oxidase have been used as chemotherapeutic agents for the treatment of depression. However, the many potent antidepressants of the hydrazine class are hepatotoxic, and for this reason have fallen into disuse. Attempts, therefore, have been made to search for other antidepressants with more rapid onset and lower toxicity.

A number of β-carbolines have been found to be potent inhibitors of monoamine oxidase (1–3), yet little is known about the way these compounds bind to the enzyme. In this phase of these studies, a number of tetrahydro and aromatic β-carbolines, mostly with methyl substituents at various positions, were synthesized and their *in vitro* inhibitory activities evaluated. Information on the mode of binding of these compounds to the enzyme will be useful in the design and synthesis of more potent inhibitors which may become useful in the treatment of depression.

DISCUSSION

A fivefold decrease in inhibitory activity was observed when a methyl group was placed on C-1 of the tetrahydro-β-carbolines I and IV (Table I) and the aromatic β-carboline (XX)(Table II) to give II, V, and XXI, respectively. Ethyl substitution on C-1 of I and XX resulted in an even greater decrease in activity; III was seven times less active as an inhibitor than I, and XIII was 16 times less than XX. This loss of inhibitory activity could be attributed to steric hindrance by the bulk of the alkyl group. When a larger group such as COOH was substituted on C-1 of tetrahydro-β-carbolines, an even greater loss of activity occurred; the activity of XV was about 18 times less than I and four times less than II. However, the possibility of a repulsion between COO^- of XV and an anion site on the enzyme could not be entirely ruled out. Likewise, a methyl substituent on C-3 caused a threefold decrease in activity when I was compared with XVII. A COOH group on C-3 gave an inactive compound (XVIII).

Introduction of a methoxy group to C-6 of the ring decreased the inhibitory activity; a fourfold loss in activity was observed in both cases when I was converted into IV, or II into V. An eightfold decrease in activity was observed when 1,9-dimethyl-β-carboline (XII) was compared to its 6-methoxy derivative (XIII). If the indole nucleus binds as an electron donor to an electron-poor locus of the enzyme in a charge-transfer complex (4), the electron-donating methoxy group should make IV and V bind better. Thus, the decreased activity of 6-methoxytetrahydro-β-carbolines can be attributed either to the bulk of the methoxy group or to its polar nature, which is unfavorable for the binding. Studies to obtain greater binding of the indole nucleus by other substituents are in progress.

Neither methyl nor propyl substitution on N-2 of II affected the inhibitory activity; VI and VII were equally as active as II, indicating that a long alkyl

Received June 12, 1967, from the Biological Research Division, Texas Research Institute of Mental Sciences, and the Departments of Biochemistry and Psychiatry, Baylor University College of Medicine, Houston, TX 77025
Accepted for publication September 12, 1967.
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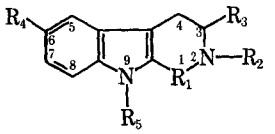
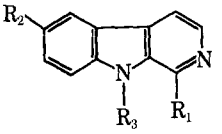
TABLE I—INHIBITION OF MONOAMINE OXIDASE BY TETRAHYDRO-β-CARBOLINES



Compd.	R_1	R_2	R_3	R_4	R_5	% Inhibition	mM Concn. Inhibitor
	Iproniazid					50	0.025
I[a]	CH_2	H	H	H	H	50	0.34
II[b]	$CHCH_3$	H	H	H	H	50	1.5
III[a]	$CHCH_2CH_3$	H	H	H	H	50	2.5
IV	CH_2	H	H	CH_3O	H	50	1.3
V[b]	$CHCH_3$	H	H	CH_3O	H	50	6.3
VI[a,b]	$CHCH_3$	CH_3	H	H	H	50	1.5
VII[a,b]	$CHCH_3$	n-C_3H_7	H	H	H	50	1.3
VIII	$CHCH_3$	CH_3CO	H	H	H	24	1.0[c]
IX	$CHCH_3$	CH_3CO	H	CH_3O	H	2	1.0
X[a]	$CHCH_3$	CH_3	H	H	CH_3	50	0.90
XI[a]	CH_2	H	H	H	CH_3	50	0.0098
XII[d]	$CHCH_3$	H	H	H	CH_3	50	0.085
XIII[a,b]	$CHCH_3$	H	H	CH_3O	CH_3	50	0.98
XIV	CHCOOH	H	H	H	CH_3	50	0.79
XV	CHCOOH	H	H	H	H	50	6.0
XVI	CHCOOH	H	H	CH_3O	H	6	1.0[e]
XVII	CH_2	H	CH_3	H	H	50	0.92
XVIII[b]	CH_2	H	COOH	H	H	50	16
XIX[b]	CO	H	H	H	H	50	2.5

[a] Hydrochloride salt. [b] Commercial sample. [c] Assay was run in the presence of 40 μl. of dimethylsulfoxide; this amount of dimethylsulfoxide alone gives 40% inhibition of the enzyme. [d] Sulfate salt. [e] Highest tested concentration.

TABLE II—INHIBITION OF MONOAMINE OXIDASE BY β-CARBOLINES



Compd.	R_1	R_2	R_3	% Inhibition	mM Concn. Inhibitor
XX[a]	H	H	H	50	0.029
XXI[a]	CH_3	H	H	50	0.14
XXII	CH_2CH_3	H	H	50	0.47
XXIII	H	H	CH_3	50	0.010

[a] Commercial sample.

chain like propyl can be tolerated by the enzyme provided the basicity of the N-2 nitrogen is not appreciably decreased. That a basic N-2 nitrogen is required for the binding of the β-carbolines to the enzyme is indicated by the fact that the acetyl derivatives (VIII and IX) of II and V show a marked decrease in activity. This was further observed in XIX where the basicity of the N-2 nitrogen was abolished by formation of a lactam linkage. Other evidence is seen from the work of McIsaac and Estevez (3), who found that replacement of the N-2 nitrogen of β-carbolines by CH completely abolished monoamine oxidase inhibitory activity.

An increase of 35-fold in inhibitory activity was observed in XI by the introduction of a methyl group at N-9 nitrogen of I. Only an eighteenfold increase was observed when a methyl group was placed at N-9 nitrogen of II; this difference is probably due to the steric interaction between the two adjacent methyl groups in XII. The effect of substitution on N-9, however, is not as great in the aromatic series. For example, the introduction of an N-9 methyl group to XX (Table II) resulted in XXIII which is only three times as active as XX.

In summary, tetrahydro-β-carbolines in general are not as good inhibitors of monoamine oxidase as aromatic β-carbolines *in vitro;* an approximately tenfold difference in inhibitory activity between these two series can be seen when I is compared to XX, and II to XXI. However, by substituting a methyl group at the N-9 nitrogen of the tetrahydro-β-carboline (I) a 35-fold increase in activity occurs, and the resulting XI becomes as potent as the aromatic β-carboline (XXIII). From the assay, XI appears to be three times more active than iproniazid (Table I). A Lineweaver-Burk plot showed that XI was a competitive inhibitor of the monoamine oxidase $Km = 8.1 \times 10^{-6}$ M and $Ki = 7.4 \times 10^{-6}$ M (Fig. 1). To our knowledge XI is so far the only

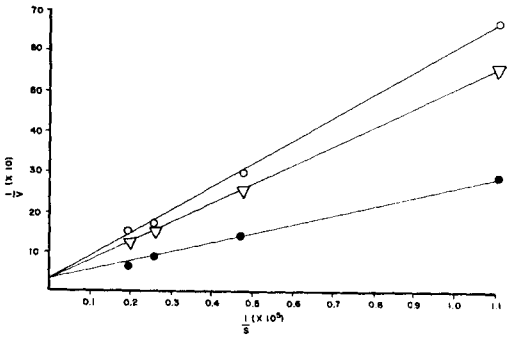


Fig. 1—Lineweaver-Burk plot of inhibition of MAO by XI. Key: ●, *no inhibitor,* ○, 1×10^{-6} M *XI,* ▽, 8×10^{-6} M *XI.*

TABLE III—PHYSICAL CONSTANTS OF β-CARBOLINES

Compd.	Method[a]	% Yield	M.p., °C.	Anal.,% C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
I	*A*	90	280[b,c]	...	...	...	...	...	...
III	*B*	45	261–262[d]	65.9[d]	66.0	7.23	7.30	11.8	12.0
XVI	*A*	50	224–226[b]	63.4	63.3	5.72	5.75	11.4	11.4
IV	*A*	87	268–269 dec.[d]	60.2[d]	60.0	6.31	6.45	11.7	11.9
VIII	*C*	30	205.5–206[e]						
IX	*C*	31	202–202.5[e]						
X	*D*	50	224–225 dec.[f,g]	52.4[f]	54.0	4.77	4.96	15.8	16.0
XI	*D*	50	257–258[d,f]						
XII	*D*	63	233 dec.[f,g]	53.1[g]	53.1	4.45	4.55	16.3	16.2
XVII	*A*	66	240–241[b]	77.4	77.2	7.57	7.65	15.0	15.2
XXII	*E*	7.2	218–219[d]	67.1[d]	66.9	5.62	5.70	12.0	11.8
XXIII	*D*	52	108–109[b]	79.1	79.2	5.53	5.57	15.4	15.5

[a] Method *A* is the condensation of the appropriate indolealkylamine with glyoxylic acid, followed by decarboxylation of the 1-carboxy derivative (see example under *Synthesis*); method *B* is the condensation of tryptamine with aldehyde; method *C* is the acetylation by acetic anhydride; method *D* is the methylation using sodium in liquid ammonia; method *E* is the cyclization and decarboxylation of *N*-acetyltryptophan with $POCl_3$ and polyphosphoric acid. [b] Free base. [c] Lit. m.p. 282–284° (7). [d] Hydrochloride salt. [e] Prepared from acetic anhydride and sodium acetate; lit. m.p. 205–207° (VIII) and 203–204.5° (IX) prepared from acetic anhydride alone (11). [f] Free base was an oil. [g] Monopicrate salt.

tetrahydro-β-carboline whose inhibitory activity is comparable to the most potent of the aromatic β-carbolines. There is no obvious explanation for the greater inhibitory activity of XI other than that the methyl group on N-9 makes the indole nucleus bind strongly to the enzyme. This methyl group may itself bind to the monoamine oxidase by a combination of van der Waals forces and hydrophobic bonding. However, the maximum increase in binding expected for one methyl group from both hydrophobic bonding and van der Waals forces would be only tenfold (5). Studies of the effect of other substituents of N-9 and N-2 of β-carbolines on inhibitory activity are in progress.

CHEMISTRY

Three tetrahydro-β-carbolines (IV, XI, and XVII, Table III) were synthesized from the condensation of the appropriate indolealkylamine with glyoxylic acid, followed by decarboxylation of the intermediates (XVI, XIV, and XXIV). Compound XVII prepared according to this method had a melting point of 240–241°, whereas 201–203° was recorded for this compound prepared by reacting α-methyltryptamine with formaldehyde (6). The synthesis of I by this route has been reported (7). The starting material for XIV, 1-methyltryptamine, was prepared according to the method of Potts and Saxton (8). In our preparation this oily amine was converted to its hydrochloride salt, m.p. 174–175°, in 42% yield. The melting point (239.5–240.5°) of the picrate of XI agreed with that in the literature (9) prepared by reduction of the 1-oxo derivative. Although the preparation of IV (10) and XI have been reported, our procedure seems to be more convenient than the reported method *via* the Na–BuOH reduction of the corresponding 1-oxo derivatives. At pH 4, 1-carboxytetrahydro-β-carboline precipitated, while glyoxylic acid and the salt of the unreacted tryptamine remained in the aqueous solution. In the next decarboxylation step, the product was obtained by precipitation from the basic medium in which unreacted amphoteric starting material, if any, was soluble in basic medium. Thus, in both steps the product can be obtained in a reasonably pure state and subsequent recrystallization should not be necessary. The decarboxylation was performed at 65–70° for 45 min. However, in a later run comparable yields were obtained when the decarboxylation was carried out at reflux temperature for only 30 min.

The condensation of tryptamine with propionaldehyde in aqueous acid medium was carried out at reflux temperature. 1-Ethyltetrahydro-β-carboline (III) was isolated as its hydrochloride salt.

The preparation of 1-ethyl-β-carboline (XXII) started from the acylation of tryptophan with propionyl chloride. Cyclization and subsequent decarboxylation of the resulting *N*-propionyltryptophan with $POCl_3$ and polyphosphoric acid gave XXII in a low yield of 7.2%. No attempt was made to improve the yield.

Alkylation of the sodium salts of 1-methyl-1,2,3,4-tetrahydro-β-carboline and its 2-methyl derivative with methyl iodide gave XII and X, respectively. 9-Methyl-β-carboline (XXIII) was prepared in the similar manner. An attempt was made to prepare X from XII. Treatment of XII with formaldehyde and formic acid did not give the desired product. Since the hydrochloride salts of both X and XII as well as the sulfate salt of X were hygroscopic, these two compounds were characterized as their picrate salts (Schemes I and II).

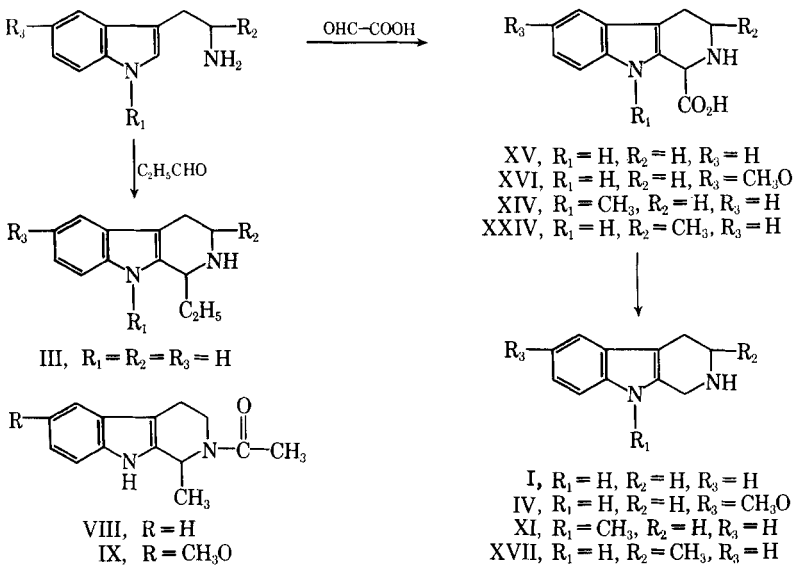
EXPERIMENTAL

Melting points were taken on a Fisher-Johns apparatus and are corrected.

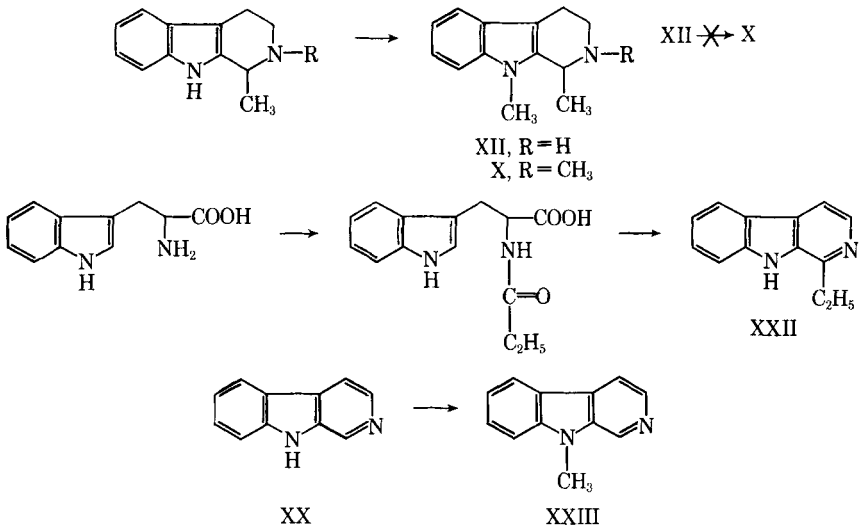
Synthesis

1-Ethyl-1,2,3,4-tetrahydro-β-carboline(III)—To a stirred suspension of 2.0 Gm. (12.5 mmoles) of tryptamine in 50 ml. of water was added 10% H_2SO_4 until the pH of the solution reached between 2–3. A stream of helium was flushed over the surface while the solution was heated to reflux. Propionaldehyde (3 ml., 40 mmoles) was added and the solution was allowed to stand for 30 min. without further heating. The condenser was removed and the solution was heated to boiling for an additional 30 min. It was made strongly basic with 10% KOH and extracted with ether (3 × 50 ml.). The ethereal extracts were combined and dried with anhydrous Na_2SO_4. Anhydrous hydrogen chloride was passed into the ethereal solution to precipitate the hydrochloride; yield, 2.2 Gm. (76%).

Thin-layer chromatography on silica using *n*-



Scheme I



Scheme II

propanol–concentrated NH_4OH (95:5) showed contamination of the product by tryptamine. One recrystallization from absolute ethanol–ether gave 1.3 Gm. (45%) of solid, m.p. 261–262°, which was free of starting material. $\nu_{max.}$ (KBr) 3240 (NH); 2950, 2900, 2760, 2670, 2620, 2575, 2480, 2380, 2290 (CH, NH^+); 1620, 1590 (C=C, NH^+); 745 cm.$^{-1}$ (indole CH). (See Table III for analytical data.)

6-Methoxy-1,2,3,4-tetrahydro-β-carboline-1-carboxylic acid (XVI)—A solution of 400 mg. (4 mmoles) of glyoxylic acid monohydrate in 22 ml. of water was added with magnetic stirring to a solution of 1 Gm. (4 mmoles) of 5-methoxytryptamine hydrochloride in 8 ml. of water. The mixture was adjusted to pH 4 with 10% KOH and the product precipitated after a short time. After being stirred for 5 hr. at room temperature, the solid was collected on a filter and washed several times with small amounts of water; yield, 450 mg. (50%), m.p. 224–226°, with evolution of CO_2. $\nu_{max.}$ (KBr) 3300 (NH) 2900–2875, 2790, 2530 (CH, NH^+); 1645, 1590, 1570 cm.$^{-1}$ (COO^-, NH^+, C=C); $\lambda_{max.}$ (1% HCl) 220, 273, 293 (s), 367 mμ. (See Table III for analytical data.)

6-Methoxy-1,2,3,4-tetrahydro-β-carboline (IV)—A mixture of 400 mg. (1.7 mmoles) of XVI and 3 ml. of concentrated hydrochloric acid in 10 ml. of water was heated at 65–70° for 45 min., during which time a white solid deposited in the solution. Just enough water was added to dissolve the solid and the product was precipitated by adding excess of 10% KOH; yield, 276 mg. (87%), m.p. 216–218°. $\nu_{max.}$ (KBr) 3270 (NH); 1625, 1590, 1560 cm.$^{-1}$ (C=C); $\lambda_{max.}$ (EtOH) 220, 273, 293 (s), 362 mμ.

A melting point of 220–223° has been recorded for this compound prepared by reduction of 6-methoxy-1-oxo-1,2,3,4-tetrahydro-β-carboline (10).

For analysis this product was converted to the

hydrochloride salt, m.p. 268–269° dec.; $\nu_{max.}$ (KBr) 3190 (NH); 2900–2880, 2780–2740, 2640, 2570, 2560, 2500, 2440 (CH, NH^+); 1620, 1590, 1580, 1565 $cm.^{-1}$ (NH^+, C=C). (See Table III for analytical data.)

1-Ethyl-β-carboline (XXII)—To a solution of 5 Gm. (24.5 mmoles) of tryptophan in 200 ml. of ice cold 10% KOH was added a solution of 20 ml. of propionyl chloride in 200 ml. of benzene and the mixture was stirred vigorously. An additional small amount of solid KOH plus crushed ice was added to make the aqueous solution strongly basic; this was followed by addition of 5 ml. of propionyl chloride. This procedure was repeated once more. The aqueous layer, after being made strongly basic with solid KOH, was separated. It was then acidified with concentrated HCl to about pH 1 and extracted with chloroform (3 × 200 ml.). The combined chloroform extracts were dried with Na_2SO_4 and the product was precipitated by adding 1 L. of petroleum ether (b.p. 60–110°); yield, 4.3 Gm. (67%), m.p. 161–161.5°. $\nu_{max.}$ (KBr) 1715 (acid C=O); 1615, 1540 $cm.^{-1}$ (amide C=O and NH).

A vigorously stirred mixture of 4.2 Gm. (16 mmoles) of the above *N*-propionyltryptophan, 42 Gm. of polyphosphoric acid, and 9 ml. of $POCl_3$ was heated at 125° for 45 min., then poured onto 100 Gm. of crushed ice. After removal of the resinous material by filtration, the solution was made strongly basic with 10% KOH and extracted with ether (3 × 15 ml.). The ethereal extracts were combined and dried with anhydrous Na_2SO_4. Anhydrous hydrogen chloride was passed into the ethereal solution to precipitate the hydrochloride; yield, 1.2 Gm. (32%), m.p. 135–145°. This crude product was recrystallized several times from absolute ethanol–ether then from ethanol–heptane giving 270 mg. (7.2%) of beige solid, m.p. 218–219°, which was chromatographically pure [solvent, *n*-propanol–concentrated NH_4OH (95:5)]. $\nu_{max.}$ (KBr) 3340 (NH); 3125, 3040, 2950, 2900, 2850, 2800, 2750 (CH, NH^+); 1630, 1620, 1570, 1525, 1505 (C=C, NH^+); 820 (pyridine CH); 755 $cm.^{-1}$ (indole CH); $\lambda_{max.}$ (H_2O) 212, 233, 250 (s), 280 (s), 287, 335, 347. (See Table III for analytical data.)

1,9-Dimethyl-1,2,3,4-tetrahydro-β-carboline (XII)—1-Methyl-1,2,3,4-tetrahydro-β-carboline (1.86 Gm., 0.01 mole) was added slowly with stirring to a sodamide solution prepared by reacting 0.25 Gm. (0.011 mole) of Na with 60–70 ml. of liquid ammonia in the presence of a catalytic amount of ferric nitrate. After all the amine had dissolved (about 15 min.), 1.56 Gm. (0.011 mole) of methyl iodide in 20 ml. of ether was added slowly over a period of 30 min. Stirring was continued for an additional 20 min. The volatile solvents were removed by a stream of helium while the flask was immersed in hot water. The residue was extracted with benzene (3 × 5 ml.) and the combined extracts dried with anhydrous Na_2SO_4. An excess amount of 5% methanolic H_2SO_4 was added to the benzene solution and the sulfate salt solidified upon addition of ether. It was collected on a filter, washed with ether, and dried *in vacuo;* yield, 1.7 Gm. (63%), m.p. 239–242° dec.. $\nu_{max.}$ (KBr), 3350, 3275, 3225, 3200, 3000, 2930, 2890, 2850, 2800 (NH, CH, NH^+); 1600, 1555 (C=C); 745 $cm.^{-1}$ (indole CH); $\lambda_{max.}$ (1% HCl) 222, 274, 281, 288 mμ. For analysis the sulfate was converted to the monopicrate which was purified by recrystallization from absolute ethanol. (See Table III for analytical data.)

9-Methyl-β-carboline (XXIII)—β-Carboline (800 mg., 4.6 mmoles) was treated with a mixture of 114 mg. (5 mmoles) of sodium in liquid ammonia and 468 mg. (6.6 mmoles) of methyl iodide as in the preparation of 1,9-dimethyl-1,2,3,4-tetrahydro-β-carboline. The reaction residue was extracted with benzene, evaporated, and the residue dissolved in the minimum amount of petroleum ether (b.p. 30–60°). After removal of the insoluble starting material by filtration, the petroleum ether was evaporated *in vacuo* leaving a white solid. Recrystallization from heptane gave 434 mg. (52%) of product, m.p. 108–109°; $\nu_{max.}$ (KBr) 1620, 1595, 1555, 1495 (C=N, C=C); $\lambda_{max.}$ (EtOH) 216, 236, 241 (s), 251 (s), 264 (s), 284, 290, 346, 357 mμ. (See Table III for analytical data.)

A m.p. of 107° (12) and 109° (13) has been recorded for this compound prepared by the treatment of 9-methyl-1-oxo-β-carboline with $POCl_3$.

Assay

Beef liver was homogenized in 10 vol. of 0.25 *M* sucrose and the cell debris was removed by centrifugation. The supernatant was centrifuged at 13,000×g for 90 min. to sediment the mitochondrial pellet which was then suspended in 0.01 *M* phosphate buffer pH 7.6 and subjected to sonication at 20 kc./sec. for 20 min. The sonicated preparation was then centrifuged at 100,000×g for 60 min., and the supernatant containing monoamine oxidase (340 mμmole/hr./mg. protein) was used as the enzyme source. This procedure gives approximately sevenfold purification.

All the stock solutions of the hydrochloride salts of inhibitors were prepared in water and the solutions of free amines in 0.01 *N* HCl. Compound VIII was dissolved in dimethylsulfoxide, IX and XIX in 50% aqueous dimethylsulfoxide, XIV in 0.01 *N* NaOH, and XVI and XVIII in 0.05 *N* NaOH. Incubation was carried out at 37° for 30 min. in a solution containing 60 mμmoles of the substrate, tryptamine-2-^{14}C hydrochloride, varying amounts of inhibitor, 20 μl. of enzyme, phosphate buffer pH 7.4, and water to make a final volume of 1 ml. The product, a mixture of indoleacetaldehyde and indoleacetic acid, was extracted with ether in strong acidic medium. After the removal of solvent, the product was assayed for ^{14}C in a liquid scintillation spectrometer and the concentration of inhibitor at which enzyme activity was 50% inhibited was determined.

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Keyphrases

Monoamine oxidase inhibitors
β-carbolines–synthesis
Methyl and methoxy substitution–activity effect
Ethyl substitution–activity effect
Propyl substitution–activity effect
IR spectrophotometry–structure
UV spectrophotometry–structure

Drug Release from Wax Matrices I

Analysis of Data with First-Order Kinetics and with the Diffusion-Controlled Model

By JOSEPH B. SCHWARTZ, ANTHONY P. SIMONELLI, and WILLIAM I. HIGUCHI

The release of drug into aqueous media from wax matrices has been investigated using benzoic acid and salicylic acid as the active ingredients. The data have been examined from the viewpoint of the first-order kinetic theory and that of a diffusion-controlled process. It was found that the experimental data are best analyzed using a diffusion-controlled model and square root of time release profiles. Parameters controlling the release are presented.

SEVERAL WORKERS (1–4) have recently reported the results of investigations regarding the factors influencing drug release from inert, insoluble matrices. Matrices composed of plastic polymers have been shown to exhibit release profiles which are best described by the linear square root of time dependence indicating that a diffusion-controlled mechanism is operative (2). Waxes, on the other hand, present a much more complicated system, both experimentally and theoretically, due to various physical-chemical factors and properties not present in the plastic systems.

As a result, the mechanism of release from wax matrices has not been as firmly established. In view of the large number of patents issued in this area, it would be highly desirable that the release rates from these systems also be quantitated by use of the diffusion model and the determination of the necessary controlling parameters made.

Theoretical treatment has shown that drug release from an insoluble, inert matrix is described by the T. Higuchi equation (5) if the rate-determining process is diffusion; and this is given by:

$$Q = \sqrt{\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s t} \qquad \text{(Eq. 1)}$$

where Q is the amount of drug released per unit area of the disk exposed to the solvent; D is the diffusion coefficient of the drug in the solvent; ϵ is the porosity of the matrix; τ is the tortuosity of the matrix; A is the concentration of solid drug in the matrix; C_s is the solubility of the drug in the solvent; and t is time. Each of the above variables lends itself to experimental determination. Any units may be used for the above variables and constants, provided they are mutually consistent.

It was the purpose of this study to establish the applicability of the diffusion model to wax systems and to determine the controlling variables.

EXPERIMENTAL

Tablets composed of a drug homogeneously distributed throughout a wax matrix were prepared by the following method. The drug was suspended or dissolved in the melted waxes contained in an evaporating dish. Following the removal of heat, continual stirring until complete solidification had

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Accepted for publication September 12, 1967.
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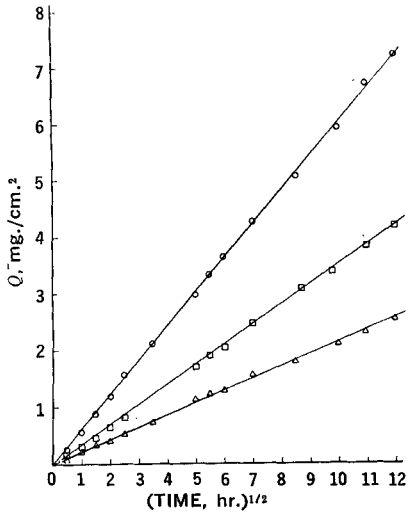

Fig. 1—Release profile of various concentrations of salicylic acid when plotted according to the diffusion model. Key: Δ, 5%; □, 10%; ○, 20%.

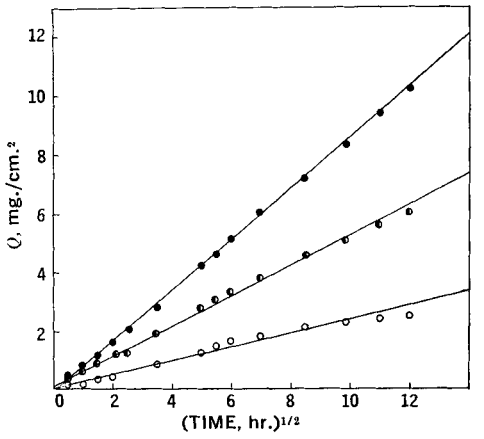

Fig. 2—Release profiles of various concentrations of benzoic acid when plotted according to the diffusion model. Key: ○, 5%; ◐, 10%; ●, 20%.

occurred prevented any separation of drug and wax. To facilitate compression, this solid was then granulated by means of a 16-mesh screen and a spatula. Tablets of approximately 250 mg. with a $^3/_8$-in. diameter were compressed from this granulation using a Carver press at a compressional force of 5,000 lb.

To measure release rate, a volumetric flask (250 ml.) containing a tablet was filled with 100 ml. of the solvent and was placed in a shaking water bath maintained at 30°. Aliquot samples were withdrawn at various times and replaced with fresh solvent. After proper dilution with water, these samples were assayed using a Beckman DU spectrophotometer (salicylic acid, 294 mμ; benzoic acid, 259 mμ).

RESULTS AND DISCUSSION

Preliminary investigations were carried out with several waxes and their combinations. The matrix chosen for these studies was composed of PGMS[1] and hydrogenated castor oil[2] in a 1:1 ratio. It was observed that either wax, when used alone, had disadvantages which disappeared when the two were combined. Tablets of PGMS exhibited relatively rapid release rates but showed a tendency to flake when run for any length of time under experimental conditions. Tablets of hydrogenated castor oil did not flake but gave an extremely slow release. The 1:1 ratio provided the optimum matrix.

Tablets containing either salicylic or benzoic acid were studied. Figures 1 and 2 show linear square root of time plots indicating that these systems are diffusion controlled. Average slopes (3 or more runs) of these curves, listed in Table I, are proportional to the concentration of drug in the tablet as predicted by Eq. 1 and support the above mechanism.

Interestingly, the data also yielded an apparently straight line when the log of the drug left in the matrix was plotted as a function of time as predicted by first-order release:

$$\log W = \frac{kt}{2.303} + \log W_0 \qquad \text{(Eq. 2)}$$

where W = amount of drug left in tablet,
W_0 = initial amount of drug in tablet,
k = first-order rate constant,
t = time.

Figure 3 illustrates the release profiles of the salicylic acid data when plotted this way. The initial curvature can be attributed to the presence of surface drug and can be ignored. This same figure shows that the rate constant appears to remain relatively constant with a change in concentration of the drug in the tablet; this fact is in agreement with a first-order analysis.

Since both first-order and square root of time plots are acceptably linear, a more stringent test was developed to distinguish between the mechanisms. The use of the predicted rate equations corresponding to Eqs. 1 and 2 can be used for this purpose as shown by the following treatment.

Equation 1 can be reduced to the following:

$$Q' = KSt^{1/2} \qquad \text{(Eq. 3)}$$

where Q' = amount released = $Q \times S$,
S = surface area of tablet,
$K = \left[\frac{D\epsilon}{\tau}(2A - \epsilon C_s)\, C_s\right]^{1/2}$

By differentiation of the above reduced equation and appropriate substitution, Eq. 4 can be obtained:

$$\frac{dQ'}{dt} = \text{rate} = \frac{K^2S^2}{2Q'} \qquad \text{(Eq. 4)}$$

which indicates that the rate will be inversely proportional to the amount of drug released, Q'.

The rate predicted by first-order kinetics, however, is given by the following relationship:

$$\frac{dQ'}{dt} = \text{rate} = kW_0 - kQ' \qquad \text{(Eq. 5)}$$

since

$$-\frac{dW}{dt} = \frac{dQ'}{dt} = kW \qquad \text{(Eq. 6)}$$

[1] Propylene glycol monostearate, Glyco Chemicals, Inc.

[2] Castorwax, Baker Castor Oil Co.

TABLE I—VALUES OF PARAMETERS FOR THE RELEASE OF SALICYLIC AND BENZOIC ACID FROM A PGMS–HYDROGENATED CASTOR OIL MATRIX IN WATER

Drug	w/w, %	ϵ	A (mg./ml.)	Slope of Q vs. $t^{1/2}$ Plot	τ
Salicylic acid	5	0.11212	47.47	0.220	16.9
$D = 3.852 \times 10^{-2}$ cm.²/hr.	10	0.12087	99.03	0.380	12.8
$C_s = 2.0$ mg./ml.	20	0.20352	201.70	0.640	15.9
Benzoic acid	5	0.09040	48.27	0.278	18.9
$D = 3.96 \times 10^{-2}$ cm.²/hr.	10	0.11745	99.33	0.513	14.7
$C_s = 4.2$ mg./ml.	20	0.19218	204.64	0.870	17.2

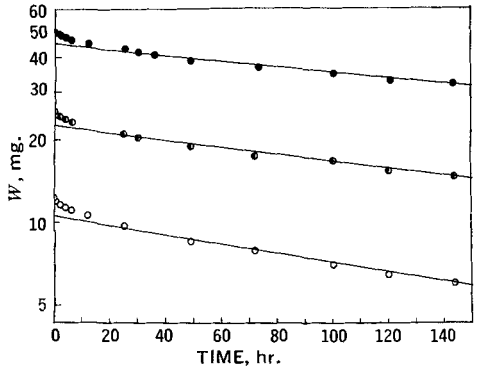

Fig. 3—Release profiles of various concentrations of salicylic acid when plotted according to first-order kinetics. Key: ○, 5%; ◐, 10%; ●, 20%.

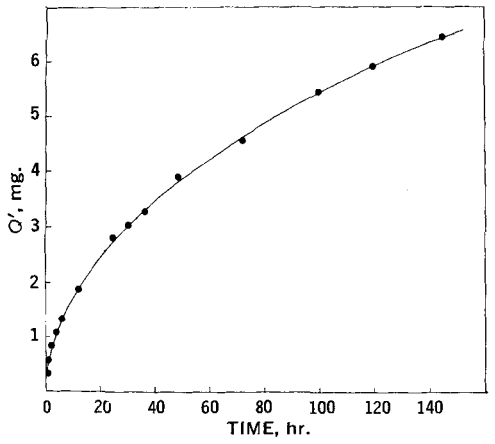

Fig. 4—Amount of drug released from the matrix as a function of time for 5% salicylic acid.

TABLE II—VALUES FOR RATE, AMOUNT RELEASED, AND THE CORRESPONDING RECIPROCAL FOR THE RELEASE OF 5% SALICYLIC ACID

Rate = dQ'/dt	Q'(mg.)	$1/Q'$(mg.)
0.3400	0.480	2.083
0.2250	0.700	1.429
0.1670	0.850	1.176
0.1185	1.105	0.905
0.0940	1.520	0.658
0.0650	2.350	0.426
0.0400	3.520	0.284
0.0290	4.670	0.214
0.0225	5.580	0.179
0.0215	6.180	0.162

and

$$W = W_0 - Q' \qquad \text{(Eq. 7)}$$

which indicates that the rate is proportional to Q' rather than inversely proportional as predicted by the diffusion model.

The rates of release were determined by obtaining slopes of a Q' *versus* time plot (see Fig. 4). These values are tabulated along with Q' and reciprocal Q' values in Table II.

By comparison of Eqs. 4 and 5, it is clear that the first predicts a linear plot when *rate* is plotted as a function of $1/Q'$ and that the second predicts a linear plot when *rate* is plotted as a function of Q'. Figures 5 and 6 show that the two mechanisms can indeed be differentiated by this rate treatment as only Fig. 5 is linear indicating a diffusion-controlled mechanism. In addition, it should be noted that the slope of the line in Fig. 5 was found to be equal to $K^2S^2/2$ as predicted by Eq. 4.

This conclusion can be further confirmed by the use of Eq. 8, which is obtained by taking logs of Eq. 3:

$$\log Q = \log K + {}^1/_2 \log t \qquad \text{(Eq. 8)}$$

This predicts that a plot of log Q *versus* log t must not only give a straight line, but must have a slope

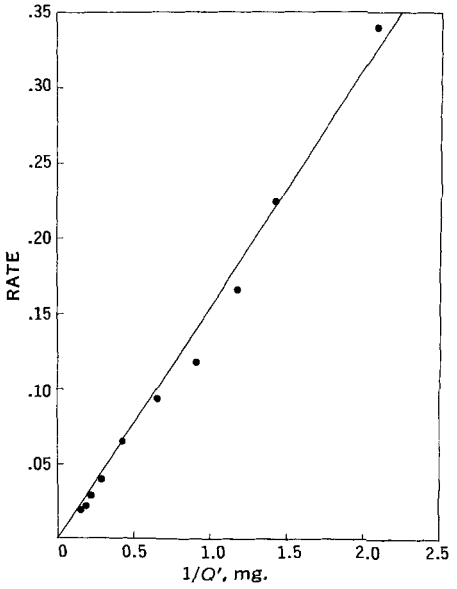

Fig. 5—Rate as a function of reciprocal drug released for 5% salicylic acid.

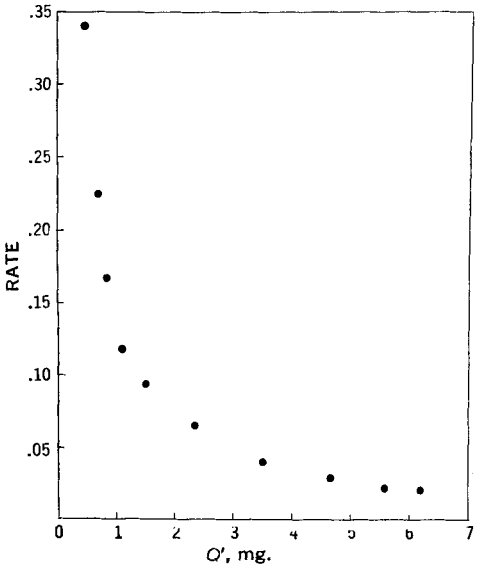

Fig. 6—Rate as a function of amount of drug released for 5% salicylic acid.

of $^1/_2$. This was found to be true and is illustrated by Fig. 7.

In addition, the various parameters for all systems were calculated by use of methods previously reported (6, 7) and are presented in Table I. The values for tortuosity, τ, are relatively constant and reasonable, as one would expect slightly higher τ values when the matrix components are relatively plastic and easily distorted.

CONCLUSIONS

Although the systems studied demonstrated apparently linear semilog plots with slopes that were apparently independent of the concentration of drug embedded in the wax matrix, more stringent tests clearly showed that the diffusion-controlled mechanism was operative and that a first-order release was incorrect.

It should be noted that one must use studies which are sufficiently long to properly evaluate matrix systems. First-order processes demand three or more half-life intervals, and the diffusion model requires an exponential increase in time with each successive sample due to the $t^{1/2}$ dependence. These studies utilized a minimum of 6 days and yet only 50 to 60% was released and can account for the linear semilog plots. This also explains the lack of significant curvature predicted in the latter points of the $t^{1/2}$ plots due to finite changes in the effective area of drug release of a whole tablet since these do not occur until greater than 60 to 70% is released.

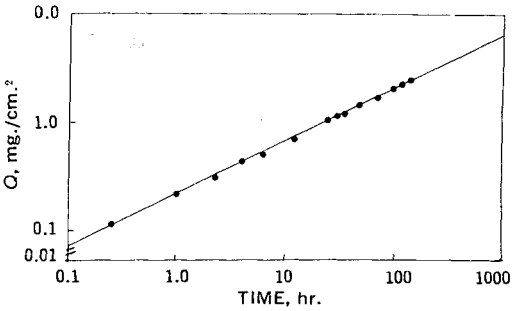

Fig. 7—Plot of the log of the amount of drug released per unit surface area against the log of time for 5% salicylic acid.

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Keyphrases

Drug release
Matrices–wax
Diffusion controlled model
UV spectrophotometry–analysis

Drug Release from Wax Matrices II

Application of a Mixture Theory to the Sulfanilamide–Wax System

By JOSEPH B. SCHWARTZ, ANTHONY P. SIMONELLI, and WILLIAM I. HIGUCHI

Using the T. Higuchi relationship for the release of a drug from a solid matrix, a hydrogenated castor oil–propylene glycol monostearate–sulfanilamide system has been investigated. Plots of the amount of drug released per unit of tablet surface area exposed as a function of the square root of time enabled comparison of the release for various concentrations of drug, and the calculation of the apparent tortuosity values. A model based on the Bruggeman mixture relationship for a two-phase system has been used and equations were derived for the prediction of theoretical tortuosity values as a function of porosity due to drug. These theoretical values for tortuosity appear to follow a skewed convex parabolic function. The experimental results obtained compared well with the theoretical predictions.

THE APPLICABILITY of applying the basic T. Higuchi relationship (2) for systems incorporating drug in a wax matrix has been previously considered (1). This relationship is given by:

$$Q = \sqrt{\frac{D\epsilon}{\tau}(2A - \epsilon C_s)\,C_s t} \qquad \text{(Eq. 1)}$$

where Q is the amount of drug released per unit area of the tablet exposed to the solvent; D is the diffusion coefficient of the drug in the solvent; ϵ is the porosity of the matrix; τ is the tortuosity of the matrix; A is the concentration of solid drug in the matrix; C_s is the solubility of the drug in the solvent; and t is time. Equation 1 predicts a linear relationship between Q and the square root of time. Each of the above variables may be determined experimentally, and these have been reported for two drug–wax systems (1).

In the present communication, a drug–wax system is described in which the drug release behavior is relatively complex. Over one range of drug–wax composition the rate appears to be diffusion controlled through the aqueous pores, but the tortuosity values are extremely high. In the second range (high drug concentrations in the tablet) the mechanism appears to change to one in which the rate is not solute diffusion controlled in the aqueous pores. A quantitative physical model is presented that appears to satisfactorily describe the data in the low drug concentration range.

THEORY

Before a theory can be developed, it is essential to first consider the various possible physical arrangements of drug and matrix and their corresponding tortuosity values. If the matrix is composed of nearly spherical particles, such as one would have in a bed of spheres, the τ values should range between about 1.5 to 3 (2, 3). On the other hand, if the matrix material is less spherical, either because the initial particles are nonspherical or because during preparation of the matrix the particles become distorted (4), then greater τ values would be expected. However, even under extreme conditions, it is difficult to imagine τ values much greater than 10 or 20 when the simple concept (2, 3) underlying the meaning of tortuosity is used.

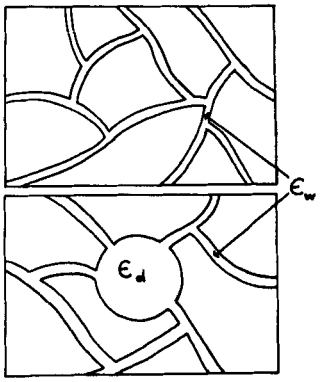

Fig. 1—Schematic diagram of proposed tablet model with air channels through the wax represented by ϵ_w and drug represented by ϵ_d.

As will be seen later, apparent τ values greater than 1,000 have been observed with compressed tablets made of drug–wax combinations. In order to explain such results the following model is proposed.

It is assumed that the drug particles are isolated in a sea of wax. This should be a reasonable approximation at low drug concentrations, since most waxes are extremely soft (plastic) and should flow around the drug particles during compression. Figure 1 schematically illustrates this model. The important feature of this situation is that diffusion is most difficult in the wax regions connecting the pores (left behind after the drug is leached) rather than in the pores themselves as might be generally assumed (2, 5). Thus the permeability of the drug is largely determined by the permeability characteristics of the wax matrix itself.

One equation which gives the permeability char-

Received April 14, 1967, from the College of Pharmacy, University of Michigan, Ann Arbor, MI 48104
Accepted for publication September 12, 1967.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
Previous paper: Schwartz, J. B., Simonelli, A. P., and Higuchi, W. I., *J. Pharm. Sci.*, **57**, 274 (1968).

acteristics of a two-phase system is the Bruggeman equation (3, 6).

$$\frac{P_2 - P_e}{P_2 - P_1} = V_1 \left(\frac{P_e}{P_1}\right)^{1/3} \quad \text{(Eq. 2)}$$

where P = permeability,
P_e = permeability of the total system,
V = volume fraction,
1 = external phase,
2 = internal phase.

In a diffusion-controlled system, permeability may be defined as:

$$P = \frac{D\epsilon}{\tau} = KD \quad \text{(Eq. 3)}$$

where $K = \epsilon/\tau$ and where all symbols have been previously defined and all are effective quantities.

The Bruggeman equation (Eq. 2) may be applied to the present system by regarding the matrix "phase" as the external phase. Then the pores left by the leached drug in the tablet constitute the internal phase of the system.

The following relationships are then known for the system:

$$D_2 = D_s \quad \text{(Eq. 4)}$$
$$\therefore P_2 = D_s$$
$$V_1 = (1 - \epsilon_d)$$

where D_2 = diffusion coefficient of internal phase,
D_s = diffusion coefficient of the drug in the solvent,
P_2 = permeability of the internal phase,
V_1 = volume fraction of the external phase,
ϵ_d = porosity due to drug.

Substituting into Eq. 2:

$$\frac{D_s - P_e}{D_s - P_1} = (1 - \epsilon_d)\left(\frac{P_e}{P_1}\right)^{1/3} \quad \text{(Eq. 5)}$$

From Eq. 3 the following relationships may be obtained:

$$P_e = \left(\frac{\epsilon_d + \epsilon_w}{\tau}\right) D_s \quad \text{(Eq. 6)}$$

Table I—Parameters[a] for Sulfanilamide–PGMS–Hydrogenated Castor Oil System

w/w% Drug	% Released by 144 hr.	Slope of Q vs. $t^{1/3}$ Plot	ϵ_d	τ
1	26.02	0.0210	.006	985
2	19.77	0.0357	.013	698
5	15.02	0.0667	.033	707
8.33	12.23	0.0830	.055	1073
10	11.01	0.0863	.067	1287
15	10.83	0.1223	.102	1343
20	11.00	0.1640	.139	1286
25	10.43	0.1910	.176	1444
30	13.87	0.2883	.213	921
35	11.88	0.3200	.257	1030
50	16.62	0.6053	.385	602
60	22.79	1.088	.489	292
70	31.50	1.833	.650	176

[a] Average values for a minimum of three runs.

since the total porosity of the system, ϵ, is composed of that porosity due to drug, ϵ_d, plus that due to air, ϵ_w; and τ is the apparent overall tortuosity.

$$P_1 = \left(\frac{\epsilon_w}{\tau_w}\right) D_s \quad \text{(Eq. 7)}$$

$$\frac{D_s - \frac{(\epsilon_d + \epsilon_w)}{\tau} D_s}{D_s - \left(\frac{\epsilon_w}{\tau_w}\right) D_s} = (1 - \epsilon_d)\left[\frac{\left(\frac{\epsilon_d + \epsilon_w}{\tau}\right) D_s}{\left(\frac{\epsilon_w}{\tau_w}\right) D_s}\right]^{1/3} \quad \text{(Eq. 8)}$$

$$\frac{1 - \left(\frac{\epsilon_d + \epsilon_w}{\tau}\right)}{\left(\frac{\epsilon_d + \epsilon_w}{\tau}\right)^{1/3}} = (1 - \epsilon_d)\frac{\left(1 - \frac{\epsilon_w}{\tau_w}\right)}{\left(\frac{\epsilon_w}{\tau_w}\right)^{1/3}} \quad \text{(Eq. 9)}$$

Letting

$$\theta = \frac{\left(1 - \frac{\epsilon_w}{\tau_w}\right)}{\left(\frac{\epsilon_w}{\tau_w}\right)^{1/3}}$$

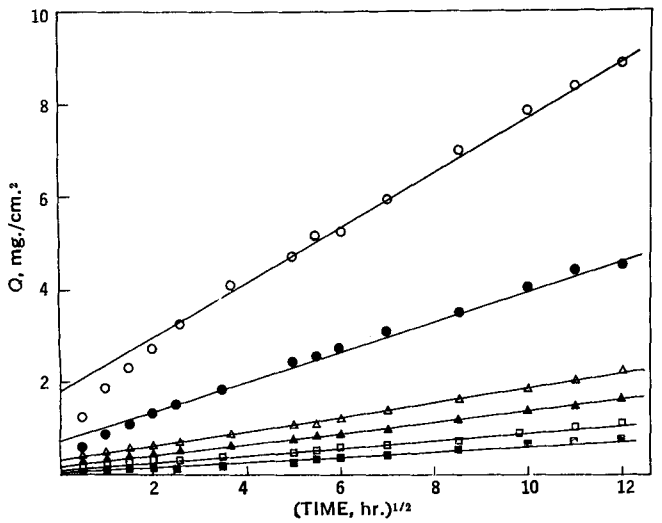

Fig. 2—Q versus $t^{1/2}$ plots for tablets with varying concentrations of drug released in distilled water. Key: ■, 5%; □, 10%; ▲, 15%; △, 20%; ●, 35%; ○, 50%.

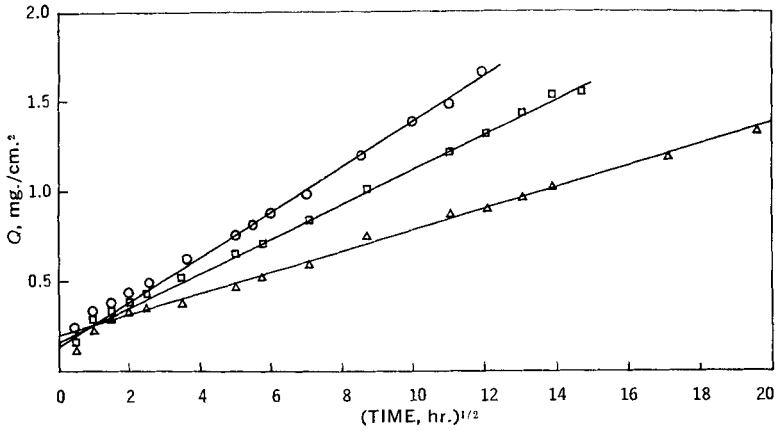

Fig. 3—Comparison of released profiles for 15% sulfanilamide released in different solvents. Key: ○, distilled water; □, 0.5 M NaCl; △, 5.0 M NaCl.

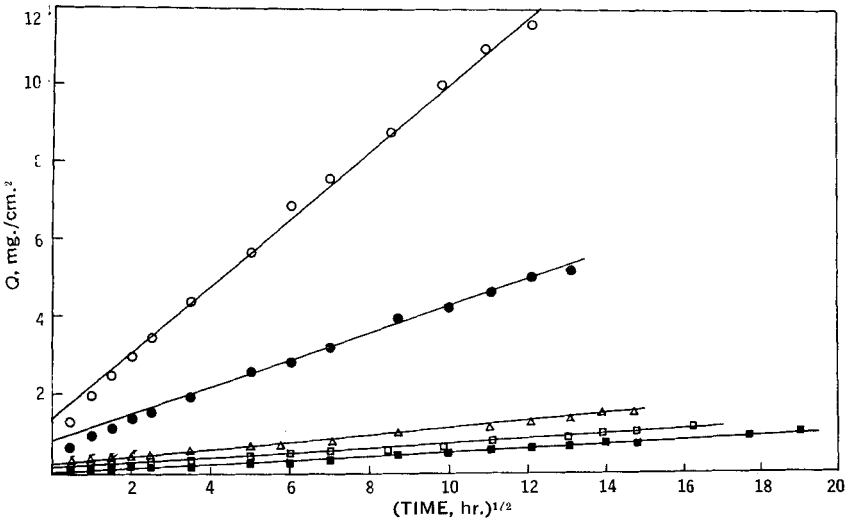

Fig. 4—Q versus $t^{1/2}$ plots for tablets with varying concentrations of drug released in 0.5 M NaCl. Key: ■, 5%; □, 10%; △, 15%; ●, 35%; ○, 50%.

$$\frac{[\tau - (\epsilon_d + \epsilon_w)]\tau^{1/3}}{\tau(\epsilon_d + \epsilon_w)^{1/3}} = (1 - \epsilon_d)\,\theta \quad \text{(Eq. 10)}$$

$$\tau^{2/3}[\tau - (\epsilon_d + \epsilon_w)] = \theta\,(1 - \epsilon_d)(\epsilon_d + \epsilon_w)^{1/3} \quad \text{(Eq. 11)}$$

If it is assumed that $\tau \gg \epsilon_d + \epsilon_w$, then the following relationship holds:

$$\tau^{1/3} = \theta\,(1 - \epsilon_d)(\epsilon_d + \epsilon_w)^{1/3} \quad \text{(Eq. 12)}$$

$$\tau = \theta^3\,(1 - \epsilon_d)^3(\epsilon_d + \epsilon_w) \quad \text{(Eq. 13)}$$

Thus, the apparent tortuosity of the system is given theoretically as a function of the permeability factors of the matrix alone, θ; the porosity due to drug, ϵ_d; and the porosity due to air, ϵ_w. It should be noted that the apparent tortuosity is independent of the diffusion coefficient of the drug in the solvent.

EXPERIMENTAL

The procedure used for these experiments is essentially the same as that presented in a previous paper in this series (1). The same components, PGMS[1] and hydrogenated castor oil,[2] were used as the matrix material and in the same 1:1 ratio. The drug used in this study was sulfanilamide which was spectrophotometrically assayed in the UV region. In addition to the usual solvent, distilled water, varying concentrations of sodium chloride solution were used to test the C_s effect.

RESULTS AND DISCUSSION

Figure 2 shows the release profiles of the tablets in water for various concentrations of sulfanilamide as a function of the square root of time. It is noted that the initial points appear to deviate from linearity, and we have tentatively attributed this to the release of surface drug before the matrix diffusion model takes control. To analyze the data, the best straight lines were drawn through the points after $t^{1/2} = 3$. The experiments had been carried out for much longer periods of time. Based on the observed slopes, the apparent values for tortuosity, τ, were calculated according to Eq. 1, these are listed in Table I along with several other parameters for the system.

Because of the surprisingly high values obtained for τ, it was decided to see whether diffusion in the aqueous phase was rate determining. This was done by changing the C_s factor (see Eq. 1). Tablets were run in NaCl solutions instead of water; because of the salting out effect, the solubility of sulfanilamide

[1] Propylene glycol monostearate, Glyco Chemicals Inc.
[2] Castorwax, Baker Castor Oil Co.

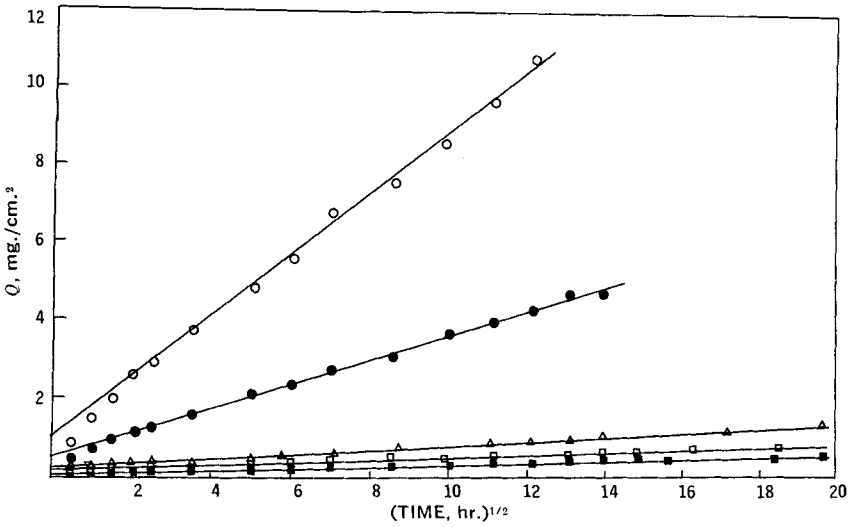

Fig. 5—Q versus $t^{1/2}$ *plots for tablets with varying concentrations of drug released in 5.0 M NaCl. Key:* ■, *5%;* □, *10%;* △, *15%;* ●, *35%;* ○, *50%.*

TABLE II—TEST FOR DIFFUSION-CONTROLLED MODEL SULFANILAMIDE-PGMS-HYDROGENATED CASTOR OIL

w/w%	Solvent	C_s (mg./ml.)	η[a]	Slope	Ratio to H_2O	Predicted[b] Ratio
5	H_2O	10.70	1	0.06667	1	1
	0.5 *M* NaCl	9.92	1.05	0.05300	0.7949	0.9396
	5.0 *M* NaCl	5.46	1.70	0.02500	0.3749	0.5479
10	H_2O	10.70	1	0.08633	1	1
	0.5 *M* NaCl	9.92	1.05	0.06900	0.7992	0.9396
	5.0 *M* NaCl	5.46	1.70	0.03410	0.3950	0.5479
15	H_2O	10.70	1	0.12233	1	1
	0.5 *M* NaCl	9.92	1.05	0.09500	0.7765	0.9396
	5.0 *M* NaCl	5.46	1.70	0 05667	0.4632	0.5479

[a] Compared to water = unity. [b] On basis of η and C_s.

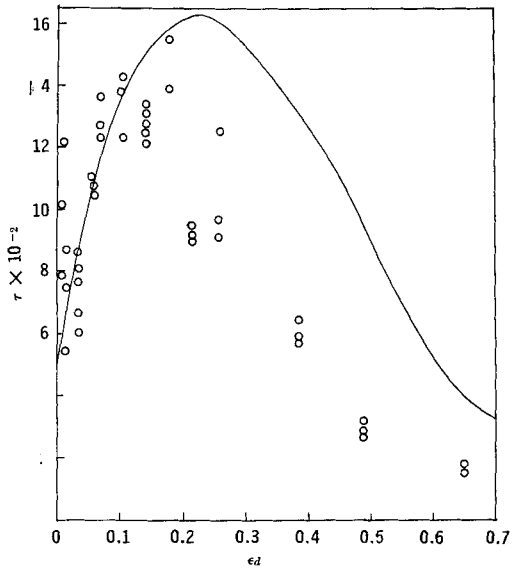

Fig. 6—Comparison of experimental values with the theoretical curve obtained by applying the Bruggeman theory.

is reduced appreciably by high concentrations of NaCl The reduced solubility was expected to reduce the slope of the *Q versus* $t^{1/2}$ plots in accordance with the predictions of Eq. 1. As can be seen in Fig. 3 and also by comparing Fig. 2 with Figs. 4 and 5, this effect was seen for those experiments at low drug concentrations.

The salt effects were not observed, however, with the tablets containing high concentrations ($\gtrsim$35%) of drug. This suggests a basic change in the mechanism of release at the high drug concentrations. Thus the proposed model would be expected to hold only at drug concentrations of less than about 35%.

Table II shows the slopes obtained in the various solvents and the slopes normalized to the distilled water values. According to the Stokes-Einstein equation, the diffusion coefficient, D, is affected by the viscosity of the solvent. Therefore, both the effect of the salt upon D and the effect upon C_s were taken into account in Table II. While the agreement is not exactly quantitative, the comparison strongly supports the diffusion-controlled concept for the low drug concentration cases.

Figure 6 shows the comparison of the experimental τ values with the Bruggeman relationship (Eq. 13). An experimentally determined (1) value for ϵ_w = .0374 was used and a τ_w value of 500 was used to give the fit as shown. A different choice of τ_w would only raise or lower the theoretical curve in Fig. 6; the shape would remain unchanged. Differentiation with respect to ϵ_d and proper analysis of Eq. 13 reveals that the maximum, calculated at 0.222, is independent of τ_w. Treatment of the second derivative, moreover, showed an inflection point at ϵ_d = 0.481 which gives the theoretical curve (see Fig. 6) a skewed convex parabolic shape.

The good agreement of the data with the theory at the lower ($\lesssim$20%) drug concentrations strongly

supports the proposed model in this concentration range. The large deviations from the theory at high drug concentration are consistent with the idea that a more rapid process takes over that does not appear to be aqueous diffusion controlled. More work is needed to explain the latter behavior.

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Keyphrases

Drug release
Matrices–wax
Sulfanilamide–wax system
Tortuosity values–apparent
UV spectrophotometry–analysis

Alpha-Chymotrypsin-Catalyzed Hydrolysis of Some Carbonate Esters

By ATUL A. SHAH* and KENNETH A. CONNORS

The kinetics of the α-chymotrypsin-catalyzed hydrolysis of several carbonate diesters, ROCOOR′, can be described by the Michaelis-Menten equation. *p*-Acetamidophenyltrichloroethyl carbonate shows a sigmoid pH-rate curve with a kinetic dependence on an enzyme function of pKa 7.2. The limiting values of the catalytic rate constant at high pH are, for *p*-acetamidophenyltrichloroethyl carbonate, 8.0×10^{-2} sec.$^{-1}$; for *p*-nitrophenyltrichloroethyl carbonate, 8.2×10^{-2} sec.$^{-1}$; for *p*-nitrophenylethyl carbonate, 0.2×10^{-2} sec.$^{-1}$. The evidence is consistent with the intermediate formation of an alkyl enzyme carbonate, the hydrolysis of which is rate controlling.

CARBONATE DIESTERS are derivatives of carbonic acid, the two hydrogens of the acid being formally replaced by alkyl or aryl groups to give the general structure ROCOOR′. The suggestion has recently been made (1) that such esters may be useful "prodrugs" because they may possess desirable pharmaceutical properties and can release the parent pharmacologically active compounds (ROH and R′OH) upon hydrolysis in the body. The mechanism of enzymatic hydrolysis of carbonate esters is therefore of pharmaceutical interest.

The enzymatic hydrolysis of carbonate esters is also of considerable fundamental importance. α-Chymotrypsin has been selected as the enzyme in the present study because it is commercially available in crystalline form, a good assay method is known, and especially because more is known about the catalytic behavior of chymotrypsin than of any other enzyme. Chymotrypsin is a member of a group of related enzymes known as the serine hydrolases; these enzymes catalyze the hydrolysis of proteins, peptides, amides, and esters. It is now well established that chymotrypsin (and probably other closely related enzymes) functions catalytically by a double displacement type of reaction. With an ester substrate, for example, the reaction may be written as shown in Scheme I.

$$\text{En—OH} + \text{RCOOR}' \rightleftarrows \text{En—OH}\cdot\text{RCOOR}'$$

$$\text{En—OH}\cdot\text{RCOOR}' \xrightarrow{\text{acylation}} \text{EnOCOR} + \text{R}'\text{OH}$$

$$\text{EnOCOR} + \text{H}_2\text{O} \xrightarrow{\text{deacylation}} \text{En—OH} + \text{RCOOH}$$

Scheme I

Here En—OH represents the enzyme, with a serine hydroxyl group specifically indicated. En—OH · RCOOR′ is the enzyme–substrate complex. In the acylation step, the enzyme is acylated with the release of the alcohol portion of the substrate. Deacylation involves hydrolysis of the acyl–enzyme intermediate EnOCOR to give the carboxylic acid portion of the substrate and to regenerate the enzyme. The evidence supporting this interpretation of chymotrypsin-catalyzed reactions has recently been reviewed by Bruice and Benkovic (2). It is now of interest to consider the possible mode of action of chymotrypsin when the substrate is a carbonate ester, ROCOOR′. It is obvious that two possible

Received August 2, 1967, from the School of Pharmacy, University of Wisconsin, Madison, WI 53706
Accepted for publication October 5, 1967.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
Supported by a grant from Smith Kline & French Laboratories, Philadelphia, Pa.
Valuable discussions with Dr. Joseph Swintosky and Dr. Lewis Dittert are gratefully acknowledged.
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By ATUL A. SHAH* and KENNETH A. CONNORS

The kinetics of the α-chymotrypsin-catalyzed hydrolysis of several carbonate diesters, ROCOOR′, can be described by the Michaelis-Menten equation. *p*-Acetamidophenyltrichloroethyl carbonate shows a sigmoid pH-rate curve with a kinetic dependence on an enzyme function of pKa 7.2. The limiting values of the catalytic rate constant at high pH are, for *p*-acetamidophenyltrichloroethyl carbonate, 8.0×10^{-2} sec.$^{-1}$; for *p*-nitrophenyltrichloroethyl carbonate, 8.2×10^{-2} sec.$^{-1}$; for *p*-nitrophenylethyl carbonate, 0.2×10^{-2} sec.$^{-1}$. The evidence is consistent with the intermediate formation of an alkyl enzyme carbonate, the hydrolysis of which is rate controlling.

CARBONATE DIESTERS are derivatives of carbonic acid, the two hydrogens of the acid being formally replaced by alkyl or aryl groups to give the general structure ROCOOR′. The suggestion has recently been made (1) that such esters may be useful "prodrugs" because they may possess desirable pharmaceutical properties and can release the parent pharmacologically active compounds (ROH and R′OH) upon hydrolysis in the body. The mechanism of enzymatic hydrolysis of carbonate esters is therefore of pharmaceutical interest.

The enzymatic hydrolysis of carbonate esters is also of considerable fundamental importance. α-Chymotrypsin has been selected as the enzyme in the present study because it is commercially available in crystalline form, a good assay method is known, and especially because more is known about the catalytic behavior of chymotrypsin than of any other enzyme. Chymotrypsin is a member of a group of related enzymes known as the serine hydrolases; these enzymes catalyze the hydrolysis of proteins, peptides, amides, and esters. It is now well established that chymotrypsin (and probably other closely related enzymes) functions catalytically by a double displacement type of reaction. With an ester substrate, for example, the reaction may be written as shown in Scheme I.

$$\text{En—OH} + \text{RCOOR}' \rightleftarrows \text{En—OH}\cdot\text{RCOOR}'$$

$$\text{En—OH}\cdot\text{RCOOR}' \xrightarrow{\text{acylation}} \text{EnOCOR} + \text{R}'\text{OH}$$

$$\text{EnOCOR} + \text{H}_2\text{O} \xrightarrow{\text{deacylation}} \text{En—OH} + \text{RCOOH}$$

Scheme I

Here En—OH represents the enzyme, with a serine hydroxyl group specifically indicated. En—OH · RCOOR′ is the enzyme–substrate complex. In the acylation step, the enzyme is acylated with the release of the alcohol portion of the substrate. Deacylation involves hydrolysis of the acyl–enzyme intermediate EnOCOR to give the carboxylic acid portion of the substrate and to regenerate the enzyme. The evidence supporting this interpretation of chymotrypsin-catalyzed reactions has recently been reviewed by Bruice and Benkovic (2). It is now of interest to consider the possible mode of action of chymotrypsin when the substrate is a carbonate ester, ROCOOR′. It is obvious that two possible

Received August 2, 1967, from the School of Pharmacy, University of Wisconsin, Madison, WI 53706
Accepted for publication October 5, 1967.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
Supported by a grant from Smith Kline & French Laboratories, Philadelphia, Pa.
Valuable discussions with Dr. Joseph Swintosky and Dr. Lewis Dittert are gratefully acknowledged.
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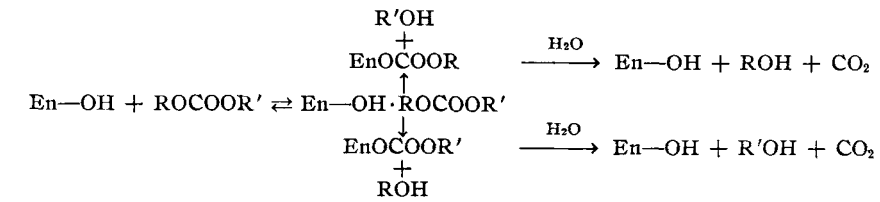


Scheme II

"acyl–enzymes" can be formed from this substrate. The simplest representation of the reaction may therefore be written as shown in Scheme II.

The deacylation must be more complicated than is implied by Scheme II. The relative rates of formation of the two possible intermediates and their rates of hydrolysis will depend upon the structures R and R'. Structural variation in the substrate should therefore provide useful information on the mechanism of this reaction.

EXPERIMENTAL

Materials—*N-trans*-Cinnamoylimidazole was prepared according to Schonbaum, Zerner, and Bender (3). It was recrystallized twice from cyclohexane, m.p. 133°. [Lit. (3) m.p. 133–135°.] *p*-Nitrophenyl acetate (*p*-NPA) was prepared by the method of Chattaway (4). The crude product was recrystallized twice from Skellysolve B, m.p. 80°. [Lit. (5) m.p. 80°.] *p*-Acetamidophenyl-2,2,2-trichloroethyl carbonate (1) (*p*-APTC), m.p. 153°; *p*-acetaminophenol, m.p. 169–170°; these products were generously supplied by Smith Kline & French Laboratories. *p*-Nitrophenylethyl carbonate (*p*-NPEC): 2.8 Gm. (0.02 mole) of *p*-nitrophenol was dissolved in 20 ml. of pyridine. The solution was cooled in an ice bath and with continuous stirring 2.5 ml. (0.025 mole) of ethyl chloroformate (Aldrich Chemical Co.) was added dropwise. The mixture was allowed to stand 15 min. The pyridine was removed by evaporation under reduced pressure. The oily brown residue was recrystallized twice from methanol–water, m.p. 66°. [Lit. (5) m.p. 67–68°.]

p-Nitrophenyl-2,2,2-trichloroethyl carbonate (*p*-NPTC): to a solution of 120 ml. of toluene and 8.2 ml. of pyridine, cooled to −10°, was added 43 ml. (3.5 mole) of phosgene solution in toluene during about 90 min. Cooling was effected in dry ice–isopropyl alcohol. The reaction mixture was allowed to reach 0°, at which time the addition of 15 ml. (0.1 mole) of trichloroethanol was initiated. This addition required 40 min., and the final temperature was 10°. The mixture was stirred at room temperature for 2 hr. and then 30 ml. of cold water was cautiously added. Considerable CO_2 gas was evolved. The solution was washed with four more 30-ml. portions of water and dried over anhydrous magnesium sulfate. The dry solution was filtered.

Seventy-five milliliters of filtrate was added to a mixture of 4.5 ml. of pyridine, 30 ml. of toluene, and 7.5 Gm. of *p*-nitrophenol; the addition required 1 hr. The mixture was stirred for 90 min. and 0.9 ml. of pyridine was added, followed by 0.5 ml. of pyridine 30 min. later. The mixture was then cooled to 5°, 75 ml. of water was added, and it was stirred for 15 min. The toluene layer was separated and dried over magnesium sulfate. The solvent was removed by reduced pressure and the yellow residue was recrystallized three times from Skellysolve B. Pale yellow needles were obtained, m.p. 58°.

Anal.—Calcd. for $C_9H_6Cl_3NO_5$: C, 34.34; H, 1.91, Cl, 34.97; N, 4.45. Found: C, 34.31; H, 1.85; Cl, 34.85; N, 4.44.

The infrared spectrum showed peaks at 5.65 μ (OCOO), 6.2 and 6.4 μ (aromatic), 8.2 μ (COOR), 9.9, 10.5, and 12.3 μ (*p*-disubstituted phenyl ring), 13 μ (C—Cl), 6.6 and 7.4 μ (aromatic nitro). The nuclear magnetic resonance spectrum yielded τ 5.05 (methyl protons) and 2.5 and 1.67 (aromatic protons). The integrated spectrum gave 1:1:1 ratio for the three types of protons.

α-Chymotrypsin, three times recrystallized, was a Worthington product. The purity of the Worthington chymotrypsin was consistently found to be 84–86% by titration against *N-trans*-cinnamoylimidazole, following the procedure of Schonbaum *et al.* (3).

Acetonitrile (Eastman Kodak technical grade) was refluxed over phosphorus pentoxide and distilled twice from P_2O_5, b.p. 81°. Tris(hydroxymethyl)aminomethane (Tris) (Sigma Chemical Co.) was recrystallized from 95% ethanol. Pentaerythritol (Aldrich Chemical Co.) and *n*-butyl alcohol (Mallinckrodt A.R.) were used directly. Buffer chemicals were reagent grade. All water was redistilled from alkaline permanganate in an all-glass still. Buffers were prepared according to published formulas (6, 7). Ionic strength was maintained constant by addition of potassium chloride.

Apparatus—pH was measured with a Radiometer model 25 SE pH meter or with a Sargent model DR pH meter. The meters were standardized against the standard buffers recommended by Bates (8).

Spectral measurements were made with a Cary model 14 recording spectrophotometer equipped with a thermostated cell compartment that maintained temperature to ±0.1°. The absorbance scale was checked against the alkaline chromate solution described by Haupt (9).

Procedures—Reactions were followed by continuous spectrophotometric monitoring at a constant wavelength, which was 240 mμ for *p*-APTC and 400 mμ for *p*-NPA, *p*-NPEC, and *p*-NPTC. The temperature was 25 ± 0.1° in all experiments. Ionic strength was 0.2 *M*, and pH was held constant with buffers. The substrate concentration was much larger than the enzyme concentration. The analysis of the operational normality of the chymotrypsin was made during the same time period as the kinetic measurements.

A typical experiment was conducted as follows. One-centimeter cells, each containing 3.0 ml. of

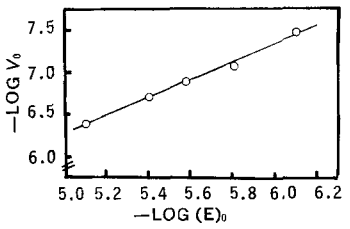


Fig. 1—Plot of $-\log v_0$ against $-\log [E]_0$ for the chymotrypsin-catalyzed hydrolysis of p-*APTC; pH = 7.54, $[S] = 1.13 \times 10^{-4}$ M, $[E]_0 = 7.8 \times 10^{-7}$ to 7.8×10^{-6} M.*

buffer solution, were placed in the reference and sample compartments of the spectrophotometer and were allowed to reach temperature equilibrium. The spectrophotometer signal was balanced and then 50 μl. of substrate stock solution (in acetonitrile) was introduced to the sample cell by means of a glass stirring rod. This was followed by 50 μl. of aqueous enzyme solution. Recording was initiated within 5–10 sec. When rates of nonenzymatic hydrolysis were significant, the enzyme solution was added prior to the substrate. Net enzymatic initial velocities were obtained by subtracting nonenzymatic initial velocities from total initial velocities.

Initial velocities were obtained from the initial zero-order portion of the absorbance-time tracing. The conversion of this quantity, $v_0' = dA/dt$ (initial velocity in absorbance units per second) to $v_0 = dc/dt$ (initial velocity in moles per liter per second) is readily accomplished through application of Beer's law to the substrate and product; the equation obtained is:

$$v_0 = v_0'/b(\epsilon_S - \epsilon_P)$$

where b is the path length, ϵ_S is the molar absorptivity of the substrate, and ϵ_P is the molar absorptivity of the product.

Beer's law is followed, at constant pH, by all substrates and products studied. The molar absorptivity of *p*-APTC at 240 mμ was 1.46×10^4 in the pH range 6.5 to 10.0. For *p*-nitrophenol the molar absorptivity at 400 mμ was 9.52×10^3, 1.75×10^4, and 1.90×10^4 at pH 7.02, 7.84, and 8.91, respectively.

RESULTS[1]

Order with Respect to Enzyme—Because carbonate esters are unusual substrates for chymotrypsin, it was considered important to investigate the validity of the usual rate relationships. When the logarithm of the initial velocity of the chymotrypsin-catalyzed hydrolysis of *p*-APTC was plotted against the logarithm of the enzyme concentration at constant substrate concentration, straight lines of unit slope were obtained. Figure 1 and Table I show these results. It can be concluded that under these conditions the chymotrypsin-catalyzed hydrolysis of *p*-APTC is first order with respect to the enzyme.

Stoichiometry—Hartley and Kilby (10, 11) studied the chymotrypsin-catalyzed hydrolysis of *p*-NPA and *p*-NPEC. They observed, with both of these substrates, an initial rapid release of *p*-nitrophenol (the "burst effect") followed by a slower, zero-order production of *p*-nitrophenol. These experiments have been repeated, with identical results. Extrapolation of the linear portions of the plot of *p*-nitrophenol concentration *versus* time to zero time gave finite intercepts. When these intercept values (expressed as molar concentration of product) are plotted against the molar concentration of enzyme, straight lines of unit slope are obtained (12). The stoichiometry of the reactions between chymotrypsin and *p*-NPA or *p*-NPEC is therefore 1:1.

The manifestation of the burst effect by *p*-NPEC is pertinent in the present study because it suggests similar mechanisms for the chymotrypsin-catalyzed hydrolyses of *p*-NPEC and *p*-NPA.

Kinetic Behavior—The adherence of carbonate ester systems to the Michaelis-Menten kinetic scheme was investigated. Plots of initial velocity against substrate concentration show the characteristic "saturation" effect (Fig. 2). Linear Lineweaver-Burk plots of $1/v_0$ *versus* $1/[S]$ were obtained, and it was from these plots that the kinetic parameters were determined. The kinetic scheme is:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} ES \xrightarrow{k_{cat.}} \text{products} \qquad \text{(Eq. 1)}$$

where ES is the enzyme–substrate complex. From the Lineweaver-Burk plots can be obtained the quantities $V_{max.}$ and $K_{m(app.)}$, where:

$$V_{max.} = k_{cat.}[E]_0 \qquad \text{(Eq. 2)}$$

$$K_{m(app.)} = \frac{k_{-1} + k_{cat.}}{k_{+1}} \qquad \text{(Eq. 3)}$$

Since the enzyme concentration is known, $k_{cat.}$ can be calculated.

Table II gives values of $k_{cat.}$ and $K_{m(app.)}$ for the chymotrypsin-catalyzed hydrolysis of *p*-APTC

TABLE I—ORDER WITH RESPECT TO ENZYME IN THE HYDROLYSIS OF *p*-APTC[a]

pH	Order (Slope)[b]	Order (Slope)[c]
7.06	1.01	0.93
7.54	1.06	1.03
8.15	0.99	1.01

[a] 25.0°; ionic strength = 0.20; 3.2% acetonitrile. [b] $[S]_0 = 1.13 \times 10^{-4}$ *M*; $[E]_0 = 7.8 \times 10^{-7}$ to 7.8×10^{-6} *M*. [c] $[S]_0 = 1.17 \times 10^{-4}$ *M*; $[E]_0 = 8.3 \times 10^{-7}$ to 8.3×10^{-6} *M*.

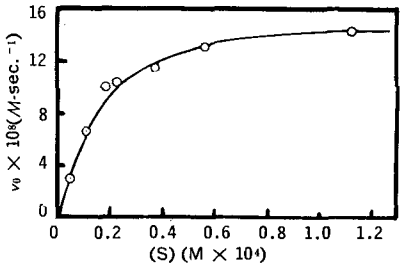


Fig. 2—Initial velocity against substrate concentration for the chymotrypsin-catalyzed hydrolysis of p-*APTC; pH = 7.05, $[E]_0 = 4.02 \times 10^{-6}$ M.*

[1] These abbreviations are used: *p*-acetamidophenyltrichloroethyl carbonate, *p*-APTC; *p*-nitrophenyl acetate, *p*-NPA; *p*-nitrophenylethyl carbonate, *p*-NPEC; *p*-nitrophenyltrichloroethyl carbonate, *p*-NPTC.

TABLE II—$k_{cat.}$ AND $K_{m(app.)}$ VALUES FOR THE CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF *p*-APTC[a]

pH	No. of Detns.	Buffer	$10^2 k_{cat.}$ (sec.$^{-1}$) Mean[b]	$10^2 k_{cat.}$ (sec.$^{-1}$) Range	$10^6 K_{m(app.)}$ (M) Mean[b]	$10^6 K_{m(app.)}$ (M) Range
6.57	1	Phosphate	1.8	—	12.5	—
6.85	2	Phosphate	3.3	0.2	9.8	1.6
7.05	2	Phosphate	3.5	0.4	11.0	0.5
7.48	2	Phosphate	5.0	0.8	13.1	—
7.79	1	Phosphate	7.8	—	—	—
8.11	5	Phosphate	7.9 (±0.8)	1.0	16.2 (±3.7)	8.5
8.11	1	Borate	7.5	—	18.6	—
8.25	1	Botate	7.0	—	13.9	—
8.36	3	Borate	7.5 (±0.6)	1.1	15.9 (±1.2)	1.5
8.49	3	Borate	6.8 (±0.4)	0.7	15.3 (±5.3)	10.5
8.75	3	Borate	7.7 (±0.4)	0.7	19.8 (±1.7)	3.1
8.95	3	Borate	8.1 (±0.3)	0.5	21.0 (±1.7)	3.2
9.17	3	Carbonate	7.5 (±0.4)	0.6	28.9 (±3.0)	5.7
9.34	1	Carbonate	6.8	—	28.0	—
9.80	1	Carbonate	6.4	—	—	—

[a] $[E]_0 = 4 \times 10^{-6} M$; $[S] = 10^{-5}$ to $10^{-4} M$; 1.6% acetonitrile. [b] Uncertainties listed are standard deviations.

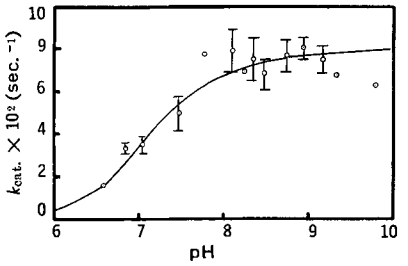


Fig. 3—pH-rate profile for the chymotrypsin-catalyzed hydrolysis of p-*APTC.*

over the pH range 6.5 to 9.8. The rate constant $k_{cat.}$ shows a sigmoid dependence on pH (the values above pH 9 are not considered as reliable as the others). If the enzyme is treated as a monobasic acid with characteristic dissociation constant, K_a, and the catalytic activity of the enzyme is assigned solely to its basic form, it is easily shown that $k_{cat.}$ is related to the pH through the equation:

$$k_{cat.} = \frac{k' K_a}{[H^+] + K_a} \qquad \text{(Eq. 4)}$$

where k' is a rate constant that is independent of pH. Figure 3 shows the data of Table II with a smooth curve calculated with Eq. 4 and the values pKa = 7.2 and $k' = 8.0 \times 10^{-2}$ sec.$^{-1}$.

The hydrolysis of *p*-NPEC and of *p*-NPTC were studied at several pH's. These results are given in Tables III and IV. It is seen that $k_{cat.}$ increases with pH in much the same manner as is observed with *p*-APTC. *p*-NPTC and *p*-APTC constitute an interesting pair of substrates because they contain the common trichloroethyl group. It is noted that the limiting value of $k_{cat.}$ (that is, the value at high pH) is 8.2×10^{-2} sec.$^{-1}$ for *p*-NPTC; this value is essentially the same as the limiting value of 8.0×10^{-2} sec.$^{-1}$ found for *p*-APTC. *p*-NPEC and *p*-NPTC are also a pair of substrates with a common group; the limiting rate constants are quite different for these compounds. The consequences of these comparisons will be discussed later.

Effects of Additives—During the pH-dependence study of the chymotrypsin–*p*-APTC system an unusual acceleration effect was observed in 0.05 M Tris buffers of pH 8 to 9. Values of $k_{cat.}$ were found to be about threefold greater in the presence of Tris than were values observed in other buffers. Figure 4 shows this effect; the constants observed in Tris buffers are plotted with the normal pH-rate profile. In attempting to account for this acceleration, some structurally related compounds were investigated as additives in the chymotrypsin–*p*-APTC system. The additives used were: (*a*) pH 8.15 ammonia buffer; (*b*) 0.15 M methanol; (*c*) pH 8.15 ammonia buffer and 0.15 M methanol; (*d*) pH 8.03 0.1 M glycine–NaOH buffer; (*e*) pH 8.0 0.1 M imidazole–HCl buffer; (*f*) 0.01 M pentaerythritol; (*g*) 0.15 M *n*-butyl alcohol. Only *n*-butyl alcohol caused an acceleration of the enzymatic reaction, but it was not nearly as effective as was Tris. Studies were carried out to evaluate $k_{cat.}$ and $K_{m(app.)}$ at several values of *n*-butyl alcohol concentration; both quantities increased with increase in the alcohol concentration (12). *p*-NPA was found to be a competitive inhibitor of the chymotrypsin-catalyzed hydrolysis of *p*-APTC. Since *p*-NPA is known to be

TABLE III—$k_{cat.}$ AND $K_{m(app.)}$ VALUES FOR THE CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF *p*-NPEC[a]

pH	Buffer	$10^3 k_{cat.}$ (sec.$^{-1}$)[b]	$10^6 K_{m(app.)}$ (M)
7.02	Phosphate	0.6	6.4
7.81	Phosphate	1.3	4.2
8.35	Borate	1.5	5.0
8.83	Borate	1.9	11.9

[a] $[E]_0 = 3.89 \times 10^{-6} M$; $[S] = 2.9 \times 10^{-6}$ to $1.4 \times 10^{-3} M$; 1.6% acetonitrile. [b] Literature value (10) is 1.31×10^{-3} sec.$^{-1}$ at pH 7.6 (turnover number = 0.078 mole *p*-NPEC/min./mole enzyme).

TABLE IV—$k_{cat.}$ AND $K_{m(app.)}$ VALUES FOR THE CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF *p*-NPTC[a]

pH	Buffer	$10^2 k_{cat.}$ (sec.$^{-1}$)	$10^7 K_{m(app.)}$ (M)
7.02	Phosphate	4.1	5.5
7.84	Borate	6.6	4.7
8.91	Borate	8.2	9.1

[a] $[E]_0 = 4.14 \times 10^{-7} M$; $[S] = 6.5 \times 10^{-7}$ to $4 \times 10^{-5} M$; 1.6% acetonitrile. The 0.0–0.1 absorbance slide wire of the spectrophotometer was used.

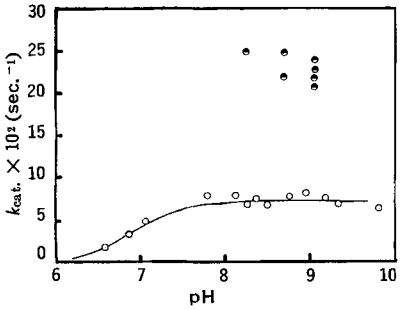


Fig. 4—Acceleratory effect of TRIS on the chymotrypsin-catalyzed hydrolysis of p-*APTC. Key:* ○, *values in phosphate, borate, and carbonate buffers;* ◒, *values in Tris buffers.*

a normal substrate of chymotrypsin, this result implies that the hydrolysis of *p*-APTC is also catalyzed in a normal manner.

DISCUSSION

The conventional Michaelis-Menten kinetic scheme is represented by Eq. 1. It has been shown above that the chymotrypsin-catalyzed hydrolysis of several carbonate diesters can be accounted for by this overall scheme, and the enzymatic behavior of these substrates is therefore normal. The relationship between initial velocity and substrate concentration is the well-known Michaelis-Menten equation, Eq. 5:

$$v_0 = \frac{k_{cat.}[S][E]_0}{K_{m(app.)} + [S]} \qquad \text{(Eq. 5)}$$

where $K_{m(app.)}$ is defined in Eq. 3.

If the reaction proceeds through an acyl–enzyme intermediate, as with ester and amide substrates of chymotrypsin, $k_{cat.}$ may be a complex constant. The reaction then becomes Scheme III.

It can be shown (2, 12, 13) that the $k_{cat.}$ of Eq. 1 is related to the constants of Scheme III by $k_{cat.} = k_{+2}k_{+3}/(k_{+2} + k_{+3})$, and the implications of this relationship for chymotrypsin reactions have been discussed in detail (14).

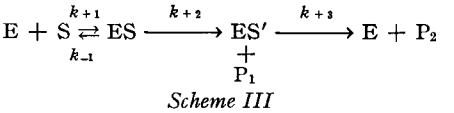
The situation is more complicated with carbonate diesters. Now the kinetic scheme is shown in Scheme IV.

Application of the steady-state approximation to ES, ES′, and ES″ (where ES′ and ES″ are the two possible "acyl–enzyme" intermediates) leads to an equation of the same form as Eq. 5, and comparison shows that the experimental $k_{cat.}$ can be expressed by Eq. 6:

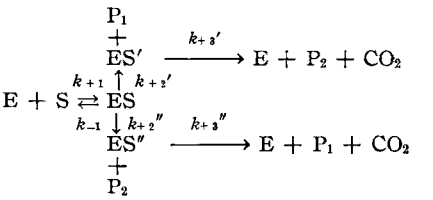
$$k_{cat.} = \frac{k'_{+3}k_{+3}''(k_{+2}' + k_{+2}'')}{k_{+2}'k_{+3}'' + k_{+2}''k_{+3}' + k_{+3}'k_{+3}''} \qquad \text{(Eq. 6)}$$

It is therefore clear that $k_{cat.}$ cannot, in general, be given a simple interpretation. If, however, one of the possible pathways in Scheme IV is greatly favored (*e.g.*, let $k_{+2}' \rangle\rangle\rangle k_{+2}''$), then Eq. 6 simplifies to $k_{cat.} = k_{+2}'k_{+3}'/(k_{+2}' + k_{+3}')$, which is identical with the corresponding equation for Scheme III. If this condition holds and, in addition, the deacylation of ES′ is rate determining (*i.e.*, $k_{+2}' \rangle\rangle\rangle k_{+3}'$) then $k_{cat.} = k_{+3}'$.

The similar behavior of *p*-NPA and *p*-NPEC suggests very strongly that the carbonate ester is



Scheme III



Scheme IV

TABLE V—LIMITING $k_{cat.}$ VALUES

Substrate	$10^2k_{cat.}$(sec.$^{-1}$)
p-APTC	8.0
p-NPTC	8.2
p-NPEC	0.2

hydrolyzed by the same overall reaction path as the carboxylic ester. Each substrate exhibits a rapid "burst" release of a stoichiometric amount of *p*-nitrophenol, which is followed by a steady-state zero-order production of *p*-nitrophenol. This behavior is consistent with Scheme III, where P_1 = *p*-nitrophenol and ES′ = $EnOCOCH_3$ (for *p*-NPA) or $EnOCOOCH_2CH_3$ (for *p*-NPEC).

The limiting $k_{cat.}$ values for *p*-APTC, *p*-NPTC, and *p*-NPEC provide the interesting comparison shown in Table V.

These relative rates can be accounted for with the assumptions that Scheme IV is the pertinent reaction scheme, that formation of the alkyl enzyme–carbonate is greatly favored over the aryl enzyme–carbonate, and that deacylation is rate controlling. Then, as shown above, $k_{cat.} = k_{+3}'$. The essential identity of $k_{cat.}$ for *p*-APTC and *p*-NPTC leads to these assumptions, for this identity is readily explicable on the basis that $k_{cat.}$ is a measure of the reaction rate of an intermediate common to the two substrates; this intermediate would be $EnOCOOCH_2CCl_3$. This interpretation is consistent with the fortyfold greater value of $k_{cat.}$ for *p*-NPTC relative to *p*-NPEC.

Because of the greater complexity of Scheme IV compared with Scheme III, the argument presented in the preceding paragraph, implicating a common intermediate, is not so compelling as it is for the carboxylic acid derivatives. However, the greatly different chemical natures of the R and R′ groups in these ROCOOR′ substrates probably favor a considerable disparity in the rate constants k_{+2}' and k_{+2}'' in Scheme IV. If R and R′ were similar (both aryl groups, for example) it is possible that very complex kinetics might be observed, and that $k_{cat.}$ could only be interpreted in terms of Eq. 6.

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Keyphrases

Carbonate ester hydrolysis
α-Chymotrypsin catalyzed hydrolysis
UV spectrophotmetry, continuous-analysis
Stoichiometry
pH-dependence of hydrolysis

Effect of Certain Drugs on Perfused Human Placenta VIII

Angiotensin-II Antagonists

By CHARLES O. WARD and RONALD F. GAUTIERI

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WHILE RESEARCH with angiotensin in the last two decades has dealt principally with its effects on innervated preparations, there still exists in the literature a paucity of information on the actions of this polypeptide in the more common nerve-free preparations, the perfused human placenta and umbilical cord. Eliasson and Astrom (1) could detect only moderate increases in perfusion pressure using angiotensin in the former preparation; others have reported little or no effect with angiotensin on either the perfused umbilical cord (2) or helical strips of both the human umbilical artery and vein (3). In contrast to these investigations, Gautieri and Mancini (4) recently reported an increase in perfusion pressure of the human placenta that ranged from 11 to 51 mm. Hg; this response to angiotensin was reduced but not completely blocked by either 3-or 6-mg. doses of phentolamine.

In addition to the effects of angiotensin on the above isolated preparations, many recent investigations have linked abnormally high blood levels of angiotensin and renin with the pathogenesis of the toxemias of pregnancy (5–8). The symptomology of clinical toxemia has been mimicked by the injection of renin-containing renal extracts into rats made hypertensive by the administration of desoxycorticosterone (DCA); renal damage similar to that of toxemia as well as convulsions, anasarca, oliguria or anuria, sodium retention, and increased blood urea nitrogen were reported (5). Also, an increased pressor response to angiotensin administration was found by Chesley (6) in women with pre-eclampsia. However, a decreased sensitivity to this autocoid was observed in normal pregnancy when this group was compared with

Received April 14, 1967, from the School of Pharmacy, Temple University, Philadelphia, PA 19140
Accepted for publication October 4, 1967.
Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
The authors express sincere thanks to the Obstetrical Staff, Temple University Hospital, for the generous supply of placentas, to Dr. David E. Mann, Jr., for his valuable suggestions and discussions, and to Mr. James C. Tatnall for his technical assistance.
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nonpregnant controls. Dexter and Haynes (7) found higher than normal concentrations of renin in the blood of patients with eclampsia and pre-eclampsia and Landesman *et al.* (8) reported increased blood levels of angiotensinase in toxic women when compared with nontoxic, pregnant controls. When high doses of renin and angiotensin were administered to the pregnant rat, a drop in placental blood pressure was noted by Burlingame *et al.* (9). Injections of renin into the rat placenta itself produced consistent increases in perfusion pressures.

Thus, because angiotensin may be involved in the etiology of the toxemias of human pregnancy and because a previous study in this laboratory (4) has shown that angiotensin exerts its pressor action on perfused human placental vessels by both a stimulation of α-adrenergic receptors and also a direct stimulation of vascular smooth muscle, it was the purpose of this investigation to screen compounds that would selectively modify the mechanisms whereby angiotensin produces vasoconstriction in these vessels, in order not only to identify angiotensin antagonists, but also to more clearly elucidate its vasopressor action in this system.

MATERIALS AND METHODS

Full-term human placentas, obtained from the hospital 15 to 20 min. after normal delivery, were used throughout this investigation. Each was transported to the laboratory in a light-resistant glass container filled with approximately 1 L. of Tyrode's solution preheated to 38°.

The apparatus used and the procedures employed in the preparation and perfusion of the placentas, in recording and maintaining the perfusion pressure, and in measuring the inflow and outflow volumes of the perfusate have been described in previous papers (10, 11). In 38 successful placental preparations, each lasting 1–4 hr., a total of 106 studies was conducted. The results from 4 placentas were discarded because the response to a test dose of angiotensin was erratic.

Throughout this investigation the agonist, angiotensin, was administered before the various antagonists for the purpose of establishing a standard response to its vasoconstrictor effect; the antagonists were then administered 2–3 min. after the pressor response to angiotensin subsided. Subsequent responses to angiotensin, at 4–6-min. intervals after the pressor or depressor effect of the various antagonists subsided, were then compared to the initial standard. The mean pressure change at maximal antagonism of the pressor effect of angiotensin, as well as the mean onset of maximal antagonism in minutes, were used as bases for comparing the relative potencies of the various antagonists. The pressor dose of angiotensin used throughout this investigation was kept at 50.0 mcg. and was based on that used in a previous investigation (4). This dose of angiotensin produced a mean increase in perfusion pressure of 20.3 mm. Hg (range 10.6–34.0 and S. D. ±6.6).

The doses of the antagonists were determined by trial and error and were kept as low as possible, yet sufficient to exhibit angiotensin antagonism. However, in the case of the α-adrenergic blocking agents, their doses were based on those used to antagonize the constrictor effect of 40-mcg. doses of norepinephrine in a previous study with this preparation (11).

The following drugs were injected into the rubber tubing, prior to entering the pump, in a volume of distilled water not exceeding 5.0 ml., except where noted: angiotensin-II,[1] 0.01%; cyproheptadine hydrochloride,[2] 0.01%; papaverine hydrochloride USP, 1.0%; sodium cobaltinitrite, 0.4%; nitroglycerin USP, 0.1%; isosorbide dinitrate,[3] 0.4%; sodium nitrite, 0.4%; dipyridamole,[4] 0.5% in 1.0 ml. of an aqueous solution containing polyethylene glycol 600, 50 mg., and tartaric acid, 2 mg.; dibenamine hydrochloride,[5] 0.1% and 0.2% in 95% ethyl alcohol USP; phentolamine methanesulfonate,[6] 0.1%; tolazoline hydrochloride,[7] 0.1%; hydralazine hydrochloride,[8] 0.1%; lidoflazine,[9] 0.25% in 0.17 *M* acetic acid; atropine sulfate, 0.1%; cocaine hydrochloride USP, 1.0%. In addition, the appropriate amounts of 95% ethyl alcohol USP, 0.17 *M* acetic acid, and an aqueous solution containing 50 mg. of polyethylene glycol 600 and 2 mg. of tartaric acid per 1.0 ml. were injected to observe their effects on the placental vessels and the normal pressor response to angiotensin.

RESULTS

The following results, which are summarized in Table I, were obtained on the vasculature of full-term human placentas perfused at pressures between 70 and 120 mm. Hg. This range of perfusion pressures corresponds to inflow rates of 17 to 62 ml. of perfusion fluid (Tyrode's solution modified by the addition of 0.525% polyvinylpyrrolidone[10] per minute.

Cyproheptadine—In a dose of 100.0 mcg. cyproheptadine produced a mean pressure decrease of 3.32 mm. Hg (range 0.4 to 6.6) in the subsequent pressor responses to the standard 50.0-mcg. dose of angiotensin. In the five experiments performed, the maximum antagonism occurred an average of 24.4 min. (range 16.0 to 30.0) after the administration of the antagonist. The 200.0-mcg. dose of cyproheptadine produced a mean decrease of 3.6 mm. Hg (range 2.2 to 7.0) in perfusion pressure at maximum effectiveness. The onset of maximum antagonism

[1] Hypertensin, valyl-5-angiotensin-II amide, lot No. B-5578, supplied through the courtesy of Ciba Pharmaceutical Co., Summit, N.J.
[2] Periactin, supplied through the courtesy of Merck Sharp and Dohme Laboratories, West Point, Pa.
[3] Isordil, supplied through the courtesy of Ives-Cameron Laboratories, New York, N.Y.
[4] Persantin, supplied through the courtesy of Geigy Chemical Corp., Ardsley, N.Y.
[5] Supplied through the courtesy of the Givaudan Corp., Delawanna, N.J.
[6] Regitine, supplied through the courtesy of Ciba Pharmaceutical Co., Summit, N.J.
[7] Priscoline, supplied through the courtesy of Ciba Pharmaceutical Co., Summit, N.J.
[8] Apresoline, supplied through the courtesy of Ciba Pharmaceutical Co., Summit, N.J.
[9] Supplied through the courtesy of McNeil Laboratories, Fort Washington, Pa.
[10] Plasdone C.

TABLE I—COMPARISON OF ANGIOTENSIN ANTAGONISM BY SEVERAL COMPOUNDS IN PERFUSED HUMAN PLACENTAL VESSELS

Drug	Dose[a]	No. of Exp.	Av. Max. Antagonism, min.	Mean Pressure Decrease, mm. Hg	S.E.M.[b]	P Value[a]
Cyproheptadine	100.0 mcg.	5	24.4	3.32	±1.10	0.025
Cyproheptadine	200.0 mcg.	5	18.4	3.60	±1.31	0.050
Papaverine	5.0 mg.	5	19.0	2.88	±1.18	0.050
Papaverine	10.0 mg.	5	8.6	3.28	±0.74	0.010
Sodium cobaltinitrite	2.0 mg.	5	20.6	2.00	±0.53	0.010
Isosorbide dinitrate	2.0 mg.	5	25.6	1.36	±0.50	0.050
Nitroglycerin	2.0 mg.	5	18.4	3.12	±1.08	0.025
Sodium Nitrite	2.0 mg.	6	19.5	6.66	±2.11	0.025
Dipyridamole	5.0 mg.	5	14.4	5.16	±0.76	0.005
Dibenamine	2.0 mg.	5	16.2	2.08	±1.57	0.150[c]
Dibenamine	4.0 mg.	5	20.2	7.72	±1.29	0.005
Phentolamine	3.0 mg.	5	14.1	7.74	±2.32	0.025
Tolazoline	5.0 mg.	5	15.4	4.90	±1.56	0.025
Hydralazine	4.0 mg.	5	17.6	2.24	±0.43	0.005
Atropine	1.0 mg.	5	17.4	2.50	±0.50	0.005
Cocaine	2.0 mg.	6	13.3	1.73	±1.36	0.150[c]
Lidoflazine	5.0 mg.	5	17.4	8.86	±3.33	0.050

[a] P value calculated from "paired t test" (17). [b] S.E.M. = standard error of the mean. [c] P value >0.050 not significant.

occurred at about 18.4 min. (range 4.0 to 32.0) after 200.0 mcg. of cyproheptadine had been administered. In one of the five preparations the 200.0-mcg. dose of the antagonist resulted in a mild increase (0.4 mm. Hg) in the pressor response to subsequent doses of angiotensin. In 7 of the 10 experiments performed, using both doses of cyproheptadine, the antagonist itself produced an increase in perfusion pressure ranging from 1.0 to 9.2 mm. Hg; in the other three experiments no pressure change was noted immediately following the administration of the antagonist.

Papaverine—In a total of 5 experiments, a 5.0-mg. dose of papaverine produced an average decrease in the pressor response to angiotensin of 2.88 mm. Hg (range 2.8 to 6.2). In one instance a 1.2 mm. Hg increase in perfusion pressure, rather than a decrease, was noted when angiotensin was given following papaverine administration. The maximum decrease in the pressor response to subsequent doses of angiotensin occurred an average of 19.0 (range 7.0 to 52.0) min. after administration of the antagonist. In two cases the antagonist produced a decrease in perfusion pressure itself; an increase was noted immediately following the administration of the antagonist in one experiment; and no change was produced by papaverine itself in the remaining two experiments. In a dose of 10.0 mg. papaverine always produced a decrease in pressure by itself of 2.0 to 6.2 mm. Hg and the mean pressure decrease at maximum angiotensin antagonism was 3.28 mm. Hg (range 1.4 to 5.4) in the five experiments performed at this dosage. The greatest degree of antagonism occurred an average of 8.6 min. (range 5.0 to 14.0) after the higher dose of papaverine had been administered.

Sodium Cobaltinitrite—Sodium cobaltinitrite, in a dose of 2.0 mg., produced a mean decrease in the pressor response to subsequent doses of angiotensin of 2.0 mm. Hg (range 0.6 to 3.6) at maximum effectiveness. In the five experiments performed the maximum degree of angiotensin antagonism occurred an average of 20.6 min. (range 16.0 to 37.0) after administration of the antagonist. In all of the experiments performed, sodium cobaltinitrite produced a decrease in perfusion pressure itself of 0.8 to 6.6 mm. Hg.

Isosorbide Dinitrate—In a dose of 2.0 mg. isosorbide dinitrate reduced the pressor response to 50.0 mcg. of angiotensin an average of 1.36 mm. Hg (range 1.6 to 2.2) at maximal antagonism. In one of the five experiments performed the pressor response to angiotensin was increased 0.6 mm. Hg when compared to the original test dose of the agonist. An average of 25.6 min. (range 15.0 to 44.0) elapsed before the maximum effect of isosorbide dinitrate was noted; each administration of the antagonist was followed by a decrease in the perfusion pressure that ranged from 1.4 to 5.2 mm. Hg.

Nitroglycerin—In a total of five experiments 2.0 mg. of nitroglycerin produced an average decrease in the pressor response to subsequent angiotensin administration of 3.12 mm. Hg (range 2.4 to 5.0). The maximal efficacy of the antagonist was noted an average of 18.4 min. (range 14.0 to 25.0) after administration. In four instances the antagonist itself produced a decrease in perfusion pressure ranging from 1.4 to 14.4 mm. Hg.

Sodium Nitrite—Sodium nitrite, in a dose of 2.0 mg., produced a mean decrease in perfusion pressure of 6.66 mm. Hg (range 2.2 to 13.7) at maximum angiotensin antagonism. In one case a 1.6 mm. Hg increase in perfusion pressure was noted following angiotensin administration, when compared to the test dose of the agonist, after sodium nitrite had been given. In the six experiments performed the maximal antagonism of angiotensin occurred an average of 19.5 min. (range 11.0 to 38.0) after administration of the antagonist. In each case sodium nitrite itself produced a decrease in perfusion pressure of 1.0 to 5.2 mm. Hg.

Dipyridamole—In a total of five experiments 5.0 mg. of dipyridamole produced an average decrease in the pressor response to subsequent angiotensin administration of 5.16 mm. Hg (range 3.5 to 7.5). The maximum effect of the antagonist was noted an average of 14.4 min. (range 8.0 to 19.0) after administration. In each instance the antagonist itself produced a decrease in perfusion pressure of 2.0 to 5.2 mm. Hg immediately following its administra-

tion. A decrease in perfusion pressure (1.0 to 3.0 mm. Hg) was also noted when 1.0 ml. of an aqueous solution containing 50.0 mg. of polyethylene glycol 600 and 2.0 mg. of tartaric acid (equivalent to the vehicle for the 5.0-mg. dose of dipyridamole) was tested in four separate experiments.

Dibenamine—In a dose of 2.0 mg. dibenamine produced a mean pressure decrease of 2.08 mm. Hg (range 0.6 to 7.6) in the pressor response to the standard 50.0-mcg. dose of angiotensin. In the five experiments performed, the greatest decrease in the pressor response to angiotensin occurred an average of 16.2 min. (range 12.0 to 24.0) after the 2.0-mg dose of dibenamine had been given. In five separate experiments of 4.0-mg. dose of dibenamine produced a mean decrease of 7.72 mm. Hg (range 3.8 to 11.2 in the pressor response to subsequent doses of angiotensin at maximum effectiveness. The onset of maximal antagonism occurred at about 20.2 min. (range 11.0 to 28.0) after dibenamine had been given. The administration of 2.0 mg. of dibenamine produced an increase of 1.8 mm. Hg in the pressor response to subsequent doses of angiotensin in one preparation. In 9 of the 10 experiments performed, using both the 2.0- and 4.0-mg. doses of dibenamine, the antagonist itself produced an increase in perfusion pressure ranging from 1.6 to 27.0 mm. Hg. The administration of 1.0 ml. of 95% ethyl alcohol, equal to the amount used to dissolve the 2.0-mg. dose of dibenamine, resulted in a comparable increase in perfusion pressure (2.6 to 2.40 mm. Hg) in four separate experiments. Also, when 4.0 ml. of 95% ethyl alcohol, equivalent to the amount used to dissolve the 4.0-mg. dose of the antagonist, were tested in four experiments, an increase in perfusion pressure of 14.0 to 26.0 mm. Hg was observed.

Phentolamine—In a total of five experiments, a 3.0-mg. dose of phentolamine produced an average decrease in the pressor response to angiotensin of 7.74 mm. Hg (range 4.0 to 16.7). The maximum decrease in the pressor response to subsequent doses of angiotensin occurred an average of 14.1 min. (range 7.5 to 18.5) after the administration of the antagonist. In three cases the antagonist itself produced an increase in perfusion pressure ranging from 1.2 to 2.2 mm. Hg.

Tolazoline—Tolazoline, in a dose of 5.0 mg., produced a mean decrease in the pressor response to subsequent doses of angiotensin of 4.90 mm. Hg (range 4.0 to 8.8) at maximal antagonism. In one experiment tolazoline had no effect on the pressor response to angiotensin. In the five experiments performed the maximum degree of angiotensin antagonism occurred an average of 15.4 min. (range 11.0 to 23.0) after administration of tolazoline. In four of the experiments tolazoline itself produced an increase in perfusion pressure of 1.8 to 4.4 mm. Hg; in the one remaining instance a decrease in perfusion pressure (2.6 mm. Hg) was noted immediately after tolazoline had been given.

Hydralazine—In a dose of 4.0 mg. hydralazine reduced the pressor response to a standard 50.0-mcg. dose of angiotensin an average of 2.24 mm. Hg (range 1.0 to 4.4) at maximal antagonism. An average of 17.6 min. elapsed (range 13.0 to 23.0) before maximum the effect of hydralazine was noted. In each of the five experiments performed hydralazine administration was followed by an increase in perfusion pressure of 0.14 to 2.6 mm. Hg.

Atropine—Atropine, in a dose of 1.0 mg., produced a mean decrease in perfusion pressure of 2.50 mm. Hg (range 1.6 to 4.4) at maximum angiotensin antagonism. In the five experiments performed, the greatest decrease in the pressor response to angiotensin occurred an average of 17.4 min. (range 7.5 to 26.0) after the antagonist was administered. Atropine, itself, had no effect on perfusion pressure in any of the placentas studied.

Cocaine—In a dose of 2.0 mg. cocaine reduced the pressor response to a standard 50.0-mcg. dose of angiotensin an average of 1.73 mm. Hg (range 2.0 to 6.4) at maximal antagonism. An average of 13.33 min. elapsed (range 6.0 to 33.0) before the maximum effect of cocaine was noted. Cocaine itself had no effect on perfusion pressure in three experiments; in one case a decrease in perfusion pressure was noted; in the remaining two instances an increase in perfusion pressure was noted immediately after the antagonist was given. In only four of the six experiments performed did cocaine show an antagonism of the pressor effect of angiotensin; in the other two placentas increases in the pressor response to angiotensin (1.4 and 2.6 mm. Hg) were observed.

Lidoflazine—In a total of five experiments, 5.0 mg. of lidoflazine produced an average decrease in the pressor response to angiotensin of 8.86 mm. Hg (range 2.6 to 21.4). The maximum effect of the antagonist occurred an average of 17.4 min. (range 11.0 to 28.0) after administration. In each instance the antagonist itself produced a decrease in perfusion pressure of 2.6 to 8.0 mm. Hg immediately following its administration. The administration of an amount of 0.17 *M* acetic acid (2.0 ml.) equal to that used to dissolve the 5.0-mg. dose of lidoflazine produced a decrease (1.6 to 4.0 mm. Hg) in the perfusion pressure itself in three separate experiments; in another case it had no effect on perfusion pressure alone.

Polyethylene Glycol 600 and Tartaric Acid—The administration of 1.0 ml. of an aqueous solution, containing 50.0 mg. of polyethylene glycol 600 and 2.0 mg. of tartaric acid, slightly decreased thd pressor response to subsequent doses of angiotensin (0.2 mm. Hg) in one experiment. In three other experiments, however, an increase in the pressor response to the agonist (0.6 to 4.0 mm. Hg) was observed after injection of this solution.

Ethyl Alcohol—The administration of both 1.0 and 4.0 ml. of 95% ethyl alcohol caused no perceptible effect on the pressor responses of the placenta to subsequent doses of angiotensin in eight separate experiments.

Acetic Acid—A dose of 2.0 ml. of 0.17 *M* acetic acid in three experiments caused a small decrease (0.4 to 0.8 mm. Hg) in the pressor response to subsequent doses of angiotensin; in one other experiment an increase (1.0 mm. Hg) in the pressor response to angiotensin was observed.

DISCUSSION

Because a previous investigation in this labora-

tory (4) had demonstrated that the vasoconstrictor effect of angiotensin in the perfused human placenta was primarily the result of a direct stimulation of vascular smooth muscle and only partially due to a stimulant effect on α-adrenergic receptors, the majority of the drugs tested as antagonists in this investigation were chosen from two pharmacologic classes: negative musculotropic agents (papaverine, sodium cobaltinitrite, isosorbide dinitrate, nitroglycerin, sodium nitrite, and dipyridamole) and compounds that selectively block α-adrenergic receptors (dibenamine, phentolamine, tolazoline, and hydralazine).

Among the direct-acting smooth muscle depressants, sodium nitrite and dipyridamole were the most potent angiotensin antagonists and exhibited a mean pressure decrease at maximum antagonism of 6.66 mm. Hg and 5.16 mm. Hg, respectively. Isosorbide dinitrate, whose depressant effect on the placental vasculature has been shown to be due to the nitrate moiety (12), proved to be the weakest antagonist in this class (mean pressure decrease of 1.36 mm. Hg). However, its onset of action was longest, averaging 25.6 min. Each of the drugs in this category produced a mild and transient decrease in perfusion pressure which always subsided before its antagonistic effect on the pressor response to angiotensin was noted.

When 1.0 ml. of the vehicle used to dissolve dipyridamole (an aqueous solution containing 50.0 mg. of polyethylene glycol 600 and 2.0 mg. of tartaric acid per 1.0 ml.) was tested as an angiotensin antagonist, a slight increase (0.6 to 4.0 mm. Hg) in the pressor response to subsequent doses of angiotensin was observed in three experiments and a small decrease (0.2 mm. Hg) was noted in another. Thus it is reasonable to assume that the antagonistic ability of dipyridamole observed in this study is due entirely to the chemical itself, and not to the solvent employed.

Since previous investigations in this laboratory have shown that angiotensin and serotonin share similar mechanisms in producing a pressor effect on the vasculature of the perfused human placenta (4), and because cyproheptadine was shown to be a potent serotonin antagonist when tested in this preparation (13), this compound was evaluated as an angiotensin antagonist in this investigation. Only moderate angiotensin antagonism was recorded when cyproheptadine was tested in this study (mean pressure decreases of 3.32 mm. Hg with the 100.0-mcg. dose and 3.60 mm. Hg decrease with 200.0 mcg.). Cyproheptadine administration was followed by an immediate rise in perfusion pressure (range 1.0 to 9.2 mm. Hg) in this investigation whereas Gokhale *et al.* (2) observed no effect with this compound on the perfusion pressure of the human umbilical artery; yet they recorded a significant decrease, as in this study, in perfusion pressure immediately after administration of papaverine and sodium nitrite. In view of the above results, the negative musculotropic action for cyproheptadine, suggested as an additional mechanism of action by Ward and Gautieri (13), does not appear to be involved with its ability to antagonize the pressor effect of angiotensin in this preparation.

Dibenamine (mean pressure decrease of 7.72 mm. Hg in a dose of 4.0 mg.) and phentolamine (mean pressure decrease of 7.74 mm. Hg) were the most effective α-adrenergic receptor blocking agents employed in this study. In our results, the mean difference between the pressor effect of angiotensin before and after the administration of the 2.0-mg. dose of dibenamine was not statistically significant when the 0.050 level of probability was chosen after computation of the paired t test (17). Our results with the 3.0-mg. dose of phentolamine agree remarkably well with those of Gautieri and Mancini (4), who used a similar dose of this agent as an angiotensin antagonist in this preparation; they found, however, that doubling the dose of phentolamine did not increase its antagonistic ability. Our results with hydralazine (mean pressure decrease of 2.24 mm. Hg), in antagonizing the pressor effect of angiotensin in this isolated organ preparation, differ from those of Page and McCubbin (14) and Gross and Turrian (15), who could detect no antagonism of the pressor effect of angiotensin in the intact dog. Meier *et al.* (16), in contrast, have reported that hydralazine, but not phentolamine, reduced the pressor response to angiotensin in the perfused vasculature of the rabbit hind leg.

Although it was observed that the immediate increase in perfusion pressure of the human placenta, after the administraton of dibenamine, was due to the 95% ethyl alcohol used as the solvent, no angiotensin antagonism could be attributed to this vehicle when appropriate amounts were tested in eight separate experiments.

The foregoing results, therefore, indicate that the negative musculotropic agents employed produced the most predictable and consistent results of all the antagonists utilized. We are, then, in agreement with Gautieri and Mancini (4) who postulated that the pressor effect of angiotensin in the perfused human placental vessels was due primarily to a direct stimulation of vascular smooth muscle and only secondarily to a stimulation of α-adrenergic receptors.

A portion of angiotensin's stimulant effect on the guinea pig ileum, *in vitro*, has been shown to be the result of acetylcholine release from the ganglion cells in the muscular wall of the gut (18). Because of the above as well as the observations that acetylcholine is present normally in the human placenta (19) and elicits a vasoconstrictor effect on these vessels which is blocked by atropine (1), atropine was tested as an antagonist in an attempt to discern whether a portion of the pressor response to angiotensin in these nerve-free vessels (20) was the result of acetylcholine release. In this investigation atropine was only a weak angiotensin antagonist and produced a mean pressure decrease of 2.50 mm. Hg at maximum efficacy. From the results it is not possible, at this time, to attribute the antagonistic ability of atropine observed in these experiments to an antagonism of acetylcholine possibly released in response to angiotensin administration. Also, the observation that atropine can produce vasodilatation by a direct effect on vascular smooth muscle (21) cannot be ignored as a mechanism of angiotensin antagonism.

Since Distler *et al.* (22) have shown that angiotensin administration may result in the release of norepinephrine, a constrictor of placental vessels

(1, 11), from helical strips of the aorta and carotid artery of the pig, cocaine was chosen for testing as an antagonist because it has been shown to block the release of norepinephrine from storage sites in the terminals of sympathetic nerves (23). Cocaine appeared to be the least effective antagonist studied in the dose employed (mean pressure decrease of 1.73 mm. Hg) and the results obtained were not statistically significant ($P > 0.050$).

Lidoflazine, a specific angiotensin antagonist, has been shown to block the noncompetitive, indirect component of angiotensin's stimulant action on the guinea pig ileum *in vitro* (24); it was chosen for this study because it was the only specific angiotensin antagonist known. In this investigation lidoflazine was the most potent agent employed, as evidenced by a mean pressure decrease at maximal efficacy of 8.86 mm. Hg. Its onset of action (17.4 min.), however, was not greatly different from the other compounds employed. In four separate experiments an appropriate amount of 0.17 *M* acetic acid was tested for angiotensin antagonism because this was the solvent for lidoflazine. Only a negligible degree of antagonism (mean pressure decrease of 0.4 to 0.8 mm. Hg) was noted, indicating that the response to lidoflazine was not the result of an action of its solvent.

The results of this investigation indicate that the pressor response to angiotensin in the vasculature of the perfused human placenta is due principally to a direct stimulation of the vascular smooth muscle and, to a lesser degree, a stimulation of α-adrenergic receptors present in these vessels (11). No release of either acetylcholine or norepinephrine by angiotensin, as a portion of its pressor action in these vessels, can be postulated from the results of this study. It seems clear, therefore, that this preparation may be used to screen compounds that have potential therapeutic efficacy in the toxemias of pregnancy and as an aid in discerning the mechanisms of these and other vasoactive substances endogenous to the human body.

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Keyphrases

Angiotensin II antagonists
Placenta–perfused human
Musculotropic agents, negative–test antagonists
α-Adrenergic blocking agents–test antagonists
Pressor response

Mechanistic Toxicology of Triethyl Citrate in Cultured Mammalian Cells by Cinematography

By ALAN B. JONES, W. L. GUESS, and J. AUTIAN

The role of triethyl citrate in growth inhibition of mouse fibroblast cells (strain L-929) in culture has been studied by the use of phase-contrast microscopy and time-lapse microphotography. Concentrations of the ester studied ranged from 7.9 to 48 m*M* of growth medium. It was found that at concentrations below 12 m*M*, the primary site of action of the toxicant was mitochondria resulting in mitochondrial swelling, inhibiting the function of this organelle, and growth inhibition. At higher dose levels, a second mechanism masks the mitochondrial involvement. The cytological picture is characterized by cytoplasmic gelation, plasma membrane contraction, and eventual lysis of the cell. The mechanism proposed was protein denaturation, but further studies are necessary for its definition. The method is a valuable tool for observing the effects of toxicants on living cells. When coupled with biochemical procedures, it offers a workable technique for the interpretation of mechanisms of actions of toxicants.

TRIETHYL CITRATE and other esters of citric acid are commonly used as plasticizers in polyvinyl chloride and cellulosic formulations. The *in vitro* toxicity of triethyl citrate has received some attention in the past as evidenced by previous studies from this laboratory (1). In these studies, Rosenbluth demonstrated the inhibitory effect of a homologous series of citric acid esters on the growth of mammalian cells in culture, and characterized triethyl citrate as being a nonspecific physical toxicant. As a physical toxicant, its biological effects could be related to the physical properties of the compound by mathematical relationships derived by Ferguson (2). In the homologous series studied, triethyl citrate represented the lowest number of the series exhibiting this phenomenon of physical toxicity since trimethyl citrate toxicity was shown to be of a chemical nature.

Compounds exhibiting physical toxicity are generally thought to accumulate in the lipoprotein of the cell due to their specific physical properties. Previous work on the mechanism of action of depressants, which fall into the category of physical action, have yielded two proposed mechanisms of action. Albert (3) suggested that the accumulation of a depressant drug caused a swelling of the plasma membrane. This swelling results in the mechanical separation of the enzyme systems necessary for active transport. Also, this swelling could result in a physical blocking of the pores in the membrane, thus again blocking the passage of material into and out of the cell. On the other hand, in 1961, Pauling (4) suggested that depressants exert their activity by forming clathrates through localized complexation of crystalline water in the cell.

A different mechanism of action for depressants was proposed by Warburg in 1921 (5). He suggested that they act by an inhibition of respiration of the cell at the level of the mitochondria. Later biochemical evidence by Butler (6), demonstrated that amobarbital specifically interfered with the cytochrome reductase system resulting in a decreased cellular respiration.

In contrast to these above noted modes of action, compounds exhibiting chemical specificity are in numerous reports in the literature. In the cases of these chemical toxicants, the biological activity cannot be related to the physical characteristics of the compound in question by mathematical relationships as previously mentioned. They are able to evoke a biological response at a much lower concentration than is predicted in Ferguson's relationships.

As a continuation of the *in vitro* studies previously mentioned, the present study was undertaken to elucidate a mechanism or mechanisms by which triethyl citrate acts at the subcellular level to inhibit cell growth. The approach taken was primarily based on the fact that dynamic and morphological changes induced by a toxicant in cellular and subcellular structures are directly related to biochemical changes which have occurred previous to the time of the noted change. Through the establishment of a sequence of events which lead to growth inhibition and possibly cell death, it was felt that the deduction of general biochemical changes which occur preceding these events might be possible and a mechanism of action obtained.

In normal cellular functions and activities,

Received April 14, 1967, from the Drug-Plastic Research and Toxicology Laboratory, College of Pharmacy, University of Texas, Austin, TX 78712

Accepted for publication October 5, 1967.

Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This research was supported in part by grant CA-06120 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md. and contract PH43-64-557 from the Division of Biologics Standards, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

the cell maintains a high degree of integrity. This maintenance is part of a dynamic system and requires a continuous source of energy. The subcellular organelle, the mitochondrion, supplies this energy in the form of adenosine triphosphate (ATP), which it manufactures through a series of metabolic reactions. Since the mitochondria are responsible for maintaining the energy system of the cell, any interference or inhibition of their functions would result in a significant effect upon cellular activity and should be manifested in altered cellular morphology (7).

The mitochondria have been shown to be quite sensitive to unfavorable conditions for cell life (8) yet at times quite resistant (9). However, other cellular organelles and cellular substructures such as the nucleus, plasma membrane, nuclear membrane, *etc.*, play a key role in maintaining the cellular integrity, and their responses to a toxicant should be evaluated in the interpretation of any of the biochemical reactions which occurred preceding the noted morphological change.

In order to obtain these data, the system of choice was the phase contrast microscope since it enabled observations to be made on the living cell. Their morphological alterations were noted under the influence of triethyl citrate administration and recorded with aid of time-lapse cinematography.

MATERIALS AND METHODS

Mouse fibroblast cells (Earle's NCTC929), a typical mixture of cell types maintained in static culture in this laboratory, were used exclusively. The cell line was last cloned in 1963 and has undergone an unknown number of passages since that time. The cells were cultured in Eagle's basal medium (10) plus 4.0 ml. of calf serum, 1.0 ml. of a 200 mM solution of L-glutamine, and 5.0 mg. of streptomycin/100 ml. of growth medium in a 5% CO_2–air atmosphere maintained at 35°. Routine subcultures were made every 4 days using a 0.05% trypsin solution.

Sykes-Moore tissue culture chambers (Belco Glass, Inc.), fitted with 2.5-mm. working distance rubber "O"-rings, were used for all microscopic work. Upon the preparation of the culture chambers at the time of subculturing, a cell suspension of approximately 10^4 cells per ml. was added to the chambers using the procedures of Sykes and Moore (11). After the chambers were filled with the cell suspension, they were placed right side up in an incubator at 35° and allowed to remain in that position for 12 hr. to allow the cells to settle to the bottom and attach to the lower coverslip.

Time-lapse experiments were begun 24 to 36 hr. after the chambers were filled. The purpose of this lapse in time after filling was to insure that the cells had recovered from any of the trauma induced by trypsinization during the subculturing procedures.

Prior to infusion of the test chemicals, study cells were observed by photography for a minimum of 30 min. for normal activity, thus serving as an internal control. Repeated media change induced no cellular alterations during control periods of study.

Concentrations of the triethyl citrate (Chas. Pfizer and Co., Inc.) used were determined by weighing the ester and dissolving it in the required amount of growth medium. The concentrations used in this study were 7.9 mM, 9 mM, 12 mM, 24 mM, and 48 mM and were prepared immediately before use. Approximately 6 mM and 12 mM represent the ID_{50} and the ID_{100} for the 72-hr. cultures previously reported (1). The introduction of the medium plus chemical was accomplished by perfusing the chamber with exactly 1.0 ml. of medium containing the drug. This resulted in a concentration in the chamber of 0.65 times the concentration of the perfusion medium (11, 12). Prior to infusion, all fluids were prewarmed to 35° and the pH of the medium was adjusted by aeration with an air mixture containing 5% carbon dioxide. In this communication, all concentrations indicated are those of the medium in the chamber and not the perfusion medium. It should also be noted that all procedures discussed to this point were conducted using aseptic techniques.

For observation, the chambers were placed on the stage of a Nikon model M inverted phase-contrast microscope housed in an electric incubator maintained at 35°. The illuminator was a low voltage lamp (6 v., 15 w.) equipped with both a green filter and a heat reflecting filter. A 100× oil immersion phase-contrast objective was chiefly used. The time-lapse equipment consisted of a Bolex SH 44 16 mm. movie camera with Nikon's connecting hardware and a time control panel with camera drive mechanism (R. J. Matthias and Associates). Other photomicrographic equipment consisted of a Nikormat FS 35 mm. camera with Nikon adaptor connections.

The individual cells for time-lapse observations were selected by scanning the culture for a cell undergoing mitosis, then waiting 8 hr. after anaphase before the administration of the triethyl citrate. At this time, all cells were well into interphase and had taken on their normal interphase characteristics and certain subcellular structures were readily visible. After the administration of the triethyl citrate, the cells were observed for 4 hr. with exposures taken every 3.6 sec.

Kodak 16 mm. Tri-X reversal movie film and Kodak 35 mm. Plus-X panatomic film were used exclusively. The processed movie film and prints from the 35 mm. film were analyzed for morphological alterations. The movie film was analyzed not only at normal projection speeds which accelerated movements in the cells by about 70× but also through the use of a single frame projection editor made in the laboratory. Replicate experiments were conducted at each dose level.

RESULTS AND DISCUSSION

The normal L-cell during interphase (Fig. 1) in a static culture is firmly attached to the glass surface of the container in which it is growing. It may be found in one of many configurations, ranging from almost circular to greatly elongated, such that its length may be 8 to 10 times its width.

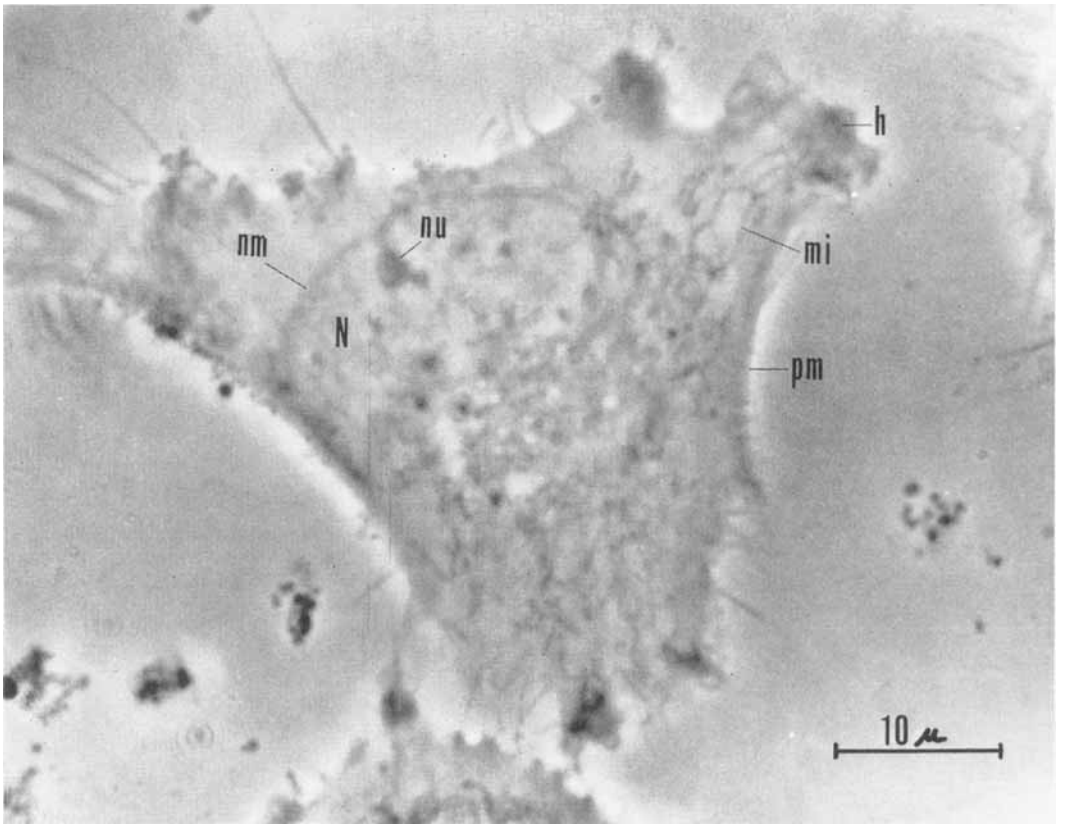

Fig. 1—Normal interphase cell illustrating the nucleus (N), *nuclear membrane* (nm), *nucleolus* (nu), *plasma membrane* (pm), *mitochondria* (mi), *and hyaloplasmic zone* (h).

Control experiments showed that in general, the cells appeared somewhat elongated (Fig. 1), such that the length was two to three times the width. The plasma membrane, which is responsible for the maintenance of concentration gradients between the cytoplasm and the exterior medium appears very irregular with many filament-like extensions. These are actual extensions of the membrane and are filled with cytoplasm. On time-lapse photography, these zones of the plasma membrane show very little movement.

Active areas of movement were seen in zones of the plasma membrane where the membrane had extended beyond the points of attachment to the glass. The entire zone, known as the hyaloplasmic zone, is noted to undergo wavelike motions when the cine records are observed. This hyaloplasmic zone is generally associated with pinocytosis and quite frequently, a small vesicle arising from pinocytosis is seen in the adjacent cytoplasm.

The normal cytoplasm is filled with small particulate material, most of which is distinguishable only as ground substance due to the limit of resolution of the light microscope. This ground substance is more concentrated in the perinuclear area with the peripheral cytoplasm being relatively free of visible particulate matter. However, among this ground substance, one major subcellular structure, the mitochondria, is readily discernible. The mitochondria generally take on long rod-shaped configurations which are especially identifiable in the peripheral cytoplasm. Other than vesicles, these mitochondria are generally the only subcellular structure which are readily identifiable in the hyaloplasmic zones.

Located near the middle of the cytoplasm, the nucleus of the normal 8-hr. cell was readily visible. It generally has a kidney-shaped cross section, the indentation being due to the presence of the rigidly fixed centrioles in the cytoplasm. The membrane boundary of the nucleus was sharply outlined with smoothly curving contours. The nucleoplasm appeared homogeneous at magnifications used in this study with one to five nucleoli spread throughout it. The nucleoli appeared as darker condensed masses with fairly well-defined peripheries.

Control experiments revealed the following additional facts about the normal cell from 8 to 12 hr. after anaphase. As has been previously indicated, in the 4-hr. observation period, there is little movement of the plasma membrane, except for the wavelike movements in the hyaloplasmic zones. The vesicles arising in these areas migrated toward the center of the cell and during the migration increased in absorbance while they decreased in size. As they joined the ground substance in the perinuclear zone, they became indistinguishable.

The mitochondria in the peripheral areas of the cytoplasma undergo active movements. These movements appeared to be associated with the localized movements of the cytoplasm. The ground substance in the perinuclear zone was observed to

migrate both in and out of the area as there is a constant transfer of material to the various locales within the cell.

The nucleus retained its general orientation with the rest of the cell. However, with time it often rotates about the centrioles. This rotation may or may not occur, since in some controls it rotated and in others it did not. When rotations did occur, the maximum angle of rotation was observed to be about 30°.

When a normal healthy cell was exposed to a medium change while under observation, there was no change in the morphological characteristics previously mentioned. However, when various concentrations of triethyl citrate were present in the infusion medium, the following responses of the individual subcellular units were noted.

Plasma Membrane—In early response to the toxicant, the plasma membrane retracted as indicated by the disappearance of the hyaloplasmic zones (Fig. 2, *A* and *B*). At all dose levels this response was observable. The onset times ranged from 2 to 3 min. at the lower concentrations to a matter of seconds at the higher doses. The initial contraction was followed by a general contraction of the plasma membrane with a decrease in cross-sectional area of the cell and an accompanying decrease in surface area of the plasma membrane. This general contraction was a gradual process as indicated by the changes in cross-sectional area of the cell as illustrated in Fig. 2, *C* and *D*. The general retraction of the plasma membrane was observable on the cine records within 15 min. at all dose levels except 7.9 m*M*. In this cine record, membrane activity appeared normal until 3 hr. after dosing. At this time, slight retraction was noticeable.

At doses of 24 m*M* and 48 m*M* the general retraction of the membrane appeared to be continuous with the retraction of the hyaloplasmic zones. Also at the 48-m*M* dose level, the retraction was followed by the appearance of superficial vacuoles (Fig. 3) at 2 hr. Lysis of the cell followed within a few minutes. Cultures at other dose levels were not observed continuously until death. However, the 12-m*M* and 24-m*M* cultures were dead within 24 hr. Their mode of death also appeared to be lysis with the superficial vacuoles still present.

Cytoplasm—As indicated above, the ground substance and the particulate matter in the cytoplasm moved about in definite patterns in the normally functioning cell. In general, triethyl citrate caused a slowing of movements of the particles through the cytoplasm. This phenomenon, called cytoplasmic gelation was seen in the cine records at higher dose levels, but appeared to be dose dependent. The onset of gelation occurs simultaneously with the general retraction of the plasma membrane. At dose levels of 9 m*M* and 12 m*M*, the gelation appeared to start from the peripheral portions of the cell and migrate toward the nucleus. At the higher concentration levels, the onset was so rapid that such a migration was not observable. Total cessation of movement was observed only at the 48-m*M* level. Immediately prior to lysis, all cellular movements ceased. Also only at this dose level did the movements of the ground substance ever appear to be inhibited in the region of the centrioles and Golgi complex.

Appearance of Dense Granules in the Cytoplasm—At all dose levels with the exception of 48 m*M*, within 20 to 30 min. after the infusion of the medium containing the drug, small dark spheres of 0.5 to 1 μ in diameter appeared in the cytoplasm (Fig. 2, *C* and *D*). At the higher dose level, lysis occurred so rapidly that this response was apparently not observed. Associated with the appearance of these spherical granules was the disappearance of the rod-shaped mitochondria. Examination of the cine records revealed that the spherical granules appeared to arise from the mitochondria. Thus these dense particles are apparently swollen mitochondria. Their appearance in different areas of the cytoplasm followed no set pattern, nor was it a totally dose-related phenomenon. However, it was uncommon to see them in the region of the centriole, the area least affected by gelation. They were generally confined to other portions of the cytoplasm.

Nucleus—The nucleus appeared to be unaffected during the period of observation at dose levels below 12 m*M*. However, at this dose and at 24 m*M*, the nuclear membrane did appear to increase in optical density as if some materials were being deposited on or in it. This nuclear involvement occurred at 3.5 hr. and 3 hr., respectively. The same response was noted at the 48-m*M* dose level at 1.5 hr. Associated with this response, the nuclear membrane lost its smooth contour and became very irregular with a scalloped configuration. However, at 48 m*M*, there followed a constriction of the nucleus with an increase in the optical density of the nuclear membrane and a decrease in the optical density of the nucleoplasm. The nucleoli also increased in their optical density (Fig. 3). Upon lysis of the cell, the nucleus remained in this fixed configuration. The same fixed nuclear morphology was noted in the lysed cells dosed at 12 m*M* and 24 m*M* which were discussed above.

DISCUSSION

In the light of the previously described results, it is concluded that there are at least two possible mechanisms of action by which triethyl citrate inhibits cell growth and results in cell death. At the lower dose levels, where growth inhibition has been noted (1), triethyl citrate acts specifically on the mitochondria, eventually resulting in a decrease in the production of ATP, and thus a decrease in the growth rate of the cultures. As the dose level was increased to 12 m*M*, another mechanism probably comes into play which at higher concentrations (48 m*M*) completely masks any mitochondrial involvement. In such situations, the triethyl citrate precipitated the soluble proteinaceous materials in the cytoplasm resulting in osmotic changes and cell lysis.

If the toxicant is to act intracellularly, it must cross the plasma membrane by one of several mechanisms. Different mechanisms by which a compound may enter the cell are pinocytosis, phagocytosis, active transport across the membrane, and simple diffusion across the membrane. Triethyl citrate does cross the cell membrane and acts intracellularly as evidenced by the various morphological alterations noted. The exact mechanism of the penetration was of course not determined in this study but it was concluded that the

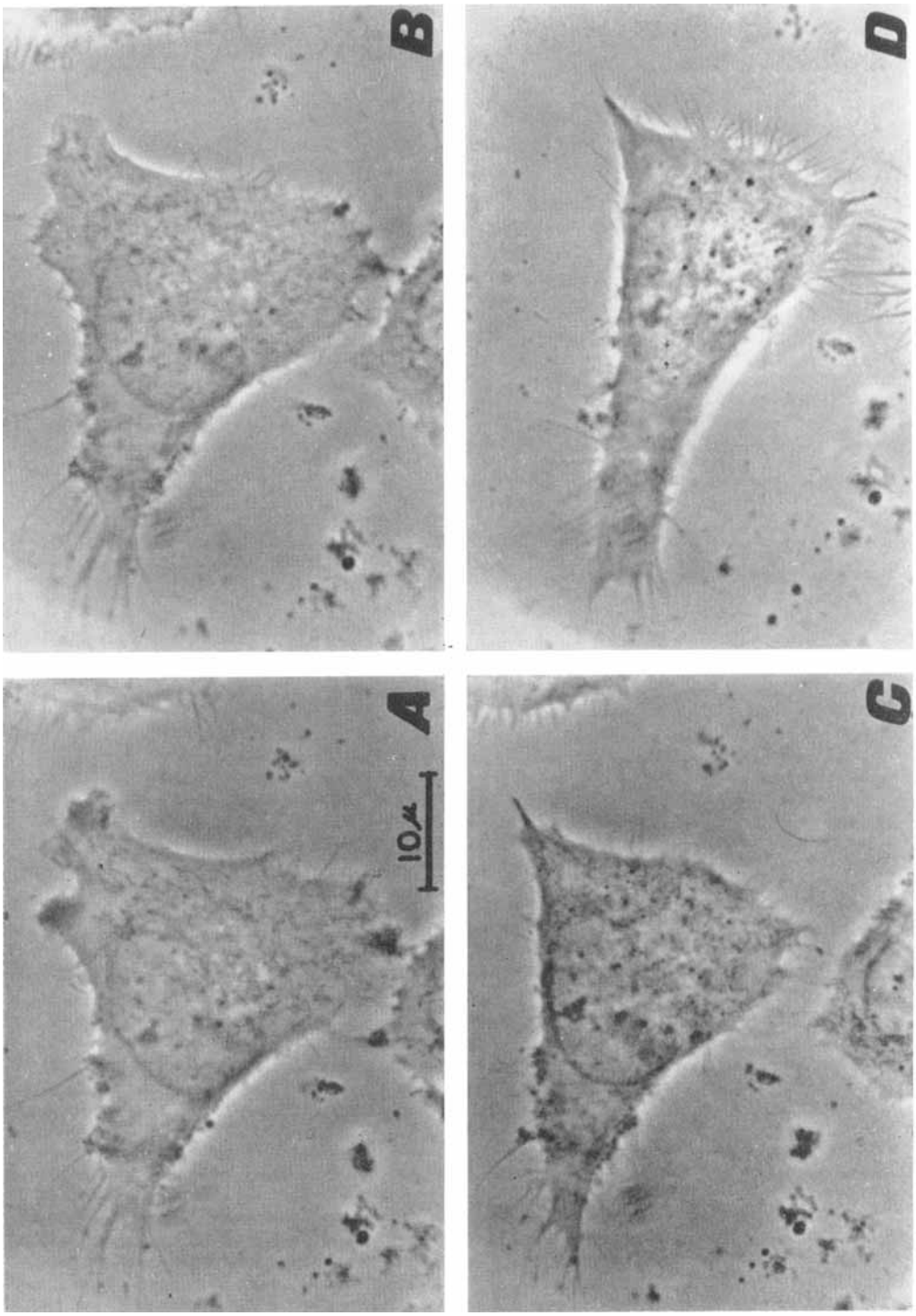

Fig. 2—Responses of a cell when dosed with a 16-mM solution of triethyl citrate. Key: A, prior to dose; B, 2 min. after dose; C, 1 hr. after dose; D, 5 hr. after dose. (See text for discussion.)

compound crossed by simple diffusion or active transport, since under the experimental conditions used with the mouse fibroblast cells, only an occasional pinocytotic vesicle appeared and no phagocytosis was observed.

The primary result of the intracellular activity of triethyl citrate at the lower concentrations (7.9 m*M* and 9 m*M*) was eventual mitochondrial swelling, since this reponse was observed well before any other responses. Similar morphological alterations in mitochondria have been noted by other workers. Biesele (8) observed this phenomenon in the treatment of embryonic mouse skin fibroblasts and mouse sarcoma 180 with 6-mercaptopurine, and Chevremont *et al.*, (13), noted it with trihydroxy-*N*-methylindole when treating primary embryonic chick

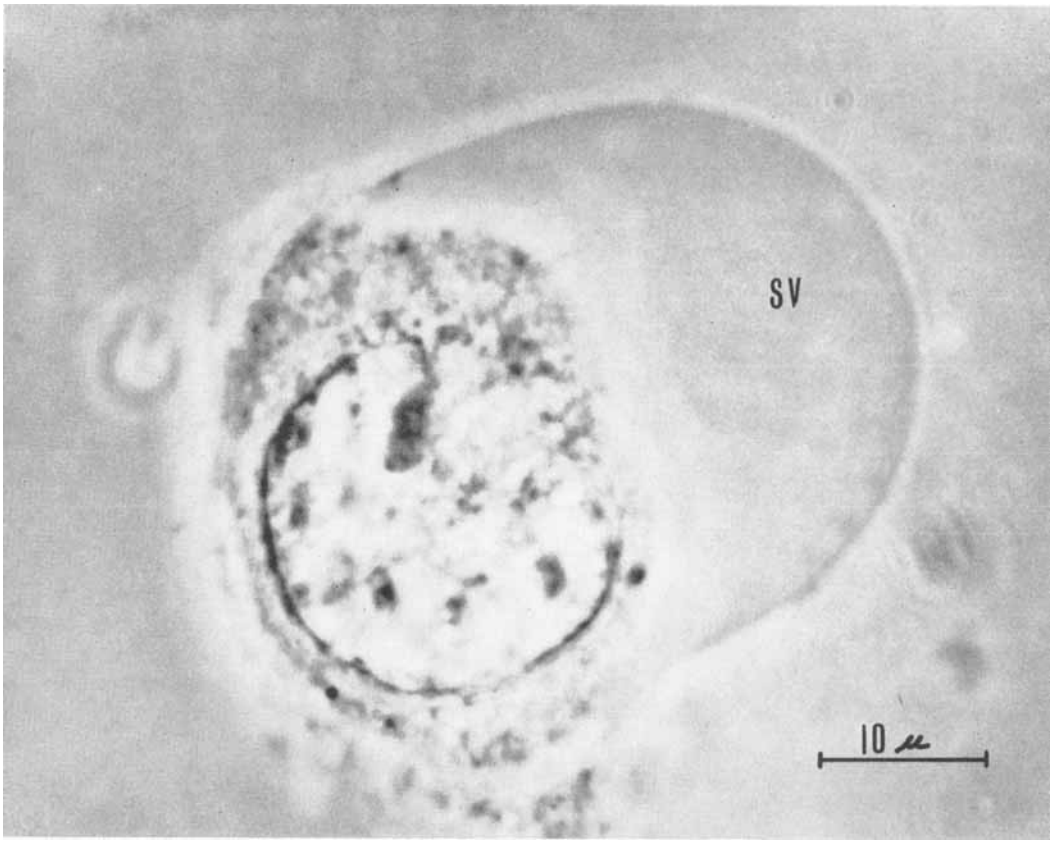

Fig. 3—Cell prior to lysis. Note superficial vacoule (sv), *rounded configuration, irregular nuclear membrane, and condensed nucleoli.*

muscle cultures. As Biesele indicated, mitochondria are notoriously sensitive to unfavorable conditions of cell life and the specificity of any morphological change in them is therefore open to question when this parameter is considered alone.

In studies with L-cells, Danes and Struthers (14), demonstrated that an iron–dextran complex interfered with the respiratory system (the mitochondria) of the cell and with only a slightly reduced respiratory activity, there was a greatly reduced growth. In similar studies with L-cells, Golaz (15) has demonstrated a decrease in the ATP production with 9m*M* and 12m*M* concentrations of triethyl citrate. These decreases were noted after 1.5 hr. of treatment.

Since ATP is produced in the mitochondria and observation of cytological changes showed mitochondrial swelling, it is concluded that this organelle is a specific point of attack for the triethyl citrate. As indicated above, only a slight depression in the respiratory system is necessary for growth inhibition. Thus, it is believed that triethyl citrate causes mitochondrial swelling and interferes with ATP production and respiration of the cell, whereby growth inhibition results.

On the other hand, at dose levels above 12 m*M*, additional response noted cannot be attributed to mitochondrial involvement. As has been indicated, plasma membrane contraction and cytoplasmic gelation are apparent at these dose levels prior to mitochondrial swelling. It is well known that many compounds of a chemical nature are capable of precipitating proteins. Pure triethyl citrate, when added to calf serum, causes an immediate precipitation of serum proteins. However, in concentrations used in the growth medium, no noticeable precipitation was noted. This points to a possible complexation of triethyl citrate with proteins in the cell, resulting in the observed progressive gelation. With high concentrations in the medium, the triethyl citrate rapidly penetrated the cell membrane. In doing so, some complexation with membrane lipoprotein could occur, but the data did not demonstrate this, since the membrane itself is well below the resolution of the phase-contrast microscope.

As the triethyl citrate entered the cytoplasm, and increased in concentration in this phase, there was an apparent interaction with the proteinaceous material there. Specifically, there appeared to be some interaction with the actin-myosin-like proteins in the cytoplasm which, among other things, are associated with the movements of the cytoplasm throughout the cell (16). Such an interaction would account for the rapid cessation of movements in the hyaloplasmic zones. Further interactions would account for the contraction of the membrane and the cessation of movements in other areas of the cytoplasm. In addition to the actin-myosin-like proteins, other cytoplasmic proteins may also be affected and thereby play a role in membrane contraction and the gelation phenomenon.

The decrease in the optical density of the nucleoplasm along with the condensation of the nucleoli and the contraction of the nuclear membrane probably follows this same nonspecific mechanism.

Occurring simultaneously with the nuclear contraction was the appearance of the superficial vacuoles followed by cell lysis. This response, however, was not related to the nuclear involvement but probably due to the complete gelation which was present. With such a gelation along with mitochondrial suppression the active transport system of the membrane was unable to obtain the required ATP necessary for maintaining concentration gradients necessary for normal cell function. The fact that gelation occurred rapidly at the higher dose levels also accounted for the lack of ATP for the membrane since the ATP could not be transported to the membrane through the gel matrix. Thus, the sodium ion diffused across the energyless membrane and obligatory water entered the cell resulting in the appearance of the superficial vacuoles with eventual lysis.

Bessis (17) observed similar sequential events which resulted in cell death and as he and other authors (18, 19) have pointed out, these sequential events can be a great aid in deciding where to look for supportive biochemical data.

SUMMARY

The role of triethyl citrate in growth inhibition of mammalian cells in culture has been studied by the use of phase-contrast microscopy with the following major findings.

(*a*) At dose levels below 12 m*M*, the mitochondria of the cell are initially inhibited in their functions resulting in the growth inhibition of the cell.

(*b*) At dose levels above 12 m*M*, a second mechanism masks the mitochondrial involvement. The cytological picture is characterized by cytoplasmic gelation, plasma membrane contraction, and eventual lysis of the cell. The mechanism proposed was protein denaturation, but further studies are necessary for its definition.

(*c*) Phase-contrast time-lapse cinematography is a valuable tool for determining sequential events occurring in the morphological patterns of living cells under the influence of toxic agents. When coupled with biochemical procedures, it offers a workable technique for the interpretation of mechanisms of action of toxicants, and should play a key role in bridging the gap between *in vitro* and *in vivo* systems.

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Keyphrases

Triethyl citrate toxicity
Tissue culture–mouse fibroblast cells
Eagle's basal medium plus calf serum
Membrane, plasma-toxicant effects
Cytoplasm-toxicant effects
Nucleus-toxicant effects
Phase-contrast microscopy
Time-lapse cinematography

Synthetic Porphyrins II

Preparation and Spectra of Some Metal Chelates of *para*-Substituted-*meso*-Tetraphenylporphines

By NIRMALENDU DATTA-GUPTA and THOMAS J. BARDOS*

Various new metalloporphyrins, including both Fe(II) and Fe(III), Co(II), and Co(III) complexes of *p*-substituted-tetraphenylporphines, were synthesized for use as possible model compounds in the study of the mechanisms of the oxidation-reduction of the heme-enzymes and cobalamines. Characteristic differences were observed in the near-ultraviolet, visible, and infrared spectra of the complexes of different metal ions. These compounds may also be of interest as specific radiation sensitizing or protecting agents.

THE IMPORTANT biological roles of metalloporphyrins (heme, chlorophyll) and of the structurally related metallocorrins (vitamin B_{12}) suggested that the appropriate metal chelates of certain synthetic *para*-substituted-*meso*-tetraphenylporphine derivatives (1) may serve as antimetabolites, or as model compounds in the study of the modes of action of these interesting natural substances. In addition, such compounds would be expected to be effective in absorbing radiation energy at certain wavelengths and may serve either as radiation sensitizing or as radiation protecting agents (2). It is of particular interest that some porphyrin derivatives apparently show selective distribution properties in the various tissues. Hematoporphyrin was reported to accumulate selectively in tumors and other rapidly growing tissues (3–5) and was used as a fluorescent indicator for the delineation of neoplastic tissue in cancer patients (6). It would seem, therefore, that certain metalloporphyrins may be used as tumor-selective radiation sensitizers or "carriers" of various cytotoxic groups and/or metals [*e.g.*, Hg (7) or ^{57}Co (8)] in the chemotherapy of neoplastic diseases.

Recently, Winkelman and co-workers reported (8, 9) that a sulfonated derivative of *meso*-tetraphenylporphine was even more selectively localized (attaining a 50–100 times higher concentration in the tumor tissues) than hematoporphyrin, but that the ^{57}Co chelate of this sulfonated compound lost its metal and only the free ligand was absorbed. This is in contrast with the reported stabilities of various injected Zn (3), Hg (7), and ^{64}Cu (10) porphyrins throughout their organ-uptake and/or excretion and is, of course, also in contradiction with the known stability of the Co-corrin derivative, vitamin B_{12}. Thus, the fate of the chelated metal during the absorption and localization of the metalloporphyrins in the tissues requires further investigation.

In the present paper, the synthesis and physicochemical properties of various metal chelates of the recently reported (1) *para*-substituted-*meso*-tetraphenylporphines are described. Due to the interest in obtaining model compounds for the study of the mechanisms of oxidation-reduction of the various heme-enzymes as well as of the cobalt in the various cobalamincoenzyme mediated reactions, particular efforts were centered on the synthesis of metalloporphyrins containing Fe and Co, respectively, at various oxidation states. The structures and chemical names of the new metalloporphyrins described in this paper are shown in Table I.

RESULTS AND DISCUSSION

The metalloporphyrins (I to VII) were synthesized by refluxing the ligand (1) and the metal salt in a suitable solvent (see under *Experimental*). Progress of the reactions was followed by observing the changes in the visible spectra of the reaction mixtures. The reactions were stopped when complete disappearance of the characteristic visible absorption maxima of the ligands occurred. The choice of solvent, due to solubility problems, was limited to pyridine, glacial acetic acid, and 88% formic acid. The suitable solvent for each preparation could be found only by carrying out small-scale reactions. The choice of metal salts was limited to the readily available chlorides or acetates. The ferrous complex (compound IV) was prepared and purified under nitrogen atmosphere, since the Fe(II) rapidly oxidized to Fe(III).

Compound VIII was prepared by two alternative methods, that is, either by direct chelation of the corresponding ligand, *meso*-tetra-(*p*-carboxyphenyl)-porphine, or by alkaline hydrolysis of *meso*-tetra-(*p*-carbomethoxyphenyl)porphinocobalt(II) (compound VII). The latter compound (VII) was solu-

Received August 7, 1967, from the Department of Medicinal Chemistry School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214, and the Roswell Park Memorial Institute, Buffalo, NY 14214

Accepted for publication September 29, 1967.

This investigation was supported by research grants CA-06695 and CA-10191-01 from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

Previous paper: Datta-Gupta, N., and Bardos, T. J., *J. Heterocyclic Chem.*, 3, 495(1966).

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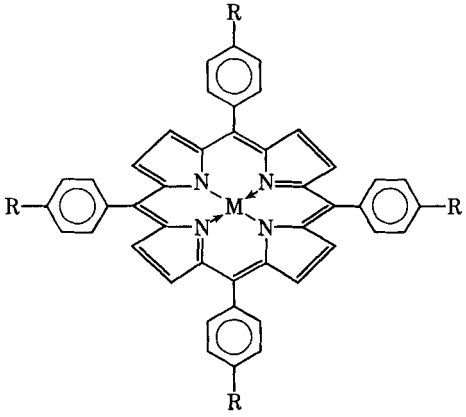
TABLE I—STRUCTURES AND CHEMICAL NAMES OF NEW SYNTHETIC METALLOPORPHYRINS



	R	M	Name
I	CH_3	Co(II)	*meso*-Tetra-*p*-tolylporphinocobalt(II)
II	CH_3	Cu(II)	*meso*-Tetra-*p*-tolylporphinocopper(II)
III	CH_3	Zn(II)	*meso*-Tetra-*p*-tolylporphinozinc(II)
IV	CH_3	Fe(II)	*meso*-Tetra-*p*-tolylporphinoiron(II)
V	CH_3	Cl-Fe(III)	*meso*-Tetra-*p*-tolylporphinoiron(III) chloride
VI	CN	Cl-Fe(III)	*meso*-Tetra-(*p*-cyanophenyl)porphinoiron(III) chloride
VII	CO_2CH_3	Co(II)	*meso*-Tetra-(*p*-carbomethoxyphenyl)porphinocobalt(II)
VIII	COOH	Co(II)	*meso*-Tetra-(*p*-carboxyphenyl)porphinocobalt(II)
IX	CH_2OH	Co(II)	*meso*-Tetra-(*p*-hydroxymethylphenyl)porphinocobalt(II)
X	CH_3	Br-Co(III)	*meso*-Tetra-*p*-tolylporphinocobalt(III) bromide

ble in dry tetrahydrofuran and was also used as the starting material for the synthesis of *meso*-tetra-(*p*-hydroxymethylphenyl)porphinocobalt(II) (compound IX). Before attempting to reduce VII with lithium aluminum hydride, *meso*-tetra-*p*-tolylporphinocobalt(II) (compound I), as a model compound, was treated with lithium aluminum hydride in dry tetrahydrofuran solution in order to determine whether the solubility of the cobalt complex and the chelation or the oxidation state of cobalt(II) would be affected by a reaction with the reagent. Only the unchanged starting material (I) was isolated; thus lithium aluminum hydride did not seem to have any effect on the chelated Co(II) ion. However, it is quite possible that the divalent cobalt in I was reduced by lithium aluminum hydride to monovalent cobalt but the latter was rapidly reoxidized to divalent cobalt during work-up (11). Thus, reduction of VII with lithium aluminum hydride gave the Co(II) chelate of the hydroxymethyl derivative (IX) in 79.3% yield.

Meso-tetra-*p*-tolylporphinobromocobalt(III) (compound X) was prepared from I in a manner analogous to the synthesis of the corresponding derivative of aetioporphyrin (I) by Johnson and Kay (12). Attempts were also made according to the procedure of the same authors (12) to synthesize *meso*-tetra-*p*-tolylporphindicyanocobaltate. The product obtained contained theoretical amount of cyanide radicals, nitrogen and hydrogen, but the analytic results for C, Co, and K were unsatisfactory. The material was stable in alcohol but slowly decomposed in chlorinated solvents. Further work on the nature of the *meso*-tetra-*p*-tolylporphindicyanocobaltate is in progress.

The near-ultraviolet and visible spectra of the metal complexes of tetraphenylporphines are characterized by a strong "Soret band," in the 400–440 mμ region, and by another strong absorption band ("K-band") in the visible spectral region; the latter is usually accompanied by much weaker vibrational bands ("R-bands") (13). Thus, these metalloporphyrins differ from the corresponding "free base" *meso*-tetraphenylporphines by the absence of the characteristic five-banded patterns in the visible spectra of the latter (1), and also from other metalloporphyrins (*e.g.*, etio-type) which generally show a different, two-banded (α and β-bands) pattern in their visible spectral region (14).

The absorption maxima of the new metalloporphyrins are shown in Table II. Molar extinction coefficients of all, except III, VI, and VIII, have been calculated in two different solvents. Compounds III and VIII could be quantitatively dissolved only in pyridine and 5% aqueous sodium carbonate, respectively, whereas compound VI could not be quantitatively dissolved in any solvent. It is apparent from Table II that a change of the solvent from benzene (or chloroform) to pyridine, results in certain changes in the positions or intensities (or both) of the absorption bands; these changes vary in magnitude from one metal chelate to another. In either solvent, the spectra show characteristically different patterns dependent on the chelated metal ion; different *p*-phenyl substituents in the ligands have relatively small influence on the spectral patterns of the metal chelates. The Soret bands of all metal chelates, with the exception of Cu^{II} and Zn^{II}, are significantly less intense than those of the corresponding free base porphyrins

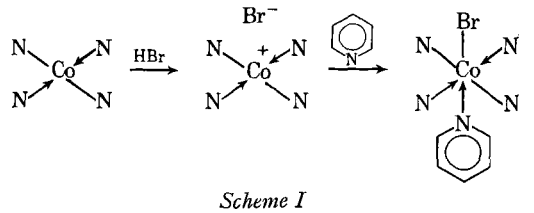
TABLE II—NEAR ULTRAVIOLET AND VISIBLE ABSORPTION SPECTRA OF NEW METALLOPORPHYRINS

Compd. (Solvent)	$\lambda_{max.}$ (mμ) ($\epsilon \times 10^{-3}$) "Soret Band"		"K and R Bands"				
I (Benzene)	415		530	612			
	(279)		(17.0)	(3.96)			
(Pyridine)	414		538				
	(147)		(9.6)				
II (Benzene)	424	523	552	592	650		
	(472)	(4.83)	(16.7)	(4.83)	(1.36)		
(Pyridine)	431	520	564	605			
	(672)	(6.5)	(20.8)	(13.6)			
III (Benzene)	424	514	549	590			
(Pyridine	422		545	570			
	(650)		(14.2)	(3.4)			
IV (Benzene)	409		572	612			
	(121)		(9.64)	(5.66)			
(Pyridine)	416		571	614			
	(112)		(8.3)	(4.8)			
V (Benzene)	420		512	547	587	650	694
	(136)		(5.62)	(2.25)	(1.5)	(1.5)	(1.6)
(Pyridine)	424	485	530		590	649	
	(218)	(10.0)	(16.2)		(2.9)	(2.1)	
VI (Chloroform)	419		527	560	598	651	
(Pyridine)	426	508	548	572	600	642	
VII (Tetrahydrofuran)	411		526				
	(253)		(14.8)				
(Pyridine)	416		534				
	(166)		(6.8)				
VIII (5% aq. Na_2CO_3)	430		547	586			
	(247)		(13.5)	(6.3)			
IX (Tetrahydrofuran)	410		528				
	(226)		(13.5)				
(Pyridine)	414		534				
	(196)		(7.6)				
X (Benzene)	410		545				
	(122)		(13.3)				
(Pyridine)	439		556	594			
	(170)		(8.2)	(5.0)			

(1). The number of the visible absorption bands of the metal chelates vary between one (Co^{II}) and five (Fe^{III}).

The spectrum of the Co(III) complex (X) very markedly differs from those of the Co(II) chelates in pyridine solution, but the difference is less pronounced when the spectra are compared in benzene. The more significant difference in the spectrum of X in pyridine is probably due to the extra ligand action of a molecule of pyridine with the trivalent cobalt. The spectrum of X in pyridine may correspond to that of an octahedral pyrimidinobromocobalt(III) complex, as proposed by Johnson and Kay (12) (Scheme I).

The spectra of the Fe(II) complex (IV) in benzene and pyridine are similar to each other, except for the position of the Soret band. The intensity of this band is remarkably low in both solvents. No other Fe(II) complex of a *meso*-tetraphenylporphine has been reported previously. The spectra of the corresponding Fe(III) complex(V) in either benzene or pyridine, are quite different from those of the Fe(II) complex. Although the spectra of V in the two solvents considerably differ from each other, their



Scheme I

composite pattern shows remarkable similarity to the spectrum of the free base porphyrin (1) in the positions of the absorption bands. However, the intensity of the Soret band of V is extremely low (particularly, in the benzene spectrum), just as in the case of the Fe(II) complex (IV).

The infrared spectra of the new metalloporphyrins, obtained in potassium bromide phase, are shown in Table III. The weak N-H stretching vibrations above 3300 $cm.^{-1}$ observable in the spectra of all the ligands (1) having no hydroxyl group, are missing from the spectra of their chelates, due to the replacement of N-H protons by metal ions. Probably the most characteristic difference between the infrared spectra of a tetraphenylporphine ligand and its metal chelates is that the three bands appearing between 960–1000 $cm.^{-1}$ in the spectra of the former (1) collapse to one strong band near 1000 $cm.^{-1}$ in the spectra of the latter. This absorption band has been associated with a C-H vibration of the pyrrole rings. In a limited series of metalloporphyrins containing certain divalent cations, Thomas and Martell (15) were able to correlate the relative frequencies of this band with the relative stabilities of chelation. In the present series, the metal complexes of *meso*-tetra-*p*-tolylporphine, (I–V, and X) show the following relative order of frequencies for the "1000 $cm.^{-1}$ band": Co(II) > Fe(II), Fe(III) > Cu(II) > Zn(II) > Co(III). Except for the relative positions of the iron chelates, this series seems to agree with the stability order of metalloporphyrins as determined by various methods (16, 17).

TABLE III—CHARACTERISTIC INFRARED ABSORPTION SPECTRAL BANDS OF METALLOPORPHYRINS I TO X

I	II	III	IV	V	VI	VII	VIII	IX	X	Assignments
								3420 (m)		O—H (stretch)
					2175 (m)					—C≡N
						1725 (vs)	1690 (vs)			—C=O
1602 (vw)	1595 (w)				1602 (w)	1600 (s)	1600 (s)	1600 (w)		—C=C— (phenyl)
1535 (m)	1520 (vw)	1525 (vw)	1524 (wsh)		1535 (m)	1565 (w)	1565 (w)	1540 (vw)		—C=C— (pyrrole)
1500 (m)	1480 (m)	1500 (m)	1480 (m)	1480 (m)		1480 (wsh)		1500 (vw)	1475 (m)	—C=C— (phenyl) —C=N
1442 (m)	1435 (w)	1439 (w)	1440 (w)	1440 (wsh)	1440 (w)	1435 (m)			1440 (m)	—C—H bend (pyrrole)
1347 (s)	1330 (s)	1340 (s)	1325 (s)	1327 (m)	1320 (s)	1360 (m)	1350 (m)	1343 (s)	1340 (s)	=C—N— stretch
1202 (m)	1202 (ms)	1205 (m)	1200 (ms)	1200 (w-m)		1207 (m)	1240–1260 (s; broad)	1200 (m)		
1178 (ms)	1176 (ms)	1180 (ms)	1178 (ms)	1178 (s)		1175 (m)	1204 (m)	1175 (m)		
1105 (m)	1104 (m)	1104 (m)	1105 (m)	1107 (w)	1113 (m)	1105 (s)	1175 (m)	1103 (w)	1102 (m)	*p*-Substituted phenyl
1070 (ms)	1064 (ms)	1070 (ms)	1065 (m)	1067 (w)			1100 (m)	1070 (m)		
1000 (vs)	995 (vs)	992 (vs)	997 (vs)	997 (vs)	996 (vs)	1002 (vs)	1002 (vs)	998 (vs)	992 (vs)	—C—H rock
			871 (s)			867 (m)	866 (m)			
847 (w)	846 (m)	845 (m)	846 (w)	844 (w)		827 (m)	879 (m)	850 (w)		
797 (vs)	794 (vs)	798 (vs)	796 (vs)	800 (vs)	800 (vs)	795 (s)	795 (vs)	796 (vs)	794 (s)	*p*-Substituted phenyl
	750 (m)	746 (w)				767 (s)	767 (m)			Pyrrole ring
720	720	719	719	722		718	715	713	713	
718 (ms)	(ms)	(ms)	(s)	(s)		710 (ms)	(m)	(ms)	(m)	
	693 (m)									

TABLE IV—SYNTHESIS OF METALLOPORPHYRINS I TO IX BY INSERTION OF METAL-ION INTO APPROPRIATE LIGAND (FREE BASE PORPHYRIN)

Compd.	Ligand	Metal Salt	Time of Reflux	Solvent: Reaction	Solvent: Recrystallization	Anal., %	
I	*p*-CH_3-TPP[a]	$CoCl_2$-$6H_2O$	22 hr.	Pyridine	Chloroform	Calcd.	C, 79.23; H, 4.95; N, 7.64
						Found	C, 79.25; H, 4.83; N, 7.71
II	*p*-CH_3-TPP	$Zn(OCOCH_3)_2$	30 min.	Pyridine	Chloroform–ethanol	Calcd.	C, 78.74; H, 4.92; N, 7.66
						Found	C, 77.91; H, 4.80; N, 7.84
III	*p*-CH_3-TPP	$Cu(OCOCH_3)_2$	30 min.	Pyridine	Chloroform	Calcd.	C, 78.54; H, 4.91; N, 7.64
						Found	C, 78.89; H, 4.90; N, 7.51
IV	*p*-CH_3-TPP	$FeCl_2$	12 hr.	Pyridine	Chloroform–ethanol	Calcd.	C, 79.57; H, 4.97; N, 7.74
						Found	C, 79.00; H, 4.82; N, 7.78
V	*p*-CH_3-TPP	$FeCl_3$	5 hr.	Pyridine	Chloroform–ethanol	Calcd.	C, 75.85; H, 4.74; N, 7.37
						Found	C, 76.27; H, 4.72; N, 7.54
VI	*p*-CN-TPP	$FeCl_3$	5 hr.	Pyridine	Pyridine	Calcd.	C, 71.69; H, 2.99; N, 13.94
						Found	C, 72.10; H, 3.33; N, 13.52
VII	*p*-CO_2Me-TPP	$CoCl_2$ $6H_2O$	3 hr.	Acetic acid	Chloroform	Calcd.	C, 69.10; H, 3.99; N, 6.20
						Found	C, 69.49; H, 4.60; N, 6.14
VIII	*p*-COOH-TPP	$CoCl_2$ $6H_2O$	3.5 hr.	90% HCOOH	AcOH–HCOOH	Calcd.	Co, 6.97
						Found	Co, 6.96
IX	*p*-CH_2OH-TPP	$CoCl_2$ $6H_2O$	1 hr.	Acetic acid	Tetrahydrofuran–chloroform	Calcd.	C, 72.82; H, 4.55; N, 7.08
						Found	C, 73.61; H, 4.93; N, 7.14

[a] TPP = *meso*-tetraphenylporphine.

EXPERIMENTAL[1]

General Method of Synthesis by Insertion of Metal Ion—In general, the metalloporphyrins were prepared by refluxing the appropriate ligand (1) with 4–5 times excess of a metal salt and a solvent, for a certain length of time (until disappearance of the free base spectral bands) as given in Table IV. After evaporation of the solvent, the excess inorganic salt was removed by washing with water, and the reaction product, obtained in the residue in essentially quantitative yield, was recrystallized from a suitable solvent or solvent mixture (Table IV).

Alternative Method for the Preparation of VIII—The *meso*-tetra-(*p*-carboxyphenyl)porphinocobalt-

[1] Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

(II) was also synthesized by basic hydrolysis of *meso*-tetra-(*p*-carbomethoxyphenyl)porphinocobalt-(II) (VII). A mixture of VII, 3 Gm., 4% aqueous potassium hydroxide solution, 100 ml., and tetrahydrofuran, 500 ml., was refluxed for 24 hr. The solvents were removed *in vacuo*. The residue was dissolved in water and neutralized with 6 *N* sulfuric acid. The purple precipitate was centrifuged and repeatedly washed with water. The material was dried over anhydrous calcium chloride. Yield: 2.66 Gm. (94.5%). This product had identical spectra with that obtained by the insertion method.

Alternative Method for the Preparation of IX—A mixture of *meso*-tetra-(*p*-carbomethoxyphenyl)-porphinocobalt(II) (VII), 1.7 Gm., lithium aluminum hydride, 1.7 Gm., and tetrahydrofuran (freshly distilled over lithium aluminum hydride), 50 ml., was refluxed for 2 hr., then 1 additional Gm. of lithium aluminum hydride and 50 ml. of dry tetrahydrofuran were added to the reaction mixture. Refluxing was continued for 2 more hr., followed by decomposition of the unreacted lithium aluminum hydride by moist ether at 0°. The slurry thus obtained was filtered and washed with 150 ml. of tetrahydrofuran. On evaporation of the solvent, 1 Gm. of purple crystalline solid was obtained. An additional 0.16 Gm. of material was obtained by further washing of the aluminum hydroxide slurry with 100 ml. of tetrahydrofuran, followed by evaporation of the solvent. Total yield: 1.16 Gm. (79.3%).

Synthesis of Meso-tetra-p-tolylporphinocobalt (III)Bromide(X)—The *meso*-tetra-*p*-tolylporphinocobalt(II) (I), 300 mg., was dissolved in 50 ml. of chloroform, and to this was added 50 ml. of methanol and 1 ml. of 47.5% hydrobromic acid. The mixture was stirred at room temperature for 2 hr., and then 50 ml. of chloroform was added. The reaction mixture was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was crystallized from a mixture of chloroform and petroleum ether (b.p. 40–60°). Yield: 300 mg. (90%). An analytical sample was obtained by recrystallization from the same solvent mixture and drying over phosphorus pentoxide at 100° for 8 hr.

Anal.—Calcd. for $C_{48}H_{36}BrCoN_4$: C, 71.38; H, 4.46; N, 6.94. Found: C, 71.29; H, 4.62; N, 6.73.

Spectral Measurements—The near ultraviolet and visible absorption spectra (Table II) were determined on Beckman DU (attached to a Gilford absorbance indicator, model 2000) and Beckman DB spectrophotometers. The infrared spectra (Table III) were determined on Perkin-Elmer model 137 and Beckman model IR-8 spectrophotometers.

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Keyphrases

Porphyrins—synthetic
Metalloporphyrins—synthesis
meso-Tetraphenylporphyrins, *para*-substituted metal chelates
UV spectrophotometry—structure
Visible spectrophotometry—structure
IR spectrophotometry—structure

Colorimetric Assay for Chloramphenicol Using 1-Naphthol

By DANIEL S. MASTERSON, JR.

Chloramphenicol heated in 6 *N* sodium hydroxide with 1-naphthol gives a blue color that is the basis for a quantitative assay method.

ASSAY METHODS for chloramphenicol are many and varied, but most of them have significant restrictions with respect to their applicability. Microbiological methods (1, 2) cannot be used for chloramphenicol palmitate, since the unhydrolyzed ester lacks antibacterial activity. Polarographic analysis is also not applicable to chloramphenicol palmitate because of unfavorable solubility characteristics (3). Reduction and diazotization methods (4, 5) give the same color response with numerous other compounds containing the aromatic nitro substituent and thus cannot specifically identify the reactant as chloramphenicol. In addition, the latter methods, dependent only on the nitrophenyl group of the molecule, will not reflect degradation involving other parts of the molecule. The ultraviolet spectrophotometric procedure (6) for the assay of chloramphenicol, its derivatives, and their many dosage forms, reflects the absorbance, irrespective of its origin, of the test solution at wavelengths from 271 to 278 mμ. Thus, the chloramphenicol ingredient must be separated from other components of its dosage forms, which can give erroneously high results. Also, the spectrophotometric procedure will not reflect degradative modifications not involving the nitrophenyl group.

In the course of his work on a colorimetric test for dihydrostreptomycin, Weiss (7) discovered that chloramphenicol, after acid and alkaline hydrolysis, produced a blue color with 1-naphthol in alkaline solution. This result proved to be unique among all the antibiotics then available. The present work was undertaken to establish the optimum conditions for the utilization of the reaction as an analytical procedure, and to check its specificity among various antibiotics and compounds structurally related to chloramphenicol.

The reduction, diazotization, and coupling method of Glazko (4) as modified by Levine and Fischbach (5), the ultraviolet spectrophotometric assay (6), and the microbiological assay (1, 2) were used as corroborative procedures. Further modification of the reduction-diazotization procedure was found to be of advantage, and the details of the method used are given below.

Received May 25, 1967, from the Division of Antibiotics and Insulin Certification, Bureau of Science, Food and Drug Administration, U. S. Department of Health, Education and Welfare, Washington, DC 20204

Accepted for publication September 11, 1967.

The author expresses his gratitude to Drs. Peter J. Weiss, William W. Wright, Daniel Banes, and Kenneth A. Freeman, and Mr. George Selzer, Food and Drug Administration, for their counsel during the course of this investigation.

The author acknowledges his debt to the Microbiological Branch, Division of Antibiotics and Insulin Certification, for the microbial assays, and to Parke, Davis and Co., Detroit, Mich., for providing compounds structurally related to chloramphenicol.

EXPERIMENTAL

Preparation of Chloramphenicol Samples

Chloramphenicol Powder—Accurately weigh approximately 50 mg. of the working standard or sample, transfer to a 250-ml. volumetric flask, dissolve in about 5 ml. of methanol, dilute to volume with water, and mix. For the analysis of chloramphenicol palmitate, dilute the standard to volume with chloroform instead of water.

Capsules—Place the contents of five 250-mg. capsules in a 250-ml. volumetric flask, swirl with approximately 25 ml. of methanol, bring to volume with water, and mix. Dilute this solution with water to an estimated concentration of 0.1 mg./ml.

Tablets—Grind five tablets to a fine powder. Accurately weigh approximately 60 mg. (equivalent to 50 mg. of chloramphenicol) and transfer to a 250-ml. volumetric flask. Add 10 ml. of methanol, swirl the mixture for several minutes, bring to volume with water, and mix.

Chloramphenicol Sodium Succinate Powder—Accurately weigh approximately 70 mg. (equivalent to 50 mg. of chloramphenicol), transfer to a 250-ml. volumetric flask, and dissolve in and bring to volume with water.

Chloramphenicol Palmitate Powder—Accurately weigh approximately 87 mg. (equivalent to 50 mg. of chloramphenicol), transfer to a 250-ml. volumetric flask, and dissolve in and bring to volume with chloroform.

Oral Suspension—Measure 1 ml. of suspension, using a syringe fitted with a 13-gauge needle, and place in a 125-ml. separator containing 25 ml. of chloroform. Shake the separator vigorously for

2–3 min., add 10 ml. of water, and shake vigorously for an additional 2–3-min. period. Allow the layers to separate and filter the chloroform layer through absorbent cotton one or more times until the filtrate is clear. Dilute an aliquot of filtrate with chloroform to a concentration of 0.2 mg. of chloramphenicol per ml.

1-Naphthol Method

Reagents—1-Naphthol, 2 mg./ml. in 6 *N* sodium hydroxide, prepared immediately before use by dissolving 1-naphthol in a small amount of methanol, adding 12 *N* sodium hydroxide and diluting 1:1 with water. Sodium hydroxide (12 *N*) prepared from a 50% solution.

Procedure—Into 100-ml. volumetric flasks place 4.0, 6.0, and 8.0 ml. of the standard solution and 6.0, 4.0, and 2.0 ml. of water to give a final volume of 10 ml. Use a flask containing 10 ml. of water as a blank.

Place 5.0 ml. of the 0.2 mg./ml. solutions of chloramphenicol or chloramphenicol sodium succinate into 100-ml. volumetric flasks and add 5.0 ml. of water.

In the case of capsules and aqueous injection, assay 10.0 ml. of solutions containing 0.1 mg./ml.

For chloramphenicol palmitate chloroform solutions, pipet 5.0 or 6.0 ml. into 100-ml. volumetric flasks and evaporate to dryness at room temperature, using a stream of air. Dissolve the residue in 1 ml. of methanol by careful swirling and then dilute with 10 ml. of water.

To each flask add 10 ml. of 12 *N* sodium hydroxide and 10 ml. of 1-naphthol solution. Swirl the flasks and place in a boiling water bath for 30 min.; then cool to room temperature within the next 5 min. in a tap water bath. Add methanol to volume and mix. After a color development period of 1 hr., measure the absorbance at 610 mμ against the reagent blank, using a suitable spectrophotometer.

Precision of the 1-Naphthol Method—A series of 75 absorbance *versus* concentration experiments, each involving from one to four concentrations, was performed and the 200 absorptivity (1 mg./ml., 1 cm.) values ranged from 24.1 to 40.4 (average 33.3), with 95% of them being between 30.0 and 37.3. The deviation of any absorptivity value from the average in those experiments with two or more samples was less than 3% for 158 of the 172 values determined. Because of the large variation in absorptivity values among different groups of two or more as opposed to the good precision obtained within groups, it was concluded that the absorptivity value of the standard should be determined each time samples are assayed. In addition, the absorptivity value obtained varied with different lots of 1-naphthol.

Effect of Changes in Conditions, Reagents, and Procedure—The blue color could not be extracted into chloroform from alkaline solution. However, when the solution was acidified and extracted with chloroform, and the chloroform extract shaken with fresh alkali, the color appeared in the alkaline phase.

When the blue solution was gradually neutralized, the blue color remained visible, though lessened, down to pH 9.6. From 9.6 to 9.1, it gradually disappeared, leaving a pink color. Addition of alkali restored the blue color.

It was found that 1-naphthol may be added to the reaction either before or after the heating step. Prior addition was found to be superior if aqueous solutions of chloramphenicol were employed, but subsequent addition was found to be advantageous when the chloramphenicol was dissolved in methanol, although the latter procedure was not sufficiently reproducible for quantitative applications.

The ability of 1-naphthol to produce the blue color decreased notably when this reagent was prepared in less concentrated (1 *N*) sodium hydroxide. When the heating was done in less concentrated alkali or the alkali was partially neutralized before dilution with methanol, the blue color was less stable, *i.e.*, the final color would be purple or bluish-pink. The same result occurred when in the final step the reaction mixture was diluted in water rather than methanol. When the solution was partially neutralized (pH 11 or 12) following hydrolysis, the blue color appeared more rapidly, but on standing it would not achieve the same final intensity and purity as when 6 *N* sodium hydroxide solution was used.

Low temperature (80–85°) reduced the reaction rate and was impractically slow, while high temperature (120–150°) experiments disclosed no apparent advantage.

In some experiments 10 ml. of water was added prior to dilution with methanol. This was done to eliminate carbonate precipitation which occurred occasionally after the addition of methanol.

Other alcohols, such as ethanol, 1-propanol, or isopropanol, either decreased the blue color (ethanol) or produced a green solution (propanol).

When dissolved in methanol, *p*-nitrobenzaldehyde reacted similarly to chloramphenicol, but when dissolved in water it produced a green color. Dichloroacetic acid, nitrobenzene, and benzaldehyde did not give a color.

2-Naphthol, 1,4-naphthoquinone, and 1-naphthol-3,6-disulfonic acid did not give a color response when substituted for 1-naphthol. When 1-naphthylamine and 1-hydroxy-2-naphthoic acid were substituted, the former gave a pink color of lower intensity and the latter gave an unstable blue color.

Mechanism of the 1-Naphthol Reaction—The determination of the chemical nature of the colored complex and of the products of the heating step was not deemed to be within the scope of this investigation. However, it is notable that *p*-nitrobenzaldehyde produces azoxy compounds (8) and chloramphenicol gives azo compounds (9) when heated in alkaline solution. A coupling reaction between 1-naphthol and azo compounds has been proposed (10). Further, the production of a blue color in concentrated sodium hydroxide by the reaction of 1-naphthol with *p*-nitrodiazobenzaldehyde has been used as a test for 1-naphthol in 2-naphthol (11).

A similar reaction, production of a blue color with thymol and chloramphenicol in alkali, is preceded by reduction of the chloramphenicol by means of aluminum wire (12).

Diazotization Method

Reagents—Five percent aqueous sodium nitrite, 5% aqueous sodium sulfamate, and 0.5% aqueous *N*-(1-naphthyl)ethylenediamine dihydrochloride solutions. Aqueous sodium hydrosulfite containing 25 mg./ml. prepared immediately before use.

Procedure—Into 100-ml. volumetric flasks pipet 1.0, 2.0, and 3.0 ml. of a 0.2 mg./ml. solution of

TABLE I—RESULTS OF FOUR ASSAY METHODS APPLIED TO CHLORAMPHENICOL SAMPLES

Sample	Label Claim	Assay Results Found by Four Methods— 1-Naphthol	Red.-Diaz.	UV	Microbiological
Chloramphenicol-1	1000 mcg./mg.[a]	992, 1016	[b]	989, 981, 981	1012
Chloramphenicol-2		997, 1003	[b]	981, 975, 981	1013
Chloramphenicol-3		1003, 973	[b]	1010, 980, 971	992
Chloramphenicol-4		1003	[b]	977	963
Capsule-1	250 mg./capsule	261, 251	[b]	250	[b]
Capsule-2		266, 257	[b]	250	[b]
Capsule-3		252, 264	[b]	252	[b]
Tablet	250 mg./tablet	250, 254, 245, 251	246, 259 246, 255	242, 249	[b]
Aqueous injection	1 Gm./vial	1.090, 1.071, 1.051 1.062, 1.065, 1.058	[b]	1.020, 1.140 1.130	[b]
Chloramphenicol sodium succinate	726 mcg./1 mg.[a]	710, 736, 746, 722, 710	[b]	747, 743, 745	[b]
Chloramphenicol palmitate-1	575 mcg./mg.[a]	562, 568	571, 577, 578	587, 587, 587	[b]
Chloramphenicol palmitate-2		599, 562	573, 541, 564	560, 560, 560	[b]
Oral suspension-1	31.25 mg./ml.	33.7, 33.2, 34.8 33.3, 33.7, 32.8	33.3, 31.8, 34.5 33.0, 34.4, 34.1	36.8, 35.4	[b]
Oral suspension-2		24.2 25.1	[b] [b]	25.6, 25.6	[b]

[a] Theoretical potency. [b] Not tested.

chloramphenicol standard, 2.0 ml. of 0.2 mg./ml. solutions of chloramphenicol powder, or the volume (1–3 ml.) equivalent to 0.4 mg. chloramphenicol of solutions prepared from other dosage forms as in the 1-naphthol method. Add 5.0 ml. of 0.1 *N* sodium hydroxide. (In the case of chloramphenicol palmitate, the chloroform solutions are evaporated to dryness, the residue is dissolved in 1 ml. of methanol, and 5.0 ml. of 0.1 *N* sodium hydroxide is added.) Place the flasks in a boiling water bath for 15 min., allow the flasks to cool to room temperature, and add 2 ml. of sodium hydrosulfite solution to each. Fifteen minutes later, add 1 ml. of sodium nitrite solution and 1 ml. of concentrated hydrochloric acid. After 3–5 min., add 2 ml. of sodium sulfamate. After another 3–5 min., during which the flasks are occasionally swirled vigorously, apply a stream of air to assure elimination of nitrous fumes. To each flask add 1 ml. of *N*-(1-naphthyl)ethylenediamine dihydrochloride solution, 25 ml. of methanol, and sufficient water to make 100 ml. After 2 hr. record the absorbance between 530 and 570 mμ. Peak absorption is observed at 553 ± 2 mμ.

RESULTS

Application of the 1-Naphthol Assay Method—Samples of chloramphenicol powder, capsules, tablets, and aqueous injection vials, chloramphenicol sodium succinate, and chloramphenicol palmitate powder and oral suspension were tested by the 1-naphthol colorimetric test and the ultraviolet spectrophotometric method, and in some cases by the reduction-diazotization procedure or the microbiological turbidimetric assay. As shown in Table I, results obtained by the 1-naphthol method compare favorably with the results of other procedures.

Tests on Degraded Material—Tests were run on a sample of chloramphenicol palmitate oral suspension which had been frozen in transit and thawed by heating. The suspension had a strong caramel odor and was dark brown (ordinarily suspensions have a pleasant flavory odor and are cream colored). As shown in Table II, there was good agreement between the 1-naphthol method and the reduction-diazotization procedure, while the ultraviolet spectrophotometric method gave considerably higher results. The former methods gave values for chloramphenicol content of a magnitude that would be reasonably expected and indicated the product was low in potency, while the latter method reflected excess nonspecific absorption due to the presence of degradation products.

TABLE II—ASSAY RESULTS ON DEGRADED CHLORAMPHENICOL PALMITATE ORAL SUSPENSION

Bottle	Extract	1-Naphthol[a]	Red.-Diaz.[a]	UV[a]
1	1	27.9	27.4	50.3
	2	27.9	27.1	56.0
2	1	24.0	26.9	41.6
	2	25.9	23.9	42.2
3	1	25.3	24.9	48.6
	2	25.0	23.7	48.4

[a] Milligrams chloramphenicol per ml. of suspension. Label claim 31.25 mg./ml.

Aqueous chloramphenicol standard solutions of various ages ranging up to 9 months were assayed by the various methods used above. The ultraviolet and reduction-diazotization methods indicated all solutions to be 100% potent. The microbiological and 1-naphthol assays reflected continuing loss of potency with age. However, the coincidence of the results produced by these two methods was only qualitative, not quantitative, in that the 1-naphthol assays did not show as much loss as the microbiological assays.

Specificity of the Methods—Several compounds structurally related to chloramphenicol were subjected to 1-naphthol, reduction-diazotization, ultraviolet, and microbiological assays. The results summarized in Table III reveal that all the chemical methods were insensitive to differences in structural configuration. The only other modifications to which the 1-naphthol method was insensitive were those involving the acyl group. The *m*-nitro analog of chloramphenicol did not give a blue color.

TABLE III—ASSAY OF CHLORAMPHENICOL AND STRUCTURALLY RELATED COMPOUNDS

Compd.	1-Naphthol Color	1-Naphthol mcg./mg.	Red.-Diaz. mcg./mg.	UV[a] mμ	UV[a] mcg./mg.	Microbiol. Assay[b] Plate[c]	Microbiol. Assay[b] Turb.[d]
Chloramphenicol FDA working std.	Blue	1000	1000	277	1000	1000	1000
Chloramphenicol project std.	Blue	982	982	276	1017	1000	970
Chloramphenicol sample	Blue	997	985	277	1014	1000	1000
p-Nitrobenzaldehyde	Yellow	0	2032	267	3027	0	12.0
p-Nitro-α-acetamino-β-hydroxypropiophenone	Blue	247	949	268	1916	23.1	11.8
L-Threo-1-*p*-nitrophenyl-2-amino-1,3-propanediol	Blue	1357	1451	275	1549	2.3	5.0
DL-Threo-1-*p*-nitrophenyl-2-amino-1,3-propanediol	Blue	1316	1503	276	1534	4.6	1.0
D-Threo-1-*m*-nitrophenyl-2-amino-1,3-propanediol	Green	0	1286	266	1211	0	8.0
L-Threo-1-*p*-nitrophenyl-2-acetamino-1,3-propanediol	Blue	1214	1288	277	1139	0	0.2
DL-Threo-1-phenyl-2-nitro-1,3-propanediol	Yellow	0	0	250	2317	0.8	2.0
D-Threo-1-phenyl-2-amino-1,3-propanediol	Yellow	0	3.5	No peak	0	50.6	5.6
L-Threo-1-phenyl-2-amino-1,3-propanediol	Yellow	0	0	No peak	0	3.2	0.3
DL-Threo-1-phenyl-2-amino-1,3-propanediol	Yellow	0	0	No peak	0	2.4	0.3
DL-Threo-1-phenyl-2-dichloroacetamido-1,3-propanediol	Yellow	0	2.7	No peak	0	0	21.0
D-Threo-1-*m*-nitrophenyl-2-dichloroacetamido-1,3-propanediol	Yellow	0	849	268	765	0	125

[a] Wavelength at which absorption maximum occurred. [b] In terms of mcg. of chloramphenicol equivalent potency per mg. [c] *S. lutea* (1). [d] *E. coli* (2).

1-*p*-Nitrophenyl-2-amino-1,3-propanediol gave 5.5 times the color response of *p*-nitro-α-acetamino-β-hydroxypropiophenone, while the reduction-diazotization and ultraviolet procedures yielded relatively little difference in response with these compounds. Otherwise, the only structural modification to which these methods responded was the loss of the nitrophenyl function. Some of the compounds structurally related to chloramphenicol possessed an antimicrobial activity ranging up to 12.5% of that of chloramphenicol.

SUMMARY

A new analytical method for chloramphenicol involves the reaction between 1-naphthol and the product of heating chloramphenicol in strong alkali. Certain advantages of specificity and applicability over present methods are obtained.

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Keyphrases

Chloramphenicol formulations
Chloramphenicol assay
Colorimetry—spectrophotometric analysis
1-Naphthol—reagent
Mechanism-1—naphthol reaction
Diazotization—analysis

Modification of USP Vitamin D Assay to Remove Interference of Vitamin E

By MODEST OSADCA and ELMER DE RITTER

When tocopherol is incorporated in multivitamin products at the 10–30 mg. levels, corresponding to the daily adult requirement, serious interference is encountered in determining vitamin D by the USP method. A modification of the USP assay method is described in which the tocopherol is separated by column chromatography on secondary magnesium phosphate prior to the normal USP chromatographic purification steps. Proper conditioning and performance testing of the secondary magnesium phosphate are essential to assure good separation of tocopherol and quantitative recovery of vitamin D.

THE RECOGNITION of a daily adult requirement for tocopherol in the range of 10–30 mg. (1) has led to increased usage of vitamin E in multivitamin supplements. For example, the formulas for decavitamin capsules and tablets in the USP XVII (2) include a suitable form of α-tocopherol at a level equivalent to 15 mg. of *dl*-α-tocopheryl acetate per capsule or tablet. Vitamin D levels, on the other hand, have tended to be lowered in such products due to increased emphasis on the desirability of avoiding intakes of vitamin D in excess of the recommended daily allowance (1). As a result of these developments, the ratio of vitamin E to vitamin D in many products has increased markedly to the point where the vitamin E causes considerable overestimation of vitamin D by the USP assay method (2).

A modified vitamin D assay procedure has been developed in which tocopherol is removed from the solvent extract by column chromatography on secondary magnesium phosphate prior to the normal chromatographic purification steps in the USP method. The use of secondary magnesium phosphate for separations in the assay of vitamin E has been described by Bro-Rasmussen and Hjarde (3). Careful conditioning of the adsorbent and performance testing before use are of critical importance in the vitamin E assay and also in the vitamin D assay described below.

Mulder *et al.* (4) separated tocopherols from vitamin D by column chromatography on alkaline alumina prior to passage through the diatomaceous earth[1] and Fuller's earth[2] columns, but the oils tested contained slightly less than 1 mg. of α-tocopheryl acetate per 1,000 units of vitamin D. Multivitamin mixtures containing 10–30 mg. of vitamin E plus 400 units of vitamin D represent ratios of vitamin E to vitamin D about 25 to 75 times as great as those reported by Mulder *et al.* (4). In our experience with alkaline alumina, when quantitative recovery of vitamin D was obtained, the removal of these higher levels of vitamin E was not complete and significant interference was encountered in the vitamin D assay.

EXPERIMENTAL

Reagents and Apparatus—These are described in USP XVII (2) with the following additions.

Diethyl Ether—must be free of peroxides; Mallinckrodt's black label has been suitable.

Petroleum Ether—Merck reagent grade benzin, boiling range 30–65°; purify by passing through a silica gel column until no absorption is found at 300 mμ.

Secondary Magnesium Phosphate—The following products have been used successfully when conditioned as directed below.

(*a*) Magnesium phosphate, dibasic, pure, E. Merck A. G., Darmstadt, West Germany—distributed by Brinkmann Instruments, Inc., Westbury, L. I., N. Y. (*b*) Magnesium phosphate, C.P., dibasic—distributed by Amend Drug and Chemical Co., Inc., New York, N. Y.

Conditioning Procedure for Secondary Magnesium Phosphate—Dry about 250 Gm. for 4 hr. at 180° and cool over concentrated H_2SO_4. Boil the powder for 10 min. with 2 L. of a solution of 16 Gm. of secondary sodium phosphate (anhydrous, reagent grade) in H_2O. Cool slightly, filter through Whatman No. 1 paper on a Büchner funnel, and wash with about 1.5 L. of H_2O. Dry the secondary magnesium phosphate for 48 hr. at 180° and cool to room temperature over concentrated H_2SO_4. Grind in a mortar, screen through a 120-mesh sieve, and store in a tightly closed container. Prior to use, transfer a weighed amount of this material to a jar, add 7% H_2O, close jar, and shake thoroughly to break up all lumps and distribute the water. Store in a tightly closed jar. Let stand at least 2–3 hr. before use and do not keep for more than 2 days after adding the H_2O.

Preparation of Column—Plug the lower, constricted end of a 17–20 mm. i.d. chromatography tube about 25 cm. long with glass wool. With the stopcock closed, fill about two-thirds with petroleum ether. Add conditioned secondary magnesium phosphate portionwise, allowing it to flow slowly into the tube and settle without pressure or vacuum until a column height of 10–12 cm. is

Received April 25, 1967, from the Products Development Department, Hoffmann-La Roche, Inc., Nutley, NJ 07110
Accepted for publication September 28, 1967.

[1] Celite, Johns-Manville Corp., New York, N. Y.
[2] Florex, Floridin Co., Pittsburgh, Pa.

obtained. Drain off the petroleum ether until the solvent is about 3 mm. above the surface of the adsorbent.

Column Performance Check—Using a mixture of 75 mg. of α-tocopheryl acetate and 25,000 USP units of vitamin A as acetate or palmitate, obtain a sample preparation as described under *Procedure*. Transfer the 10 ml. of petroleum ether extract onto the prepared column and open the stopcock. Wash down with two 5-ml. aliquots of petroleum ether, being careful not to let the surface of the column run dry. Elute with a 6:94 (v/v) mixture of diethyl ether–petroleum ether until the vitamin A zone (seen as a green-yellow fluorescence with the aid of a long wavelength UV light) reaches the glass wool plug. This should require 35–60 ml. of eluant and all of the tocopherol which precedes vitamin A is then in the eluate receiver. Change the receiver and elute the vitamin A with a 3:7 (v/v) mixture of diethyl ether–petroleum ether. Vitamin A elution as observed with the UV light should be complete with about 50 ml. of eluant.

Check recovery of vitamin D from a second, similar column of secondary magnesium phosphate by adding 10 ml. of a petroleum ether solution containing 2,000 USP units of vitamin D and no vitamin A or vitamin E. Proceed with chromatography as described above, using the volume of 6:94 diethyl ether–petroleum ether mixture determined above to be necessary to bring the vitamin A down to the glass wool plug. Change the receiver and elute the vitamin D with 70 ml. of the 3:7 mixture of diethyl ether–petroleum ether. Evaporate this second eluate and measure the vitamin D colorimetrically as per USP XVII. Quantitative recovery of vitamin D should be obtained with this procedure.

When a new lot of secondary magnesium phosphate is received, a portion should be conditioned and checked for performance as described above. Drying times and moisture content of the adsorbent influence its activity. If the above conditions are not optimal for a particular lot of secondary magnesium phosphate, they may have to be modified until the above performance criteria are met. If vitamin A spreads all through the column and moves down too rapidly, the adsorbent is not sufficiently active. Longer drying or less water addition may then be needed. If the vitamin A does not move down or moves too slowly, addition of more water is indicated.

Procedure—Obtain a sample preparation as described on p. 892 of USP XVII except to add 50 mg. of hydroquinone instead of 2 ml. of cottonseed oil prior to saponification and to take up the residue after evaporation of the solvent hexane extract in 10 ml. of petroleum ether rather than solvent hexane. The vitamin D level in the original sample taken may be as low as 2,000 units. Transfer an aliquot of the petroleum ether extract containing 2,000–4,000 USP units of vitamin D onto the prepared secondary magnesium phosphate column. Open stopcock and allow solution to drain into column without pressure or suction. Do not let top of column run dry. Wash down with two 5-ml. portions of petroleum ether. Elute tocopherol with 6:94 mixture of diethyl ether–petroleum ether until the vitamin A zone reaches the glass wool plug. Change the receiver and elute vitamin D along with vitamin A with 70 ml. of a 3:7 mixture of diethyl ether–petroleum ether. Evaporate the eluate just to dryness under a stream of nitrogen with the flask immersed in a water bath at 40°. Dissolve the residue in 5 ml. of solvent hexane. Take a 2 or 4-ml. aliquot containing about 1,600 USP units of vitamin D for "first column chromatography" as described in USP XVII and continue to follow the USP procedure.

Table I—Comparison of USP and Modified USP Assays for Vitamin D in Presence of Vitamin E

Vitamin D, USP units	Vitamin E, mg. *dl*-α-Tocopheryl Acetate	Vitamin A, USP units	—Vitamin D (or Equivalent) Found, USP units— USP XVII	Modified USP XVII	Difference
		Model Systems			
0[a]	15	0	150	10	140
			270	20	250
0[a]	30 (*dl*-α-Tocopherol)	4,000	300	30	270
400	15	5,000	580	401	179
			700	404	296
		Multivitamin Tablets			
(Added per tablet[b])					
A 600	15	25,000	790	580	210
			820	630	190
			810	600	210
			810	610	190
B 600	15	5,000	710	580	130
		Multivitamin Drops			
(per 0.6 ml.[b])					
500	10	2,500			
		Initial	612	480	132
		2 mo. at 45°	654	450	204
		3 mo. at 45°	636	444	192

[a] Samples without vitamin D were treated as if they contained 400 USP units per specified quantity of vitamin E. [b] Vitamin D content represents the actual amount added in manufacturing; vitamin A and E values represent label claims.

Applications of Procedure—Both the USP XVII procedure for vitamin D assay and the present modified procedure have been applied to (*a*) samples of vitamin E only, (*b*) a laboratory mixture of vitamins A, D, and E, (*c*) multivitamin tablets and drops, the latter including samples aged at 45°. In all cases the equivalent of 2,000 USP units of vitamin D based on label claim was taken as the original sample and the entire 2,000 units taken for the first column chromatography in both procedures.

RESULTS

The results are summarized in Table I. When only vitamin E is carried through the USP vitamin D assay procedure, a significant amount of brown color is obtained and measured as vitamin D. The additional chromatography on secondary magnesium phosphate in the modified procedure reduces this nonspecific blank to a very low level. In the model mixture of vitamins A, D, and E, the USP method overestimated the 400 units of vitamin D by 45–74% in the presence of 15 mg. of α-tocopheryl acetate. Recoveries in the two trials of the modified procedure with this combination of vitamins were 100 and 101%. Similarly, with multivitamin products the USP method overestimates the vitamin D content to a considerable degree (130–210 USP units on a total of 500–600 units). The results by the modified procedure are close to the actual levels of addition of vitamin D.

The magnitude of the interference due to tocopherol in the USP assay for vitamin D is not sharply reproducible from one sample or product to the next. On the basis of the results in Table I, it does not appear that a generally applicable correction to the USP assay value could be calculated based on the relative proportions of tocopherol and vitamin D and the dilutions used. Preliminary removal of the tocopherol *via* column chromatography on secondary magnesium phosphate provides an effective and reliable method of removing this source of interference.

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Keyphrases

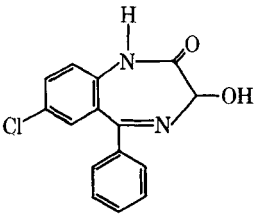
Vitamin D analysis
Vitamin E—interference elimination
Column chromatography—separation
Magnesium phosphate, secondary—column adsorbent
UV light—vitamin A fluorescence
Colorimetry—analysis

Qualitative and Quantitative Tests for Oxazepam

By EDWARD F. SALIM*, J. L. DEUBLE†, and G. PAPARIELLO†

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

7-CHLORO-1,3-DIHYDRO-3-HYDROXY-5-PHENYL-2H-1,4-BENZODIAZEPIN-2-ONE; $C_{15}H_{11}ClN_2O_2$; mol. wt. 286.72. The structural formula of oxazepam may be represented as I.



I

Physical Properties—Oxazepam occurs as a creamy white to pale yellow, practically odorless powder. It is practically insoluble in water, slightly

Received May 24, 1967, from the *Drug Standards Laboratory, American Pharmaceutical Association Foundation, Washington, DC 20037
Accepted for publication September 20, 1967.
† Wyeth Laboratories, Philadelphia, PA 19101. Wyeth Laboratories has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.
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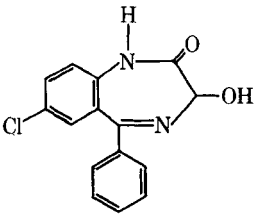

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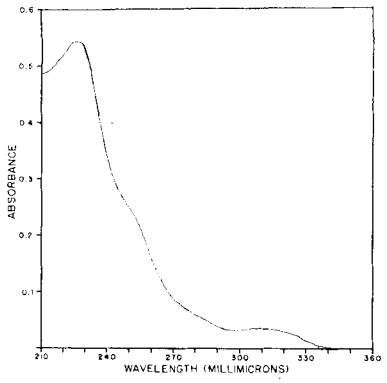

Fig. 1—Ultraviolet absorption spectrum of oxazepam in alcohol (4 mcg./ml.); Beckman model DK-2A spectrophotometer.

soluble in alcohol and in chloroform, and very slightly soluble in ether. The pH of a 2% suspension in carbon dioxide-free water is between 4.8 and 7.0.

Identity Tests—A 1 in 250,000 solution of oxazepam in alcohol exhibits an ultraviolet absorbance maximum at about 229 mμ [absorptivity (*a*) about 124]. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.5% dispersion of oxazepam in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 2.

Purity Tests—Dry about 1 Gm. of oxazepam, accurately weighed, at 105° in vacuum, maintaining the pressure below 5 mm. of mercury, for 3 hr.; it loses not more than 2% of its weight.

Char about 1 Gm. of oxazepam, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.3%.

Assay—Transfer about 400 mg. of oxazepam, accurately weighed, to a tall-form beaker, and dissolve in 100 ml. of dimethylformamide. Using a magnetic stirrer and taking precautions against absorption of atmospheric carbon dioxide in the sample and titrant, titrate the solution with 0.1 *N* tetrabutylammonium hydroxide [prepared in benzene–methanol (9:1)] to a potentiometric end point using glass *versus* calomel electrodes. Perform a blank determination, and make any necessary correction. Each milliliter of 0.1 *N* tetrabutylammonium hydroxide is equivalent to 28.67 mg. of $C_{15}H_{11}ClN_2O_2$. The amount of oxazepam found is not less than 98% and not more than 102%, calculated on the dried basis.

DOSAGE FORMS OF OXAZEPAM

Oxazepam Capsules

Identity Test—The ultraviolet absorption spectrum of the final solution prepared from the capsule sample in the *Assay* is similar to the spectrum obtained from the oxazepam standard solution.

Assay—Remove, as completely as possible, the contents of not less than 20 oxazepam capsules, and weigh. Transfer a quantity of mixed powder, equivalent to about 50 mg. of oxazepam, to a medium-porosity, sintered-glass funnel that is fitted into a small suction flask. Extract the sample with five 25-ml. portions of alcohol, transfer the combined

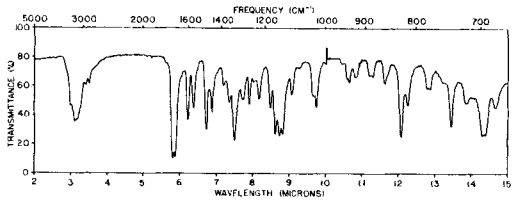

Fig. 2—Infrared spectrum of oxazepam in potassium bromide disk (0.5%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

extracts quantitatively to a 250-ml. volumetric flask, make to volume with alcohol, and mix. Transfer 2.0 ml. of the solution to a 100-ml. volumetric flask, dilute to volume with alcohol, and mix. Concomitantly determine the absorbance of this solution and of a standard solution of oxazepam, in the same medium, at a concentration of about 4 mcg./ml., in 1-cm. cells, at the maximum at about 229 mμ, with a suitable spectrophotometer, using alcohol as the blank. Calculate the quantity, in milligrams, of $C_{15}H_{11}ClN_2O_2$ in the portion of capsules taken by the formula $12.5 \times C(A_u/A_s)$, in which C is the exact concentration of the standard solution, in mcg./ml., A_u is the absorbance of the sample solution, and A_s is the absorbance of the oxazepam standard solution. The amount of oxazepam found is not less than 90% and not more than 110% of the labeled amount.

DISCUSSION

USP and NF terminology for solubility, melting range, reagents, *etc.*, has been used wherever feasible.

Oxazepam[1] is a psychotropic agent in the benzodiazepine class which is useful in the management and control of anxiety, tension, agitation, irritability and such related symptoms commonly seen in patients with a diagnosis of psychoneurotic or psychophysiological reaction. Oxazepam is structurally related to chlordiazepoxide and diazepam and the relative effectiveness of these compounds as tranquilizing agents has been reported (1, 2).

Identity Tests—The infrared spectrum of oxazepam constitutes an acceptable identification test for the compound. Ultraviolet absorption spectra in alcohol and 0.1 *N* alcoholic sulfuric acid can be used to differentiate the important benzodiazepine derivatives. The following tabulation indicates the wavelengths of maximum absorption for each compound in each solvent system.

	Wavelength, mμ	
	Alcohol	0.1 *N* Alcoholic Sulfuric Acid
Oxazepam	229	229
Diazepam	227	235, 280
Chlordiazepoxide hydrochloride	243, 262	247, 310

Quantitative Tests—Potentiometric nonaqueous titration of oxazepam with tetrabutylammonium hydroxide gave an average value of 98.9 ± 0.4%.[2] Preparation, standardization, and storage of the titrant have been detailed by Cundiff and Markunas (3). Determination of the end point potentiometrically is preferred to a visual titration with thymol

[1] Marketed as Serax by Wyeth Laboratories, Philadelphia, Pa.

blue T.S. since the indicator change which is somewhat gradual produces a greater variation in results. Similar titrations using azo violet or thymolphthalein were noted to give sharper end points but in each case a green color was observed at the stoichiometric point. The initial color of these indicators when added to a dimethylformamide solution of oxazepam is yellow and this leads to a green end point instead of the characteristic blue associated with the indicators.

Analysis of commercial 10-mg. oxazepam capsules by the spectrophotometric method gave a value of 97.8 ± 0.6%[2] of the labeled amount. A spectrophotometric approach can be used for analysis of bulk oxazepam provided suitable reference standard material is available for absorbance measurement.

[2] Maximum deviation from the mean value.

REFERENCES

(1) Geller, I., *Arch. Intern. Pharmacodyn.*, **149**, 243(1964).
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Keyphrases

Oxazepam
UV spectrophotometry–structure
IR spectrophotometry–structure
Potentiometric titration–analysis
Tetrabutylammonium hydroxide–titrant

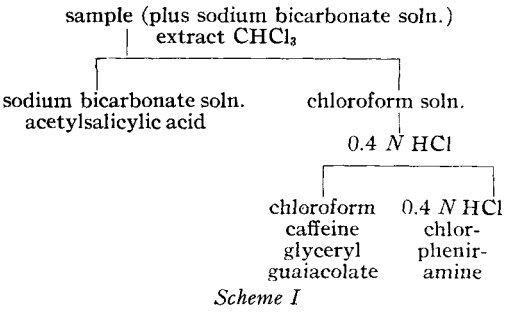
Paper Chromatographic Assay of Glyceryl Guaiacolate in a Pharmaceutical Formulation

By S. AHUJA*

A preliminary extraction scheme, which permits the separation of glyceryl guaiacolate [3-(*o*-methoxy-phenoxy)-1,2-propanediol] and caffeine from acetylsalicylic acid and chlorpheniramine maleate and other tablet excipients, has been developed. The separation of glyceryl guaiacolate and caffeine is achieved by paper chromatography. Glyceryl guaiacolate is then assayed on the basis of its absorbance in the UV region, after elution from paper chromatographic strips.

IN THIS LABORATORY, a simple and specific method was desired for assaying glyceryl guaiacolate in tablets containing glyceryl guaiacolate, acetylsalicylic acid, caffeine, and chlorpheniramine maleate in addition to the usual tablet excipients. Glyceryl guaiacolate can be determined titrimetrically (1, 2) or by gas chromatography of its acetate derivative (3). Giebelmann (4) described paper chromatographic methods (by the ascending technique) for qualitative detection of glyceryl guaiacolate. Since paper chromatography provides a good combination of a simple and specific method, this technique was investigated. This report describes a quantitative method for determination of glyceryl guaiacolate with paper chromatography (by the descending technique) with a newly developed solvent system. This solvent system provides good separations within 3 hr. Successful separation of the various constituents of the formulation is shown in Scheme I.

Glyceryl guaiacolate and caffeine are separated by paper chromatography and glyceryl guaiacolate is assayed spectrophotometrically after elution from paper chromatographic strips.



Scheme I

EXPERIMENTAL

Materials and Methods—An accurately weighed sample (equivalent to 195 mg. of glyceryl guaiacolate) is transferred to a separator. Sodium bicarbonate solution (25 ml. of 4% solution) is added and the separator is shaken well to ensure dissolution of the sample. This solution is extracted with chloroform (30 ml.) and the chloroform extract is transferred to another separator and shaken with 0.4 *N* hydrochloric acid (25 ml.). The chloroform extract is saved for paper chromatographic assay of glyceryl guaiacolate. This process is repeated

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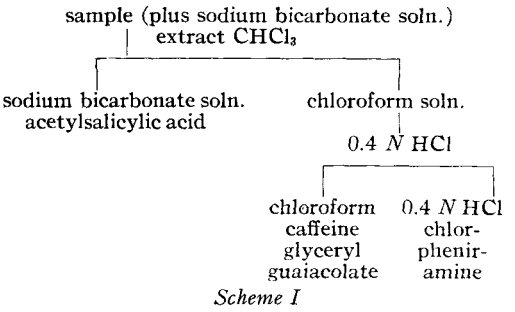

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TABLE I—RECOVERIES WITH THE PAPER CHROMATOGRAPHIC METHOD

Batch	mg. Glyceryl Guaiacolate/Tablet—Actual	Found	% Recovery
A	195.0	195.2	100.1
		191.0	98.0
B	195.0	193.6	99.3
		194.0	99.5
C	195.0	195.0	100.0
		193.2	99.1

four more times using fresh portions of chloroform, but using the same 0.4 *N* hydrochloric acid solution. The combined chloroform extracts are diluted to yield a concentration of 1 mg./ml. of glyceryl guaiacolate. One hundred microliters of this extract is placed on paper chromatographic strips[1] (20 in. long) impregnated with the lower phase of the following solvent system: benzene–ethanol–water (2:1:1). Blank strips and strips with a standard solution of the same concentration (1 mg./ml.) are prepared similarly. The strips are allowed to equilibrate for 2 hr. in a tank saturated with the above-mentioned solvent system, and developed with the upper phase of the solvent system (developing time approximately 3 hr.). Glyceryl guaiacolate and caffeine spots are located with a UV lamp (R_f of glyceryl guaiacolate is 0.52, R_f of caffeine, 0.70).

Glyceryl guaiacolate spots from two strips are combined and eluted with 10 ml. ethanol. The absorbance of these solutions is read at 275 mμ with a spectrophotometer and corrected for the paper blank. (The absorbance readings of the sample without correction are generally over 13 times the magnitude of the paper blank reading. Paper blanks and placebos yield absorbance readings of approximately 0.020 units.) The concentration of glyceryl guaiacolate is calculated against the standard, similarly corrected for the paper blank.

[1] Whatman No. 1.

The results for three different batches of glyceryl guaiacolate tablets, with a known concentration of glyceryl guaiacolate, are given in Table I.

CONCLUSIONS

An average recovery of 99.3% (S.D. = ±1.05) may be achieved by the paper chromatographic method of assay described for glyceryl guaiacolate tablets. This method is simple, reliable, and specific. The specificity is achieved by the separation of the desired component by the paper chromatography followed by its determination at the appropriate wavelength in the ultraviolet region. The concentrations of glyceryl guaiacolate estimated were in the order of 195 mg. However, the design of this method permits estimations down to approximately 0.5 mg. of glyceryl guaiacolate or as high as might be desired. Furthermore, this method permits the quantitative determination of the other ingredients present in the formulation, such as acetylsalicylic acid, chlorpheniramine maleate, and caffeine, by the conventional methods.

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Keyphrases

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Paper chromatography–separation
UV spectrophotometry–analysis

Colorimetric Assay and Improved Method for Identification of Vancomycin Hydrochloride

By JAMES R. FOOKS, IAIN J. MCGILVERAY, and ROBERT D. STRICKLAND*

Procedures are described for the qualitative and quantitative determination of vancomycin hydrochloride by means of spectrophotometry and thin-layer chromatography. The Folin-Ciocalteau reaction which forms the basis of both procedures is also amenable to the estimation of vancomycin factor A, a commonly occurring impurity.

VANCOMYCIN, an antibiotic obtained from *Streptomyces orientalis* (1), is retained for use against Gram-positive cocci resistant to the more common antibiotics and for treatment of patients allergic to penicillins and cephalosporins (2). The structure of this drug has yet to be fully established, but chemical studies (3–5) have demonstrated the presence of carboxyl, amino, and phenolic groups. Glucose and amino acid fragments have also been identified after partial hydrolysis. Vancomycin as obtained from *S.*

Received August 4, 1967, from Research Laboratories, Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada.
Accepted for publication September 27, 1967.
The authors are grateful to Miss K. Fitzpatrick, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, for the microbiological results.
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By JAMES R. FOOKS, IAIN J. MCGILVERAY, and ROBERT D. STRICKLAND*

Procedures are described for the qualitative and quantitative determination of vancomycin hydrochloride by means of spectrophotometry and thin-layer chromatography. The Folin-Ciocalteau reaction which forms the basis of both procedures is also amenable to the estimation of vancomycin factor A, a commonly occurring impurity.

VANCOMYCIN, an antibiotic obtained from *Streptomyces orientalis* (1), is retained for use against Gram-positive cocci resistant to the more common antibiotics and for treatment of patients allergic to penicillins and cephalosporins (2). The structure of this drug has yet to be fully established, but chemical studies (3–5) have demonstrated the presence of carboxyl, amino, and phenolic groups. Glucose and amino acid fragments have also been identified after partial hydrolysis. Vancomycin as obtained from *S.*

Received August 4, 1967, from Research Laboratories, Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada.
Accepted for publication September 27, 1967.
The authors are grateful to Miss K. Fitzpatrick, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, for the microbiological results.
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orientalis may contain an unidentified fraction, factor A, which has similar chemical properties (5).

The USP XVII (6) refers to the Federal Register (7) for identity and assay procedures. Tests described in B.P. 1963 (8) and the Federal Register for identity and potency of vancomycin depend on microbiological assay using *Bacillus subtilis*. The official paper chromatographic methods (7, 8) for identifying vancomycin and estimating vancomycin factor A also employ this microorganism.

Betina included vancomycin in a systematic study of antibiotics by paper chromatography (9). Lightbown and de Rossi (10) described the separation of four biologically active compounds from vancomycin international reference standard by electrophoresis in agar gel. No reports could be found in the literature of a substitute for the time-consuming paper chromatographic and bioautographic procedures used in the characterization of vancomycin hydrochloride.

The object of this paper is to supplement and improve the present standards by providing chemical methods for the identification and assay of the antibiotic and a more rapid and sensitive means of separating it from closely related substances.

EXPERIMENTAL

Vancomycin hydrochloride injectable and vancomycin factor A used in this study were kindly supplied by the Eli Lilly & Co., Indianapolis, Ind. The "fresh" sample was dated 1966 and the "aged" had an expiration date in 1960. Vancomycin hydrochloride USP reference standard was obtained from USP Reference Standards, New York, N. Y.

Colorimetric Assay—In the Folin-Ciocalteau method the reagents were sodium carbonate 5% aqueous and Folin-Ciocalteau phenol reagent.[1]

A solution of vancomycin USP reference standard (1 ml.) representing 10 mcg./ml. was treated with sodium carbonate (1 ml.) reagent followed by diluted 1:3 Folin-Ciocalteau reagent (0.5 ml.). The solution was then made up to a volume of 5 ml. The development of color in this solution was followed by absorbance measurements at 725 mμ every 15 min. for 2 hr. A reagent blank was prepared by replacing the antibiotic with distilled water.

A calibration curve was obtained with standard amounts of vancomycin hydrochloride USP reference standard. The Folin-Ciocalteau and sodium carbonate reagents were added as described above and the absorbance of each solution at 725 mμ was measured after 1 hr.

Two commercial samples of vancomycin hydrochloride were assayed using this standard curve.

Microbiological Assays—The assay procedure for vancomycin hydrochloride described in the Federal Register was used (7).

Tests for Identity and Purity Using Thin-Layer Chromatography (TLC)—*Chromatographic Plates*—(*a*) Glass plates (20 × 20 cm.) were coated to a thickness of 250 μ with a slurry of Kieselguhr G (Merck) (40 Gm.) in distilled water (80 ml.) according to the method of Stahl (11) and left to dry overnight. They were used within 2 days without preactivation. (*b*) Glass plates were coated as above with Silica Gel G (Merck), but activated at 105° for 10 min. before use.

Solvent Systems—(*a*) Benzene–*n*-butanol–water (20:20:100) aqueous phase. (*b*) Methanol–water (1:99).

Detection Agent—(*a*) Sodium carbonate 20% aqueous and (*b*) Folin-Ciocalteau phenol reagent diluted 1:2 with distilled water.

Samples of an aqueous solution of vancomycin hydrochloride and factor A each representing 10 mcg. were applied to the same point on a Kieselguhr-coated plate by means of a Hamilton microsyringe. After drying in a stream of warm air, the plates were inserted in a previously equilibrated, filter paper-lined tank saturated with solvent system (*a*) and chromatographed to a height of 15 cm. (approximate time taken 50 min.). The residual solvent was removed immediately in a stream of warm air and the spots located by spraying with 20% sodium carbonate solution followed by Folin-Ciocalteau reagent. Gray-blue areas were observed for both vancomycin hydrochloride and factor A.

The sequence was repeated for 5 mcg. of vancomycin and factor A on Silica Gel G with solvent system (*b*). Approximate R_f values in each system were calculated from 10 plates. The limit of detection was determined by spotting dilutions of vancomycin hydrochloride USP reference standard and factor A separately in amounts ranging from 10 to 0.01 mcg. on five plates. Both compounds were visible at concentrations below 0.1 mcg.

The approximate R_f values of a number of drugs which also react with Folin-Ciocalteau reagent were compared by TLC in the above systems.

Quantitative TLC by Densitometry—The instrument used to scan the plates was a Photovolt densitometer (model 520M) equipped with a TLC stage, attached to a Varicord recorder (model 42B), a search unit C, and an Integraph integrator. A blue filter (max. 420 mμ) and a slit 0.1 mm. wide and 6 mm. long were used. The distance between plate and search unit was maintained at 1 mm. with a response setting of 10.

Thin-layer chromatograms of 2–10 mcg. quantities of vancomycin hydrochloride were developed with solvent system (*a*) on Kieselguhr. The plates were dried without delay in a stream of warm air and then sprayed immediately with 20% sodium carbonate solution. After drying thoroughly, they were sprayed with freshly prepared Folin-Ciocalteau reagent (1:3) and dried for 15 min. in a stream of warm air.

Densitometric scanning began as soon as the sprayed plate was dry and each spot was measured separately. The densitometer was vectored to the highest reading at the center of the spot, the plate clamped firmly in position, and the densitometer readjusted to the lowest background reading. Subsequently automatic scanning was effected in the direction of solvent flow. The operation was performed in a darkened room to prevent interference from stray light.

[1] Folin-Ciocalteau phenol reagent manufactured by Hartman-Ledden Co., Philadelphia, Pa.

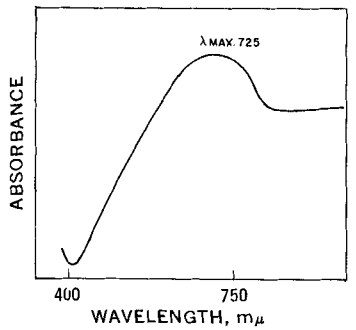

Fig. 1—Reaction of vancomycin with Folin-Ciocalteau reagent. Absorption curve of the product.

TABLE I—TIME OF COLOR DEVELOPMENT *vs.* ABSORBANCE FOR VANCOMYCIN WITH FOLIN-CIOCALTEAU REAGENT

Time, min.	Absorbance
0	0.075
15	0.116
30	0.148
45	0.162
60	0.166
75	0.173
90	0.178
105	0.180
120	0.186

The integrated areas were evaluated to give a standard curve. The process was repeated and some mixtures of vancomycin and factor A were estimated from the curve.

RESULTS AND DISCUSSION

The presence of phenolic groups in vancomycin has been demonstrated (1, 5). The phenolic moieties have been found to give the typical Folin-Ciocalteau reaction resulting in a blue-gray color (absorption maximum at 725 mμ) (Fig. 1). Color intensity was found to increase rapidly up to 1 hr. and, though a slight increase occurred after this time (Table I), the readings taken were sufficiently reproducible for assay purposes. The pH of the solutions produced by the reaction conditions averaged 9.6. When the pH was increased using more concentrated sodium carbonate solutions (*i.e.*, 20%), the color intensity of the product was lower and carbon dioxide was evolved which interfered in the measurement of color. Under the conditions used, Beer-Lambert's law was obeyed over the range 0.4–100 mcg./ml. Experimental results showing a comparison of the three vancomycin samples are shown in Table II which also lists the microbiological results. Both sets of results compare favorably.

Higgins *et al.* (3) observed that vancomycin, after hydrolysis in acid or alkaline solution, reacted with ninhydrin to give a positive test for amino groups. In our efforts to obtain a quantitative response with this reaction, various conditions were investigated for acid hydrolysis of vancomycin and for color development with the ninhydrin reagent. Results using the method of Yemm and Cocking (12) devised for amino acids were found to be inconsistent, and this reaction cannot be recommended as useful for the determination of vancomycin.

TABLE II—DETERMINATION OF SAMPLES OF VANCOMYCIN—COMPARISON OF COLORIMETRIC AND MICROBIOLOGICAL RESULTS

Sample	Absorbance	Concn. Found, mcg./ml.	Concn. Expected, mcg./ml.	%	% Microbiological
USP (reference)	0.151	10	10.0	100	100
Aged	0.129	8.50	10.0	85	85
Fresh	0.156	10.25	10.0	102.5	102

TABLE III—R_f VALUES OF SEVERAL ANTIBIOTIC DRUGS IN TWO SOLVENTS[a]

	System (*a*)	System (*b*)
Vancomycin	0.98	0.54
Factor A	0.87	0.24
Bacitracin	...	0.10
Chlortetracycline HCl	0.13	0.18
Gramicidin	...	0
Griseofulvin	...	0.4[b]
Sodium methicillin	0.98	0.80
Novobiocin	0.98[b]	0 to 0.4[b]
Oxytetracycline hydrochloride	0.87[b]	0.15
Polymyxin B sulfate	...	...
Tyrocidine hydrochloride	0	0
Tyrothricin	0	0

[a] Spray reagent: Folin-Ciocalteau. [b] Streaking; no reaction.

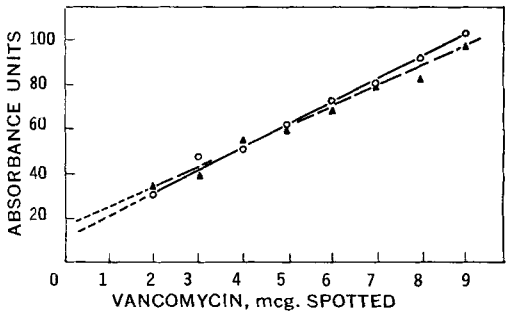

Fig. 2—Standard curves from TLC densitometry. Key: ○, USP vancomycin; ▲, USP vancomycin with 1 mcg. factor A.

The systems described for thin-layer chromatography were chosen after an empirical study of many solvent-adsorbent variations. Solvent system (*a*) was satisfactory for the separation of vancomycin from factor A up to the 20-mcg. level. Adverse tailing effects were observed with greater concentrations. With solvent system (*b*) on silica gel, effects of overloading were apparent with even less material and the system, though very rapid, is recommended only as an auxiliary for separation of vancomycin from other drugs as seen in Table III. From this table it is evident that system (*a*) does not differentiate methicillin and novobiocin from vancomycin. In solvent system (*b*), however, they

exhibit different R_f values. Table III shows the average R_f value of vancomycin and factor A in the two solvent systems.

Vancomycin and factor A could be determined semiquantitatively by densitometry over a range from 2 to 10 mcg. Reliable scanning was difficult with greater amounts because of streaking, especially where mixtures of the two components were involved. Figure 2 shows the linear curve of vancomycin (USP reference standard) concentration *versus* average spot density calculated from five plates and the effect of factor A on this curve. Factor A has little or no effect on the lower range (3 to 6 mcg.) but appears to inhibit color development at higher levels.

The vancomycin factor A used in this work was impure, as it contained approximately 20% vancomycin as estimated by TLC. The R_f values quoted as "factor A" are for the major, slower running spot. In making the densitometer readings for vancomycin in mixtures it was necessary to begin the scan from the lowest background reading between the spots for vancomycin and factor A.

The Folin-Ciocalteau assay is obviously not specific for vancomycin hydrochloride and should not be used in the absence of other criteria of identity. One such procedure is thin-layer chromatography which also detects the presence of interfering materials including factor A. Where nonphenolic impurities occur, the Folin-Ciocalteau method is recommended as the most convenient assay. Where impurities such as factor A are encountered, the semiquantitative assay of vancomycin by TLC with Folin-Ciocalteau spray followed by densitometry provides an alternative.

These data show that the chemical methods may supplement the current microbiological procedures for the qualitative and quantitative determination for this drug.

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Keyphrases

Vancomycin HCl
Colorimetry—analysis
Folin–Ciocalteau reaction
Microbiological analysis
TLC–densitometric scanning
Factor A–vancomycin—TLC separation

Technical Articles

Design and Evaluation of a Miniature Air-Suspension Coating Apparatus

By HAL N. WOLKOFF, GEORGE PINCHUK, and PAUL H. SHAPIRO

Experiments were conducted, using a miniature air-suspension coating apparatus designed and built in these laboratories, to determine the feasibility of uniformly coating gram quantities of various sized and shaped particles. The coating chamber used has an inner diameter of 1.22 in. and an overall length of 4.875 in. A schematic drawing of the apparatus is presented and the function of each component as well as its critical dimensions are discussed. The coating capabilities of the apparatus were explored using nonpareil seeds, tablets of several sizes and shapes, and hard gelatin capsules. Data are presented to compare the relative coating efficiency of the miniature unit with the widely used 6-in. Wurster apparatus. The results indicate that minor differences do exist in terms of coating efficiency but that the two units are comparable with respect to the uniformity of coating which can be achieved.

AIR-SUSPENSION coating techniques have generated considerable interest since Wurster's disclosures in 1953 and 1957 (1, 2). The process has been widely used on an experimental basis and, to a lesser extent, in commercial production. It has as its major advantages simplicity of operation, versatility, rapidity, and uniformity of the final product. As opposed to conventional pan coating techniques, this process provides for rigorous control over the many factors responsible for batch-to-batch variability.

Received April 14, 1967, from the Department of Pharmaceutical Research, Schering Corp., Bloomfield, NJ 07003
Accepted for publication September 11, 1967.
Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
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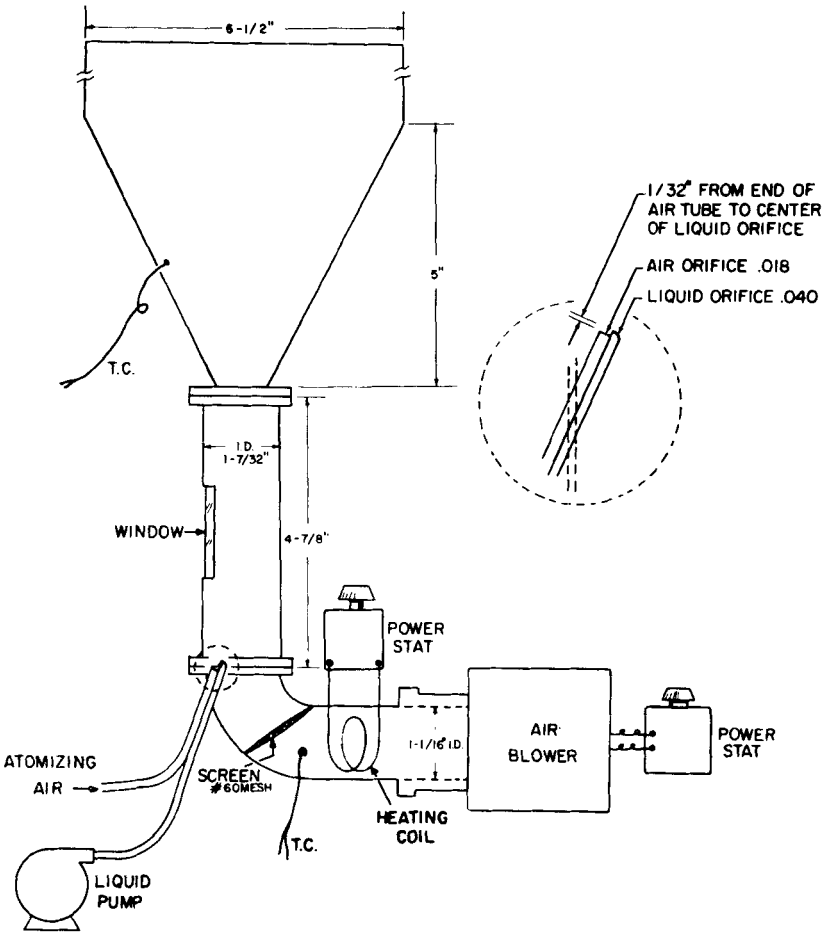

Fig. 1—Schematic diagram of the miniature air-suspension coating apparatus.

Several reports have appeared in the literature concerning the design and utility of laboratory air-suspension coating devices (3–6). The optimum load for each of these units is in the kilogram range. In the authors' experience, it has been frequently desirable to work with much smaller quantities of material. This may be the case when a drug is in short supply during early biopharmaceutical studies, or when new coating agents which are not yet available in sizeable quantities are being evaluated.

An air-suspension coating apparatus, capable of processing gram quantities of material, ideally satisfies these needs. This report deals with the design and evaluation of such a unit, referred to as a miniature air-suspension coating apparatus.

EXPERIMENTAL

Apparatus—A schematic diagram of the miniature air-suspension coating apparatus is shown in Fig. 1. The air supply is provided by a Master heat gun, model HG 301 (Master Appliance Corp., Racine, Wis.). The heating element and blower were rewired to allow for independent control of air volume and temperature. The unit has a maximum capacity of approximately 35 cu. ft. of air per min. Air output can be controlled by either varying the voltage delivered to the blower or by adjusting the air intake shield.

A Sigmamotor, model T8, equipped with a Westinghouse hi-torque speed control, type 902, was used to deliver the coating solutions through Tygon tubing ($^1/_{32}$ or $^1/_{16}$ in. i.d. × $^1/_{32}$ or $^1/_{16}$ in. wall thickness) to the atomizing nozzle. The coating solution was aerosolized at the point of entry into the coating chamber by the construction shown in the insert of Fig. 1. The air-liquid tubes of the atomizer head, coating chamber, and expansion chamber were constructed of No. 316 stainless steel. A clear, solvent-resistant plastic window[1] was inserted into the coating chamber to permit observation of the coating process.

The inlet and outlet air temperatures are determined by the use of electrical thermometers model 380 (Radio Frequency Labs, Inc., Boonton, N. J.).

A photograph of the apparatus and auxiliary equipment are shown in Fig. 2. The panel board is equipped with inlet and outlet air temperature gauges, atomization air pressure control knob and gauge, and a master on-off switch. The variable transformers shown on the right side of the photo-

[1] Saranex, trademark of Dow Chemical Co.

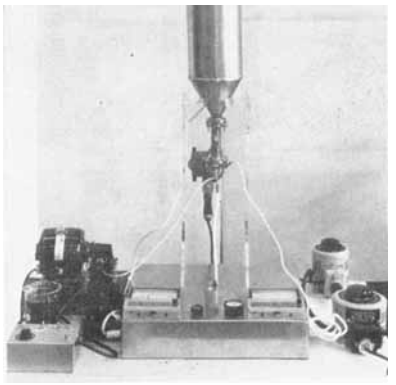

Fig. 2—Miniature air-suspension coating apparatus and ancillary equipment.

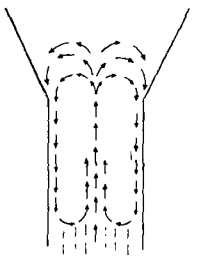

Fig. 3—Schematic representation of optimum particulate flow pattern.

graph are for air volume and temperature control. The control box to the left governs the race at which the Sigmamotor delivers coating solution to the atomizing head.

Materials—Eastman cellulose acetate phthalate, Distillation Products Industries; ethylcellulose, 50 cps., pharmaceutical grade, Dow Chemical Co.; isopropyl alcohol, acetone, and chloroform, reagent grade; castor oil USP; syrup USP; D&C Red No. 21, H. Kohnstamm and Co.; chlorpheniramine maleate tablets, 4 mg.;[2] and nonpareil seeds coated with chlorpheniramine maleate, 4.0 mg./1.64 Gm. (prepared by applying a sugar solution containing the drug to 20-mesh nonpareil seeds in a coating pan).

Coating Solutions—Ethylcellulose solution was prepared by dissolving 12.5 Gm. of polymer and 0.05 Gm. D&C Red No. 21 in 1 L. of a 50/50 acetone–isopropyl alcohol mixture.

Cellulose acetate phthalate solution was prepared by dissolving 25 Gm. of CAP and 7.5 Gm. of castor oil in 500 ml. acetone and then adding sufficient isopropyl alcohol to adjust the volume to 1 L.

RESULTS AND DISCUSSION

Equipment Characteristics—The feasibility of coating particles using the air-suspension technique depends, in addition to the surface characteristics and strength of the particle, on establishing an appropriate flow pattern. The desired flow pattern is schematically presented in Fig. 3. The extent to which a circulation pattern of this type can be established is determined by the particle size, shape, and density, the total weight of material in the coating chamber, and the air distribution in the operating parts of the unit.

The general utility of the miniature air-suspension apparatus was determined by evaluating the flow patterns established with a number of particles having different sizes and shapes. This information is presented in Table I. Obviously, variations in either the atomization air pressure or fluidizing air velocity would result in different optimum and maximum chamber loads. As the load is decreased from optimum, the circulation pattern becomes erratic.

TABLE I—CONDITIONS FOUND TO ESTABLISH CIRCULATORY FLOW PATTERNS WITH VARIOUS SIZED AND SHAPED PARTICLES[a]

Particle Description	Optimum Load, Gm.	Max. Load, Gm.
$^5/_{16}$ in. Std. concave tablet Av. gauge = 0.145 in. wt. = 200 mg.	4.00	8.00
$^7/_{16}$ in. Std. concave tablet Av. gauge = 0.220 in. wt. = 550 mg.	1.65	5.50
Oval concave tablet (0.200 × 0.300 in.) Av. gauge = 0.150 in. wt. = 170 mg.	4.00	8.00
Clover leaf tablet ($^5/_{16}$ in.) Av. gauge = 0.150 in. wt. = 170 mg.	3.91	7.82
Hexagonal tablets ($^7/_{16}$ in.) Av. gauge = 0.220 in. wt. = 570 mg.	2.85	5.70
No. 1 gelatin capsule wt. = 410 mg.	2.05	2.46
No. 0 gelatin capsule wt. = 530 mg.	1.59	2.12
Nonpareil seeds (20 mesh)	10.00	16.00

[a] The atomization air pressure was maintained constant at 20 p.s.i. and the transformer setting for the fluidizing air was 90 (intake shield at maximum opening).

Coating Studies—During the course of biopharmaceutical testing, it is often desirable to evaluate enteric-coated dosage forms. At this stage of new drug research materials are frequently in short supply, and therefore a technique for handling small amounts of material is highly desirable. The miniature air-suspension coating apparatus was evaluated as a tool for applying cellulose acetate phthalate (CAP) to a variety of solid dosage forms.

The typical coating procedure used was as follows. The fluidizing air is turned on and brought to the appropriate temperature range by the adjustment of the heating coil transformer. The coating solution is pumped through the feeding tube to the atomizer head. The atomization air is turned on and the particles introduced into the coating chamber by way of the exhaust air duct. The coating rate is controlled so that about a 20° differential exists between the inlet and outlet air temperatures. When all of the solution has been applied, the pump and atomization air is turned off. A few minutes of additional drying time are provided to minimize solvent retention. The heating coil and fluidizing air are then turned off, the unit disassembled, and the coated material recovered.

The enteric properties of the CAP-coated tablets and capsules were evaluated using the USP XVII procedure (7).

A modification of this procedure was used to evaluate the enteric-coated nonpareil seeds. The seeds were placed into a stainless steel cylinder

[2] Chlor-Trimeton Maleate, trademark of Schering Corp.

(25 × 64 mm.) covered at each end with an 80-mesh stainless steel screen. The cylinder was moved in a vertical plane beneath the surface of the test fluid by means of a Stoll-Gershberg apparatus (8). The nonpareil seeds were examined after 1 hr. in simulated gastric fluid at 37 ± 2°. Any visual evidence of nonpareil seed disintegration was interpreted as a failure of the enteric coating. The same procedure was repeated using simulated intestinal fluid and the time for total disintegration (more than 90% of material passing through the screen) was recorded.

The results presented in Table II demonstrate the versatility with which the apparatus can be used to enteric coat a spectrum of solid pharmaceutical dosage forms. It should be pointed out, however, that the uniform disintegration times of the various dosage forms in simulated intestinal fluid is probably best interpreted as due to the high solubility of cellulose acetate phthalate in this media rather than as an indication of uniform film thickness.

The data presented in Table III clearly demonstrate the effect of coating suboptimal chamber loads. Coating efficiency decreases markedly with decreasing chamber loads, probably due to an erratic flow pattern and decreased bed density. Coating efficiency for very small chamber loads can be increased by decreasing the diameter of the coating chamber. This was done by inserting a 0.75-in. i.d. tube mounted with rubber stoppers into the coating chamber. One gram of 5/16-in. standard concave tablets was coated with 2.5 ml. of 2.5% CAP solution. The coated tablets weighed 1.065 Gm., indicating a coating efficiency of approximately 80% as opposed to 12% when the same quantity of tablets was coated in the 1.22-in. chamber.

Table II—Enteric Dosage Forms Prepared in Miniature Air-Suspension Coating Apparatus

Batch No.	Chamber Load, Gm.	CAP Coating Applied, %	Disintegration After 1 hr. in Simulated Gastric Fluid at 37°	Disintegration Time (min.) in Simulated Intestinal Fluid at 37° (Range)
	Std. Concave Tablets, 5/16 in.			
1	4.0	8	none	12–15
2	4.0	8	none	12–15
3	4.0	16	none	12–15
4	4.0	16	none	12–15
5	4.0	16	none	12–15
6	2.0	16	none	10–11
7	2.0	16	none	8–9
8	2.0	16	none	8–9
9	1.0	16	none	11–12
10	1.0	16	none	11–12
11	1.0	16	none	11–12
12	1.0	16	none	11–12
13	1.0	16	none	11–12
14	1.0	16	none	11–12
	Oval Concave Tablets, 0.2 × 0.3 in.			
15	4.0	12	none	10–15
	Clover Leaf Tablets, 5/16 in.			
16	3.91	12	none	10–15
	Hexagonal Tablets, 7/16 in.			
17	5.70	9	none	10–15
	Std. Concave Tablets, 7/16 in.			
18	2.75	9	partial	...
19	2.75	18	none	10–15
	No. 1 Gelatin Capsules			
20	2.05	16	none	5–10
	No. 0 Gelatin Capsules			
21	1.60	20	none	5–10
	Nonpareil Seeds, 20 mesh			
22	10.00	5	partial	...
23	10.00	7	none	5–10
24	10.00	13	none	5–10
25	10.00	16	none	5–10

Table III—Relative Efficiency of Coating 5/16-In. Standard Concave Tablets as a Function of Chamber Load

Expt. No.	Chamber Load, Gm.	Solids Applied, mg.	Wt. Gain, mg.	Coating Time, min.	Coating Efficiency,[a] %
1	4.0	650	390	8	60
2	4.0	650	400	8	62
3	2.0	325	130	4	40
4	2.0	325	130	4	40
5	1.0	162	20	2	12
6	1.0	162	20	2	12

[a] Defined as wt. gain (mg.)/solids applied (mg.) × 100.

Correlation with 6-in. Wurster Apparatus—Experiments were carried out to determine whether information generated using the miniature unit could be extrapolated to larger air-suspension coating units. The quantity of ethylcellulose required to retard the release of chlorpheniramine maleate from tablets and seeds was determined in the miniature unit. Tablets and seeds were then coated in the 6-in. unit using an amount of coating solution proportional to the increased batch size.

Coating Procedure—Twenty milliliters of 1.25% ethylcellulose applied to 10 Gm. of seeds yielded a product which released chlorpheniramine maleate for over 16 hr. Four liters of the same solution was then applied to 2 Kg. of seeds in the 6-in. unit.

Twenty chlorpheniramine maleate tablets (5/16-in. standard concave, 200 mg.) were coated with 3 ml. of 1.25% ethylcellulose solution in the miniature coater. Similarly, 750 ml. was applied to 5,000 tablets in the large unit.

Release Rate Determination—The release rates were determined at 37 ± 0.1° in 150 ml. of 0.01 *N* HCl in a 3.5 × 16-cm. test tube. The sample (one tablet or 1.64 Gm. of seeds) was placed in a 24 × 64 mm. stainless steel cylinder covered at each end with an 80-mesh screen. The cylinder was oscillated in a vertical plane with a Stoll-Gershberg apparatus. The chlorpheniramine maleate was assayed spectrophotometrically at 264 mμ with a Beckman DU spectrophotometer modified with a Gilford model 220 absorbance converter. A continuous record was made of chlorpheniramine maleate from the seeds by pumping the solution through a 1-cm. flow cell with a Sigmamotor pump and recording the output of the absorbance converter on a Gilford-Honeywell recorder. In the case of the tablets, only a single point determination was made at 4 hr.

Discussion—The chlorpheniramine maleate release rates from the nonpareil seeds are shown in Fig. 4. The results of the tablets are given in Table IV. The variation in release rates is much smaller with the nonpareil seeds than with the tablets be-

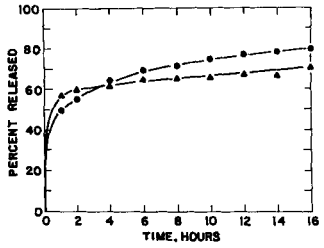

Fig. 4—Release of chlorpheniramine maleate from ethylcellulose-coated nonpareil seeds in 0.01 N HCl maintained at 37 ± 0.1°. Key: ●, miniature; ▲, 6-in. model.

TABLE IV—MINIATURE *Versus* 6-in. WURSTER CHLORPHENIRAMINE MALEATE TABLETS COATED WITH 1.25% ETHYLCELLULOSE SOLUTION

Expt. No.	1.22-in. Chamber	6-in. Chamber I	6-in. Chamber II
	% Release in 4 hr.		
1	54	18	17
2	33	18	14
3	50	17	33
4	46	36	31
5	60	51	50

cause the measurement of the release rates from 1.64 Gm. of seeds gives the average of a large number of individual assays, while the tablets were assayed individually. However, although the release rates are not precisely identical, the results do show that it should be possible to scale-up from the 1.22-in. coater to the 6-in. model with very little additional experimentation.

SUMMARY

An apparatus has been described which is capable of processing gram quantities of material in much the same manner as the larger 6-in. model Wurster unit. The miniature device has been evaluated and found to be highly versatile with respect to the types of discrete particles for which it can effect a continuous coating. Coating experiments indicate the miniature unit to be qualitatively comparable with the larger 6-in. model, although some quantitative differences were observed. The data suggest that minimal scale-up problems would probably accompany transition to the large unit.

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Capsule coating
Enteric dosage forms–coating
Drug release–coated dosage forms

Notes

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By THOMAS J. HALEY and FRANK D. CARTWRIGHT

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These studies were supported by contract AT(04-1)GEN-12 between the Atomic Energy Commission and the University of California.
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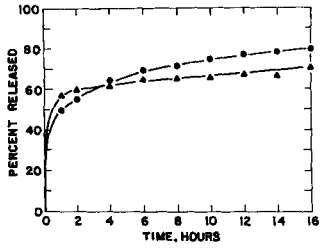

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hemogram of rats. The chemicals did cause a transient tachycardia and a slight hypotension in dogs. Inasmuch as this is the total information on the biological effects of perrhenates and rhenium trichloride and because the element and its compounds are coming into greater usage (3), the authors have re-evaluated the pharmacological and toxicological properties of $KReO_4$ and Re_2Cl_6.

METHODS

The samples of potassium perrhenate ($KReO_4$) and rhenium trichloride (Re_2Cl_6) had a minimum purity of 99.9%. The intraperitoneal LD_{50}'s were obtained with male CFl mice weighing 21 to 34 Gm.

The method of Draize *et al.* (4) was used to study eye and skin irritation in rabbits. In the eye irritation studies, two rabbits were used, and each animal had one eye exposed to 3 mg. of crystalline compound while the other eye served as a control. The rabbit skin irritation studies used six animals per compound and 0.5-Gm. amounts of each salt were applied.

The effects of the compounds on guinea pig ileal strips bathed in Ringer-Locke solution were studied in a thermostatically regulated 25-ml. bath using the Trendelenburg (5) method. Studies were also made on the isolated rabbit ileum in the presence of either 2.5 mcg. of acetylcholine or 0.5 mcg. of nicotine.

For the study of the effects of intravenous administration of these compounds, 15 cats of both sexes, weighing 2.3 to 4.6 Kg. were anesthetized with 0.5 ml./Kg. of allobarbital[1] urethan solution intraperitoneally. A six-channel recorder with transducers was used to record carotid arterial pressure, respiration, nictitating membrane contraction, ECG lead II, femoral arterial pressure, and femoral arterial flow. The latter was obtained with a 25-ml. flowmeter (6). Preganglionic stimulation of the cervical sympathetic fibers and the contralateral vagus fibers was accomplished with a stimulator at 8 v./10 sec. One hour was allowed to elapse prior to beginning drug administration. Intravenous dosages of the drugs used were: potassium perrhenate, 10–90 mg./Kg.; potassium chloride 10–140 mg./Kg.; rhenium trichloride, 10–20 mg./Kg.; hydrochloric acid, 1 ml. of 7.5, 15, and 30%; epinephrine, 5 mcg./Kg.; acetylcholine, 5 mcg./Kg.; histamine, 2 mcg./Kg.; and atropine, 2 mg./Kg. All injections were made at a constant volume of 1 ml., except at the highest doses where solubility dictated the total volume injected. Where appropriate, the results were analyzed statistically by the method of Litchfield and Wilcoxon (7).

RESULTS

Acute Toxicity—The LD_{50}/7 days for $KReO_4$ was 2.8 Gm./Kg. with a range of 2.5 to 3.05 Gm./Kg.; slope value was 1.07 (1.06–1.08). Toxic symptoms were sedation and severe ataxia with death or recovery within 7 days. No convulsions were observed. The LD_{50}/7 days for Re_2Cl_6 was 280 mg./Kg. with a range of 264.4 to 296.5 mg./Kg.; slope value was 1.1 (1.08–1.13). Toxic symptoms were sedation and abdominal irritation with death or recovery within 7 days.

Eye Irritation—Potassium perrhenate caused no detectable damage to the cornea, iris, or conjunctiva throughout the 72-hr. observation period. Rhenium trichloride caused an immediate irritation of the cornea, congestion and swelling of the iris, redness of the conjunctiva, and a very slight lacrimal discharge. However, all these ocular effects cleared up within 24 hr. and no permanent damage resulted from the exposure.

Skin Irritation—Neither potassium perrhenate nor rhenium trichloride had any effect on abraded or unabraded rabbit skin but the latter compound interacted with the hair to produce a permanent black stain. Similar staining was also observed on human skin and was probably caused by deposition of the metal on skin proteins.

Effects on Isolated Ileum—Potassium perrhenate in the dosage range of 2.4 to 952.4 mcg./ml. had no effect on the tonus or contractility of the isolated rabbit ileum and did not block the responses to either acetylcholine or nicotine. Similar results were obtained on the Trendelenburg guinea pig enteric ganglia preparation. Within the dosage range of 0.24 to 2.14 mg./ml., rhenium trichloride had no effect on the isolated rabbit ileum but at a dose of 2.38 mg./ml. the compound decomposed and the ileum went into spasm from the acid liberated.

Pharmacological Effects—Doses of 10–50 mg./Kg. of $KReO_4$ produced a transient hypertension with tachycardia but had no effect on respiration. Hypotension and bradycardia as well as bradypnea occurred at doses of 60 and 70 mg./Kg. Atropinization did not affect the above responses but did increase the toxic dose by 10 mg./Kg. Femoral blood flow decreased at doses of $KReO_4$ of 40 to 70 mg./Kg. and atropinization shifted the responses to doses of 60 to 80 mg./Kg. Death from cardiovascular collapse coupled with respiratory failure occurred in 4 of 5 cats at 70 mg./Kg. and atropinization increased the dosage to 80 mg./Kg. for three cats and 90 mg./Kg. for the other two animals. Electrocardiographic changes caused by $KReO_4$ included: elevated P-waves, bradycardia, dropped beats, absent P-waves, absent T-waves, increased S-T interval ventricular tachycardia, transient auricular fibrillation, transient ventricular fibrillation, and 2 to 1 heart block. Atropinization had no influence on the cardiac changes produced by the chemical. $KReO_4$ did not block the pharmacological responses to acetylcholine, epinephrine, or histamine. Control studies with KCl indicated that the K^+ ion contributed little or nothing to the responses obtained with $KReO_4$ because KCl required 110 to 120 mg./Kg. to affect the cats, whereas $KReO_4$ produced its effects at much lower doses.

$KReO_4$ caused contraction of the nictitating membrane of the nonatropinized preparations but not of the atropinized ones, indicating a direct effect on the superior cervical ganglion because the responses resembled those obtained with acetylcholine and electrical stimulation rather than an epinephrine response and because atropine blocks at the ganglionic synapse.

Rhenium trichloride produced a slight hypertension at a dose of 10 mg./Kg. and was lethal to the cat when 20 mg./Kg. was given. However, these effects were related to the acid liberated by decomposition of the Re_2Cl_6 and were duplicated by the administration of the same concentration of hydrochloric acid to a control animal.

[1] Trademarked as Dial by Ciba Pharmaceutical Co., Inc., Summit, N. J.

DISCUSSION

The low order of toxicity of potassium perrhenate (1) and the high order of toxicity of rhenium trichloride (2) have been confirmed and extended to determination of their intraperitoneal LD_{50}'s. The rhenium compounds tested showed no real irritating properties on the eyes or skin of animals and no effects on isolated intestine. This is a striking contrast to observations on other rare elements (8–10). The chemical composition of the rhenium compounds, their solubility, and their rate of decomposition determines their lethality to animals. Rhenium trichloride, which decomposes readily with the liberation of hydrochloric acid, was 10 times more toxic than potassium perrhenate.

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Skin irritation
Ileum, isolated–effect on tonus, contractility

Photodisintegration Studies of ^{14}C-Carboxyl 2,3,5-Triiodobenzoic Acid

By R. H. JARBOE, JR., J. B. DATA, and J. E. CHRISTIAN

^{14}C-Carboxyl labeled 2,3,5-triiodobenzoic acid was examined at various intervals of time during exposure to ultraviolet irradiation. Photodisintegration occurred and the amount of degradation increased in quantity with increased duration of exposure. 2,5- and 3,5-diiodobenzoic acid were identified as two products resulting from the irradiation. Other unidentified products are also present.

IN CERTAIN PLANTS 2,3,5-triiodobenzoic acid (TIBA) has pronounced hormonal effects on the flowers, fruits, yields, metabolism, and translocation of natural plant constituents (1–8). Because these plants serve as a source of food to man or indirectly by first serving as food for animals which ultimately constitute man's diet, the question naturally arises as to the safety of this material to man. Furthermore, when TIBA is used on plants, it could be questioned whether the photodisintegration products from TIBA might be a health hazard. If sunlight causes photochemical deterioration either before or after absorption in the plant, the products of degradation could be translocated throughout the plant and eventually, directly or indirectly, become incorporated in foods.

EXPERIMENTAL

Materials—Glass chromatographic plates, 20 × 20 cm., utilizing silica[1] of 250 μ thickness, were used. A plastic preservative for thin-layer chromatograms, Brinkmann Instruments Co., Westbury, L. I., N. Y. was used.[2] The ultraviolet source (Ultra-Violet Products, Inc., South Pasadena, Calif., long-wave UV model S1 3660) was equipped with a long wavelength filter to approximate normal sunlight. The samples were counted in a Packard model 3003 Tri-Carb liquid scintillation spectrometer. The solvent system used was composed of a ratio of 10 parts of petroleum ether (b.p. 30–60°) and 1 part of propionic acid. Solvents and chemicals used in this study were of reagent grade or their equivalent.

Purification of TIBA-^{14}C—TIBA labeled in the carboxyl position with ^{14}C (9) was purified utilizing thick-layer chromatographic techniques prior to the photodisintegration studies. The purification procedure consisted of pipeting an alcoholic solution of approximately 93% TIBA-^{14}C onto previously prepared 1-mm. silica plates and air dried. The plates were developed in a solvent system of petroleum ether–propionic acid in the ratio of 10 parts of petroleum ether (b.p. 30–60°) and 1 part of propionic acid and subsequently exposed to photographic film (Eastman Kodak No Screen medical X-ray film). The resultant autoradiogram was used to identify TIBA-^{14}C. The TIBA-containing silica was scraped from the plate and placed in a Soxhlet extractor and extracted with anhydrous ether for a period of 72 hr. The Soxhlet extractor was wrapped with black paper to protect the contents from exposure to light. After the extraction period the ethereal solution was then concentrated to dryness under reduced pressure without heat in the dark.

Photochemical Degradation of TIBA-^{14}C—The TIBA-^{14}C used for the preparation of 30 ml. of a 2.1 × 10^{-2} % aqueous suspension was the same as that used in a study conducted on field grown soybeans by Spitznagle (9). About 5 ml. of the suspension was

Received May 29, 1967, from the Departments of Bionucleonics and of Medicinal Chemistry, School of Pharmacy and Pharmacal Sciences, Institute for Environmental Health, Purdue University, Lafayette, IN 47907
Accepted for publication September 15, 1967.
This investigation was supported in part by a grant from the International Minerals and Chemical Corp., Skokie, Ill.
[1] Adsorbosil-1, Applied Science Laboratories, Inc., State College, Pa.
[2] Neaton

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By R. H. JARBOE, JR., J. B. DATA, and J. E. CHRISTIAN

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IN CERTAIN PLANTS 2,3,5-triiodobenzoic acid (TIBA) has pronounced hormonal effects on the flowers, fruits, yields, metabolism, and translocation of natural plant constituents (1–8). Because these plants serve as a source of food to man or indirectly by first serving as food for animals which ultimately constitute man's diet, the question naturally arises as to the safety of this material to man. Furthermore, when TIBA is used on plants, it could be questioned whether the photodisintegration products from TIBA might be a health hazard. If sunlight causes photochemical deterioration either before or after absorption in the plant, the products of degradation could be translocated throughout the plant and eventually, directly or indirectly, become incorporated in foods.

EXPERIMENTAL

Materials—Glass chromatographic plates, 20 × 20 cm., utilizing silica[1] of 250 μ thickness, were used. A plastic preservative for thin-layer chromatograms, Brinkmann Instruments Co., Westbury, L. I., N. Y. was used.[2] The ultraviolet source (Ultra-Violet Products, Inc., South Pasadena, Calif., long-wave UV model S1 3660) was equipped with a long wavelength filter to approximate normal sunlight. The samples were counted in a Packard model 3003 Tri-Carb liquid scintillation spectrometer. The solvent system used was composed of a ratio of 10 parts of petroleum ether (b.p. 30–60°) and 1 part of propionic acid. Solvents and chemicals used in this study were of reagent grade or their equivalent.

Purification of TIBA-^{14}C—TIBA labeled in the carboxyl position with ^{14}C (9) was purified utilizing thick-layer chromatographic techniques prior to the photodisintegration studies. The purification procedure consisted of pipeting an alcoholic solution of approximately 93% TIBA-^{14}C onto previously prepared 1-mm. silica plates and air dried. The plates were developed in a solvent system of petroleum ether–propionic acid in the ratio of 10 parts of petroleum ether (b.p. 30–60°) and 1 part of propionic acid and subsequently exposed to photographic film (Eastman Kodak No Screen medical X-ray film). The resultant autoradiogram was used to identify TIBA-^{14}C. The TIBA-containing silica was scraped from the plate and placed in a Soxhlet extractor and extracted with anhydrous ether for a period of 72 hr. The Soxhlet extractor was wrapped with black paper to protect the contents from exposure to light. After the extraction period the ethereal solution was then concentrated to dryness under reduced pressure without heat in the dark.

Photochemical Degradation of TIBA-^{14}C—The TIBA-^{14}C used for the preparation of 30 ml. of a 2.1 × 10^{-2} % aqueous suspension was the same as that used in a study conducted on field grown soybeans by Spitznagle (9). About 5 ml. of the suspension was

Received May 29, 1967, from the Departments of Bionucleonics and of Medicinal Chemistry, School of Pharmacy and Pharmacal Sciences, Institute for Environmental Health, Purdue University, Lafayette, IN 47907
Accepted for publication September 15, 1967.
This investigation was supported in part by a grant from the International Minerals and Chemical Corp., Skokie, Ill.
[1] Adsorbosil-1, Applied Science Laboratories, Inc., State College, Pa.
[2] Neaton

placed in a quartz cell used in spectrophotometric analyses work, and the solution exposed continuously to the ultraviolet source at a distance of 20 cm. for 8 weeks. The cell was equipped with a plastic lid which was sealed by vacuum grease to prevent atmospheric effects.

Twenty-five microliter samples were removed at 0, 1, 2, 4, 8, 12, 24, 48, and 72 hr. and at 1, 2, 3, 4, 5, 6, 7, and 8 weeks. These samples were spotted on thin-layer silica plates. After development in the petroleum ether–propionic acid solvent, the plates were air dried and sprayed with undiluted plastic preservative to prevent sloughing of the silica during the photographic exposure period. To prepare the autoradiogram the thin-layer plates were exposed for about 5 days to Eastman Kodak No Screen medical X-ray film and then developed and fixed in Eastman Kodak liquid developer and liquid fixer, respectively.

The radioactivity of each labeled compound, separated on the thin-layer plates, was quantitatively determined in the Packard Tri-Carb liquid scintillation spectrometer. The radioactive areas were scraped from the plates and placed into a counting vial which contained a scintillator fluid composed of 0.4% PPO (2,5-diphenyloxazole) and 0.01% dimethyl POPOP [1,4-bis(4-methyl-5-phenyl-2-oxazoyl)benzene] in an equal volume each of toluene and 2-ethoxyethanol. Because TIBA was proved to be soluble in the scintillator fluid, suspension gel was not needed. A preliminary determination of possible quenching indicated no appreciable change in the counting rate with the amounts of silica contained in the samples.

Figure 1 is a drawing of the autoradiogram of the thin-layer chromatogram showing the separated degradation products of TIBA-^{14}C. Identification of the compounds was accomplished using unlabeled standards of TIBA, 2,5-, and 3,5-diiodobenzoic acid and location with bromocresol green reagent (10).

Figures 2 and 3 show the quantitative relationships between labeled TIBA and the products resulting from the deterioration as the irradiation time is increased.

Results—The TIBA-^{14}C at zero time contained 2.15% impurity after the thick-layer chromatographic purification process. This situation results from either the impurity not being completely removed in the purification process or that some type of degradation occurs during the isolation

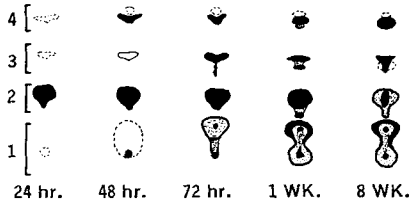

Fig. 1—Autoradiogram of the thin-layer chromatogram of TIBA-^{14}C and its products of photodisintegration at different time intervals. Key: spot 1, unidentified material at the origin; spot 2, TIBA-^{14}C; spot 3, 2,5-diiodobenzoic acid; spot 4, 3,5-diiodobenzoic acid. Unidentified impurities associated with these spots were not separated further nor identified. The spot with its associated impurities (represented by bracketed area) was removed, assayed, and calculated as one component.

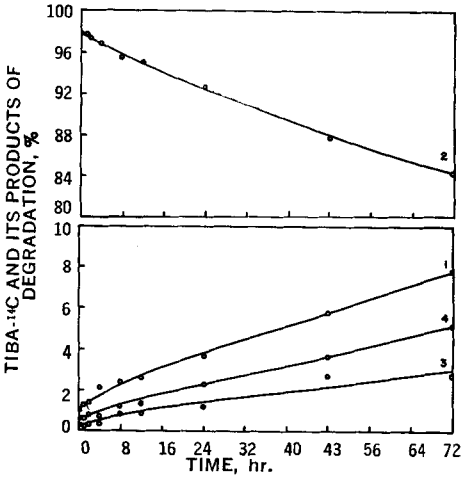

Fig. 2—Graphic relationship between the percentage of TIBA-^{14}C (curve 2) and the percentage of its products of degradation (curves 1, 3, and 4) at different hourly intervals. The curve number corresponds to the spot number in Fig. 1 where the nature of the products of degradation is explained.

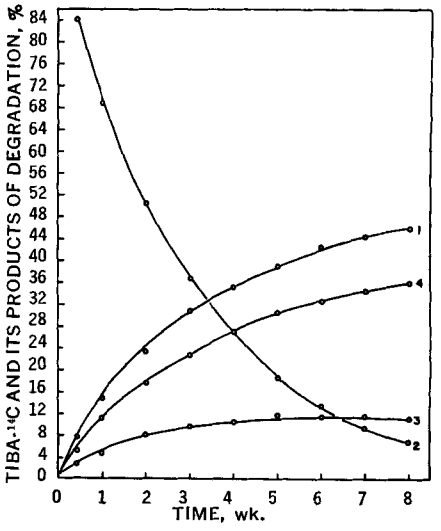

Fig. 3—Graphic relationship between the percentage of TIBA-^{14}C (curve 2) and the percentage of its products of degradation (curves 1, 3, and 4) at different weekly intervals. The curve number corresponds to the spot number in Fig. 1 where the nature of the products of degradation is explained.

which makes it difficult to obtain the desired product in absolutely pure form. Control samples of the TIBA-^{14}C kept in a light-resistant container showed no significant change at the end of 5 and 8 weeks.

It was observed that with increased time of exposure to the ultraviolet light the products resulting from the degradation increased in quantity and number since the solvent system used in the separation no longer sharply or completely separated the components of the mixture (compare the degree of resolution in Fig. 1 for the same spot at different time intervals of exposure). Impurities associated with the identified components were not separated but were counted as the entire spot (identified compound

plus its associated impurity). This was done for the purpose of evaluating the relationship between the concentration of TIBA-^{14}C and the products resulting from the photodisintegration. The unidentified compound(s) remaining at the origin were treated in an analogous manner.

The trace of unidentified impurities (spot 1) remaining at the origin increased in quantity to almost 8% of the total radioactivity at the end of 72 hr. of exposure and to 46% after 8 weeks. The quantity of TIBA-^{14}C (spot 2) decreased to 84% at the end of 72 hr. of exposure and to 7% at the end of 8 weeks. Spot 3 of the control (0 time) was identified as 2,5-diiodobenzoic acid. Spot 3 increased to 3% at the end of 72 hr. and 11% at the end of 8 weeks. *o*-Iodobenzoic acid, using the petroleum ether–propionic acid system for separation, has an R_f value very close to that of 2,5-diiodobenzoic acid and may be one of the impurities associated with it. Spot 4 in the control (0 time) was identified as 3,5-diiodobenzoic acid. This compound along with other products of photodisintegration associated with it increased to 5% at the end of 72 hr. and 36% at the end of 8 weeks. *m*-Iodobenzoic acid and also benzoic acid have R_f values using the above solvent system close to that of 3,5-diiodobenzoic acid and are probably impurities associated with the latter. At subsequent time intervals additional unidentified products from the degradation of TIBA-^{14}C became apparent.

CONCLUSIONS

2,3,5-Triiodobenzoic acid in a 2.1×10^{-2}% aqueous suspension is photochemically degraded by ultraviolet light. Ninety-three, 88, 84, 69, and 7% of TIBA are recovered from solution after 24, 48, 72 hr., 1 and 8 weeks of exposure, respectively. Two products of degradation were identified as 2,5- and 3,5-diiodobenzoic acid. From this study, it can be concluded that TIBA might be degraded in a like manner by sunlight after its application to plants.

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Keyphrases

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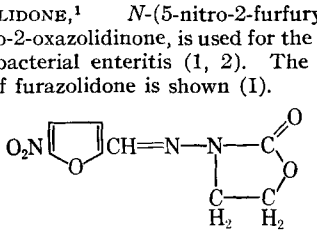
Degradation products of ^{14}C-carboxyl 2,3,5-triiodobenzoic acid
TLC separation
Liquid scintillation spectrometry—analysis

Method Specific for Determination of Furazolidone in Urine: Evidence for Drug-Related Metabolites

By R. D. HOLLIFIELD and JOHN D. CONKLIN

Furazolidone, *N*-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidinone, is a chemotherapeutic drug used orally for the treatment of bacterial enteritis. A new, more specific analytical method for the determination of furazolidone in urine is described. Utilizing this procedure, furazolidone was not detected in urine samples collected from dogs and humans following oral administration of the drug. Evidence is provided for the presence of drug-related metabolites.

FURAZOLIDONE,[1] *N*-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidinone, is used for the oral treatment of bacterial enteritis (1, 2). The structural formula of furazolidone is shown (I).



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Previously, urinary concentrations of furazolidone were measured either by bioassay (1) or by the method of Nakamura and Inoue (3), which is based on the conversion of the drug to 5-nitrofurfural phenylhydrazone. A new analytical procedure, more specific for the determination of furazolidone in urine, is described in this report. Results obtained by this procedure and by the Nakamura and Inoue method are presented regarding furazolidone concentrations in dog and human urine following oral drug administration.

EXPERIMENTAL

Drug Administration—Micronized furazolidone (about 5 μ or less) in gelatin capsules was administered orally to unfasted, adult, male beagle dogs at 1.25 mg./Kg. q.i.d. at 4-hr. intervals (5 mg./Kg./day). Urine samples were then collected by catheterization at selected intervals. Furazolidone as a

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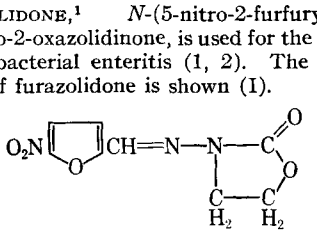

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EXPERIMENTAL

Drug Administration—Micronized furazolidone (about 5 μ or less) in gelatin capsules was administered orally to unfasted, adult, male beagle dogs at 1.25 mg./Kg. q.i.d. at 4-hr. intervals (5 mg./Kg./day). Urine samples were then collected by catheterization at selected intervals. Furazolidone as a

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TABLE I—RECOVERIES OF FURAZOLIDONE FROM DOG AND HUMAN URINE

Fluid	Range of Control	Absorbance at 600 mμ[a] — Furazolidone, mcg./ml. 2	5	10	30	50	Av. Recovery[b] Mean ± S.D.
Water	0.000–0.002	0.030	0.069	0.138	0.418	0.719	
Dog urine	0.007–0.011	0.029	0.066	0.138	0.427	0.708	98.6 ± 2.6
Human urine	0.005–0.008	0.032	0.071	0.140	0.420	0.718	102.2 ± 2.3

[a] Control corrected data based on a mean from at least three samples. [b] Based on furazolidone concentrations in water.

TABLE II—DETERMINATION OF FURAZOLIDONE CONCENTRATIONS IN DOG URINE

Dog	Dose[a] mg./Kg./day	Interval, hr.	Method — DMF–DDAH, mg./L.	Nakamura and Inoue, mg./L.
A	5	0–4	None detected	2.7
		4–8	None detected	15.6
		8–12	None detected	9.0
		12–24	None detected	0.1 (1.2)[b]
B	5	0–4	None detected	3.4
		4–8	None detected	8.9
		8–12	None detected	6.5
		12–24	None detected	1.6 (2.4)[b]

[a] Micronized furazolidone (about 5 μ or less) in gelatin capsules was administered orally at 1.25 mg./Kg. q.i.d. every 4 hr. [b] Represents the percentage of dosed material convertible to 5-nitrofurfural phenylhydrazone recovered in the urine within 24 hr.

100-mg. tablet[2] or as 100 mg. of macrocrystalline drug (about 75 μ) in a gelatin capsule was administered orally to healthy adult, human males q.i.d. at 4-hr. intervals (5 mg./Kg./day for an 80-Kg. adult). Voided urine specimens were then obtained at selected intervals.

Drug Analysis—The reagents include: crystalline furazolidone (Eaton Laboratories); saturated ammonium sulfate solution, pH 6.0, special enzyme grade (Mann Research Lab., Inc.); spectro-quality nitromethane and *N*,*N*-dimethylformamide (DMF), reagent grade (Matheson, Coleman, and Bell); and p-diisobutylcresoxyethyoxyethyl dimethylbenzyl ammonium hydroxide[3] (DDAH) 1 *M* in methanol (443 Gm./L. methanol, Packard Instrument Co., catalog No. 6003005). One milliliter of this DDAH solution is diluted to 25 ml. with DMF to produce a 0.04 *M* solution. A Beckman DU spectrophotometer was used to measure absorbance.

Reference drug solutions are prepared by dissolving 50 mg. of furazolidone in 50 ml. of DMF and diluting this with water to obtain the necessary drug concentrations. One milliliter of urine and 2 ml. of water are mixed together, 3 ml. of saturated ammonium sulfate solution added, and the contents mixed. Ten milliliters of nitromethane is then added, the contents mixed vigorously for 1 min. and centrifuged for 10 min. at 2,000 r.p.m. Eight milliliters of the nitromethane extract (top layer) is transferred to a test tube containing 1 Gm. of sodium sulfate, and the contents are mixed vigorously for 1 min. to dry the solvent. Six milliliters of the nitromethane extract is removed and placed in a 50-ml. flask, and the solvent is evaporated under vacuum at 50° using a flash evaporator. Following evaporation, the residue is dissolved in 3 ml. of DMF. Exactly 2.9 ml. of this solution is transferred to a test tube, 0.5 ml. of 0.04 *M* DDAH in DMF (freshly prepared) added, and the absorbance determined at 600 mμ. Since the presence of water inhibits the color formation, water contamination of the DMF should be avoided. It is recommended that the absorbance of each sample be measured within 10 min. following addition of the DDAH solution since the color formed deteriorates slowly with time.

Some of the urine samples were examined chromatographically. The sample was extracted with nitromethane, the extract spotted on Whatman No. 1 paper, and subjected to ascending paper chromatography for 15 hr. at room temperature. After drying, the papers were examined under UV light and the R_f of each spot determined. The solvent systems used were: 95% ethanol, *n*-butanol, and either 0.5 *N* acetic acid or ammonium hydroxide (1:4:1).

RESULTS AND DISCUSSION

DMF-DDAH Procedure—The furazolidone–DDAH complex in DMF exhibits an absorbance maximum at 600 mμ. The standard curve for this color complex in DMF follows Beer's law to 50 mg./L. The drug recoveries from dog and human urine (Table I) indicate that the reference standards and internally corrected standards are identical, and that the sensitivity of the method is at least 2 mg./L.

Urinary Drug Concentrations—The method described was applied to the determination of furazolidone in urine samples collected after drug administration. Micronized furazolidone (about 5 μ or less) in gelatin capsules was administered orally to dogs. Furazolidone as a 100-mg. tablet or as macrocrystalline drug (about 75 μ) in gelatin capsules was administered orally to human subjects. Urine samples were collected at designated intervals. As shown by the results (Tables II and III), furazolidone was not detected in any of the urine samples analyzed by the DMF–DDAH method.

For comparison, the urine samples were also analyzed for drug by the Nakamura and Inoue procedure (3), which revealed the presence of material(s) convertible to 5-nitrofurfural phenylhydrazone. It has been reported that 5-nitrofurfural phenylhydrazone in toluene exhibits a red band when eluted on a column of aluminum oxide (4). Some of the urine samples (Tables II and III) were subjected to the colorimetric procedure of Buzard *et al.* (5), in which furazolidone is converted to 5-nitrofurfural phenylhydrazone and extracted with toluene. The characteristic red band was observed when these toluene extracts were examined on aluminum oxide columns, providing additional evidence for the presence of material(s) convertible to 5-nitrofurfural phenylhydrazone in dog and human urine following furazolidone dosage.

Recently, interest has been directed toward drug particle size and its related effects *in vivo* with regard to the absorption of nitrofuran derivatives (6). Relative to this, it was observed that greater amounts of material(s) convertible to 5-nitrofurfural phenylhy-

[2] Furoxone tablet.
[3] Hyamine hydroxide.

TABLE III—DETERMINATION OF FURAZOLIDONE CONCENTRATIONS IN HUMAN URINE

Subject	Dose[a] 400 mg./Day	Interval, hr.	Method: DMF–DDAH mg./L.	Method: Nakamura and Inoue, mg./L.
A	Tablet	0–4	None detected	7.7
		4–8	None detected	54.6
		8–12	None detected	25.8
		12–24	None detected	12.0 (6.6)[b]
B	Tablet	0–4	None detected	20.4
		4–8	None detected	26.1
		8–12	None detected	44.1
		12–24	None detected	9.9 (8.7)[b]
C	Gelatin capsule	0–4	None detected	8.1
		4–8	None detected	15.6
		8–12	None detected	3.1
		12–24	None detected	9.5 (3.0)[b]
D	Gelatin capsule	0–4	None detected	7.0
		4–8	None detected	5.2
		8–12	None detected	13.6
		12–24	None detected	8.5 (3.1)[b]

[a] Furazolidone as a 100-mg. tablet or as 100 mg. of macrocrystalline drug (about 75 μ) in a gelatin capsule was administered orally q.i.d. every 4 hr. [b] Represents the percentage of dosed material convertible to 5-nitrofurfural phenylhydrazone recovered in the urine within 24 hr.

TABLE IV—PAPER CHROMATOGRAPHY OF DOG AND HUMAN URINE

Sample	R_f Values: Acidic, Dog	Acidic, Human	Basic, Dog	Basic, Human
Control urine	0.14	0.12	0.12	0.16
	0.37	0.36	0.31	0.35
	0.58		0.59	
		0.74		
Control urine plus furazolidone	0.16	0.12	0.17	0.16
	0.36	0.37	0.30	0.34
	0.59		0.58	
	0.66[a]	0.68[a]	0.65[a]	0.67[a]
		0.73		
Exptl.[b]	(0.05)[c]	(0.05)	(0.07)	(0.07)
	0.15	0.13	0.19	0.13
	0.37	0.38	0.29	0.35
	(0.51)	(0.55)	(0.43)	(0.44)
	0.59		0.58	
		0.74		

[a] R_f value for furazolidone. [b] Dog *A* (4–8 hr.) Table II, subject C (4–8 hr.) Table III. [c] Figures in parentheses represent drug-related metabolites.

drazone were recovered in the urine of humans administered the furazolidone tablet than were found in the urine of subjects administered the macrocrystalline drug (Table III).

Chromatography—A chromatographic comparison of urine collected from dogs and humans administered furazolidone and of urine to which furazolidone had been added was conducted. Although furazolidone was detected when added to the urine (Table IV), the drug was not detected in either dog or human urine following oral furazolidone administration. However, the presence of two drug-related metabolites, one yellow and the other orange in appearance under UV light, in both dog and human urine, was established.

Following chromatography the drug-related spots were eluted and their absorbance spectra determined. These eluates were also analyzed chemically. When the spot representing drug added to urine (R_f 0.65–0.68, Table IV) was eluted, it exhibited an absorbance spectrum with a maximum at 367 mμ in water, which is characteristic of that reported for furazolidone (1). This eluate yielded the expected blue color when subjected to either the DMF–DDAH or Nakamura and Inoue procedures. As shown in Table V, each of the metabolites exhibited an absorbance spectrum with a maximum near 415 mμ in water. Apparently, the spot which appears orange under UV light (Table V) represents one of the material(s) in dog and human urine which is convertible to 5-nitrofurfural phenylhydrazone. The presence of drug-related metabolites which absorb near 415 mμ in the urine of animals administered certain nitrofuran derivatives has been reported previously (7–9).

On the basis of spectral analysis, the yellow material appears to be present in a greater amount than the orange material in dog urine, while the orange material is present in greater amounts than the yellow material in human urine. This indicates that although the two drug-related metabolites are each present in dog and human urine, they apparently are present in different relative amounts. This may explain the twofold difference observed between dog and man (Tables II and III) in the percent excretion of dosed micronized material convertible to 5-nitrofurfural phenylhydrazone.

Under *in vitro* conditions, furazolidone exhibits significant inhibitory activity against *Escherichia coli* (1). Urine samples collected from dogs and humans following furazolidone administration demon-

TABLE V—CHARACTERISTICS OF THE DRUG-RELATED URINARY METABOLITES

Sample	R_f Value[a]: Acidic	Basic	Appearance: White Light	UV Light	Absorbance Max. in Water, mμ	Chemical Assay: DMF–DDAH Method	Nakamura and Inoue Method
Dog urine	0.05	0.07	Yellow	Yellow	415	No color	No color
	0.51	0.43	Yellow	Orange	415	No color	Blue color
Human urine	0.05	0.07	Yellow	Yellow	415	No color	No color
	0.55	0.44	Yellow	Orange	415	No color	Blue color

[a] See Table IV.

strate little activity against *E. coli* (1), suggesting that only small amounts of furazolidone are excreted in dog and human urine. This also indicates that the urinary drug-related metabolites observed in the present study are not significantly active against *E. coli*.

In summation, when urines collected from dogs and humans administered furazolidone orally were analyzed by a new analytical procedure, furazolidone was not detected. Chromatographic examination of these urine samples verified this conclusion and also revealed the presence of two drug-related metabolites in both dog and human urine.

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Metabolites, furazolidone–analysis in urine
Colorimetry–analysis
Paper chromatography–analysis

Fractionation of Fatty Acids of *Cucurbita maxima* Seed Oil With Urea

By J. P. TEWARI and M. C. SRIVASTAVA

The mixed fatty acids of *Cucurbita maxima* seed oil have been fractionated by liquid–solid countercurrent distribution with urea. The percentage fatty acid composition of oil is: palmitic, 21.5; stearic, 8.4; oleic, 27.0; and linoleic, 43.10.

UNDER the Indian Council of Medical Research inquiry on anthelmintic activity of *Cucurbita maxima* seeds (family, *Cucurbitaceae*), the anthelmintic activity and the chemotherapeutic actions of aqueous, alcoholic, and ethereal extracts of the decorticated seeds of *C. maxima* have been reported (1, 2).

A large amount of the oil was obtained as a by-product during the defatting of the seeds prior to the isolation of cucurbitin. In view of the importance of this oil in the Indian system of medicine (3, 4) the mixed fatty acids of *C. maxima* oil were fractionated by the liquid–solid countercurrent distribution of fatty acids with urea employing the method of Sumerwell (5).

The results of fractionation have been recorded in Table I and agree closely with those obtained by Chowdhury *et al.* spectrophotometrically (6).

TABLE I—LIQUID–SOLID COUNTERCURRENT DISTRIBUTION OF *C. maxima* FATTY ACIDS WITH UREA

S. No. of Fraction	Wt. of Fraction	S.E.	I.V.	Palmitic Acid	Stearic Acid	Oleic Acid	Linoleic Acid
1	3.28	205.2	0.82	1.20	2.04	0.03	...
2	2.61	215.8	2.42	2.26	0.28	0.07	...
3	2.08	215.2	2.52	1.72	0.19	0.18	...
4	1.56	216.2	6.92	1.38	0.06	0.12	...
5	...	...	...	...	...	...	...
6	0.61	195.4	90.1	...	...	0.61	...
7	...	...	...	...	...	...	...
8, 9, 10	0.91	196.4	90.2	...	...	0.91	...
11	4.85	197.8	121.2	...	...	3.20	1.65
Raffinate	14.60	198.5	162.0	...	...	3.10	11.50
Total	30.50	...	...	6.56	2.57	8.22	13.15
Percentage of acids	...	...	...	21.5	8.4	27.0	43.1
Percentage of acids by Chowdhury (6)	...	...	...	←——29.9——→		26.4	43.7

EXPERIMENTAL

The oil from the seeds of *C. maxima* was saponified and fatty acids were obtained from the soap after removing the unsaponifiable matter. The mixed fatty acids (30.5 Gm. I.V., 103.2; N.V., 198.2) were fractionated by liquid–solid countercurrent distribution of fatty acid with urea employing the method

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Keyphrases

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Neutralization value
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Isolation of Candicine Chloride, Laurifoline Chloride, and Xanthoxyletin from the Bark of *Zanthoxylum elephantiasis*

By JOZEF TOMKO*, ALBERT T. AWAD†, JACK L. BEAL, and RAYMOND W. DOSKOTCH

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The NMR spectra of xanthoxyletin is consistent with its structure showing a singlet at δ = 1.47 (6H) for the two methyl groups at C-2′, a singlet at 3.88 (3H) for the methoxy group, and one at 6.52 (1H) for the aromatic proton at C-8. The protons at C-3 and C-4 appeared as a pair of doublets at δ = 6.20 and 7.87 (J = 9.5 c.p.s.), respectively, and those at C-3′ and C-4′ also as a pair of doublets were found at δ = 5.73 and 6.61 (J = 10 c.p.s.), respectively.

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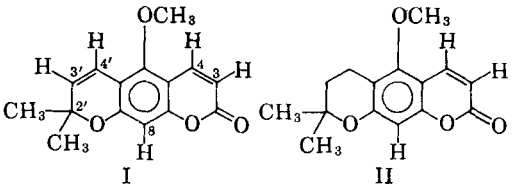

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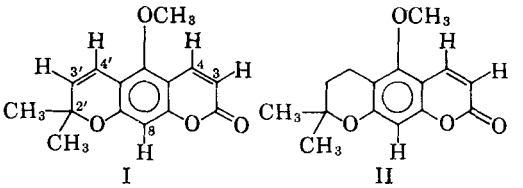

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TABLE I—SILICIC ACID AND DIATOMACEOUS EARTH CHROMATOGRAPHY OF QUATERNARY ALKALOIDS

Fraction No.	Eluant	Quantity, ml.	Alkaloid, mg.
1	Chloroform	500	...
2	Chloroform–methanol (10%)	1000	...
3	Chloroform–methanol (20%)	500	...
4	Chloroform–methanol (30%)	400	...
5	Chloroform–methanol (30%)	100	Trace, laurifoline chloride
6	Chloroform–methanol (40%)	100	86, laurifoline chloride
7	Chloroform–methanol (40%)	100	62, laurifoline chloride
8	Chloroform–methanol (40%)	100	84, mixture of laurifoline chloride and candicine chloride
9	Chloroform–methanol (40%)	100	77, mixture of laurifoline chloride and candicine chloride
10	Chloroform–methanol (50%)	300	220, candicine chloride
11	Chloroform–methanol (70%)	100	Trace, candicine chloride

ceous earth[2] (4:1). This was placed on top of a column (2.6 cm. diameter) of 100 Gm. of the same adsorbent which was prepared in the manner described in a previous publication (3). The results are given in Table I.

Fractions 6 and 7 were dissolved in methanol, filtered, and acetone added to point of cloudiness to obtain 90 mg. of laurifoline chloride, m.p. 249–251° dec., $[\alpha]_D^{29}$ + 40° (c 0.985 in methanol). The compound was identical in IR, UV, and R_f (0.16 for solvent system *1* and 0.68 for *2*) with that of an authentic sample.

The material in fraction 10 was crystallized from methanol–ether to give 180 mg. of candicine chloride, m.p. 278–279° dec. The IR, UV, and R_f (0.23 for solvent system *1* and 0.75 for *2*) were the same with those values of an authentic sample.

Isolation of Xanthoxyletin—The residue from the petroleum ether extract was placed on a column of acid alumina (Woelm, activity grade I, 100 Gm.) and elution started with benzene. Elution with benzene yielded xanthoxyletin (2 Gm.) which crystallized from a mixture of chloroform and diethyl ether, m.p. 133–134° [lit. (2) m.p. 133°].

Anal.—Calcd. for $C_{15}H_{14}O_4$: C, 69.75; H, 5.46. Found: C, 70.28; H, 5.58.

UV spectrum: $\lambda_{max.}^{MeOH}$ 221 mμ (log ε = 4.37); 269 (4.42), 346 (4.10), unaffected by dilute acid or alkali.

[2] Celite 545. Johns-Manville Corp., New York, N. Y.

Hydrogenation of 165 mg. of xanthoxyletin with 50 mg. of PtO_2 in 12 ml. of ethanol showed an uptake of 1 *M* equivalent of hydrogen and the crystalline dihydroxanthoxyletin (120 mg.) was obtained from ethanol–diethyl ether, m.p. 145–146°; [lit. (2) m.p. 144.5–145.5°]; UV spectrum showed $\lambda_{max.}^{MeOH}$ 332 mμ (log ε = 4.15).

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Keyphrases

Alkaloids, quaternary–*Zanthoxylum elephantiasis*

Candicine Cl, Laurifoline Cl, and Xanthoxyletin–isolation

Column chromatography–separation

NMR spectrometry

UV spectrophotometry–structure

Optical rotation–identification

Muscarine in *Clitocybe* Species

By K. GENEST, D. W. HUGHES, and W. B. RICE

Results of chemical and bioassays for muscarine in a sample of *Clitocybe dealbata* indicate the absence of other muscarine-like substances or muscarine potentiators. Evidence was found for the presence of muscarine in herbarium samples of *C. illudens*, *C. cerussata*, *C. dealbata*, and *C. rivulosa*. The age of samples ranged from 12 to 26 years.

A PREVIOUS COMMUNICATION (1) reported the isolation and determination of muscarine in *Clitocybe dealbata* and qualitative results on the physiological action of mushroom preparations injected into rats. It was of interest to know whether comparable results for muscarine content could be determined by chemical and biological assay, since muscarine-like potentiating factors have been reported in some species of *Inocybe* (2, 3). Other *Clitocybe* species have frequently been described as being toxic or of doubtful edibility (4–8). A collection of herbarium samples of such species was also examined for muscarine content.

EXPERIMENTAL

Material and Methods

Clitocybe dealbata—1965 crop: 57 Gm.; 1966 crop: 1.5 Gm.[1] of dry powder, passing 100-mesh sieve. Both samples from same location in Ottawa, Ontario.

Herbarium Samples—Samples were obtained from the Herbarium, Taxonomy and Mycology Section, Plant Research Institute, Department of Agriculture, Ottawa, Ontario. Relevant authenticative data are listed in Table I.

Chemical Determination—Muscarine was determined in accordance with the method described previously (1). For herbarium samples 0.25–0.5 Gm. of dried mushroom powder was used.

Biological Examination—The bioassay method of Malone *et al.* (9) was applied to the 1965 sample of *C. dealbata*. The standard for the bioassay was *dl*-muscarine iodide (Geigy). Healthy, nonfasted, adult male albino rats of the Wistar strain were used.[2] Muscarine was injected intraperitoneally into three groups of 10 rats each at 0.0125, 0.025, and 0.05 mg./Kg. (calculated as *l*-muscarine, free base). Mushroom preparations containing 27.3, 54.7, and 109.4 mg. of mushroom powder (100 mesh) per 15 ml. were injected into three groups of 10 rats each (1 ml./200 Gm.). Agar (0.25%) in water was used as dosage vehicle. Scores of the salivation metameter, as defined by Malone *et al.* (9), were measured. Preparations from herbarium samples were injected in amounts shown in Table II.

TABLE I—HERBARIUM SAMPLES OF *Clitocybe* SPECIES

Herbarium No.	Botanical Name	Common Name	Place and Time of Collection
DAOM 11834	*Cantharellus aurantiacus* (Wulf.) Fr. = *Clitocybe aurantiaca* (Fr.) Studer	Orange-yellow Chanterelle	Sept. 24, 1944, Chalk River, Ontario
DAOM 55728	*Clitocybe cerussata* var. *difformis* (Fr.) sensu Bres.	...	July 24, 1949, Yukon Territory
DAOM 33896	*Clitocybe dealbata* (Sow. ex Fr.) Kummer	Ivory clitocybe	Oct. 18, 1951, Vancouver, British Columbia
DAOM 10018	*Clitocybe illudens* Schw.	Jack-o'-lantern	Sept. 2, 1940, Oakville, Ontario
DAOM 24168	*Clitocybe illudens* Schw.	Jack-o'-lantern	Aug. 17, 1950, Ottawa, Ontario
DAOM 113652	*Clitocybe illudens* Schw.	Jack-o'-lantern	Aug. 10, 1954, Kentville, Nova Scotia
DAOM 64791	*Clitocybe rivulosa* (Pers. ex Fr.) Kummer	...	Sept. 16, 1946, Uppsala, Sweden

RESULTS AND DISCUSSION

Comparison of Chemical and Bioassays for C. dealbata—The muscarine content of the 1965 sample of *C. dealbata* was found to be 0.18% (0.16–0.21)[3] ($N = 16$) as determined by the chemical method (1) and calculated as *l*-muscarine (free base). The statistical analysis of the bioassay results is given in Table III. By this method, the sample was found to contain 0.26% (0.23–0.30)[3] (30 rats) of muscarine, also calculated in terms of the naturally occurring isomer. The λ value for this assay was found to be 0.2308 which agrees well with that reported by Malone *et al.* (9). The chemical and bioassays are in general agreement and the difference between the two is probably not due to the presence of other muscarine-like agents and/or potentiators, reported to occur in *Inocybe* species (2, 3), for which values obtained by bioassays were 3 to 13 times higher than those obtained by chemical assays. This interpretation is strengthened by the fact that the authors used *dl*-muscarine as standard and

Received July 10, 1967, from Research Laboratories, Food and Drug Directorate, Ottawa, Ontario, Canada.
Accepted for publication September 19, 1967.
The authors thank Dr. D. B. O. Savile, Plant Research Institute, Department of Agriculture, Ottawa, for herbarium samples, Dr. Leo Levi for encouragement, and Messrs. G. Bélec and W. Skakum for technical assistance.

[1] 1966 was an extremely poor year for *C. dealbata* in our region.

[2] From the animal colony of the Food and Drug Directorate.

[3] 95% confidence limits for the percentage figure.

TABLE II—MUSCARINE ASSAY AND BIOLOGICAL TESTS OF *Clitocybe* SAMPLES

Sample	Age, Yr.	Chemical Assay, % of Dry Wt.	Biological Tests: Amt. of Mushroom Used, mg./ml.	Biological Tests: Reaction
C. aurantiaca	$22^1/_2$	0.00	200/3	No reaction
C. cerussata	$17^1/_2$	0.012	200/4	Slight to moderate salivation and lacrimation
C. dealbata	$15^1/_3$	0.01	100/3	Moderate to profuse salivation, slight to profuse lacrimation, chromodacryorrhea
C. dealbata	$1^1/_4$	0.18	[a]	
C. dealbata	$^1/_4$	0.083	...	
C. illudens	$26^1/_2$	0.0075	150/3	Slight salivation ...
			450/7.5[b]	moderate salivation, slight lacrimation
C. illudens	$16^1/_2$	0.005	200/3	No reaction
C. illudens	$12^1/_2$	0.00	200/3	No reaction[c]
C. rivulosa	$20^1/_2$	0.035	30/3	Moderate to profuse salivation, moderate to profuse lacrimation, chromodacryorrhea

[a] Sample was bioassayed, see text. [b] 1.5, 2.25, and 3.75 ml. injected per rat. [c] One rat showed slight salivation 30 min. after injection.

TABLE III—ANALYSIS OF VARIANCE AND FACTORIAL ANALYSIS OF BIOASSAY

Term	*df*	*SS*	*MS*	*F*	*P*
Between treatments	5	176.588	35.317	14.468	<0.001
S vs. U	1	0.104	0.104	0.042	>0.20
Slope	1	166.057	166.057	68.028	<0.001
Departure from parallel	1	3.906	3.906	1.600	>0.20
Curvature	1	1.519	1.519	0.622	>0.20
Opposite curvature	1	5.002	5.002	2.049	0.10–0.20
Residual error	54	131.825	$s^2 = 2.441$		
Total	59	308.413			

calculated results for the bioassay on the basis that the racemic mixture has half the activity of naturally occurring *l*-(+)-muscarine (10). Some pharmacological studies, however, indicate that this is only an approximation (11).

Herbarium Samples—Various *Clitocybe* species have been classified as poisonous or of doubtful edibility. The older mycological literature can be confusing, mainly due to nonuniformity of nomenclature (12). *C. dealbata*, for instance, was classified without qualification as edible (4). Recent sources, however, list *C. dealbata*, *C. illudens*, *C. rivulosa* (6, 7), and *C. cerussata* var. *difformis* (7) as poisonous, and *C. aurantiaca* (6) as a mushroom of possible toxicity. Herbarium samples of these species were investigated for the presence of muscarine by the chemical method. Results of these tests and of the investigation of two recently collected samples of *C. dealbata* are given in Table II. Muscarine could be detected in all species which are considered poisonous. Two samples of *C. illudens* were muscarine positive, but the alkaloid was not detected in a third specimen. *C. aurantiaca* was negative also. The classification of this mushroom appears to be controversial, and while it is considered poisonous by some mycologists, others consider its edibility established (6, 13). Farnsworth (14) pointed out that the age of herbarium samples limits the usefulness of chemical tests, but the same author cites cases where 1300-year-old plants were still found to give positive alkaloid tests. A decline of muscarine content with time in herbarium samples is suggested by the results on samples 3 months, 15 months, and 15 years old (Table II). Yet compositional differences have also been observed between crops of the same year. Among the herbarium samples investigated *C. rivulosa* is the *Clitocybe* species exhibiting the highest potency, a finding which confirms recent work on surface-grown cultures of that species (15, 16). Results of chemical tests carried out on the herbarium samples were confirmed by biological tests (Table II). Since very little herbarium material was available, bioassays could not be performed. Qualitative muscarine symptoms in rats were afforded by all samples containing 0.0075% or more of muscarine.

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Keyphrases

Alkaloids—*Clitocybe dealbata*
Muscarine—isolation
Biological analysis—muscarine
Chemical analysis—muscarine
Clitocybe species—muscarine, aging effect

Fluorometric Determinations Comparable to Eye Response

By JOSEPH E. SINSHEIMER, JAMES T. STEWART*, and JOSEPH H. BURCKHALTER

A filter-photomultiplier tube system approaching the spectral response of the human eye has been selected, its spectral response recorded, and the system evaluated with a series of model fluorescent compounds.

FLUORESCENT tagging agents are being synthesized in these laboratories for potential use in fluorescent antibody-antigen techniques (1). To determine more rationally structures to be synthesized, it was desirable to direct these studies by an objective and sensitive comparison of fluorescence. Cognizant that the antibody-antigen technique ultimately depended upon visual observation of fluorescence, a filter-photomultiplier tube detection system that approached the spectral response of the human eye (2, 3) was developed for use with commercially available fluorometers. As this system has general application for the comparison of materials to be employed for their visible fluorescence, the selection and evaluation of this system is reported.

EXPERIMENTAL

Apparatus—The Aminco-Bowman spectrophotofluorometer No. 4-8106, equipped with an Electro Instruments X-Y recorder No. 1620-809, and a detection system consisting of a Kodak Wratten filter No. 106 and a RCA 1P28 phototube were employed in this procedure.

Spectral Response of the Detection System—In order to describe the spectral response of a 106 filter–1P28 photomultiplier detection system, the following comparison of experimental to reference corrected spectra was employed. Relative quantum intensity data were obtained from a corrected fluorescent spectrum of quinine sulfate[1] by dividing the intensity at 10-mμ increments by the maximum intensity. The relative quantum intensity for the aluminum chelate of an azonaphthalene sulfonic acid dye (aluminum-PBBR chelate) has been described by White (4) and served as the second reference corrected spectrum.

Solutions of quinine sulfate (10×10^{-6} Gm./ml. in 0.1 N H_2SO_4) and the aluminum-PBBR chelate were placed in the fluorometer equipped with the 106 filter and their fluorescence emission spectra were taken in the usual manner at the appropriate excitation wavelengths (365 and 540 mμ, respectively).

Based upon these experimental spectra, values at 450 mμ for quinine sulfate and 630 mμ for aluminum-PBBR chelate were each set equal to 100% quantum intensity and values at the other wavelengths for each compound were made relative to it. Dividing these percentages by the relative quantum intensity from the corrected spectra gave quantum values which were then divided by their respective wavelengths to give energy per wavelength data. The energy data were plotted so that the values for both compounds were made equal at 580 mμ (a common wavelength nearest to that of 560 mμ, the peak for the average human eye). Values at the other wavelengths for both compounds were made relative to 100% for the maximum at 575 mμ of the combined curves. (See Fig. 1.)

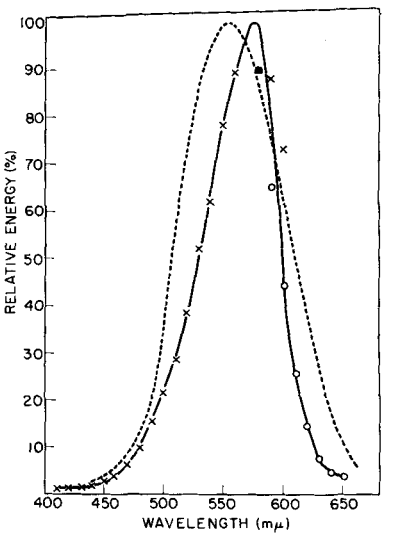

Fig. 1—Relative energy response of the 106 filter-1P28 photomultiplier system as compared to the average human eye. Key: ------, human eye responses; ——, 106-1P28 response; X, quinine sulfate data; O, Al-PBBR data.

Received June 15, 1967, from the College of Pharmacy, University of Michigan, Ann Arbor, MI 48104
Accepted for publication October 6, 1967.
This investigation was supported in part by research grant AI 05817 and training grant GM 1367 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
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[1] Spectrum supplied by Dr. R. E. Phillips, G. K. Turner Associates, Palo Alto, Calif.

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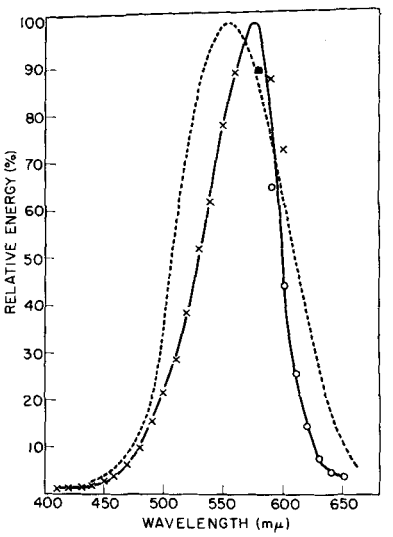

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Visual Detection Measurements—A fluorescent compound was dissolved in an appropriate solvent and its emission spectrum determined in the Aminco-Bowman spectrophotofluorometer equipped with the 106 filter. Concentrations were chosen to be in a range linear with fluorescence and the proper excitation wavelengths were employed for each compound. The approximate area under an emission peak for a particular substance (peak height by width at half height in cm.) was divided by the concentration of that compound in moles per milliliter to provide intensity in terms of cm.^{2} mole^{-1} ml. data for each compound.

DISCUSSION AND RESULTS

A comparison of the blue fluorescence of a series of compounds synthesized in these laboratories to that of quinine sulfate by the quinine reference unit procedure (5) proved successful for estimating relative fluorescence of these compounds. However, this same comparison was not satisfactory for predicting success in the fluorescent antibody technique. That is, compounds found by this procedure to possess fluorescence of medium intensity compared to fluorescein isothiocyanate, a widely used antibody tagging reagent (6), did not provide observable fluorescent conjugates in the fluorescent antibody technique. Thus, a need for a detection system approaching that of the human eye was indicated.

The nine-stage photomultiplier tubes (RCA 1P21, 1P28, and 931A) commonly employed with commercially available fluorometers were investigated. As the relative spectral response of these tubes approach that of the eye at wavelengths above 500 mμ, there was only the need to modify the response at the lower wavelengths. Both a Kodak Wratten filter No. 106 and a Corning filter No. CS 3-124 were evaluated for this purpose with RCA 1P21, 1P28, and 931A tubes. A comparison of these systems for the spectral response of fluorescence obtained from quinine sulfate to the corrected relative response of quinine sulfate as reported by Lippert (7) was made. For the individual tubes used in the present study, the 106 filter was the most satisfactory and gave approximately the same relative response curves for each of the three types of tubes. However, as the 106–1P28 combination was two times more sensitive than the other system, it was used for more extensive study.

In a further description of the 106–1P28 detection system, quinine sulfate and the aluminum–PBBR chelate, suggested by White (4), were used as reference sources. These compounds covered the range of fluorescence emission that was of interest in approximating the eye response, and their corrected emission spectra have been established (4, 7). Emission spectra of these compounds were determined in the Aminco-Bowman spectrophotofluorometer with the 106 filter in front of a 1P28 photomultiplier tube. In this manner, the standard materials serve as a defined source so that the ratio of the experimental curves to their corresponding standard corrected curves serve to describe the effect of filter, photomultiplier tube, and emission monochromator. This procedure is analogous to the use of such standard compounds for the calibration of the emission portion of a fluorometer (4).

The relative quantum intensity data obtained in this manner were expressed in energy units by dividing by wavelength to give a direct comparison to the relative energy data available for the human eye. Finally, in order to form a continuous curve, the intensity values obtained for each reference compound were made relative to their values at 580 mμ as an equal value. This wavelength was chosen as being common to both reference spectra and the wavelength nearest to the 560 mμ maximum for the average human eye.

TABLE I—FLUORESCENCE RELATIONSHIPS TO RHODAMINE B

Compd.	Excitation λ (mμ)	Normal Emission λ (mμ)	Relative Fluorescence Without Filter	Relative Fluorescence With Filter[g]
Rhodamine B[a]	540	580	1.000	1.000
Rhodamine B isothiocyanate[b]	540	580	0.300	0.260
Disodium fluorescein[c]	490	520	12.500	5.000
Fluorescein isothiocyanate[b]	490	520	10.000	3.330
4-Naphtho-(1,3;4,5)-triazolylstilbene-2-sulfonic acid, sodium salt[d]	365	460	6.330	0.510
4,4′-Bis(4-anilino-6-di-ethanolamine-*s*-triazin-2-ylamino)-2,2′-stilbene-disulfonic acid disodium salt[e]	365	435	1.800	0.024
Quinine sulfate[f]	365	455	4.900	0.051

[a] Allied Chemical Co. [b] Baltimore Biological Laboratories. [c] Eastman Kodak. [d] Geigy Chemicals. [e] American Cyanamid Co. [f] Mallinckrodt Chemical Works. [g] All values in this column could be multiplied by a factor of 0.35 to compare fluorescence relative to rhodamine B determined without filter.

The relative energy responses of the 106–1P28 detection system used in these studies and derived as just described is given in Fig. 1. For purpose of comparison, the relative energy response of the human eye is also given in this figure.

An application of the 106–1P28 system for the evaluation of compounds whose fluorescence is of interest in the visible range is given in Table I. All compounds were compared at concentrations in the linear portion of their concentration-fluorescence curves. Two series of fluorescent intensities were compared to those of rhodamine B with and without the presence of the 106 filter. Of the compounds evaluated, rhodamine B with an emission maximum at 580 mμ nearest to the 575 mμ maximum of the system showed the least effect (35% reduction) with the presence of a 106 filter. While fluorescein shows a significant change in relative fluorescence in the two systems, this change is even more pronounced with compounds of blue fluorescence at the lower wavelengths.

Compounds exhibiting fluorescence at these lower wavelengths are of particular interest in our studies, so that the 106 filter–1P28 photomultiplier system has proven of value in our evaluation of the fluorescence of potential antibody tagging agents. This same system should have general application where a fluorescence detection system approaching that of the human eye is required.

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Keyphrases

Fluorometry—analysis
Eye response—fluorometry, comparable
Quinine—azonaphthalene sulfonic acid dye aluminum chelate, reference source
Rhodamine B—fluorescence standard

Quantitative Analysis and Alkaline Stability Studies of Allopurinol

By PHILIP D. GRESSEL and JOSEPH F. GALLELLI

Methods for quantitative chromatographic separation and spectrophotometric analysis are reported for allopurinol [4-hydroxypyrazolo(3,4-*d*) pyrimidine] in the presence of its alkaline decomposition products. Stability data are presented using rate constants obtained from Arrhenius-type plots; $t_{90\%}$ for an unbuffered sodium allopurinol solution is approximately 150 days at 25°. The formate salt of 3-amino-4-pyrazolecarboxamide accounts for essentially all the degradation under pharmaceutically usable conditions.

ALLOPURINOL, 4-hydroxypyrazolo(3,4-*d*)pyrimidine, is a new, potent inhibitor of xanthine oxidase used in the treatment of hyperuricemia associated with gout. Extensive studies with the parenteral form of this drug have been performed by the National Cancer Institute to evaluate its effectiveness in the prevention of hyperuricemia and uric acid nephropathy in patients treated with X-ray and/or chemotherapy. The chemical stability studies in the present work were oriented toward conditions likely to be encountered in the clinical studies.

Some preliminary work (1) had been done previously on the assay of allopurinol solutions, using the ratio of optical absorbance at 262 mμ:255 mμ. It is found, however, that the UV spectrum of the major decomposition product does not differ enough from that of allopurinol, at any pH, to enable one to perform accurate determinations (see Fig. 1). The preliminary work (1) utilizing simple spectrophotometric analysis had not resulted in the detection of any decomposition of a refluxing solution of sodium allopurinol. However, thin-layer chromatography conducted in this laboratory on similar samples clearly showed the presence of two compounds in significant amounts. This led to the search for a larger scale quantitative method of separation of the components in aqueous solutions.

A specific method of assay was developed for allopurinol in the presence of its decomposition products, utilizing ion-exchange chromatographic separation and spectrophotometric analysis. Kinetic studies employing the assay method were done for some alkaline conditions.

Received June 30, 1967, from the Pharmaceutical Development Service, Pharmacy Department, Clinical Center, National Institutes of Health, Bethesda, MD 20014
Accepted for publication September 19, 1967.
The authors express their appreciation to Miss Gertrude B. Elion and Dr. George H. Hitchings, Burroughs Wellcome & Co., Tuckahoe, N. Y., for their assistance in the identification of the degradation product and for supplying authentic samples. They are also indebted to Dr. Henry Fales, National Heart Institute National Institutes of Health, for performing and interpreting NMR and mass spectra.

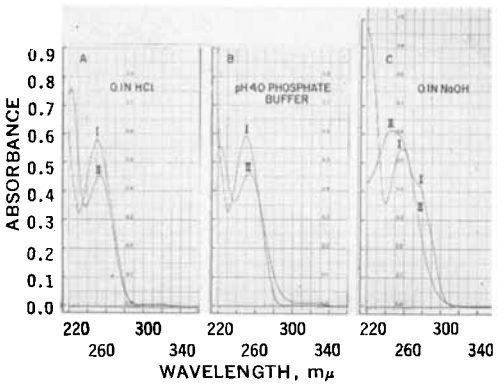

Fig. 1—Ultraviolet spectra of allopurinol (I) *and 3-amino-4-pyrazolecarboxamide* (II), *1.04 × 10⁻² M, in 0.1 N HCl, pH 4.0 phosphate buffer (0.1 M phosphate, 0.2 M NaCl), and 0.1 N NaOH.*

EXPERIMENTAL

Reagents—Allopurinol,[1] m.p. greater than 320°, was confirmed by IR spectrum (no official standards exist).

A strongly basic ion-exchange resin,[2] 200–400 mesh, was used; it was washed alternately with large volumes of 2 *N* HCl, water, and 2 *N* NaOH, (2) until the effluent was clear both by visual inspection and by ultraviolet spectrum. Then the procedure was repeated with the phosphate buffers, visual inspection. The pH 10 washings were continued until there was no change in pH. Stock solutions of phosphate buffers were prepared as follows: (*a*) pH 10 buffer, 0.100 *M* Na_2HPO_4, 0.200 *M* NaCl, adjusted to pH 10.00 with NaOH; (*b*) pH 4 buffer, 0.100 *M* $NaH_2PO_4 \cdot H_2O$, 0.200 *M* NaCl, adjusted to pH 4.00 with HCl.

Silica gel containing a fluorescent material[3] was

[1] Obtained from Burroughs Wellcome & Co., Tuckahoe, N. Y.

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[3] Silica gel GF_{254} according to Stahl for thin-layer chromatography, manufactured by E. Merck Ag., Darmstadt, Germany; distributed by Brinkmann Instruments Inc., Westbury, L. I., N. Y.

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Keyphrases

Fluorometry—analysis
Eye response—fluorometry, comparable
Quinine—azonaphthalene sulfonic acid dye aluminum chelate, reference source
Rhodamine B—fluorescence standard

Quantitative Analysis and Alkaline Stability Studies of Allopurinol

By PHILIP D. GRESSEL and JOSEPH F. GALLELLI

Methods for quantitative chromatographic separation and spectrophotometric analysis are reported for allopurinol [4-hydroxypyrazolo(3,4-*d*) pyrimidine] in the presence of its alkaline decomposition products. Stability data are presented using rate constants obtained from Arrhenius-type plots; $t_{90\%}$ for an unbuffered sodium allopurinol solution is approximately 150 days at 25°. The formate salt of 3-amino-4-pyrazolecarboxamide accounts for essentially all the degradation under pharmaceutically usable conditions.

ALLOPURINOL, 4-hydroxypyrazolo(3,4-*d*)pyrimidine, is a new, potent inhibitor of xanthine oxidase used in the treatment of hyperuricemia associated with gout. Extensive studies with the parenteral form of this drug have been performed by the National Cancer Institute to evaluate its effectiveness in the prevention of hyperuricemia and uric acid nephropathy in patients treated with X-ray and/or chemotherapy. The chemical stability studies in the present work were oriented toward conditions likely to be encountered in the clinical studies.

Some preliminary work (1) had been done previously on the assay of allopurinol solutions, using the ratio of optical absorbance at 262 mμ:255 mμ. It is found, however, that the UV spectrum of the major decomposition product does not differ enough from that of allopurinol, at any pH, to enable one to perform accurate determinations (see Fig. 1). The preliminary work (1) utilizing simple spectrophotometric analysis had not resulted in the detection of any decomposition of a refluxing solution of sodium allopurinol. However, thin-layer chromatography conducted in this laboratory on similar samples clearly showed the presence of two compounds in significant amounts. This led to the search for a larger scale quantitative method of separation of the components in aqueous solutions.

A specific method of assay was developed for allopurinol in the presence of its decomposition products, utilizing ion-exchange chromatographic separation and spectrophotometric analysis. Kinetic studies employing the assay method were done for some alkaline conditions.

Received June 30, 1967, from the Pharmaceutical Development Service, Pharmacy Department, Clinical Center, National Institutes of Health, Bethesda, MD 20014
Accepted for publication September 19, 1967.
The authors express their appreciation to Miss Gertrude B. Elion and Dr. George H. Hitchings, Burroughs Wellcome & Co., Tuckahoe, N. Y., for their assistance in the identification of the degradation product and for supplying authentic samples. They are also indebted to Dr. Henry Fales, National Heart Institute National Institutes of Health, for performing and interpreting NMR and mass spectra.

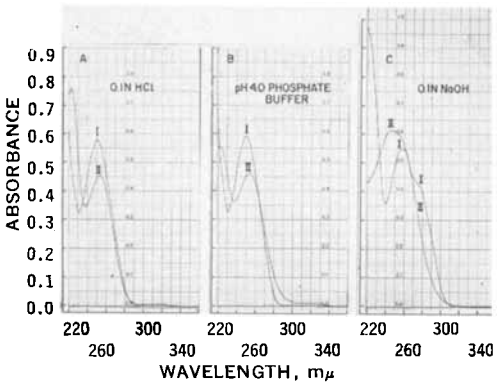

Fig. 1—Ultraviolet spectra of allopurinol (I) *and 3-amino-4-pyrazolecarboxamide* (II), *1.04 × 10⁻² M, in 0.1 N HCl, pH 4.0 phosphate buffer (0.1 M phosphate, 0.2 M NaCl), and 0.1 N NaOH.*

EXPERIMENTAL

Reagents—Allopurinol,[1] m.p. greater than 320°, was confirmed by IR spectrum (no official standards exist).

A strongly basic ion-exchange resin,[2] 200–400 mesh, was used; it was washed alternately with large volumes of 2 *N* HCl, water, and 2 *N* NaOH, (2) until the effluent was clear both by visual inspection and by ultraviolet spectrum. Then the procedure was repeated with the phosphate buffers, visual inspection. The pH 10 washings were continued until there was no change in pH. Stock solutions of phosphate buffers were prepared as follows: (*a*) pH 10 buffer, 0.100 *M* Na_2HPO_4, 0.200 *M* NaCl, adjusted to pH 10.00 with NaOH; (*b*) pH 4 buffer, 0.100 *M* $NaH_2PO_4 \cdot H_2O$, 0.200 *M* NaCl, adjusted to pH 4.00 with HCl.

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TABLE I—THIN-LAYER CHROMATOGRAPHIC SEPARATION OF ALLOPURINOL FROM ITS DEGRADATION PRODUCTS

Solute	Eluent	R_f
Allopurinol	Chloroform–USP ethanol, 3:2	0.64
3-Amino-4-pyrazolecarboxamide	Chloroform–USP ethanol, 3:2	0.47
Degradation product of allopurinol	Chloroform–USP ethanol, 3:2	0.47
3-Amino-4-pyrazolecarboxamide hemisulfate hydrate	Chloroform–USP ethanol, 3:2	0.47
Degradation product of allopurinol from H_2SO_4 soln.	Chloroform–USP ethanol, 3:1	0.35
Partially degraded allopurinol soln.	Chloroform–USP ethanol, 3:1	0.14, 0.35
Partially degraded allopurinol soln.	Acetone, A.R.	0.33, 0.57
Allopurinol	Acetone, A.R.	0.57

Ion-Exchange Chromatographic Separation Procedure—Columns were packed with slurries of the anion-exchange resin in pH 10 phosphate buffer. Methods of packing and eluting the column and detecting the solutes in the effluent are essentially the same as those used in previous work conducted in this laboratory (3). The pH 10 buffer was used to elute the pure allopurinol fraction; then pH 4 buffer was substituted to elute the decomposition product from the columns. Effluent fractions corresponding to individual peaks were pooled and quantitatively diluted in pH 4 phosphate buffer to obtain ultraviolet spectra for quantitative analysis.

Columns were regenerated for reuse by pumping 300 ml. of the pH 4 phosphate buffer, then 300 ml. of the pH 10 phosphate buffer, through the apparatus.

Kinetic Studies—Chemical assays using the separation techniques above were performed on aliquots of solutions kept under various conditions (see Table II) at time intervals varying with observed reaction rates. Studies at the listed conditions were conducted at 37° in a constant-temperature water bath, and at 52° in a constant-temperature oven. One study was done at those temperatures as well as at 76° in a constant-temperature oven.

TABLE II—STABILITY STUDIES: CONDITIONS AND RESULTS
(k = RATE CONSTANTS IN RECIPROCAL HOURS)

Soln.	Concn. mg./ml.	pH at 25°	Solvent	Observed $k_{76°}$	Observed $k_{52°}$	Observed $k_{37°}$	Extrap. $k_{25°}$	Calcd. $t_{90\%}$ 25°, Days
A	5	10.70	0.2 *M* PO_4^- buffer	...	2.57×10^{-3}	5.68×10^{-4}	1.54×10^{-4}	28.5
B	5	11.50	0.2 *M* PO_4^- buffer	...	3.85×10^{-3}	7.48×10^{-4}	1.84×10^{-4}	23.9
C	5	10.80	NaOH, *q.s.*	1.22×10^{-2}	1.09×10^{-3}	1.38×10^{-4}	2.92×10^{-5}	150
D	5	11.50	NaOH, *q.s.*	1.74×10^{-2}	1.96×10^{-3}	...	1.03×10^{-4}	42.6
E	1	10.80	*A* × 20/100 in 0.9% NaCl	...	1.57×10^{-3}	3.19×10^{-4}	7.82×10^{-5}	56.3
F	1	11.50	*B* × 20/100 in 0.9% NaCl	...	2.20×10^{-3}	5.36×10^{-4}	1.82×10^{-4}	24.3
G	1	10.50	*C* × 20/100 in 0.9% NaCl	...	4.32×10^{-4}	1.13×10^{-4}	4.00×10^{-5}	110

used in the thin-layer chromatographic procedures.

All other chemicals were reagent grade, except as specified.

Apparatus—Thin-layer chromatography plates were prepared with a Desaga thin-layer chromatography kit.[4] Chromatographic column elution under positive pressure was performed with a Sigmamotor pump.[5] The eluent was monitored with a GME ultraviolet absorption meter, model UV 265 IF;[6] percent transmittance was recorded on a Texas Rectilinear recording milliameter.[7] Borosilicate glass columns, 1 cm. inner diameter, 25 cm. in length, and fitted with ground-glass connections for a reservoir for packing or eluting under air pressure, were used to hold the ion-exchange resin. Ultraviolet analyses were carried out on either a Beckman model DU spectrophotometer or a Cary 11 recording spectrophotometer. Infrared spectra were obtained on a Beckman IR5A spectrophotometer. Melting points were obtained on a Thomas-Hoover melting point apparatus.[8] Constant-temperature ovens (±1°) and a constant-temperature water bath (±.02°) were used in stability studies.

Thin-Layer Chromatographic Separation Procedure—Activated layers 250 μ thick, of Silica Gel GF_{254}, were used for qualitative studies. Spots were detected by their quenching of fluorescence under ultraviolet light. (See Table 1).

Determination and Identification of the End Product of Degradation—Recovery of the major end product was accomplished by two methods. In one procedure, solutions in the phosphate eluents were vacuum distilled to dryness; the residue was extracted with *n*-butanol to isolate the free base. In the second procedure, solutions were made acidic (pH 1–2) with sulfuric acid, concentrated to about one-half their original volume by vacuum distillation, then refrigerated for several days to obtain crystals of the sulfate salts. Ultraviolet, infrared, NMR, and mass spectral analyses were performed, as well as elemental analyses. Retention of the unknown on a cation-exchange resin[9] was observed. Duclaux values (4) were obtained on a dilute solution of distillate from a sample of allopurinol solution which had been refluxed for 15 hr. in sufficient excess NaOH to make the solution pH 11.7 at 25°. Melting point data were obtained on the sulfate salt of the unknown, known 3-amino-4-pyrazolecarboxamide hemisulfate hydrate, and on an intimate mixture of the known and unknown.

Results and Discussion

End Products of Reaction—All the evidence points to 3-amino-4-pyrazolecarboxamide as the

[4] Distributed by Brinkmann Instruments, Inc., Westbury, L. I., N. Y.

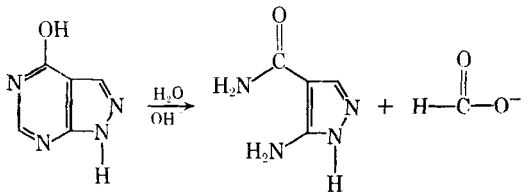
[5] Sigmamotor, Inc., Middleport, N. Y.

[6] Gilson Medical Electronics, Middleton, Wis.

[7] Texas Instruments, Inc., Houston, Tex.

[8] Arthur H. Thomas Co., Philadelphia, Pa.

[9] Dowex-50.



Scheme I

only significant end product. The overall reaction is shown in Scheme I.

Spectral evidence further confirms 3-amino-4-pyrazolecarboxamide; the IR spectra of known and unknown 3-amino-4-pyrazolecarboxamide are identical in either the free basic form or the salt form, although the spectra of the free form of either differ significantly from those of the salt form. UV spectra are identical throughout the normal pH scale. High resolution mass spectra of the degradation product show a parent peak with mass 126.0526. Calculated formula weight for 3-amino-4-pyrazolecarboxamide is 126.0541. The melting point of an admixture of its known and unknown hemisulfate hydrate is not depressed: known, 220–221°; unknown, 219–220°; equal mixture, 221–222°, all uncorrected.

The reaction scheme was confirmed indirectly by the confirmation of formate in a rapidly degraded solution. Duclaux values (3) obtained on the distillate were 2.7%, 2.9%, and 3.1%, respectively. The values for a correspondingly low concentration (0.2%) of authentic formic acid were 2.8%, 3.3%, and 3.6%.

Ion-Exchange Recovery of Allopurinol—The acidic nature of allopurinol, as well as the basic nature of its degradation product, coupled with their solubility in very polar solvents, made allopurinol a suitable candidate for ion-exchange chromatographic separation. In this work allopurinol was quantitatively retained on the column as the anion while 3-amino-4-pyrazolecarboxamide was being eluted off along with nonionic constituents. (See Fig. 2.) Then the pure allopurinol fraction was quantitatively removed in the pH 4 elution.

The pKa of allopurinol is approximately 9.4. Thus a pH of at least 10 was required to have the molecule substantially in its anionic form. The choice of anion-exchange resins, therefore, was limited to those which have strongly basic groups whose ionization was not materially affected at the required pH's.

Calculation for Intact Allopurinol—Allopurinol follows Beer's law in absorbance range 0–2 under controlled conditions. At pH 4, using the phosphate eluent, $\lambda_{max.} = 250$ mμ. Molar absorptivity (ϵ) = $7.74 \times 10^3/M$; absorptivity (a) = 56.5. No interference is encountered from nonionic compounds, which have been eluted in the first fraction.

Stability of Allopurinol Solutions—Results of the kinetic studies are found in Table II. In each case there was reasonably good adherence to first-order kinetics with respect to allopurinol. (See Fig. 3.) The trials at different pH's under otherwise similar conditions indicate a first-order dependency on hydroxyl ion concentration. Arrhenius-type plots were used to extrapolate higher temperature data to 25°. (See Fig. 4.) Activation energy of the reactions was about 25,500 cal./M.

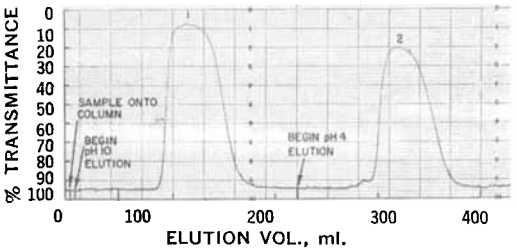


Fig. 2—Typical ion-exchange column chromatogram of a partially degraded sample of allopurinol.

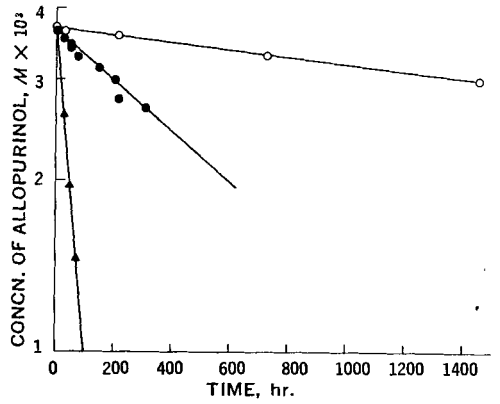


Fig. 3—Typical first-order plots showing the disappearance of allopurinol as a function of time at 37°(○), 52° (●), and 76° (▲), under otherwise similar conditions (solution C, Table II).

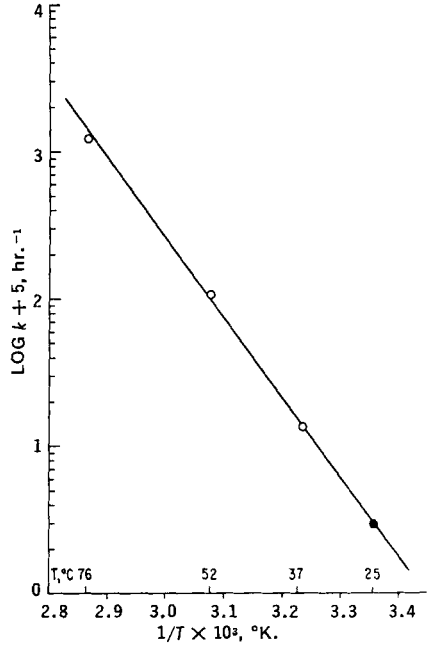


Fig. 4—Arrhenius-type plot of a typical stability study (solution C, Table II).

CONCLUSIONS

A quantitative method of assay has been found for allopurinol in the presence of inorganic salts, carbohydrate fillers, and its only significant alkaline degradation product. The method utilizes anion-exchange chromatography followed by spectrophotometric analysis.

The major product of alkaline degradation of allopurinol under pharmaceutically usable conditions is 3-amino-4-pyrazolecarboxamide, as the formate salt.

The lowest pH commensurate with the desired solubility should be used in formulating injections. At pH 10.8, an unbuffered solution of allopurinol has a $t_{90\%}$ of about 150 days.

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Keyphrases

Allopurinol–assay
Stability—alkaline solution
Degradation products—identity
Column chromatography—separation
TLC—separation
UV—structure, analysis
IR—structure
NMR
Mass spectroscopy

Investigation of the Use of Derivative Neutron Activation Analysis for Drug Assay

By W. A. SKINNER, M. A. LEAFFER, and R. M. PARKHURST

The use of neutron activation analysis for assay of chlorine- or bromine-containing drugs or derivatives of drugs was investigated. The ubiquitous nature of chloride greatly limited the use of this analytical method for chlorine-containing derivatives because of the background problem. Bromine-containing derivatives of aspirin and salicylic acid were prepared for neutron activation analysis, but again background bromide and interfering substances limited detection to 1×10^{-2} mcg. of bromide. Sensitivity of this analytical method is not comparable to that attainable with gas chromatography or spectrofluorometry.

In 1964, Steim and Benson (1) studied the use of derivative activation chromatography as a method for analysis of bromine-containing derivatives of amino acids, carboxylic acids, keto acids, sugars, and unsaturated fatty acids. The lower limits for detection of most amino acids separated as *N*-brosyl derivatives on paper were 0.01 mcg., as were the limits for citric or lactic acid as their bromophenacyl esters. The major limitation in sensitivity was the background arising from activation of contaminants in the paper. Conventional G-M counting was used.

There has been interest in sensitive methods for the assay of drugs in plasma or urine and it was decided to investigate the use of derivative neutron activation analysis for this purpose.

EXPERIMENTAL

Activation Source and Counting Methods—The Triga Mark I nuclear reactor at General Atomics, San Diego, Calif., with a thermal neutron flux of 1.8×10^{12} n/cm.^{2}-sec. was used for these studies. In the case of chloride determinations, the 1.64 Mev. gamma ray of 37.3 min. ^{38}Cl was used for identification and quantitative measurements while with bromide, the 0.78 Mev. gamma ray of 35.7 hr. ^{82}Br was used. Measurements were by the use of multichannel gamma ray spectrometry. Counting for bromide samples was on the fourth day after irradiation to allow short-lived isotopes to decay. Activation time in the reactor was 30 min.

Assay of Chlorine-Containing Drugs—In order to investigate the problems of background related to chloride analyses, chlorpromazine was used as a model drug. The paper selected for paper chromatographic and neutron activation studies was Schleicher and Schuell No. 589 which reportedly (1) has a lower background than others. The solvent system used was *n*-butanol–acetic acid–water (60:15:25). Chlorpromazine hydrochloride was found to move near the solvent front (R_f, 0.93) in this system. The high R_f has the advantage that any oxidation products of the drug are more polar and will trail the drug on the paper. Detection of 1 mcg. of chlorpromazine hydrochloride on Silica Gel G thin-layer plates could be made with ultraviolet light, ceric sulfate spray, or 50% sulfuric acid spray.

The chloride background of SS No. 589 white C paper was investigated prior to initiating studies with the drug. Neutron activation was for 30 min. at a flux of 1.8×10^{12} n/cm.^{2}-sec. Wide variation in chloride content of various sections of the paper

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TABLE I—ANALYSIS OF BROMIDE IN THE *p*-BROMOPHENACYL DERIVATIVE OF ASPIRIN BY NEUTRON ACTIVATION ANALYSIS AFTER CHROMATOGRAPHY

Section (cm. from Bottom)	Wt. of Silica Gel G, mg.	mcg. Br (Found)
4–6	33.7	ND[a] < 0.071
6–8	32.8	ND < 0.067
8–10	43.4	8.88 ± 0.15
10–12	35.0	0.238 ± 0.032
12–14	49.2	ND < 0.085

[a] Not detected.

TABLE II—ANALYSIS OF BROMIDE IN THE *p*-BROMOPHENACYL DERIVATIVE OF ASPIRIN BY NEUTRON ACTIVATION ANALYSIS

Wt. of Silica Gel G, mg.	mcg. Br (Found)	mcg. Br. (Theoret.)
26.0	0.947 ± 0.034	1.00
34.3	ND ± 0.072	0.10
41.7	ND ± 0.071	Blank

was noted. Two-by-two inch strips from bottom to top had chloride contents of 20.4 ± 0.5 mcg., 21.0 ± 0.7 mcg., 37.0 ± 0.7 mcg., and 41.7 ± 0.7 mcg. Washing the paper with deionized, distilled water for 8 hr. reduced the background of the 2 × 2-in. strips to 5 mcg. Other papers besides SS No. 589 white C paper were investigated for their chloride content and best results were obtained with SS 589 orange paper. Washing the paper for 3 days with 10% acetic acid followed by 7 additional days of washing with deionized, distilled water resulted in chloride values of 0.5 to 2.7 mcg. for 0.5 × 1.0-in. sections of paper.

Various silica gels for TLC were analyzed for chloride content by neutron activation. Values ranged from 0.3 to 3.5 mcg. of chloride per 5 mg. of Silica Gel GF (typical quantity per spot). The presence of manganese in Silica Gel H greatly decreased the sensitivity of the chloride assay.

Assay of Bromine-Containing Derivatives of Drugs—In contrast to the case of chloride, the bromide background of the paper was lower and more uniformly distributed. The average bromide background on SS No. 589 white C paper was 0.10 ± 0.02 mcg. per 2 × 2-in. section. Washing the paper for 8 hr. with deionized, distilled water reduced the background of the 2 × 2-in. sections to nondetectable levels, *i.e.*, <0.03 mcg.

Silica gels for TLC were analyzed by neutron activation analysis for bromide background. A 5-mg. sample of Silica Gel GF had nondetectable levels of bromide present.

In order to ascertain the problem of chromatographic separation and spreading on the plates, bromine-containing derivatives of aspirin and its metabolite were prepared. Reaction of acetylsalicylic acid with *p*-bromophenacylbromide yielded the *p*-bromophenacyl derivative of the drug. Similarly, reaction of *p*-bromophenacylbromide with salicylic acid yielded the corresponding derivative of a metabolite of the drug. The *p*-bromophenacyl derivative of aspirin has not been previously reported in the literature and was thus analyzed, m.p. 133–135°, uncorrected (capillary tube).

Anal.—Calcd. for $C_{17}H_{13}BrO_5$: C, 54.13; H, 3.45. Found: C, 54.25; H, 3.59.

The aspirin derivative was spotted on a plate in an amount so that 10 mcg. of bromide was at the origin. The chromatogram was developed in chloroform, the plate sectioned, and sent for neutron activation analysis of bromide. The results are shown in Table I.

Samples of the bromine-containing derivative of aspirin were spotted on a Silica Gel G plate which was not developed, but the silica gel was scraped off and analyzed for bromide. Table II shows these results.

Manganese in the silica gel interfered with the detection and reduced the sensitivity. It was found that bromine-containing derivatives could be removed from the silica gel plates with analytical grade acetone and the acetone evaporated in the polyethylene vials used for the neutron activation thus eliminating the interfering manganese. However, the polyethylene vial blank analyzed for 0.0435 ± 0.0087 mcg. of bromide due to interfering substances present in the polyethylene. A tube in which analytical grade acetone was evaporated analyzed for 0.0303 ± 0.0101 mcg. of bromide indicating that the solvent does not contribute to the background. The experience of General Atomics has been that polyethylene is the best available substance for these studies.

An empty polyethylene vial used for sample activation by General Atomics was activated and after removal from the reactor, the vial was washed with analytical grade acetone, and the washings transferred to a nonirradiated vial, evaporated to dryness, and counted for gamma rays. Less than 1.3×10^{-2} mcg. equivalent of bromide was detected. Maximum upper limits were established on the basis of 3 σ of the count rate in the area of the 0.777 Mev. photopeak of 35.8 hr. ^{82}Br. To determine whether any extractable bromide was present in Silica Gel HF, two samples were extracted with acetone and treated as above. The results were $<8.8 \times 10^{-3}$ mcg. bromide and $<1.22 \times 10^{-2}$ mcg. of bromide indicating that extraction of bromide from the silica gel did not contribute to the background.

SUMMARY

Background problems of paper or silica gel used for chromatography greatly limit the use of neutron activation analysis for chlorine-containing drugs. Washing of the paper lowered the background to a level of 0.5 to 2.7 mcg. for 0.5 × 1.0-in. sections of paper while Silica Gel GF values ranged from 0.3 to 3.5 mcg. per 5 mg. of gel.

Although background problems encountered in the neutron activation analysis of bromide in bromine-containing derivatives of drugs were much less than in the case of chloride, they were quite limiting. Silica gel was preferred to the use of paper for separation of the drug derivatives studied. Due to interference of substances present in the polyethylene vials used for sample holding during activation, it was necessary to extract the sample after activation and count in another vial after

solvent removal. With these precautions, limits of detection of bromide were approximately 1×10^{-2} mcg. which is far less sensitive than spectrofluorometric and gas chromatographic methods for certain drugs. With longer activation times perhaps the sensitivity could be increased but costs of analyses would also increase and, perhaps, background problems.

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(1) Steim, J. M., and Benson, A. A., *Anal. Biochem.*, **9**, 21(1964).

Keyphrases

Neutron activation, derivative—analysis
Chlorpromazine—test compound
Aspirin, bromine derivatives—test compounds
Paper chromatography—separation, degradation products
TLC—separation, degradation products

Effects of Some Adrenergic Agents on Low Frequency Electroshock Seizures

By PENG NAM YEOH and HAROLD H. WOLF

The effect of several adrenergic agents on low frequency electroshock seizures was studied in normal, reserpine and α-mmT pretreated mice. Seizure threshold was elevated by pronethalol and propranolol and reduced by phenoxybenzamine and isoproterenol. Both reserpine and α-mmT antagonized the threshold altering activity of pronethalol and phenoxybenzamine. Reserpine plus pronethalol or propranolol completely abolished the central stimulation induced by isoproterenol. A central adrenergic system involving antagonistic types of receptors appears to be involved.

It has been shown that β-adrenergic blocking agents possess anticonvulsant activity when evaluated by maximal electroshock and chemoshock techniques (1, 2). Preliminary studies in these laboratories (unpublished) indicate that the β-adrenergic blocking agents pronethalol and propranolol also reduce seizure susceptibility in audiogenic seizure susceptible mice, whereas phenoxybenzamine, an α-adrenergic blocking agent, enhances this phenomenon.

It was the objective of this study to determine whether such drug effects reflect general changes in the level of brain excitability which could be readily quantitated by measuring alterations in low frequency electroshock seizure threshold (l.f. EST). Increasing evidence indicates that endogenous brain amines may play an important role in the expression of seizure states (3–6). The experiments were designed to facilitate an evaluation of the relationship between the adrenergic drug activity described and the existing level of endogenous brain amines.

EXPERIMENTAL

Adult, male, albino mice of a random bred Swiss strain (Maxfield Animal Supply, Cincinnati, Ohio) ranging from 16 to 28 Gm. were used as experimental animals. They were housed in groups of 9 to 11 animals in plastic cages (26 × 15 × 12 cm.) with wire mesh or perforated metal tops. Except during periods of experimentation, they were maintained on Purina laboratory chow and given free access to tap water.

Pronethalol [(2-isopropylamino)-1-(2-naphthyl) ethanol hydrochloride] and propranolol [(1-isopropylamino)-3-(1-naphthyloxy)-2-propanol hydrochloride] were employed as β-adrenergic blocking agents and phenoxybenzamine hydrochloride as an α-adrenergic blocking agent. The catecholamine depleting agents used were reserpine (2 mg./Kg., 24-hr. pretreatment) and α-methyl-metatyrosine (α-mmT; 400 mg./Kg., 24-hr. pretreatment).

All drugs were given intraperitoneally as solutions in distilled water except pronethalol which was administered in 0.2% methylcellulose.[1] They were prepared so that 10 ml./Kg. contained the required dose. Requisite volumes of saline or methylcellulose (0.2%) were administered to control animals. In studies where two or more drugs were employed in sequence, the control animals received only the drug (or drugs) used for pretreatment. Except for fluorometric assays, all experiments were conducted between 4 and 11 p.m. at 23–25°.

Neurotoxicity Studies—The method of Weaver and Miya (7) with slight modifications was employed to obtain the time of peak effect and the dose of each of the adrenergic agents which produced overt evidence of neurological toxicity in 50% of mice (TD_{50}). Mice were placed on a rolling bar, 2.5 cm. in diameter, rotating at 6 r.p.m. An animal was considered to be unaffected if it could stay on the bar continuously for 1 min. in any one of three consecutive trials. Data obtained in this test were statistically analyzed by the method of Litchfield and Wilcoxon (8). In all further experiments (unless otherwise stated) the dose of all adrenergic agents used was $^1/_2$ TD_{50}.

Low Frequency Electroshock Seizure Threshold (l.f. EST) Test—A Grass stimulator (model S4G, Grass Instrument Co., Quincy, Mass.) was used to apply a unidirectional current (6 pulses per second, 0.2 msec. duration, 3 sec. stimulus duration) through corneal electrodes (9). Mice were shocked at various intensities according to the staircase method of Finney (10). The presence of a "stunning" response (at least 3 sec. of immobility) or minimal clonus was con-

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TABLE I—NEUROTOXICITY OF SOME ADRENERGIC BLOCKING AGENTS

Drug	Time of Peak Effect, min.	Toxic Dose$_{50}$, mg./Kg.
Pronethalol	5	52.0 (35.9–75.4)[a]
Propranolol	15	59.5 (53.1–66.6)
Phenoxybenzamine	60	74.0 (64.9–84.4)

[a] The figures within parentheses represent 95% fiducial limits.

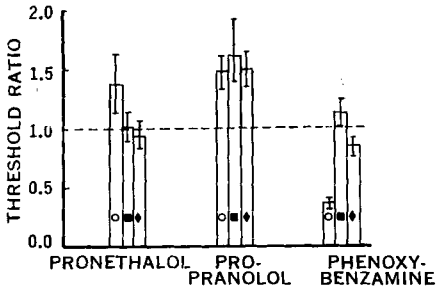

Fig. 1—Effect of some adrenergic blocking agents on l.f. EST. Key: ○, *drug* per se; ■, *reserpine + drug;* ◆, *α-mmT + drug.*

sidered indicative of a seizure. Groups of at least 24 mice each were administered $^1/_2$ TD_{50} of the various adrenergic agents and subjected to the l.f. EST test at the time of peak neurotoxicity. Control groups were always tested concurrently with drug-treated animals. The l.f. EST was determined as previously described (11). The threshold ratio (threshold of drug group/threshold of control group) was calculated and the 95% confidence limits for this ratio determined by the method of Litchfield and Wilcoxon (8).

Catecholamine Assays—The amount of depletion of brain catecholamines (norepinephrine and epinephrine) produced by reserpine and α-mmT (under experimental conditions where the depleting agents, *per se*, do not alter seizure threshold) was determined. Fluorometric assays of whole brains (3 brains per sample) were conducted according to a modified method of Anton and Sayre (12). Fluorescence was read using an Aminco-Bowman spectrophotofluorometer with photomultiplier tube IP21. The excitation–emission wavelengths for norepinephrine at pH 7.0 and for epinephrine at pH 2.0 were (395–505) and (410–520), respectively. The level of brain catecholamines (mcg./Gm. wet tissue) in reserpine and α-mmT pretreated mice was compared statistically to that of control animals by means of a *t* test.

RESULTS

A summary of the results obtained in the neurotoxicity studies is presented in Table I.

The effects of $^1/_2$ TD_{50} of the various adrenergic blocking agents employed on l.f. EST can be seen in Fig. 1. In this and Fig. 2, the vertical bracketed lines represent 95% confidence intervals for the calculated ratios and any line which does not cross 1.0 indicates that the treatment employed had a significant ($p < 0.05$) effect on convulsive threshold.

Thus it can be calculated that pronethalol and

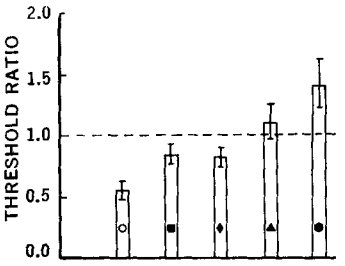

Fig. 2—Effect of isoproterenol on l.f. EST. Key: ○, *isoproterenol* per se; ■, *reserpine + isoproterenol;* ◆, *α-mmT + isoproterenol;* ▲, *reserpine + pronethalol + isoproterenol;* ●, *reserpine + propranolol + isoproterenol.*

propranolol elevate seizure threshold by 37% and 47%, respectively, whereas phenoxybenzamine lowers it by 65% when compared to saline-treated controls. Pretreatment with either reserpine or α-mmT antagonizes most of the central effects of pronethalol and phenoxybenzamine, but not that of propranolol.

No significant change in l.f. EST was demonstrated in control mice treated with either reserpine (2 mg./Kg., 24-hr. pretreatment) or α-mmT (400 mg./Kg., 24-hr. pretreatment). However, under these same experimental conditions, significant depletion of brain catecholamines was demonstrated. An examination of the results of the fluorometric assay of brain catecholamines indicated that the dose of reserpine employed produces 85.8% and 63.2% depletion of norepinephrine and epinephrine, respectively, whereas that of α-mmT induces 70.0% depletion of norepinephrine and 31.3% depletion of epinephrine.

DISCUSSION

The results of this investigation indicate that the anticonvulsant effects of pronethalol and propranolol do extend to low frequency electroshock seizures. Furthermore, phenoxybenzamine appears to lower markedly the threshold for such seizures. While the central activity seen with propranolol does not seem to be related to the existing brain level of endogenous catecholamines, that observed following treatment with either pronethalol or phenoxybenzamine is significantly altered in animals whose catecholamines have been partially depleted by reserpine or α-mmT.

Since essentially opposite effects on l.f. EST were produced by the α- *versus* β-adrenergic blocking agents examined, it was decided to investigate the central effects of an agent known to stimulate selective adrenergic receptors in the periphery. For this purpose the β-receptor stimulant isoproterenol was chosen and the same experimental procedure outlined above was followed. The TD_{50} for isoproterenol was found to be 470 (401.7–549.9) mg./Kg. with a time of peak effect of 10 min. The effect of isoproterenol on l.f. EST may be seen in Fig. 2.

Isoproterenol *per se* markedly lowers seizure threshold at a dose of $^1/_2$ TD_{50}. Pretreatment with either reserpine or α-mmT significantly reduces (but does not abolish) this effect. Finally, this same dose of isoproterenol does not induce any evident central stimulation in animals pretreated

with reserpine and a β-adrenergic blocking agent (pronethalol or propranolol). In fact, it can be seen that the propranolol plus reserpine pretreated mice have elevated seizure thresholds following the administration of isoproterenol.

The results of this preliminary study suggest the presence of central adrenergic receptors which may be involved in the expression of l.f. EST. This concept is supported by the previous work of Swinyard *et al.* (3, 4), who demonstrated that the threshold for both chemically and electrically induced seizures in mice can be altered by prior administration of epinephrine and norepinephrine. Moreover, Baldessarini and Kopin (13) have recently reported that a marked release of ^{3}H-norepinephrine from rat brain occurs in response to electrical stimulation and Scudder *et al.* (14) have demonstrated the existence of a positive correlation between the level of endogenous brain amines and maximal electroshock seizure latency in mice.

The failure to observe consistently significant alterations in l.f. EST when reserpine or α-mmT were given *per se* may reflect incomplete amine depletion, lack of inhibition of amine biosynthesis (in the case of reserpine), or production of active metabolites from α-mmT, *e.g.*, *m*-hydroxyamphetamine, metaraminol, or *m*-methoxyamphetamine (15). Nevertheless, the results observed with the adrenergic agents employed seem to imply that threshold for low frequency electroshock seizures may be markedly influenced by the degree of drug-induced differential stimulation applied to opposing central adrenergic receptors. In order to substantiate this hypothesis, additional data of a similar nature must be collected for a variety of adrenergic drugs which are known to interact specifically with α and β receptors. Furthermore, the work must be conducted in animals that have been subjected to a more selective inhibition of brain amine biosynthesis than that reported above. Such studies are currently in progress.

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Keyphrases

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Fluorometry—brain tissue analysis

Ion-Exchange Resins and Cinchona Alkaloids I. Exchange Equilibria

By S. S. KANHERE, R. S. SHAH, and S. L. BAFNA

The exchange equilibria of four cinchona alkaloid sulfates with sulfonic acid cation-exchange resins of different degrees of cross-linking and particle size have been studied and the results are discussed.

THE AVAILABLE ion-exchange studies with cinchona alkaloids (1–21) do not seem to cover detailed physicochemical studies and hence it should be of interest to undertake such work. This paper summarizes the study of exchange equilibria of four cinchona alkaloid sulfates with styrene divinylbenzene copolymer-based sulfonic acid cation-exchange resins (in hydrogen form) of different degrees of cross-linking (percent nominal divinylbenzene content) and particle size.

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EXPERIMENTAL

Resins (22) and chemicals (23) were from the samples used in earlier work. The stock solution of the alkaloid sulfate (as $Q_2H_2SO_4 \cdot nH_2O$, where Q denotes the alkaloid base) was prepared in distilled water and its concentration in gram equivalents (half the molecular weight of the alkaloid sulfate) per liter was evaluated by sulfate estimation (as barium sulfate).

To study the exchange equilibria of the alkaloid sulfates with the resins, weighed amounts of air-dried resins were placed in contact with suitable volumes of the aqueous sulfate solution of known concentration, in well-stoppered flasks, with frequent shaking at room temperature (~30°). Preliminary work was carried out to find the approximate time within which equilibrium was attained. After sufficiently more time than this (about 4 to 35 days, depending

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on X, the relative cross-linking of the resins), the solutions were analyzed for alkaloid sulfate concentration by taking out a known volume from each flask, diluting suitably with distilled water, and measuring the absorbance at the invariant wavelengths (23). The total sulfate in the equilibrium mixture was estimated gravimetrically for each resin and it showed no measurable difference in the initial and equilibrium values. Preliminary work also indicated that the values of P_R at room temperature, 35°, and 45° did not differ significantly; also the lowering of the pH to about 2.5 (by addition of sulfuric acid) did not significantly affect the value of P_R.

RESULTS AND DISCUSSION

The selectivity coefficients for alkali metal ions with resins in hydrogen form in sulfate solution had been studied earlier (22). When calculations were carried out for exchange in solutions of the cinchona alkaloid sulfates studied, with resins in the hydrogen form, the calculated values of P_R (Tables I and II; P_R = 100 times the mole fraction of counter ions in the resin phase at equilibrium) were practically constant for resins of higher degree of cross-linking and varied only to a relatively small extent for resins of lower degrees of cross-linking when P_A was varied.

The results were not measurably different when either the amount of alkaloid sulfate solution was held constant and the amount of added resin varied or when the amount of added resin was held constant and the amount of alkaloid sulfate solution was varied, provided the ratio of the initial concentration (in meq./L.) of the resin to the initial concentration of the alkaloid sulfate was the same. This suggests that the value of the equilibrium constant for the exchange (24–32) of the cation QH^+ is large and the equilibrium $HR + QH^+ \rightleftharpoons RQH + H^+$ is shifted very much to the right. However, all the replaceable hydrogen ions in the resin phase are not accessible for exchange, or the available capacity is only a fraction of the total capacity, probably due to the size of the organic counter ions. It is likely that the marked shift of the equilibrium to the right is aided by the operation of nonexchange interactions (31–33) and that the interactions tend to increase the extent of exchange with increase in $(A)_e$. This may be feasible, to some extent, by further expansion of and/or further separation between some segments in the swollen (34,35) or expanded resin network and should depend on the degree of cross-linking and the extent to which exchange has already occurred. If the further expansion and/or segment separation is sufficient to accommodate some more organic cations by exchange, P_R should increase with increase in $(A)_e$ or decrease in P_A; this is so for lower cross-linked resins in hydrogen form. On the other hand,

TABLE I—EQUILIBRIUM OF AQUEOUS QUININE SULFATE AND QUINIDINE SULFATE WITH SULFONIC ACID CATION-EXCHANGE RESINS

		Quinine Sulfate					Quinidine Sulfate				
Resin	a (mm.)	$(\bar{H})_i$	$(A)_i$	$(\bar{A})_e$	P_A	P_R	$(\bar{H})_i$	$(A)_i$	$(\bar{A})_e$	P_A	P_R
X1	0.215	0.86	2.12	0.47	22.1	54.7	0.85	2.00	0.48	24.0	56.5
		1.69	2.12	0.89	42.0	52.7	1.70	2.00	0.87	43.5	51.2
		2.52	2.12	1.28	60.4	50.9	2.13	2.00	1.05	52.5	49.3
		3.38	2.12	1.69	79.7	50.0	2.92	2.00	1.37	68.5	46.9
		4.22	2.12	2.05	96.7	48.6	3.61	2.00	1.64	82.0	45.4
X2	0.215	0.84	2.12	0.44	20.8	52.7	1.68	2.00	0.90	45.0	53.6
		1.68	2.12	0.86	40.5	51.1	2.14	2.00	1.08	54.0	50.5
		2.49	2.12	1.25	59.0	50.2	3.33	2.00	1.62	81.0	48.6
		3.35	2.12	1.64	77.4	49.0	4.18	2.00	1.93	96.5	46.2
		4.18	2.12	2.01	94.8	48.1	...	...	...	...	...
X4	0.215	0.70	2.07	0.34	16.5	49.1	1.28	2.00	0.61	30.5	47.6
		1.38	2.07	0.69	33.3	49.9	1.98	2.00	0.95	47.5	48.0
		2.77	2.07	1.38	66.7	49.8	2.54	2.00	1.22	61.0	48.0
		3.44	2.07	1.69	81.6	49.1	3.83	2.00	1.80	90.0	47.0
X8	0.215	0.98	2.07	0.39	19.1	40.1	1.38	2.00	0.56	28.0	40.6
		1.46	2.07	0.60	28.8	40.9	1.83	2.00	0.70	35.0	38.1
		2.00	2.07	0.80	38.8	40.2	2.76	2.00	1.05	52.5	38.0
		4.01	2.07	1.56	75.4	39.0	3.64	2.00	1.44	72.0	39.6
X12	0.215	1.44	2.12	0.37	17.3	25.5	2.86	2.00	0.71	35.5	24.8
		2.87	2.12	0.72	34.0	25.1	4.13	2.00	1.02	51.0	24.7
		5.73	2.12	1.48	69.8	25.8	5.72	2.00	1.39	69.5	24.3
		7.16	2.12	1.84	86.8	25.7	7.22	2.00	1.79	89.5	24.8
X16	0.215	2.15	2.12	0.38	18.1	17.8	4.26	2.00	0.73	36.5	17.1
		6.44	2.12	1.17	55.2	18.2	6.39	2.00	1.12	56.0	17.5
		8.64	2.12	1.59	75.0	18.4	7.76	2.00	1.54	77.0	19.8
		10.76	2.12	1.95	92.1	18.1	10.65	2.00	1.78	89.0	16.7
X20	0.215	6.10	2.02	0.33	16.3	5.4	5.53	2.00	0.27	13.5	4.9
		11.19	2.02	0.72	35.5	6.4	8.12	2.00	0.47	23.5	5.8
		...	...	...	...	...	11.10	2.00	0.70	35.0	6.3
IR-120	0.37	2.62	2.02	1.06	52.5	40.5	2.62	2.00	0.95	47.5	36.2
		3.81	2.02	1.54	76.2	40.4	3.84	2.00	1.40	70.0	36.5
		5.00	2.02	1.97	97.5	39.4	5.05	2.00	1.87	93.5	37.1
IR-200	0.37	2.87	2.02	0.90	44.7	31.5	2.89	2.00	0.88	44.0	30.4
		4.29	2.02	1.31	64.9	30.5	4.33	2.00	1.31	65.5	30.3
		5.70	2.02	1.70	84.1	29.8	5.74	2.00	1.68	84.0	29.3
Am-15	0.37	2.65	2.10	0.86	40.9	32.4	2.11	2.00	0.67	33.5	31.8
		3.52	2.10	1.12	53.3	31.8	3.52	2.00	1.09	54.5	31.0
		4.40	2.10	1.39	66.1	31.6	4.34	2.00	1.37	68.5	31.1
		5.29	2.10	1.66	79.0	31.4	5.25	2.00	1.61	80.5	30.7

TABLE II—EQUILIBRIUM OF AQUEOUS CINCHONINE SULFATE AND CINCHONIDINE SULFATE WITH SULFONIC ACID CATION-EXCHANGE RESINS

Resin	a (mm.)	Cinchonine Sulfate					Cinchonidine Sulfate				
		$(\bar{H})_i$	$(A)_i$	$(\bar{A})_e$	P_A	P_R	$(\bar{H})_i$	$(A)_i$	$(\bar{A})_e$	P_A	P_R
X1	0.215	1.35	2.00	0.77	38.5	57.0	1.34	2.18	0.79	36.2	58.9
		1.69	2.00	0.91	45.5	53.8	1.67	2.18	0.94	43.1	56.3
		2.09	2.00	1.08	54.0	51.7	2.31	2.18	1.21	55.1	52.4
		3.39	2.00	1.68	84.0	49.6	2.94	2.18	1.49	68.3	50.7
		4.00	2.00	1.94	97.0	48.5	3.48	2.18	1.74	79.8	50.0
X2	0.215	1.27	2.00	0.72	36.0	56.7	1.26	2.18	0.74	33.9	58.7
		1.59	2.00	0.87	43.5	54.7	1.58	2.18	0.89	40.8	56.3
		1.97	2.00	1.03	51.5	52.3	2.17	2.18	1.14	52.3	52.5
		3.18	2.00	1.59	79.5	50.0	2.75	2.18	1.41	64.7	51.3
		3.96	2.00	1.94	97.0	49.0	3.27	2.18	1.65	75.7	50.5
X4	0.215	1.43	2.00	0.77	38.5	53.9	1.54	2.18	0.84	38.5	54.5
		2.21	2.00	1.14	57.0	51.6	2.11	2.18	1.12	51.4	53.1
		2.83	2.00	1.43	71.5	50.5	2.71	2.18	1.40	64.0	51.6
		4.27	2.00	1.96	98.0	45.8	3.26	2.18	1.65	75.7	50.6
X8	0.215	1.42	2.00	0.62	31.0	43.7	1.49	2.18	0.67	30.7	45.0
		2.22	2.00	1.00	50.0	45.0	2.06	2.18	0.93	42.7	45.1
		2.83	2.00	1.27	63.5	44.9	2.61	2.18	1.19	54.6	45.6
		3.72	2.00	1.66	83.0	44.6	3.16	2.18	1.42	65.1	44.9
X12	0.215	3.00	2.00	0.81	40.5	27.0	3.00	2.18	0.86	39.5	28.7
		4.31	2.00	1.18	59.0	27.4	4.49	2.18	1.31	60.1	29.2
		7.54	2.00	1.97	98.5	26.1	5.98	2.18	1.77	81.2	29.6
X16	0.215	4.27	2.00	0.91	45.5	21.3	4.46	2.18	0.99	45.4	22.2
		6.37	2.00	1.39	69.5	21.8	5.33	2.18	1.19	54.6	22.3
		...	...	...	...	...	7.10	2.18	1.60	73.4	22.5
X20	0.215	5.53	2.00	0.36	18.0	6.5	11.10	2.18	0.78	35.8	7.0
		8.14	2.00	0.62	31.0	7.6	13.70	2.18	1.03	47.2	7.5
		11.06	2.00	1.00	50.0	9.0	16.66	2.18	1.34	61.5	8.0
IR-120	0.37	1.89	1.91	0.83	43.5	43.9	1.96	2.18	0.83	38.1	42.3
		2.76	1.91	1.27	66.5	46.0	2.81	2.18	1.22	56.0	43.4
		3.62	1.91	1.69	88.5	46.7	3.68	2.18	1.58	72.5	42.9
IR-200	0.37	2.06	2.00	0.70	35.0	34.0	2.73	2.18	0.93	42.7	34.1
		2.75	2.00	0.92	46.0	33.5	3.62	2.18	1.21	55.6	33.5
		4.14	2.00	1.35	67.5	32.6	4.52	2.18	1.50	68.8	33.2
		5.49	2.00	1.74	87.0	32.4	5.42	2.18	1.77	81.2	32.7
Am-15	0.37	2.64	1.91	0.93	48.7	35.2	...	...	...	...	...
		3.52	1.91	1.21	63.4	34.4	3.52	2.15	1.24	57.7	35.3
		4.42	1.91	1.50	78.5	33.9	4.39	2.15	1.53	70.9	34.7
		...	...	...	...	...	5.28	2.15	1.81	84.2	34.4

if the further expansion and/or segment separation is too small to accommodate more organic cations by exchange, P_R should remain practically constant with increase in $(A)_e$ or decrease in P_A; this is so for higher cross-linked resins in hydrogen form.

For the resins X1 and X2 (Tables I and II), the value of P_R increases to a small extent with increase in $(A)_e$ or decrease in P_A. For the resin X4, the variation in the values of P_R is to a lesser extent than that for resin X2, for cinchonine sulfate and cinchonidine sulfate; for quinine sulfate and quinidine sulfate, the value of P_R is practically independent of P_A. For the resin X8 the values of P_R for all the four cinchona alkaloid sulfates are practically independent of P_A and for higher values of P_A are less than those for the resin X4. The decrease is more for quinine sulfate and quinidine sulfate than that for cinchonine sulfate and cinchonidine sulfate. This decrease may be attributed to the reduced pore size because of higher X; the higher value of P_R for cinchonine sulfate and cinchonidine sulfate than that for quinine sulfate and quinidine sulfate should be due to the smaller size of cinchonine and cinchonidine than that of quinine and quinidine. For resins of higher degree of cross-linking, X12, X16, and X20, the behavior is similar and the value of P_R decreases with increase in X.

Similar studies were carried out with resins IR-120, IR-200, and Am-15 of different particle size (IR-120: a = 0.58, 0.37; IR-200: a = 0.84, 0.58, 0.37, 0.23; Am-15: a = 1.13, 0.84, 0.58, 0.37). It was observed that the values of P_R were practically independent of a. Tables I and II give some representative data. The macromolecular resins IR-200 and Am-15 presumably have the value of X as approximately 20 but have expanded structure and hence are more porous than the gel-type resin X20; the values of P_R for IR-200 and Am-15 are therefore higher than those for the resin X20.

Nomenclature—

$(A)_i$ = the initial concentration of alkaloid sulfate solution in meq./L.,

$(A)_e$ = the equilibrium concentration of alkaloid sulfate in external solution in meq./L.,

$(\bar{A})_e$ = the meq. of alkaloid in the resin phase per liter of solution, at equilibrium,

$(\bar{H})_i$ = the meq. of resin per liter of the solution in the hydrogen form, initially,

$P_A = 100 \cdot (\bar{A})_e/(A)_i$ = the percent exchange of alkaloid sulfate at equilibrium,

$P_R = 100 \cdot (\bar{A})_e/(\bar{H})_i$ = the percent resin capacity exchanged at equilibrium.

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Keyphrases

Cinchona alkaloids
Ion-exchange resins—alkaloids, exchange equilibria
Sulfate, total—equilibrium mixture
Alkaloid sulfate concentration

Antiradiation Compounds IX. Dithiocarbamates of Strongly Basic Pyridines and Pyrimidines

By WILLIAM O. FOYE and DOUGLAS H. KAY

Dithiocarbamates of exceptionally strong bases in the pyridine and pyrimidine series, *e.g.*, the 1-alkyl-2- and 4-imino derivatives, have been obtained. Significant protection in mice against ionizing radiation was provided by a dithiocarbamate of one of the strong bases, whereas the dithiocarbamates of weaker bases have generally shown a lower level of protection.

BROWN (1) reported that the introduction of an alkyl group on one of the ring nitrogens of aminopyrimidines greatly increases the basicity of the molecule, the amino group being converted to the imino form. For example, 2-aminopyrimidine has a pKa of 3.54 and 1,2-dihydro-2-imino-1-methylpyrimidine has a pKa of 10.75. Likewise, in the aminopyridine series Albert (2) reported that 2-aminopyridine and 4-aminopyridine have pKa values of 6.9 and 9.2, respectively, whereas the alkylated derivatives, 1,2-dihydro-2-imino-1-methylpyridine and 1,4-dihydro-4-imino-1-methylpyridine have pKa values of 12.2 and 12.5, respectively.

Dithiocarbamates of these exceptionally strong bases were desired as potential antiradiation agents. Such compounds could be considered cyclic dithio acid analogs of mercaptoethylguanidine, which is also strongly basic. Of the compounds mentioned above, only 1,2-dihydro-2-imino-1-methylpyridine has previously been treated with carbon disulfide (3), giving a compound described as 1,2-dihydro-1-methyl-2-pyridinylimmonium 1,2-dihydro-1-methyl-2-pyridinyldithiocarbamate (I). The reactions of amino and imino pyrimidines and pyridines with carbon disulfide described here also gave dithiocarbamate salts rather than zwitterions. The latter might be expected since dithiocarbamate formation takes place preferentially on the weaker of two competing bases and cation formation on the stronger (4). Infrared absorption spectra of the dithiocarbamates of the strongly basic imines showed stretching frequencies for both C=S (~1000 cm.$^{-1}$) and C=N (1650–1675 cm.$^{-1}$), which supports the structures proposed (I). Dithiocarbamates of the corresponding weak (nonalkylated) bases were also prepared for comparison of radiation-protective activity.

Antiradiation testing results show that the dithiocarbamate of one of the imines protects mice at a higher level of radiation (1,000 r) than has generally

Received June 9, 1967, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston, MA 02115
Accepted for publication October 2, 1967.
Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This investigation was supported by the U. S. Army Medical Research and Development Command contract No. DA-49-193-MD-2029 and research grant RH00297 from the National Center for Radiological Health, U. S. Public Health Service, Bethesda, Md.

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Antiradiation Compounds IX. Dithiocarbamates of Strongly Basic Pyridines and Pyrimidines

By WILLIAM O. FOYE and DOUGLAS H. KAY

Dithiocarbamates of exceptionally strong bases in the pyridine and pyrimidine series, *e.g.*, the 1-alkyl-2- and 4-imino derivatives, have been obtained. Significant protection in mice against ionizing radiation was provided by a dithiocarbamate of one of the strong bases, whereas the dithiocarbamates of weaker bases have generally shown a lower level of protection.

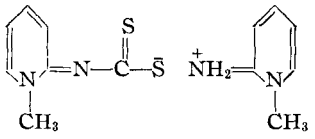
BROWN (1) reported that the introduction of an alkyl group on one of the ring nitrogens of aminopyrimidines greatly increases the basicity of the molecule, the amino group being converted to the imino form. For example, 2-aminopyrimidine has a pKa of 3.54 and 1,2-dihydro-2-imino-1-methylpyrimidine has a pKa of 10.75. Likewise, in the aminopyridine series Albert (2) reported that 2-aminopyridine and 4-aminopyridine have pKa values of 6.9 and 9.2, respectively, whereas the alkylated derivatives, 1,2-dihydro-2-imino-1-methylpyridine and 1,4-dihydro-4-imino-1-methylpyridine have pKa values of 12.2 and 12.5, respectively.

Dithiocarbamates of these exceptionally strong bases were desired as potential antiradiation agents. Such compounds could be considered cyclic dithio acid analogs of mercaptoethylguanidine, which is also strongly basic. Of the compounds mentioned above, only 1,2-dihydro-2-imino-1-methylpyridine has previously been treated with carbon disulfide (3), giving a compound described as 1,2-dihydro-1-methyl-2-pyridinylimmonium 1,2-dihydro-1-methyl-2-pyridinyldithiocarbamate (I). The reactions of amino and imino pyrimidines and pyridines with carbon disulfide described here also gave dithiocarbamate salts rather than zwitterions. The latter might be expected since dithiocarbamate formation takes place preferentially on the weaker of two competing bases and cation formation on the stronger (4). Infrared absorption spectra of the dithiocarbamates of the strongly basic imines showed stretching frequencies for both C=S (~1000 cm.$^{-1}$) and C=N (1650–1675 cm.$^{-1}$), which supports the structures proposed (I). Dithiocarbamates of the corresponding weak (nonalkylated) bases were also prepared for comparison of radiation-protective activity.

Antiradiation testing results show that the dithiocarbamate of one of the imines protects mice at a higher level of radiation (1,000 r) than has generally

Received June 9, 1967, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston, MA 02115
Accepted for publication October 2, 1967.
Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This investigation was supported by the U. S. Army Medical Research and Development Command contract No. DA-49-193-MD-2029 and research grant RH00297 from the National Center for Radiological Health, U. S. Public Health Service, Bethesda, Md.

been shown by the dithiocarbamates of weaker bases.



I

DISCUSSION

The method of Brown (1) was used to prepare 1,2-dihydro-2-imino-1-methylpyrimidine hydriodide, and the free base was obtained in benzene by a modified extraction procedure. A minimum amount of water was found necessary in the neutralization step to prevent rearrangement to 2-methylaminopyrimidine which occurs readily in warm alkaline solution (1). The free base in benzene was treated with carbon disulfide, without heating, to yield the desired dithiocarbamate, obtained as the imine salt. The next higher homolog, 1,2-dihydro-2-imino-1-ethylpyrimidine was converted similarly to the dithiocarbamate salt, obtained by very slow precipitation from toluene. It should be noted that these dithiocarbamates were not recrystallized, thus avoiding the possibility of such further condensations as were recently reported by Takeshima *et al.* (5) for the immonium salt of a dithio acid.

The preparation of 1,2-dihydro-2-imino-1-ethylpyrimidine hydriodide (m.p. 191–193°) has not previously been described. However, Kogon (6) has described an isomer, 2-ethylaminopyrimidine (m.p. 50–51°), its hydriodide (m.p. 154–155°), and the picrate (m.p. 160–161°). The latter hydriodide was obtained by refluxing a solution of 2-aminopyrimidine and ethyl iodide in absolute ethanol. By using a less polar solvent, dioxane, the desired 1,2-dihydro-2-imino-1-ethylpyrimidine hydriodide was obtained. However, this hydriodide was found to rearrange readily in 10% aqueous alkali to 2-ethylaminopyrimidine. A minimum amount of water in the neutralization step was found necessary to prevent this rearrangement. Proof that it did not occur is shown by the formation of the picrate (m.p. 178–179°) of the free base in toluene just prior to treatment with carbon disulfide.

N-2-Pyridyldithiocarbamate was obtainable as the triethylammonium salt by the procedure of either Fairfull and Peak (7) or Knott (8). This product was found to be light-sensitive. The preparation of 2-pyridylammonium *N*-2-pyridyldithiocarbamate was previously reported, using the sodium salt of 2-aminopyridine in nonaqueous media with carbon disulfide (9).

4-Pyridylammonium *N*-4-pyridyldithiocarbamate was more conveniently prepared than the triethylammonium salt (8). In regard to the former compound, Camps (10) reported that reaction of 4-aminopyridine, ethanol, and carbon disulfide yielded a product melting at 152°, which he suspected to be the pyridylammonium salt of the dithiocarbamate. No elemental analyses were reported, and it is probable that Camps' product was a thiourea derivative rather than the pyridylammonium salt of the dithiocarbamate, which was found to melt at 134–135°.

The methods of Chichibabin were used to prepare 1,2-dihydro-2-imino-1-methylpyridine hydriodide (11) and 1,4-dihydro-4-imino-1-methylpyridine hydriodide (12). The free base of the 2-imino isomer was obtained by extracting an aqueous alkaline solution of the hydriodide with benzene. The other isomer was obtained by triturating the hydriodide with powdered potassium hydroxide in benzene and extracting with warm benzene. The dithiocarbamate salt of 1,2-dihydro-2-imino-1-methylpyridine (I) was prepared by the procedure of Topchiev (3). The corresponding 4-imino isomer was prepared by treating a benzene solution of the free base with carbon disulfide.

Although the tendency for a shift of the methyl group from the ring nitrogen of an aminopyridine to form a methylamino derivative appears to be much less than in the pyrimidine series, proof that this shift did not occur was obtained by preparing the picrates of the free bases in benzene prior to treatment with carbon disulfide. These picrates differed substantially from those of the rearranged bases. Additional evidence that rearrangement did not occur to give the dithiocarbamates of the *N*-methylaminopyrimidines was shown by the failure of the rearranged 2-methylaminopyrimidine to give a dithiocarbamate under the reaction conditions employed. 2-Aminopyrimidine also failed to undergo dithiocarbamate formation using any of the methods described.

Isomeric with these imines are 3-aminomethylpyridine and 4-aminomethylpyridine. Treatment of these amines in absolute ethanol with carbon disulfide gave the respective dithiocarbamate salts.

Antiradiation Properties—Antiradiation screening for several of the compounds has been carried out at the Walter Reed Army Institute of Research under the direction of Dr. D. P. Jacobus. Tests were done as previously indicated (13) in mice *versus* 1,000 r (γ-rays), and results received so far show that 1,2-dihydro-1-methyl-2-pyridinylimmonium 1,2-dihydro-1-methyl-2-pyridinyldithiocarbamate (I) provided up to 24% protection with doses up to 350 mg./Kg. The dithiocarbamates of the corresponding 4-iminopyridine and of the 2-imino-1-methylpyrimidine showed no protection at this radiation level with doses of less than 50 mg./Kg. A previous report (14) of radiation-protective activities of dithiocarbamates of weaker bases showed activity only at a lower level of radiation dosage (600 r, X-rays).

EXPERIMENTAL

Analyses for carbon, hydrogen, and nitrogen were done by Weiler and Strauss, Oxford, England, or by Carol K. Fitz, Needham, Mass. Sulfur analyses done by Parr bomb peroxide fusion. Melting points were taken on a Mel-Temp apparatus and are corrected. Infrared absorption spectra were obtained with a Perkin-Elmer model 137B spectrometer.

1,2 - Dihydro - 1 - methyl - 2 - pyrimidinylimmonium 1,2 - Dihydro - 1 - methyl - 2 - pyrimidinyldithiocarbamate—Fifteen grams (0.063 mole) of 1,2-dihydro-2-imino-1-methylpyrimidine hydriodide (1) was placed in a glass mortar immersed in an ice bath. The solid was covered with 50 ml. of dry benzene, and powdered potassium hydroxide (15 Gm.) was added slowly during 30 min. to the cooled mixture (below 10°) with trituration. The yellow benzene extract was decanted, and the residue was triturated with an additional 150 ml. of anhydrous benzene in three portions during 1 hr. The benzene

extracts were combined and dried, and the residue was stored in a stoppered container with 40 ml. of anhydrous benzene at 0° for 2 days.

The combined benzene extracts were dried and filtered into a flask equipped with a drying tube. The benzene solution of 1,2-dihydro-2-imino-1-methylpyrimidine was ice-cooled, and 2 ml. (0.03 mole) of carbon disulfide in 10 ml. of anhydrous benzene was added slowly with stirring during 15 min. Stirring with ice-cooling was continued for 4 hr., and the mixture was allowed to stand overnight at room temperature.

An additional milliliter of carbon disulfide was added and the mixture was stirred for 3 hr. at room temperature. The orange-colored solid was collected quickly, was washed three times with 40-ml. portions of anhydrous benzene, and dried *in vacuo*, giving 1.7 Gm. of product that melted at 111–113°. Further washing with benzene gave 1.5 Gm. melting at 115–117° with slight decomposition beginning about 110°; $\nu_{min.}^{Nujol}$ 970, 1000, 1140, 1180, 1325, 1540, 1600, 1650, 1670.

Anal.—Calcd. for $C_{11}H_{14}N_6S_2$: C, 44.89; H, 4.76; N, 28.57. Found: C, 45.09; H, 4.69; N, 28.40.

1,2 - Dihydro - 2 - imino - 1 - ethylpyrimidine Hydriodide—Ten grams (0.105 mole) of 2-aminopyrimidine and 130 ml. of dioxane were heated to boiling, filtered, and refluxed with 20 ml. (0.25 mole) of ethyl iodide for 1 hr. The mixture was kept at room temperature for 24 hr., and this procedure was repeated twice. The precipitated solid was removed prior to each reflux period. After recrystallization from 95% ethanol 8.0 Gm. (30.3%) of product was obtained, m.p. 191–193°.

Anal.—Calcd. for $C_6H_{10}IN_3$: C, 28.70; H, 4.01; I, 50.56; N, 16.73. Found: C, 29.15; H, 4.07; I, 49.90; N, 16.28.

The picrate was crystallized from 95% ethanol, m.p. 178–179°.

Anal.—Calcd. for $C_{12}H_{12}N_6O_7$: C, 40.92; H, 3.43. Found: C, 41.35; H, 3.26.

1,2 - Dihydro - 1 - ethyl - 2 - pyrimidinylimmonium 1,2 - Dihydro - 1 - ethyl - 2 - pyrimidinyldithiocarbamate—Ten grams (0.04 mole) of 1,2-dihydro-2-imino-1-ethylpyrimidine hydriodide was placed in a glass mortar containing 10 Gm. of powdered potassium hydroxide. The mixture was triturated with 200 ml. of toluene, in four portions, during 1 hr. The combined toluene extracts were filtered and then dried.

The toluene extract (5 ml.) was treated with 5 ml. of a saturated solution of picric acid in toluene, giving a picrate melting at 178–179°, the value for the picrate of 1,2-dihydro-2-imino-1-ethylpyrimidine.

The toluene solution of 1,2-dihydro-2-imino-1-ethylpyrimidine was treated with 5 ml. (0.07 mole) of carbon disulfide, and mechanically stirred during a 3-week period. A yellow solid slowly precipitated which became orange in color by the end of the first week. The orange solid was collected, washed with two 25-ml. portions of anhydrous ether, and dried. About 1.2 Gm. of orange solid was obtained that melted at 101–105°.

Anal.—Calcd. for $C_{13}H_{18}N_6S_2$: C, 48.42; H, 5.62; N, 26.06. Found: C, 48.73; H, 5.80; N, 25.55.

Triethylammonium N-2-Pyridyldithiocarbamate—The procedure of Fairfull and Peak (7) was used. From 6.27 Gm. (0.07 mole) of 2-aminopyridine, 7.0 Gm. (39%) of product was obtained that melted at 88–89°, which agrees with the reported value (7); $\nu_{min.}^{KBr}$ 980, 1030, 1090, 1150, 1235, 1300, 1500, 1575, 2450, 2600.

4-Pyridylammonium N-4-Pyridyldithiocarbamate—A mixture of absolute ethanol (30 ml.) and 9.4 Gm. (0.1 mole) of 4-aminopyridine was refluxed on a water bath until the 4-aminopyridine dissolved. Then 14 ml. (0.2 mole) of carbon disulfide was added slowly during 10 min. Refluxing was stopped after 15 min. when hydrogen sulfide was evolved.

The reaction solution was stirred with ice-cooling for 2 hr., and then refrigerated overnight. A yellow solid was collected and washed with absolute ethanol. After refluxing the filtrate for 30 min. and cooling, a total of 5.0 Gm. (38%) of product was obtained, m.p. 134–135°. $\nu_{min.}^{KBr}$ 1000, 1060, 1200, 1250, 1300, 1500, 1600, 1650, 2050, 2500.

Anal.—Calcd. for $C_{11}H_{12}N_4S_2$: C, 49.97; H, 4.57; N, 21.19; S, 24.27. Found: C, 50.16; H, 4.55; N, 21.40; S, 24.53.

1,2 - Dihydro - 2 - imino - 1 - methylpyridine—Eighteen grams (0.076 mole) of 1,2-dihydro-2-imino-1-methylpyridine hydriodide (11) dissolved in 50 ml. of 2 *N* sodium hydroxide solution was extracted with 150 ml. of warm benzene for 9 hr. in a liquid-liquid extraction apparatus. The extraction was repeated twice. The combined benzene extracts were evaporated *in vacuo* at 45°/20–25 mm., giving 7.5 Gm. (88%) of a red oil that boiled at 110°/20 mm., which agrees with the reported value (11) for the free base. The picrate of the free base was prepared, and it melted at 205–207° after recrystallization from 95% ethanol. [Lit. m.p. 201° (11).]

1,2 - Dihydro - 1 - methyl - 2 - pyridinylimmonium 1,2 - Dihydro - 1 - methyl - 2 - pyridinyldithiocarbamate—The procedure of Topchiev (3) was used. From 7.5 Gm. (0.07 mole) of 1,2-dihydro-2-imino-1-methylpyridine, 7.2 Gm. (67%) of product was obtained that melted at 153–154° with decomposition beginning about 145°, which agrees with the reported value (3); $\nu_{min.}^{Nujol}$ 950, 1030, 1050, 1070, 1160, 1235, 1560, 1600, 1650, 1670.

1,4-Dihydro-4-imino-1-methylpyridine Hydriodide—The method of Chichibabin (12) was used. From 20 Gm. (0.21 mole) of 4-aminopyridine, 40 Gm. (80%) of product was obtained that melted at 190–192°. [Lit. m.p. 187–188° (12).]

1,4 - Dihydro - 1 - methyl - 4 - pyridinylimmonium 1,4 - Dihydro - 1 - methyl - 4 - pyridinyldithiocarbamate—Thirty grams (0.13 mole) of 1,4-dihydro-4-imino-1-methylpyridine hydriodide was placed in a glass mortar. Anhydrous benzene (50 ml.) was added, and 25 Gm. of powdered potassium hydroxide was added slowly during 30 min. with trituration. The cloudy benzene extract was removed by decantation and filtered under nitrogen. The residue was triturated thoroughly with 350 ml. of warm benzene in seven portions during 1 hr.

About 20 ml. of the combined benzene extract was evaporated with a stream of nitrogen to yield a white hygroscopic solid that melted at 150–151°, which agrees with the reported value (12) for the free base. The picrate of the free base was prepared, m.p. 192–194°, after recrystallization from 95% ethanol. [Lit. m.p. 188–189° (12).]

The cloudy benzene extract was treated with 2 ml. (0.03 mole) of carbon disulfide, which was added

dropwise during 5 min. with constant stirring. The mixture was stirred for 30 min., and the orange solid was collected, washed with two 50-ml. portions of benzene, and dried *in vacuo*. About 5.5 Gm. of product was obtained, m.p. 170–172°. $\nu_{min.}^{Nujol}$ 960, 980, 1040, 1135, 1200, 1540, 1650.

Anal.—Calcd. for $C_{13}H_{16}N_3S_2$: C, 53.39; H, 5.52; N, 19.16; S, 21.93. Found: C, 53.08; H, 5.59; N, 19.32; S, 21.10.

4-Pyridylmethylammonium N-4-Pyridylmethyldithiocarbamate—4-Aminomethylpyridine (11 Gm., 0.1 mole) (Reilly Tar and Chemical Corp.) in 30 ml. of absolute ethanol was ice-cooled and stirred while 3.5 ml. (0.05 mole) of carbon disulfide was added dropwise. The mixture was stirred for 15 min. and the solid was collected and washed with two 25-ml. portions of absolute ethanol. About 12 Gm. (80.5%) of a white, light-sensitive solid was obtained, m.p. 131–132°. $\nu_{KBr}^{min.}$ 940, 1000, 1070, 1115, 1225, 1300, 1600.

Anal.—Calcd. for $C_{13}H_{16}N_4S_2$: C, 53.39; H, 5.52; N, 19.16; S, 21.93. Found: C, 53.19; H, 5.58; N, 18.70; S, 21.87.

3-Pyridylmethylammonium N-3-Pyridylmethyldithiocarbamate—The same procedure was used as above. From 3-aminomethylpyridine (11 Gm., 0.1 mole) was obtained 13.8 Gm. (92.6%) of a white solid that melted at 130–132°. $\nu_{KBr}^{min.}$ 920, 975, 1050, 1185, 1250, 1300, 1575.

Anal.—Calcd. for $C_{13}H_{16}N_4S_2$: C, 53.39; H, 5.52; N, 19.16; S, 21.93. Found: C, 53.19; H, 5.27; N, 18.55; S, 21.89.

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Keyphrases

Antiradiation compounds
Pyridine dithiocarbamates—synthesis
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IR spectrophotometry—structure
Radiation protective properties

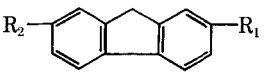
New Compounds: Fluorene Derivatives as Potential Carcinogens

By NELLIE W. PITZER and FRANCIS E. RAY*

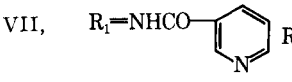
A series of eight new *N*-substituted fluorenamides have been synthesized for evaluation of carcinogenic activity.

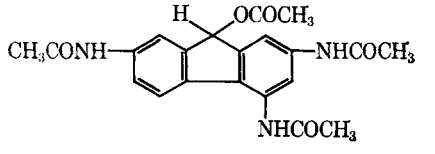
IT HAS BEEN reported that diethylnitrosamine produces hepatic carcinomas in rats (1, 2). Since *N*-2-fluorenylamine is also a carcinogen (3), it was thought that *N*-2-fluorenylethylnitrosamine (I) might have a similar or stronger activity.

Because *N*-2-fluorenylsuccinamic acid is a potent carcinogen (4), additional derivatives (II, III, IV, V and VI) were synthesized. Since nicotinic acid is known to be involved in the biological oxidation systems (5), carcinogenic activity might be found in *N*-2-fluorenylnicotinamide (VII). In the generally less carcinogenic fluorenone series, *N,N',N''*-2,4,7-fluorenylene(9-acetoxy)trisacetamide (VIII) was obtained.[1]



I, R_1 = N·(NO)CH_2CH_3, R_2 = H
II, R_1 = $NHCOCH_2CH_2COOH$, R_2 = $NHCOCH_2CH_2COOH$
III, R_1 = $NHCOCH_2C$:$(CH_2)COOH$, R_2 = H
or R_1 = NHCOC:$(CH_2)CH_2COOH$ (Structural studies on this compound will be presented in a future communication.)
IV, R_1 = $NHCO(CH_2)_6CH_3$, R_2 = H
V, R_1 = $NHCO(CH_2)_6CH_3$, R_2 = $NHCO(CH_2)_6CH_3$
VI, R_1 = $NHCO(CH_2)_{14}CH_3$, R_2 = H

VII, 

VIII, 

EXPERIMENTAL

Ultraviolet spectra were obtained on a Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer in 95% ethanol. Microanalyses were performed by Schwarzkopf Microanalytical Laboratories.

Received July 26, 1967, from the Pharmaceutical Chemistry Research Laboratory, University of Florida, Gainesville, FL 32601
Accepted for publication September 12, 1967.
This investigation was supported by research grant RO1 CA 07737 from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
The authors thank Dr. Danuta Malejka for her assistance in preparing this manuscript.
* Deceased.
[1] Compounds I, II, III, and VIII were proved to be carcinogenic in the preliminary investigation by Dr. Harold P. Morris, National Cancer Institute, Bethesda, Md. All compounds are subject of further studies in this institute.

dropwise during 5 min. with constant stirring. The mixture was stirred for 30 min., and the orange solid was collected, washed with two 50-ml. portions of benzene, and dried *in vacuo*. About 5.5 Gm. of product was obtained, m.p. 170–172°. $\nu_{min.}^{Nujol}$ 960, 980, 1040, 1135, 1200, 1540, 1650.

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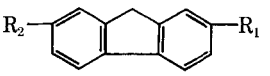
By NELLIE W. PITZER and FRANCIS E. RAY*

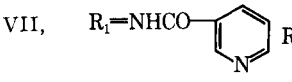
A series of eight new *N*-substituted fluorenamides have been synthesized for evaluation of carcinogenic activity.

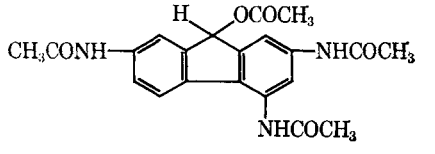
IT HAS BEEN reported that diethylnitrosamine produces hepatic carcinomas in rats (1, 2). Since *N*-2-fluorenylamine is also a carcinogen (3), it was thought that *N*-2-fluorenylethylnitrosamine (I) might have a similar or stronger activity.

Because *N*-2-fluorenylsuccinamic acid is a potent carcinogen (4), additional derivatives (II, III, IV, V and VI) were synthesized. Since nicotinic acid is known to be involved in the biological oxidation systems (5), carcinogenic activity might be found in *N*-2-fluorenylnicotinamide (VII). In the generally less carcinogenic fluorenone series, *N,N',N''*-2,4,7-fluorenylene(9-acetoxy)trisacetamide (VIII) was obtained.[1]



I, R_1 = N·(NO)CH_2CH_3, R_2 = H
II, R_1 = $NHCOCH_2CH_2COOH$, R_2 = $NHCOCH_2CH_2COOH$
III, R_1 = $NHCOCH_2C$:$(CH_2)COOH$, R_2 = H
or R_1 = NHCOC:$(CH_2)CH_2COOH$ (Structural studies on this compound will be presented in a future communication.)
IV, R_1 = $NHCO(CH_2)_6CH_3$, R_2 = H
V, R_1 = $NHCO(CH_2)_6CH_3$, R_2 = $NHCO(CH_2)_6CH_3$
VI, R_1 = $NHCO(CH_2)_{14}CH_3$, R_2 = H

VII, 

VIII, 

EXPERIMENTAL

Ultraviolet spectra were obtained on a Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer in 95% ethanol. Microanalyses were performed by Schwarzkopf Microanalytical Laboratories.

Received July 26, 1967, from the Pharmaceutical Chemistry Research Laboratory, University of Florida, Gainesville, FL 32601
Accepted for publication September 12, 1967.
This investigation was supported by research grant RO1 CA 07737 from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
The authors thank Dr. Danuta Malejka for her assistance in preparing this manuscript.
* Deceased.
[1] Compounds I, II, III, and VIII were proved to be carcinogenic in the preliminary investigation by Dr. Harold P. Morris, National Cancer Institute, Bethesda, Md. All compounds are subject of further studies in this institute.

N-2-Fluorenylethylnitrosamine (I)—*N*-2-Fluorenylethylamine (6) (6 Gm., 0.03 mole) was heated in 300 ml. of water until the solid melted; 20 ml. of hydrochloric acid was added, and the mixture was cooled to 0–4°. Sodium nitrite (3.5 Gm. in water), 200 ml. of water, and 100 ml. of ether were added. With stirring, more ether was added. The aqueous and ether layers were separated, and the aqueous layer was extracted with ether. The combined ether extracts were washed with water, 10% sodium bicarbonate, and water; dried over sodium carbonate and evaporated *in vacuo*. The yellow product (I) was washed with pentane and dried. Yield, 1.7 Gm. (25%); m.p., 129–134°. Recrystallization from 250 ml. hexane gave 1.3 Gm., m.p. 132–134°. $\lambda_{max.}$, mμ 266–268 (ε 13,000), 304–307 (ε 18,000).

Anal.—Calcd. for $C_{15}H_{14}N_2O$: C, 75.60; H, 5.92; N, 11.76. Found: C, 75.70; H, 5.86; N, 11.66.

N,N'-2,7-Fluorenylenebis-succinamic Acid (II)—To a solution of 20 Gm. (0.1 mole) of *N,N'*-2,7-fluorenylenediamine in 50 ml. of pyridine was added 22 Gm. (0.22 mole) of succinic anhydride, and the mixture was heated with stirring. The precipitate was filtered, washed successively with pyridine and ethanol, and dried. The solid was heated in 0.2 *N* NaOH with charcoal, the solution was filtered hot, and acidified with HCl. The white precipitate (II) was filtered, washed with water, stirred 15 min. in glacial acetic acid, filtered, washed, and dried. Yield, 20 Gm. (55%); m.p. 251–260°. A sample for analysis was dissolved in pyridine, precipitated with hexane, collected, and extracted with toluene, m.p. 256–257°; $\lambda_{max.}$, mμ 303–304, 312–315 sh, 320–322 sh.

Anal.—Calcd. for $C_{21}H_{20}N_2O_6$: C, 63.63; H, 5.09; N, 7.07. Found: C, 63.71; H, 5.02; N, 7.02.

N-2-Fluorenylitaconamic Acid (III)[2]—To a solution of 4.6 Gm. (0.025 mole) of *N*-2-fluorenylamine in 50 ml. of chloroform at 50–55° was added rapidly a solution of 2.8 Gm. (0.025 mole) of itaconic anhydride also at 50–55°, to obtain a white precipitate, 6.8 Gm. (93%), m.p. 207–208°. Recrystallization from ethanol gave a m.p. 219°. $\lambda_{max.}$, mμ 289–292 (ε 28,600), 300–302 sh (ε 20,700), 314.5 (15,800).

Anal.—Calcd. for $C_{18}H_{15}NO_3$: C, 73.70; H, 5.15; N, 4.78. Found: C, 73.63; H, 5.12; N, 4.88.

N-2-Fluorenyloctanoylamide (IV)—To a solution of 4.5 Gm. (0.035 mole) of *N*-2-fluorenylamine in 350 ml. of dry benzene was added 5.5 ml. of octanoyl chloride, and the mixture was refluxed 5–6 hr. The white precipitate was filtered and washed with hexane. Yield, 5.4 Gm. (91%), m.p. 157–160°. Recrystallization from ethanol gave a m.p. 160–161°. $\lambda_{max.}$, mμ 289 (45,600), 303 (34,300), 314 (28,900).

Anal.—Calcd. for $C_{21}H_{25}NO$: C, 82.04; H, 8.20; N, 4.56. Found: C, 82.15; H, 8.20; N, 4.68.

N,N'-2,7-Fluorenylenebis-octanoylamide (V)—To a suspension of 10 Gm. (0.05 mole) of *N,N'*-2,7-fluorenylenediamine in 200 ml. of dry benzene was added 18 ml. of octanoyl chloride, and the mixture was refluxed 2 hr. Yield, 18 Gm. (74%); m.p. 238–246°. Recrystallization from dimethylformamide gave a m.p. of 254°. $\lambda_{max.}$, mμ 304 (ε 53,000), 320–322 sh (ε 44,400).

[2] Prepared by Miss Sandra Rose in this laboratory.

Anal.—Calcd. for $C_{29}H_{40}N_2O_2$: C, 77.63; H, 8.99; N, 6.24. Found: C, 77.62· H, 9.20; N, 6.06.

N-2-Fluorenylpalmitoylamide (VI)—To a solution of 9 Gm. (0.05 mole) of *N*-2-fluorenylamine in 500 ml. of dry benzene was added 15.5 ml. of palmitoyl chloride and the mixture was refluxed 2 hr. to give a white precipitate. Yield, 16 Gm. (76%); m.p. 148–151°. Recrystallization from ethanol gave a m.p. of 150–151°. $\lambda_{max.}$, mμ 289 (ε 36,100), 314 (ε 19,600).

Anal.—Calcd. for $C_{29}H_{41}NO$: C, 82.99; H, 9.85; N, 3.34. Found: C, 82.78; H, 9.69; N, 3.50.

N-2-Fluorenylnicotinamide (VII)[3]—To 12 Gm. (0.08 mole) of nicotinoyl chloride was added a solution of 18 Gm. (0.14 mole) of *N*-2-fluorenylamine in 125 ml. of dry benzene alternately with 21 Gm. of Na_2CO_3. The thick mixture was refluxed 1 hr., filtered, and washed with benzene. The dried cake was broken up and, in 30-Gm. portions, was stirred and heated in water for 20 min. Yield, 20 Gm. (70%). Recrystallized from ethanol, the m.p. was 203–205°; $\lambda_{max.}$, mμ 268–278 sh (ε 16,800), 314–316 (ε 25,700).

Anal.—Calcd. for $C_{19}H_{14}N_2O$: C, 79.70; H, 4.93; N, 9.78. Found: C, 79.66; H, 4.89; N, 9.80.

N,N',N'' - 2,4,7 - Fluorenylene - (9 - acetoxy)-tris-acetamide (VIII)—2,4,7-Trinitrofluorenone (5.25 Gm. 0.017 mole) in 125 ml. of glacial acetic acid was heated in a pressure bottle, 0.06 Gm. PtO_2 was added, and the hot mixture was placed quickly on the Parr pressure reaction apparatus. Hydrogen was added at 40 p.s.i. The pressure dropped to 25.5 p.s.i. in 20 min. The reaction mixture was filtered into 20 ml. of acetic anhydride and left for 1 hr. It was heated 2 hr., the volume was reduced, and the mixture was allowed to stand overnight. The dark red precipitate was filtered, washed with acetic acid and then benzene, and extracted with hot ethanol. Yield, 3.5 Gm. (52%); m.p. over 400°. It was recrystallized from dimethylformamide: $\lambda_{max.}$, mμ 286 (ε 31,100).

Anal.—Calcd. for $C_{21}H_{21}N_3O_5$: C, 63.79; H, 5.35; N, 10.63. Found: C, 64.33; H, 5.12; N, 10.39.

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[3] Prepared by Mrs. Margaret B. Stone in this laboratory.

Keyphrases

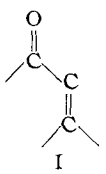
Fluorene derivatives
N-fluorenamides—synthesis
UV spectrophotometry—structure

Structural Studies on Complexes I. Postulation of Polarization Bonding in Xanthine Complexes

Sir:

A number of workers have been engaged in research attempting to elucidate the exact nature of the complex formation observed between xanthine derivatives and various drugs containing unsaturated ring systems. Foremost among these studies are those of Higuchi *et al.* (1–3), whose phase solubility diagrams on caffeine and theophylline complexes led to his postulation that hydrophobic bonding was playing an important role in these association complexes. In attempts to further characterize the nature of the hydrophobic bonding in xanthine complexes, workers postulated different types of mechanisms.

Guttman and Athalye (4) studied the solubilization of riboflavin by using various xanthines and felt their results were in agreement with the ideas of Foley and Harbury (5) whose spectral studies on complexes of various isoalloxazine derivatives indicated a charge transfer mechanism might be operative. The idea of an electron donor-acceptor mechanism has also been presented by Eckert (6) whose spectroscopic measurements on a series of procaine–xanthine complexes indicated this possibility. In the latter study a charge transfer band was observed only when the concentrations of the complex ingredients were quite large, which possibly indicates that the interaction was a weak one. Eckert concluded that in order to form a complex with procaine, the electron acceptor should have the structural feature shown in I.



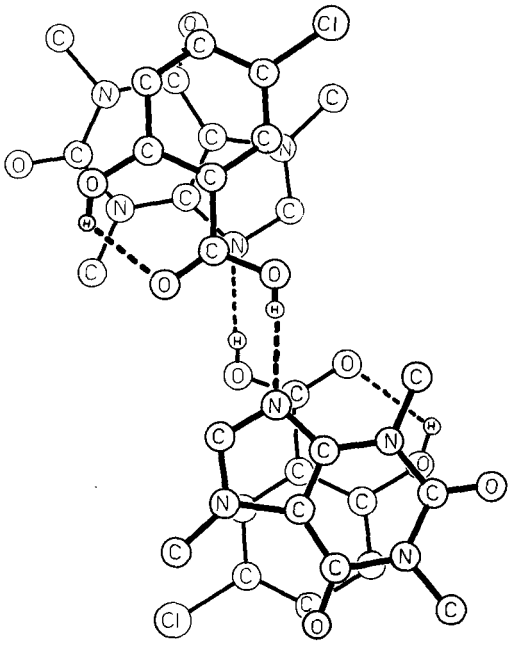
I

In another vein, the Pullmans (7) using quantum mechanical calculations, found that caffeine and other xanthines should act as good electron "donors." In a recent review article by Schnaare and Martin (9), a model for the interaction between caffeine and benzocaine was presented using an approach similar to the Pullmans. This model is quite different to the one presented by Eckert. To the best of the author's knowledge, there has been no experimental evidence reported in the literature to substantiate such a mechanism. It is interesting to note that the recent solubility studies of Jan and Donbrow (8) on caffeine–benzoic acid complexes seem to favor caffeine as an electron acceptor.

In the remaining portion of this communication, I shall present evidence that "polarization bonding," as described by Wallwork (10), appears to be a fundamental force behind some of these xanthine complexes. These interactions are described as pi–pi ones that range in gamut from the charge transfer bonding of Mulliken (11) on the stronger side, to the weaker interactions between polar groups on one component and a polarizable second component [Briegleb (12)]. An example of this type of bonding is found in the crystal structure of 2-phenol-1-quinone (13), where the carbonyl groups of the quinone are found to lie over the centers of the phenyl rings of the phenols. Interestingly enough, other crystal structures of complexes involving planar moieties, one containing a carbonyl group and the other an unsaturated ring system, show similar molecular packing (14, 15).

Recently, the structure of the 1:1 complex of tetramethyl uric acid and pyrene was reported (16). The authors suggested that polarization bonding is possibly the dominating force as they found that there is a "smaller degree of overlap, which seems to indicate the charge transfer forces, if any must be weak." Though no charge transfer band was positively identified for this compound, it is interesting to compare the structure to the conclusions of Eckert. The packing of the molecules in the crystal lattice is such that the C(6) carbonyl of the tetramethyl uric acid occupies a position directly above the center of the pyrene pi system.

The crystal structure of a 1:1 complex of caffeine and 5-chlorosalicylic acid has recently been completed in this laboratory. Crystal structures do not pretend to represent the exact molecular arrangement in solution, but they are of value in determining the types of forces that can be present in solution. Structure II shows two important features. One is a relatively strong hydrogen bond between the carboxyl hydrogen and N9 of caffeine, shown by the dashed line. The other feature is the relative position of the phenyl ring



II

in relation to the caffeine molecule. This arrangement is consistent with the ideas presented above for polarization bonding. A more detailed account of this material will be published at a later date.

In conclusion, it is felt that in order to propose molecular models for xanthine complexes of pharmaceutical interest, one should take into account polarization interactions. Studies on other pyrimidine and purine complexes are being carried out in this laboratory to shed more light on this problem.

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Received October 13, 1967.
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Support of various phases of this research were obtained from the Public Health Service (grant numbers CA-10104-01, A-3909, and 4F2-GM-22-830-02) and the National Science Foundation (G202-07 and GB 2029).

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Keyphrases

Complexes–xanthine
Xanthine complexes–bonding mechanism
Polarization bonding–complex formation effect
Caffeine, 5-chlorosalicylic acid complex–crystal structure

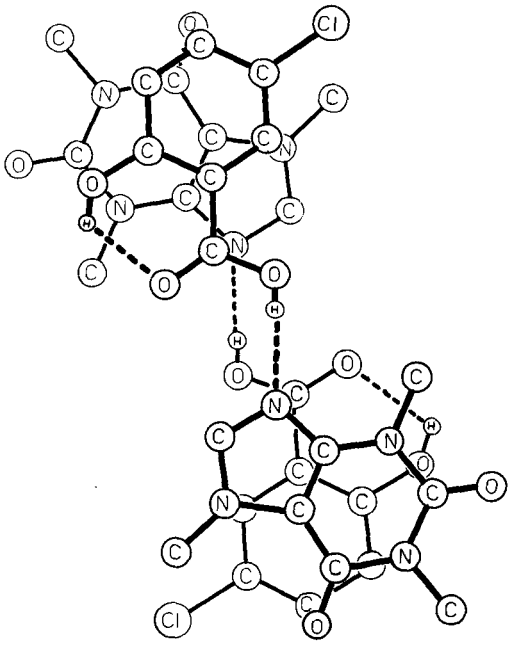
Effect of Certain Tetracycline Analogs on Phenylalanine-^{14}C Incorporation by *Escherichia coli* B Cell-free Extracts

Sir:

This communication reports the effects of several tetracycline analogs on messenger RNA (mRNA) and polyuridylic acid (poly U)-directed phenylalanine-^{14}C incorporation by *E. coli* B cell-free extracts. This study was undertaken to determine (*a*) if mRNA and poly U-directed amino acid incorporation are differentially sensitive to inhibition by the tetracyclines, and (*b*) to examine structure-activity relationships in this series of drugs.

The tetracyclines have been observed (1) to inhibit protein synthesis *in vivo* (*Staphylococcus aureus*). Franklin (2) has studied the incorporation of leucine-^{14}C into polypeptides by rat liver or *E. coli* cell-free extracts and has observed that the tetracyclines inhibit the transfer of amino acids from the aminoacyl-tRNA complex to the growing polypeptide. Suarez and Nathans (3) showed that tetracycline inhibits protein synthesis in *E. coli* cell-free extracts and impedes the binding of aminoacyl-tRNA to mRNA-ribosome complex. Hierowski (4) and Maxwell (5) have made similar observations.

The relative inhibitory activity of the tetracycline analogs was examined at a drug concentration of 1.79×10^{-4} *M*. *E. coli* cell-free extracts and reaction mixtures were prepared according to Nirenberg (6). Incubations were terminated after 70 min. by the addition of 5%



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The relative inhibitory activity of the tetracycline analogs was examined at a drug concentration of 1.79×10^{-4} *M*. *E. coli* cell-free extracts and reaction mixtures were prepared according to Nirenberg (6). Incubations were terminated after 70 min. by the addition of 5%

TABLE I—EFFECT OF TETRACYCLINE ANALOGS ON mRNA-DIRECTED PHENYLALANINE-^{14}C INCORPORATION BY *E. Coli* B CELL-FREE EXTRACTS[a]

Inhibitor	Phenylalanine-^{14}C Incorporation (dpm./mg. protein ± S.E.)	Percent Inhibition
Experiment 1		
Control	476 ± 47	..
Isotetracycline	367 ± 39[b]	23
Tetracycline	316 ± 20[b]	34
4-Epitetracycline ammonium salt	478 ● 7[c]	..
Pyrrolidinomethyl-tetracycline	402 ± 6[c]	16
4-Dedimethylamino-tetracycline	424 ± 47[c]	11
Experiment 2		
Control	968 ± 45	..
Tetracycline	603 ± 64[b]	38
7-Chlorotetracycline	697 ± 52[b]	30

[a] Each incubation flask contained in 0.70-ml. total volume: *E. Coli* B cell-free extract (1.40–2.0 mg. protein), phosphoenolpyruvate (5.4 × 10^3 μM), 2-mercaptoethanol (4.2 μM), magnesium acetate (5.2 × 10^3 μM), phosphoenolpyruvate kinase (1.2 EU), aminoacid-^{12}C mixture (143 μM in each of 19 aminoacids, phenylalanine excepted), D, L-3′-phenylalanine-^{14}C (143 μM, 0.57 μc.), adenosine-5′-triphosphate (761 μM), guanosine-5′-triphosphate (23 μM), potassium chloride (3.6 × 10^4 μM), and Tris (7.1 × 10^4 μM). pH = 7.8. Four or five samples per group. [b] $p < 0.05$. [c] Not significantly different from control, $p > 0.05$.

trichloroacetic acid. Protein content was determined by the Lowry method and radioactivity determination made using a liquid scintillation spectrometer. Data were corrected for quenching by use of the internal standard technique.

In the mRNA-directed system (Table I), with tetracycline assigned an activity of 100, the relative activities of the analogs were: (*a*) 7-chlorotetracycline (80), (*b*) isotetracycline (67), (*c*) pyrrolidinomethyltetracycline (47), (*d*) 4-dedimethylaminotetracycline (32), and (*e*) 4-epitetracycline ammonium salt (0). These findings suggest that the nature of the substituents on C_2, C_3, and C_4 are relatively more important than those on C_{11} and C_{12} of the drug molecule.

In the case of poly U-directed phenylalanine-^{14}C incorporation (Table II) the relative activities of each analog *versus* tetracycline (100) were: (*a*) pyrrolidinomethyltetracycline (102), (*b*) 7-chlorotetracycline (98), (*c*) 4-epitetracycline ammonium salt (93), (*d*) 4-dedimethylaminotetracycline (43), and (*e*) isotetracycline (0). The relatively low inhibition by 4-dedimethylaminotetracycline and isotetracycline suggested that the C_4-dimethylamino group and/or C_{11}-keto and C_{12}-hydroxyl groups are important in the presence of artificial messenger.

TABLE II—EFFECT OF TETRACYCLINE ANALOGS ON POLY U-DIRECTED PHENYLALANINE-^{14}C INCORPORATION BY *E. Coli* B CELL-FREE EXTRACTS[a]

Inhibitor	Phenylalanine-^{14}C Incorporation (dpm. mg. protein ± S.E.)	Percent Inhibition
Control	2174 ± 108	...
Isotetracycline	2178 ± 144[c]	...
Tetracycline	1009 ± 53[b]	54
4-Epitetracycline ammonium salt	1082 ± 67[b]	50
Pyrrolidinomethyl-tetracycline	980 ± 36[b]	55
4-Dedimethylamino-tetracycline	1660 ± 131[b]	23
7-Chlorotetracycline	1018 ± 104[b]	53

[a] Incubation mixtures prepared as in Table I, except that 1.04 × 10^4 μM magnesium acetate and polyuridylic acid (20 mcg./ml.) were used. Five samples per group. [b] $p < 0.01$. [c] Not significantly different from control, $p > 0.05$.

These data suggest that there may be different modes of action of the tetracycline antibiotics in the inhibition of mRNA and poly U-directed phenylalanine-^{14}C incorporation by *E. Coli*. Further studies on the nature of the differential inhibition are in progress.

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Received October 9, 1967.
Accepted for publication December 18, 1967.

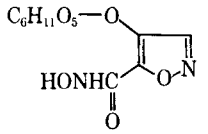
Keyphrases

Tetracycline analogs
Escherichia coli B cell-free extracts
^{14}C–Phenylalanine incorporation–inhibition
Liquid scintillation spectrometry–analysis

Structure of Hiptagin as 1,2,4,6-Tetra-*O*-(3-nitropropanoyl)-β-D-glucopyranoside, Its Identity with Endecaphyllin X, and the Synthesis of Its Methyl Ether

Sir:

Although *Hiptage madablota* Geartn. (family *Malpighiaceae*) does not grow widely in Java, the plant was apparently well known to the natives, who ascribed aphrodisiacal properties to an extract of the roots and provided detailed instructions for dispensation to bulls (1). Ritsema (1) reported the isolation in rather high yield of a white, crystalline, nitrogenous compound from this extract but was unable to duplicate the purported physiological effects when the substance was administered subcutaneously.[1] Ritsema's compound was named hiptagin by Gorter (2), who repeated and improved the original isolation procedure, assigned the (incorrect) formula $C_{10}H_{14}N_2O_9 \cdot {}^1/_2H_2O$, and carried out a number of hydrolysis experiments with this substance, m.p. 110°. Hiptagin could be hydrolyzed under a variety of acidic and basic conditions and Gorter was able to identify a formidable array of products, among them glucose. Aside from glucose, however, the product most directly relevant to the structure of hiptagin was hiptagenic acid, a compound subsequently shown by Carrie (3) to be identical with the aglycone derived from karakin and finally identified by Carter and McChesney (4) as the long known (5) 3-nitropropanoic acid. The plethora of hydrolytic transformations observed by Gorter were somewhat ingeniously rationalized by his proposal (2) of structure I for hiptagin although, in retrospect, they are readily traced to the presence of the 3-nitropropanoyl moiety. In view of the challenge to structure I inherent in Carter's formula-

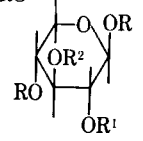


I

tion (6) of karakin, a closely related glucoside, as 1,4,6-tri-*O*-(3-nitropropanoyl)-β-D-glucopyranoside (II), the structure of hiptagin was clearly in need of revision.

We were led naturally to a consideration of this problem in connection with work with the endecaphyllins (7), a group of glucose polyesters of 3-nitropropanoic acid isolated from a toxic extract of *Indigofera endecaphylla* Jacq. (family (*Leguminosae*, subfamily *Papilionatae*). Following the directions of Gorter (2), we have easily isolated from an acetone extract[2] of defatted root-bark of *H. madablota* a white, crystalline glucoside, m.p. 102–104°, which we believe to be hiptagin and for which the structure 1,2,4,6-tetra-*O*-(3-nitropropanoyl)-β-D-glucopyranoside (III) is required by the evidence presented below.

At the outset, this substance proved to be identical with endecaphyllin X (7) by mixed melting point, optical rotation, and infrared spectral comparisons and thus could be formulated as a glucose tetraester of 3-nitropropanoic acid ($C_{18}H_{24}N_4O_{18}$).[3] In addition, acid-catalyzed diazomethylation (7,8) provided a monomethyl ether, m.p. 139–141° (IV), which was shown to be indistinguishable from the derivative similarly prepared from endecaphyllin X. The NMR spectra of III and IV (hexadeuteroacetone solution) revealed the anomeric proton as a doublet centered at τ4.10 with $J_{1,2}$ = 8.3 c.p.s. These values indicate that the C-1 hydroxyl group is acylated and is in the β-configuration. Detailed analysis of these spectra allowed assignment of a triplet centered at τ5.90 (separation, 8.5 c.p.s.) to the C-3 proton in III which corresponds to a triplet at τ6.1 (separation, 9 c.p.s.) in the spectrum of IV. This upfield shift of the C-3 proton on methylation requires that the free hydroxyl group in III be located at the 3-position.



II, karakin; R = $O_2NCH_2CH_2CO$-
$R^1 = R^2 = H$

III, hiptagin; R = $R^1 = O_2NCH_2CH_2CO$-
$R^2 = H$

IV, hiptagin methyl ether;
R = $R^1 = O_2NCH_2CH_2CO$-
$R^2 = CH_3$

[1] As pointed out by Gorter (2), this result must be considered inconclusive since the pure compound is insoluble in water and, insofar as we are aware, no additional biological data have been reported.

[2] This extract was kindly supplied by Dr. G. Ganguli, Department of Chemistry, University College of Science, Calcutta, India. A solid which precipitated during this acetone extraction has been identified as mangiferin. Finnegan, R. A., Stephani, R. A., Ganguli, G., Ganguly, S. N., and Bhattacharya, A. K., to be published.

[3] Interestingly, we have also repeated the isolation of karakin from the kernels of *Corynocarpus laevigata* Forst. (family *Anacardiaceae*) and find it to be identical with endecaphyllin B (7). Although the data upon which Carter based the assignment of structure II to karakin (6) are equivocal, we have adduced spectroscopic evidence in support of II.

This conclusion was verified by a synthesis of IV which made use of the known (9) 3-*O*-methyl-α-D-glucose, m.p. 164–166°. Acylation with 3-nitropropanoyl chloride in *N*-methylpyrrolidone solution provided an oily tetra-ester whose NMR spectrum showed it to be an anomeric mixture (C-1 proton of α-anomer at τ3.63, $J_{1,2}$ = 4 c.p.s.; C-1 proton of β-anomer at τ4.10 $J_{1,2}$ = 8.3 c.p.s.). However, crystallization from methylene chloride solution afforded an authentic sample of the β-anomer, m.p. 136–140°, whose infrared and NMR spectra were indistinguishable from those of naturally derived IV and whose mixed melting point was undepressed.

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A few errors in structures are so obvious that they cannot cause trouble. The appendix leaves a little to be desired because of its brevity. A list of references is presented in a usable manner. The book will be valuable to a varied audience. It is sufficiently broad to serve as supplementary reading in graduate courses, and it appears to be commendable to either a pharmacologist or medicinal chemist contemplating research in the area of ganglion-active compounds. For the active researcher in the area, it is a valuable review through 1962 (although the book was ostensibly "brought up to date" for the 1967 edition); for these persons, the book's most valuable asset is the collection of data from Russian sources.

Reviewed by J. C. Kellett, Jr.
National Science Foundation
Washington, D. C.

Mass Spectrometry of Organic Compounds. By H. Budzikiewicz, C. Djerassi, and D. H. Williams. Holden-Day, Inc., 500 Sansome Street, San Francisco, Calif., 1967. xv + 690 pp. 19 × 26 cm. Price $17.95

This book is aimed at two groups of readers. The first includes students who may wish to become proficient in the interpretation and use of mass spectral data. The second group includes organic chemists who are familiar with mass spectrometry and employ it from time to time in their research. The authors use the "mechanistic" approach to the interpretation of mass spectra and this should be particularly appealing to the modern organic chemist.

A general introduction is devoted to the mass spectrometry aspects of ionization, localization of charge, bond fissions, and metastable peaks. In addition, mention is also made of some specific techniques employed, such as element mapping, the kinetic approach to mass spectra, and the combined vapor phase chromatography–mass spectrometry. Each of the 27 chapters that follow deals with a particular functional group (or related functional groups) in organic compounds. In each chapter, the aliphatic members are first discussed, followed by a discussion of the aromatic members. This organization is a distinct improvement over that of the first volume.

Although this volume appears at first glance to be only a revision of the authors' earlier book, "Interpretation of Mass Spectra of Organic Compounds," the reader soon becomes aware that the extensive revisions and new material discussed, indeed, make this a new book warranting the new title. New material includes lactones (chapter 4), acids and anhydrides (chapter 5), *N*-oxides, nitrosamines, and quaternary ammonium salts (chapter 8), nitrogen-containing carbonyl derivatives (chapter 10), epoxides (chapter 13), alcohol derivatives (chapter 14), carbonates and ureas (chapter 15), sulfoxides, sulfones, and related compounds (chapter 19), organic phosphorus compounds (chapter 26), and organo-metallic compounds (chapter 27). The extent of this new material demonstrates the need for this new volume and is reinforced by the need to update the other topics.

The book is well written and the coverage is thorough and up-to-date. The references listed for each chapter include early 1967 papers as well as several private communications of work not yet published. Such a reference list is extremely valuable to the mass spectrometrist and renders this book a must in his personal library. The book has a few typographical errors which do not detract from its overall value in any way. Another special value of the book is the listing of summaries and conclusions wherever possible. For example, the reader is given general rules for mass spectral behavior for alkyl eliminations (p. 264), hydrogen losses (p. 282), acetylnaphthoquinones (pp. 533–534), and metal complexes of β-diketones (pp. 664–665). These rules should prove to be particularly useful in the general interpretation of mass spectra.

Finally, the authors frequently point out areas requiring additional study either by high-resolution mass spectrometry, deuterium labeling, or the need to study more compounds (or derivatives) containing a particular functional group. The suggestions offered should stimulate further research in these areas.

In summary, this excellent work extends and amplifies the application of mass spectrometry in organic and biological chemistry. The nonexpert in the field will receive an introduction into this dynamic area and those knowledgeable in the field will find this to be a useful reference.

Reviewed by Marvin F. Grostic
The Upjohn Company
Kalamazoo, Mich.

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Although this volume appears at first glance to be only a revision of the authors' earlier book, "Interpretation of Mass Spectra of Organic Compounds," the reader soon becomes aware that the extensive revisions and new material discussed, indeed, make this a new book warranting the new title. New material includes lactones (chapter 4), acids and anhydrides (chapter 5), *N*-oxides, nitrosamines, and quaternary ammonium salts (chapter 8), nitrogen-containing carbonyl derivatives (chapter 10), epoxides (chapter 13), alcohol derivatives (chapter 14), carbonates and ureas (chapter 15), sulfoxides, sulfones, and related compounds (chapter 19), organic phosphorus compounds (chapter 26), and organo-metallic compounds (chapter 27). The extent of this new material demonstrates the need for this new volume and is reinforced by the need to update the other topics.

The book is well written and the coverage is thorough and up-to-date. The references listed for each chapter include early 1967 papers as well as several private communications of work not yet published. Such a reference list is extremely valuable to the mass spectrometrist and renders this book a must in his personal library. The book has a few typographical errors which do not detract from its overall value in any way. Another special value of the book is the listing of summaries and conclusions wherever possible. For example, the reader is given general rules for mass spectral behavior for alkyl eliminations (p. 264), hydrogen losses (p. 282), acetylnaphthoquinones (pp. 533–534), and metal complexes of β-diketones (pp. 664–665). These rules should prove to be particularly useful in the general interpretation of mass spectra.

Finally, the authors frequently point out areas requiring additional study either by high-resolution mass spectrometry, deuterium labeling, or the need to study more compounds (or derivatives) containing a particular functional group. The suggestions offered should stimulate further research in these areas.

In summary, this excellent work extends and amplifies the application of mass spectrometry in organic and biological chemistry. The nonexpert in the field will receive an introduction into this dynamic area and those knowledgeable in the field will find this to be a useful reference.

Reviewed by Marvin F. Grostic
The Upjohn Company
Kalamazoo, Mich.

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JOURNAL OF Pharmaceutical Sciences

March 1968 volume 57, number 3

Review Article

Biopharmaceutical Considerations in Subcutaneous and Intramuscular Drug Administration

By BERTON E. BALLARD

THE PURPOSES of this review are to consider some of the factors affecting the absorption rates of drugs administered subcutaneously and intramuscularly, and to consider some of the literature pertaining to their formulation and administration. This review will largely supplement rather than duplicate references contained in earlier reviews on these subjects (1–4). As a result, certain important but previously covered topics may be omitted entirely, or be discussed from different points of view. The parenteral route of drug administration is an important one for the preclinical screening and evaluation of drugs as well as their clinical use in human and veterinary medicine. The rational design of products intended for parenteral use depends upon many factors including the physical, chemical, pharmaceutical, pharmacological, and toxicological properties of the drug and the adjuvants used in its formulation. Certain anatomical or physiological factors, such as injection site and body movement, may influence the absorption rates of some parenteral products.

ENDOTHELIAL TISSUE

Although this review will not go into extensive detail on the anatomy and histology of the microcirculation at the many possible injection sites, a few key points are worth mentioning. At present there are many investigations of the several mechanisms involved in the passage of drugs and other substances through capillary and lymphatic vessels and the ground substance surrounding them. The subcutaneous region is well supplied by capillary and lymphatic vessels (5). Muscle tissue also has a rich supply of capillary vessels. However, it is generally agreed that there are few, if any, lymph vessels in muscle tissue proper (5, 6). There is an abundant supply of lymphatic vessels in connective tissue sheaths and tendons. Lymph vessels usually exist where fascial planes enter muscles, and the fluid moves through spaces along fascial planes between muscle fibers. One important difference among lymphatics in several regions of the body concerns the state of their intercellular junctions. In active regions of the body one junction in two to five may be open, but in motionless regions there may be only one open junction in 50 to 100. Normal lymphatics in motionless regions and normal blood vessels allow very few particles to pass through their junctions. In contrast, lymphatics in regions where there is much movement, injured lymphatics, and injured blood vessels all allow much more material to pass through their frequently opened junctions. Mild trauma near a lymphatic vessel in a motionless region can result in a marked increase in the vessel's permeability, probably due to the opening of many of the normally closed junctions (7).

Received from the School of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122

This paper was supported by general research support grant FR 05453 from the Division of Research Facilities and Resources, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

The author wishes to dedicate this paper to Dean T. C. Daniels, whose leadership at the University of California has done so much to raise the standards of education and research in the pharmaceutical sciences.

The passage of various particles and fluid from one to the other side of blood and lymphatic endothelial tissue has been shown to occur in part by means of vesicles. Particles and fluid contacting one endothelial surface enter cells in small vesicles, and then leave the opposite surface from the same organelles. This process has been called cytopemphis (6, 7). There is evidence that these vesicles are not directed in their intracellular passage, but may move and discharge their contents randomly on either side. Cytopemphis permits the net transport of material to be proportional to the concentration difference of the substance on either side of the endothelial tissue. The various paths by which particles of different sizes are thought to traverse the lymphatic endothelium have been summarized by Casley-Smith (7). A classification of the types and sizes of apertures found in normal and injured capillary and venous walls has been compiled by Landis (8). As to particles, there are theoretically only four possible paths that can be taken through endothelial tissue and these include: intercellular, intracellular in organelles, cytoplasmic matrix, or fenestrae paths. It appears that all four of these paths are used to some extent in various endothelial tissues (7). For lipid-soluble molecules there is abundant evidence that they diffuse through capillary walls and can pass through regions of the wall that are relatively impermeable to lipid-insoluble molecules (9).

DIFFUSION

From a macroscopic point of view, many drugs in solution injected into subcutaneous or intramuscular sites behave as if their absorption were taking place passively by diffusion. For most drugs it has not been determined whether the microscopic absorption route is *via* capillaries, lymphatics, or both. Insofar as drug molecules penetrate endothelial tissues rapidly by passive diffusion, the penetration rate of the drug from the injection site can be described by Fick's Law in one direction.

$$dN/dt = \bar{D}A(dC/dx) \qquad \text{(Eq. 1)}$$

where dN/dt is the penetration rate, N is the amount of drug penetrating the tissue, and t is time. In a well-stirred system, the rate is proportional to the mean diffusion coefficient (10) of the drug in the membrane, $\bar{D}$, the area, A, of the absorbing membrane exposed to the solution, and the concentration gradient (dC/dx) of drug across the membrane. Equation 1 may be approximated (11) by:

$$dN/dt = \frac{\bar{D}AK}{\delta}(C_s - C_b) \qquad \text{(Eq. 2)}$$

where the dx term of Eq. 1 has been replaced by δ, the thickness of the thin subcutaneous or intramuscular membrane that is assumed to be constant for each animal and site, and the dC term of Eq. 1 has been replaced by the terms K and $(C_s - C_b)$. The term K is the equilibrium distribution ratio or partition coefficient of the lipid-soluble drug between the membrane lipids and the aqueous phases found at the injection site or body fluids. The term $(C_s - C_b)$ is the difference between the drug concentration at the injection site, C_s, and the drug concentration, C_b, in the body fluids, *e.g.*, blood and lymph, flowing past the absorption site at any time.

The term C_b can usually be ignored, since it can be reasonably assumed that the drug's concentration in the fluids of distribution is negligible compared to its concentration at the absorption site at any time. Thus, Eq. 2 can be written as:

$$dN/dt = \left(\frac{\bar{D}AK}{\delta}\right) C_s \qquad \text{(Eq. 3)}$$

When the volume of the drug solution at the absorption site, V_s, remains nearly constant throughout the experiment, the rate of penetration will be equal to:

$$dN/dt = \left(\frac{\bar{D}AK}{\delta V_s}\right) A_s \qquad \text{(Eq. 4)}$$

where A_s is the amount of drug at the site at any time. Equation 4 can be further reduced to:

$$dN/dt = PA_s \qquad \text{(Eq. 5)}$$

where P is the penetration coefficient which includes all the terms in parentheses in Eq. 4, and has the units of time^{-1}. The penetration coefficient or absorption rate constant has the same magnitude as the clearance constant, but the opposite sign. Thus, it can be seen from Eq. 4 that the absorption rate is directly proportional to $\bar{D}$, A, and K, but is inversely proportional to δ and V_s. The half-life of the drug at the absorption site, $t_{0.5}$, can be calculated from a plot of the logarithm of the fraction of drug remaining at the site at any time *versus* time. The half-life and penetration coefficient are related by:

$$P = \ln 2/t_{0.5} \qquad \text{(Eq. 6)}$$

Area—The local distribution of solutions injected subcutaneously or intramuscularly is of interest, because the penetration rate of the drug depends in part upon the geometry and the resulting area of the depot exposed to the tissue. When the water-immiscible silicone, dimethylpolysiloxane,[1] was injected subcutaneously into animals, most of it was limited to the tissue planes of the subcutaneous region.

[1] Dow Corning 360 medical fluid.

Pathologic findings in the rat, mouse, and guinea pig following the administration of massive doses of the silicone were essentially similar. In most instances the silicone was contained in innumerable thin-walled spherical or ellipsoidal sacs and several larger pools within the adipose tissue (12, 13). Brown *et al.* (14) studied the disposition of subcutaneous injections of a radiopaque water-in-oil emulsion in guinea pigs at the injection site. The horizontal and vertical aspects of the injection site were X-rayed over a period of days. A cross-section of the tissue showed on the film a lateral spread of the radiopaque material in the subcutaneous tissue that progressed over a period of days.

What is the immediate distribution of substances injected intramuscularly? Shaffer (15) injected radiopaque substances into the gluteus muscle of humans. He found that iodized oil and bismuth salicylate in oil were confined to the planes of fascia or connective tissue surrounding muscles and groups of muscles. Injections of metallic bismuth suspended in isotonic dextrose solution were similarly distributed in the fascial planes of the muscle. Fluoroscopic studies showed that viscous oily solutions, such as iodized oil, tended to form a sphere-shaped deposit about the needle point and then spread out more slowly. It did not spread as extensively along the fascial septums as did the aqueous systems studied. The oily solutions continued to spread from 1 to 5 min. before they became fixed in position. The aqueous suspension of bismuth spread to its final location almost as soon as the injection procedure was completed. Some authors (16) have suggested that the term "intramuscular" is a misnomer and should be called "intermuscular" when it refers to injections in this region, because of the spread of solutions along the fibrous tissue *between* muscle fibers. Gruhzit (17) also noted that water-soluble bismuth tartrate injected intramuscularly was precipitated in the tissues and distributed along the connective tissue fasciculi between the muscle fibers. Zelman (18) has stated that deep, firm massage of the muscle tissue following an intramuscular injection favors the spread of the medication through a wider area of tissue, thus increasing the area for absorption to take place.

From the foregoing examples it can be seen that with the usual needle-injection technique, it is difficult, if not impossible, to control the area of an injected solution, suspension, or emulsion in contact with the tissues. If other factors are equal (*e.g.*, metabolic and excretion rates), one would expect that higher initial serum levels of drug would result when the solution has a larger area exposed to the tissue, since drug penetration rate is directly proportional to this area.

Recently, an experimental technique in animals has been devised (19) to study the *in vivo* subcutaneous absorption rate of about 1% (w/v) benzyl alcohol in normal saline where the area of tissue exposed to the solution is held constant. A medical silicone adhesive is used, which is capable of affixing a cylindrical, hollow glass absorption cell to moist subcutaneous tissue. This adhesive prevents the spread of the solution from the site, and is reported to be completely inert to living tissue and will not cause irritation or sensitization. With this procedure it is possible to keep the solution in the cell constantly stirred, which cannot be conveniently done when needle-injection techniques are used. Furthermore, it is convenient when removing drug samples periodically for analysis.

Volume—The subcutaneous absorption cell just described has another advantage. The volume of solution in the cell, as well as the area exposed, can be held constant. Thus, the penetration coefficient, P, defined in Eqs. 4 and 5 will be constant for fixed values of $\bar{D}$, K, and δ. One would predict that if the volume of the solution in the cell were increased over control values, a decrease in the magnitude of P would occur. If so, the result would be that the penetration rate of the drug would also decrease. Similarly, a decrease in volume over controls should result in an increase in both P and the penetration rate. These predictions have not been verified experimentally using the subcutaneous absorption cell just described, but Sund and Schou (20) have shown that the intramuscular clearance rates of radioactive mannitol and sucrose solutions were inversely proportional to the volume of solution injected.

When radioactive drugs or ions are used in absorption studies, it has been observed that plots of the logarithm of the fraction of drug (*e.g.*, radioactive counts) remaining at the site *versus* time may not be linear (20). The absorption half-life at the beginning of the experiment is often shorter than that calculated at some later time. This may indicate that a bi- or polyexponential equation would fit the data better. A few of the reasons for this phenomenon are: (*a*) blood or lymph flow from the site may be impaired at the later time, (*b*) the solution at the site is not uniformly mixed and there is a longer diffusion path for molecules at the center of the injected solution, or (*c*) the magnitude of the penetration coefficient may not remain constant with time. For example, if the area of tissue exposed to the liquid progressively decreases, or if the volume of solu-

tion progressively increases at the site, the value for the penetration coefficient will decrease with time to give rise to a longer absorption half-life toward the end of the experiment. Either hydrostatic (*e.g.*, injection pressure), osmotic pressure effects, or both may also be factors in determining the net solvent flow between the fluids at the site and in the vessels.

Concentration—Sund and Schou (20) studied the effects of different drug concentrations on clearance rate. On the one hand, with a pharmacologically inert, neutral, water-soluble substance like sucrose, the clearance rate from rat muscle was independent of drug concentration over the range of 0.19–9.6 mg./ml. This is the predicted result on the basis of Eqs. 4 and 5 when the injection volume is constant as it was in their experiment. On the other hand, the absorption rate of a substance like atropine depends markedly upon its concentration at the injection site. The relative clearance rate for atropine decreases with increasing concentrations of the drug over a threshold value of about 0.5 mg./ml. Atropine and several other anticholinergic drugs when mixed with sucrose solutions decreased the clearance rate of sucrose. The self-depression of atropine absorption and the inhibited absorption of sucrose are probably both due to the local pharmacological action of atropine. Sund and Schou (21) conclude that although the exact mechanism is not known, atropine probably interferes with local blood flow at the injection site. Atropine also has interesting local effects on the inflammatory process. Houck (22) administered intradermal injections of 50% croton oil in peanut oil to rats. Croton oil, a water-immiscible vesicant, produces wounds that are reproducible. He found that the microcirculatory insufficiency characteristic of injuries produced by the diluted croton oil was eliminated by the subcutaneous administration of atropine. He proposed that one of the primary effects of atropine was to accelerate wound healing by restoring the biological continuity of the wound with the circulation and surrounding tissue.

Molecular Size—As a first approximation, the diffusion coefficient of a spherical or nearly spherical drug molecule that is larger than the solvent molecules is inversely proportional to its molecular radius (or weight). Thus, in a diffusion-controlled process, large molecules would be expected to have slower penetration rates than smaller ones.

However, none of the equations so far presented in this paper give the investigator any indication as to whether an injected molecule would be absorbed primarily *via* capillary vessels, lymphatic vessels, or both. Table I summarizes some of the literature relating molecular or formula weights of injected substances and their probable primary absorption routes. From the subcutaneous site it appears that molecules or ions having low molecular weights are absorbed primarily *via* the capillaries, while molecules having high molecular weights appear to be absorbed primarily *via* lymph vessels. Sund and Schou (20) followed the clearance rate of labeled carbohydrates from rat muscle. Table II lists the substances used, their molecular weights, aqueous diffusion coefficients, and the fraction of drug cleared 5 min. after the injection. As expected, it can be seen from this table that with an increase in molecular weight, there is a decrease in clearance rate. The fact that the fraction cleared at the end of 5 min. correlates with the diffusion coefficient, they state, is evidence that diffusion is the main driving force for the absorption. These investigators did not determine experimentally which of the molecular species was cleared primar-

TABLE I—RELATIONSHIP BETWEEN MOLECULAR OR FORMULA WEIGHT OF CHEMICAL SPECIES AND PROBABLE ROUTE OF ABSORPTION FOLLOWING AN INTRAMUSCULAR OR SUBCUTANEOUS INJECTION

Mol. Species	Mol. or Formula Wt.	Route of Administration	Probable Primary Absorption Route	Ref.
$^{24}NaCl$	58	i.m.	Capillary	(145)
Strychnine (salt?)	>334	s.c.	Capillary	(38)
$^{59}FeCl_3$	270	s.c.	Capillary	(146)
^{59}Fe-labeled plasma	?	s.c.	Lymphatic	(146)
Black tiger snake venom	>20,000	s.c.	Lymphatic	(38)
India cobra venom	2500–4000	s.c.	Capillary	(38)
Russell viper venom	~30,000	s.c.	Lymphatic	(38)
Diphtheria toxin	~70,000	s.c.	Lymphatic	(38)
Tetanus toxin	?	s.c.	Lymphatic	(38)
Iron polysaccharide complexes	10,000–20,000	i.m.	Lymphatic	(37)
Neolymphins[a]	High	i.m.	Lymphatic	(24)
Iron–sorbitol–citrate complexes	<5000	i.m.	~16% lymphatic ~50–60% capillary	(147)

[a] The neolymphins are neomycinpolymetacrillate, neomycindextransulfate, and neomycincarboxymethyl starch.

TABLE II—CARBOHYDRATE ABSORPTION FROM RAT MUSCLE (20)

Substance	Mol. Wt.	Aqueous Diffusion Coefficient × 10^6	Fraction of Drug Cleared 5 min. After i.m. Injection × 10
D-Mannitol-1-^{14}C	182	8.7	~7
Sucrose-^{14}C	342	7.5	~6
Inulin-methoxy-^{3}H	3000–4000	2.1	~2
Inulin-carboxyl-^{14}C	3000–4000	2.1	~2
Dextran-carboxyl-^{14}C	60,000–90,000	~0.5	~0.7

ily by the capillary or by lymphatic routes, although they pointed out that the drainage through lymph channels could be of relatively greater importance for the absorption of large molecules compared to smaller ones.

Lewis (23) attempted to explain why sudden anaphylactic deaths occasionally occurred in humans after subcutaneous injections of macromolecules like diphtheria antitoxin when absorption of this substance from the subcutaneous site was known to be slow. The test substance he used was horse serum. Table III shows that the serum was absorbed slowly from the subcutaneous region of dogs, probably because the absorption took place largely *via* the lymphatics. Massage of the wheal produced by the injection and the use of high injection pressures resulted in a more rapid appearance of the serum in the thoracic duct lymph. Lewis concluded that the serum's absorption rate was too slow to account for cases of anaphylactic death in humans, and that the probable reason for this phenomenon was an accidental intravenous injection of the serum.

The effect that molecular size or weight can have on the route of drug clearance from an injection site has been demonstrated by Málek and co-workers (24). They prepared salts of an antibiotic base like streptomycin or neomycin with high molecular weight anionic substances like polyacrylic acids, sulfonic or phosphorylated polysaccharides, and natural polycarboxyl acids. After an intramuscular injection of neomycin sulfate into a dog, a peak blood level of about 20 mcg./ml. was obtained at 2 hr., which fell to zero at 12 hr. After an intramuscular injection of neomycindextransulfate (neolymphin II), a peak neomycin blood level of about 3 mcg./ml. was obtained at 2 hr., and its concentration in the blood remained nearly constant at 1 mcg./ml. from 8 to 24 hr. They termed these macromolecular salts "antibiolymphins." According to the authors, the antibiolymphins were absorbed from the injection site primarily *via* the lymphatic system in contrast to the corresponding sulfate salt, which presumably was absorbed *via* the capillaries, although the authors did not state this.

TABLE III—APPEARANCE OF SUBCUTANEOUSLY ADMINISTERED HORSE SERUM IN LYMPH AND BLOOD OF DOGS UNDER VARIOUS CONDITIONS (23)

Conditions	Time to Detect —Presence of Horse Serum in— Thoracic Duct Lymph	Blood
No massage at site	40 min.	3.5 hr.
	...[a]	1.5 hr.[a]
Massage at site	15–20 min.	2 hr.
High-pressure injection	<5 min.	40 min.

[a] Thoracic duct not cannulated.

pH—Madison and Christian (25) studied the influence of pH on the absorption rate of $^{22}NaCl$ and $^{23}NaCl$ administered subcutaneously in rats. The magnitude of the absorption rate was evaluated on the basis of the amount of radioactivity present in a sample of heart blood removed 45 min. after the injection. From pH 2.5 to 10 inclusive there was little effect on the normal absorption rate of sodium ion. At pH values of 1.0 and 2.0, a decrease in sodium ion absorption rate was observed, while at pH values of 11.0 and 12.0 an increase in rate occurred.

In the case of organic bases, the pH of the injected solution can often have a profound effect on absorption rate. Cutts and Walker (26) repeated the work of White and Claflin (27). They confirmed that in mice intraperitoneal injections of the nitrogen mustard HN_2, methyl-bis(β-chloroethyl)-amine hydrochloride, at two different pH values resulted in different LD_{50} values. At pH 2 the LD_{50} was about 5 mg./Kg., while at pH 8 it was about 2 mg./Kg. White and Claflin (27) showed under their injection conditions that little HN_2 would have undergone cyclization. Since HN_2 has a pK′ of 6.45 at 15° (28), the molecule exists almost entirely as the water-soluble protonated form at pH 2. But at pH 8 a significant fraction of the amine exists in the undissociated form. On the basis of pH-partition considerations (29), the drug's absorption and distribution at the higher pH value should be facilitated resulting in the lower value found for the LD_{50}.

PHAGOCYTOSIS

Although passive diffusion is an important mechanism for drug absorption, the process of phagocytosis may also be involved in drug absorption from subcutaneous and intramuscular sites. Rees and co-workers (30) studied the systemic distribution of dimethylpolysiloxane,[2] following its subcutaneous administration, in mice. They stated that while the mechanism of absorption and systemic distribution of the silicone fluid in mice is at present unknown, it may be distributed to the viscera by gaining entrance to the general circulation (*via* capillaries?) or lymphatic channels. However, the authors suggest that the most likely mechanism is by the process of phagocytosis by histocytes.

When sodium urate crystals are injected subcutaneously into animals and man, an acute inflammatory response occurs accompanied by phagocytosis of the crystals by mononuclear and

[2] Dow Corning MDX 4-4011.

polymorphonuclear leucocytes (31). A similar acute inflammatory response occurs after subcutaneous injections of microcrystalline ammonium and potassium urates, uric acid, xanthine, hypoxanthine, calcium carbonate, and creatinine. Seegmiller and co-workers (32) have shown that when microcrystalline sodium urate and sodium orotate suspended in a 50% glycerol–water vehicle were injected subcutaneously in man, 24 hr. later the site in most subjects showed warmth, swelling, erythema, induration, and tenderness. There was essentially no reaction to control subcutaneous injections of the vehicle after the immediate pain and wheal had subsided 6 to 8 hr. after the injection. Malawista and Seegmiller (33) have shown, when humans were pretreated with colchicine, the inflammatory response to subcutaneously administered monosodium urate monohydrate and sodium orotate microcrystals was substantially reduced over controls not so pretreated. Malawista (34) has shown that colchicine interferes with the amoeboid motility of individual human polymorphonuclear leucocytes. Other substances like actinomycin D, 6-mercaptopurine, and puromycin have been found to block lymphocyte emigration into a region of inflammation (35). An interesting but as yet unexplained finding is that the inflammatory response to subcutaneous injections of amorphous sodium urate particles was much less than that found with microcrystalline preparations of the same salt (32).

Gómez-Luz (36) showed that when microcrystals of insoluble oxytetracycline were administered subcutaneously into man, crystals could be found in the peripheral blood either intracellularly or free and in human bone marrow 6 to 14 days following the injection. Patients suffering from nonspecific urethritis received the insoluble oxytetracycline subcutaneously, and microcrystals were found in their urethral discharges. Gómez-Luz explained these observations by suggesting that polymorphonuclear leucocytes and monocytes engulfed the microcrystals by the process of phagocytosis, and these crystals then were transported to the general circulation and finally to the site of infection.

Beresford and co-workers (37) showed that most of the absorption of iron polysaccharide complexes injected into rabbit muscle tissue occurred during the initial 72 hr. The absorption during this time was mediated in part by the inflammatory reaction evoked, and in part by lymphatic transport of the iron complexes. The iron complexes remaining after 72 hr. were fixed by tissue macrophages that seemed to impede further absorption.

BIOLOGICAL FACTORS

There are several biological factors, in addition to those mentioned, which can influence the rates of drug absorption. Some of these factors include body movement, pre-existing disease conditions, the age of the animal, the anatomical region into which the injection is made, and the condition of the tissue at the site.

Body Movement—If a molecule is absorbed primarily *via* the lymphatic route, body movement or exercise can often have a profound effect on how rapidly it reaches the general circulation. Muscular movement has long been known to increase the flow of lymph fluid along the lymphatic vessels. When an animal is at rest, little or no lymph flows (5). The effect that body movement has on the absorption rate of a substance in solution can be seen from the work by Barnes and Trueta (38). They injected black tiger snake venom subcutaneously into rabbits. Since this venom has a relatively large molecular weight (>20,000), it is transported from the injection site almost exclusively by the lymphatic drainage. Control animals lived no longer than 150 min. following the injection, while animals whose injected limb was immobilized by a plaster cast lived more than 8 hr.

Controlled physical activity has also been shown to have an effect on the rate at which subcutaneously implanted procaine penicillin G implants were absorbed in rats. Ballard (39) found that the mean absorption rate per mean pellet area[3] in animals rotated in a rodent activity cage at 3.83 r.p.m. was 2.57×10^{-3} Gm. hr.$^{-1}$ cm.$^{-2}$, while that of nonrotated control animals was 1.93×10^{-3} Gm. hr.$^{-1}$ cm.$^{-2}$. These differences were shown to be statistically significant.

Disease Conditions—Bauer and co-workers (40) noted that patients with cardiac failure and patients with myxedema had prolonged absorption half-lives following a jet injection of ^{131}I solutions into their thighs. When the myxedemic condition was reversed by appropriate thyroid medication, the absorption half-life of the drug returned to the normal range. De Groot and co-workers (41) noted delayed intramuscular absorption of a triiodothyronine formulation in one hypothyroid subject studied. Using the same jet injection technique, Bauer *et al.* (42) studied the absorption half-life of ^{131}I from the thighs of patients suffering from myocardial infarctions. None of the patients had congestive heart failure. In one patient the half-lives were

[3] An error in the caption at the top of column five of Table I (39) indicated that all the rates per unit area were tenfold slower than they should be.

60, 29, 14, and 8 min., respectively, on the first, eighth, fourteenth, and thirty-first days after the infarction. In healthy controls the mean absorption half-life for ^{131}I was 9 (range: 6–14) min. Irons (43) followed the blood levels of penicillin in patients suffering from a variety of bacterial infections after injections of an aqueous suspension of procaine penicillin G. He commented that a depression of penicillin blood levels tended to occur in a patient with severe cardiac failure because there was poor absorption of the drug from the injection site.

Animal Age—Lee (44) studied the effect of age on the acute toxicity of subcutaneously administered chlorpheniramine maleate and diphenhydramine hydrochloride in rats. He found that the LD_{50} of chlorpheniramine during the first 16 days of life varied between 175 and 230 mg./Kg., but after 25 days of life the LD_{50} was 360–365 mg./Kg. A similar trend was found for diphenhydramine. There was no apparent difference in the activity of enzymes concerned with the metabolism of both drugs in the livers of 15- and 40-day-old rats. Lee concluded that the development of greater resistance to both drugs with increasing age could be explained by a decrease in the amount of drug absorption from the subcutaneous region. The reason why age should influence drug absorption from the subcutaneous region is not known at present. However, if one may speculate, there are at least two possible reasons for this phenomenon.

Ballard and Menczel (19) studied the subcutaneous absorption half-life of aqueous benzyl alcohol solutions in rats. The mean absorption half-life for four animals was 1.6 hr., but the range was from 0.96 to 2.58 hr. under conditions where the tissue area exposed to the drug solution was constant. They suggested that one probable reason for the large interanimal variation in $t_{0.5}$ could be attributed to differences in the membrane thickness exposed to the drug solution in the abdominal region for the several animals. It is well known that the thickness of the subcutaneous tissue among humans is not constant (45). In Eqs. 4 and 6 it can be seen that the penetration coefficient is inversely proportional to δ, while $t_{0.5}$ is directly proportional to δ. It is reasonable to suppose that as young animals age the thickness of their subcutaneous tissue may increase as well, resulting in increases in $t_{0.5}$ and increases in LD_{50} values for drugs whose acute toxicity can be correlated with drug blood-level.

Another hypothesis among several others deals with the question: what influence does animal age have on the chemical composition of subcutaneous fat tissue? The penetration rate of a lipid-soluble drug should be directly proportional to the lipid/water partition coefficient of the drug. (See Eq. 4.) If the chemical composition of this tissue changes with age, and the apparent partition coefficient of the drug differs in younger and older tissue, then variations in absorption rate would be expected. Using gas chromatographic analysis Bondrup *et al.* (46) studied the triglyceride composition of subcutaneous fat obtained from premature stillborn and full-term stillborn children, and normal, lean, and obese adult patients. Between the children and adult groups they found that there was a pronounced difference in the relative percentages of C_{12} to C_{18} triglycerides in the two groups. The testing of these two hypotheses must await more data on the relative thicknesses of the subcutaneous tissue surrounding injected solutions in young and older animals, and apparent partition coefficient data for drugs in these triglyceride systems.

Anatomical Region and Tissue Condition—The absorption rate of subcutaneously or intramuscularly administered drug can often be profoundly affected by the anatomical region into which the drug is injected and the condition of the tissue found at the site. Nora and co-workers (47) studied the effect of the route of insulin administration in the management of diabetes mellitus in children. They found no significant difference in absorption rate of ^{131}I-labeled lente insulin from subcutaneous and intramuscular tissues at a given anatomical region, but they did find significant differences at different sites. For example, the mean absorption half-life for the drug following intramuscular arm injections was 224 min., and that for the subcutaneous arm injections was 232 min. By comparison, the mean absorption half-life of the drug for intramuscular thigh injections was 314 min., and that for the subcutaneous thigh injections was 310 min. They concluded that variability in diabetic control potentially could be encountered when alternating the anatomical region of the injection between arm and thigh, but such a problem would not be anticipated between the subcutaneous and intramuscular sites at a given anatomical region.

Burchell and Swasdio (48) compared the absorption rates of atropine injected by various routes. The absorption rate of drug administered by various routes could be compared by recording the time interval between drug injection and the advent of tachycardia. Maximum pulse-rate elevation was noted at about 7 min. after intravenous administration of atropine, 28 min. after broad ligament administration, and

40 min. for both subcutaneous and intramuscular drug administration. They did not discuss the reasons for the unexpected rapid absorption of drug from the broad ligament.

Braid and Scott (49) studied the systemic absorption of the local anesthetic drugs, lidocaine and prilocaine, in humans after using two types of regional nerve block, namely, lumbar epidural block and intercostal regional block. Their work showed that much higher serum levels of both drugs follow intercostal block compared to epidural block. The authors ascribed the higher serum levels found with the intercostal block to differences in the relative vascularity of the tissues at the two injection sites. Although the epidural space contains numerous blood vessels, there are comparatively few capillaries available for drug absorption.

The condition of the tissue at the injection site can also affect drug absorption rate. Root and co-workers (50) studied the subcutaneous absorption of insulin labeled with radioactive iodine in diabetics and volunteers. In the controls and patients with uncomplicated diabetes, the absorption rates of tagged insulin were nearly identical. Drug absorption rate was retarded in diabetics when insulin was injected into regions where induration or an insulin pad had formed. The pads disappeared when injections in these regions were discontinued. After a recovery period, the tissue regained its ability to absorb insulin rapidly once again. Their results agreed with the clinical observation that insulin tends to lose its effectiveness when injected in insulin pads.

Chavarría *et al.* (51) administered cycloguanil pamoate intramuscularly to patients with dermal leishmaniasis. One of the treatment failures occurred in a child with a suppurating abscess at the injection site. When the abscess was drained of pus, particles of the yellow drug were found indicating that it had not been completely absorbed. The authors suggested that this complication might explain the treatment failure in this patient.

ENZYME EFFECTS

It has been known for years that when the enzyme hyaluronidase is added to a drug solution intended for intramuscular use, the onset of drug action is more rapid than when the drug alone is used (52–54). Hyaluronidase hydrolyzes hyaluronic acid, a component of tissue ground substance, which limits the spread of fluids at the injection site. Thus, after hydrolysis, the area of drug distribution in the tissue is increased with the result that absorption rate is also increased (55). The enzyme chymotrypsin, on the other hand, influences drug absorption by a mechanism not yet understood. Seneca and Peer (56) administered orally enteric-coated tablets of this enzyme to volunteers. Four hours later the subjects again received chymotrypsin orally, and 100 mg. of tetracycline hydrochloride intramuscularly. A control group was treated in the same manner except that its members received placebo tablets instead of the enzyme. At the end of 6 hr. the placebo groups had excreted about 13% of the injected dose of tetracycline in the urine, while the enzyme groups excreted about 37% of the dose over the same time. Serum levels of drug at various times following its injection were not reported for these subjects. However, when tetracycline hydrochloride and chymotrypsin were given orally, the mean level of antibiotic in the serum and the cumulative amount of drug excreted up to 12 hr. were significantly higher than when tetracycline hydrochloride was given orally alone. The authors state that chymotrypsin either interferes with the excretion of tetracycline or it increases drug absorption rate. On the basis of the evidence provided in the above experiment and the literature (57, 58), the authors concluded that interference with drug excretion was probably not the mechanism for the enhanced blood levels.

FORMULATION—PHYSICAL ASPECTS

The preceding section discussed some of the mechanisms and factors affecting drug absorption In this section some aspects of parenteral drug formulation will be considered. Nash (59) pointed out that most reviews written about pharmaceutical suspensions devote little space to a discussion of the formulation of parenteral suspensions. There are formulation problems associated with these dispersed systems which other suspension systems do not share. Some of these factors are product sterility, syringeability, ease of resuspension, and drainage.

The manufacture of sterile parenteral suspensions requires careful control at the following stages: (*a*) final drug recrystallization, (*b*) drug micronization, (*c*) drug sterilization, (*d*) vehicle sterilization, (*e*) aseptic wetting of the sterile powder with a portion of the sterile vehicle, (*f*) aseptic dispersion and milling of the bulk suspension, and (*g*) aseptic filling of the sterile containers with the finished suspension. The use of sound parenteral formulation techniques and sterility controls in the first place results in reductions in the foreign matter content of such powders and the chances of contaminating the formulation with pyrogenic materials.

One of the most important physical properties of a good parenteral suspension is its syringeability, or the ability of a solution or suspension to pass easily through a hypodermic needle. Properties that tend to reduce the formulation's syringeability can be traced to increases in the vehicle's viscosity and density, increases in the particle size of solids in the suspension, and increases in the percentage of drug suspended in the formulation. Nash states that probably the most important of the above factors contributing to a decrease in syringeability is the viscosity of the formulation. He believes one practical standard for syringeability is that the entire contents of a syringe should pass easily through a 25-gauge hypodermic needle.

The resuspendability of a suspension may be defined in terms of the minimum amount of shaking that is required to suspend particles that have settled in the container. A practical consideration is how much effort will a busy nurse or physician exert to resuspend settled particles? Ideally, the best answer to this problem is to formulate initially a "permanent suspension," or a physically stable, flocculated suspension.

A parenteral suspension should exhibit good drainage. Good drainage can be seen when the suspension separates cleanly from the inner walls of the container. The current practice of coating containers and plugs with silicone preparations makes good suspensions drain better and helps minimize the tendency toward bad drainage of over-flocculated systems.

Rheology—The pharmaceutical industry in recent years has come to rely heavily on sophisticated rheological measurements in order to help solve some of the difficult stability problems encountered in pharmaceutical systems. However, Boylan (60) points out that pharmaceutical literature is nearly devoid of rheological studies involving parenteral products. In his review, he gives examples of the effect that the formula and vehicle can have on the rheological properties of some commercially available procaine penicillin G suspensions.

Polymorphism—In recent years drug product formulators, government agencies, and others have become increasingly aware of the importance of identifying and characterizing the physical and biological properties of drug polymorphs. For example, both chloramphenicol palmitate and stearate have two polymorphic forms, one form of the ester in each case is therapeutically active and the other is not (61). In 1965 the Food and Drug Administration (FDA) amended its regulations to include a limit of not more than 10% of polymorph A, the therapeutically inactive form, in chloramphenicol palmitate suspension (62).

Hayden and co-workers (63) studied the absorption spectra of 175 reference standard compounds under controlled conditions. Except in resolution and slight relative intensity reversals, they observed no significant infrared spectral changes when potassium halide disks containing 150 of the standards were remeasured after periods of 2 weeks to 6 months. However, 25 compounds[4] displayed significant spectral variations after standing. They attributed the changes exhibited by these compounds primarily to crystalline form transitions. The conditions of crystallization, force of grinding, or the disk storage period influenced the crystalline forms and their infrared spectra. Lees (64) cites other examples where drugs were converted from one polymorphic form to another by apparently innocuous grinding procedures.

Mesley and Johnson (65) studied the infrared spectra of pharmaceutically important steroids with particular reference to the occurrence of polymorphism. Of 35 steroids examined 19 had two or more polymorphic forms.[5]

The articles by Macek (3) and Collard (66) consider some of the physical problems associated with the formulation of drugs intended for parenteral use. Polymorphic changes and particle size control in suspensions are among the topics discussed. Identification of the various polymorphic or solvate forms of drugs is also important because it has been shown that the subcutaneous absorption rate of one form may differ from that of another (2, 67).

Scheindlin (68) has briefly reviewed the use of stabilizers in parenteral drug formulations. He discusses the following classes of stabilizers: buffers, antioxidants, chelating agents, protective colloids, and miscellaneous stabilizers such as nonaqueous solvents. Anschel (69) reviewed the literature dealing with some of the methods of solubilizing some steroid hormones and antibiotics.

FORMULATION—BIOLOGICAL ASPECTS

Apart from the physical aspects of parenteral

[4] Betazole hydrochloride, calcium panthothenate, carbinoxamine maleate, dihydrostreptomycin sulfate, epinephrine bitartrate, ergonovine maleate, estradiol, estradiol benzoate, hydroxystilbamidine isethionate, menadione, mercaptopurine, nifuroxime, nitrofurantoin, oxytetracycline, phenindamine tartrate, phentolamine methanesulfonate, probenecid, pyrilamine maleate, sodium diatrizoate, sulfamethazine, sulfamethoxypyridazine, tetracaine hydrochloride, thiamine hydrochloride, trimethaphan camphorsulfonate, and zoxazolamine.

[5] Compound (number of solid forms identified): betamethasone (2), cortisone acetate (7), dexamethasone acetate (4), dimethisterone (2), ethinyl estradiol (3), fludrocortisone acetate (4), fluocinolone acetonide (2), hydrocortisone (2), methylprednisolone (2), norethandrolone (2), norethynodrel (2), prednisolone (2), prednisolone trimethylacetate (3), prednisone (2), prednisone acetate (2), progesterone (2), spironolactone (3), testosterone (3), and triamcinolone (2).

formulation, the test of whether a given formulation will be acceptable also depends upon performance *in vivo*. It has been known for some time that formulation variables can affect the animal's response to the product under study, sometimes to a large degree.

Particle Size—The effect of particle size on the availability of a suspension has been demonstrated by Foglia and co-workers (70). They administered the estrogenic substance *p*-hydroxypropiophenone (PHP) in saline subcutaneously to spayed female rats. When the size of the suspended particles was about 10,000 μ^3, and a high dose was maintained for a long time, they observed no estrus. When samples of the same powder were recrystallized to yield a particle size of about 2,000 μ^3 or less, they observed estrogenic activity. After samples of these active microcrystals were recrystallized to yield particles having the original size of about 10,000 μ^3, they became inactive again. The authors concluded that PHP appears to be a weak estrogenic substance, and its activity depends upon the crystal size injected.

Solubilizers—Kopáčová and Vrba (71) studied the local anesthetic activity of 1.5, 3, and 6% concentrated solutions of benzocaine in highly concentrated aqueous solutions of the solubilizing agents antipyrine, dimethylformamide, diacetone alcohol, and butane-1,2,4-triol. They assumed that the depot activity of the benzocaine was due to prolonged absorption following the drug's precipitation at the injection site. All benzocaine solutions caused undesirable local reactions. Reactions were greatest with the solubilizers butane-1,2,4-triol and antipyrine, and least with dimethylformamide. As to the degree of anesthesia, benzocaine in dimethylformamide was somewhat less effective than benzocaine in the solubilizers antipyrine and diacetone alcohol.

Viscosity—Coles *et al.* (72) studied the effect of aluminum monostearate–paraffin gels on the antigen response to *Clostridium welchii* type D toxoid. When these formulations were injected subcutaneously into rabbits and guinea pigs, the level and duration of the antitoxin titer in the blood was directly related to the viscosity of the vaccine formulations studied. In attempting to explain why an iron–polysaccharide complex (IPH) marketed for human use was poorly absorbed from muscle tissue of rats, Beresford and co-workers (37) surmised that it had a higher viscosity than the other iron–polysaccharide complexes tested. Also, its molecular weight might lie beyond the range capable of penetrating the lymphatics.

Base and Salt Forms—Monash and Gibbs (73) studied the acute toxicities of a number of local anesthetics administered intraperitoneally and subcutaneously to mice. They dissolved the salts in normal saline, and prepared a suspension of the base using polysorbate 80 and normal saline. The LD_{50} data are shown in Table IV. Intraperitoneal injections of both bases and salts resulted in approximately equal toxicity. This suggests that these forms of the drugs were absorbed from the peritoneal region into the circulation at nearly similar rates. In general, absorption from the subcutaneous tissue was not as rapid as it was from the intraperitoneal region. The result was that the acute toxicity of the salt and base were both reduced in most cases. After subcutaneous injections of the two forms of the drug, the acute toxicity of the base was always less than that for the salt form of the drug. The authors stated that this occurred because the base was less water soluble and hence was absorbed more slowly. However, their results raise some unanswered questions. Why should the salt and base forms of the drug have substantially the same degree of toxicity when administered by the intraperitoneal route, yet have different degrees of toxicity when administered subcutaneously? Should not the argument concerning the lesser solubility of the base compared to its salt and the differences in their absorption rates apply equally well to both injection sites? Also, what possible influence does the polysorbate 80 and the particle size distribution of the suspension have on drug absorption rate from either absorption site?

Solvent Effects—Foldes and co-workers (53) studied the influence of solvent on the myoneural effects of succinylcholine administered intramuscularly to humans. The chloride salt of the drug was made into a 10% solution by dissolving it in water and in 0.9% saline. When succinylcholine was dissolved in water, the onset of action was more rapid and its intensity greater than when the solvent was normal saline. In the water group apnea developed in 155 (S.E. ± 15.6) sec. and lasted

TABLE IV—ACUTE TOXICITY OF SALTS AND BASES OF SOME LOCAL ANESTHETICS IN MICE (73)

	Median Lethal Dose, mg./Kg.			
	i.p. Injection		s.c. Injection	
Anesthetic	Base	Salt	Base	Salt
Tetracaine	60	60	85	50
Benoxinate	37	30	75	37
Dibucaine	50	35	60	35
Lidocaine	200	160	275	225
Procaine	225	225	1000	500
Pramoxine	300	300	900	750

881 (S.E. ± 93.4) sec. in 9 out of 10 subjects. In the saline group apnea developed in 253 sec. and lasted 993 sec. in only 3 out of 10 subjects. The authors suggested that the delayed onset and lesser intensity of action of the intramuscularly injected succinylcholine dissolved in normal saline were due to the retardation of its rate of absorption that prevented the development of a sufficiently high plasma concentration. There are at least two possible reasons for this phenomenon. First, both succinylcholine chloride solutions are hypertonic with respect to the body fluids. However, of the two, the saline solvent is more hypertonic. Thus, there should be a greater net flow of water toward the injection site in the case of the saline vehicle with the result that drug absorption rate is decreased due to the greater solvent drag effect. Second, in water the diffusing electrolyte is moving under the influence of the chemical potential gradient for that ionic species, and the electrical field produced by the motion of the oppositely charged ion or ions. The more mobile Cl^- will tend to diffuse more rapidly than the succinylcholine^{2+} species. In so doing, a microscopic charge separation will tend to occur. Since it is an experimental fact (10) that macroscopic charge separation does not occur, the diffusion velocity of the slower ion will increase, while that of the Cl^- will decrease. When succinylcholine chloride is diffusing in an aqueous medium having an appreciable sodium chloride concentration, the microscopic conditions prevailing in the solution are different from what they were before. Now there is no symmetry of the "ionic atmosphere" which was found where the drug was diffusing in water. The succinylcholine^{2+} (or tracer ion) is moving relative to a background of nondiffusing Cl^-, while in the former case all ions are diffusing with the same velocity (10). Regardless of the mechanisms involved, on the basis of the biological data provided by Foldes *et al.* (53) it would appear that the diffusion rate of the succinylcholine ion is retarded in the saline vehicle resulting in a decreased absorption rate.

Potency Testing—There exists a danger that a parenteral product will be evaluated in terms of the "active" ingredient contained in the formulation without due consideration to other pharmaceutically important substances present in the preparation, such as solubilizers, preservatives, buffering agents, and the like. Bueno-Montano *et al.* (74) studied what effect chlorobutanol, ethanol, phenol, sodium acetate, and propylene glycol might have on the *in vitro* spontaneous contractile patterns of isolated human uterus tissues. They found all of these agents to be depressant to isotonically contracting uterine smooth muscle. The authors point out that the presence of agents commonly used in commercial preparations may complicate the *in vitro* appraisal of a given product.

Foreign Particulate Matter—The parenteral product formulator must determine the origin and nature of foreign particulate matter which might be found in a product and eliminate the sources of this contamination. This problem is so important that the FDA sponsored a symposium in July 1966 entitled "The Safety of Large Volume Parenteral Solutions." Abstracts of the papers presented at this meeting and other pertinent work are included in *References 75–80.*

Scherr (81) and Zolov (82) have suggested that a possible cause of abscess formation found in some patients receiving subcutaneous emulsion therapy is due to metal particles introduced into the formulation during emulsification. In addition, Caplin (83) proposed that other foreign bodies such as cotton gauze and rubber stopper particles might give rise to untoward local reactions during emulsion therapy, although he does not cite experimental evidence for his statement.

Another source of foreign matter may arise as a result of coring when the needle is passed through the rubber closure of a vial or the skin. Magath and McClellan (84) noted when needles were inserted through stoppered vials, that rubber fragments might be cut out. They injected these particles into the gluteal muscle of guinea pigs and found that they migrated quickly from the injection site into the popliteal space. Magath and McClellan concluded that it was unlikely that the fragments would produce much damage unless they were accidently injected into a blood vessel or if they gave rise to an infection. Gibson and Norris (85) directed their research toward finding the answers to these questions: How often does a hollow needle piercing the skin core out a fragment of tissue? What is likely to cause this to occur? And what is the possible fate of the fragments? Using needles having bores in the range commonly used for intravenous solutions, they recovered skin fragments 69% of the time. They noted no real difference between new and resharpened needles. This needle coring is probably due to a very sharp or ragged back bevel. However, attempts to blunt the back bevel did not greatly reduce the number of skin fragments. Some of the fragments may remain in the needle bore, partially or completely blocking it. Some may become sus-

pended in the injection fluid or lodged in the barrel causing the piston to stick. The remainder will be ejected along with the fluid. Gibson and Norris (85) state that it is difficult to assess the clinical significance of their findings, since many thousands of injections are given daily and no harmful effects have been attributed to skin emboli. This fact suggests that little is to be feared from the injected skin fragments *per se*. However, the authors suggest that unless the skin is carefully prepared prior to the injection, there is a risk that an infection may occur at the subcutaneous, intramuscular, or pulmonary sites. Parenthetically, Dann (86) recently has provided evidence that the routine procedures for skin preparation before injections are unnecessary, unless the skin is obviously dirty. Also, pretreatment of the skin with an antiseptic was judged to be unnecessary when measle vaccine was injected intradermally and subcutaneously by means of a dermojet instrument (87). Some investigators have attempted to avoid this potential problem. Thompson *et al.* (88) used 18- to 20-gauge Huber point hypodermic needles to facilitate the injection of suspensions and to minimize the likelihood of introducing skin fragments into animals. Charlebois (89) says the problem of coring skin or closures by needles is of particular importance now with the growing popularity of epidural injections. To eliminate the possibility of injecting a core of skin into the epidural space, the physician should make the actual injection with a needle other, and smaller, than the one used to pierce the skin. To eliminate the possibility of injecting a core of the closure into the epidural space, the local anesthetic drug should *not* be obtained by piercing a pliable membrane. The container closure should be removed before withdrawing the solution. The author further concludes that no drug that has been obtained by piercing a pliable membrane should be injected into a patient, and multiple-dose vials should not be used by the medical profession.

Carcinogenesis—The product formulator should always be alert to the potential problem of carcinogenesis. Roe (90) has reviewed the evidence for carcinogenic activity of various pharmaceutical agents and preparations. Table V shows his categorization of these substances into high, medium, and low carcinogenic risk groups. Several substances listed are of interest to the parenteral product formulator. Roe comments on the significance of tumor induction in subcutaneous tissues of rats and mice. The induction of cancers in these rodents at a subcutaneous site may well be a result of physical rather than chemical carcinogenesis similar to the induction of neoplasms following the implantation of chemically inert materials. Also, rats and mice are especially susceptible to the induction of local tumors following the subcutaneous or intramuscular injection of various substances. For these reasons Roe states that it is generally agreed that not much weight can be given to positive results obtained from injections in rats and mice unless they are supported by positive results from other experiments employing other methods of exposure.

Nevertheless, the subcutaneous route is frequently used to screen substances for their possible carcinogenic activity. For example, the colors, blue VRS, green S, and brilliant blue FCF

TABLE V—ATTEMPT TO CATEGORIZE SPECIFIC PHARMACEUTICAL AGENTS AND PREPARATIONS INTO HIGH, MEDIUM, AND LOW CARCINOGENIC RISK GROUPS (90)

HIGH RISK
All biological alkylating agents, especially β-naphthyl-di-2-chloroethylamine
Urethan
Isonicotinic acid hydrazide
Arsenic
Phenylbutazone
Creosote and coal tar preparations
Estrogens
Materials such as plastic sponges used in surgery
Combination of ^{131}I and goitrogens
3-Phenyl-5β-diethylaminoethyl-1,2,4-oxadiazole
MEDIUM RISK
Penicillins given by s.c. or i.m. injection
Actinomycins given by s.c. or i.m. injection
Iron dextran, saccharated iron oxide, ferric sodium gluconate complex, and ferrous glutamate given by s.c. or i.m. injection
Tannic acid and tannins
Medicinal paraffin
Goitrogens
Cortisone
Pronetalol[a]
8-*o*-Hydroxyquinoline
Vaccine preparations from which the presence of oncogenic viruses has not been excluded
Griseofulvin
LOW RISK
Methotrexate
Sulfonamide
Chloramphenicol
Chlorpromazine
Reserpine
Thalidomide
Carbon tetrachloride and chloroform (in doses which do not cause obvious liver damage)
Injections of colloidal gold or silver
Spermicidal contraceptive preparations, except possibly those containing 8-*o*-hydroxyquinoline
Carrageenin
Carboxymethylcellulose
Progesterone and oral contraceptives
Cocarcinogenic agents including phenols, essential oils, and surface active agents

[a] Alderlin.

were tested subcutaneously in rats to determine whether they might be safe for use as food colors (91). Poirier and co-workers (92) studied the carcinogenic activities of *N*-hydroxy-2-acetylaminofluorene (*N*-hydroxy-AAF) and some of its metal chelates after subcutaneous administration into rats. They noted an approximate correlation between the retention times of the potassium salt of *N*-hydroxy-AAF and its various metal chelates and the incidence of sarcomas found at the injection site. Certain chelates (*e.g.*, Ni^{2+}, Co^{2+}, Fe^{3+}, and Cu^{2+}) had moderate to high incidences of sarcomas following one to four injections. For these chelates the half retention time, or the time when only one-half of the administered *N*-hydroxy-AAF species remained at the site, ran from 4 to 50 days. The potassium salt and the Mn^{2+} and Zn^{2+} chelates showed less activity at the injection site. Their half retention times ranged from 2.5 hr. to 2.5 days.

Hueper (93) investigated the carcinogenic potential of hypertonic (25%) solutions of the sugars: *l*-arabinose, anhydrous *d*-dextrose, *d*-galactose, lactose, *d*-levulose, *d*-maltose, *l*-sorbose, and *d*-sucrose. These solutions were administered subcutaneously to rats and mice twice a week for periods up to 2 years. Only 2 rats out of 60 animals given the sorbose solution developed sarcomas at the injection site. No tumors were found at this site in rats and mice injected with the other sugar solutions, injected with water, or traumatized by needle puncture. One conclusion of interest was that nonspecific chronic irritation (*e.g.*, repeated water injections and needle trauma) did not represent a mechanism for carcinogenesis in this case.

It is of interest that Roe (90) catagorized mineral oil as being in the medium carcinogenic risk group as shown in Table V, since it is used in the food-processing industry of this country (94). Boitnott and Margolis (95) studied lymph nodes taken from the porta hepatis at autopsy. They found in 38 cases (78%) that the nodes contained large oil droplets that were deposits of liquid saturated hydrocarbons. They state that in all likelihood these hydrocarbons were derived in large part from absorbed mineral oil from the intestinal tract. The high incidence of oil droplets found in these lymph nodes seemed out of proportion to laxative and other medicinal uses of mineral oil, although quantitative data on this point are not available. They cite evidence that the use of mineral oil in the food-processing industry is currently estimated to result in the ingestion of an average of 47.5 Gm. of mineral oil per capita per year in this country. Although there are no data on the absorption of mineral oil at dosage levels comparable to those present in foods, they felt that this yearly intake seemed adequate to explain the amounts and incidence of the oil found in their autopsy specimens.

The FDA recently terminated many of the investigational new drug (IND) applications for clinical investigations of a mineral oil-emulsifier product. Allergists had been adding antigens to the product in order to treat specific allergies of patients. The mineral oil and emulsifier, generally mannide monooleate, tended to act as a sustained-release medium for the antigens, permitting less frequent injection of the patient. Tests used for measuring the biological safety of mannide monooleate have been detailed by Berlin (96). Fiero (97) has reviewed the industrial procedures used to purify the grade of mineral oil used in medicine and pharmacy. The position of the FDA and the Committee on Mineral Oil Adjuvant Preparations concerning the hazards of mineral oil adjuvants has been detailed in a letter sent to an allergist (98). Allergists (99–102) have responded to the action taken by the FDA in letters and editorials. It appears that considerably more testing will be required before a safe, generally accepted repository material will be available for routine clinical use.

Injection Pain—The intensity of pain resulting from some intramuscular or subcutaneous injections may range from a mild or transient unpleasant sensation to a severe reaction which might necessitate the termination of the therapy. Since pain may arise from parenteral injections to these regions, the product formulator should be aware of some of the causes of such pain. Travell (103) has classified the different causes of the immediate pain following injections. Immediate pain may arise from: (*a*) local irritation due either to the antiseptic used on the skin or the properties of the parenteral formulation (*e.g.*, pH, tonicity, or chemical action on the tissues), (*b*) mechanical trauma due to the introduction of the needle to the injection site, or to the sudden distension of the tissues resulting from a too rapid discharge of the syringe contents, and (*c*) abnormal sensitivity of the tissue at the injection site. She states that some of the chief causes of delayed pain of injection include infection, aseptic irritation and necrosis, antigenic reactions, reactions to pyrogens, and painful muscle spasm.

The pain may be classified according to its origin as cutaneous, subcutaneous, or intramuscular pain. Cutaneous pain originates from the

stimulation of sensory nerve receptors found in the skin. To eliminate skin pain, Travell recommends that a sharp needle, free of barbs, having the smallest diameter compatible with the viscosity of the solution be used. A cooling spray (*e.g.*, ethyl chloride) should be applied to the site for 2 or 3 sec. before the injection, and the instant the liquid disappears, the needle should be quickly inserted through the skin. Weeks and Travell (104) found that at a skin temperature of 15° one-third of the subjects had anesthesia to a needle prick, while at 10° or below all had anesthesia to the same stimulus. Travell (103) states that the subcutaneous tissues are relatively insensitive to the movement of the needle unless the fascia of the underlying muscle tissue is entered and pulled. Subcutaneous pain can be induced by the injection of an irritant material or a hypotonic solution such as distilled water. The addition of a local anesthetic to the vehicle injected may reduce, but not entirely eliminate, the immediate pain response from the subcutaneous tissues. In contrast to skin, normal voluntary muscle tissue is practically insensitive to the insertion of a needle. However, hypersensitive or "trigger" areas may exist in the muscle tissue of some patients. When these areas are stimulated by pressure, palpation, or the insertion of a needle, pain may result which can be referred to adjacent regions. It can be seen from this brief account that pain derived from subcutaneous or intramuscular injections may arise not only from the formulation injected, but from the techniques used in making the injection, as well as certain predisposing conditions found in the target tissue.

A classic method used by the parenteral product formulator to minimize injection pain has been to add a local anesthetic such as benzyl alcohol, procaine, lidocaine, and the like to the preparation (103, 105). Miller (106) has been critical of the use of such local anesthetics in parenteral products on the grounds that the pain reflects tissue damage, and this is not corrected merely by blocking of the sensory nerves in the region. Perhaps it would be better if the offending substance could be identified and the product modified in its formulation so that the amount of irritation caused could be reduced.

Untoward Reactions—Fortunately, over the years there have been remarkably few serious untoward reactions reported in the literature directly resulting from the techniques used in injecting parenteral formulations. Such injuries may provide the basis for malpractice suits. In a recent case over a quarter of a million dollars was awarded to a patient for damages incurred after an intragluteal injection of penicillin (107). Table VI lists some of the drugs associated with severe complications following intra-arterial or intramuscular injections. The injuries include nerve damage (*e.g.*, sciatic), gangrene of extremities, progressive fibrosis of muscle (*e.g.*, quadriceps), and others. Lachman (45) states there is complete agreement among all authors that damage to the sciatic nerve after intragluteal injection is essentially independent of the drug used. While the damage has in large measure been attributed to the techniques used for making the injection, physical and possibly chemical properties of the injected substances must also have contributed to the injuries reported. A number of authors have discussed injection procedures that should be followed to minimize complications (18, 45, 108–116).

Tables VII and VIII list some of the reported milder local reactions after the administration of selected drug solutions and suspensions. The preparation of Tables VI–VIII was facilitated by recent advances in the abstracting of adverse drug reaction reports (117–119).

At present the causes of many of the local tissue reactions following drug administration are not well understood. Part of the difficulty stems from the fact that the mechanisms by which body

TABLE VI—SUBSTANCES ADMINISTERED IN REPORTED COMPLICATIONS FOLLOWING INTRA-ARTERIAL AND INTRAMUSCULAR INJECTIONS

Drug	Ref.
Ascorbic acid	(150)
Barbital and aprobarbital mixture	(151)
Bismuth tartrate potassium	(152)
Caffeine sodium benzoate	(153)
Chloramphenicol and the succinate ester	(153, 154)
Chlorpromazine	(18)
Digitoxin	(153)
Epinephrine	(155)
Erythromycin	(154)
Gamma globulin	(153)
Iron dextran	(153)
Meperidine HCl and promethazine HCl mixture	(149)
Paraldehyde	(157)
Penicillin and its salts in various vehicles	(107, 109, 150, 151, 153, 154, 158–166)
Promazine and salts	(149, 153)
Quinine and salts	(167, 168)
Sodium amobarbital	(148, 149)
Sodium thiopental	(121, 149, 151, 172)
Streptomycin and salts	(150, 153, 158, 161, 166)
Sulfonamides	(169–171)
Tetracycline and salts	(108, 153, 154, 166)
Thialbarbital[a]	(156)
Vitamin K	(150, 153, 154)

[a] Trademarked as Kemithal by Imperial Chemical Industries, Ltd., Wilmslow, Cheshire, England.

TABLE VII—LOCAL REACTIONS REPORTED FOR SELECTED DRUG SOLUTIONS[a]

Active Ingredient	Vehicle	Route of Administration and Untoward Local Reactions in One or More Humans or Animals	Species	Ref.
Ampicillin (as sodium salt)	.....	i.m.: severe pain at injection site	Human	(173)
Bis-(4-hydroxyiminomethyl-pyridine-1-methyl) ether dichloride[b]	.....	i.m.: dull, burning pain following injection using plastic self-injection ampul, which is manually emptied, but painless when automatic type is used. Pain assumed to be caused by needle movement	Human	(135)
Butyl-*p*-aminobenzoate 5%, procaine 1%, procaine HCl 0.25%[c]	Propylene glycol 78%, polyethylene glycol 300 2%, in distilled water	Intercostal nerve block: neuritis, inflammatory reactions, and lasting absence of nerve function resulted	Human	(174, 175)
Capreomycin	.....	i.m.?: bleeding at injection site	Human	(176)
Capreomycin	.....	i.m.: s.c. "lumps" at injection site from first week on	Human	(177)
Capreomycin	.....	i.m.: mild pain or stinging initially, palpable nodules, diffuse induration at injection site	Human	(178)
Capreomycin	.....	i.m.: sterile abscess of hip (due to injection technique?)	Human	(179)
Cephaloridine	Sterile water or isotonic saline	i.m.: moderately severe pain at site	Human	(180)
Chloramphenicol sodium succinate	Aqueous or saline	i.m.: local pain at injection site, sometimes severe	Human	(181)
Dihydrotriazine hydrochloride[d]	40% benzyl benzoate in castor oil	s.c.: local induration and frequently open lesions at injection site	Mice	(88)
Diphtheria–tetanus vaccine	No lidocaine	s.c.: initial pain occurring immediately after the injection and reaching a peak within a few minutes. Pain partly due to mechanical distension caused by the fluid, but mainly due to some unknown factor in the vaccine	Human	(105)
	Lidocaine added	s.c.: 12 of 13 volunteers felt less pain with the added lidocaine; one noted no difference		
Emulsion therapy with various pollens or molds	Adjuvant: 35% mannide monooleate, 65% mineral oil	s.c.: abscess at injection site	Human	(82)
Iron–dextran complex[e]	Aqueous	i.m.: injection abscess (may be due to faults in aseptic technique). Injections very painful and produced staining of skin	Human	(183)
Iron–sorbitol–citrate complexes[f]	Aqueous	i.m.: pain and skin discoloration at injection site	Human	(184, 185)
Nafcillin[g]	.....	i.m.: local pain and induration when given frequently in the same area	Human	(186)
Oxytetracycline ethanol–ammonium magnesium salt	80% propylene glycol in water, 2% lidocaine	i.m.: single injections almost invariably well tolerated, with only occasional complaints of mild pain lasting 15–30 min. Multiple injections usually well tolerated when given at 24-hr. intervals. More frequent injections occasionally associated with discomfort and more lasting pain	Human	(187)
N-(Pyrrolidino-methyl) tetracycline	Water, lidocaine	i.m.: local pain or discomfort at injection site	Human	(189)
N-(Pyrrolidino-methyl) tetracycline[h]	Water, lidocaine, ascorbic acid	i.m.: little discomfort at time of injection, but 15–20 min. later subjects complained of moderately severe pain at injection site lasting 1.5–2.5 hr. Without lidocaine same formulation caused moderate to severe pain from 1 min. to 2–3 hr. after	Human	(190)
Sodium fusidate[i]	Aqueous	s.c., i.p.: local tissue injury with necrosis at injection site, probably this irritating effect connected with the surface-active properties of the drug	Mice	(182)

(*Continued on next page*)

TABLE VII (*Continued.*)

Active Ingredient	Vehicle	Route of Administration and Untoward Local Reactions in One or More Humans or Animals	Species	Ref.
Sodium pentobarbital	2% benzyl alcohol in 60% propylene glycol in water, pH = 10.5	s.c.: pain usually present, firm nodule at injection site; i.m.: usually no pain	Human	(188)
Thiamine hydrochloride	Aqueous	i.m.: intense pain lasting on av. 122 sec. Addition of 0.5% procaine HCl reduced duration of pain to av. of 20 sec.	Human	(103)
7-(Thiophene-2-acetamido) cephalosporanate sodium[j]	Normal saline	i.m.: slight to moderately severe pain at injection site	Human	(191)
Trypsin	Gelatin in double-blind (DB) study Saline in alternating (A) study	i.m.: in "DB" study *ca.* 30% of subjects developed local tenderness at injection site with nodule formation. In "A" study local reaction was 10%, no nodule formation occurred	Human	(201)
Vitamin D_3 hydrosol	Water, ethanol polyoxyethylene ricinoleic acid	i.m.: hemorrhagic focal area 1 week after initial injection. After 3 weeks calcification occurred on muscle surface and dermis of skin in contact with musculature	Rat	(192)

[a] Consult references cited for pharmacologic or possible toxic effects in other tissues. [b] Toxigonin. [c] Efocaine. [d] 4,6 Diamino-1-(*p*-chlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride (DHT). [e] Imferon. [f] Jectofer. [g] Unipen. [h] Syntetrin. [i] Fucidin. [j] Keflin.

TABLE VIII—LOCAL REACTIONS REPORTED FOR SELECTED DRUG SUSPENSIONS[a]

Active Ingredient	Vehicle	Route of Administration and Untoward Local Reactions in One or More Humans or Animals	Species	Ref.
Betamethasone sodium phosphate + betamethasone acetate[b]	Aqueous	i.m.: stinging, burning, or acute pain at injection site	Human	(193)
Cycloguanil pamoate	Lipid: 40% benzyl benzoate in castor oil	s.c.: transient palpable masses, and open lesions	Mice	(88)
	Aqueous: 1.5% pectin + 0.1% polysorbate 60	s.c., i.m.: palpable masses occurred only with very large injections	Monkey	(88)
Cycloguanil pamoate	40% benzyl benzoate in castor oil	i.m.: suppurating abscess developed at injection site	Human	(51)
Hydrocortisone acetate	0.9% NaCl, 0.5% Na carboxymethylcellulose, 0.1% methylcellulose, 0.19% polysorbate 80, 0.24% methylparaben, 0.026% propylparaben, water *q.s.*	i.t.[c]: pain at injection site or referred pain to face or behind eye	Human	(194)
Insulin	Aqueous	s.c.: local reactions may occur in the form of red painful indurated areas at injection site and may persist for 2–3 weeks, then gradually disappear. Insulin atrophy i.c.[d]: induration, necrosis, ulceration and scarring	Human	(195)
Monosodium urate	5% dextrose in water	s.c., i.d.[e]: erythema, induration, local temperature elevation	Human	(196)
Monosodium urate monohydrate	50% glycerin in water	s.c.: erythema, induration, warmth, tenderness	Human	(33)
Sodium orotate	50% glycerin in water	s.c.: erythema, induration, warmth, tenderness	Human	(33)
Tetracycline phosphate	Lidocaine, monoacetin	i.m.: local discomfort or pain	Human	(189)
Triamcinolone acetonide	Aqueous	i.m.: subcutaneous fat atrophy	Human	(197)
Triamcinolone acetonide	0.9% benzyl alcohol, 0.75% Na carboxymethylcellulose, 0.04% polysorbate 80 in water, NaCl *q.s.* for tonicity	i.m.: atrophy in area of injection, s.c. fat necrosis at injection site	Human	(198)
Triamcinolone acetonide	Aqueous	i.m.: atrophy of s.c. fat, local abscess	Human	(199)
Triamcinolone acetonide	Aqueous	s.c.: atrophy of keloids	Human	(200)

[a] Consult references cited for pharmacologic or possible toxic effects in other tissues. [b] Celestone Soluspan. [c] Intraturbinate. [d] Intracutaneous. [e] Intradermal.

cells recognize injected foreign materials are complex. An explanation of these local tissue reactions is not generally possible at the molecular level. Some of the research concerned with foreign body reactions and the mechanisms of phagocytic discrimination has been reviewed by Boyden (120). On a macroscopic level some of the causes for these local tissue reactions include the following. After injections of sodium thiopental, Davies (121) believed that necrosis of tissue at the site was caused in part by direct toxic action of the solution, and in part by infection. Subcutaneous nodule formation following "intramuscular" injections has been attributed to erroneous deposition of the formulation into the subcutaneous region (45). After an intramuscular injection, painful subcutaneous nodules (15) sometimes form because leakage of the solution occurs back along the needle tract. In other cases the subcutaneous nodule formation is caused by the deposition of material into the subcutaneous region because needles of insufficient length were used (18). McLean *et al.* (122) discusses some of the causes of local untoward reactions to repository therapy; they conclude that a sterile abscess forms because a foreign body type of irritative process occurs. Goldman (123) discusses the local reactions following intra- and sublesional injections of corticosteroid suspensions. While local reactions are uncommon, they include (*a*) pain and discomfort, (*b*) hemorrhage, (*c*) atrophy, (*d*) secondary infection (local and diffuse), (*e*) pigmentation changes, (*f*) hypersensitivity reactions, and (*g*) panniculitis. If a relatively large amount of corticosteroid crystals is injected superficially, a slough may develop which appears to be due to a blockage of local circulation as well as changes in the local ground substance. If sloughing does not occur, the depot may remain visible in the skin for long periods of time.

Hanson (124) investigated some of the local toxic effects of the antibiotics: chloramphenicol, chloramphenicol sodium succinate, oxytetracycline, tetracycline, and their vehicles after subcutaneous and intramuscular injections into rabbits. Cioc and von Schilling (125) describe histological changes in rabbit and pig muscle tissue produced by injections of a number of drugs.[6]

Newer Techniques—Up to this point, the traditional syringe-and-needle method of introducing drugs subcutaneously and intramuscularly has been discussed almost exclusively. The history of the development of injection therapy has been thoroughly discussed by several authors (1, 126–128) and will not be reviewed again at this time. However, newer devices are appearing on the market. Under certain circumstances they may have advantages over the more conventional devices. Some of these instruments involve the principle of jet injection whereby the drug solution is forced through the epidermis and dermis as a fine dispersion under high pressure (40, 42, 87, 129–133). Some reports in the literature indicate that dosing errors might result from the use of some jet-injection instruments due to calibration problems (130, 132). (See Table IX.)

TABLE IX—SUMMARY OF RECORDINGS COMPARING THE DOSAGE-DELIVERANCE RELIABILITY OF TWO JET-INJECTION INSTRUMENTS AND A PIPET TECHNIQUE (132)

Anticipated Vol. Delivered, ml.	Mean % of Anticipated Vol. Actually Delivered		
	Jet No. 1	Jet No. 2	Pipet
1.0	82	113	100
0.7	94	119	101
0.5	97	128	102
0.2	39	170	98

Another instrument using the syringe-and-needle principle is the Hypule, which is designed for unit dosage systems. It is a prefilled, disposable device which delivers an exact volume of solution subcutaneously (134). Erdmann *et al.* (135) studied the absorption and excretion of an alkyl-phosphate antidote,[7] following its injection muscularly into humans, using two types of sterile, self-injection ampules: a plastic type,[8] which the user empties manually after puncture, and an automatic type,[9] which is self-emptying.

Prolonged-Action Formulations—One of the problems of evaluating the absorption rates of solids administered as suspensions *via* the subcutaneous or intramuscular routes is that no convenient method exists today for estimating the surface area of the solid exposed to the absorbing membranes while *in situ*. The effective area of the suspension exposed to the absorbing tissues varies according to the injection techniques used and the physical properties of the suspension. Since the effective area of solid exposed to tissues is not known, precise estimates of the drug absorption rate

[6] Dipyrone (50%), thiamine, ferro dextran complex, liver extract, vitamin B_6, dihydroergocornine-cryptine-cristine methanesulfonate (ergot fluid extract), trimethyl 3-oxyphenyl ammonium bromide, liver extract with vitamins B_1 and B_{12}, α-(*N*-β-diethylaminoethyl)aminophenylacetic acid, calcium bromide, and eucalyptol, guaiacol, and sage compound.

[7] Toxogonin, E. Merck AG, Darmstadt, Germany.

[8] Gotole, Penicillin-Gesellschaft Dauelsberg, Göttingen, Germany.

[9] Autule, Penicillin-Gesellschaft Dauelsberg, Göttingen, Germany.

cannot be made, since the absorption rate is directly proportional to this area at any time. Where applicable one quantitative method of evaluating the absorption rate of solids is the use of the pellet implantation technique of drug administration. For example, suppose the parenteral product formulator intends to formulate a suspension and has several salt, polymorphic, or solvate forms of a given drug. He should be able to establish in a given species and a given absorption site the rank order for the mean absorption rates per mean pellet areas. From this and other information at his disposal, the investigator could make a preliminary estimate as to which of the drug forms should be studied in detail for possible use in the suspension formulation.

For a single implanted sphere of a drug the weight of solid at any time can be calculated (136) from:

$$W = \frac{\pi\rho}{6}(D^0 - kt)^3 \qquad \text{(Eq. 7)}$$

and for an implanted disk:

$$W = \frac{\pi\rho}{4}(D^0 - kt)^2(h^0 - kt) \qquad \text{(Eq. 8)}$$

where W is the weight of the solid at any time, ρ is the apparent density of the solid, D^0 is the initial diameter of the disk or sphere, h^0 is the initial height of the disk, k is the mean absorption constant having dimensions of length $\times$ time^{-1}, and t is the duration of pellet implantation. It is assumed that D^0 or $h^0 \geqslant kt$, and that the shape of the solid is not distorted during the implantation period. Once the value of k has been determined for one geometric shape of a particular drug in a given animal species at a given absorption site, it can be used to estimate the probable absorption rate characteristics for other geometric shapes of the same drug.

Recently, Ballard and Goyan (137) made use of an analog computer to study the kinetics of drug absorption, distribution, metabolism, and elimination of a model drug, sulfadiazine, in all forms following its administration as subcutaneous disk-shaped implants in rats. Since the partially absorbed solid was recovered from the implantation site, the mean absorption constant, k, could be determined by Eq. 8. The cumulative amount of free and acetylated drug was followed as a function of time. The kinetic model used is shown in Fig. 1. The term Si is the amount of drug in the disk at the implantation site, S is the amount of free drug in the fluids of distribution in the animal, Se is the cumulative amount of free drug excreted unchanged in the urine, Sa is the amount of acetylated drug in the fluids of distribution in the animal, and Sae is the cumulative amount of acetylated drug excreted into the urine up to any time. For purposes of achieving material balance between the amount of drug absorbed and that actually recovered in the urine as free and acetylated drug, it was necessary to postulate an amount of drug or metabolite, Su, in some unknown compartment. If Su represents free drug, then it is the cumulative amount excreted to any time by a route other than *via* the urine. In this case k_5 would be ignored. If Su is the amount of undetected metabolite to any time, then Sue is the cumulative amount of that metabolite excreted by some route to any time. While the absorption constant, k, has units of length $\times$ time^{-1}, the constants k_1, k_2, k_3, k_4, and k_5 are assumed to be first-order rate constants for the respective processes in units of time^{-1}. In Fig. 2, curve 1 (Se) shows the cumulative amount of free drug, and curve 2 (Sae) shows the cumulative amount of acetylated drug excreted *via* the urine as a function of time. Curve 3 (Su or Sue) is defined by material balance considerations and is the cumulative amount of free drug (Su), or the cumulative amount of an unspecified metabolite (Sue) excreted *via* an unknown route to any time. In the model the constant k_2 is assumed to be very large compared to k_1, and k_5 is very large compared to k_4. Curve 4 (S) represents the amount of free drug in the fluids

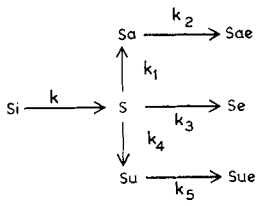

Fig. 1—Kinetic model showing disposition of sulfadiazine in all forms following its administration as a subcutaneously implanted disk-shaped pellet in a rat (137). Consult text for details. [Reprinted with permission from Med. Biol. Eng., *4, 483 (1966).]*

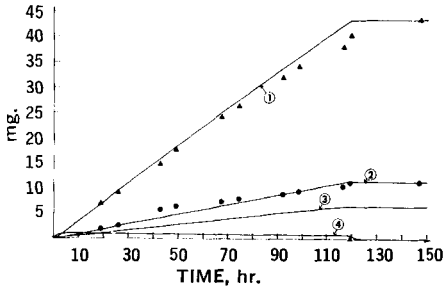

Fig. 2—Analog computer plots for rat B. Consult text for details concerning curves 1–4. Unpublished data (137). Key: ▲, free sulfadiazine; ●, acetylated sulfadiazine.

of distribution in the animal at any time. With curve 4 (S) one notes that after the peak amount of free drug is reached in the fluids of distribution about 10 hr. after implantation, there is a very gradual decline in amount until the pellet is removed (vertical arrow at 118.3 hr.), whereupon an exponential drop occurs. This gradual decline in amount would be expected because the pellet in losing mass with time, is also changing area very slowly.

There are several advantages in using the analog computer in *in vivo* drug kinetic studies such as the one illustrated here. First, it is generally simple to write out the differential equations for the proposed steps in the kinetic model shown in Fig. 1, and it is often possible to integrate these equations (137). However, their direct solution becomes extremely difficult by ordinary digital methods, particularly when one or more rate constants are not known exactly. Second, it is possible with an analog program to plot the probable amount-time course of drug or metabolites in compartments *not* analyzed directly in the investigation (*e.g.*, curves 3 and 4, Fig. 2). Third, once the pharmacokinetic constants have been determined for a particular shape of a drug implant in a given species and site of implantation, it should be possible to simulate with the computer the distribution and elimination time courses of the drug and its metabolites with other implant shapes and implantation times, even though the experiment was not actually undertaken *in vivo*.

A striking example of how the usefulness of a drug can be enhanced by preparing a repository formulation can be seen with the antimalarial drug cycloguanil pamoate. Shortly after chlorguanide was introduced, *in vitro* evidence indicated that the intact drug was essentially inactive, and that an active metabolite, 4,6-diamino-1-(*p*-chlorophenyl) - 1,2 - dihydro - 2,2 - dimethyl - *s* - triazine (DHT), was responsible for the activity of the drug. The hydrochloride salt of DHT is less active than chlorguanide against *P. cynomolgi* in monkeys when given orally or parenterally. The decreased activity of DHT compared to chlorguanide in monkeys is believed to be due to the rapid excretion of the soluble metabolite (88).

The pamoate salt of DHT, cycloguanil pamoate, has a solubility of 0.03 mg./ml. in pH 7, 0.1 *M* phosphate buffer. The drug has been formulated as parenteral suspensions in a lipid vehicle consisting of 40% benzyl benzoate and 60% castor oil, and in an aqueous vehicle consisting of 1.5% pectin and 0.1% polysorbate 60[10] in water. The drug was injected intramuscularly or subcutaneously as a 10 to 25% suspension in the vehicles described above. As a result of their experiments, the authors (88) made the following tentative generalizations regarding the repository effects of cycloguanil pamoate against *P. cynomolgi* in monkeys: the type of vehicle had little influence, the period of protection was related to the amount of drug per injection site, injection of a given amount at a single site was as effective as similar injections at multiple sites, and either intramuscular or subcutaneous routes were used without markedly different results.

From a pharmacokinetic standpoint, the rate-limiting step in the disposition of cycloguanil pamoate in the body is the dissolution rate of the salt in the fluids present at the injection site. As a consequence of the slow release of the drug from this site and the rapid excretion of the active metabolite *via* the urine, low plasma levels have been found. The plasma level of DHT following the injection of cycloguanil pamoate would mimic in many ways an extremely slow intravenous infusion of a soluble salt of DHT. If cycloguanil pamoate were administered as an implanted disk instead of as a suspension having an ill-defined area in contact with the tissues, the amount of free DHT in the fluids of distribution in the animal at any time would be represented qualitatively by the shape of curve 4 in Fig. 2. Since certain strains of malaria parasites have become increasingly resistant to some of the antimalarial drugs, attempts have been made to prepare parenteral repository preparations containing mixtures of drugs. This work has been reviewed by Powell (138).

The use of computers in pharmacokinetic studies has been discussed by Wagner (139). Analog, digital, and hybrid computers have been used principally for the rapid numerical analysis of data and for pharmacokinetic simulation. Wagner (139) points out that the equations and models used are always oversimplifications of the real system and their appropriateness in a given experiment can be judged only by their ability to describe the observed data and the accuracy with which they make predictions of future observed data. Some of the examples described in Wagner's paper include a pharmacokinetic analysis of the serum concentrations of linocomycin following an intramuscular injection of the drug hydrochloride, an analog computer prediction of the multiple-dose serum levels of lincmycin observed after a series of constant-rate intravenous infusions, and the digital computer simulation of serum levels produced by depot intramuscular preparations of lincomycin.

[10] Tween 60, Atlas Chemical Industries, Inc., Wilmington, Del.

In the past few years a number of other prolonged-action formulations have been reported on in the literature. Rizkallah and Taymor (140) gave, to normal females, intramuscular injections of various combinations of dihydroxyprogesterone aceophenide and estradiol enanthate in an attempt to discover which formulation would give the most satisfactory cyclic uterine bleeding response, yet produce minimum side effects. Kowarski and Bar-Natan (141) reported details of the biological responses to a number of prolonged-action diethylstilbestrol formulations intended for subcutaneous administration. Laffan and co-workers (142) studied the prolonged-action effects of fluphenazine enanthate dissolved in sesame oil after subcutaneous administration to animals. One preliminary report (143) casts doubt on the effectiveness of this formulation given intramuscularly in treating some schizophrenic patients. Linna *et al.* (144) managed to produce experimental hypertension in rats by prolonged subcutaneous administration of norepinephrine bitartrate in an oily vehicle.

In summary, this review has attempted to consider some of the biopharmaceutical aspects of drug absorption from subcutaneous and intramuscular routes of administration. A portion of the literature dealing with the formulation and administration of such parenteral formulations also has been discussed. From the foregoing it is clear that there are many areas where more basic research is needed. If this review stimulates others to conduct such research, one of the several goals of this paper will have been achieved.

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Keyphrases

Biopharmaceutics, drugs–parenteral
Drug administration—subcutaneous, intramuscular
Endothelial tissue—drug absorption
Diffusion—drug absorption
Phagocytosis—drug absorption
Biological factors—drug absorption
Drug formulations—absorption, biological factors

Research Articles

Procaine Interaction with the Corneal Surface and its Relation to Anesthesia

By VICTOR F. SMOLEN and FREDERICK P. SIEGEL*

The effects of pH and procaine on the apparent density of charged groups fixed to the corneal surface of guinea pigs were studied. The net density of fixed charge was found to be negative over the pH range of 5 to 9 and decreased in the presence of procaine. These results are interpreted in terms of procaine interaction with the surface and its implications with regard to the factors that may influence the characteristics of the anesthetic response. The electrometric method employed allowed the study to be performed under physiological conditions using live animals. The method is general and can be equally applied to study other drug–tissue interactions.

THE CORNEA possesses a moist surface primarily composed of amphoteric colloids; pH and the interaction of solutes determine the extent to which attached groups are ionized and the resulting net density of fixed charge on the surface. The present study is an effort to elucidate the role of these factors in the mechanism by which the effectiveness of procaine in the eye is increased in alkaline solution (1–4).

An electrometric method modified from Joseph *et al.* (5, 6) was used to determine the apparent fixed charge density of the corneal surface of the guinea pig in response to variations in the pH of applied buffer solutions in the presence and absence of procaine. The cornea is particularly suited to this approach due to its relative simplicity and accessibility. It has also been the subject of many investigations with local anesthetics. Reliable pharmacological data particularly with procaine were found to be readily available (1). The method is generally applicable to the study of other tissue surfaces and other drugs as well. It allows the results to be obtained under nearly physiological conditions without damage to the tissues involved. Thus, in the present study, the apparent density of fixed charge of the corneal surface was determined under the same conditions that are known to influence its anesthetic response to procaine.

THEORETICAL

The dissociation of counterions from ionogenic groups covalently bonded to a tissue surface or the absorption of ions onto neutral surface sites gives

Received May 2, 1967, from the Department of Physical and Industrial Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907
Accepted for publication October 18, 1967.
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THE CORNEA possesses a moist surface primarily composed of amphoteric colloids; pH and the interaction of solutes determine the extent to which attached groups are ionized and the resulting net density of fixed charge on the surface. The present study is an effort to elucidate the role of these factors in the mechanism by which the effectiveness of procaine in the eye is increased in alkaline solution (1–4).

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Hg	Hg_2Cl_2	Saturated KCl_1	Reference solution (0.15 *M* NaCl)	Reference surface (hind foot of guinea pig)	Ⓟ Experimental surface (guinea pig cornea)	Experimental solution or its 10-fold dilution	Saturated KCl	Hg_2Cl_2	Hg

Fig. 1—Circuit diagram corresponding to the experimental arrangement depicted in Fig. 2. Ⓟ represents the potentiometer.

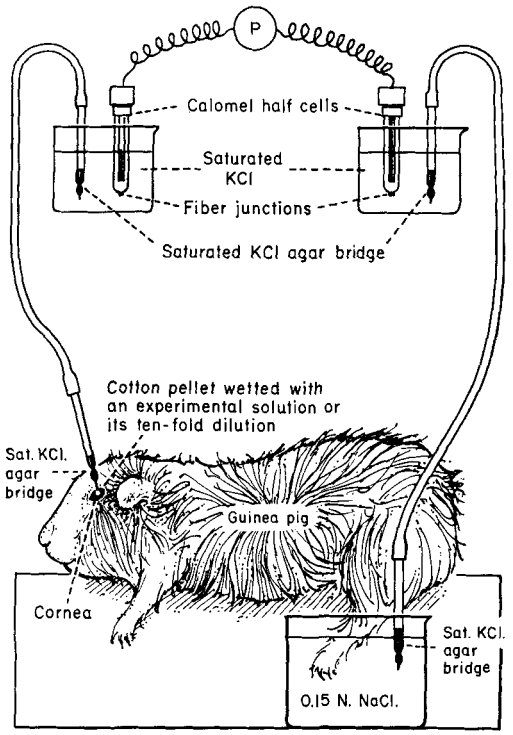

Fig. 2—Diagram of the experimental arrangement for the determination of the fixed charge density of the guinea pig cornea. Ⓟ represents the potentiometer.

rise to an unequal distribution of free ions present at the surface as compared to the bulk of a solution with which it is in contact. An electrical potential difference also develops across the colloid-aqueous boundary. The magnitude of the distribution ratios (r) for the mobile ions, present at the colloid surface as compared to the bulk of the applied solution, as well as the sign and magnitude of the electrical potential, depend upon the net density of the charge fixed to the tissue surface. The measurement of a potential, E_1, includes this equilibrium phase boundary potential as well as all other potentials developed in the circuit (see Figs. 1 and 2 for a circuit diagram and the experimental arrangement, respectively). These extraneous potentials have been found to remain constant for the short period of time required to replace the initial solution in contact with the tissue surface with a dilution of this same solution followed by the immediate recording of a second potential, E_2. The difference of these two measured potentials ($E_2 - E_1$), termed the dilution potential E_d, is thus devoid of the extraneous potentials and includes only those potential differences developed between the aqueous medium at the tissue surface and the bulk of the applied solutions. This consideration allows the interpretation of E_d in terms of the familiar Donnan equilibrium (10, 11) and its treatment as a diffusion potential (5). Under the conditions of its measurement, E_d is characteristic only of the tissue surface rather than reflecting the properties of the bulk or interior of the tissue phase. If this were not the case, some question might be raised regarding the physical significance of the measurements (11).

Following an approach similar to that of Joseph *et al.* (5) the principal author has derived equations (Eqs. 1 and 2 below) for E_d and the fixed charge density (f) (12). These equations are presented in their general form.

Equation 1 expresses E_d in terms of a ratio (r) defined as the ratio of the concentration (m_{i+c} or m_{i-c}) of an i'th cation or anion at the charged colloid surface to its concentration in the bulk of the applied solution (m_{i+a} or m_{i-a}), raised to a power equal to the reciprocal of its valence (z_{i+}) for cations, and the negative of the reciprocal of its valence (z_{i-}) for anions. The value of r, obtained from the solution of Eq. 1, is substituted into Eq. 2 from which the fixed charge density (f) is obtained. In Eq. 1 the symbols λ^0_{i+} and λ^0_{i-} denote the limiting ionic conductances of the i'th cation and anion, respectively, while D is the factor by which the experimental solution is diluted for the measurement of E_2. In the present study its value is 10. The symbol F refers to the Faraday constant; R is the gas constant and T is the absolute temperature.

Equation 1 is derived on the basis of considering the Gibbs free energy changes accompanying the transfer of charged species between the colloid surface and the bulk of the solution and expressing the transport numbers for the ions in terms of Kohlrausch's law of independent ion migration (13). Equation 1 resembles equations describing liquid junction potentials (13) which in effect it does when applied to the measurements described in this study. Values of E_d calculated from Eq. 1 agree closely with values calculated from Henderson's equation (13), which describes a liquid junction potential developed across a continuous mixture boundary. Equation 2 is merely an expression of macroscopic electroneutrality at the tissue surface.

The described electrometric method is of general applicability. It has been verified quantitatively with wool (6); the results obtained were comparable

$$E_d = \left[\frac{\sum\left(r^{z}_{i+}\cdot m_{i+a}\cdot\lambda^0_{i+}\right) - \sum\left(\frac{m_{i-a}}{r^{z}_{i-}}\lambda^0_{i-}\right)}{\sum\left(z_{i+}\cdot r^{z}_{i+}\cdot m_{i-a}\cdot\lambda^0_{i+}\right) + \sum\left(\frac{z_{i-}\cdot m_{i-a}}{r^{z}_{i-}}\lambda^0_{i-}\right)}\right]\frac{2.303RT}{F}\log D \qquad \text{(Eq. 1)}$$

$$f = \sum\frac{z_{i-}m_{i-a}}{r^{z}_{i-}} - \sum z_{i+}\cdot r^{z}_{i+}\cdot m_{i-a} \qquad \text{(Eq. 2)}$$

to titration curves of wool obtained directly (22). When the method is used *in vivo*, it conveniently and rapidly yields results not readily attainable by other means.

MATERIALS AND METHODS

Materials—All reagents were of analytical grade, except procaine hydrochloride USP.[1] Male guinea pigs of approximately 3 months of age were used.

All electrical potentials were read from a Sargent model SR recorder. Saturated calomel electrodes[2] were inserted together with KCl-agar salt bridges into 100-ml. beakers containing saturated KCl. The salt bridges consisted of 3–4 ft. lengths of transparent tygon tubing filled with saturated KCl solution. The ends were blocked with 6-in. lengths of glass tubing drawn at one end into a capillary filled with hardened KCl-agar gel and constricted in the middle to prevent loosening of the gel and subsequent leakage. The transparency of the tubing allowed ready detection of air bubbles which can profoundly interfere with conductance through the bridges.

Solutions—The composition of the solutions and the pH range selected for this study were chosen so that the results could be compared with those of Hiter (7) on the duration of corneal anesthesia. However, the pH 9.0 borate buffered solutions used by Hiter were found to give anomalous results in that observed and calculated diffusion potentials differed widely. As a consequence, 0.1 *M* phosphate buffers prepared from KH_2PO_4 and NaOH were used throughout the pH range of 5–9. The procaine solutions were always freshly prepared at the time of the experiment. Each buffered procaine solution below pH 9 contained 1% (36.7 m*M*) procaine hydrochloride. The pH 9.0 solution was saturated and the total concentration of procaine was calculated to be 4.35 m*M*. The calculation was based on the solubility of the free base given by Eisenbrand and Picher (8) as 0.13%, and from the degree of association, 44.2%, based on an acidic pK of 8.85 (9). An aliquot of each of the solutions was diluted 10-fold for use in the measurement of the potentials E_2 as described below.

The pH of the solutions was determined with a Beckman model 76 pH meter. The concentrations of the ionic species in each solution were calculated from the measured pH and the pK's of the dissociable substances (9).

Procedure—The position of the guinea pig in the experimental set-up is illustrated in Fig. 2. Figure 1 is a corresponding circuit diagram. A saturated KCl salt bridge was placed in a 0.15 *N* NaCl solution along with an immobilized shaved hind leg of the guinea pig which served as the reference surface. The animals were maintained immobile by the i.p. administration of 30–40 mg./Kg. pentobarbital.

The right eye of the guinea pig was used in the control study. The buffer solutions containing procaine were applied to the left eye. This order was reversed and alternated in subsequent experiments with the same animals which were allowed a minimum recovery time of 7 days. Animals observed to develop any abnormalities during this time were discontinued from further use.

The experimental procedure adopted for routine use involved a preliminary rinsing of the eye with isotonic NaCl solution to remove any secreted film on the cornea. This was followed by rinsing with the experimental solution to remove any solution remaining from a previous determination or treatment. Excess solution was removed by absorption into small strips of filter paper wick and gentle swabbing with dry cotton. A cotton dental pellet with a diameter less than the cornea was placed on the capillary end of a KCl-agar salt bridge and moistened with the experimental solution. The pellet was then carefully placed in contact with the cornea and the potential, E_1, was recorded for approximately 30 sec. The pellet was removed and the cornea swabbed lightly with dry cotton. A similar but smaller pellet was wetted with a 10-fold dilution of the previous solution and placed in the center of the area previously covered. A second potential, E_2, was recorded *immediately* upon contact. This procedure was repeated for all other pH values studied in a series. In developing this procedure to yield reproducible results, it was found necessary to carefully control the amount of solution applied to the cotton pellets and to prevent it from overflowing onto surrounding tissues by removing any excess onto filter paper wicks or cotton swabs. In more recent work the principal author has found that replacing the cotton pellets with solution wetted filter paper strips applied to the cornea with a micromanipulator, gave improved control and reproducibility.

The moist hydrated surface of the cornea allows diffusion and mixing of the solutions to readily occur at the surface causing initial changes in recorded potentials. The application of a procaine solution to a previously untreated cornea produces potentials (E_1) which approached relative constancy within approximately 15 sec. and remained at nearly the same value until the boundary was disturbed indicating the establishment of equilibrium at the surface. The application of diluted solutions may cause variations in recorded potential lasting up to several minutes, however in this case the initial value (E_2) is of interest. The effects of procaine were diminished or obliterated by rinsing the eye with isotonic NaCl or one of the control solutions indicating its effects are reversible.

RESULTS

Verification of Eq. 1—Equations for E_d and f specifically applicable to the solutions used in the present study were obtained by substituting appropriate values into Eqs. 1 and 2. The limiting ionic conductances of the various ions present were obtained from the literature (9, 13). The ionic conductance of procaine cation was experimentally determined by the measurement of liquid junction potentials (13). The accuracy of the resulting equations in describing the dilution potentials corresponding to each solution and its 10-fold dilution was experimentally checked by the measurement of diffusion potentials. Free diffusion-type boundaries were formed either within a capillary or by means of a filter paper bridge. The liquid junction potentials were found to be nearly inde-

[1] Supplied by Abbott Laboratories.
[2] Beckman fiber junction type calomel reference electrodes.

TABLE I—COMPARISON OF EXPERIMENTALLY OBSERVED DIFFUSION POTENTIALS TO VALUES CALCULATED FROM EQ. 1

pH of Solution	Diffusion Potential Calculated, mv.	Diffusion Potential Obs., mv.	pH of Solution	Diffusion Potential Calculated, mv.	Diffusion Potential Obs., mv.
	Buffer Solutions			Buffer Solutions Plus 1.0% Procaine HCl	
5.18	20.56	20.5 ± 0.6	5.18	8.26	9.0 ± 1.0
6.06	20.35	20.2 ± 0.4	6.06	8.51	9.9 ± 1.1
6.70	19.09	19.0 ± 0.8	6.84	9.29	10.6 ± 0.8
8.18	16.95	17.6 ± 0.2	7.58	10.12	10.8 ± 0.9
9.00	16.82	17.0 ± 0.5	8.15	10.21	10.8 ± 0.5
			9.00	9.53	11.1 ± 1.5

pendent of the method which was employed to form the boundary. The electrical potentials measured in this manner are of the same magnitude as the corresponding dilution potentials at zero density of fixed charge, *i.e.*, when the ratio r equals one. The averages of 10 separate determinations performed with each experimental solution are listed in columns 3 and 6 of Table I along with maximum deviations. The agreement of these results with calculated values, listed in columns 2 and 5 of Table 1, was found in most cases to be within a mv.

Results of Applying Eqs. 1 and 2 to Measurements on the Cornea—Values of r corresponding to experimental values of E_d were obtained by graphical solution of Eq. 1. Eleven calibration curves, corresponding to the 11 buffer solutions used, were constructed by plotting calculated values of E_d as a function of r over a chosen range. Values of r corresponding to experimentally observed values of E_d were obtained from these curves and checked by calculation. An average value of E_d, observed from a minimum of six different determinations using six different animals, was used in each case. These values of E_d and corresponding values of r are listed in Table II for both the control buffer solutions and the buffered procaine solutions. The corresponding values of f are listed in columns 4 and 8 in Table II. They were calculated by substituting the values of r into Eq. 2. Figure 3 contains plots of f as a function of pH. Although the control and

TABLE II—RESULTS OF ELECTROMETRIC DETERMINATIONS ON GUINEA PIG CORNEAL SURFACE

pH of Buffer Solutions	Av. Obs. Dilution Potential[a] (E_d), mv.	Ion Distribution Ratio[b] (r)	Fixed Charge Density on Corneal Surface[c] (f), meq./L.
	Control Buffer Solutions		
5.18	21.8 ± 0.9	1.020	−4.10
6.06	22.2 ± 1.0	1.035	−7.40
6.70	22.0 ± 1.5	1.054	−15.1
8.18	23.0 ± 1.7	1.095	−51.3
9.00	33.0 ± 1.3	1.300	−140.
	Buffered Procaine Solutions (1%)		
5.18	9.0 ± 1.0	1.013	−3.60
6.06	9.9 ± 1.1	1.023	−6.80
6.84	10.6 ± 0.8	1.022	−8.50
7.58	10.8 ± 0.9	1.010	−5.50
8.15	10.8 ± 0.5	1.010	−6.20
9.00	11.1 ± 1.5	1.025	−17.1

[a] E_d is the difference of two measured potentials, $E_2 - E_1$, see text. The deviations are the maximum observed differences from the mean of a minimum of 6 determinations of E_d using different animals. [b] The values of r are obtained from E_d by graphical solution of Eq. 1. [c] The values of f in columns 4 and 8 are calculated by substituting the corresponding values of r into Eq. 2.

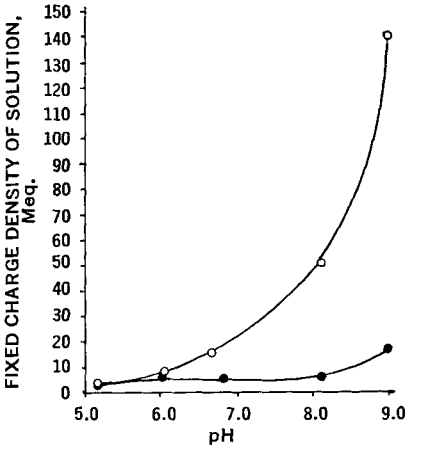

Fig. 3—Upper curve is the control titration curve of the guinea pig cornea; lower curve is the procaine displaced titration curve.

procaine displaced curves differ in shape, a negative net fixed charge density is common to each throughout the pH range studied. The isoelectric point (the pH below which the surface has a net positive charge) was found to be approximately 2.5. At normal physiological pH the anionic fixed charge density obtained from the control curve is 28 meq./L. of solution.

The procaine displaced curve lies entirely below the control curve indicating the anionic fixed charge density was decreased at every pH. The number of titratable groups is noted to be decreased by 90% in the presence of procaine. The changes at the corneal surface were generally found to be reversible as indicated by the fact that the shapes of the curves in Fig. 3 are independent of the order in which the corresponding E_d values are experimentally observed.

DISCUSSION

Interpretation of Titration Curves—The curves in Fig. 3 are analogous to titration curves observed for wool (14–15) and other insoluble proteinaceous structures (5, 6, 16, and 17). The net negative charge of the corneal surface that is apparent even at relatively low pH may in part be attributed to the strongly acidic sulfate groups of mucopolysaccharides (16). The titratable groups in the pH range of 5–9 may be assumed to be phosphatic, imidazolyl, imino, and α-amino groups. Carboxyl and sulfate groups are likely dissociated below pH 5 while ϵ-amino and phenolic groups are generally titratable above pH 9. Generally the tendency of

any group to dissociate is influenced by the composition of its medium and the state of the colloids as a whole (18, 19).

Mechanism of Procaine Effect—There are several mechanisms that could operate alone or in combination to bring about the lowering of anionic fixed charge density observed in the presence of procaine. Among them are a possible increase in the acidic pK's of the dissociable groups, an increase in the specific affinities of fixed anionic sites for binding the metal ions (Na^+ and K^+), the rupture of salt linkages between fixed groups of opposite charge followed by binding of mobile cations to the freed anionic groups, the absorption of procaine or other cations to uncharged sites on the colloids, and direct salt binding of procaine to fixed anionic sites. The complex nature of the possible interactions and the probable heterogeneity of the binding sites allows only speculation as to the relative contribution of each mechanism. However, lacking evidence to the contrary and considering that the two curves were obtained with solutions differing only in that one series contained procaine, it may be reasonably assumed that the lowering of the anionic charge density results primarily from the binding of procaine cations. Presumably the neutral species of procaine which is in equilibrium with the cationic form could also interact with the corneal surface. Its binding would not alter the net charge of the surface except perhaps indirectly by an inductive effect (18). The binding of the cationic species would be reinforced by long-range electrostatic forces which render its approach to binding sites increasingly favorable with an increasing density of negative fixed charge. Procaine cations taken up by the surface in a particular pH range need not necessarily bind to the anionic groups dissociating in that range. In fact, binding to uncharged sites on the surface would also lead to a stoichiometric decrease in anionic fixed charge density. That amine binding can occur is demonstrated by the fact that basic amino dyes such as toluidine blue are good tissue stains. Sawinski and Rapp (20) have shown that two binding sites are available for procaine on human serum albumin. Glassman (21) has concluded that long chain organic cations form a stoichiometric complex with plasma albumin. Steinhart and Zaiser (22) have also demonstrated binding of organic cations to wool and egg albumin.

In their studies of colloidal surfaces, Catchpole *et al.* (16, 17) have attributed the effect of cations on the fixed charge density of colloidal surfaces to a direct neutralization of fixed anionic groups due to salt binding. They also assumed a stoichiometric relation between the equivalents of bound cation and the observed decrease in negative fixed charge density. Adopting a similar approach to the treatment of the present results, the outermost surface of the cornea can be described to behave essentially as a cation exchanger having a variable affinity for procaine cations which is dependent upon the pH of the environment. Variations in the apparent affinities for association of procaine cations with the surface may be expected since the binding sites are probably dissimilar and thier intrinsic association affinities may be affected by the state of the colloids as a whole.

The overall equilibria pertinent to this model are shown below, where fHP, PH^+, and P refer to bound procaine, free procaine cations, and base, respectively, while f^- refers to the sites and fH to sites occupied by protons, H^+.

$$fHP \rightleftharpoons f^- + PH^+ \rightleftharpoons P + H^+ \quad \text{(Eq. 3)}$$

$$fH \rightleftharpoons f^- + H^+ \quad \text{(Eq. 4)}$$

In accordance with this scheme the extent to which procaine is bound and the accompanying decrease in fixed negative charge density depends upon the availability of anionic binding sites and procaine cations. Both factors depend upon pH. The increasing rate of lowering of fixed anionic charged density—evidenced by the widening vertical gap between the two curves in Fig. 3—with increasing pH, may in part be ascribed to the greater rate of availability of binding sites relative to the decrease in procaine cations occurring with pH.

The concentration of procaine bound to the corneal surface at each pH can be estimated from the assumed stoichiometric decrease in the negative charge density, Δf, which corresponds to the vertical difference between the two curves presented in Fig. 3. These values are listed in column 2 of Table III. The concentration of free procaine cations present at the corneal surface, $[P^+]_c$, can be calculated using the appropriate value of r and the concentration present in the applied solutions, $[P^+]_s$, *i.e.*, $[P^+]_c = r[P^+]_s$. Using these values apparent association constants, K_B, have been calculated from Eq. 5 where f' denotes the negative charge density taken from the lower, procaine, curve in Fig. 3. The values of K_B are listed in column 3 of Table III. As expected these values are not constant but increase with pH.

$$K_B = \frac{\Delta f}{f'[P^+]_s \cdot r} \quad \text{(Eq. 5)}$$

The volumes of the applied procaine solutions placed in contact with the cornea can be assumed to be relatively large in comparison to the volume of the corneal surface with which they equilibrate; the concentrations of the various components of the solutions can also be assumed to remain constant throughout the equilibration process. The total concentration of procaine occurring at the corneal surface, $[P]_t$, at each pH studied (taken as the sum of the concentrations of free procaine cation, free base, and bound procaine), can be estimated using Eq. 6.

$$[P]_r = r[P^+]_s + [P]_s \quad \text{(Eq. 6)}$$

TABLE III—CALCULATED RESULTS RELATING TO THE PROCAINE–CORNEAL SURFACE INTERACTION

pH of Solutions	Bound Procaine[a] (Δf), mmole/L.	Association Constant (K_b), L./mmole	Total Procaine Concentration at Corneal Surface, mmole/L.
5.18	0.4	3.0	37.6
6.06	0.6	3.0	38.1
6.84	9.5	30	46.9
7.58	27	140	64.0
8.15	44	231	81.0
9.00	123	461	133.

[a] Values of Δf corresponding to each pH listed were obtained from the vertical differences in the curves presented in Fig. 3.

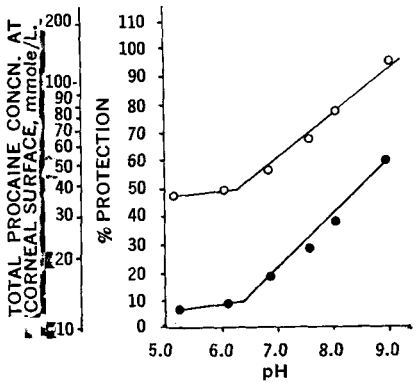

Fig. 4—Curve A represents the relationship of the logarithm of the total procaine concentration at the corneal surface to the pH of the applied solutions. Curve B represents the relationship of the duration of procaine-induced corneal anesthesia (plotted as percent protection) to the pH of the applied solutions.

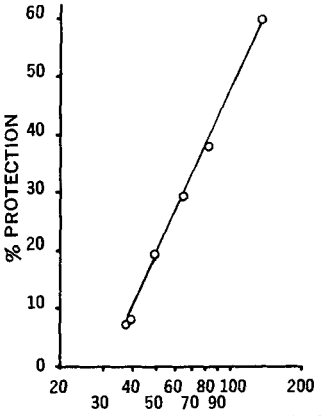

Fig. 5—Relationship of the logarithm of total procaine concentration at the corneal surface to duration of procaine-induced corneal anesthesia plotted as percent protection.

Since we are concerned with only the outermost surface of the cornea which resides in the same aqueous medium, the concentration of procaine free base at the surface can be assumed as equal to that in the bulk of the applied solutions $[P]_s$.

Values of $[P]_t$, listed in column 4 of Table III, show nearly a fourfold increase in the pH range of 5.18–9.0 arising primarily from increasing concentrations of bound procaine.

Relation of Procaine Binding to Duration of Anesthetic Effect—The procaine solutions used in the present study are similar to those used by Hiter in his study of the duration of procaine anesthesia induced upon the guinea pig cornea as a function of pH. His experimental approach was based upon a modified Chance and Lobestein technique (23). The procaine solutions were instilled into the eye and allowed to remain in contact with the cornea for 15 sec., a time found to be sufficient for the establishment of equilibrium between the procaine solution and the corneal surface. Excess solution was then removed from the cornea by the initiation of a blink reflex. The anesthetic effect was noted with a loss of the blink reflex when the cornea was stimulated at 1-min. intervals. The duration of the anesthetic effect was recorded as percent protection.[3]

The durations of anesthetic responses Hiter observed with 1% procaine solutions in the pH range of 5–9 are plotted in the lower curve in Fig. 4. The upper curve in Fig. 4 is a semilogarithmic plot of $[P]_t$ *versus* pH. The marked similarity in the two curves may be explained if the fact is considered that under conditions of constant pH, the duration of surface anesthesia in the eye is approximately a linear logarithmic function of the concentration of the local anesthestic in solution (1, 2). It may be expected that the duration of the anesthestic effect is also an approximately linear function of the logarithm of the concentration of the local anesthetic at the corneal surface itself. That this prediction is realized is evidenced by the curve shown in Fig. 5 where this relationship is demonstrated.

[3] The actual duration of the anesthetic effect was recorded in minutes, divided by 60 and multiplied by 100.

The treatment of the data which have been presented has in effect converted the pH dependency of the duration of anesthesia into a concentration dependency. The significance of this result lies in that it indicates that the duration of surface anesthesia can be related to and is dependent upon the total concentration of procaine estimated to be present at the corneal surface. Since the reversibly bound procaine is primarily responsible for the increasing total concentrations and is in equilibrium with free procaine, it may be assumed to act as a reservoir for the drug allowing it to be slowly released from its binding sites. By this means the bound procaine could serve in a manner analogous to continuous release dosage forms in prolonging the anesthetic response by maintaining threshold or suprathreshold levels of procaine at the biological sites of action for extended periods of time.

The role of lachrymal fluid in the onset and duration of the anesthetic response in relation to the effects of pH and buffer capacity (2) will be discussed in a future paper.

CONCLUSIONS

Although much of this treatment has been qualitative, it provides an example of the role that tissue structures may have in modulating their response to drugs. It also points out factors, in addition to the liberation of free base, which may be operating to increase the effectiveness of procaine in alkaline solution.

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Potential Anticonvulsant Agents

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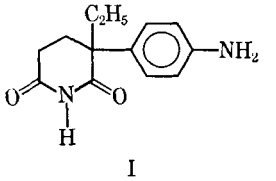

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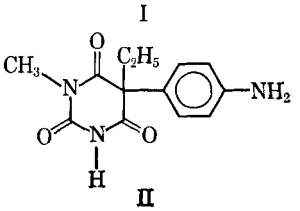

II

Received July 21, 1967, from the Department of Pharmaceutical Chemistry, University of Rhode Island, College of Pharmacy, Kingston, RI 02881
Accepted for publication October 6, 1967.
Abstracted in part from a thesis submitted by Harry Carl Clemson to the Graduate School, University of Rhode Island, in partial fulfillment of Doctor of Philosophy degree requirements.
Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
The authors express their sincere appreciation to Dr. A. J. Bej for his critical review of this manuscript.
This investigation was supported by NIH predoctoral research fellowship award, fellowship No. 5-F1-GM-19, 760.
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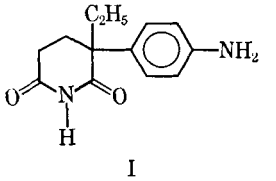

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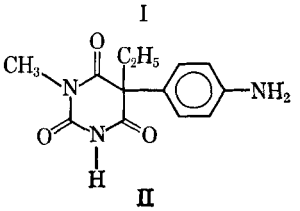

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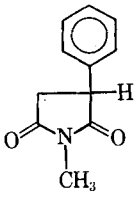
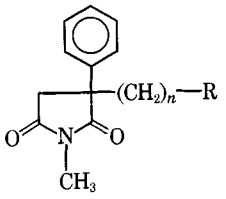
protective index (median toxic dose/median effective dose) than mephobarbital against electroshock seizures in mice. Mainly as a result of this apparent beneficial effect of an amino group on the anticonvulsant properties of these compounds, the authors were prompted to investigate the effect of more basic amino moieties on the anticonvulsant properties of phensuximide (III),



III

a known antiepileptic agent. Specifically, the interest was in introducing aminoalkyl groups into the 2-position of III. The resulting compounds would have the general structure IV, in which R may be either dialkylamino or heterocyclic amino groups and $n = 1$ or 2.



IV

Prior to the beginning of this project, substituted succinimides were prepared by the cyclization of appropriately substituted succinic acid intermediates with ammonia or a primary amine (3–13). The intent was to substitute aminoalkyl groups directly into the 2-position of III. Direct substitution into the ring appeared only recently in the literature (14), but this was not until several products had already been prepared.

DISCUSSION

Mannich Bases—It was envisioned that the synthesis of the alkylaminomethyl derivatives of *N*-methyl-2-phenylsuccinimide (IV, $n = 1$) may be accomplished by subjecting the parent compound (III) to Mannich conditions. A careful search of the literature revealed that there is no report of the successful application of the Mannich reaction to either *N*-substituted-2-phenylsuccinimides or *N,N*-disubstituted-2-phenylamides. Therefore, the authors became interested in establishing if, indeed, *N*-methyl-2-phenylsuccinimide (III) would undergo the Mannich reaction.

The most common solvent used in the Mannich reaction is ethanol. This solvent was initially employed, along with paraformaldehyde and dimethylamine hydrochloride under acidic conditions, in an attempt to prepare 2-dimethylaminomethyl-*N*-methyl-2-phenylsuccinimide hydrochloride (compound *1*, Table I). Several attempts under these conditions with various reaction times proved unsuccessful. Only starting material could be isolated. It was then decided to try higher boiling solvents which differ widely in their polarities. Four solvents were investigated: 2-butanol, glacial acetic acid, cyclohexanol, and toluene.

Substitution of 2-butanol for ethanol was also unrewarding. However, initial results were obtained when glacial acetic acid was used as the solvent; five Mannich bases were prepared: 2-dimethylaminomethyl - *N* - methyl - 2 - phenylsuccinimide hydrochloride (compound *1*, Table I), 2 - diethylaminomethyl - *N* - methyl - 2 - phenylsuccinimide hydrochloride (compound *2*, Table I), *N*-methyl-2-phenyl-2-(1-pyrrolidinomethyl) succinimide hydrochloride (compound *3*, Table I), *N*-methyl - 2 - (4 - morpholinomethyl) - 2 - phenylsuccinimide hydrochloride (compound *5*, Table I), and *N*-methyl-2-(4-methyl-1-piperazinomethyl)-2-phenylsuccinimide dihydrochloride (compound *7*, Table I). The yields, however, were generally poor.

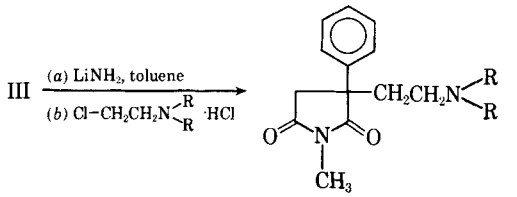
Several attempts to synthesize *N*-methyl-2-phenyl - 2 - (1 - piperidinomethyl)succinimide hydrochloride (compound *4*, Table I) using the glacial acetic acid method failed, but this compound was finally obtained in cyclohexanol.

N - Methyl - 2 - phenyl - 2 - (1 - pyrrolidinomethyl)succinimide hydrochloride (compound *3*, Table I), which was prepared previously in glacial acetic acid, was obtained in a significantly higher yield in cyclohexanol. The synthesis of 2-(3-azabicyclo[3.2.2]non - 3 - ylmethyl) - *N* - methyl - 2-phenylsuccinimide hydrochloride (compound *8*, Table I) was also accomplished in cyclohexanol. The use of 3-azabicyclo[3.2.2]nonane as the amine in the Mannich reaction has been reported only in recent years (15).

Four Mannich bases (compounds *2*, *4*, *6*, and *8*, Table I) were prepared in toluene. In every case but one, the yields were substantially increased over those obtained in glacial acetic acid or cyclohexanol.

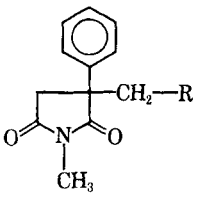
In reviewing the data, it was observed that in every case in which ethanol was used as the solvent, paraformaldehyde was the source of formaldehyde. Therefore, in order to ascertain if the source of formaldehyde might have made a difference, the synthesis of compound *2*, Table I, was again attempted in ethanol, but 36% formalin was substituted for paraformaldehyde. The product was obtained in a 49% yield which is significantly lower than the 67% yield obtained in toluene.

New Higher Homologs—The next higher homologs of the Mannich bases (IV, $n = 2$) were prepared according to the reaction shown in Scheme I.



Scheme I

TABLE I—MANNICH BASES OF *N*-METHYL-2-PHENYLSUCCINIMIDE



Compd.	R	Method	Yield, %	M.p., °C.	Formula	Anal. Calcd.	Anal. Found	% Protection[k] (Electro-shock)
1	—$N(CH_3)_2$·HCl	*A*	20	210–212 dec.	$C_{14}H_{19}ClN_2O_2$	C, 59.47 H, 6.77 N, 9.91 O, 11.32	C, 59.28 H, 6.63 N, 9.87 O, 11.50	60
2	—$N(C_2H_5)_2$·HCl	*A*[a] *B*[b, c] *D*	10 67 49	181–183 dec.	$C_{16}H_{23}ClN_2O_2$	C, 61.87 H, 7.40 N, 9.02 O, 10.30	C, 62.06 H, 7.34 N, 9.12 O, 10.40	30
3	—N⟨ ⟩·HCl	*A* *C*[d]	14 42	211–213 dec.	$C_{16}H_{21}ClN_2O_2$	C, 62.23 H, 6.85 N, 9.07 O, 10.36	C, 62.41 H, 6.89 N, 9.03 O, 10.39	0
4	—N⟨ ⟩·HCl	*B*[e, f] *C*[g]	56 34	188–189 dec.	$C_{17}H_{23}ClN_2O_2$	C, 63.25 H, 7.13 N, 8.68 O, 9.91	C, 63.38 H, 7.25 N, 8.68 O, 9.85	90
5	—N⟨ ⟩O·HCl	*A*	42	178–182 dec.	$C_{16}H_{21}ClN_2O_3$	C, 59.17 H, 6.52 N, 8.62 O, 14.78	C, 59.35 H, 6.42 N, 8.76 O, 14.87	0
6	—N⟨ ⟩O	*B*[h, f]	79	131–133	$C_{16}H_{20}N_2O_3$	C, 66.65 H, 6.99 N, 9.72 O, 16.64	C, 66.47 H, 6.90 N, 9.62 O, 16.72	...
7	—N⟨ ⟩N—CH_3 ·2HCl	*A*	18	210–235 dec. (sealed capillary)	$C_{17}H_{25}Cl_2N_3O_2$	C, 54.55 H, 6.73 N, 11.23 O, 8.55	C, 54.36 H, 6.58 N, 11.05 O, 9.13	66
8	—N⟨ ⟩·HCl	*B*[i, f] *C*[i]	16 16	183–184 dec.	$C_{20}H_{27}ClN_2O_2$	C, 66.19 H, 7.50 N, 7.72 O, 8.82	C, 66.11 H, 7.29 N, 7.63 O, 9.38	30

[a] The starting amine, diethylamine, was used in the form of its hydrochloride; total reaction time was 4 hr. [b] Recrystallized from 95% ethanol. [c] 36% formalin. [d] Total reflux time 2 hr. [e] Recrystallized from isopropanol. [f] 95% paraformaldehyde. [g] Total reflux time 24 hr. [h] Isolated as free base and recrystallized from 95% ethanol. [i] Recrystallized from isopropanol. [j] Total reflux time 18 hr. [k] Dose level = 200 mg./Kg; see body of paper for experimental protocol.

The addition of III to a suspension of commercial lithium amide in anhydrous toluene generated its lithium salt, which was allowed to react with the appropriately substituted β-chloroethylamine hydrochlorides. Since the starting amines were used in the form of their hydrochlorides, it was necessary to use at least two equivalents of the lithium amide. In subsequent preparations of some of the compounds, it was found that an increase in the number of equivalents of lithium amide used in the reaction produced an increase in the yields of the products. In specific cases the increases were quite significant (Table II).

EXPERIMENTAL

All melting points have been determined on either a Fisher-Johns block or the Kofler micro-hot stage and have been corrected. The infrared spectra were obtained using a Beckman IR-8 spectrophotometer. All imide derivatives displayed the two characteristic carbonyl stretching frequencies between 1775 and 1690 $cm.^{-1}$ (16–18). Also, the "ammonium bands" characteristic of tertiary amine hydrochlorides were present between 2700 and 2250 $cm.^{-1}$ (19).

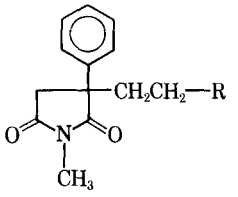
The elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn., and Micro-Analysis, Inc., Wilmington, Del.

All chemicals were obtained commercially unless specified otherwise.

Intermediates

2-Phenylsuccinic Acid—The preparation of this compound was according to the method of Allen *et al.* (20).

TABLE II—NEXT HIGHER HOMOLOGS OF MANNICH BASES



Compd.	R	Yield, % (Equiv. of LiNH2)		M.p., °C.	Formula	Anal. Calcd.	Anal. Found	% Protection[e] (Electroshock)
1	—N(CH3)2·HCl	39[a] (3.4)	...	198–200	$C_{15}H_{21}ClN_2O_2$	C, 60.71 H, 7.12 N, 9.44 O, 10.78	C, 60.69 H, 7.05 N, 9.55 O, 10.90	20
2	—N(C2H5)2·HCl	37[b] (2.5)	54 (3.7)	209–210	$C_{17}H_{25}ClN_2O_2$	C, 62.86 H, 7.76 N, 8.62 O, 9.85	C, 62.90 H, 8.03 N, 8.79 O, 9.90	50
3	—N⟨ ⟩·HCl	32[b] (2.6)	40 (3.7)	184–185 dec.	$C_{17}H_{23}ClN_2O_2$	C, 63.25 H, 7.18 N, 8.68 O, 9.91	C, 63.18 H, 7.23 N, 8.80 O, 9.90	70
4	—N⟨ ⟩·HCl	45[c] (2.4)	72 (5.1)	219–220 dec.	$C_{18}H_{25}ClN_2O_2$	C, 64.18 H, 7.48 N, 8.32 O, 9.50	C, 64.30 H, 7.58 N, 8.53 O, 9.55	44
5	—N⟨ O ⟩·HCl	38[d] (2.2)	41 (3.4)	215–218 dec.	$C_{17}H_{23}ClN_2O_3$	C, 60.26 H, 6.84 N, 8.27 O, 14.17	C, 60.52 H, 6.84 N, 8.29 O, 14.18	44
6	—N⟨ ⟩N—CH3·2HCl	12[a] (3.0)	...	262–266 dec.	$C_{18}H_{27}Cl_2N_3O_2$	C, 55.67 H, 7.01 N, 10.82 O, 8.24	C, 55.55 H, 7.05 N, 10.95 O, 8.36	44
7	—N⟨ ⟩·HCl	39[a] (2.0)	54 (5.4)	240–245 dec.	$C_{21}H_{29}ClN_2O_2$	C, 66.91 H, 7.76 N, 7.43 O, 8.49	C, 67.09 H, 7.81 N, 7.59 O, 8.41	11

[a] After addition of the amine hydrochloride, the reflux time was 24 hr. [b] After addition of the amine hydrochloride, the reflux time was 18 hr. [c] After addition of the amine hydrochloride, the reflux time was 37 hr. [d] After addition of the amine hydrochloride, the reflux time was 42 hr. [e] Dose level = 200 mg./Kg., except compound 4 (100 mg./Kg.); see body of paper for experimental protocol.

N-Methyl-2-phenylsuccinimide (III)—Made by the procedure of Miller and Long (4).

4-Methyl-1-(2-hydroxyethyl)piperazine—The following procedure is a modification of Ratouis, Boissier, and Dumont's method for the preparation of phenoxyalkylpiperazines (21). A mixture of 23.0 Gm. (0.230 mole) of *N*-methylpiperazine, 16.0 Gm. (0.200 mole) of 2-chloroethanol, 31.8 Gm. (0.230 mole) potassium carbonate, and 700 ml. of 2-butanol was heated to 100° for 15 hr. The mixture was allowed to cool to room temperature and filtered to remove the inorganic salts. The 2-butanol was then removed *in vacuo*. The residue, 4-methyl-1-(2-hydroxyethyl)piperazine, was used without further purification in the following procedure.

4-Methyl-1-(2-chloroethyl)piperazine Hydrochloride—The 4-methyl-1-(2-hydroxyethyl)piperazine, prepared by the procedure above, was treated according to the method of Billinghurst (22). That is, a chloroform solution of the alcohol was treated with thionyl chloride and the solid product was recrystallized from methanol to yield 22.4 Gm. (78%, based on 2-chloroethanol) of the title compound.

3-(2-Chloroethyl)-3-azabicyclo[3.2.2]nonane Hydrochloride—The procedure of Eastman Chemical Products, Inc., was modified in the preparation of this compound (23). To 31.2 Gm. (0.250 mole) of 3-azabicyclo[3.2.2]nonane was added, dropwise, 20.1 Gm. (0.250 mole) of 2-chloroethanol over a period of 20 min. After the addition was complete the reaction mixture was warmed on a steam bath. The entire reaction mixture formed a solid mass after 5 min. To this dark solid was added, with stirring, 150 ml. of a 10% solution of sodium hydroxide and 50 ml. of ether. The layers were separated and the basic aqueous solution was washed twice with 25-ml. portions of ether. The combined ethereal washings were dried over anhydrous mag-

nesium sulfate and filtered. The ether was removed *in vacuo* to give 30.1 Gm. (71.2%) of 3-(2-hydroxyethyl)-3-azabicyclo[3.2.2]nonane, which was used without further purification in the next step.

In 200 ml. of chloroform was dissolved 20.1 Gm. (0.178 mole) of the crude 3-(2-hydroxyethyl)-3-azabicyclo[3.2.2]nonane. To this solution was added slowly 30 ml. of thionyl chloride dissolved in 200 ml. of chloroform. The reaction was exothermic. After the addition was complete, the mixture was refluxed for 3 hr. and then allowed to stand at room temperature overnight. The solid was collected by filtration and recrystallized from absolute ethanol to yield 14.3 Gm. (35.8%) of 3-(2-chloroethyl) - 3 - azabicyclo[3.2.2]nonane hydrochloride, m.p. 214–215° dec.; lit. m.p. 198–225°.

Mannich Bases (Table I)

Method A—To 25 ml. of cold glacial acetic acid was added slowly, with stirring, 0.120 mole of the amine as a 40% aqueous solution (for an exception, see compound *2*, Table I). To this solution was added 10 ml. of 36% formalin (0.12 mole formaldehyde), followed by 18.9 Gm. (0.100 mole) of *N*-methyl-2-phenylsuccinimide. The mixture was heated on a steam bath for 1–2 hr.

The reaction mixture was cooled in an ice bath and made basic with 10% sodium hydroxide solution, and then extracted with chloroform. The chloroform extracts were dried over anhydrous magnesium sulfate and filtered, and the solvent was removed *in vacuo*. The residue was dissolved in anhydrous ether and filtered to remove any undissolved material. The ethereal solution was saturated with dry hydrogen chloride and the solid was collected by filtration, washed with anhydrous ether, and recrystallized from absolute ethanol.

Method B—A mixture of 0.10 mole of the amine, 18.9 Gm. (0.100 mole) of *N*-methyl-2-phenylsuccinimide, 60 ml. of toluene, and either 8.0 ml. of 36% formalin (2.9 Gm., 0.10 mole formaldehyde) or 3.4 Gm. (0.10 mole, calculated as formaldehyde) of 95% paraformaldehyde was heated at reflux for 20–24 hr. The volatile liquids were then removed *in vacuo*. An ethereal solution of the residue was dried over anhydrous magnesium sulfate, filtered, and saturated with dry hydrogen chloride. Sometimes a tacky material precipitated, which upon further addition of dry hydrogen chloride, with stirring, usually solidified to a white solid. However, if this treatment failed, then triturating the material with fresh anhydrous ether accomplished the same thing. The hydrochlorides were recrystallized from either 95% ethanol or isopropanol.

Method C—The amine (0.055 mole), 5.0 ml. (1.8 Gm., 0.060 mole formaldehyde) of 36% formalin and 9.4 Gm. (0.050 mole) of *N*-methyl-2-phenylsuccinimide were added to 50 ml. of cyclohexanol. The mixture was heated at reflux for several hours and then allowed to cool to room temperature.

The mixture was then dissolved in ether and the resulting solution was washed four times with 50-ml. portions of 10% hydrochloric acid solution. The combined acidic solutions were made basic with 10% sodium hydroxide solution, which usually precipitated an oil. The basic mixture was then extracted with ether, and the ethereal layer was dried over anhydrous magnesium sulfate, filtered, and saturated with dry hydrogen chloride. The solid was recrystallized from absolute ethanol. As in the previous method, occasionally a tacky material precipitated from the acidified ethereal solution. Treating the material in a manner similar to that in method *B* yielded an amorphous solid which then could be easily recrystallized from absolute ethanol.

Method D—Since only 2-diethylaminomethyl-*N*-methyl-2-phenylsuccinimide hydrochloride (compound *2*, Table I) was prepared by this method, the detailed procedure follows. To 3.7 Gm. (0.050 mole) of diethylamine were added 5.0 ml. (1.8 Gm., 0.060 mole formaldehyde) of 36% formalin, 9.4 Gm. (0.50 mole) of *N*-methyl-2-phenylsuccinimide, and 50 ml. of 95% ethanol. The solution was heated at reflux for 24 hr., and the volatile liquids were then removed *in vacuo*. An ethereal solution of the residue was dried over anhydrous magnesium sulfate, filtered, and saturated with dry hydrogen chloride. The solid was recrystallized from absolute ethanol to yield 2-diethylaminomethyl-*N*-methyl-2-phenylsuccinimide hydrochloride.

Next Higher Homologs (Table II)

To a suspension of lithium amide in 60 ml. of anhydrous toluene, under nitrogen, was added 4.7 Gm. (0.025 mole) of *N*-methyl-2-phenylsuccinimide. The mixture was heated at reflux for 5 hr., and then allowed to cool to room temperature. The amine hydrochloride (0.028 mole) was then added. After having been heated at reflux for several hours, the mixture was cooled in an ice bath and hydrolyzed with 20 ml. of water. The layers were separated and the organic layer was washed with water until the aqueous extracts were neutral. The toluene solution was dried over anhydrous magnesium sulfate, filtered, and the toluene removed *in vacuo*.

An ethereal solution of the residue was saturated with dry hydrogen chloride. If a tacky material precipitated, it was treated according to one of the procedures mentioned under *Method B*. The products were recrystallized from either absolute ethanol or isopropanol.

In some subsequent preparations it was found that if the number of equivalents of lithium amide were increased, the yields could also be increased.

PHARMACOLOGY

Pharmacological tests were conducted to determine if the tertiary amino derivatives of III were effective in abolishing the tonic hind leg extensor seizure of convulsions induced electrically and by pentylenetetrazol. A comparative pharmacological study was conducted on all the experimental compounds using male, albino, Charles River mice weighing 19–26 Gm. The mice were divided into groups of 10 and were fasted for 24 hr. before intraperitoneal injection of the test compound. All the compounds were soluble in water except compound *8* (Table I) which, like III, was suspended in a 7% solution of gum acacia. One hour after the compounds were administered, the mice were challenged with electroshock or pentylenetetrazol.

The electroschock procedure used in this study was a modification of the supramaximal seizure technique of Toman *et al.* (24). A 60-c. alternating current of fixed voltage was delivered for 0.2 sec. to each mouse through two attached ear clips. In order to screen the compounds for antipentylene-

tetrazol activity, 100 mg./Kg. of pentylenetetrazol was injected subcutaneously into each mouse. This dose is considered to be approximately twice the CD_{50} in untreated mice (25). In both tests, an animal was considered protected if the tonic extensor component of the seizure was abolished. The control animals, which were injected with 7% gum acacia or distilled water, showed 0% protection against electroshock and subcutaneous pentylenetetrazol.

All of the test compounds were evaluated at a dose level of 200 mg./Kg. with the exception of compound 4 (Table II, 100 mg./Kg.). However, none of the derivatives of III were shown to be effective in preventing the tonic hind leg extension produced by pentylenetetrazol. It was not possible to test higher doses of the experimental compounds since amounts greater than 200 mg./Kg. produced toxic symptoms.

The results from the electroshock testing demonstrated that several of the analogs were more protective against electroshock than was the parent compound (III). At a dose level of 200 mg./Kg., III protected 20% of the test animals against electroshock. The electroshock data appear in Tables I and II.

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Keyphrases

N-Methyl-2-phenylsuccinimide derivatives–synthesis
Mannich reaction
Lithium amide–higher homolog synthesis
IR spectrophotometry–structure
Anticonvulsive activity against electroshock, pentylenetetrazol

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show it to be a relatively slow but effective sterilizing agent (6–9). Other workers have found PMN to be less than effective in the case of fluorescein. Anderson *et al.* (10) found that 0.002% PMN failed to sterilize fluorescein drops after several days contact, but 0.004% PMN achieved sterility within a day. These workers did not use contact times of less than a day and the recovery media did not contain inactivators.

EDTA enhanced the activity of chlorhexidine, benzalkonium, and polymyxin against *Ps. aeruginosa* growing both in serum and broth (11). Furthermore, acquired resistance by this organism to a quaternary ammonium compound was eliminated using EDTA (12, 13).

The present work was done to obtain quantitative information about survival of *Ps. aeruginosa* in fluorescein and other aqueous solutions and to study the preservative properties of 0.1% EDTA (13) used in combination with PMN.

EXPERIMENTAL

Ps. aeruginosa NCTC[1] 6750, NCTC 8203, and NCTC 7244 were used as test bacteria. The growth medium was oxoid[2] nutrient broth No. 1 and incubation was at 37°. B.D.H.[3] fluorescein sodium (microscopical quality) was used. Other chemicals were Analar quality (B.D.H.). Cell numbers in broth cultures were estimated by measuring the absorbance at 420 mμ with a Unicam 600 spectrophotometer and by colony counts using broth as a diluent. Experimental details have been described previously (14).

Survival in Solutions Without Preservative

Survival experiments were made using screw-capped 20-ml. and 100-ml. glass bottles in a water bath at 22°. Inocula were added to fluorescein sodium solution 2% in distilled water (Fig. 1). Colony counts were made at intervals during the first day, then daily, then weekly, until a consistent pattern of results occurred. Representative suspensions were then counted at intervals for about a year. Samples were examined microscopically for cell clumping. An identical control procedure was carried out, adding inocula to distilled water (Fig. 2), to nutrient broth (Fig. 2), and to tris buffer pH 8.6 (pH of fluorescein solution) (Fig. 3). A total of about 50 suspensions was stored.

Preparation of Inocula—A technique similar to that described previously (14) was used to obtain a reproducible growth cycle in nutrient broth. Twenty-four hour cultures were used. The original culture was diluted with fresh broth (culture OR) so that the storage liquids contained initially about 10^3 cells/ml. and duplicates contained 10^6/ml. In addition, the contribution of the original growth medium to survival was assessed. This was done by using inocula washed by centrifugation (3 times) with either fresh broth (culture WB) or 0.2 *M* NaCl then water (culture WW). Preliminary experiments showed that the volume of broth added as part of the cell inoculum contributed to the survival of *Ps. aeruginosa* in the various storage liquids. Preliminary work also suggested that the size of container was significant. Consequently, inocula

[1] National Collection of Type Cultures, Colindale, London, England.
[2] Oxo Ltd., London, England.
[3] B.D.H., Poole, Dorset, England.

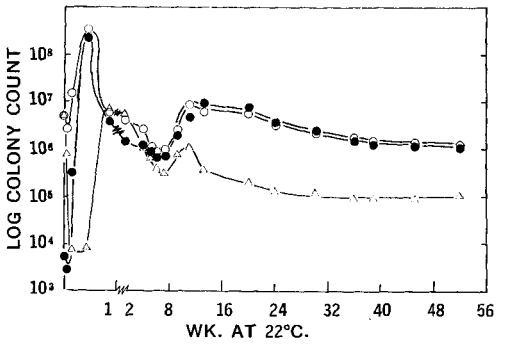

Fig. 1—Survival of Ps. aeruginosa *in fluorescein solution. Key: ●, 10 ml. broth inoculum in 90 ml. fluorescein solution (10^3/ml.); ○, 10 ml. broth inoculum in 90 ml. fluorescein solution (10^6/ml.); △, 0.5 ml. broth inoculum in 99.5 ml. fluorescein solution (10^6/ml.).*

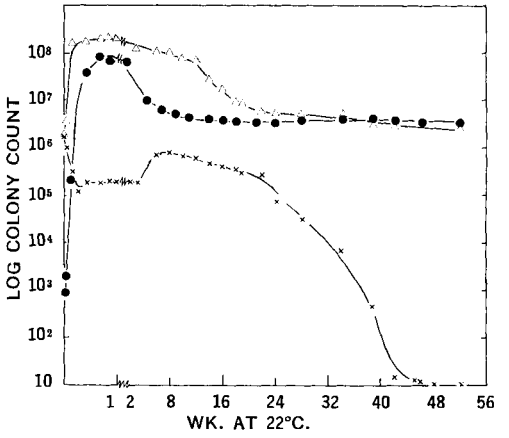

Fig. 2—Survival of Ps. aeruginosa *in water and broth. Key: ×, 0.5 ml. broth inoculum in 99.5 ml. water, (10^6/ml.); ●, 10 ml. inoculum washed in fresh broth and added to 90 ml. water (10^3/ml.); △, 0.5 ml. broth inoculum in 99.5 ml. broth (10^6/ml.).*

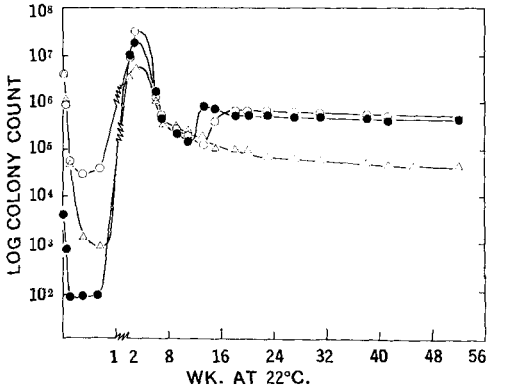

Fig. 3—Survival of Ps. aeruginosa *in tris buffer (pH 8.6). Key: ●, 10 ml. broth inoculum in 90 ml. tris buffer (10^3/ml.); ○, 10 ml. broth inoculum in 90 ml. tris buffer (10^6/ml.); △, 0.5 ml. broth inoculum in 99.5 ml. tris buffer (10^6/ml.).*

TABLE I—SURVIVAL TIMES FOR *Ps. aeruginosa* IN SOLUTIONS PRESERVED WITH PMN AND EDTA

Soln.	Organism Strain (NCTC)	Replicate[a]	Contact Time: 45 min.	90 min.	3 hr.	5 hr.	1 Day	2 Days	7 Days
Fluorescein + 0.002% PMN	6750	1	+	+	−	−	−	−	−
		2	+	+	−	−	−	−	−
	8203	1	+	+	−	−	−	−	−
		2	+	+	−	−	−	−	−
	7244	1	+	+	−	−	−	−	−
		2	+	+	−	−	−	−	−
Fluorescein + 0.002% PMN + 0.1% EDTA	6750	1	+	+	+	−	−	−	−
		2	+	+	+	−	−	−	−
	8203	1	+	+	+	−	−	−	−
		2	+	+	+	−	−	−	−
	7244	1	+	+	−	−	−	−	−
		2	+	+	−	−	−	−	−
Fluorescein + 0.004% PMN	6750	1	+	+	−	−	−	−	−
		2	+	+	−	−	−	−	−
	8203	1	+	+	+	−	−	−	−
		2	+	+	+	−	−	−	−
	7244	1	+	+	+	−	−	−	−
		2	+	+	−	−	−	−	−
Fluorescein + 0.004% PMN + 0.1% EDTA	6750	1	+	+	−	−	−	−	−
		2	+	+	−	−	−	−	−
	8203	1	+	+	+	−	−	−	−
		2	+	+	+	−	−	−	−
	7244	1	+	+	−	−	−	−	−
		2	+	+	−	−	−	−	−
0.002% PMN	6750	1	+	+	+	+	−	−	−
		2	+	+	+	−	−	−	−
	8203	1	+	+	+	−	−	−	−
		2	+	+	+	+	−	−	−
	7244	1	+	+	+	−	−	−	−
		2	+	+	+	−	−	−	−
0.002% PMN + 0.1% EDTA	6750	1	+	+	+	+	−	−	−
		2	+	+	+	+	+	−	−
	8203	1	+	+	+	+	−	−	−
		2	+	+	+	+	+	−	−
	7244	1	+	+	+	+	+	+	−
		2	+	+	+	+	+	−	−
0.004% PMN	6750	1	+	+	+	−	−	−	−
		2	+	+	+	−	−	−	−
	8203	1	+	+	+	−	−	−	−
		2	+	+	+	−	−	−	−
	7244	1	+	+	+	−	−	−	−
		2	+	+	+	−	−	−	−
0.004% PMN + 0.1% EDTA	6750	1	+	+	+	+	+	−	−
		2	+	+	+	+	+	−	−
	8203	1	+	+	+	+	+	−	−
		2	+	+	+	+	+	−	−
	7244	1	+	+	+	+	−	−	−
		2	+	+	+	+	+	−	−

[a] +, −, visible growth or not after 1-week incubation in recovery broth. Duplicate controls separately containing 2% fluorescein, 0.1% EDTA, and recovery broth alone were also inoculated and all gave growth.

consisted of 1 ml. added to 99 ml. storage liquid (*A*); 1 ml. to 9 ml. (*B*), in 20-ml. bottles; 10 ml. to 90 ml. (*C*); 0.5 ml. to 99.5 ml. (*D*). Inoculation was always made to give both 10^3 cells/ml. and 10^6/ml. approximately.

Storage Liquids—*Sodium Fluorescein Solution, 2%*—Inoculum OR was used to give suspensions *B*, *C*, and *D*; WB to give *B*, *C*, and *D*; WW to give *A* and *B* (Fig. 1).

Distilled Water—Inoculum OR gave suspensions *B* and *D*; WB gave *B*, *C*, and *D* (Fig. 2).

Nutrient Broth—Inoculum OR gave suspensions *A* and *D* (Fig. 2).

Tris Buffer—Inoculum OR gave suspensions *B*, *C*, and *D* (Fig. 3). Storage was at 22°.

Survival in Preserved Solutions

One-half milliliter broth inocula of all three test strains to a final concentration of about 10^6 cells/ml. were added to various combinations of fluorescein, EDTA, and PMN in 20-ml. solutions in a water bath at 22° (Table I). One-half milliliter samples were taken at various time intervals, added to 10 ml. recovery broth, and incubated at 37° for 7 days. As a control procedure, samples from all positive tubes were incubated on a milk agar medium (10% dried, defatted milk,[4] and 1.5% agar) and examined for pigment and clearing of the casein.

Recovery Broth—The recovery broth of Kohn *et al.* (9) for *Ps. aeruginosa* after contact with PMN was used, with *M*/372 $CaCl_2 \cdot 6H_2O$ added to inactivate EDTA (0.1% equivalent to *M*/372). The broth was composed of: 0.3 Gm. lecithin, 3 Gm. polysorbate 80,[5] 2.93 Gm. dehydrated thioglycollate medium USP, 59 mg. $CaCl_2 \cdot 6H_2O$ (autoclaved separately), water to 100 ml. This medium was selected after preliminary experiments using colony counts with the basic lecithin–polysorbate–thiogly-

[4] Marvel milk granules, Cadbury Bros. Ltd., Bournville, England.

[5] Tween 80. Atlas Chemical Industries, Inc., Wilmington, Del.

collate medium in the presence and absence of $M/372$, $M/37$, and $M/3.7$ $CaCl_2 \cdot 6H_2O$.

RESULTS AND DISCUSSION

Solutions Without Preservative

Fluorescein Solution—Unwashed broth inocula of *Ps. aeruginosa* added to fluorescein solution rapidly dropped in count when diluted 100-fold or more (Fig. 1, open triangle). The count rose again after this initial drop and remained relatively constant. This pattern was the same for inocula of 10^6/ml. and 10^3/ml. The initial inhibitory effect of fluorescein solution was eliminated when the inoculum was in a sufficiently large volume of broth (Fig. 1, open and closed circles). The larger volume of broth protected the cells from the initial drop in colony count and also allowed the count to rise and be maintained at a higher level for inocula of both 10^6/ml. and 10^3/ml. The results from inocula washed in fresh broth (not illustrated) were similar to those with unwashed cells and showed that the washing process with broth had no significant effect upon subsequent survival in fluorescein solution. Inocula of cells washed in water showed rapid death and were sterile in less than a day.

Water, Broth, and Tris Buffer—The pattern of survival of *Ps. aeruginosa* in water was similar to that in fluorescein and representative results are given in Fig. 2. Inocula with the greater amounts of broth were protected in comparison to those with less, and viability was maintained at higher levels. The effect was similar for both 10^6/ml. and 10^3/ml. inocula. Figure 2, times sign, shows that 0.5 ml. broth inoculum added to 99.5 ml. water retained a relatively constant count for about 6 months; this dropped until the storage liquid was sterile after about 1 year. An inoculum of 10 ml. broth added to 90 ml. water initially showed a rise in count to about 10^8/ml. This dropped for about 2 months when a count of about 4×10^6/ml. was maintained for the rest of the experimental period. A similar pattern occurred with cells washed in fresh broth (Fig. 2, closed circle). The pattern for broth as a storage liquid (Fig. 2, open triangle) was similar to that of water with the high broth inoculum. All inocula into tris buffer showed an initial drop in count (Fig. 3). Inocula with the greater amounts of broth (Fig. 3, open and closed circles) were protected against initial death and ultimately viability was maintained at higher levels when compared to results with small broth inocula.

Broth exerted a protective effect in all the storage liquids and also supported growth. The protection and growth were apparently related to the amount of broth present. The size of cell inoculum and size of storage container apparently had no influence on survival. All three strains gave similar results.

The magnitude of the initial drop in count (about 99.9%) when broth inocula was diluted 100-fold in fluorescein solution, tris buffer (pH 8.6), and water was unexpected. This result, together with that using water-washed inocula (sterilized within a day) shows that the kind of inoculum used in studies with *Ps. aeruginosa* is crucial. It would seem that washing in water has a damaging effect upon *Ps. aeruginosa* and that the use of water-washed cell inocula may give misleading results in antibacterial studies.

In practice it is likely that contamination from *Ps. aeruginosa* would be from cells present with nutrient material. The results from this study show that aqueous vehicles will support viability of large populations of this organism for periods of a year or more. Inocula maintained viability to about the same extent in fluorescein solution, tris buffer, and water.

Preserved Solutions

PMN alone was consistently effective in sterilizing inocula of about 10^6 cells/ml. in fluorescein solution (Table I). There was little difference between the effects of 0.004% and 0.002% PMN, and sterility was achieved with all strains between 1.5 and 5 hr. Kohn *et al.* (9) obtained closely similar results. In the absence of fluorescein, sterility was also achieved within several hours using PMN alone in water. It might be anticipated that a high molecular weight anion such as fluorescein might react with a cation such as the phenylmercuric ion. This possibility is not excluded by the results which, nevertheless, show PMN to be effective within the experimental limits.

In every instance except one (0.004% PMN) solutions with EDTA took longer to achieve sterility than those without it. This apparent antagonism may be due to EDTA chelating the mercury, making it less available for antibacterial action. These results confirm the efficacy of PMN as a relatively slow acting preservative againt *Ps. aeruginosa* and suggest that EDTA exerts a slight antagonistic action in combination with PMN in the test circumstances.

It is questionable whether this preservative effect of PMN is a sufficient protection, especially for drops used with damaged eyes. The difficulty of preserving eye drops against *Ps. aeruginosa* is well known (4) and the present work shows the dangers of long-term contamination of inadequately preserved drops including fluorescein. The use of sterile, single-dose units for damaged eyes or, in the case of fluorescein, sterile, impregnated paper strips would seem to reduce this hazard.

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Keyphrases

Fluorescein solution
Pseudomonas aeruginosa–fluorescein solution
Cells, *P. aeruginosa*–water, broth washed
Phenylmercuric nitrate–preservative activity
EDTA effect–phenylmercuric nitrate activity

Mechanism of Action of Starch as a Tablet Disintegrant III

Factors Affecting Starch Grain Damage and Their Effect on Swelling of Starch Grains and Disintegration of Tablets at 37°

By JAMES T. INGRAM and WERNER LOWENTHAL

A study was undertaken to determine the effect of the tableting procedure on starch grain swelling and tablet disintegration. The swelling of damaged starch grains in simulated gastric fluid USP is demonstrated. Submersion of starch samples that had been ball milled from 10 to 48 hr. produced increases in grain diameters of 40 to 80 percent. Damage to the grains resulting from compression of pure cornstarch is shown to be insignificant. The effect of compressional force and hardness of tablet ingredients was examined and in each formulation the starch grain damage increased with compressional force. There was no correlation between degree of starch grain damage and hardness of the tablet ingredients. It appeared that the crystalline form of the ingredient may have exerted a greater influence on starch damage than the hardness. There was no correlation between starch damage and stress produced by elastic recovery of the tablet. Contrary to what might be expected, there was an inverse relation between the amount of stress and the degree of starch grain damage for all of the formulations except aspirin. There was no evident relationship demonstrated between disintegration time and starch grain damage. Outside of compressional force, the inherent effect of the tablet ingredient was the only factor that appeared to affect disintegration. The long-accepted swelling mechanism of starch as a tablet disintegrant was not demonstrated in this study. The results of the investigation revealed no measurable correlations between starch grain damage and disintegration or between starch swelling and compressional force.

FROM THE PREVIOUS STUDIES, it does not seem probable that untreated or unmodified starch will swell sufficiently at 37° to cause tablet disintegration (1, 2). If swelling is the primary mechanism whereby starch exerts its disintegrant effect, then this property may be imparted to the starch by the tableting procedure.

Since swelling of the grains may occur reversibly or irreversibly, the effect of each type of swelling on disintegration should be considered. It is known that the starch grains have a limited capacity for absorbing cold water and swelling reversibly (3). When a suspension of starch is subjected to heat or appropriate chemical treatment, the micellar network within the grain is weakened by disruption of hydrogen bonds, and irreversible swelling, or gelatinization, occurs. Irreversible swelling results in partial solubilization of the starch molecules and formation of an adhesive paste. This adhesiveness should tend to hinder rather than promote disintegration. This hindrance was observed by submersing an aspirin tablet containing 10% cornstarch in hot water, which will cause starch to gelatinize. The tablet would not disintegrate and examination of the tablet revealed that the interior was essentially dry. This could indicate formation of a starch paste "sealer" coat around the exterior of the tablet, preventing penetration of water into the tablet.

Cooper and Brecht (4) also suggested starch gelatinization as a possible explanation for the hindrance to disintegration observed when starch is incorporated into a sodium salicylate tablet. Sodium salicylate in aqueous solution causes starch to gelatinize.

The detrimental effect of irreversible swelling on disintegration is further supported by the observation that any substantial amount of pregelatinized starch (*e.g.*, 5%) in the tablet formulation hinders disintegration (5).

From this it would appear that only reversible swelling could exert a favorable effect on disintegration. The literature indicated that reversible swelling results in 5 to 10% increases in grain diameters (5). Assuming that starch grains are spheres, a 10% diameter increase corresponds to a 33.1% volume increase. This volume increase has no relation to tablet disintegration until it can be correlated to the increase it would produce in the tablet volume. The maximum proportion of starch normally used in tablet formulations is 10% by weight. The percent of the total tablet volume occupied by this amount of starch will depend on the density of the other in-

Received July 28, 1967, from the School of Pharmacy, Medical College of Virginia, Richmond, VA 23219
Accepted for publication October 4, 1967.
Abstracted in part from the thesis presented by James T. Ingram to the Graduate School, Medical College of Virginia, in partial fulfillment of Master of Science degree requirements.

gredients. The range for densities of tablet formulations is usually from 0.5 to 2.5 Gm./ml. The percent increase in tablet volume (%V) corresponding to a 33.1% starch volume increase may be calculated by:

$$\%V = 0.331 \times 0.1 \times \frac{d_t}{d_s} \times 100$$

where d_t is the density of the tablet formulation, d_s is the density of starch, and the two constants, 0.331 and 0.1, refer to the 33.1% starch volume increase and the 10% by weight starch in the formulation. Based on these conditions, a starch volume increase of 33.1% should produce a tablet volume increase of only 1.1 to 5.5%. Since most tablets probably contain more than 5.5% void space, it seems unlikely that starch grains would produce sufficient strain within the tablet to promote disintegration.

It is known that untreated starch is insoluble in cold water but may absorb 25 to 30% of its weight in water with no appreciable swelling (6). Sjostrom (7) states that if a starch grain is mechanically damaged it will gelatinize in cold water.

A possible explanation for swelling occurring in starch grains incorporated into a tablet is in a report on the compressional behavior of potato starch. Fuhrer (8) reported that the grains are deformed plastically under the pressure and suggested that at a suitable degree of deformation the stability of the grains is lowered so that they may swell in water at room temperature.

EXPERIMENTAL

Since no substantial evidence was found by changing the chemical environment to support the hypothesis that starch effects tablet disintegration through swelling, this study was planned to demonstrate the effect of damage on starch grain swelling and to determine the influence of the tableting procedure and tablet ingredients on starch grain damage and tablet disintegration.

Since the nature of the tablet constituents may affect swelling, the addition of an ingredient to cornstarch was studied. The following general formula was used for the tablet formulations:

Active ingredient	10.00 Gm.
Cornstarch[1]	0.75 Gm.

The materials chosen as the ingredient in the formulations were aspirin, talc, calcium carbonate, calamine, sodium chloride, and sucrose. Aspirin was selected because of its widespread use and because of its use in previous disintegration studies. Talc, calcium carbonate, and calamine were selected to include materials of varying hardness, because hardness may influence the degree of damage imposed on the starch grains during tableting. Mohs' hardness scale values for these three materials are: talc, 1; calcium carbonate, 3; and calamine, 5 (9). Sucrose and sodium chloride were included to determine the effect of water-soluble substances and their crystalline structure on starch damage. Tablets of pure cornstarch were included as controls.

The force of compression used in the tableting procedure influences void space, disintegration time, and may influence starch grain damage. These factors were studied and comparisons among them were made to determine what significance could be attributed to each factor.

The extent of elastic recovery after compression for each of the formulations and for pure cornstarch was investigated. The greater the elastic recovery of the tablet after release of pressure, the greater is the stress within the tablet. This stress might affect starch grain damage.

Procedure for Preparation and Examination of Tablets—The tablet formulations were mixed in a mortar and then compressed as a powder in a Carver laboratory press model B[2] using standard, flat-faced, stainless steel punches and die of $^3/_8$ in. diameter.

The die and lower punch were fixed at a definite position throughout the experiment and the powder was manually placed in the die and leveled with the top. This procedure gave uniform weights for the tablets from a particular formulation.

Tablets of each formulation were prepared using varying compressional forces. The individual tablets were measured for thickness and diameter with a vernier caliper and weighed. Utilizing the tablet dimensions and weights, the percent void space (% e) was calculated by:

$$\%\ e = \left(1 - \frac{d_b}{d_t}\right) \times 100$$

where

$$d_b = \frac{W}{V}$$

and

$$d_t = \frac{10.75\ \text{Gm.}}{10.00\ \text{Gm.}/d_i + 0.75\ \text{Gm.}/d_s}$$

where d_b and d_t are the bulk density and true density of the formulation, d_i is the density of the ingredient (10, 11), d_s is the density of starch, W is the weight of the tablet in Gm., and V is the volume of the tablet in cm.^{3}.

Procedure for Determination of Disintegration Time—The disintegration time for each tablet in simulated gastric fluid USP (SGF) at 37° was determined using a modification of the USP disintegration apparatus, so that only one tablet was disintegrated at a time and the time required for all the granules to pass through the 10-mesh screen determined. The disintegration times reported represent an average of five determinations.

Procedure for Production and Determination of Starch Grain Damage—Sandstedt and Mattern (12) state that starch grains are easily damaged by pressure, shear, or strain such as that applied by grinding procedures. Damage to the grains was effected by ball milling in a 1-quart porcelain ball mill using 0.5-in. diameter porcelain balls and a single tier roller mill.[3] Samples were removed at various time intervals and the extent of damage to the starch grains was determined.

[1] Marketed as Melojel (MJ) by National Starch and Chemical Corp.

[2] Fred S. Carver, Inc., Hydraulic Equipment, Summit, N.J.

[3] Phipps and Bird, Inc., catalog No. 62-502.

A qualitative method suggested by Schoch (5) consisted of suspending the starch in water and examining the grains under a polarizing microscope. Schoch states that severe damage gives grains which swell in cold water and hence show no polarization cross. Lesser degrees of damage result in a darkened area at the center of the grain when viewed under normal light.

If the damage is severe enough that the paracrystalline structure of the grain is disrupted, the unswollen grain should also lose its polarization cross. Lesser degrees of damage resulting in less disruption of the paracrystalline nature of the grain should allow passage of proportional amounts of polarized light. In order to eliminate the possibility that reversible swelling in water may affect the polarization characteristics of starch, glycerin was used as the slurrying medium, since starch does not swell in glycerin (13).

The percent damage of the various samples removed from the ball mill was calculated from observation of the polarization crosses of 200 grain. Each grain was assigned a weighted degree of damage, dependent on the number of quadrants within the polarization cross which presented a darkened appearance. For example, if the polarization cross contained two quadrants which exhibited a darkened appearance, the grain was assigned a degree of damage of 0.5. Since it was not possible to determine the absolute extent of a darkened area within the cross, these percentages reflect only a relative degree of damage, which is useful for comparison of samples.

The effect of pressure on starch swelling was determined from tablets of pure cornstarch (MJ). The tablets were prepared on a Carver press utilizing various compressional forces. The tablets were slurried in SGF and in glycerin, and swelling was determined on 100 grains' diameters from each sample, using the procedure described previously (1).

Procedure for Determination of Starch Grain Damage in Tablets—The starch grain damage produced by tableting of the different formulations was determined by examination of 200 grains under the polarizing microscope using the procedure described previously. Two tablets of each formulation prepared at each pressure were used for preparation of the slurries. The procedure for preparation of the slurries was modified, because the starch as a tablet constituent was not available for direct slurrying. The aspirin tablets were slurried in absolute ethyl alcohol, which dissolved the aspirin. The alcohol was decanted and the residual starch grains were slurried in glycerin. Talc, calamine, sucrose, and sodium chloride tablets were disrupted in absolute ethyl alcohol to release the starch for preparation of slurries. Glacial acetic acid was used to effect release of starch from calcium carbonate tablets. Glycerin, absolute ethyl alcohol, and glacial acetic acid were used because these liquids have no effect on starch swelling.

Procedure for Determination of Elastic Recovery—The elastic recovery after compression for each formulation was determined on a Carver press. The powder was placed in the die and the distance the upper punch moved to produce each pressure was measured by dial micrometers attached to the upper punch.

After the maximum pressure had been applied and released, the micrometer reading for the tablet at zero was determined. The tablet thickness was measured with a vernier caliper immediately after removal of the tablet from the die in order to exclude any creep recovery. The sum of these two values minus the micrometer reading at each pressure gave the tablet thickness under pressure. By comparison of the final tablet thickness with the tablet thickness while under pressure, the elastic recovery could be determined.

The elastic recovery for the pure cornstarch tablets was determined by the same procedure at each pressure level studied, instead of only at the maximum pressure applied.

TABLE I—EFFECT OF STARCH GRAIN DAMAGE AND TIME ON MEAN GRAIN SIZES OF CORNSTARCH (MJ) IN SIMULATED GASTRIC FLUID

Ball Mill Time, hr.	% Damage	Time in SGF, min. 0	5	30
0	1.1	6.13[a]	6.41	6.22
1	15.8	5.43	7.08	6.87
2	14.8	5.61	6.27	6.25
3	18.0	5.58	7.18	6.44
4	17.1	5.74	7.58	7.17
5	18.4	5.95	6.64	7.28
10	41.6	5.59	7.10	8.07
15	44.7	6.50	7.79	7.48
20	50.8	6.52	7.53	7.79
36	67.6	7.18	8.37	8.77
48	82.8	6.91	12.24	12.53

[a] Mean grain sizes in scale divisions, one scale division = 1.8 μ.

RESULTS AND DISCUSSION

Investigation of Relationships Among Compressional Force, Starch Grain Damage, and Starch Swelling—Since the compressional force may damage the starch grains, the effect of damage on swelling of starch grains was first studied. Cornstarch (MJ) grains were damaged by ball milling and samples were removed after various time intervals up to 48 hr. and the degree of damage determined by observation of 200 grains under the polarizing microscope. The samples were also examined for the effect of damage on swelling in SGF in a 3 × 12 full factorial experiment. The damage determinations and the mean grain sizes are shown in Table I. The analysis of variance showed that the degree of damage and submersion time both produced significant differences in grain diameters at the 1% level. Duncan's multiple range test (14) for the time effect shows that the grain diameters of unsubmerged starch are significantly different from those of submerged starch, with no difference being demonstrated between the two submersion times. Duncan's multiple range test for the damage effect shows a significant difference between the samples ball

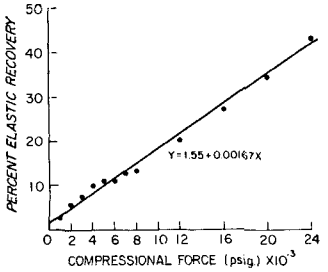

Fig. 1—Effect of compressional force on percent elastic recovery of cornstarch (MJ).

TABLE II—EFFECT OF PRESSURE ON PERCENT STARCH GRAIN DAMAGE

Pressure, psig	Ingredient					
	Aspirin	Calcium Carbonate	Calamine	Talc	NaCl	Sucrose
2000	12.8[a]	16.5	13.9	8.0	31.5	22.4
4000	17.4	28.5	18.8	12.1	59.4	50.4
6000	16.9	35.0	26.1	14.0	56.6	53.8
8000	19.2	38.1	27.5	15.1	68.1	56.3

[a] Percent damage.

TABLE III—EFFECT OF PRESSURE ON PERCENT VOID SPACE

Pressure, psig	Ingredient					
	Aspirin	Calcium Carbonate	Calamine	Talc	NaCl	Sucrose
2000	2.3[a]	38.2	6.2	17.7	7.6	12.6
4000	0.3	29.9	1.6	15.9	3.8	9.6
6000	0.1	27.8	0	13.9	2.9	6.5
8000	0	24.0	0	10.5	3.4	8.8

[a] Percent void space.

milled for 48 hr. and the samples ball milled for 36 hr. or less. This means more than two-thirds of the starch grains had to be damaged before there was a statistically significant increase in swelling.

To determine the extent of swelling produced by various compressional forces, a 3 × 8 full factorial experiment was designed. Cornstarch (MJ) tablets were prepared on a Carver press using 0.5-in. flat-faced punches and mean grain sizes determined in SGF after 5 and 10 min. submersion. The mean grain sizes of the starch grains were determined from a glycerin slurry to include unsubmerged starch (zero time) as a basis for comparison. Eight pressures between 0 and 5000 psig were investigated. The analysis of variance of the mean grain sizes revealed no significant differences among the variables investigated.

Using 0.5-in. diameter punches, the maximum compressional force used in the previous experiment (5000 psig) corresponds to an actual pressure of 25,480 p.s.i. Because the actual pressures produced in tableting may vary over a wide range depending on the punch sizes that are used, cornstarch samples subjected to an extremely high compressional force were investigated for evidence of damage. Tablets of cornstarch were prepared on a Carver press utilizing $^3/_8$-in. diameter flat-faced punches and compressional forces of 500 and 24,000 psig, which correspond to actual pressures of 4530 and 217,440 p.s.i., respectively. The percent starch grain damage as determined from glycerin slurries, were 7.8% for the tablet prepared at 500 psig and 5.5% for the tablet prepared at 24,000 psig.

The results of the elastic recovery determinations for cornstarch are shown in Fig. 1. Cornstarch exhibited a high degree of elastic recovery when subjected to high compressional forces. The strain produced by this elastic recovery showed no apparent correlation to starch grain damage as indicated by the above data.

From the observations of strain in starch tablets after subjected to pressure and the extent of starch grain damage produced by pressure, along with the observations in the experiment on pressure and swelling, it seems that starch grains are not significantly damaged by the forces involved during and after compression.

Effect of Tableting Procedure on Starch Properties and Tablet Disintegration—Since starch grain damage resulting from compression of cornstarch alone was shown to be insignificant and when a high percent of starch grains are damaged there is an increase in swelling, the combined effect of other tablet ingredients and compression on starch grains was investigated. If these variables could be related to starch grain damage and hence swelling, this could be considered indirect evidence for a swelling mechanism for tablet disintegration.

Effect of the Hardness of an Added Ingredient and Compressional Force on Tablet Properties—To determine how the added ingredient and force of compression affect such properties as disintegration time and void space in the tablet and damage to the starch grains, tablets were compressed over a pressure range of 2000 to 8000 psig. In each formulation the percent void space decreased[4] and the degree of starch grain damage increased with compressional force. The ingredient apparently had no effect on the proportionality of these relationships, but only on the degree to which they occur. Variation in the degree of starch damage among the different formulations is shown in Table II and variation in void space is shown in Table III.

Damage varied from 8.0 to 15.1% for talc to 31.5 to 68.1% for sodium chloride. The ingredients did not produce correspondingly greater degrees of damage as hardness increased. Calamine, with a hardness of 5, produced a maximum starch grain damage of 27.5%; whereas calcium carbonate, with a hardness of 3, produced a maximum damage of 38.1%. The crystalline form or the brittleness of the ingredient may exert a greater influence on starch grain damage than hardness, as illustrated by the damage produced by sodium chloride. The void space percentages in the different formulations ranged from 0 to 2.3% for aspirin tablets to 24.0 to 38.2% for calcium carbonate tablets.

The results of the elastic recovery determinations for the different formulations after compression at 24,000 psig were: sodium chloride, 15.9%; sucrose, 19.5%; aspirin, 34.6%; calcium carbonate, 70.4%; calamine, 76.2%; and talc, 77.7%. There is no apparent correlation between the strain produced

[4] Void space in the sodium chloride and the sucrose tablets decreased with increasing pressure up to 6000 psig and then anomalously increased at 8000 psig. Outside of experimental error, no explanation could be found for this behavior and this increase is disregarded in forming generalizations on compressional effects. Fusion of some of the crystals at the higher pressures and subsequent recrystallization after removal of pressure might effect a void space increase, if expansion of the tablet occurs during recrystallization.

TABLE IV—EFFECT OF PRESSURE ON DISINTEGRATION TIME

Pressure, psig	Ingredient					
	Aspirin	Calcium Carbonate	Calamine	Talc	NaCl	Sucrose
2000	16.5[a]	25.6[a]	5.6[a]	>8[b]	9.4[a]	92.6[a]
4000	30.9	17.2	5.7	>8	15.7	351.2
6000	60.5	9.0	5.0	>8	18.2	440.4
8000	51.5	9.6	4.7	>8	19.4	427.6

[a] Time in sec. [b] Time in hr.

by elastic recovery and the extent of damage to the starch grains. Surprisingly, the results indicated an inverse relationship between strain and starch grain damage for all of the formulations except aspirin.

The relationship between compressional force and disintegration time varied with the ingredients. Disintegration times for the aspirin, sodium chloride, and sucrose tablets increased with increasing compressional force; whereas disintegration times for the calcium carbonate and the calamine tablets decreased with increasing compressional force. These relationships are shown by the data in Table IV. Since the disintegration times for the talc tablets compressed at all pressures were greater than 8 hr., no correlations for this formulation could be made.

As shown above, the relationships between disintegration time and void space or starch grain damage varied and no correlations could be made. The ingredients appear to be the main source of the variation.

Disintegration times for the aspirin, sodium chloride, and sucrose tablets increased with increasing starch grain damage and decreasing void space. Disintegration times for the calcium carbonate and the calamine tablets decreased with increasing starch grain damage and decreasing void space.

It had been previously demonstrated that the logarithm of disintegration time varies directly with compressional force (15). This study revealed a direct relationship between pressure and dissolution time for the tablet formulations containing sodium chloride and sucrose. It is questionable whether the small decreases in void spaces found justify the large increases in dissolution time. The extensive starch grain damage noted would indicate an increased swelling ability for the starch grains. This was not borne out with the two soluble tablet formulations. The increased damage and hence swelling ability of the starch grains may have actually exerted a detrimental effect on dissolution by formation of a paste which then would hinder penetration of SGF into the tablet.

The tablet formulations containing insoluble ingredients varied in the relationships between pressure and disintegration time. The greatest damage range observed in this group was in the calcium carbonate tablets. Starch grain damage in this formulation ranged from 16.5% to 38.1%. The disintegration times for calcium carbonate tablets decreased from 25.6 to 9.6 sec., indicating that the swelling may have exerted a favorable influence on disintegration. The large void space in this formulation, however, could overshadow any effect on disintegration produced by the increased swelling capability of the damaged starch grains. Also, the results from the ball milling experiment indicated that greater than 67% of the starch grains must be damaged before a significant increase in swelling is observed.

The range of disintegration times for calamine tablets was too small to allow any significant correlations to disintegration time.

The results of this investigation disclosed no correlation between starch grain damage and tablet disintegration. Outside of pressure, the inherent effect of the added ingredient was the only factor that appears to affect tablet disintegration. Starch grain damage, void space, and hardness of the ingredients are at best of secondary importance.

Effect of Compressional Force on Aspirin Tablet Properties—In order to obtain more information on the effect of compressional force, aspirin tablets prepared over a pressure range of 1000 to 24,000 psig were examined for relationships existing among disintegration time, void space, and damage to the starch grains.

The inverse relationship between compressional force and void space observed in the previous study is again obvious over the wider pressure range. Figure 2 shows that void space decreased rapidly with increases in pressure up to 3000 psig. Above 3000 psig the void space decreased slowly up to 7000 psig, at which point zero percent void space was attained.

No relationship between compressional force and degree of starch grain damage was observed over the pressure range studied. Damage varied from 9.4 to 19.2%, but did not consistently increase as the pressure increased.

The increase in disintegration time with increasing

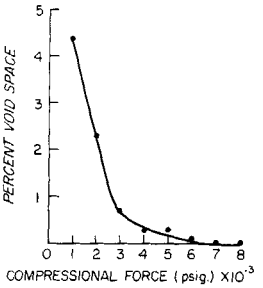

Fig. 2—Influence of compressional force on percent void space in aspirin tablets.

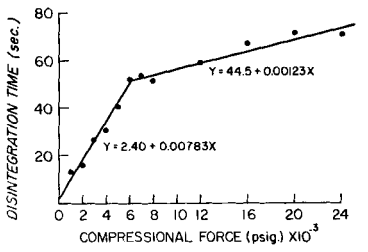

Fig. 3—Influence of compressional force on disintegration time of aspirin tablets.

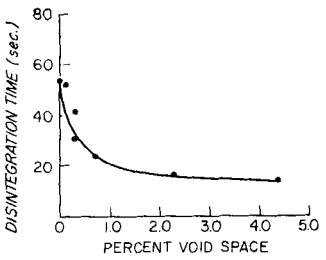

Fig. 4—Relationship between disintegration time and percent void space in aspirin tablets.

compressional force for the aspirin tablets is shown in Fig. 3. Disintegration times increased linearly with compressional forces between 1000 and 6000 psig. Tablets produced with greater than 6000 psig pressure also had disintegration times that were linearly dependent on compressional force; however, the disintegration rate was slower.

Disintegration times increased slowly with decreasing void space until 0.7% void space (3000 psig) was attained. Below 0.7% void space disintegration times increased rapidly until zero percent void space was reached at 6000–7000 psig. After zero percent void space was reached, disintegration times continued to increase with pressure. It appears, at least with aspirin, that the disintegration time is little affected until a certain value at about 0.7% void space is attained, and that further application of pressure affects disintegration greatly. Figure 4 illustrates the relationship between void space and disintegration time in aspirin tablets.

Since there was no correlation between compressional force and degree of damage to the starch grains, the percent damage could not be related to disintegration. Damaged starch grains do not appear to play a significant role in disintegration of aspirin tablets. The tablet that had the shortest disintegration time produced a starch grain damage of only 11.9%.

Since compaction pressure produced by impact due to the rapid action of a tableting machine might effect greater damage than that produced in the slower, hand-operated Carver press, aspirin tablets prepared on a Colton model 321 tablet press[5] were examined for evidence of starch grain damage. Observation of 200 grains showed only 2.6% damage. Disintegration time for these tablets was 15.4 sec. This again indicates that starch grain damage apparently has no significant effect on disintegration.

SUMMARY AND CONCLUSIONS

A procedure for estimation of starch grain damage is described. Swelling of damaged starch grains in SGF at 37° was demonstrated by experiments with cornstarch damaged by ball milling.

Damage to the grains, resulting from compression of pure cornstarch at pressures up to 24,000 psig was shown to be not significant.

The effect of compressional force and hardness of the tablet ingredients on starch grain damage was examined together with the relationships among compressional force, elastic recovery, void space, and disintegration time for the formulations.

In each formulation the starch grain damage increased as the compressional force was increased. The ingredient apparently had no effect on the direct proportionality of this relationship, but only on the degree to which it occurs. There was no correlation between degree of starch grain damage and hardness of the ingredient. The crystalline form of the ingredient may exert a greater influence on starch damage than hardness of the ingredient. This was illustrated by the sodium chloride and sucrose formulations, which produced the greatest degree of starch damage. There was no correlation between starch damage and stress produced by elastic recovery. Contrary to what might be expected, there was an inverse relation between the amount of stress and the degree of starch grain damage for all of the formulations except aspirin.

There was no evident relationship demonstrated between disintegration time and starch grain damage. Dissolution times for sucrose and sodium chloride tablets increased as the degree of starch grain damage increased. Disintegration times for the calcium carbonate tablets decreased with increasing starch damage, but the large void space in this formulation could overshadow any effect of swelling due to damage. Outside of compressional force, the inherent effect of the tablet ingredient was the only factor that appeared to affect disintegration, although starch grain swelling cannot be eliminated as a possible mechanism.

Most compressed tablets are composed of more than one ingredient and require several steps in manufacture. Ingredients commonly used in tablets represent a wide range of chemical and physical properties. Thus, it is possible that disintegration is influenced by a wide variety of factors which are specific for only a given tablet formulation and it is difficult to determine the mechanism of action of starch as a tablet disintegrant. The long-accepted swelling mechanism of action of starch as a tablet disintegrant could not be demonstrated in this study. The results of the investigation revealed no measurable correlations between starch grain damage and disintegration or between starch grain swelling and compressional force.

The proposals of Patel and Hopponen (16) and Nogami *et al.* (17) on the effect of capillaries, starch wettability, and liquid penetration into tablets become more substantial.

[5] Arthur Colton Co., Cedar Rapids, Iowa.

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Keyphrases

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Tablet disintegration–starch grain damage
Crystalline substance–starch grain damage
Compression force–starch grain damage
Starch swelling–tablet disintegration

Metabolic Fate of Orally Administered 2,3,5-Triiodobenzoic Acid in Lactating Animals

By RODNEY D. ICE*†, JOHN E. CHRISTIAN*, and MILLARD P. PLUMLEE**

The whole body retention, excretion, distribution, thyroid uptake, and metabolism of 2(^{131}I),3,5-triiodobenzoic acid (TI*BA) were studied in goats and a cow. A single oral dose of TIBA exhibited a two-component whole body radioactivity retention curve and was excreted primarily in the urine. TIBA plus nine metabolites, four of which were identified, were found in the urine. The major metabolite was 2,5-diiodobenzoic acid (2,5-DIBA). Trace amounts of 2,3-diiodobenzoic acid (2,3-DIBA), orthoiodobenzoic acid (OIBA), and iodide ion were found. TIBA was metabolized by deiodination. Iodide ion was concentrated in the thyroid and excreted by way of milk and urine.

In soybeans, 2,3,5-triiodobenzoic acid (TIBA) affects plant morphology and flowering response (1, 2). When properly used, soybean production is increased through a better utilization of photosynthate, lodging is decreased, and a more compact plant results (3). Spitznagle (4) has reported a residue in soybeans treated with TIBA. Soybean products are used for animal and human food consumption and the question arises as to the environmental health safety of TIBA. The metabolic fate of TIBA in lactating animals is important to a thorough understanding of the potential hazards.

Ice *et al.* (5) reviewed TIBA and using TIBA labeled with ^{131}I in the two position, orally administered to rats, observed two whole body retention components—one with a biological half-life of 11.8–17.9 hr. and the second of 395–403 hr. Of the administered dose, 70–78% was excreted in the urine, while 3–4% was excreted in the feces over 4 days. Three metabolites plus TIBA and iodide ion were located in the urine. Whole body retention of radioactivity was evident. Distribution studies in rats indicated a marked thyroid uptake of radioactivity.

Ebert and Ware (3) and Barker *et al.* (6) using carboxyl labeled TIBA-^{14}C orally administered to rats, found TIBA and/or its metabolites in all organs analyzed. Seventy-five percent of radioactivity was excreted *via* the urine and 29% *via* the feces. Chromatographic studies indicated two to six metabolites plus TIBA in the urine.

Gutenmann *et al.* (7) found no TIBA in the milk or feces of the dairy cow during a period of 4 days. In urine, 13.5% of the dose excreted was TIBA. A second compound in the urine was identified as 2,5-DIBA and accounted for 53.5% of the TIBA fed.

EXPERIMENTAL

Instrumental Methods—Whole body radioactivity was measured in goats using large volume liquid scintillation counting techniques. The basic mechanisms and characteristics of the 2 π liquid scintillation detector have been previously reported (8). The detector has since been modified by the addition of an upper detecting tank, which allows 4 π geometry. Dosages were adjusted to provide negligible coincidence loss.

A 3-in. diameter standard NaI(Tl) crystal, centrally located in a steel vault, was used for counting liter samples of excreta and tissue. A 3-in. diameter NaI(Tl) well crystal was used to determine the radioactivity in 3-ml. samples of tissue, to count eluent fractions from chromatography columns, to

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quantitate metabolites scraped from thick-layer chromatograms, and to count milk samples. Thyroid uptake was determined using a standard 2-in. diameter NaI(Tl) crystal in a collimator, DS-5.[1] Each crystal was connected to appropriate electronics and used in a differential mode. All radioactivity determinations were corrected for background, counter efficiency, and decay as required.

Gel filtration chromatography columns were made by placing a slurry of prewashed 100 Gm. (dry weight) Sephadex G-15[2] into a glass column 122 cm. long and 2.2 cm. i.d. A pledget of glass wool was used at the base and top of the dextran gel bed. Characteristics of the column were: bed length 32 cm.; void volume 135 ml.; and flow rate 0.4 ml./min.; deionized water was the eluent. Five-milliliter fractions were collected in a fractionator[3] with volumetric attachment. Sample size placed on the column was 5 ml.

The thick-layer (1.0-mm.) chromatographic and autoradiographic procedures used were similar to those reported earlier (5). The adsorbent was changed to 85 Gm. of MN-Kieselgel G-HR/UV[4] mixed with 137 ml. of deionized water to form a slurry capable of coating 5 plates each 20 × 20 cm. The solvent systems used were: (*a*) petroleum ether–propionic acid, (10:1, v/v) (3); (*b*) *n*-butanol–acetic acid–water, (4:1:1, v/v) (5); (*c*) ethyl acetate–methanol–2 *N* ammonium hydroxide, (100:40:60, v/v, upper phase) (9); (*d*) ethyl acetate–methanol–0.2 *N* acetic acid (100:40:60, v/v upper phase) (9). Unlabeled standard compounds were detected visually under ultraviolet light (254 mμ).

Radioactive TIBA was synthesized and purified as previously reported (5) immediately prior to experimental use. Radiochemical purity was established by thin-layer chromatography in each solvent system followed by autoradiography.

A precison level of experimental work was established so that an error greater than 5% (at the 95% confidence level) would not be accepted. The mean standard error and the Pearson product-moment correlation coefficient were calculated according to the method outlined by Downie (10).

Whole Body Retention—Five adult female milk goats in the 8–9 month of lactation were used. The average daily milk volume was 300 ml. per goat. The goats weighed 39.5–60.4 Kg. and were maintained on a daily lactation ration consisting of 1.9 Kg. of commercial goat chow and legume hay *ad libitum*.

Prior to dosing, a whole body radioactivity measurement was made on each goat to determine the quantity of natural radioactivity appearing in the differential counting window used. This base level of natural radioactivity was subtracted from whole body determinations made after dosing.

Four of the goats were each given a single dose of 0.1 mg. TI*BA, representing 0.3 μCi. The doses were prepared by pipeting 10 μl. of an alcoholic solution of TI*BA into a No. 000 gelatin capsule and allowing the alcohol to evaporate. Each capsule administered with a balling gun to avoid mastication. Thirty minutes were allowed to elapse before the first whole body radioactivity determination was made. For each determination, the individual goat was immobilized in a steel container (11) without tranquilizers or anesthesia. Twelve 1-min. radioactivity determinations were made on each goat at each time interval. To prevent counting the capsule as a point source, the first net count rate 30 min. after dosing was assigned 100% retention. To measure any possible cross external contamination from excreta, a fifth goat was used as a control. This animal received similar treatment as the four other goats with the exception that a placebo was administered.

Excretion—Five adult goats in the third month of lactation weighing 47.8–62.3 Kg. were acclimatized to metabolism cages (12) for 2 days prior to experimentation. Each goat was catheterized with a Bardex Foley catheter with Bard inflation valve[5] (size 16 French, 5-ml. balloon) and the urinary bladder drained. The goat was milked dry and then given a dose of 5.0 mg. TI*BA representing 5.4 μCi. After dosing, all urine, feces, and milk were recovered at 6 hr., and then at 12-hr. intervals thereafter up to 114 hr. All excreta were weighed and samples 1 L. size taken by weight for radioactivity determinations.

The procedure was repeated using a 3-year old Holstein cow weighing 477 Kg. The cow had calved for the first time 5 months earlier. During the study, the cow was maintained on a daily lactation ration of 18.2 Kg. corn silage, 11.4 Kg. of a 16% protein concentrate, and 2.3 Kg. hay. The single dose to the cow was 1.197 Gm. of TI*BA representing 55.92 mCi. The dose, in a No. 12 veterinary capsule, was administered to the cow with a balling gun. Average daily milk volume was 17.5 Kg. After dosing, all excreta were recovered and weighed at 6 hr. and then at 12-hr. intervals up to 162 hr. One-liter samples by weight were taken for radioactivity determination. Total residue determinations were made on the feces according to "Standard Methods" (13).

Thyroid Uptake—During the excretion study a concomitant determination of thyroid uptake was made on the same five lactating goats dosed with TI*BA. The collimated NaI(Tl) crystal was centered 10 cm. from the thyroid gland of each goat and two radioactivity measurements were made every 12 hr. through 210 hr. after administration of TI*BA. The procedure was repeated on the flank of each goat.

Distribution—Four adult lactating goats weighing 51.4–75.0 Kg. were used. Each goat was milked dry, catheterized, and the urinary bladder drained as in the excretion study. The catheter was then sealed. Each goat was given a single dose of 150 mg. of TI*BA containing 5.0 mCi. Eight hours after dosing, the catheter was reopened, the bladder drained, and the goat milked dry. The goat was stunned by a sharp blow on the head and sacrificed by bleeding from a severed jugular vein. A 1-L. blood sample was collected. The goat was dissected, with the kidneys, thyroid, liver, spleen, brain, heart, and gastrointestinal tract being removed. Lean and fat tissue were obtained from the hind quarter and inner surface of the loin, respectively.

[1] Nuclear-Chicago, Des Plaines, Ill.
[2] Pharmacia Fine Chemicals Inc., Piscataway, N. J.
[3] Gilson Medical Electronics, Middleton, Wis.
[4] Macherey, Nagel and Company, Duren, Germany.
[5] Davol Rubber Company, Providence, R. I.

The gastrointestinal (G.I.) tract was emptied of its contents, weighed, and mixed uniformly. One-liter samples of fat, liver, lean, blood, and G.I. tract were taken by wet weight. The heart, spleen, kidney, and brain were weighed and frozen. Two samples of frozen tissue were removed, weighed, and placed in counting vials. The thyroid gland was weighed, minced, and a 3-Gm. sample taken. Radioactivity measurements were made on all samples and compared to a standard of similar geometry with known specific activity. The percent of the administered dose of TIBA and its metabolites was determined for each organ. Also calculated was the percent of dose per kilogram of organ tissue. A normalized concentration was determined by setting the percent per kilogram of blood equal to 100.0 and correcting all other concentrations to this normalized value.

Metabolism—Accumulated 8-hr. urine, milk, and the thyroid gland from the goats and accumulated 6–18 hr. urine and milk from the cow were analyzed for metabolites. Gel filtration columns and thick (1.0-mm.) layer chromatograms were used. Five-milliliter samples of urine, treated[6-8] urine, skim milk, treated skim milk,[9] and digested thyroid tissue[10] were separated on gel filtration columns.

The identification of TIBA and iodide ion in the column eluent was accomplished by adding TI*BA and iodide-^{131}I ion as internal standards to the urine and milk samples placed on the column. The eluent fractions under each peak and duplicated fractions that had been acidified were forced air evaporated to 0.5 ml. and spotted on thick-layer chromatograms in aliquots of 50 μl. Also chromatographed were treated[6-8] urine samples, skim milk pancreatin,[9] digested thyroid tissue,[10] standard solutions of suspected metabolites, whole urine, and skim milk. A chromatogram was developed in each solvent system. Each chromatogram was autoradiographed 16 days, and the position of metabolites compared to appropriate standards.

Milk Analyses—The skim portion of the milk was obtained by centrifuging 10 ml. of whole milk for 10 min. at 4500 r.p.m. The cream layer was penetrated with a 5-in., 20-gauge needle attached to a syringe and the skim milk portion removed. The partition of radioactivity in cream and skim milk was determined by comparing the relative concentrations of radioactivity in whole milk and skim milk.

Protein bound iodide in milk was determined by adding 5 ml. of 20% trichloroacetic acid (TCA) to 5 ml. of whole milk, stirring, and centrifuging 10 min. at 4500 r.p.m. The nonprotein bound fraction (supernatant) was removed. The protein fraction was treated similarly two more times with 5 ml. of TCA. The nonprotein fractions were combined and the quantity of nonprotein bound radioactivity was compared with the radioactivity of whole milk.

[6] Acidified urine. Urine was acidified to pH 3 using 1 *N* HCl, shaken, and allowed to stand 24 hr.

[7] Alkaline urine. Urine was made alkaline to pH 11 using 1 *N* NaOH, shaken, and allowed to stand 24 hr.

[8] Urine–beta glucuronidase. Ten milliliters of urine and acetate buffer solution pH 4.5 were mixed with 100,000 u. (454 mg.) beta-glucuronidase (Type B-1, Bovine Liver, Sigma Chemical Co., St. Louis, Mo.). The solution was stirred, shaken, and incubated at 37° for 48 hr. The resultant suspension was centrifuged 10 min. at 4500 r.p.m. and 5 ml. of supernatant taken as the sample.

[9] Skim milk–pancreatin. An aliquot of whole milk was added to pancreatin (20 mg./ml.), shaken, and incubated at 37° for 48 hr. The resultant suspension was centrifuged 10 min. at 4500 r.p.m. and a 5-ml. supernatant was taken as the sample.

[10] Digested thyroid tissue. To a cold finger condenser was added 2 Gm. of minced thyroid tissue, 1 ml. of 0.1% thiourea solution, and 10 ml. of 1 *N* NaOH. The condenser was air evacuated, sealed, and heated on a steam bath 4 hr. The digested tissue was centrifuged and 5 ml. of supernatant used for the sample.

RESULTS AND DISCUSSION

The results of the whole body retention study are shown in Table I. The data indicated at least 2 biological half-life components, represented by the equation

$$Y = 19.9e^{(-0.0164)(X \text{ hr.})} + 71.8e^{(-0.0512)(X \text{ hr.})},$$

where Y is the percent retained at X hours. A long biological half-life component of 423 hr. was indicated and had a correlation coefficient of $r = 0.87$. A short biological half-life component of 13.5 hr. was found and had a correlation coefficient of $r = 0.99$. The derived whole body retention equation assumes milk production to be invariant at 300 ml. daily.

The results of the excretion study are shown in Table II. During the acclimatization period prior to experimentation, total excretion records were maintained. Total excreta before and during the study were compared and no significant difference was seen in the quantity of milk, urine, or feces produced due to the TIBA treatment.

Urinary excretion was the primary route of excretion accounting for 76.1% of the administered radioactivity in goats after 114 hr. Milk and fecal excretion accounted for 12 and 2.7%, respectively. Using the derived whole-body retention equation, the accountable dose was 107.4% of the administered dose.

The whole body retention equation was derived from goats with an average daily milk production of 300 ml. In the excretion study, however, the average daily milk production per goat was markedly higher with 1930 ml. With increased milk production during the excretion study, there was increased excretion by the mammary gland at the expense of whole body retention. Weaver (14) has shown that iodide ion excretion in milk varies directly as does the quantity of milk produced. In the cow, 57.5% of the administered dose was excreted in the urine, 12.2% in the milk, and 11.4% in the feces.

Thyroid and flank uptake studies of TIBA

TABLE I—PERCENT WHOLE BODY RETENTION OF ORALLY ADMINISTERED TI*BA IN LACTATING GOATS

Time, hr.	Mean Retention,[a] %	Mean S. E.
0[b]	100.0[c]	0.0
8	76.3	12.5
16	47.8	9.9
24	40.5	8.0
32	30.3	7.2
40	27.2	8.1
52	22.9	7.2
64	20.6	6.9
76	19.6	6.4
88	18.0	6.5
112	16.8	6.0
136	15.7	6.0
160	15.4	5.7
184	14.8	5.4

[a] Percent of administered dose calculated as equivalent TIBA. [b] Thirty min. after dosing. [c] Arbitrarily assigned 100.0%.

TABLE II—PERCENT EXCRETION OF TIBA AND ITS METABOLITES FOLLOWING ORAL ADMINISTRATION OF TI*BA IN LACTATING ANIMALS

	5 Goats[a]						Cow[b]			
	Urine		Milk		Feces					
Time, hr.	Mean %	Mean S. E.	Mean %	Mean S. E.	Mean %	Mean S. E.	Urine %	Milk %	Feces %	Fecal Solids %
6	29.2	5.2	2.0	1.0	...[c]	...[c]	8.2	0.6	...[c]	16.4
18	33.1	0.6	3.1	1.3	0.4	0.1	25.7	2.6	0.8	20.0
30	8.2	1.3	2.6	1.0	0.6	0.1	9.2	2.1	3.1	19.6
42	2.9	0.9	1.6	0.4	0.6	0.2	7.4	1.8	2.2	22.3
54	1.0	0.3	1.1	0.4	0.3	0.2	2.5	1.4	1.7	20.3
66	0.7	0.4	0.6	0.1	0.3	0.1	1.5	1.1	1.3	20.2
78	0.3	0.2	0.4	0.1	0.2	0.1	1.0	0.8	0.7	26.2
90	0.3	0.2	0.3	0.1	0.1	0.1	0.7	0.6	0.6	18.9
102	0.2	0.1	0.2	0.1	0.1	...[c]	0.3	0.3	0.4	19.0
114	0.2	0.2	0.1	0.1	0.1	...[c]	0.3	0.3	0.2	19.6
126	...	...	...	...	...	...	0.2	0.2	0.1	20.8
138	...	...	...	...	...	...	0.2	0.2	0.1	22.3
150	...	...	...	...	...	...	0.2	0.1	0.1	24.1
162	...	...	...	...	...	...	0.1	0.1	0.1	23.4
Total	76.1		12.0		2.7		57.5	12.2	11.4	

[a] Weighed 47.8–62.3-Kg. dose was 5 mg. TI*BA representing 5.4 μCi. [b] Weighed 477 Kg. dose was 1.197 Gm. TI*BA representing 55.2 mCi. [c] Less than 0.05%.

and/or its metabolites were done concomitantly on the five goats used in the excretion study. Thyroid uptake occurred for 5 days after a single dose before declining in radioactive content. Comparison of the flank uptake curve with the thyroid uptake curve, indicated a definite thyroid involvement in TI*BA metabolism.

The distribution of radioactivity in each goat after 8 hr. is shown in Table III. Of the administered dose, 41% remained in the G.I. tract after 8 hr. TIBA and/or its metabolites were distributed throughout all tissue samples analyzed. The kidney was indicated as the primary organ excreting absorbed TIBA. The thyroid contained 0.19%, of the administered dose and had a relative concentration of radioactivity 28 times the blood concentration after 8 hr. In the organs examined, the lowest level of radioactivity was found in the brain, which accounted for less than 0.01% of the dose administered.

Column chromatographic separation of whole urine produced a pattern typified by Fig. 1. Internal standards of iodide (^{131}I) ion and TI*BA were eluted with peaks 2 and 8, respectively. Column chromatography of acidified urine produced the iodide peak and an enlarged peak 9 at the expense of peaks 3 to 8. The effect may be due to the release of iodinated benzoic acids from conjugation and a change of the iodinated benzoic acids in the ionized form to the molecular form. The urinary metabolites were quantitated by summing the radioactivity associated in each peak and determining the relative percent of each peak contributing to the total radioactivity. The results are shown in Table IV.

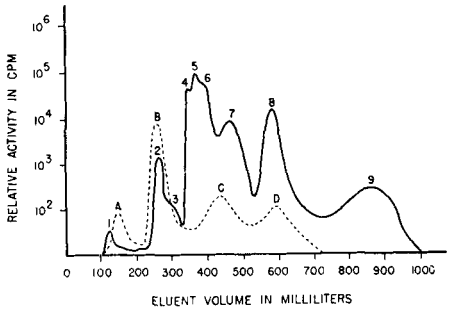

*Fig. 1—Typical column chromatogram of urine (_____) and skim milk (.....) radioactivity after oral TI*BA administration to goats, using a Sephadex G-15 column and deionized water as the eluent.*

TABLE III—TISSUE DISTRIBUTION OF TI*BA AND ITS METABOLITES[a] IN FOUR LACTATING GOATS

	% Dose in Organ		Relative Concentration[b]	
Organ	Mean	S. E.	Mean	S. E.
Kidney	0.72	0.16	270.2	48.5
Heart	0.11	0.02	34.0	5.2
Thyroid	0.19	0.03	2,835.0	1,357.0
Blood	...[c]	...[c]	100.0[d]	0.0
Spleen	0.02	0.00	16.2	2.3
G. I. tract	41.35	8.43	405.2	168.7
Liver	0.46	0.14	23.8	1.9
Fat	...[c]	...[c]	7.0	1.8
Lean	...[c]	...[c]	9.5	2.2
Brain	0.01	0.00	2.9	0.3

[a] Animals were sacrificed 8 hr. after oral administration of TI*BA. [b] Calculated using the equation: [(% dose in organ/Gm. × 10^3)(100)/(% dose in blood/Gm. × 10^3)]/4 = Relative Concn. [c] Not calculated, as the total organ weights were unknown. [d] Arbitrarily set equal to 100.0.

TABLE IV—RELATIVE PERCENT OF TI*BA AND ITS METABOLITES FOUND IN THE URINE AND SKIM MILK OF LACTATING GOATS[a] AND A COW[a] AFTER ORAL ADMINISTRATION OF TI*BA

Peak No.	Eluent Vol., ml.	Goat No. 11	Goat No. 12	Goat No. 13	Goat Mean	Cow
	Relative % Metabolites in Urine[b]					
1,2,3	0–320	2.4	1.7	0.8	1.6	0.3
4,5,6,7	321–525	39.9	33.5	49.0	40.8	70.0
8,9	526–995	57.7	64.8	50.2	57.6	29.7
	Relative % Metabolites in Skim Milk[b]					
A	0–250	6.6	7.6	5.0	6.4	0.4
B	251–320	76.2	92.4	87.8	85.5	97.8
C	321–525	...	0.0	3.8	...[d]	0.7
D	526–995	17.2[c]	0.0	3.3	...[d]	1.1

[a] Accumulated 8 hr. urine or skim milk sample from goats and accumulated 6–18 hr. urine or skim milk from a cow. [b] Column chromatographic separation using Sephadex G-15 with deionized water as the eluent. [c] Represents combined peaks C and D. [d] Data unavailable.

Components of the urine eluent, acidified urine eluent, and urine eluent peaks acidified after passing through the column were identified using thick-layer chromatography. The eluent of peak 9 from

acidified urine indicated a mixture, primarily of 2,5-DIBA and TIBA with traces of 2,3-DIBA and OIBA. The concentrated eluents of whole urine fractionated into peaks 4, 5, and 6, consisted of predominately 2,5-DIBA with trace quantities of 2,3-DIBA. Peak 7 was seen only as a trace in two of the examined goats but was as shown in the third goat and the cow. When eluent from peak 7 was acidified, it was converted to TIBA.

Column chromatographic separation of skim milk metabolites produced the typical pattern illustrated in Fig. 1. Internal standards of iodide (^{131}I) ion and TI*BA were eluted with peaks B and D, respectively. Peak A may be explained as a physical absorption of radioactivity on some high molecular weight compound in milk as it was eluted with the white color of milk near the void volume. Peak A was seen in all skim milk samples including controls, but was not seen in the pancreatin-treated skim milk. The identity of peak C is unknown. The skim milk metabolites were quantitated by summing the radioactivity associated with each eluted peak. The results are shown in Table IV. While goat 12 showed only iodide ion, peak B, all other goats and the cow examined produced a predominate iodide ion peak and traces of two other radioactive components, peaks C and D.

Digested thyroid tissue from goats was also chromatographed on gel filtration column into two major peaks, one of which appeared at the void volume indicating a high molecular weight compound being passed directly through the column. This peak tailed, giving a positively skewed curve which carried into a second peak consisting of iodide ion. The large amount of peak overlapping prevented any quantitative evaluation of the results. The first peak probably represents the iodine-containing compounds found in the thyroid that result from iodide ion concentration by the thyroid gland.

Thick-layer chromatography in solvent system *a*, separated whole urine into three fractions; origin, TIBA area (R_f 0.37), and 2,5-DIBA area (R_f 0.53). Whole urine eluent from the column chromatogram, representing peaks 4, 5, and 6, was acidified and concentrated. Quantitative evaluation indicated 2,5-DIBA (R_f 0.53) to be the major metabolite. Traces of the metabolite 2,3-DIBA (R_f 0.29) were evident. In goat 13 and the cow a trace metabolite at R_f 0.15 was also seen. Concentrated peak 9 eluent from the column chromatogram of acid hydrolyzed urine consisted of 2,5-DIBA, TIBA, and the metabolite OIBA (R_f 0.41). The three major areas on the chromatogram were quantitated by scraping from the plates and counting the radioactivity associated with each area. The results are shown in Table V. No migration of the metabolites in skim milk or in digested thyroid occurred in this solvent system.

TABLE V—RELATIVE PERCENT OF TIBA AND ITS METABOLITES FOUND IN THE URINE OF LACTATING GOATS AND A COW AFTER ORAL ADMINISTRATION OF TI*BA[a]

Location		Relative % Metabolites in Urine				
		Goat[b] No.				
R_f	Area	11	12	13	14	Cow[b]
0.00–0.29	Base	24.24	23.16	89.98	34.67	28.26
0.30–0.44	TIBA	49.58	59.59	8.63	46.94	27.16
0.45–0.59	2,5-DIBA	26.17	17.25	1.39	18.39	44.58

[a] Thick-layer chromatograms on MN-Kiesel gel and a solvent of petroleum ether (30–60°); propionic acid (10:1 v/v) (16). [b] Accumulated 8-hr. urine sample in goats and accumulated 6–18-hr. urine sample in a cow.

Thick-layer chromatography in solvent system *b* separated urine metabolites into seven positions. Iodide ion (R_f 0.47) and 2,5-DIBA (R_f 0.86) were found along with TIBA (R_f 0.83). The trace at R_f 0.80 was seen in concentrated eluents of peak 9 from the column and gave an R_r of 1.0 with OIBA standard. Three unknown metabolites appeared at R_f 0.54, 0.64, and 0.70.

Chromatograms of skim milk in solvent system *b* showed a major spot corresponding to iodide ion plus a trace of radioactivity at the origin. Pancreatin-treated skim milk contained the iodide ion without the residue at the origin. Digested thyroid supernatant remained at the origin in this solvent system except for a trace at R_f 0.54 and the iodide ion at R_f 0.47. Extraction of digested thyroid with acidified *n*-butanol indicated the presence of iodide ion (R_f 0.47), a metabolite at R_f 0.54, and traces at R_f 0.80 and 0.89.

Thick-layer chromatography using solvent system *c* separated urine metabolites into TIBA (R_f 0.73), 2,5-DIBA (R_f 0.76), iodide ion (R_f 0.30), and six additional metabolites. A ninth metabolite, OIBA (R_f 0.79), was obtained from the concentrated column eluent of peak 9. The six unknown metabolites were located at R_f 0.39, 0.42, 0.44, 0.49, 0.60, and 0.69.

Chromatograms of skim milk in solvent system *c* indicated a predominate iodide ion and trace radioactivity at the origin. Pancreatin-treated skim milk removed the residue at the origin and increased the concentration of the iodide ion. Two other unidentified trace metabolites were seen in the milk of both the cow and the goats (with the exception of goat 12) at R_f 0.44 and 0.76. Digested thyroid produced only iodide ion and a residue at the origin. Digested thyroid tissue extracted with acidified *n*-butanol produced additional metabolites at R_f 0.39, 0.44, 0.73, and 0.76.

Thick-layer chromatography of urine using solvent system *d* indicated TIBA (R_f 0.54), 2,5-DIBA (R_f 0.52), iodide ion (R_f 0.42), three major metabolites at R_f 0.50, 0.46, and 0.38, and three minor metabolites at R_f 0.95, 0.60, and 0.28. The metabolite at R_f 0.95 was only seen in goat 13. A spot at R_f 0.60 was found in the urine of all animals examined and corresponded to an impurity found in the crude synthesis product of TIBA-^{14}C and TI*BA.

Skim milk from the treated animals was separated by solvent system *d* into a major metabolite of iodide ion (R_f 0.42) and a nonmigrating residue. The residue at the origin was not seen in pancreatin-treated skim milk. Except for goat 12, traces of two other metabolites in skim milk at R_f 0.54 and 0.38 were found. Chromatograms of the supernatant of the thyroid digest of goats left a residue at the origin, the iodide ion (R_f 0.42) and traces at R_f 0.52 and 0.54. The spots at R_f 0.52 and 0.54 were also evident in the acidified *n*-butanol extracts of the digested thyroid tissue.

The mean recovered volume of skim milk from whole milk for the four goats was 89.8% (±3.9) and contained 91.0% (±2.3) of the radioactivity of whole milk. Accordingly, the radioactivity was seen to be evenly concentrated in the skim milk and cream portions of goat's milk. A similar relationship was evident in the cow's milk. Protein-bound

radioactivity in whole milk was 19.4% (±10.2) in the goats and 6.2% in the cow of the total radioactivity in whole milk.

SUMMARY AND CONCLUSIONS

1. Whole body retention studies of TI*BA in four adult lactating goats showed a two-component system—one with a biological half-life of 13.5 hr. and the second of 423 hr.

2. Excretion studies in five adult lactating goats and one cow showed that the primary route of excretion was *via* the kidney. For the goats, 76, 12, and 3%, respectively, of the administered dose was excreted in the urine, milk, and feces. Corresponding values for the cow were: 57.5, 12.2, and 11.4%.

3. Distribution studies, 8 hr. after oral administration of TI*BA to goats, indicated TIBA and/or its metabolites were located in the kidney, heart, thyroid, blood, spleen, G.I. tract, liver, fat, lean, and brain. The G.I. tract contained 41% of the administered dose at 8 hr. The average relative concentration of radioactivity in the thyroid was 28 times the average blood concentration. Thyroid uptake occurred for 5 days after oral TIBA administration.

4. Metabolite studies of the urine indicated TIBA and nine metabolites. Four of the metabolites were identified as 2,5-DIBA, 2,3-DIBA, OIBA, and iodide ion. In the 6–18 hr. urine sample of the cow, TIBA accounted for 28% of the radioactivity while the predominate metabolite, 2,5-DIBA, accounted for 45% of the radioactivity. In goats, 21% of the radioactivity in the accumulated 0–8 hr. urine sample was attributable to 2,5-DIBA while 55% was TIBA. The remaining (24%, goat and 27%, cow) metabolites in the urine are explained as representing iodide ion and conjugated forms of OIBA, 2,3-DIBA, 2,5-DIBA, and TIBA.

5. Milk analyses indicated iodide ion as the predominant (82.8–100.0%) metabolite. Eighty-one percent of the radioactivity within the milk was nonprotein bound in the goat while 94% was nonprotein bound in the cow. The two unknowns in skim milk ranged from 0% up to 17.2% of the radioactivity in the milk of goats and was 1.8% of the cow's milk.

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Keyphrases

2,3,5 Triiodobenzoic acid (TIBA)
Metabolic fate, TIBA-lactating cow, goats
Thyroid uptake—TIBA
Excretion, TIBA-urine, milk, feces
Scintillation counting-whole body
Column chromatography—TIBA identification
Thick-layer chromatography—component identification

Complexing Behavior of Starches with Certain Pharmaceuticals

By ZEYAD MANSOUR and EARL P. GUTH

A study has been made of the complexing behavior of a number of starches and starch fractions with benzoic acid, *p*-hydroxybenzoic acid, sorbic acid, and other selected molecules in aqueous solutions at 30°. By means of the solubility method of analysis it was found that the low molecular weight polymer, amylose, is the main complexing component of starch. The starches showed different affinities for the same drug according to their content of amylose. A correlation of these complexes to those of "starch-alcohols" and "starch-iodine" was made.

STARCH has a multitude of applications in many areas of our life; *e.g.*, foods, drug therapy, and many industries. It has a long history of usefulness in medicine and pharmacy. Its use, however, has been entirely empirical. It is described as a protective, an adsorbent, and as a diluent in texts on pharmacology, with no explanation of the mode of action. Starch appears

Received September 18, 1967, from the College of Pharmacy, Ohio State University, Columbus OH 43210
Accepted for publication November 30, 1967.
From a dissertation submitted at The Ohio State University in partial fulfillment of Doctor of Philosophy degree requirements.

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Keyphrases

2,3,5 Triiodobenzoic acid (TIBA)
Metabolic fate, TIBA-lactating cow, goats
Thyroid uptake—TIBA
Excretion, TIBA-urine, milk, feces
Scintillation counting-whole body
Column chromatography—TIBA identification
Thick-layer chromatography—component identification

Complexing Behavior of Starches with Certain Pharmaceuticals

By ZEYAD MANSOUR and EARL P. GUTH

A study has been made of the complexing behavior of a number of starches and starch fractions with benzoic acid, *p*-hydroxybenzoic acid, sorbic acid, and other selected molecules in aqueous solutions at 30°. By means of the solubility method of analysis it was found that the low molecular weight polymer, amylose, is the main complexing component of starch. The starches showed different affinities for the same drug according to their content of amylose. A correlation of these complexes to those of "starch-alcohols" and "starch-iodine" was made.

STARCH has a multitude of applications in many areas of our life; *e.g.*, foods, drug therapy, and many industries. It has a long history of usefulness in medicine and pharmacy. Its use, however, has been entirely empirical. It is described as a protective, an adsorbent, and as a diluent in texts on pharmacology, with no explanation of the mode of action. Starch appears

Received September 18, 1967, from the College of Pharmacy, Ohio State University, Columbus OH 43210
Accepted for publication November 30, 1967.
From a dissertation submitted at The Ohio State University in partial fulfillment of Doctor of Philosophy degree requirements.

to be quite effective in "drying-up" dermatitis lesions where there is a watery exudate; as a consequence, starch is found as a component in such pharmaceuticals as dusting powders, pastes, ointments, emulsions, and aerosols. The importance of starch inclusion in these products has not been questioned. Starch has also been used in the manufacturing of compressed tablets as a disintegrator, as a diluent, and as an excipient.

Although vast numbers of researches have been published on the physical and chemical properties of the various species of starch, very little work has been published regarding the possible involvement of the properties of starch regarding the stability and availability of the drug when included in a pharmaceutical preparation.

Guth and Goudah (1) reported a study of the complexing behavior of potato and arrowroot starches with a number of pharmaceutical compounds most of which are used as preservatives. By means of the solubility method of analysis it was found that starches form complexes in solution with benzoic acid with a number of its derivatives. All the complexing drugs were polar organic molecules, and the mechanism of interaction was believed to be a combination of inclusion formation and attractive forces like hydrogen bonding and dipole–dipole interactions.

In this work further investigation was undertaken to determine the affinities of various starches for a drug molecule, and the affinity of each of the starch fractions, amylose and amylopectin, for the drug. Dry starch adsorption of fatty acids and fatty alcohols from their methanol solutions was also investigated.

Reviews on starch chemistry (2, 3) show that it is now established that starch is a polymolecular system containing two components, amylose and amylopectin. The former is a linear polymer composed of α-D-glucopyranose residues linked by α-D-(1–4) glucosidic bonds. The latter, amylopectin, is the major component (75–85% in most starches) and consists of high molecular weight multibranched polysaccharide composed of α-D-glucopyranose residues linked by α-D-(1–4) glucosidic bonds. The chains normally contain 20–25 D-glucose residues and are interlinked to form a ramified or bush-like structure by means of α-D-(1–6) glucosidic linkages. Evidence regarding the manner in which the glucose units are combined in the two starch components has been obtained from methylation studies (4), from terminal group determination by periodate oxidation (5), and from hydrolysis studies (6). When a suspension of starch in cooled water is heated, the starch granules swell to several hundred times in original volume forming a viscous sol or paste. The swelling occurs at a specific temperature after which the granules undergo a complete rupture. Thus, a starch paste or sol is a collodial system in which there are highly swollen granules, free molecules of the starch, two molecular components, and empty sacs (7). Starch sols are physically unstable and tend to become less soluble on standing. This phenomenon has been termed as "retrogradation" and explained to be caused by aggregation of amylose molecules. Amylose stable form is suggested to be an α-helix (8); in retrogradation aggregates of the α-helical molecules are formed. The properties and behavior of amylose chains have been subjected to extensive investigations (9, 10). The rates of retrogradation in a given starch sol depend on the amylose percentage in the starch, on the size of the amylose molecule, and on the method in which the starch sol is prepared (11). The occurrence of amylopectin aggregates is improbable, since it was shown that amylopectin is physically stable in aqueous solutions (12).

EXPERIMENTAL

Materials—Three amylose starches, designated as amylose-A,[1] amylose-B,[2] and amylose-C,[3] are starches containing varying amounts of amylose (National Starch and Chemical Corp., Chicago). All other starches were commercially available from different sources, potato starch (Mallinckrodt Chemical), corn starch (Argo), arrowroot and rice (S. B. Penick and Co.), pure amylose, and amylopectin (amylose-free) (Calbio Chem. Co.). All other chemicals used were commercially available and of reagent grade.

Procedures—Determinations of the "Weight Average" Molecular Weights of the Starches—Light scattering measurements were applied on dilute solutions of the starches in 0.5 *M* potassium hydroxide solution. The starch solutions were prepared by stirring the starches or starch fractions in the potassium hydroxide solution while nitrogen is bubbled through the system. The turbidities of the solutions (τ) were measured indirectly by measuring the transmitted light at 430 mμ on a Beckman DU spectrophotometer and a Cary 15 recording spectrophotometer, using 1-cm. cells. The turbidity is defined by Debye (13) as the fractional decrease in the transmitted light intensity:

$$I/I_o = e^{-\tau L} \qquad \text{(Eq. 1)}$$

where I_o = the intensity of the incident beam, I = the intensity of the transmitted beam, τ = the turbidity in cm.$^{-1}$, L = the length of the scattering medium (1 cm. in this case).

The following equation was used for molecular weight determinations taken from the theoretical treatment given by Debye.

$$\tau = H \cdot M_w \cdot C \qquad \text{(Eq. 2)}$$

where τ = turbidity, cm.$^{-1}$, M_w = molecular weight

[1] Trademarked as Amioca.
[2] Trademarked as Amylon.
[3] Trademarked as Amylon VII.

of the solute, C = the concentration of the solute in Gm./cm.^{3}, H = a constant given by the following equation:

$$H = (32\pi^3/3)\,(\gamma^2 \cdot n_o^2/\lambda^4 N) \qquad \text{(Eq. 3)}$$

where γ = the refractive increment which can be considered constant if the dilution is sufficiently great, n_o = the refractive index of the solvent, N = Avogadro's number, λ = the wavelength of light.

Equation 2 is corrected to show the effect of solvent–polymer interaction and is finally written:

$$HC/\tau = \frac{1}{M_w} + 2BC \qquad \text{(Eq. 4)}$$

where B = a constant which depends on the degree of solvent–polymer interaction.

Using Eq. 3 the values of the constant H for three different starches were calculated and averaged into one constant to be used in the study. The only unknown is the refractive index increment γ. A sample determination of that value is shown in Fig. 1. The slope of the line was calculated by the least-squares method to give the value $\gamma = 0.1141$.

The average value of H was calculated to give $H = 3.71 \times 10^{-6}$. This value was used for all subsequent calculations, using Eq. 4. The values HC/τ are plotted against C (Figs. 2–4). The least-squares method was used to calculate the intercepts $(1/M_w)$.

Fractionation of Potato Starch to Obtain Crystalline Amylose—The method based on complexing amylose with *n*-butanol was followed in this work. A starch sol was prepared by autoclaving, as described later in the complexing study. The sol was saturated with *n*-butanol after it was removed from the autoclave. The mixture was stirred while placed in the hood until it was cooled to room temperature. The precipitate of "amylose–butanol" complex was isolated by centrifuging at 7,000 r.p.m. for 20 min. This precipitate was stirred in 500 ml. of *n*-butanol saturated water and recentrifuged as before, the precipitate was resuspended several times in distilled water and centrifuged at 10,000 r.p.m. as recommended for the removal of *n*-butanol from the complex. The amylose, thus obtained, was dried and used for preparation of amylose sols for the complexing study. Although some residual *n*-butanol was left in the amylose crystalline powder, the bubbling of nitrogen through the sols during their preparation was one of the procedures recommended for the removal of the residual *n*-butanol (14).

Complexation in Aqueous Solutions—The starch sols were prepared by autoclaving as described in a previous work (1). The required amount of starch or starch fraction to make 500 ml. of the sol was accurately weighed into a 125-ml. beaker, smoothed into a slurry with distilled water, then poured all at once into a liter beaker containing 400-ml. boiling water. The gelatinized paste was stirred and transferred to an autoclave to be heated at the pressure of 20 p.s.i. for 3 hr. The resulting sol was then stirred until cooled and distilled water added to obtain 500 ml. of the sol.

Excess quantities of the drug were added to various starch sols of different concentrations in stoppered bottles. The bottles were agitated in a constant temperature water bath at 30° until the system reached equilibrium (24–48 hr.). Aliquots of 2–3 ml. were removed from each bottle using 10-ml. hypodermic syringes kept at 30° temperature. The aliquots were filtered through a Swinny filter adapter placed on the tip of the syringe. The adapter was equipped with S&S filter paper. The filtrates were placed in tubes kept in the 30° water bath. The filtered solutions were left 20 min. in the bath. Aliquots of these were removed using pipets kept at 30° temperatures and appropriately diluted for spectrophotometric analysis. Dilution factors varied from 1:100 to 1:1000 depending on the solubility and the molar absorbancy of the drug tested. The wavelengths of maximum absorbance were determined on a Cary 15 recording spectrophotometer. Each drug showed the same wavelength of maximum absorbance in water and in the presence of starch solution. Readings of absorbance were made using proper dilution of a starch sol (without the drug) as a blank in the reference cell.

Determination of the Adsorption of Long Chain Fatty Acids and Fatty Alcohols on Dry Unruptured Potato Starch Granules—Solutions of the fatty materials were prepared in two different solvents, methanol and petroleum ether. All solutions had the same concentration (15 Gm./L.), since it was shown that adsorption of fatty acids on potato starch follows a typical Freundlich isotherm (15). Fifty grams of potato starch was placed in a bottle, 100 ml. of the fatty material solution was added. The bottles were tightly stoppered and agitated in a water bath at 30° for 24 hr. All samples were filtered and the filtrates were tested for the excess of fatty acid or fatty alcohol in solution. The starch collected on the filter papers was washed with 25 ml. of petroleum ether three times. The washed starches were transferred into clean beakers and hydrolyzed with 500 ml. of 1 *N* hydrochloric acid while heated on a steam bath and stirred slowly. A blank sample of potato starch was hydrolyzed in a similar manner. Two hours of hydrolysis showed a complete disappearance of the iodine reaction in the mixture. The mixtures were cooled and placed overnight in the refrigerator. The cold

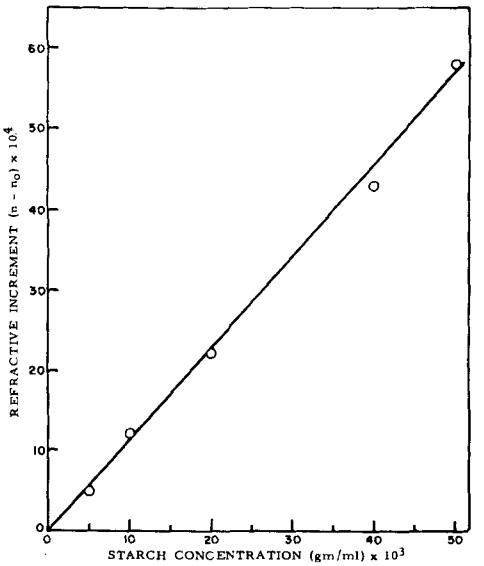

Fig. 1—Refractive index increments of potato starch solutions in 0.5 M KOH.

mixtures were than filtered through a fluted filter paper, and the beakers and the filters were repeatedly washed until the filtrates were free of hydrochloric acid as shown with methyl orange T.S. The inside and the lip of the beakers were scrubbed with small pieces of damp filter paper to pick up adherent fatty materials, and these pieces were placed in the fluted filter of each beaker. The papers were allowed to dry overnight and then were extracted in a Soxhlet extractor with petroleum ether. The solvent was removed and the amount of the fatty material was determined by weighing the dry flask containing the extracted fatty material.

RESULTS

Molecular Weight Determination—Figure 1 shows a sample determination of the refractive index increment for a series of dilute potato starch solutions. The constant γ is the slope of the line of $(n - n_o)$ *versus* (C) where n_o is the refractive index of the solvent (0.5 M KOH), n is the refractive index of the solution using light source of 430-mμ wavelength, and C is the concentration of the starch solution.

Figures 2–4 are those of HC/τ plotted against C as given by Debye (Eq. 4). The least-squares method was used to calculate the intercepts $(1/M_w)$, and Table I shows the values of the molecular weights obtained.

Complexation in aqueous solutions—Figures 5–7 represent the solubility behavior of benzoic acid, *p*-hydroxybenzoic acid, and sorbic acid in the presence of various concentrations of the starches in aqueous solutions at 30°. Figures 8 and 9 represent the solubility behavior of a number of organic molecules in the presence of potato starch aqueous solutions at 30°. Figure 10 shows the same plots on Figs. 8 and 9 plotted on the same ordinate scale to compare the slopes.

Table II shows the unruptured starch granules adsorption of fatty acids and fatty alcohols. Figure 11 shows the amounts of fatty materials adsorbed per 1 Gm. of potato starch plotted against the number of carbons of the fatty chain.

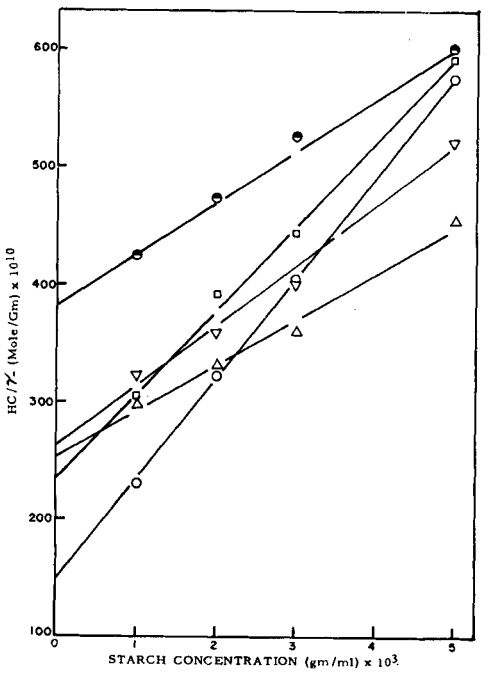

Fig. 2—Debye equation plots of turbidimetric data of various starches. Key: ◓, corn; □, amylose-A; ○, amylopectin; ▽, potato starch; △, arrowroot.

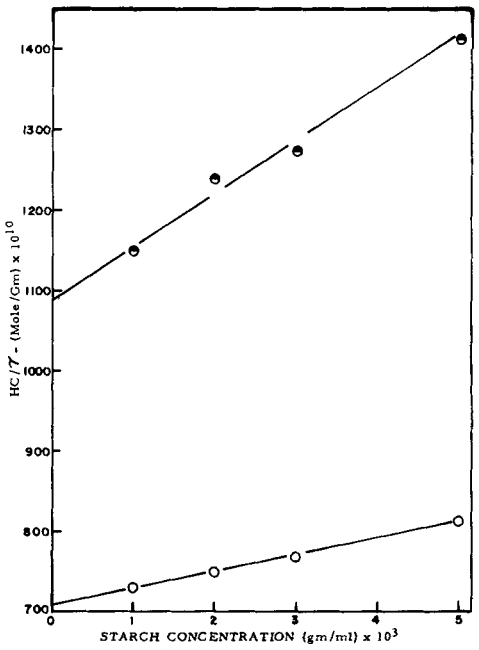

Fig. 3—Debye equation plots of turbidimetric data of rice starch and amylose-B starch. Key: ◓, amylose-B; ○, rice starch.

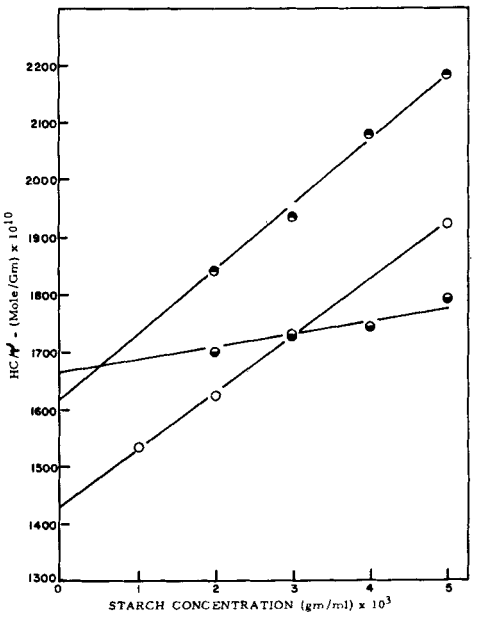

Fig. 4—Debye equation plots of turbidimetric data of several starches. Key: ◓, amylose (potato); ○, amylose-C; ◒, commercial amylose.

TABLE I—"WEIGHT AVERAGE" MOLECULAR WEIGHTS (M_w) AS CALCULATED FROM TURBIDIMETRIC DATA BY LEAST-SQUARES METHOD

Starch	Mw
Amylopectin	6.719×10^7
Amylose-A	4.288×10^7
Potato	3.959×10^7
Arrowroot	3.807×10^7
Corn	2.626×10^7
Rice	1.411×10^7
Amylose-B	9.195×10^6
Amylose-C	6.991×10^6
Amylose (fractionated)	6.171×10^5
Amylose (commercial)	6.004×10^5

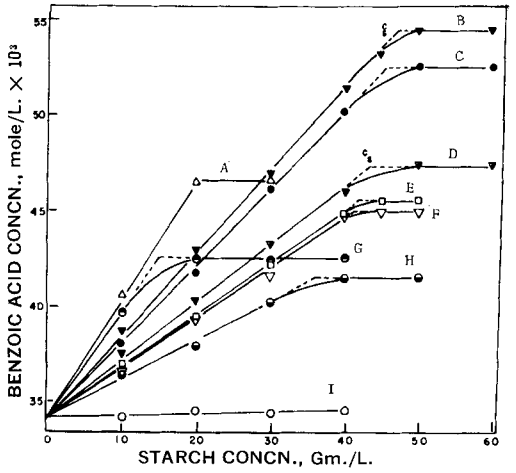

Fig. 5—Effect of various concentrations of starches on the apparent solubility of benzoic acid at 30°. Key: A, amylose; B, amylose-C; C, amylose-B; D, rice; E, corn; F, arrowroot; G, amylose-A; H, potato starch; I, amylopectin.

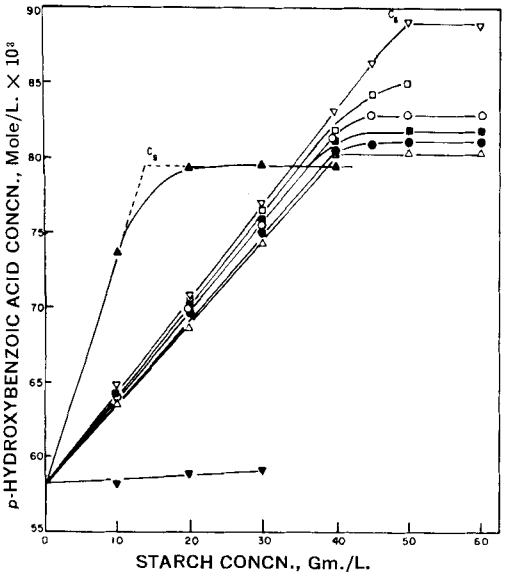

Fig. 6—Effect of various concentrations of starches on the apparent solubility of p-hydroxybenzoic acid, at 30°. Key: ▽, amylose-C; □, amylose-B; ○, rice; ■, corn; ●, arrowroot; ▲, amylose-A; △, potato; ▼, amylopectin.

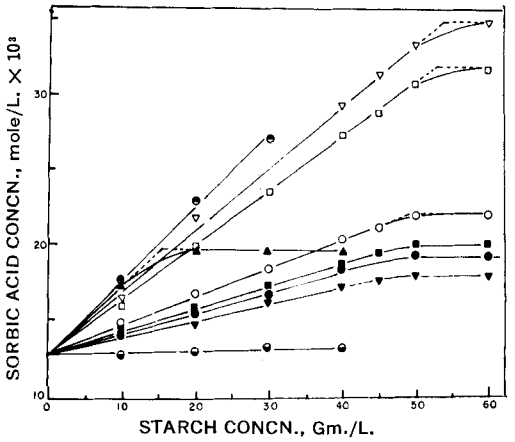

Fig. 7—Effect of various concentrations of starches on the apparent solubility of sorbic acid at 30°. Key: ◒, amylose (fractioned); ▽, amylose-C; □, amylose-B; ▲, amylose-A; ○, rice; ■, corn; ●, arrowroot; ▼, potato; ◒, amylopectin.

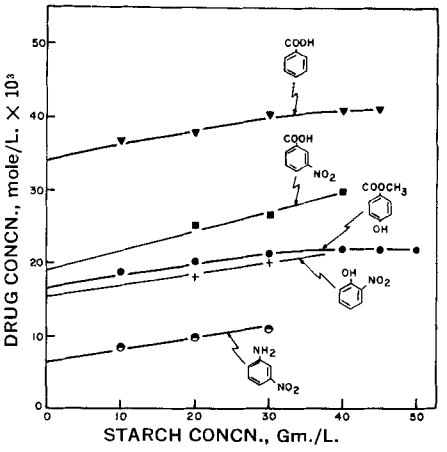

Fig. 8—Effect of various concentrations of potato starch on the apparent solubilities of several organic acids in water at 30°.

DISCUSSION

Using the light scattering method "weight average" molecular weights of the various starches were obtained. These molecular weights count the various molecules in the polymolecular starch system according to their masses. Each of the starch fractions, amylose and amylopectin, is known to be a polymolecular system consisting of a number of subfractions (16). Thus, the molecular weight of a given starch as obtained in this work can be considered a mean value which accounts for all the polymolecular components of that starch according to their respective masses. The amyloses showed the lowest molecular weights, and amylopectin the highest. As the rest of the starches are heterogeneous systems, the molecular weight values varied according to the starch content of low molecular weight amylose and according to the sizes of the amylose and amylopectin fraction. In each starch the two polymer fractions (and even their subfractions) contributed to the value of (M_w) according to their percentage and weight distribution.

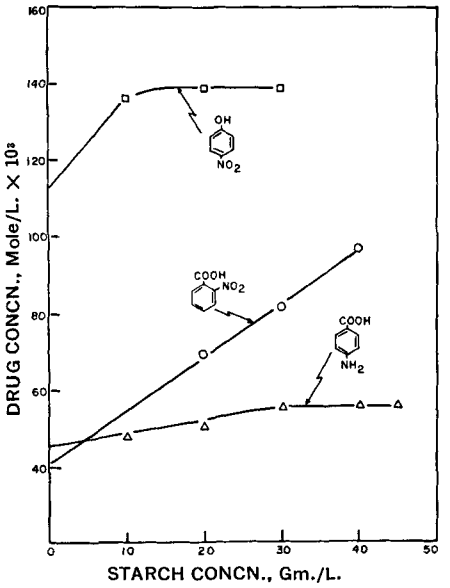

Fig. 9—Effect of various concentrations of potato starch on the apparent solubilities of several organic acids at 30°.

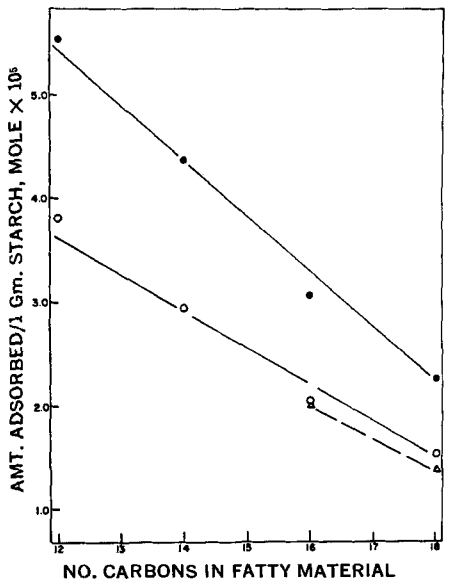

Fig. 11—Dry starch adsorption of several fatty acids and fatty alcohols from methanol or petroleum ether solutions. Key: ●, saturated fatty acids in methanol; O, fatty acids in petroleum ether; △, alcohols in petroleum ether.

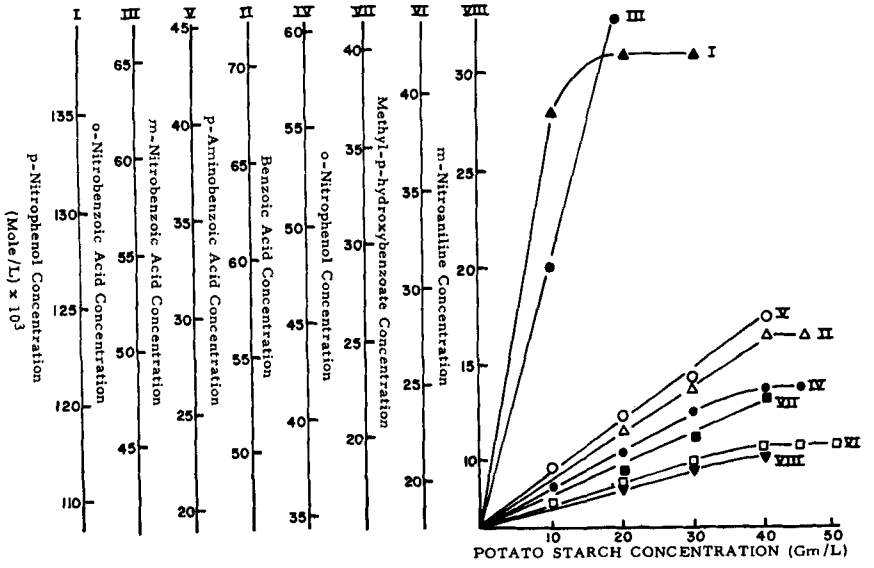

Fig. 10—Solubility behavior of a number of organic compounds in the presence of potato starch.

TABLE II—POTATO STARCH ADSORPTION OF FATTY ACIDS AND FATTY ALCOHOLS IN PETROLEUM ETHER OR IN METHANOL

Fatty Acid or Alcohol	Total Amt. Adsorbed per 1-Gm. Dry Starch, mole × 10⁵	
	Petroleum Ether Solution	Methanol Solutions
Stearic acid	1.51	2.26
Stearyl alcohol	1.35	...
Oleic acid	1.37	2.07
Palmitic acid	2.03	3.06
Myristic acid	2.94	4.36
Lauric acid	3.80	5.54
Cetyl alcohol	2.01	...

The solubility method for determining interactions between starch and the selected drugs indicated complex formation evidenced by an increase of the solubility of the drug with the increase of a given starch concentration. The phase diagrams of benzoic acid, *p*-hydroxybenzoic acid, and sorbic acid in the presence of various starches and starch fractions can be explained briefly by (*a*) pure amylopectin showed little or no interaction with the three drugs. (*b*) The various starches showed complexation represented by a linear increase of the solubility of the drug with the increase of a given starch concentration up to a certain limit where no more drug could be detected in the solution. The limit can be called the saturation concentration of the complex (C_S), at which the turbidity of the

solution was notably increased and the "starch-drug" complex precipitated out of the solution. (*c*) Amylose sols showed a high uptake of the complexing drug into solution. In sols containing more than 20 Gm./L. of amylose a cake-like product precipitated out of the solution. This precipitate was successfully centrifuged at 7,000 r.p.m. A similar precipitation occurred in potato starch when the complexing drug was *p*-nitrophenol or *p*-aminobenzoic acid (Fig. 9).

It can be said in summary that when the same solid drug was added to the solutions of the various starches (each being a polymolecular system of its own), the amounts of the drug that entered the solution before precipitating the complex were dependent on the type of the starch. These amounts can be calculated by subtracting the amount of drug in water at 30° (C_o) from the total drug in the system at the plateau region (C_s). It must be noted that these values (C_s-C_o) for the same drug decreased with the increase of the "weight average" molecular weight of the starch.

These values varied from one starch to the other for the same complexing drug. The lower the average molecular weight of the starch the greater the amount of the complexing drug needed to reach the plateau region. Such a relationship can only be explained when one understands that the lower the molecular weight of a starch the richer the starch in amylose, thus the precipitation of an amylose complex from the system at the plateau region is influenced by the average molecular weight of the starch which can be considered related to the amylose content of the starch. (*d*) Finally, one can recognize that the low molecular weight polymer, amylose, is the main complexing component of starch with the drug. The amylose tendency to crystallize in a complex form with the drugs tested is noted to be far stronger than that of amylopectin.

The complexing of potato starch sols with selected molecules occurs with wide variance as seen in Figs. 8–10. It appears at first that the tendency of drugs to complex with the starch matches their solubility, *i.e.*, the higher the solubility of a drug the higher its tendency to complex; however, arranging the tendencies for complexing, as given by the slopes of the lines on Fig. 10, in a decreasing order shows that those tendencies do not match the solubilities in water (Table III). The effect of groups, like nitro or amino, in increasing the tendency to complex is noted. For example, the water solubility of *p*-aminobenzoic acid is higher than that of *o*-nitrobenzoic acid but the latter has a higher tendency to complex with starch than the former.

TABLE III—SOLUBILITY RELATIVE TO COMPLEXING TENDENCY OF DRUG MOLECULES

Decreasing Order of Solubility in Water	Decreasing Order of Tendency to Complex[a]
I	I
II	III
III	V
IV	II
V	IV
VI	VII
VII	VI
VIII	VIII

[a] I = *p*-nitrophenol, II = *p*-aminobenzoic acid, III = *o*-nitrobenzoic acid, IV = benzoic acid, V = *m*-nitrobenzoic acid, VI = methyl-*p*-hydroxybenzoate, VII = *o*-nitrophenol, VIII = *m*-nitroaniline.

Correlations of the complexes reported in this work with those of "amylose-alcohols" used for the fractionation of starch and "amylose-iodine" can be made after extensive X-ray studies as reported in the cases of "amylose-fatty acids" complexes (19). A general similarity is noted in the behavior of the complexes reported in this work with those of "alcohols-amylose," and those reported by Whistler (20) that are obtained with nitrobenzene, pyridine, 2-heptanone, and many other water soluble compounds possessing groups capable of hydrogen bonding and used for the fractionation of starch. Such similarity in the complexing behavior makes one assume that the complexing takes the same route and mechanism as that of "starch-iodine" and "starch *n*-butanol" complexes, which are explained to be consisting of an entrapment of the "guest" molecule in the α-helical structure of amylose with a supplementary stabilization by dipole–dipole interactions (21).

The adsorption affinity of starch is demonstrated in the case of the unruptured starch granules interacting with fatty acids and fatty alcohols. The affinity was decreased with the increase of the hydrophobic chain of the fatty material. Starch affinity for a given fatty material in methanol solution was higher than its affinity for the same material in a petroleum ether solution of the same concentration. This cannot be explained easily. One must notice that the starch used contained 10–12% water. It is conceivable that the fatty acid can be carried into the granule polymers by an exchange process between the water content and the organic solvent; thus, the polarity of the solvent proved to be of value.

It is important to appreciate that the understanding of the many applications of a fundamental phenomenon, like starch complexation can help the pharmaceutial compounder to predict any unfavorable incompatibilities. A drug complexed with starch might differ from the free drug with respect to solubility, diffusivity, partition coefficient, and ability to penetrate biological membranes. A drug pharmacological effectiveness might be altered if the drug is bound in such a complex; when the complex dissociates by the hydrolysis of the macromolecular polysaccharide at some stage in the body, the drug can then be available for its usual absorption and effect. In this respect the availability of a drug for external therapeutic treatment and its effectiveness should be investigated when the drug is included in a starch paste, sol, or any base containing polysaccharides in general.

With the fundamental understanding of the high affinity of starch for molecules varying from phenols and alcohols to weak carboxylic acids, the pharmaceutical compounder should investigate the effects of the complexation of the various drugs introduced in very small quantities in a tablet containing starch, especially when starch paste or polysaccharide excipients increase the magnitude of the complexation in the wet granulation technique. In general, the possible interactions between starch and many organic molecules found in small concentration dosage forms where starch is used as a pharmaceutical adjunct, must be considered carefully. The complexation of the starch with a preservative like benzoic acid, for example, is expected to decrease its preservative activity.

Finally, drug complexation with starch can be a useful method of retarding absorption of a very irritant drug that might complex with starch and thereby reduce its toxicity. Starch has been used in this sense as an antidote for iodine poisoning. In a similar manner, the starch complexing affinity could explain the possible binding to bacterial excretion products in certain dermatitis conditions, thus providing the rationale of the wide use of starch as anti-irritant in dusting powders.

As the precipitation and isolation of "amylose-drug" complex is possible, a highly important question about the stability of a drug enclosed in such a complex form should not remain long unanswered. The nature of these complexes appear to suggest their possible use in a sustained dosage form, since the release of the drug from the complex is possibly caused by the enzymatic hydrolysis of the macromolecule.

SUMMARY AND CONCLUSION

Amylopectin (amylose-free) was found to have very little interaction for benzoic acid, *p*-hydroxybenzoic acid, and sorbic acid, while amylose was found to produce complexes rapidly. Potato starch, arrowroot starch, corn starch, rice starch, and a number of commercial starches rich in amylose were all found to complex with the three drugs tested. A number of selected pharmaceuticals showed interaction with potato starch sols, and in many cases, depending on the solubility of the pharmaceutical, the complex precipitated off the solution after reaching a saturation concentration.

Unruptured potato starch granules were found to adsorb fatty acids and fatty alcohols from methanol or petroleum ether solutions.

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Keyphrases

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Fatty acids–starch affinity
Molecular weight determination–starches
Spectrophotometry turbidity analysis
Solubility–complex formation analysis

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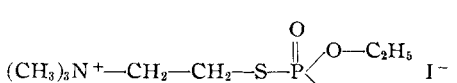
ALTHOUGH ECHOTHIOPHATE IODIDE (I), a potent long-acting cholinesterase inhibitor, has been recognized in the USP (1) to be unstable in aqueous solution, the chemical degradation of the compound has not been thoroughly investigated. However, it is known that I



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Received April 28, 1967, from the Pharmaceutical Development Department, Ayerst Laboratories, Inc., Rouses Point, New York, NY 12979
Accepted for publication October 26, 1967.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas Meeting, April 1967.
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Finally, drug complexation with starch can be a useful method of retarding absorption of a very irritant drug that might complex with starch and thereby reduce its toxicity. Starch has been used in this sense as an antidote for iodine poisoning. In a similar manner, the starch complexing affinity could explain the possible binding to bacterial excretion products in certain dermatitis conditions, thus providing the rationale of the wide use of starch as anti-irritant in dusting powders.

As the precipitation and isolation of "amylose-drug" complex is possible, a highly important question about the stability of a drug enclosed in such a complex form should not remain long unanswered. The nature of these complexes appear to suggest their possible use in a sustained dosage form, since the release of the drug from the complex is possibly caused by the enzymatic hydrolysis of the macromolecule.

SUMMARY AND CONCLUSION

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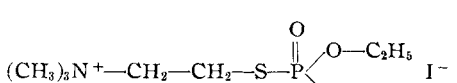

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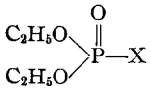
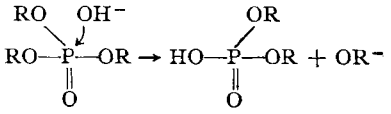
hydrolyzes quantitatively in alkaline media to (2-mercaptoethyl)trimethylammonium iodide and diethylphosphoric acid (1, 2).

Most of the past studies on the degradation of organophosphorus compounds related to I have dealt with the effect of substituents on their alkaline rates of hydrolysis (2, 3). Heath (3) found that the rate of alkaline hydrolysis of the X group from compounds of the type $RR'P(O)X$, where R and R' are alkoxy or alkylamine groups and X is an acidic group, is influenced greatly by the nature of the X group. Thus, the second-order rate constant for the hydrolysis of

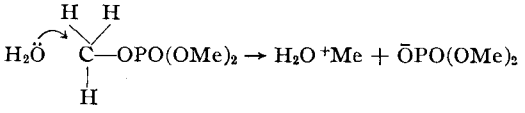


where X is OC_2H_5 is 4.86×10^{-2} M^{-1} min.$^{-1}$ at 25° compared to 81 M^{-1} min.$^{-1}$ where X is $S—C_2H_4S—C_2H_5$.

Trialkylphosphoric acid esters, however, have been thoroughly investigated by many workers (4–6). Barnard *et al.* (4) stated that in a trisubstituted phosphate ester, in which both carbon and phosphorus centers are subject to attack, hydroxide and water will tend to be selective for phosphorus and carbon, respectively. From their isotope experiments using water enriched in oxygen-18, they concluded that trimethylphosphate undergoes hydrolysis in alkaline medium with phosphorus-oxygen fission. There action was postulated as a simple nucleophilic displacement at the phosphorus atom:



In neutral and acidic media, however, the hydrolysis proceeded with carbon-oxygen bond fission. Since the reaction rate in water was not significantly changed by the presence of up to 3 M perchloric acid, it was presumed by these authors that this reaction proceeded *via* an S_{N2}-type displacement on carbon with water as the attacking species:



On the basis of the above information and the structure of (I), with both phosphorus and carbon centers open to attack, similar reactions may be anticipated, as shown in the following scheme. It was the primary purpose of this investigation to determine the mechanism of degradation of echothiophate iodide in neutral and weakly acidic media. A further objective was the determination of reaction rate constants and activation energies over a wide pH range.

RESULTS AND DISCUSSION

Order and Nature of the Degradative Steps—The results of this study indicated that in aqueous buffered solutions, the degradation of echothiophate iodide was first order with respect to the compound over a broad pH and temperature range.

In alkaline media, above pH 9, the reaction was followed measuring (2-mercaptoethyl)trimethylammonium iodide (II) formation by iodometric and spectrophotometric determinations, and by measuring consumption of hydroxyl ion on a pH stat. These results are shown in Figs. 1–3. As expected, from the ionic character of the reactant species (Scheme I), a negative primary salt effect was observed in the alkaline pH range. This is evident in Fig. 4, where the logarithm of the observed pseudo first-order rate constant, k_1 [OH^-], determined at pH 11.5, was plotted against the

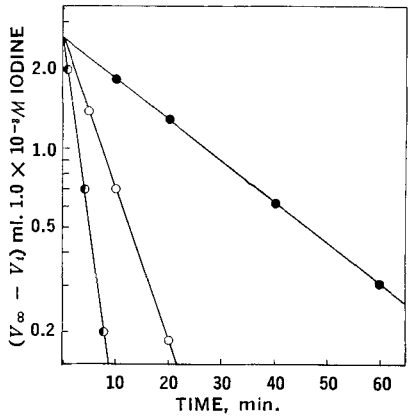


Fig. 1—Hydrolysis of echothiophate iodide as determined iodometrically at pH 10.85 and at ●, 25°; ○, 37°; and ◐, 45°.

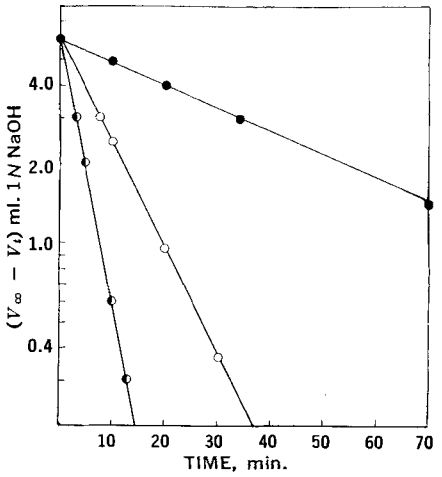
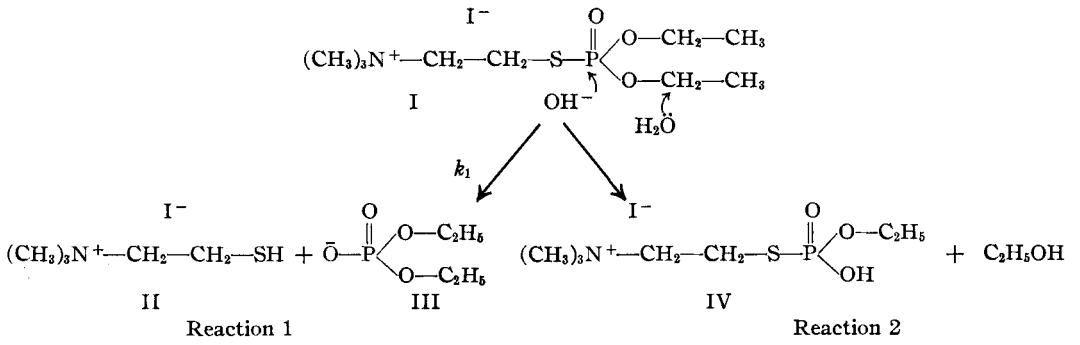


Fig. 2—Hydrolysis of echothiophate iodide as determined from pH stat measurements at pH 10.5 and at ●, 25°; ○, 37°; and ◐, 45°.



Scheme I

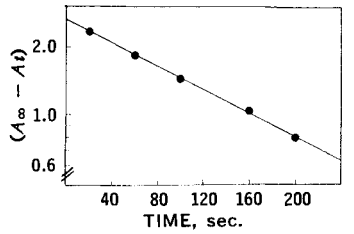


Fig. 3—Spectrophotometric observation at 226 mμ of the hydrolysis of echothiophate iodide at pH 11.8 and 25°.

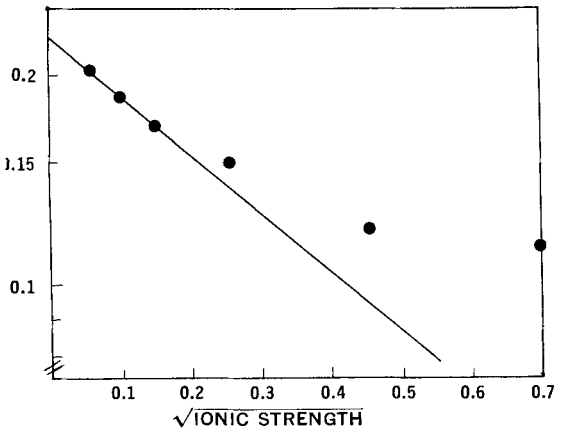


Fig. 4—Log $k_1[OH^-]$, the observed first-order rate constant of thiol formation, as a function of square root of ionic strength at 22° at pH 11.5. pH maintained with dilute NaOH, ionic strength changed by varying concentration of NaCl.

square root of ionic strength according to the Brönsted-Bjerum equation. Since this relationship was derived on the assumption of low ionic strength, the experimental values deviate rapidly from linearity. The initial slope, however, has a value of 0.9, close to the theoretically predicted value of 0.98 for sodium chloride. Since the rate of degradation at these hydroxyl ion concentrations was not influenced by changes in concentration or species of buffer, as shown in Figs. 5 and 8, the hydrolysis appears to be a specific hydroxide ion catalyzed reaction.

In acidic media, pH range 2.4–4.6, there was no detectable free thiol in degraded echothiophate iodide solutions, although solutions of thiol alone are stable under these conditions (less than 1% lost in 24 hr. at 70°). It was found, however, that in this pH range 1 mole of ethanol was formed for every

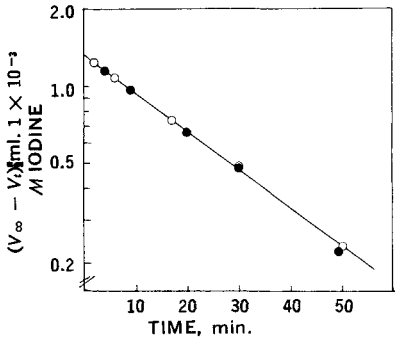


Fig. 5—Effect of change of molarity of phosphate buffer on the rate of hydrolysis of echothiophate iodide at pH 10.85 and ionic strength of 1.0 at 25°. Key: O, 0.1 M; ●, 0.2 M.

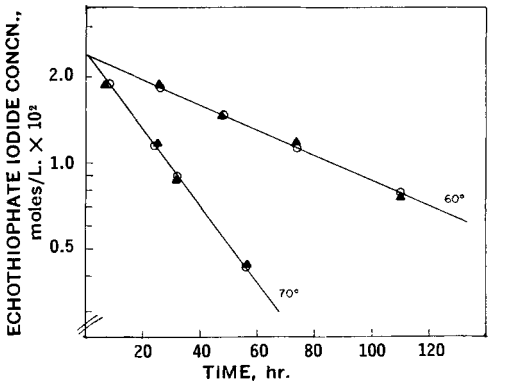


Fig. 6—Disappearance of echothiophate iodide at pH's 2.4 and 4.6 and at 60° and 70°. Key: O, found by direct analysis of echothiophate; ▲, calculated from the concentration of ethanol and based on the observed fact that one molecule of ethanol was formed for every molecule of echothiophate iodide degraded.

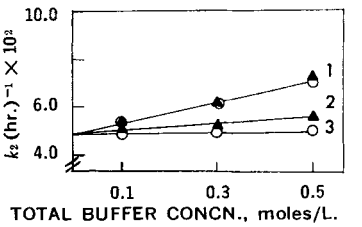


Fig. 7—Plot showing the effect of buffer concentration upon the rate of ethanol formation from echothiophate iodide at 74° and total ionic strength of 0.5. Key: ▲, 1, pH 5.2 acetate and pH 6.3 phosphate; O, 2, pH 4.6 acetate and 3, pH 2.4 phosphate.

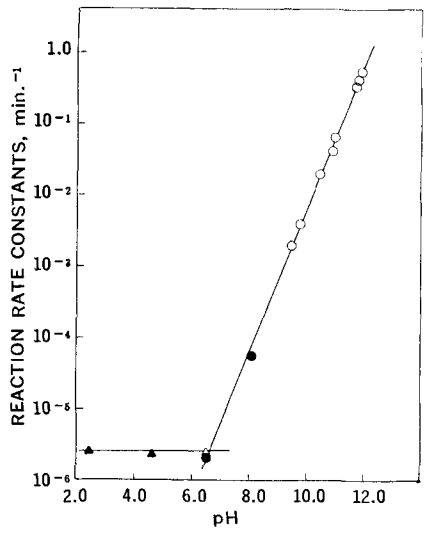


Fig. 8—pH profile of $k_1[OH^-]$ *and* k_2 *at 25°. Key:* O, $k_1[OH^-]$, ▲, k_2 *determined directly;* ●, $k_1[OH^-]$, △, k_2 *determined using the observed overall rate constant* $k_1[OH^-] + k_2$ *and Eq. 5.*

mole of I lost. Furthermore, the rate of loss of I from solution and the rate of appearance of ethanol were essentially the same. As shown in Fig. 6, the residual concentrations of I obtained by direct analysis are the same as those calculated from the concentrations of ethanol liberated. It is evident that for all practical purposes the reaction involving ethanol formation was responsible for the entire observed loss of I.

A slight influence of buffer concentration on the rate of ethanol formation determined at different hydroxyl ion concentration is seen in Fig. 7. At pH's below 5, the buffer effect was minimal. Since the rate constants were determined at low buffer concentration below pH 5, the contribution of buffer to the overall degradation was insignificant.

Thus, it appeared on the basis of these results that echothiophate iodide degrades in aqueous solution by at least two different mechanisms, depending upon the pH.

Variation of Reaction Rate Constants with pH—The pseudo first-order rate constants for formation of thiol and ethanol are shown as a function of pH in Fig. 8. These rate constants were determined directly from first-order concentration–time plots at pH's greater than 9 for thiol formation and pH's less than 5 for ethanol formation. It is seen from Fig. 8 that the plot has a zero slope at low pH and slope of unity at high pH.

In the intermediate pH range of 5–9, where both reactions contribute to the overall degradation, the individual rate constants were calculated from the overall rate of degradation of (I) and the final concentration of ethanol.

A rate equation for loss of I, based on Scheme I and Fig. 8, can be written:

$$-\frac{d[I]}{dt} = k_1[I]\,[OH^-] + k_2[I] = k_{obs.}[I] \quad \text{(Eq. 1)}$$

and

$$k_{obs.} = k_1[OH^-] + k_2$$

where [I] is the concentration of echothiophate iodide, k_1 is the rate constant for formation of thiol

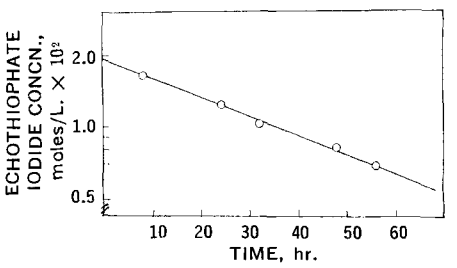


Fig. 9—Plot showing the disappearance of echothiophate iodide as a function of time at pH 6.3 and 60°.

TABLE I—FINAL CONCENTRATION OF ETHANOL AS A FUNCTION OF pH AT 60°, $[I]_o = 2.4 \times 10^{-2}$ *M*

pH	Ethanol Concentration × 10²
2.4	2.4
4.6	2.4
6.3	1.2
8.1	0.24
9.5	0.0

(II), k_2 is the rate constant for formation of ethanol and $k_{obs.}$ is the observed rate constant for loss of echothiophate iodide.

Since pH was maintained constant during an experiment, Eq. 1 can be integrated to give,

$$\log [I]_t = \log [I]_o - \frac{(k_1[OH^-] + k_2)t}{2.303} \quad \text{(Eq. 2)}$$

It can be shown (7) that for the case $[E_o] = 0$ at $t = 0$, the formation of ethanol with time may be described by,

$$[E]_t = \frac{k_2[I]_o}{(k_1[OH^-] + k_2)}\,(1 - e^{-(k_1[OH^-] + k_2)t}) \quad \text{(Eq. 3)}$$

where $[E]_t$ is the concentration of ethanol at time t. At $t = \infty$ Eq. 3 becomes,

$$[E]_\infty = \frac{k_2[I]_o}{(k_1[OH^-] + k_2)} \quad \text{(Eq. 4)}$$

$(k_1\,[OH^-] + k_2)$ can be obtained from the slope of log $[I]_t$ *versus* t plots, Fig. 9, according to Eq. 2. Since $[I]_o$ is known, and $[E]_\infty$ can be measured after $[I]_t = 0$, $k_1\,[OH^-]$ and k_2[1] can be calculated for the intermediate pH range.

According to Eq. 4, the final concentration of ethanol will be a function of pH. At high pH, k_1 $[OH^-]$ is large compared to k_2 and $[E]_\infty$ is very small, while at low pH, k_1 $[OH^-]$ is small compared to k_2 and $[E]_\infty$ approaches $[I]_o$. This is shown in Table I.

In the above scheme and equations, it was assumed that ethanol production was due solely to direct hydrolysis of echothiophate iodide as caused by Reaction 2, and the formation of ethanol from diethylphosphoric acid (III) was negligible. This assumption seems entirely valid in view of the much slower rate of hydrolysis of dialkylphosphates under these conditions (8, 9).

Both the USP assay method and the method used in this study for echothiophate iodide are based upon alkaline hydrolysis followed by iodometric determination of the liberated thiol. Although one might

[1] k_2 at pH 6.3 was determined by extrapolation of the data shown in Fig. 8 to zero buffer concentration.

expect that the degradation product (IV) produced by Reaction 2 would behave similarly, it appears that IV is not readily hydrolyzed to thiol under these basic conditions, probably because the negative charge acquired in basic solution serves to hinder attack by the negatively charged hydroxyl ion. It is known, for example, that subsequent stages of alkaline hydrolysis of triethylphosphoric acid are extremely slow after the first ethanol group is cleaved (10).

Temperature Dependency—Since the hydrolysis reaction may proceed by either of the two pathways, Reactions 1 and 2, it was necessary to determine the temperature dependency for each. The effect of temperature on Reaction 1 was determined iodometrically at pH 10.85 and on a pH stat at pH 10.5, since at these pH's, Reaction 2 was negligible. Arrhenius-type plots of log k_1 OH^- *versus* $1/T$ were observed to give straight lines, as shown in Fig. 10. The apparent activation energy was calculated from the slopes and found to be 22 Kcal./mole with a standard error of 1 Kcal./mole. Since this apparent energy of activation includes the heat of ionization of water (approximately 12 Kcal./mole), the energy of activation for the hydroxyl reaction would be 10 Kcal./mole.

The effect of temperature on Reaction 2 was determined at pH 2.4 and pH 4.6, since at these pH's Reaction 1 was negligible. The results obtained are shown in Fig. 11 where the log k_2, the observed first-order rate constant, was plotted against $1/T$. The heat of activation calculated from the slope of these lines is 23 Kcal./mole. The activation energy of the hydrolysis of trimethylphosphate in neutral and acidic solution was found to be 22.7 Kcal./mole (4).

The observed overall first-order rate constants for the degradation of echothiophate iodide, calculated at 25°, are shown as a function of pH in Table II.

In Fig. 12, the observed overall first-order rate constant $k_{obs.}$ was plotted against the pH of the solution. The line was calculated from Eq. 1 using $k_1 = 59.7\ M^{-1}$ min.$^{-1}$ and $k_2 = 2.3 \times 10^{-6}$ min.$^{-1}$ determined at pH 12 and pH 2.4, respectively, and the points were experimentally determined.

Degradation Products in Acidic Media—The degradation products in solutions at pH 4.6 were identified as ethanol and the monoethylester (IV).

The ethanol was determined quantitatively by gas chromatography. The monoethylester (IV) was isolated as the sodium salt (Na IV), and found to possess no cholinesterase inhibitor properties (11). Thin-layer chromatographic analysis of (Na IV)

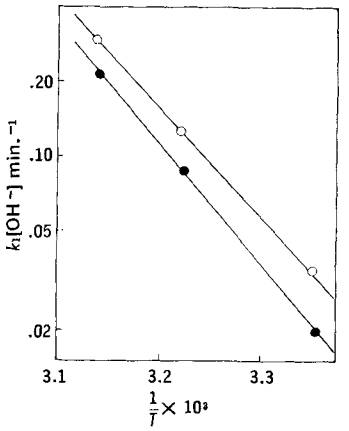


Fig. 10—Arrhenius plots showing the temperature dependency of Reaction 1. Key: ○, pH stat at pH iodometrically at pH 10.85; ●, pH stat at pH 10.5.

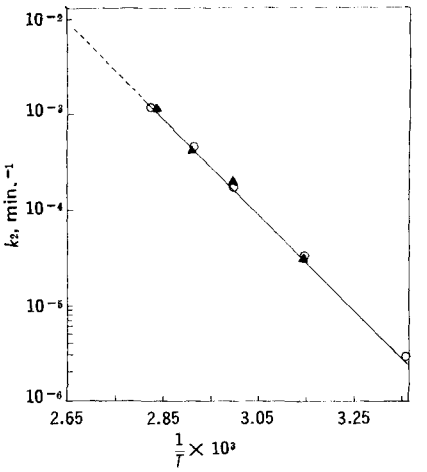


Fig. 11—Temperature dependency of Reaction 2. The log of the observed rate plotted against the reciprocal of the absolute temperature. Key: ○, pH 2.4; ▲, pH 4.6.

TABLE II—OBSERVED OVERALL FIRST-ORDER RATE CONSTANT AS A FUNCTION OF pH AT 25°

pH	$k_{obs.}$ min^{-1}
2.4	2.4×10^{-6}, 2.2×10^{-6}[a]
4.6	2.2×10^{-6}[a]
6.4	4.4×10^{-6}[a]
8.1	5.9×10^{-6}[a]
9.5	2.1×10^{-3}
9.8	4.0×10^{-3}
10.5	2.2×10^{-2}
10.9	4.1×10^{-2}
11.0	6.1×10^{-2}
11.7	3.5×10^{-1}
11.9	5.5×10^{-1}

[a] Calculated for 25° using the apparent activation energies.

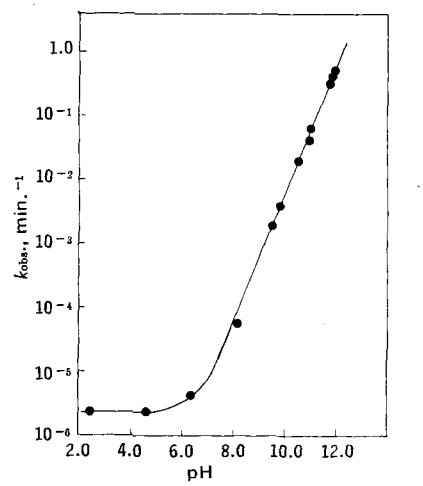


Fig. 12—Curve relating the observed overall first-order rate constant with pH at 25°. The solid line was calculated from Eq. 1, circles represent experimentally determined values.

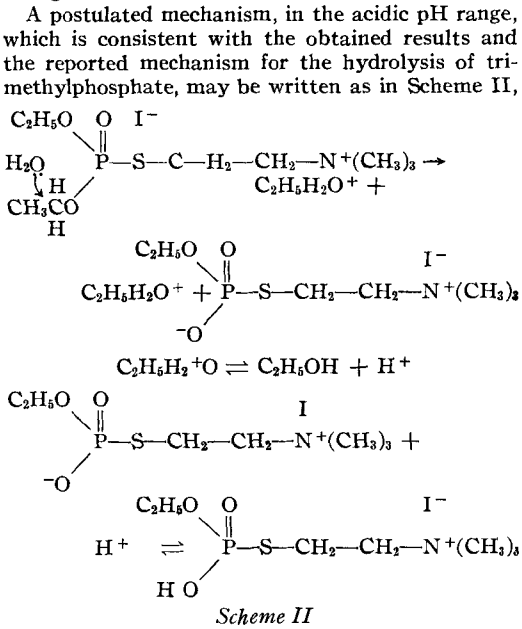
showed one spot with R_f value 0.7. The NMR spectra in D_2O was consistent with the structure of the monoethylester (12). Molecular weight determinations using perchloric acid as a titrant, according to the method described by Pifer *et al.* (13), gave a value of 377.3, corresponding to that of Na IV.

Elemental analysis of the sodium content corresponded to the theoretical value calculated for (Na IV) and the X-ray diffraction pattern showed no sodium iodide, indicating existence of Na IV, as such, in the solid state. A solution of Na IV was degraded at 121° in 3 *N* HCl, and the presence of free thiol (II) was detected by thin-layer chro matography.

Mechanism of Degradation in Weakly Acidic Media—The observed rate of formation of ethanol from echothiophate iodide was not influenced by changes in concentration of hydroxyl ion. The slight buffer catalysis observed was considered insignificant in the overall reaction.

The rate of hydrolysis of triethylphosphate (5) and trimethylphosphate (4) were also investigated in neutral and acidic media. The reported values for the observed first-order rate constants for formation of ethanol from triethylphosphate at 101° at pH 1.0 and 4.9 were 3.85×10^{-3} min.$^{-1}$ and 4.2×10^{-3} min.$^{-1}$, respectively. The observed first-order rate constants for formation of methanol from trimethylphosphate at 101° in water and in 3 *M* perchloric acid were reported to be 2.2×10^{-3} min.$^{-1}$ and 2.0×10^{-3} min.$^{-1}$, respectively. The observed first-order rate constant for the formation of ethanol from echothiophate iodide at pH 2.6 and 101° calculated by extrapolation from data shown in Fig. 11 was found to be 6.1×10^{-3} min.$^{-1}$.

A postulated mechanism, in the acidic pH range, which is consistent with the obtained results and the reported mechanism for the hydrolysis of trimethylphosphate, may be written as in Scheme II,



Scheme II

where the rate determining step would be the initial attack by H_2O on echothiophate iodide.

EXPERIMENTAL

Reagents and Apparatus—Echothiophate iodide (98% potency) and (2-mercaptoethyl)trimethylammonium iodide were obtained from the Research Department, Ayerst Laboratories, Division of Ayerst, McKenna and Harrison Ltd., Montreal.

Buffer, standard sodium hydroxide, iodine, and perchloric acid solutions were prepared using reagent grade materials.

Water used in the studies was prepared by distilling tap distilled water from an acidic permanganate solution.

The instruments used in this study were a Cary model 14 spectrophotometer, a pH stat, catalog No. S-30240, an Aerograph 204B gas chromatograph, and a Beckman pH meter.

Kinetic Procedures—The hydroxyl and hydrogen ion concentrations of the system were maintained by using buffer solutions or excess base. Buffer systems used in the study were: phosphate, pH's 2.4, 6.3, 10.8–11; acetate, pH 4.6; glycine, pH 8.1; borate, 9.5–10; and dilute NaOH (carbonate free) for pH's above 11.

The ionic strength of the solutions was adjusted to the desired value by the addition of sodium chloride. Most of the determinations were run at a total ionic strength of 0.5 unless otherwise specified.

Rate of Formation of (2-Mercaptoethyl)trimethylammonium Iodide—The rate of degradation of echothiophate iodide in alkaline pH range was determined by measuring the rate of formation of (2-mercaptoethyl)trimethylammonium iodide by three independent methods: iodometrically, spectrophotometrically, and on a pH stat.

When the reaction was followed iodometrically, solutions of 0.200 Gm. of the compound in 50 ml. of water and 50 ml. of 0.20 *M* buffer of the desired pH were equilibrated in separate flasks in a constant-temperature bath and then mixed. Periodically, 5-ml. samples (2 mg./ml.) were quickly discharged into a 50-ml. conical flask containing 5 ml. of 0.1 *M* phosphoric acid in order to quench the reaction. The thiol was then titrated with 1×10^{-3} *M* iodine solution under a blanket of nitrogen using starch as an indicator to obtain V_t (ml. of iodine solution at time t). A final titration was performed to obtain V_∞ (ml. of iodine solution at infinite time which corresponds to essentially complete hydrolysis).

Spectrophotometric determinations were made at 226 mμ. Although the ultraviolet absorption spectra of echothiophate iodine and its hydrolytic product, the thiol (pKa 7.6 determined spectrophotometrically), are practically identical in acid media (molar absorptivity values for echothiophate iodide and the thiol at pH 4.2 are 1.3×10^4 and 1.4×10^4 M^{-1} cm.$^{-1}$, respectively), the salt form of the thiol has sufficiently higher molar absorptivity that it can be differentiated in alkaline media (2×10^4 M^{-1} cm.$^{-1}$ at pH 9.1). A fresh 2.6×10^3 *M* solution of echothiophate iodide was prepared in water and a 0.1-ml. portion was transferred to a 1.0-cm. light path silica cell. Three milliliters of 0.1 *M* phosphate buffer of the desired pH was injected into the cell, and the change in absorbance at 226 mμ was followed in the Cary model 14 recording spectrophotometer. After the run was completed, the pH of the reaction mixture was determined to be unchanged.

The rate of the alkaline hydrolysis was also determined from data obtained on a pH stat. In 50 ml. of water 0.200 Gm. of the compound was dis-

solved and the consumption of hydroxyl ion, under nitrogen, was followed at constant temperatures and fixed pH. Not more than 1 ml. of 1.0 N sodium hydroxide was added totally to maintain the pH throughout the reaction.

The effect of buffer concentration upon the alkaline rate of hydrolysis was studied iodometrically at pH 10.85 using 0.1 and 0.2 M phosphate buffers and total ionic strength of 1.0.

The effect of ionic strength upon the rates of hydrolysis was studied in 3.5×10^{-3} N sodium hydroxide containing sodium chloride to give the desired ionic strength. The reaction was followed spectrophotometrically by adding the sodium hydroxide solutions to the echothiophate iodide as described above.

The effect of temperature on the rate of the alkaline hydrolysis was determined iodometrically at pH 10.85, 0.1 M phosphate buffer, and on a pH stat at pH 10.5 as described above.

Rate of Loss of Echothiophate Iodide and Rate of Formation of Ethanol—The degradation of echothiophate iodide below pH 9 was determined following the rate of loss of the compound and the rate of formation of ethanol.

Echothiophate iodide solutions, of concentration 2.4×10^{-2} M were prepared using phosphate, acetate, and glycine buffers of the desired pH and ionic strength of 0.5. The solutions were filled into hard glass ampuls and saturated with nitrogen before sealing. The ampuls were then placed in a constant-temperature bath which was maintained at the desired temperature ±0.05°. After allowing 15 min. for temperature equilibration, the 0-hr. samples were removed and further samples were withdrawn at time intervals suitable to the nature of the system. The reaction was quenched by immersion of the samples in ice water. The samples were then frozen till the end of each experiment.

The residual concentration of echothiophate iodide in the ampuls was determined using a modified USP method, in which potassium iodate as a titrant was replaced by standard iodine solution. A sample containing approximately 10 mg./ml. of echothiophate iodide was transferred into a conical flask. Two milliliters of 1.0 N sodium hydroxide was added, the flask was flushed with nitrogen, and stoppered. The solution was allowed to stand for 10 min. at room temperature. Two milliliters of 1 M phosphoric acid was then added, and the liberated thiol was titrated with 2×10^{-3} moles/L. standard iodine solution using starch as indicator. A residual blank titration for the thiol in solution was performed using the same procedure except the alkaline hydrolysis was omitted. The difference between the volume of iodine solution consumed in the two determinations is equivalent to the amount of thiol literated from the intact molecule. The concentration of echothiophate iodide can then be calculated as follows:

$$\text{moles/L. echothiophate iodide} = \frac{\text{ml. } I_2 \times 2 \times 10^{-3}}{\text{ml. sample size}}$$

The concentrations of ethanol in the ampuls were determined by gas chromatography using a 5-ft., 1/8-in. o.d. stainless steel column packed with 8% w/w didecylphthalate on 80/100 mesh Gas-chrom Z.

The following instrument parameters were used. Nitrogen carrier, 40 ml./min., column temperature 80°, sample size, 3 μl.

The amount of ethanol in the sample was determined by comparison with a standard aqueous ethanol solution.

The effect of buffer concentration on the rate of hydrolysis in the acidic pH range was determined at pH's 2.4, 6.3 phosphate and pH's 4.6 and 5.2 acetate, and ionic strength of 0.5.

The effect of temperature on the acid rate of hydrolysis was determined at elevated temperatures at pH's 2.4 and 4.6. Samples were also left at room temperature, pH 2.4, and the reaction was followed over a 1-year period.

Stability of (2-Mercaptoethyl)trimethylammonium Iodide—The stability of (2-mercaptoethyl)-trimethylammonium iodide was determined at 70° at pH's 2.4, 4.6, 6.3, and 8.1. Fresh 2.4×10^{-2} M solutions were stored in ampuls under nitrogen. The concentration of thiol was determined periodically by titrating with 2×10^{-3} M iodine to the blue starch end point.

Isolation and Characterization of Trimethyl (2-hydroxyethyl) Ammonium Iodide, S-ester with Phosphorothioic Acid, O-ether Ester (Na IV)—Three grams of echothiophate iodide was dissolved in 30 ml. of 1 M acetate buffer pH 4.6. The solution was transferred into a 50-ml. ampul and saturated with nitrogen before sealing. The ampul was kept for 4 days at 80° to ensure complete degradation. The solution was then transferred into a round-bottom flask and evaporated to dryness under vacuum at 80°. The solid residue was extracted with hot isopropanol (3 × 30 ml.), and the extracts were left at refrigerator temperature overnight. The separated crystalline compound had an m.p. 119°.

Thin-layer Chromatographic Analysis—Alcoholic solutions of (Na IV) and echothiophate iodide were prepared by dissolving 10 mg. in 1 ml. of methanol. Ten microliters of each solution was spotted directly on a chromatographic plate prepared with Silica Gel G. The plate was developed using a solvent mixture of two parts methanol, two parts water, and one part ammonium hydroxide. When placed in an iodine chamber, two spots were visible, one with R_f 0.7 corresponding to that of (Na IV), and the other of R_f 0.48 corresponding to echothiophate iodide. Twenty milligrams of (Na IV) was hydrolyzed with 3.0 N HCl at 121° and the hydrolytic product was identified using the above chromatographic procedure, as (2-mercaptoethyl)trimethylammonium iodide.

Sodium Analysis—The sodium content of the compound was determined by flame photometric analysis. Sodium calculated for $C_7H_{14}INO_3PSNa$, 6.1, found 6.2.

Resistance to Mild Alkaline Hydrolysis—To 20 mg. of (Na IV) in 3 ml. of water, 2 ml. of 1.0 N sodium hydroxide was added. No thiol was detected after 10 min. at room temperature as determined iodometrically with 2×10^{-3} M iodine solution.

SUMMARY

1. The overall degradation rate of echothiophate iodide in aqueous solution has been shown to be first order with respect to the compound over a wide pH and temperature range.

2. In alkaline media above pH 9, the main degradative pathway was the hydrolytic cleavage at

the sulfur–phosphorus bonds for form (2-mercaptoethyl) ammonium iodide. The rate of this reaction was found to be first order with respect to hydroxyl ion. The second-order rate constant at 25° was calculated to be 59.7 M^{-1} min.$^{-1}$. The apparent activation energy was determined to be 22 Kcal./mole with a standard error of 1 Kcal./mole. Since this energy of activation includes the heat of ionization of water, the energy of activation for the hydroxyl reaction would be 10 Kcal./mole.

3. Below pH 5 the main degradative route was the hydrolytic cleavage at the carbon oxygen bonds to form ethanol and trimethyl (2-hydroxylethyl) ammonium iodide, *S*-ester with phosphorothionic and *O*-ethyl ester. The rate of this reaction was not influenced by changes in concentration of hydroxyl ion but was increased slightly by increased buffer concentration. The attacking species were postulated to be a water molecule and the activation energy was determined to be 23 Kcal./mole.

4. The overall first-order rate constant for echothiophate iodide in aqueous solution was presented at 25° as a function of pH.

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Keyphrases

Echothiophate iodide degradation
pH effect—echothiophate iodide degradation
Hydrolysis, echothiophate iodide—temperature effect
Degradation products—acidic, alkaline media
GLC—analysis
TLC—analysis
UV spectrophotometry—analysis
Flame photometry—analysis
Iodometric analysis

Inhibition of Acetylcholinesterase by Chelates III

By ERNEST MARIO* and SANFORD BOLTON†

Inhibition of acetylcholinesterase by 1–1 cupric chelates of 1,3-diaminopropanol-2 and 1,3-diaminopropane is analyzed in the pH range of 8.0–9.0. The terdentate chelate of 1,3-diaminopropanol-2 exhibits weak inhibitory activity at pH values below 8.5 and increased activity at pH values above 8.5. The bidentate chelate of 1,3-diaminopropane exerts significant inhibition at pH 8.0. The bidentate system exhibits essentially noncompetitive inhibition while the terdentate system appears to be essentially competitive. Increased inhibition at elevated pH in the terdentate system is further evidence that the chelate is interacting with an ionizing group(s) on the enzyme surface, as previously reported.

As PART of a continuing study to investigate metal chelate inhibition of the acetylcholinesterase–acetylcholine enzyme reaction system, copper chelates of 1,3-diaminopropanol-2 (AOH) and 1,3-diaminopropane (AH) were prepared. These two structurally similar ligands were chosen because (*a*) the cupric chelates of AOH and AH had been clearly elucidated in an earlier investigation (1) and (*b*) it was of interest to examine the comparative effects of the two ligands, one of which is capable of forming both a 1–1 and 2–1 and the other only a 1–1 chelate.

In the first papers of this series, Bolton (2, 3) described some of the problems which are incurred in a study of this type. Since the chelate solutions contain an equilibrium mixture of several species, it is necessary to monitor closely the absolute concentrations of free ligand, free metal, 1–1 chelate, and 2–1 chelate under specific experimental conditions to determine which specie(s) is responsible for enzyme inhibition. The information obtained from the first two papers in this series may be briefly summarized as follows: (*a*) metal chelates inhibit the acetylcholinesterase–acetylcholine interaction; (*b*) in general, free ligand does not inhibit the interaction; (*c*) cupric

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ion alone is an inhibitor above concentrations of approximately $3 \times 10^{-5} M$ (the inhibitory effects of free metal ion may be eliminated if the chelate stability constant is large, or high pH values are used); (*d*) available coordination sites on the metal ion appear to be necessary prerequisites for inhibition; (*e*) overall chelate charge has a significant increasing effect on enzyme inhibition at elevated pH values; and (*f*) the 1–1[1] chelate species of Cu^{2+} appears to inhibit the enzyme system in a primarily "noncompetitive" manner.

The concentrations of individual species in an equilibrium mixture of ligand and metal under fixed conditions of concentration and pH may be calculated with a knowledge of the total metal, total ligand, chelate formation constants, ligand dissociation constants, and (H^+). The equations used to calculate the individual species in cupric chelate mixtures have been described (2). The values for free ligand (L) and free metal (M) can be solved from these equations by a method of successive approximations.[2] The values of (L) and (M) may then be used to calculate the concentrations of the chelate species present. In the case of the AOH-cupric interaction, a very strong 1–1 terdentate chelate is formed (1) with no discernible amount of 2–1 chelate species present. In the AH-cupric interaction, both a 1–1 bidentate chelate and a 2–1 tetradentate chelate are formed. The 1-1 AOH-Cu chelate carries a net (+1) charge while the 1–1 AH-Cu chelate carries a net (+2) charge. Thus, the two systems present an opportunity to compare the effects of metal coordination vacancies and overall chelate charge on enzyme inhibition.

EXPERIMENTAL

Reagents—AOH and AH (Aldrich Chemical Corp.) were obtained in the impure, free amine form. The di-HCl salts of these two ligands were prepared by dissolving the appropriate diamine species in excess concentrated hydrochloric acid (analytical reagent grade), flash evaporating to dryness and recrystallizing several times from methanol–ethanol mixtures (for AOH) and methanol–ethanol–water mixtures (for AH). Salt purity was checked by titration with standard base and the pKa values were compared to literature values, where available. Acetylcholine iodide and acetylcholinesterase (Nutritional Biochemicals Corp.) were used. Sodium chloride, magnesium chloride, and cupric nitrate, all analytical reagent grade, were also employed. The cupric nitrate solutions prepared were standardized according to the chelatometric titration method described by Wilson and Wilson (4) using murexide as an indicator. The diluent in all studies was prepared as previously reported (2).

[1] The 1–1 and 2–1 chelate species refer to (ligand–metal) and (ligand$_2$–metal) complexes, respectively.

[2] An IBM 7094 computer was used to solve the equations. The solution yielded values so that (MT) and (M) + (ML) + (ML_2) agreed to 10 decimal places.

Procedure—The experimental conditions and procedures were essentially the same as previously described (2). The values of V_0/V, (velocity with no inhibitor/velocity with inhibitor), and the values of $1/V$ were taken as the relative time necessary to consume 0.30 ml. of approximately 0.005 *N* base if the hydrolysis was linear as a function of time. The data were analyzed using the Michaelis-Menten theory of enzyme kinetics as extended by Friedenwald and Maengwyn-Davies to the case of a dissociable inhibitor (3, 8).

RESULTS AND DISCUSSION

Acid Dissociation and Chelate Stability Constants—The values of the constants required to determine the concentrations of species present in the chelate equilibrium mixtures are shown in Table I. Literature values are included where available.

Inhibition by Chelate Mixtures—Ligand-metal mixtures of different ratios[3] were used to inhibit the enzyme-substrate reaction at various pH values in the range of 8.0–9.0. The selection of pH values and chelate mixture ratios was governed by the desire to obtain one chelate species in predominance to all other species and the necessity of keeping the free metal concentration below approximately 3×10^{-5} *M* to prevent free metal initiated enzyme inhibition. Typical results of enzyme inhibition by solutions containing a 2.1–1 ligand–metal ratio of AOH-Cu at several pH values are shown in Table II. The concentration of 1–1 chelate is directly related to V_0/V. The choice of chelate mixture ratios with this system was not critical; the formation constant of the terdentate chelate attachment being very large, results in constant molar values for the 1–1 chelate species and extremely minute quantities of free metal. The values for V_0/V shown in Table II indicate a decrease in inhibition from pH 8.0 to pH 8.5 and then an increase in inhibition in the pH range of 8.5 to 9.0. Since the 1–1 chelate species formed has been shown to be stable up to pH 10, the increased inhibition cannot be due to chelate dissociation and may therefore be attributed to charge changes on the enzyme surface. The strong possibility exists that the pKa of the group(s) on the enzyme surface directly responsible for chelate attachment or proximal to the point of attachment has been reached in the pH range of 8.5–9.0. That the anionic site of the enzyme or some other negatively charged site is probably involved, was the hypothesis Bolton offered in the first paper of this series (2), and the data shown in Table II appears to support this proposed mechanism of inhibition.

The results of enzyme inhibition by several ligand-metal ratio solutions of AH–Cu^{2+} at pH 8.0 are shown in Fig. 1. The concentration of 1–1 chelate species is seen to be directly related to V_0/V. It was not possible to obtain meaningful data at pH values above 8.0 using the above-mentioned chelate mixture ratios. There was no apparent linear relationship between any of the species present and V_0/V. Some possible explanations for this behavior are: (*a*) the double plus charge in Cu^{2+} chelates of AH changes either the site or mechanism of binding on the enzyme surface and/or (*b*) the concentration of 1–1 chelate species was so small compared to 2–1 chelate species (at pH values above 8.0) that a

[3] Chelate mixture ratios refer to (LT)/(MT)

TABLE I—ACID DISSOCIATION CONSTANTS AND CHELATE STABILITY CONSTANTS FOR AOH AND AH AND THEIR CUPRIC CHELATES

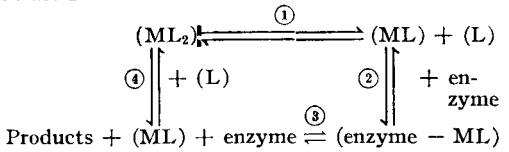
Ligand	pKa_1	pKa_2	pKa_3	Ionic Strength	Temp., ° C.	Ref.
AOH	8.01	9.58	14.20	0.162	30	...
AH	8.50	10.24	...	0.162	30	...
			Literature Values			
AOH	8.23	9.68	...	0.100	20	(5)
AH	8.88	10.64	...	0.100	20	(6)

Ligand	log K_1	log K_2	Ionic Strength	Temp., ° C.	Ref.
AOH	18.40	...	0.162	30	...
AH	9.29	6.96	0.162	30	...
		Literature Values			
AOH	...	...	...	..	...
AH	9.62	7.00	1.000	20	(7)

TABLE II—INHIBITION BY 1-1 AO-Cu^+ CHELATE IN A 2.1-1 CHELATE MIXTURE RATIO SOLUTION

pH	Molar Concn. Chelate × 10^{-3}	Molar Concn. 1-1 Chelate × 10^{-3}	Molar Concn. Free Cu^{2+} × 10^{-10}	V_0/V
8.0	1	0.9	0.45	1.021
	2	1.9	0.45	1.048
	3	2.9	0.45	1.072
	4	3.9	0.45	1.096
8.25	1	0.9	0.11	1.020
	2	1.9	0.11	1.037
	3	2.9	0.11	1.058
	4	3.9	0.11	1.080
8.50	1	0.9	0.031	1.016
	2	1.9	0.031	1.034
	3	2.9	0.031	1.057
	4	3.9	0.031	1.070
8.75	1	0.9	0.028	1.019
	2	1.9	0.028	1.039
	3	2.9	0.028	1.060
	4	3.9	0.028	1.080
9.00	1	0.9	0.003	1.042
	2	1.9	0.003	1.089
	3	2.9	0.003	1.134
	4	3.9	0.003	1.184

rate-determining equilibrium was achieved between the enzyme and the chelate solution according to Scheme I.



Scheme I

Analysis of Inhibition—The inhibition of acetylcholinesterase by the 1–1 cupric chelate of AOH at pH 8.5 was analyzed according to the methods suggested by Friedenwald and Maengwyn-Davies (8). The results of this study are tabulated in Table III and shown graphically in Fig. 2. The inhibition by the AOH chelate appeared to be essentially competitive at pH 8.5 with a small, noncompetitive component. This small noncompetitive component may in fact be the result of experimental error since the inhibitory properties of the 1–1 AO-Cu^+ chelate were relatively weak. The inhibition of acetylcholinesterase by the 1–1 cupric chelate of AH at pH 8.0 was analyzed by the same method, and the results of this study are tabulated in Table IV and shown graphically in Fig. 3. The inhibition by the 1-1 AH-Cu^{2+} chelate appeared to be essentially

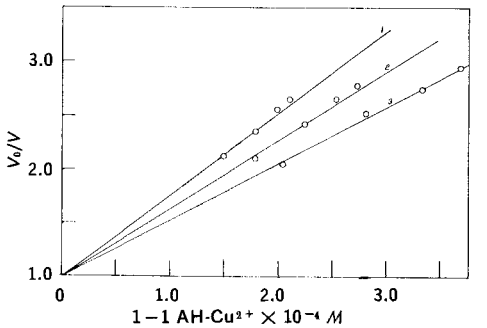


Fig. 1—Inhibition by 1–1 AH–Cu^{2+} chelate in several ligand–metal ratios at pH 8.0. Key: 1, ligand-metal ratio = 2.3–1; 2, ligand–metal ratio = 2.2–1; 3, ligand–metal ratio = 2.1–1.

TABLE III—INHIBITION BY 1–1 AO–Cu^+ CHELATE IN A 2.2–1 CHELATE MIXTURE RATIO SOLUTION AT pH 8.5 WITH VARYING SUBSTRATE CONCENTRATIONS

Molar Concn. of Substrate × 10^{-3}	Molar Concn. of Chelate Mixture × 10^{-3}	Molar Concn. of 1-1 Species × 10^{-3}	Reaction Velocity $(1/V)$
1.22	0	0.0	49.0
	1	0.9	49.5
	2	1.9	51.0
	3	2.9	52.0
	4	3.9	53.5
0.99	0	0.0	50.0
	1	0.9	52.0
	2	1.9	53.0
	3	2.9	54.5
	4	3.9	57.0
0.73	0	0.0	53.5
	1	0.9	55.0
	2	1.9	57.0
	3	2.9	58.5
	4	3.9	62.0
0.49	0	0.0	58.0
	1	0.9	61.0
	2	1.9	63.5
	3	2.9	66.0
	4	3.9	69.0

noncompetitive at pH 8.0 in agreement with earlier results reported by Bolton (3).

Relation of Inhibition to Net Chelate Charge and Coordination Vacancies—While the concentration of the 1–1 AO–Cu^+ chelate did not change between

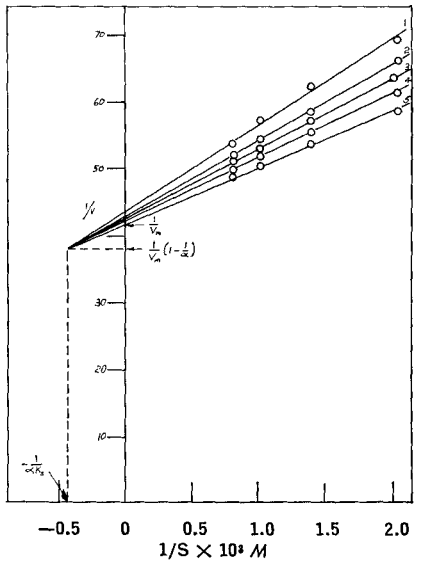


Fig. 2—Analysis of inhibition by 1–1 AO–Cu⁺ chelate at pH 8.5 using 1/S values. Key: 1, (I) = 3.9 × 10⁻³ M; 2, (I) = 2.9 × 10⁻³ M; 3, (I) = 1.9 × 10⁻³ M; 4, (I) = 0.9 × 10⁻³ M; 5, (I) = 0.

TABLE IV—INHIBITION BY 1-1 AH-Cu²⁺ CHELATE IN A 2.1-1 CHELATE MIXTURE RATIO SOLUTION AT pH 8.0 WITH VARYING SUBSTRATE CONCENTRATION

Molar Concn. of Substrate × 10⁻³	Molar Concn. of Chelate Mixture × 10⁻³	Molar Concn. of Free Cupric Ion × 10⁻⁴	Molar Concn. of 1-1 Species × 10⁻⁴	Molar Concn. of 2-1 Species × 10⁻³	Reaction Velocity 1/V
1.22	0	0.00	0.00	0.00	50.5
	1	2.50	2.06	0.79	101.0
	2	2.17	2.82	1.72	124.5
	3	1.96	3.34	2.67	138.0
	4	1.79	3.73	3.63	149.0
0.99	0	0.00	0.00	0.00	53.0
	1	2.50	2.06	0.79	111.0
	2	2.17	2.82	1.72	133.0
	3	1.96	3.34	2.67	148.0
	4	1.79	3.73	3.63	161.0
0.73	0	0.00	0.00	0.00	57.5
	1	2.50	2.06	0.79	125.0
	2	2.17	2.82	1.72	148.5
	3	1.96	3.34	2.67	162.0
	4	1.79	3.73	3.63	170.5
0.49	0	0.00	0.00	0.00	65.6
	1	2.50	2.06	0.79	142.0
	2	2.17	2.82	1.72	169.0
	3	1.96	3.34	2.67	192.0
	4	1.79	3.73	3.63	211.0

pH 8.0 and 9.0, the inhibition of the enzyme system increased when the pH exceeded 8.5 (see Table II). Since there is only one available coordination vacancy in the terdentate 1–1 AOH chelate, the increased inhibition may be attributed to the net +1 charge on the chelate being attracted to some negatively charged site on the enzyme surface with a pKa value between 8.5 and 9.0. At pH values below 8.5 the inhibition was slight. This may be expected considering the assumption that metal coordination vacancies are necessary at low pH values for inhibition to occur (the 1–1 AOH chelate having only one available coordination site, access to which is spacially hindered by the strong terdentate attachment). The results of studies with the AH system lend support to this hypothesis. The 1–1 AH chelate has two coordination vacancies and

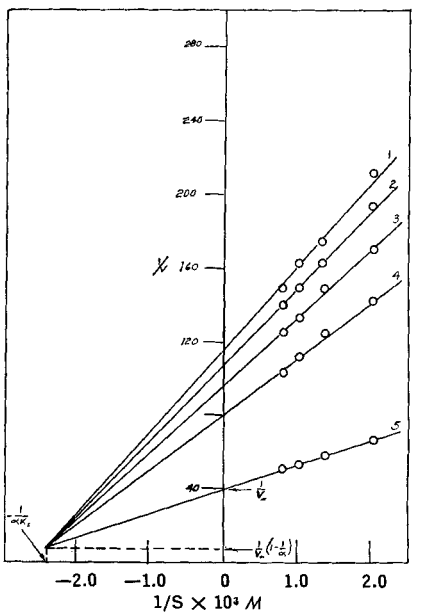


Fig. 3—Analysis of inhibition by 1–1 AH–Cu²⁺ chelate at pH 8.0 using 1/S values. Key: 1, (I) = 3.73 × 10⁻⁴ M; 2, (I) = 3.34 × 10⁻⁴ M; 3, (I) = 2.82 × 10⁻⁴ M; 4, (I) = 2.06 × 10⁻⁴ M; 5, (I) = 0.

is a stronger inhibitor than the 1–1 AOH chelate at pH 8.0 (see Fig. 1). At elevated pH values it was not possible to correlate inhibition with any individual species concentration present. This was of particular interest since the 1–1 AH chelate carries a net +2 charge and would probably be attracted to a negatively charged site on the enzyme surface more strongly than the single +1 charge of the AO–Cu⁺ chelate.

SUMMARY

Previous analyses of enzyme-chelate interactions have shown that 1–1 chelate species of ethylenediamine and glycine yield noncompetitive enzyme inhibition at pH 8.0 (see *Reference 3*). This study has shown that the 1–1 chelate species of AH also inhibits acetylcholinesterase in an essentially noncompetitive manner at pH 8.0. This was not unexpected since all of the above-mentioned chelate species are similar in that they have two metal coordination vacancies. The 1–1 chelate species of AOH was found to inhibit the enzyme weakly in an essentially competitive manner that may be attributed to the single, hindered, metal coordination vacancy present in the terdentate chelate attachment. At pH values above 8.5, the 1–1 chelate species of AOH exhibited stronger inhibitory properties. This can be explained on the basis of the predominance of a charge attraction between the chelate and the enzyme surface compared to a purely metal–enzyme attachment *via* the coordination vacancy. At pH values above 8.0, the 1-1 chelate species of AH was no longer linearly related to inhibition. Since this chelate carries a doubly positive net charge, the magnitude of any charge affinity between the chelate and a negatively charged

site on the enzyme surface would be large. If in fact, the mechanism of chelate–enzyme interaction changes from metal coordination attachment to charge attraction at elevated pH values, as the data would appear to indicate, it may be possible to identify the specific amino acid(s) responsible for the charge attraction from the estimated pKa. Further investigations are being contemplated to pursue these avenues.

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(5) Gonick, E., Doctoral dissertation, The Pennsylvania State College, 1951.
(6) Schwarzenbach, G., Maissen, B., and Ackerman, H., *Helv. Chim. Acta*, **35**, 2333(1952).
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Keyphrases

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Cupric chelate synthesis—1,3-diaminopropanol and 1,3-diaminopropane
Dissociation constants—acid
Chelate stability constants
Inhibition, acetylcholinesterase—analysis
pH Effect—acetylcholinesterase inhibition

Comparison of the Absorption and Excretion of Calcium from Precipitated Calcium Carbonate USP XVII and Egg Shells

By ARMEN H. MIRZAIAN and E. BLANCHE SOMMERS

Experiments were conducted to evaluate relative absorption and excretion of calcium from precipitated calcium carbonate USP and powdered egg shells (93.7 percent). Samples from 24-hr. specimens of urine and feces were collected after the fourth day of a repeated daily diet of the same food containing approximately 1 Gm. of calcium. The subjects were continued on the same regimen and samples were collected for 10 consecutive days from each person tested. The calcium content of the samples was determined by means of a flame photometer and a spectrophotometer. This procedure was repeated with addition of 2.5 Gm. of the official precipitated $CaCO_3$ and again with 2.6 Gm. of powdered egg shells to each daily diet. The calcium balance, that is, the difference between the quantity of calcium ingested and the total amount excreted, was positive for each type of experiment. When the diet was supplemented with the precipitated $CaCO_3$ and egg shells, calcium retention was increased 86.3 and 97.9 percent, respectively. Increased calcium absorption was reflected in increased urinary calcium. Egg shells compared favorably with the precipitated calcium carbonate as a source of absorbed calcium.

EXTENSIVE STUDIES have been concerned with nutritional standards for calcium. The National Research Council recommends a daily dietary allowance of 0.8 Gm. of calcium for an adult (1). Goodman and Gilman state that the average adult's daily requirement is about 10 mg./Kg. of body weight (2).

Calcium is the fifth most abundant element in the body, and the major fraction is in the bony structure (99%). It is present in small quantities in the extracellular fluid and to a minor extent in the structure and cytoplasm of cells of soft tissue. In the instance of hypocalcia, daily intake is frequently supplemented with a calcium salt. Wohl (3) prescribed the clinical use of calcium carbonate in preference to other calcium salts since it has a higher percentage of available calcium (40%).

The high calcium content of egg shells—*Gallus domesticus* Temminck (family, *Phasianidae*): calcium carbonate, 93.7%; magnesium carbonate, 1.3%; phosphorus pentoxide, 0.76%; and organic matter, 4.15% (4)—prompted investigation in regard to their value as a source of calcium. Limited studies of this nature have been done. However, they were primarily concerned with the palatability of foods containing powdered egg shells.

The studies presented here are concerned with comparison of calcium balances when the diet is

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Accepted for publication October 17, 1967.
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supplemented first with precipitated calcium carbonate USP XVII and then with egg shells containing an equivalent amount of calcium.

ABSORPTION AND EXCRETION OF CALCIUM

The problem of defining in a precise manner the relationship between the quantity of calcium ingested and that which is excreted in the stool in terms of absorption alone is exceedingly difficult, owing to the fact that calcium is not only absorbed from the gut lumen but secreted into the luminal contents as well. Therefore, the quantity of fecal calcium represents the net effects of the amounts ingested, absorbed, and secreted. Although considerable progress has been made in understanding some of these interrelations with the use of isotopes of calcium, much remains to be elucidated.

The urinary excretion of calcium appears to be the net effect of the quantity filtered and the amount reabsorbed. There is no evidence of renal tubular secretion of calcium. The mechanisms for the renal absorption of calcium are unknown (5).

According to Best and Taylor (6), the calcium of the feces amounts daily to from 0.4 to 0.8 Gm.; this, though a considerable amount is endogenous, is mainly the unabsorbable calcium of the food. About 150 mg. is excreted daily in the urine. An increase or decrease in the absorption of calcium is reflected in parallel changes in the urinary excretion. The urinary calcium constitutes, therefore, a convenient index of calcium absorption.

Krantz and Carr (7) state that about 30% of the calcium ingested by man is absorbed and that the remainder is excreted in the feces. The excess of the absorbed calcium is excreted in the urine, mainly as calcium phosphate. From 0.1 to 0.4 Gm. of calcium is excreted daily in the urine, dependent upon the dietary intake.

Grollman (8) reports that approximately 80% of ingested calcium is excreted in the feces. The remaining 20% of the ingested calcium in an individual, in calcium equilibrium, is excreted in the urine. With low and moderate levels of intake, 0.1–0.5 Gm. daily, approximately 30–50% is eliminated in the urine, while with high levels, 1 Gm. daily, approximately 25% is excreted. However, considerable deviations from these values occur, dependent upon variable dietary, metabolic, and gastrointestinal factors (8).

EXPERIMENTAL

Ten men ranging from 20 to 25 years of age served as the test subjects. All had normal renal function and no evidence of malabsorption.

Representative samples from pooled 24-hr. specimens of urine and of feces were obtained from each individual. This procedure was initiated after the fourth day of a repeated daily diet of the same food containing approximately 1 Gm. of calcium. The subjects were continued on the same regimen and samples were collected for 10 consecutive days thereafter. After intervals of 10 days, this procedure was repeated first with the addition of 2.5 Gm. of the official precipitated calcium carbonate and then with the addition of 2.6 Gm. of powdered egg shells to each daily diet. Preliminary studies indicated that pretreatment levels of excreted calcium were regained within 10 days after discontinuation of calcium therapy. Accordingly, a 10-day interval before the administration of each of the supplemented diets was allowed. Samples were refrigerated in tightly closed containers until they were assayed for calcium content.

The *Heinz Handbook of Nutrition* (9) was used in the selection of the basic unsupplemented diet which contained approximately 1 Gm. of calcium. Considerations other than calcium content were caloric content, balance of food elements, and food preferences of the 10 subjects. Food portions were weighed and each meal was prepared in the same manner by the same personnel.

This basic diet (Table I) was used for each of the 10 subjects in each of the three phases of the study. During the first phase of the study, it was administered without supplementation. In the second and the third phases of the study, it was supplemented with calcium carbonate and egg shells, respectively. The daily dose of each of these substances was administered in a portion of the milk consumed at breakfast. All of the 10 subjects first received the basic diet, then the calcium carbonate supplemented diet, and finally the egg shell supplemented diet.

A search of the literature concerned with experiments in regard to calcium balance did not reveal control of water intake. Accordingly, this study was not concerned with this aspect. However, total daily urine and feces for each subject were collected and tested for calcium content.

Egg shells used for the experiment were washed and their membranes were removed. The shells after being air dried were reduced to the consistency of the official calcium carbonate, and the powder thus obtained was thoroughly mixed. The official assay specified for calcium carbonate was used to determine the total calcium content of this powder

TABLE I—BASIC, UNSUPPLEMENTED DIET CONTAINING APPROXIMATELY 1 GM. OF CALCIUM

	Weight, Gm.	Calories	Calcium, mg.
Breakfast			
Bread, whole wheat	23	55	22
Butter	7	50	10
Milk	488	332	576
Orange juice, fresh	246	108	47
Coffee, black	230	9	4
Egg	54	77	29
Bacon	32	194	8
Lunch			
Bread, whole wheat	23	55	22
Butter	14	100	20
Potato, baked	100	97	13
Beef, roast	172	378	28
Peas, canned	60	111	24
Tomato, fresh	150	30	16
Peaches, canned	258	175	13
Cola	180	83	...
Dinner			
Bread, whole wheat	23	55	22
Butter	7	50	10
Chicken, roasted	115	227	16
Beans, lima	249	176	67
Salad, tossed green	144	168	38
Pie, apple	220	330	15
Ginger ale	180	63	...
Total		2,923	1,000

TABLE II—EFFECTS ON CALCIUM BALANCE OF EXTRA CALCIUM FROM PRECIPITATED CALCIUM CARBONATE USP XVII AND EGG SHELLS

	Unsupplemented Diet[a] —Calcium, 1,000 mg./Day—				Calcium Carbonate, 2.5 Gm. + Unsupplemented Diet = —Calcium, 1,980 mg./day—				Egg Shells, 2.6 Gm. + Unsupplemented Diet = —Calcium, 1,980 mg./day—			
Subject	Av. Urinary Output, mg./Day	Av. Fecal Output, mg./Day	Av. Calcium Balance, mg./Day	± S.D.	Av. Urinary Output, mg./Day	Av. Fecal Output, mg./Day	Av. Calcium Balance, mg./Day	± S.D.	Av. Urinary Output, mg./Day	Av. Fecal Output, mg./Day	Av. Calcium Balance, mg./Day	± S.D.
1	92	708	+200	±30.4	113	1,566	+301	±10.3	228	1,403	+349	± 22.4
2	110	763	+127	±20.0	183	1,621	+176	±14.6	188	1,458	+334	± 14.5
3	115	754	+131	±16.7	240	1,651	+ 89	± 9.4	165	1,550	+265	± 14.4
4	65	803	+132	±10.0	228	1,598	+154	± 9.6	169	1,573	+238	± 14.0
5	20	850	+130	±11.8	77	1,486	+417	±15.5	215	1,608	+157	± 5.4
6	79	896	+ 25	± 2.8	157	1,590	+233	±24.4	193	1,314	+473	± 28.5
7	141	797	+ 62	± 5.4	181	1,538	+261	±24.1	199	1,610	+171	± 19.5
8	117	683	+200	±17.8	199	1,594	+187	±18.9	160	1,541	+279	± 15.0
9	207	577	+216	±21.9	168	1,530	+282	±27.3	196	1,710	+ 74	± 4.7
10	128	823	+ 49	± 6.2	136	1,573	+271	±28.2	158	1,645	+177	± 14.3
Av.	107	765	+127	±66.1	168	1,575	+237	±91.4	187	1,541	+252	±115.0

[a] The unsupplemented diet (control diet) containing approximately 1 Gm. of calcium was the same in all three cases.

TABLE III—COMPARISON OF CALCIUM RETENTION FROM OFFICIAL PRECIPITATED CALCIUM CARBONATE AND FROM EGG SHELL SUPPLEMENTED DIETS TO CALCIUM RETENTION FROM THE UNSUPPLEMENTED DIET

	Av.[a] Intake, mg.	Av. Output, mg.	Av. Retention, mg.	± S.D.	P[e]	Increase, %
Diet 1[b]	1,000	873	127	± 66.1	...	...
Diet 2[c]	1,980	1,743	237	± 91.4	<.05	86.3
Diet 3[d]	1,980	1,728	252	±115.0	<.05	97.9

[a] Average for the 10 subjects. [b] Unsupplemented diet. [c] Calcium carbonate supplemented diet. [d] Egg shell supplemented diet. [e] Significance determined by subjecting data to student *t* test.

which proved to be 94.1% when calculated as calcium carbonate. The egg shell and calcium carbonate powders were stored in tightly closed containers.

Determination of Calcium—Even though various methods are available for the determination of calcium from organic sources, the assay of some of these compounds is difficult. Urine and feces present difficulties due to the presence of phosphates and coloring matter.

In this study, the determination of calcium in urine was first attempted according to the official direct EDTA titration method specified for calcium carbonate. Interfering phosphate ions of the urine were previously removed by elution of the samples through anion-exchange resin columns containing an exchanger with a high capacity for phosphates. Satisfactory end points could not be obtained so hydroxy naphthol blue, "Indicator of Choice," according to Butler *et al.* (10), was unsuccessfully substituted for the naphthol green screened murexide. Eriochrome black T was also tried as an indicator; however, due to the interference of the coloring matter of the urine, reliable end points were not obtainable.

Flame photometry proved to be a more rapid and accurate method for the determination of the calcium content of urine and feces. The samples were assayed by means of a Coleman model 21 flame photometer and a Coleman model 6D junior spectrophotometer according to the directions of the Coleman operating manual for urinary and fecal calcium (11). Results are shown in Table II.

The calcium retention that occurred when the diet was supplemented first with the official calcium carbonate and then with egg shells was compared with the calcium retention from the basic diet. Results are shown in Table III. The average daily

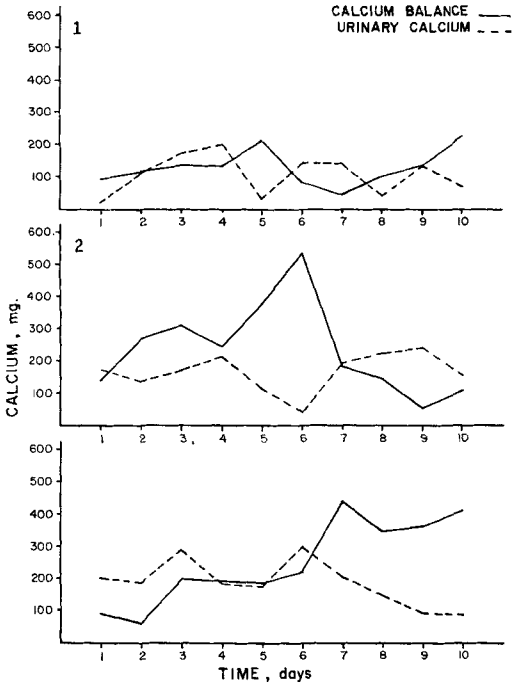

Fig. 1—Comparison of calcium balance with urinary calcium for each of 10 days. Key: 1, unsupplemented diet containing 1 Gm. calcium; 2 and 3, diets supplemented with official calcium carbonate and egg shells, respectively, each containing 1.98 Gm. of calcium (milligrams of calcium represent an average for the 10 cases studied); ——, calcium balance; ------, urinary calcium.

amounts of urinary calcium and daily calcium

balance for the 10 subjects studied are shown for the three types of experiments in Fig. 1.

DISCUSSION AND CONCLUSION

Calcium Balance—Based upon the limited number of subjects and the observed values, one cannot definitely say which source of calcium is superior. However, it is obvious that calcium from egg shells is assimilated to approximately the same extent as calcium from the official calcium carbonate. The use of egg shells as a source of dietary and therapeutic calcium warrants further investigation.

Absorption—According to Best and Taylor (6), urinary calcium constitutes a convenient index of calcium absorption. In agreement with their statement, calcium carbonate and egg shell supplemented diets, when compared to the unsupplemented diet, provide greater calcium content in excreted urine.

Some investigations, such as that of Briscoe and Ragan (12), indicate that increased calcium absorption occurs in the instance of increased calcium retention. Briscoe and Ragan also stated, "In studies of calcium balance, if maintained calcium balance is observed, it is assumed that the retained calcium is used for bone mineralization" (13).

Daily Comparison of Calcium Balance with Urinary Calcium—Since Best and Taylor consider urinary calcium a convenient index of calcium absorption (6), the purpose of this phase of the study was to determine if calcium excreted in the urine could also be used as an index of calcium balance.

The experiment concerned with the unsupplemented diet revealed that on days 4, 5, 6, 8, and 10 of the 10-day study increased calcium balances varied indirectly with urinary calcium values. In the instance of the calcium carbonate supplemented diet, this same relationship existed for 8 days of the 10-day period. The diet supplemented with egg shells, except for days 7, 9, and 10, demonstrated direct variation of calcium balance and urinary calcium. The daily calcium balance for this third type of experiment showed an irregular but overall continued increase of calcium balance during the 10-day period.

Little, if any, correlation can be derived for the three types of experiments in regard to the daily averages of calcium balances and calcium excreted in the urine. However, according to Table II, the averages for the total of each of the 10 days for the three types of experiments indicated that calcium balance does vary directly with urinary calcium. According to this study, the average urinary calcium value for a period of time can be used as an index for calcium balance. However, periodic 1-day urinary calcium values can be misleading as a clinically acceptable diagnostic index for calcium balance.

The validity of data obtained in human metabolism studies depends on the constancy of the regimen, an adequate number of subjects, and the length of time covered by the study. These studies were of short but of sufficient duration in order to substantiate the value of egg shells as a source of dietary and/or therapeutic calcium.

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Keyphrases

Calcium carbonate
Absorption, excretion—calcium
Egg shell calcium vs. precipitated calcium carbonate USP—absorption, excretion
Colorimetric analysis—excreta
Flame photometry—analysis, excreta

Determination of Phenaglycodol in Biologic Specimens by Ultraviolet Spectrophotometry

By JACK E. WALLACE

A rapid spectrophotometric method for the determination of phenaglycodol in body fluids and tissues is presented. Separation from other drugs is not required. The substituted glycol is oxidized by means of acid dichromate to a carbonyl derivative which has a high molar absorptivity in the ultraviolet region. The carbonyl product is converted to a semicarbazone which exhibits an even greater sensitivity to the absorption of ultraviolet radiations. The spectra of the phenaglycodol derivatives permit enough specificity for use of the procedure in forensic chemistry. The method is sufficiently sensitive for determination of phenaglycodol in urine 5 days after the ingestion of a single therapeutic dose. The distribution pattern of phenaglycodol in the rat and the nature of its excretion in man are presented.

PHENAGLYCODOL[1] (2-*p*-chlorophenyl-3-methyl-2, 3-butanediol) is claimed to have a distinct tranquilizing effect. Its calming and muscle relaxing properties are probably comparable to that of meprobamate (1, 2). The literature contains few methods for the analysis of this drug. Rose (3) described a technique for determining phenaglycodol by electrolytic cleavage of the glycol group to yield *p*-chloroacetophenone, which has a sensitive ultraviolet absorption spectrum. The procedure, however, is not applicable to determination of the drug in biologic specimens. Cima and Fassina (4) have published a method that utilizes both chromatographic and colorimetric techniques for determining phenaglycodol in urine. However, the method is applicable only to urine and the colorimetric assay, which utilizes a phosphomolybdic reagent, is subject to interference by many carbohydrates and polyphenols. Goldbaum (5) determined neutral drugs by gas chromatography, but his procedure does not provide positive identification nor does it afford routine application in the analysis of biologic specimens. In addition, no mention of phenaglycodol is made. In hexane, phenaglycodol absorbs radiation strongly at 222 mμ; therefore, small amounts of the drug in pure solution can be determined by spectrophotometric analysis (6). In biologic extracts, however, the ultraviolet spectral curve of the drug at 222 mμ is so affected by background absorption that direct analysis of unconverted drug, even in high concentrations, is not feasible. In addition, the solubility characteristics of this drug make difficult any procedure for obtaining it in an environment suitable for direct spectrophotometric assay. The need for a specific quantitative method for determining phenaglycodol in body fluids and tissues is apparent.

This report describes a rapid and specific method for the quantitative determination of phenaglycodol. Separation of phenaglycodol from other neutral drugs is not required. The method permits a reliable evaluation of the drug intake by patients receiving treatment with phenaglycodol.

EXPERIMENTAL

Instrumentation—A Beckman DK-2A ratio-recording spectrophotometer with linear presentation was used for the ultraviolet absorption measurements. A sample path length of 10 mm. was used throughout the study. Infrared functional group assignments were made with a Beckman IR 4 spectrophotometer.

Method—At any pH phenaglycodol is extracted from blood, urine, and tissues into most organic solvents. In this procedure 10 ml. of whole oxalated blood or serum or 10 Gm. of homogenized tissue is shaken vigorously for 3 to 5 min. with 100 ml. of chloroform. For urine, 10 ml. of specimen is refluxed for 10 min. with 1 ml. of concentrated hydrochloric acid prior to chloroform extraction. After the two phases have separated, the chloroform layer is removed and filtered through Whatman No. 41 or equivalent filter paper. The filtrate is subsequently washed with 10 ml. of 1 *N* sodium hydroxide solution and then with 10 ml. of 1 *N* hydrochloric acid solution. The volume of washed chloroform is recorded and the amount lost is considered in the final calculations. The chloroform is subsequently evaporated to dryness at 50° under vacuum. To the residue are added 20 ml. of 0.1 *N* potassium dichromate in 10 *N* sulfuric acid and 50 ml. of spectroquality hexane. The contents are refluxed for 30 min. with constant mechanical stirring, after which the hexane layer is cooled, separated, and read in a spectrophotometer against a hexane blank. When moderate or low levels of the drug are indicated, conversion of the reaction product to a semicarbazone derivative is desirable. This is accomplished by reacting 4–40 ml. of the hexane containing reaction product with 4 ml. of 0.5 *M* semicarbazide hydrochloride buffered to pH 3.5 with sodium acetate. The reaction is done conveniently at room temperature in a suitable size flask connected to a vacuum

Received October 4, 1967, from the Forensic Toxicology Branch, USAF Epidemiological Laboratory, Lackland Air Force Base, TX 78236

Accepted for publication November 13, 1967.

The author expresses gratitude to John D. Biggs and Richard W. Walker, Jr., for their technical assistance.

[1] Marketed as Ultran by Eli Lilly and Co., Indianapolis, Ind.

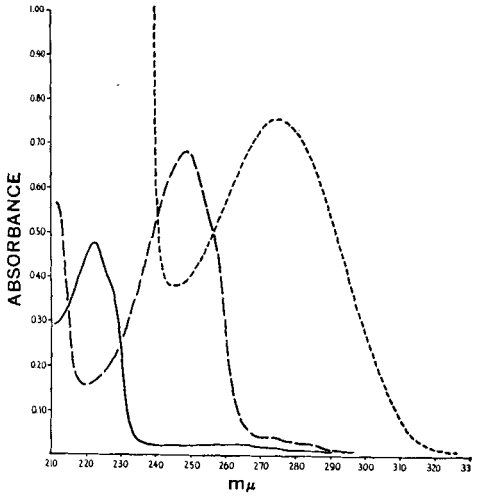

Fig. 1—Ultraviolet absorption spectra of phenaglycodol in hexane, of the phenaglycodol reaction product in hexane, and of the semicarbazone of the reaction product in water, each corresponding to a phenaglycodol concentration of 8 mcg./ml. Key: ——, phenaglycodol; — —, phenaglycodol product; ---, semicarbazone of phenaglycodol product.

TABLE I—STANDARD CURVE OF PHENAGLYCODOL REACTION PRODUCT

Phenaglycodol in Sample, mcg./ml.	Absorbance of Reaction Product[a]	Absorbance/ Concn.	Absorbance of Semicarbazone of Reaction Product[b]	Absorbance/ Concn.
10	0.843	0.084	0.954	0.095
8	0.700	0.087	0.739	0.093
6	0.500	0.083	0.589	0.098
4	0.323	0.081	0.366	0.092
2	0.168	0.084	0.201	0.101
Av.		0.084		0.096

[a] In hexane. [b] In aqueous semicarbazide.

rotary evaporator. Semicarbazone formation is complete when all the hexane has been removed. The remaining aqueous layer is read at 273 mμ against a 0.5 *M* semicarbazide blank.

The amount of phenaglycodol in the original sample is calculated from a standard curve prepared from aqueous solutions of the drug in appropriate concentrations, which have been carried through the procedure described, including the concentration technique for solutions containing small amounts of the drug.

RESULTS AND DISCUSSION

The ultraviolet absorption curve of the reaction product of phenaglycodol is well defined. It has an absorption maximum at 247 mμ in hexane and at 254 mμ in water. The unconverted drug absorbs maximally at 222 mμ. The high absorbance at 247 mμ for the reaction product and at 273 mμ for the semicarbazone of the product (Fig. 1), provides increased sensitivity and specificity over that of techniques which rely on direct spectrophotometric analysis of unconverted phenaglycodol. At 247 mμ a linear relationship exists between the absorbance of the reaction product and the concentration of the drug in the original sample. At 273 mμ a similar relationship exists for the semicarbazone of the product (Table I). Phenaglycodol extracted from urine of man and from tissues of animals that have received the drug yields reaction products that have identical ultraviolet absorption spectra to that obtained from the pure drug.

Infrared spectra of phenaglycodol (Fig. 2) and its reaction product (Fig. 3) show several distinct differences, of which the most significant is a band at 1692 cm.$^{-1}$ in the spectrum of the product. Absorption at this wavelength is entirely absent in the infrared spectrum of phenaglycodol. This band is believed to be the result of a carbonyl group formed by dichromate oxidation of the glycol group in the parent compound. The conversion of the

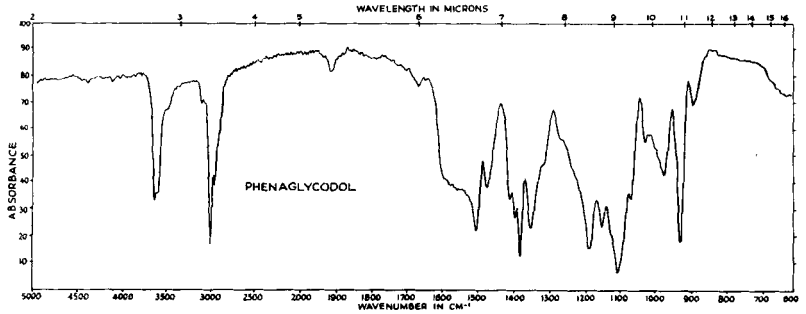

Fig. 2—Infrared absorption spectrum of phenaglycodol, 10 mg./ml. in carbon tetrachloride.

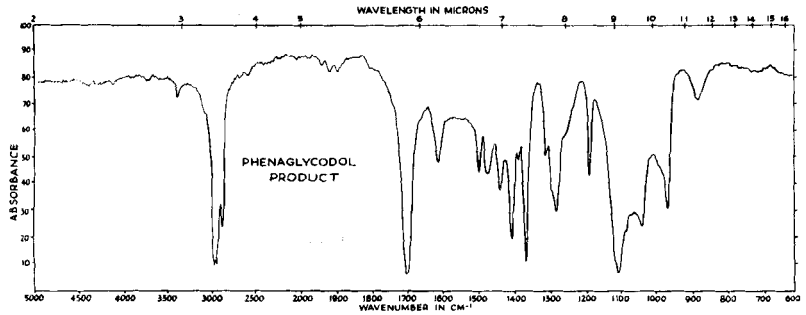

Fig. 3—Infrared absorption spectrum of the phenaglycodol reaction product, 10 mg./ml. in carbon tetrachloride.

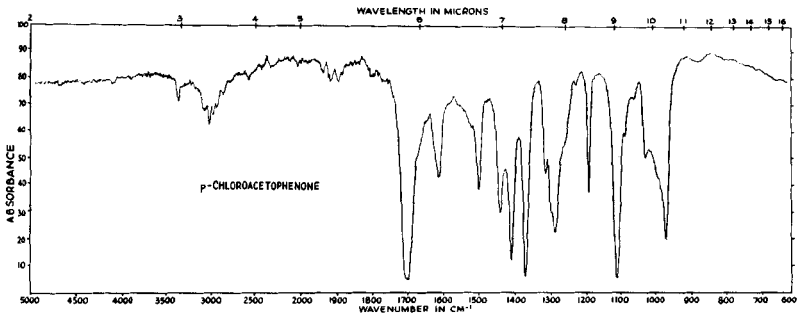

Fig. 4—Infrared absorption of p-*chloroacetophenone, 10 mg./ml. in carbon tetrachloride.*

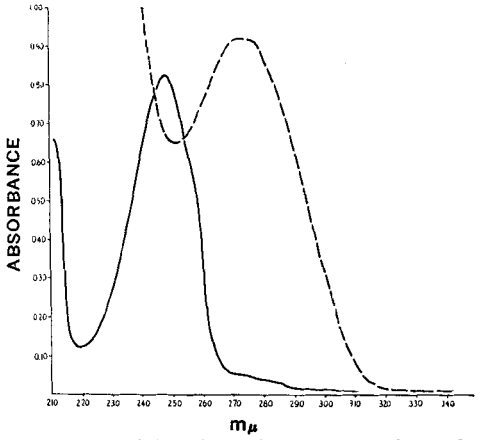

Fig. 5—Ultraviolet absorption spectra of p-*chloroacetophenone in hexane and of the semicarbazone of* p-*chloroacetophenone in water, each corresponding to a* p-*chloroacetophenone concentration of 7 mcg./ml. Key: ——, p-chloroacetophenone; ---, semicarbazone of* p-*chloroacetophenone.*

TABLE II—COMPOUNDS INVESTIGATED FOR INTERFERENCE WITH THE DETERMINATION OF PHENAGLYCODOL[a]

Compd.	Absorbance of Product[b]	Absorbance of Product Semicarbazone[c]
Phenaglycodol	1.69	1.90
Blank	0.01	0.01
Bisacodyl	0.05	0.01
Caffeine	0.01	0.01
Glutethimide	0.04	0.01
Hydroxyphenamate	0.05	0.08
Isonicotinic acid hydrazide	0.02	0.01
Isopropyl meprobamate	0.01	0.01
Meprobamate	0.01	0.01
Methyprylon	0.02	0.01
Phenacemide	0.04	0.03
Phenacetin	0.15	0.04
Phenol	0.03	0.01
Trimethadione	0.06	0.01

[a] Each compound was determined from whole blood. The level corresponds to a concentration of 20 mcg. of the compound per ml. of hexane. Each value is the average of three determinations. [b] Read at 247 mμ. [c] Read at 273 mμ.

glycol in the oxidation process is further substantiated by the lack of significant hydroxyl absorption at 3600 cm.$^{-1}$ in the spectrum of the product.

With the exception of strong carbon-hydrogen vibrations exhibited in the spectrum of the phenaglycodol product, the infrared spectra of the product and *p*-chloroacetophenone (Fig. 4) are very similar.

TABLE III—RECOVERY STUDIES OF PHENAGLYCODOL

Phenaglycodol Added, mcg./ml.	No. Determinations	Recovery,[a] Mean ± S.D. mcg./ml. Whole Blood	Urine[b]
50	12	45.5 ± 1.1	46.2 ± 1.7
25	10	23.7 ± 1.2	23.8 ± 1.3
10	9	9.7 ± 0.3	9.6 ± 0.2
5	11	4.6 ± 0.3	5.0 ± 0.2
Av. Recovery		93.7%	95.9%

[a] Determined from semicarbazone of reaction product. [b] Hydrolyzed prior to analysis.

TABLE IV—PHENAGLYCODOL LEVELS IN THE RAT[a]

Time, hr.	Phenaglycodol Recovered,[b] mcg./ml. or Gm. Blood	Brain	Kidney	Liver	Muscle
3	11.3	36.8	29.4	43.2	22.9
6	19.9	58.7	46.9	77.2	39.3
12	21.6	62.6	60.9	79.2	38.4
24	2.5	8.0	5.3	10.4	5.8

[a] Rats were given 104 mg. of phenaglycodol per Kg. body wt. [b] Determined from semicarbazone of reaction product.

TABLE V—PHENAGLYCODOL[a] DISTRIBUTION IN THE RAT

Tissue	Distribution Ratio,[b] Mean ± S.D. Concn. in Tissue/Concn. in Blood
Blood	1.0
Brain	3.2 ± 0.4
Kidney	2.6 ± 0.5
Liver	3.9 ± 0.3
Muscle	2.0 ± 0.4

[a] Unconjugated phenaglycodol. [b] Average of 15 rats.

TABLE VI—CONCENTRATION OF PHENAGLYCODOL[a] IN HUMAN BLOOD

Hours After Ingestion	Phenaglycodol Concentration,[b] mcg./ml. whole blood Adult I	Adult II	Adult III	Adult IV
0	0.0	0.0	0.0	0.0
0.5	0.5	0.4	...[c]	...
1	6.1	1.7	2.9	7.7
2	6.5	3.8	6.8	8.4
3	5.8	6.1	5.7	7.0
4	5.3	5.2	4.3	6.5
6	4.1	4.0	3.3	5.6
8	3.7	3.2	3.1	5.1
12	2.9	2.4	2.6	4.3
24	2.1	1.6	1.9	2.8

[a] Unconjugated phenaglycodol. [b] Each subject ingested a single 800-mg. dose after 12 hr. of fasting. [c] Not determined.

Ultraviolet spectral curves of the two are identical (Figs. 1 and 5) and gas-chromatographic retention data suggest that they are the same compound. Calculations utilizing the ultraviolet spectra of the phenaglycodol product and of *p*-chloroacetophenone

TABLE VII—PHENAGLYCODOL IN URINE AFTER INGESTION OF 800 MG. PHENAGLYCODOL EXCRETED[a]

Hr. After Ingestion	Adult I		Adult II		Adult III		Adult IV	
	Conjugated	Unconjugated	Conjugated	Unconjugated	Conjugated	Unconjugated	Conjugated	Unconjugated
4	30.2	0.49	32.2	0.29	19.9	0.38	23.7	0.51
8	36.1	0.19	16.3	0.23	15.0	0.16	27.4	0.47
12	22.5	0.11	42.8	0.14	8.4	0.04	32.9	0.46
24	57.6	0.44	42.0	0.11	42.2	0.30	60.1	0.56
48	69.0	0.56	60.3	0.07	40.6	0.36	83.2	0.93
72	32.9	0.24	35.4	0.06	32.6	0.51	30.2	0.29
96	13.1	0.26	15.1	0.03	12.5	0.22	13.7	0.19
120	6.3	0.34	6.3	0.02	7.5	0.19	4.3	0.14
Total	267.7	2.63	250.4	0.95	178.6	2.16	275.5	3.55

[a] Determined from semicarbazone of reaction product.

(Fig. 5) indicate that approximately 100% of the phenaglycodol is converted to *p*-chloroacetophenone.

Several drugs were investigated for interference with the determination of phenaglycodol. None produced a reaction product or semicarbazone which absorbed significantly throughout the range 220–360 mμ. The results of the study for interfering substances are presented in Table II. Standard alcoholic solutions of phenaglycodol were added to biologic material to provide concentrations from 0.50 to 5.0 mg. %. Ten milliliters of blood or hydrolyzed urine with added drug was then examined by the above method. Since phenaglycodol is predominantly conjugated in urine specimens (Table VII), hydrolysis provides a better appraisal of the method's ability to recover the drug from that specimen. Average recovery from blood was approximately 94% and from urine 96% (Table III).

To determine the tissue distribution pattern of the drug, rats were fed, *via* stomach tube, 104 mg. of phenaglycodol per kilogram of body weight. Blood and tissue levels after 3, 6, 12, and 24 hr. are shown in Table IV. Levels of the drug found in blood and tissues are given in decreasing order: liver, brain, kidney, muscle, and blood. Average distribution ratios for 15 rats are given in Table V.

Each of four adult human males who had fasted for 12 hr. received 800 mg. of phenaglycodol by mouth. Thereafter, phenaglycodol concentrations were determined in venous blood specimens drawn at predetermined intervals. The highest concentration of drug in the blood occurred 2–3 hr. after ingestion (Table VI). Phenaglycodol in the urine was predominantly excreted in the conjugated form (Table VII). Urine analysis without prior hydrolysis of the conjugated derivative indicates that less than 0.5% of the administered drug is eliminated unchanged in man during the first 120 hr. Five to 8% of the drug, however, was found in the urine during the first 24 hr. as the conjugated form. The concentration of conjugated drug measured in the urine over a 120-hr. period varied from one subject to another, but the total amount excreted in the urine by the four subjects was less variable: 178.6–275.5 mg. Cima and Fassina (4) found in their study with rabbits that in 4 days about 3% of the unchanged drug is excreted in the urine and an additional 31% is found as conjugated products, one of which they claim to be a glucuronide. Their report gives no tissue or blood levels of the drug.

The method described distinguishes between phenaglycodol and its conjugated metabolites, and affords the only available spectrophotometric analysis of the drug in blood and tissues. The sensitivity of the method permits the analyst to detect the drug in blood and urine after an individual has taken a therapeutic amount of the drug. The drug and its metabolites can be detected in the urine of adult humans for at least 5 days after the ingestion of a single 800-mg. dose.

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Keyphrases

Phenaglycodol
Body fluids, tissue—phenaglycodol determination
Semicarbazone, phenaglycodol—analysis
UV spectrophotometry—analysis
IR spectrophotometry—analysis

Allenic Amines: A New Class of Nonhydrazine MAO Inhibitors

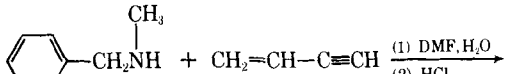
By R. P. HALLIDAY, C. S. DAVIS, J. P. HEOTIS, D. T. PALS, E. J. WATSON*, and R. K. BICKERTON

N-2,3-Butadienyl-*N*-methylbenzylamine HCl (U-1247) and *N*-2,3-butadienyl-*N*-methyl-*o*-chlorobenzylamine HCl (U-1379) have been synthesized and potent *in vivo* monoamine oxidase inhibition demonstrated. Antihypertensive activity comparable to that produced by pargyline was demonstrated in hypertensive rats with U-1247. When administered to mice, U-1247 and U-1379 produced initial stimulation of motor activity in contrast with pargyline which produced initial depression.

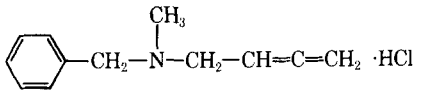
Swett *et al.* (1) in 1963 described the structural requirements necessary for monoamine oxidase inhibitory (MAOI) activity within the pargyline series. These workers indicated that a triple bond was essential for MAOI activity. It was the purpose of this study to synthesize and evaluate the activity of two allenic derivatives of pargyline which contained no triple bond.

N-2,3-Butadienyl-*N*-methylbenzylamine hydrochloride (U-1247) was prepared by a modification of the general method reported by Engelhardt (2). Engelhardt condensed water-soluble amines with vinylacetylene (I) in water to produce allenic amines. Since methylbenzylamine (II) is only slightly soluble in water, a mixture of *N*,*N*-dimethylformamide and water was used as the reaction solvent.

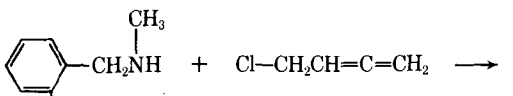
N - 2,3 - Butadienyl - *N* - methyl - *o* - chlorobenzylamine hydrochloride (U-1379) was prepared by the method of Vartanian and Badanian (3). *o*-Chloro-*N*-methylbenzylamine (II*a*) was alkylated with 4-chloro-1,2-butadiene (III) to give U-1379. U-1247 may be prepared by alkylation of *N*-methylbenzylamine (II) with III.



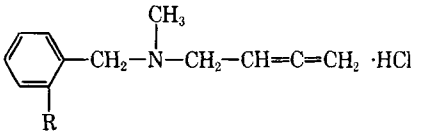
(II) (I)



U-1247



(II or II*a*) (III)



R = H (U-1247) and Cl (U-1379)

EXPERIMENTAL

N-2,3-Butadienyl-N-methylbenzylamine Hydrochloride (U-1247)—Into a 1-L. Parr bomb (capacity about 854 ml.) was placed a mixture of benzylmethylamine (121 Gm., 1.0 mole), 130 ml. of water, and 170 ml. of *N*,*N*-dimethylformamide. The homogeneous mixture was cooled by placing the bomb in a dry ice–acetone bath while nitrogen was bubbled through the mixture. After the mixture had cooled to −30°, vinylacetylene (52 Gm., 1.0 mole) was condensed into the bomb. The bomb was closed, pressurized to 150 p.s.i. with nitrogen, and heated to 70–98° for 20 hr. with shaking.

The bomb was allowed to cool for 1 day before venting and purging with nitrogen. The contents of the bomb were removed and steam distilled immediately. The steam distillate was transferred to a separator and extracted with two 1-L. portions of ether. The ethereal extracts were dried over anhydrous sodium sulfate and then concentrated under vacuum. The residue was distilled, b.p. 85–94°/2.0 mm., yield 21 Gm. (12%). The infrared spectrum indicated a strong band at 5.10 μ which is characteristic of the allene group. No absorption bands characteristic of acetylenic compounds were observed. Vapor-phase chromatography indicated the product consisted of a single material.

One gram of distilled material was treated with an ethereal solution of hydrogen chloride. An oil separated which crystallized with rubbing. The solid was recrystallized from ethyl acetate to give a material with a melting point of 118–120°.

Anal.—Calcd. for $C_{13}H_{16}ClN$: C, 68.72; H, 7.69; Cl, 16.91; N, 6.68. Found: C, 68.70, 68.70; H, 7.54, 7.63, 6.12; Cl, 17.21, 17.08; N, 6.11.

N-2,3-Butadienyl-N-methyl-o-chlorobenzylamine Hydrochloride (U-1379)—*o*-Chloro-*N*-methylbenzylamine (Sapon Labs.) (122 Gm., 0.8 mole), 5 ml. of water, and 4-chloro-1,2-butadiene (3) (75 Gm., 0.85 mole) were allowed to stand for 5 days at room temperature. To the mixture was added, with vigorous shaking, 200 ml. of a saturated solution of sodium carbonate. The organic layer was extracted with two 150-ml. portions of ether. The

Received September 8, 1967 from the Research and Development Department, The Norwich Pharmacal Company, Norwich, NY 13815

Accepted for publication November 13, 1967.

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ethereal extracts were dried over anhydrous sodium sulfate and then concentrated under vacuum. The residue was distilled, b.p. (1st fraction) 45–78°/1 mm.; (2nd fraction) 79–105°/1 mm. There was about 80 Gm. in the second fraction which was redistilled. The material boiling at 81–91°/0.2 mm. was taken as product, yield 45 Gm. (27%). The infrared spectrum showed a strong allenic band and no acetylenic band.

A small amount of the free base was dissolved in dry ether and treated with an ethereal solution of hydrogen chloride. An oil separated that crystallized with rubbing. The solid was recrystallized from benzene to give a material with a melting point of 121–122°.

Anal.—Calcd. for $C_{12}H_{15}Cl_2N$: C, 59.03; H, 6.19; N, 5.74. Found: C, 58.72, 58.79; H, 6.19, 6.32; N, 5.68, 5.67.

N-2,3-Butadienyl-*N*-methylbenzylamine hydrochloride (U-1247) may be prepared by the above method, yield 9%.

Monoamine Oxidase Inhibition—The colorimetric method of Stern *et al.* (4) was used to determine monoamine oxidase activity. MAO in the presence of oxygen catalyzes the conversion of tyramine to *p*-hydroxyphenylacetaldehyde (*p*HPA) which is trapped as the semicarbazone in the incubation mixture. Thus, inhibition of MAO activity by a drug results in a decrease in the production of *p*HPA. Potassium cyanide is added to the incubation mixture to inhibit competing oxidative reactions.

For *in vitro* studies, stock solutions (6×10^{-3} or 6×10^{-4} *M*) of pargyline, U-1247, and U-1379 prepared in 0.2 *M* phosphate buffer (pH 7.4) were serially diluted with buffer. Half-milliliter aliquots of the drug in buffer were added to 2.0-ml. aliquots of a 2.5% rat liver homogenate. Control mixtures were prepared by adding 0.5-ml. aliquots of phosphate buffer without drug to 2.0-ml. aliquots of the same rat liver homogenate. The mixtures were preincubated for 10 min. before the addition of 0.5 ml. of 0.06 *M* semicarbazide–0.006 *M* KCN solution containing 30 μM of tyramine. The reaction mixtures were shaken at 37° in an O_2 atmosphere for 10 min. At the end of the incubation period, 0.5 ml. of a 50% trichloroacetic (TCA) solution in water was added to each homogenate to stop the reaction.

For the *in vivo* study, rats ranging in weight from 250 to 300 Gm. were given a single oral dose of either pargyline, U-1247, or U-1379 in water. Each drug was administered to rats at various dosages using three rats per level of drug. Control rats were dosed with water. After 4 hr. the rats were sacrificed and their livers and brains removed to determine monoamine oxidase activity. Liver and brain homogenates, 2.5% (w/v), were prepared in 0.2 *M* phosphate buffer (pH 7.4); 2.0 ml. of homogenate was added to an additional 0.5 ml. of the phosphate buffer and treated as described for the *in vitro* studies.

Colorimetric analysis of *p*HPA in the TCA treated supernatant solution was made by diluting 0.5 ml. of the solution to 3.0 ml. with water and adding 1.0 ml. of 1.5% 4-nitrophenylhydrazine hydrochloride in 1.0 *N* HCl. After extraction of the 4-nitrophenylhydrazone of *p*HPA into 2.0 ml. of chloroform, re-extraction of 1.0 ml. chloroform with 5 ml. of 0.1 *N* NaOH, and dilution of 3.0 ml. of this basic solution to 8.0 ml. with water, the absorbances were determined in a colorimeter at 420 mμ. Percent of inhibition of MAO activity by pargyline, U-1247, and U-1379 *in vivo* and *in vitro* was calculated by comparing the absorbance of the inhibited MAO samples with the appropriate control absorbance.

Comparative Acute Toxicity in Mice—LD_{50} values were determined in CFW male mice with U-1247, U-1379, and pargyline by three routes of administration. Groups of 5 mice received graded doses of drug, and the LD_{50} value was based on deaths which were observed within a 48-hr. period. These animals were also observed for gross pharmacologic effects of the compounds.

Hypotensive and Antihypertensive Activity—The hypotensive and antihypertensive activity of U-1247 and pargyline was investigated in unanesthetized rats.

Thirty rats were divided into six groups of five rats each. Three of these groups consisted of rats made hypertensive by the method of Grollman (5). Systolic blood pressures were recorded twice daily from the tail using a pneumatic pulse pickup and an electrosphygmograph (E & M instruments).

The animals were arranged so that each testing unit consisted of five normotensive and five hypertensive rats. The following procedure was adopted: Days 1 and 4. Control blood pressures were recorded from each animal twice a day. Days 5 through 11. During this, the treatment period, the animals received daily the following: Unit 1—saline 1 ml./Kg., i.p. Unit 2—pargyline 20 mg./Kg., i.p. Unit 3—U-1247 20 mg./Kg., i.p. Days 12 through 15. This was the postdrug observation period.

Effect of U-1247 and Pargyline on the Pressor Effects of Tyramine—Rats were anesthetized with urethan (1.25 Gm./Kg., i.p.) a tracheotomy performed, and polyethylene tubing inserted. A glass cannula was inserted into one of the carotid arteries for direct blood pressure recording through a Statham pressure transducer onto a Grass polygraph. A femoral vein was cannulated for injection of drugs. Two groups of five animals each were pretreated 48, 24, and 2 hr. before i.v. tyramine (0.01 mg./Kg.) challenge with either U-1247 (100 mg./Kg. *per os*) or pargyline HCl (100 mg./Kg. *per os*). Control responses to tyramine (0.01 mg./Kg., i.v.) were obtained using seven animals.

In a second study, the protocol of Tedeschi and Fellows (6) for demonstrating augmentation of peroral tyramine by monoamine oxidase inhibition was followed, with the exception that anesthesia was accomplished with intraperitoneal urethan (1.25 Gm./Kg.) instead of intravenous pentobarbital. The treated rats were given 100 mg./Kg. of U-1247 or pargyline orally 72, 48, and 24 hr. prior to challenge with orally administered tyramine.

RESULTS

MAO Inhibition—*In vitro* U-1247, U-1379, and pargyline greatly inhibited rat liver homogenate MAO activity (Table I). Fifty percent inhibition (IC_{50}) of the MAO activity in liver homogenate was elicited by these drugs at the following molar concentrations: 9.5×10^{-7} *M* for U-1247, 2.3×10^{-7} *M* for U-1379, and 4.0×10^{-8} *M* for pargyline. The IC_{50} values for liver MAO activity must be

interpreted with caution, however, since the length of the preincubation period of MAO with these inhibitors, before the addition of substrate, may affect the degree of inhibition obtained (7).

In vivo peroral administration of U-1247, U-1379, and pargyline resulted in approximately the same magnitude of inhibition of both rat liver and brain MAO (Table I). Graphs of the percent inhibition of rat liver MAO activity by U-1247, U-1379, and pargyline *versus* the logarithms of the doses resulted in the classic sigmoid curves usually obtained in this type of study. The ID_{50} values obtained from these curves were: 0.13 mg./Kg. for pargyline, 0.24 mg./Kg. for U-1247, and 0.51 mg./Kg. for U-1379. The difference between these ID_{50} values is not considered significant. Construction of the same type of graph for the *in vivo* brain data resulted in curves which were too variable to be clearly defined as sigmoid curves. U-1247, U-1379, and pargyline produced a 50% inhibition of rat brain MAO within a 1–3 mg./Kg. dose range.

Acute Toxicity in Mice—The comparative LD_{50} values and 95% confidence limits obtained in CFW mice with U-1247, U-1379, and pargyline by three routes of administration are presented in Table II.

These LD_{50} values were based on deaths which were observed within 48 hr. following drug administration.

The gross observable effects of these three drugs also differed. Deaths among the U-1247 treated mice occurred within 2–15 min. of drug administration and no delayed deaths were observed. The toxic signs observed were: clonic convulsions, dyspnea, cyanosis, and exophthalmus. Sublethal doses produced hyperexcitability which lasted approximately 1 hr.

Pargyline, by contrast, caused little excitability at sublethal doses while minimal lethal doses produced a 1–2 hr. depression followed in 3–4 hr. by marked hyperexcitability. When large lethal doses were given death occurred in 3–10 min. with accompanying cyanosis, dyspnea, and weak (cyanotic) clonic convulsions. Deaths following oral administration were delayed as long as 48 hr.

The gross and toxic effects of U-1379 resembled those observed following either pargyline or U-1247 treatment. Marked initial stimulation, which resembled the effects of U-1247, and latent stimulation and delayed deaths as were seen following pargyline, were observed with this compound.

Hypotensive and Antihypertensive Activity—The effect of U-1247 and pargyline on the systolic blood pressure of normotensive and hypertensive rats is summarized in Table III. The control animals, both normotensive and hypertensive, maintained a steady level of systolic blood pressure over the 16-day period. The systolic blood pressure of the hypertensive animals receiving pargyline or U-1247 decreased over the 7-day treatment period. This decrease in systolic pressure was approximately equal in the two groups and no difference in potency of the two drugs could be demonstrated. At the termination of treatment, the blood pressure rose to predrug levels in approximately 3 days. The normotensive animals which received either U-1247 or pargyline did not appear to demonstrate as marked a hypotensive effect; however, there was a

Table I—Effect of U-1247, U-1379, and Pargyline on Rat Monoamine Oxidase

	U-1247	U-1379	Pargyline
In vitro[a]			
Liver			
$IC_{50}(M)$	9.5×10^{-7}	2.3×10^{-7}	4×10^{-8}
Slope	0.30	0.68	0.58
In vivo[b]			
Liver			
ID_{50}, p.o. (mg./Kg.)	0.24	0.51	0.13
Slope	0.75	1.2	0.91
Brain			
ID_{50}, p.o. (mg./Kg.)	1.9	2.18	3.1
Slope	0.87	1.68	0.81

[a] The drugs were preincubated for 10 min. with the rat liver homogenate before addition of the substrate tyramine. [b] These results were obtained on livers or brains excised 4 hr. after peroral administration of the drugs.

Table II—Acute Toxicity of U-1247, U-1379, and Pargyline in Mice[a]

Route	Pargyline, mg./Kg.	U-1247, mg./Kg.	U-1379, mg./Kg.
p.o.	726 (613–800)	339 (318–363)	305 (289–322)
i.p.	494 (430–566)	156 (136–173)	235 (211–266)
i.v.	99 (91–106)	31.6 (29.7–35.0)	59 (54–63)

[a] LD_{50} and confidence limit values, mg./Kg.

Table III—Effect of U-1247 and Pargyline on the Systolic Blood Pressure of Normotensive and Hypertensive Rats

		Systolic Blood Pressure[a] (mm. Hg)					
		Saline		Pargyline		U-1247	
Cond.	Day	Hypertensive	Normotensive	Hypertensive	Normotensive	Hypertensive	Normotensive
Pretreatment period	1	175	109	170	112	161	119
	4	165	109	174	114	155	114
Treatment period, 20	6	161	123	147[b]	109	152	123
mg./Kg. i.p. daily or	7	168	117	147[b]	114	144	113
equivalent volume of	8	168	114	142[b]	99[b]	138	106[b]
saline	11	169	110	136[b]	106	129[b]	104[b]
Posttreatment period	12	169	112	134[b]	98[b]	122[b]	98[b]
	13	163	112	150	111	143	109
	14	171	115	181	117	150	113
	15	169	121	176	118	154	116

[a] Blood pressure values are a mean of five animals tested twice each day specified. [b] Average daily blood pressure significantly lower than pretreatment period ($P < 0.05$).

TABLE IV—EFFECT OF U-1247 AND PARGYLINE ON TYRAMINE BLOOD PRESSURE RESPONSES

Treatment[a]	Tyramine Dose, mg./Kg.	ΔxBP, mm. Hg	±S.E.	No. Rats in Group
Control	Saline	12.0	2.16	6
Control	12.5	16.2	4.00	6
Control	50	38.5	5.35	6
Pargyline	12.5	37.0	11.05	6
U-1247	12.5	33.8	10.80	6

[a] Rats pretreated with U-1247 or pargyline (100 mg./Kg. p.o.) 72, 48, and 24 hr. prior to tyramine challenge (p.o.).

significant blood pressure reduction in both groups.

Effect of U-1247 and Pargyline on the Pressor Response to Tyramine—The mean blood pressure response to 0.01 mg./Kg. i.v. of tyramine in 7 rats was 7.7 (±2.5). After pretreatment with pargyline or U-1247, the mean response to tyramine in 5 rats was increased to 24.4 (±10.4) and 32.6 (±8.5), respectively. These differences from control were significant at the 0.1 level for pargyline and the 0.01 level for U-1247.

The effect of U-1247 and pargyline on perorally administered tyramine is summarized in Table IV.

In control rats there was a significant difference in the blood pressure response between 12.5 mg./Kg. and 50 mg./Kg. of tyramine. After pretreatment with either pargyline or U-1247, the response to 12.5 mg./Kg. of tyramine was increased, and no longer was significantly different from the response to the 50 mg./Kg. dose.

These data indicate that U-1247 and pargyline are capable of potentiating both intravenous and peroral tyramine.

DISCUSSION

This study indicates that within the pargyline series a triple bond is not essential for *in vitro* or *in vivo* MAOI activity. The *in vitro* data suggest that U-1247 is approximately 25 times less active than pargyline, while *in vivo* the two compounds are approximately equal in potency. The *in vivo* conversion of U-1247 to a more active form is one possible explanation of these results. The bioconversion of the allenic compounds to acetylenic derivatives, however, is unlikely as Swett (1) has indicated both the *N*-2 and *N*-3 butynyl-*N*-methylbenzylamine are inactive *in vivo*. The possibility of U-1247 and pargyline being converted to a common active form cannot be discounted from the data and is worthy of further study.

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Keyphrases

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Allenic amines—synthesis
LD_{50} value—allenic amines
Hypotensive, antihypertensive activity
Vapor-phase chromatography—identity
IR spectrophotometry—structure

Influence of Various Pretreatments (Carriers, Desiccation, and Relative Cleanliness) on the Destruction of *Bacillus subtilis* var. *niger* Spores with Gaseous Ethylene Oxide

By JOHN E. DOYLE and ROBERT R. ERNST

The ethylene oxide resistance of *Bacillus subtilis* var. *niger* spores inoculated on a variety of carriers was determined. After initial experiments indicated that cleanliness of the spores was important, desiccated and undesiccated spores of varying degrees of cleanliness on aluminum foil and chromatography paper were studied. Under the experimental conditions, desiccation of the spore population had no influence on susceptibility to ethylene oxide. The degree of cleanliness of the spore preparation was the significant factor in excessive resistance. The bacteriological controls used for testing ethylene oxide sterilization processes should be adequately cleaned so that they may accurately monitor the process.

GASEOUS ETHYLENE OXIDE is used for the sterilization of materials which cannot be sterilized by steam. As the utilization of heat- and moisture-sensitive materials increases, the importance of ethylene oxide sterilization increases for the hospital and pharmaceutical related fields.

Received August 18, 1967, from the Research Laboratories, Castle Company, Rochester, NY 14623
Accepted for publication October 13, 1967.
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Essential to an ethylene oxide process is proper monitoring. The USP XVII, page 811 states: "The efficacy of any procedure for the sterilization of pharmaceutical products by ethylene oxide should be tested thoroughly by inclusion of items contaminated with spores of known microorganisms in representative locations in typical sterilizer loads and by subsequent demonstration that these microorganisms have not survived the treatment."

Since the evaluation of the efficiency of an ethylene oxide process depends a great deal on the bacteriological controls, the controls chosen should be uniform as regards to resistance to the sterilant. This paper deals with the effect of cleanliness and desiccation on the resistance of spores to ethylene oxide.

METHODS

Bacillus subtilis var. *niger* 356 S. C. No. 4 (1) was used in this study. The culturing methods have been described previously (2). Washing the spores was accomplished by centrifugation at 20,500 × g for 10 min., decanting the supernate, followed by resuspension in sterile distilled water. This procedure was repeated for further washings when indicated. Dilutions were made in sterile distilled water to produce the desired inoculum. Inoculums were 0.01 ml. per carrier materials.

The procedures similar to those used by Ernst and Shull (3) were used in this study for determination of ethylene oxide resistance.

RESULTS

Initially in the investigation, it was of interest to determine the resistance of *Bacillus subtilis* var. *niger* on a variety of carrier materials. However, a spore suspension was used that had been washed only two times. Table I describes the variety of resistances obtained. There appeared to be little effect due to absorbency or nonabsorbency of the carrier. No consistent difference of resistance between hard and porous surfaces was observed

TABLE I—RESISTANCE OF *Bacillus Subtilis* VAR. *Niger* SPORES[a] ON VARIOUS CARRIERS

Carrier Material	Total Survival, min.	Partial Survival, min.	Total Kill, min.
Acetate fabric, 100%	5	45	60
Acetate-rayon fabric	45	60	90
Aluminum foil	5	7	10
Bibulous paper	3	4	5
Chromatography paper	3	4	5
Fluorohalocarbon film	5	10	15
Heavy paper	20	45	60
Nylon fabric	5	10	15
Nylon film	5	7	10
Polypropylene film	5	7	10
Polyvinyl chloride film	4	25	25
Rubber glove film	5	10	15
Wax paper	5	10	15

[a] Spore suspension (10^8 spores/carrier) washed two times in sterile distilled water. Ethylene oxide concentration, 1,200 mg./L.; relative humidity, 40%; temperature, 130°F.

TABLE II—RESISTANCE OF *Bacillus subtilis* VAR. *Niger* SPORES[a] ON VARIOUS CARRIERS

Carrier Material	Total Survival, min.	Partial Survival, min.	Total Kill, min.
Acetate fabric, 100%	1	3	5
Acetate-rayon fabric	5	7	10
Aluminum foil	5	10	15
Balsa wood	5	7	10
Chromatography paper	5	10	15
Cotton fabric, 100%	5	10	15
Glass tubing	3	7	10
Polyethylene film	3	7	10
Rayon fabric, 100%	5	10	15
Reinforced cellulose	5	10	15
Rick-rack	3	7	15
Rubber glove film	3	10	20
Wax paper	3	7	10
Yellow sponge ("Ivalon")	5	10	15

[a] Spore suspension (10^8 spores/carrier) washed eight times in distilled water. Ethylene oxide concentration, 1,200 mg./L.; relative humidity, 40%; temperature, 130°F.

However, a large zone of partial survivors and high resistance was observed. This may have been due to the fact the spore suspension was only washed twice. A synergistic effect of the carrier material and the residual media may have produced high ethylene oxide resistance.

Therefore, a new suspension of *Bacillus subtilis* var. *niger* was prepared as before. However, this suspension was washed eight times. As shown in Table II, once again there appeared to be little effect on the resistance regardless of whether the carrier was absorbent or nonabsorbent. High resistance due to any carrier effect was not observed. The high degree of cleanliness appeared to reduce the relative resistance of the spores.

Since the cleanliness of a spore suspension appeared to be an important aspect of its relative resistance, the effect of the relative degree of cleanliness on the resistance of spores to ethylene oxide with respect to both nonabsorbent and porous carriers was investigated. Since hard surfaces have been reported to produce a greater resistance than porous surfaces, an aluminum foil carrier was compared with a porous paper carrier. Also because desiccation (4, 5) has been reported to increase the resistance of spores, this aspect was investigated as well.

A new suspension of *Bacillus subtilis* var. *niger* was prepared as before. Equal aliquots of the spore culture broth were separated into five groups. Washing was accomplished as described previously. One suspension was not cleaned, one was cleaned once, *etc.* Then the aluminum foil and chromatography paper strips were inoculated with the five suspensions. A portion of each was allowed to dry and equilibrate at room temperature. The other portion was placed in a desiccator and dried over calcium sulfate desiccant at room temperature in excess of 1 month. Hence there were "normal" dried and desiccant dried aluminum foil and paper carriers of varying degrees of cleanliness as shown by the number of resuspensions in distilled water.

Figure 1 compares the relative resistance of spores with relative degrees of cleanliness when placed upon aluminum foil carriers. If the suspension is not washed at all, a high resistance (over 48 hr.) to sterilization is obtained, probably due to the occlusion of the spores in the media. With one resuspension a broad partial survival region was observed,

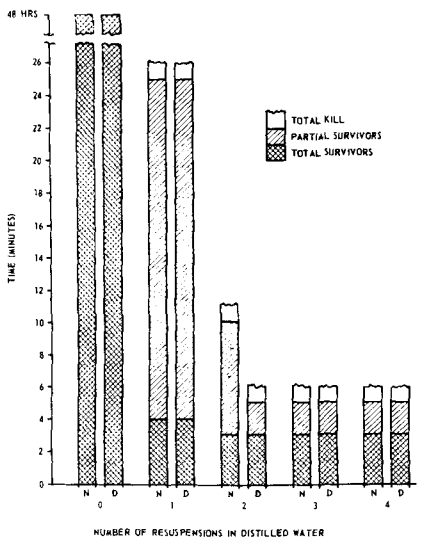

Fig. 1—Effect of relative cleanliness and desiccation on the ethylene oxide resistance of Bacillus subtilis *var.* niger *spores (10^6 spores/carrier) on aluminum foil. N = not desiccated, D = desiccated. Ethylene oxide concentration, 1,200 mg./L.; relative humidity, 40%; temperature, 130° F.*

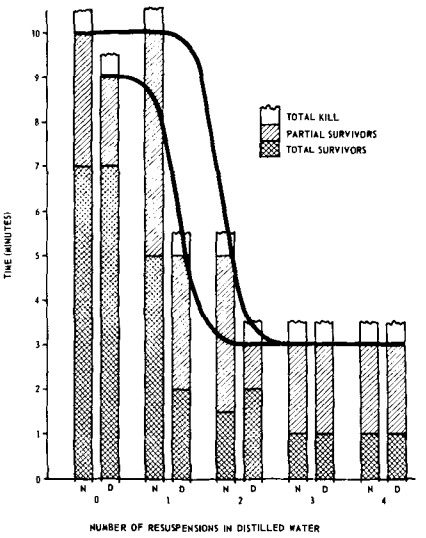

Fig. 2—Effect of relative cleanliness and desiccation on the ethylene oxide resistance of Bacillus subtilis *var.* niger *spores (10^6 spores/carrier) on porous paper. N = not desiccated, D = desiccated. Ethylene oxide concentration, 1,200 mg./L.; relative humidity, 40%; temperature, 130° F.*

indicating that some spores were being randomly protected. However, sterility was obtained in 25 min. With a second resuspension there was still some effect due to the relative uncleanliness of the suspension, except in the case of the desiccated carriers. After the third resuspension, the resistance dropped to 3 min. total survival, requiring only 5 min. for total kill. The partial survival region is approximately 2 min. Desiccation appeared to have little effect on the resistance of the spores.

Figure 2 compares the resistance of spores of relative degrees of cleanliness when placed upon a porous paper carrier. Complete resistance to ethylene oxide with the porous paper was never obtained under these conditions, even when the suspension was not washed at all. It appeared that the desiccated carriers were less resistant than the undesiccated carriers when the spore inoculum was washed less than three times. Upon further washing, undesiccated and desiccated strips had equal resistances.

Some experiments were performed with the spore preparation which was not washed, at 10^7 spores per paper carrier, and an even greater partial survival region was observed. One would expect more resistance with carriers contaminated with a greater number of spores. However, the larger region of partial survival shows the greater likelihood of protection by occlusion due to the larger amount of media as well as the larger number of spores.

When one compares the degrees of cleanliness of spores on the aluminum foil and paper carriers, it is evident that the cleanliness of the spore suspension used determines great differences in relative susceptibility. In every degree of cleanliness the aluminum foil carrier resulted in a greater resistance than the porous paper with a comparative difference in resistance becoming quite small with three washings.

The small difference of greater resistance imparted by a hard surface over a porous one is easy to understand, since diffusion is impossible or hindered through a hard surface carrier material, whereas the gases can diffuse in all directions through a porous one. Also the spores would tend to protect each other for they tend to ball up on a hard surface material whereas the spores become somewhat separated when inoculated on a porous paper or any porous material.

Desiccation of spores did not appear to play a large role in the experimental conditions. Even at 10% RH, 130°F., and 1,200 mg./L. ethylene oxide, little differences in resistance between clean desiccated and undesiccated spores of *Bacillus subtilis* var. *niger* were observed. The sterilization time for both, however, was extended to 1 hr.

Since calcium sulfate desiccation had no effect on ethylene oxide resistance, spores were dried in the following manner: (*a*) Acetone drying of spores prior to inoculation on chromatography paper. (*b*) Vacuum drying of cleaned spores on chromatography paper at 100μ for 24 hr. (*c*) Chromatography paper carriers were inoculated with clean spores at 10^7 spores/carrier. Then the carriers were exposed to 100°C. in an oven for 12 hr., reducing the spore population per carrier to 10^6. These remaining viable spores should have been extremely dry. None of these means of drying clean spore preparations produced any difference in resistance to ethylene oxide. The uncleanliness of a spore preparation seems to be the major reason for unusual resistance.

If the spore crop is clean, the region of partial survivors is narrow. However, if the spore crop is not thoroughly washed, the region of partial survivors is broad. Materials inoculated with the spore suspension are randomly protected by occlusion in crystals, encapsulation in organic debris, or both. The broader the region of partial survivors, the more unreliable the sterility monitor becomes.

One can artificially produce conditions in which microorganisms become extremely resistant to ethylene oxide. When *Staphylococcus aureus* or *Bacillus subtilis* var. *niger* were suspended in blood

and then inoculated on aluminum foil, they failed to be sterilized with ethylene oxide. Likewise, *Bacillus subtilis* var. *niger* spores suspended in peptone and dried on aluminum foil with a contamination level of only 10 spores/carrier were very difficult to sterilize with ethylene oxide. This would be similar to the protective effect found by previous investigators (6), *i.e.*, occlusion of the spores in crystals.

It is also possible to produce artificial conditions which would seem to make the organisms extremely susceptible to ethylene oxide. Spores suspended in 2% glycerin and then dried on polystyrene were less resistant to ethylene oxide than spores suspended in distilled water (7). *Bacillus subtilis* var. *niger* spores were suspended in 10% glycerin, inoculated on chromatography paper strips, and dried at room temperature. After drying at room conditions, the glycerin will contain a certain amount of water depending on the room relative humidity, for it will gain or lose moisture depending on its surroundings. At 130°F., 1,200 mg./L. ethylene oxide, these strips showed the same resistance (3 min. for inactivation) at 40% RH as at 10% RH even though the strips at 40% RH would contain a greater amount of moisture after equilibration. A similar situation would indicate to the unwary that no optimum RH was required. However, clean spores inoculated from distilled water required 1 hr. at 10% RH and 3 min. at 40% RH for inactivation.

SUMMARY

The evaluation of the efficiency of an ethylene oxide process depends a great deal on the type of bacteriological control used. It is therefore recommended that an evaluation be made of the type of load to be sterilized, so that the bacteriological controls chosen will accurately monitor the process. If one uses unwashed spores for preparation of sterility monitors when sterilizing clean materials, then he will likely obtain erratic indications of nonsterility rather than sterility. If, however, one does attempt to sterilize dirty materials, contaminated with dirt, blood, feces, *etc.*, he must realize that the process at its best will sterilize occasionally and should only be considered a decontamination procedure. The only dependable method of testing a sterilization process is to determine if it kills living microorganisms. The microbial control chosen should simulate actual conditions of the materials being sterilized.

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Keyphrases

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Staph. aureus—ethylene oxide resistant
Sterilization, ethylene oxide—variables affecting

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By NORMAN F. H. HO and W. I. HIGUCHI

A theoretical study of preferential coalescence and aggregation of small particles in heterodispersed systems has been carried out where moderate electrical barriers exist between the particles. Equations based on the concepts of Derjaguin, Verwey, and Overbeek were employed. Computation over a wide range of conditions has shown that small particles may aggregate (or coalesce) with themselves or with larger particles at rates that are 10 to 50 orders of magnitude faster than particles 10 times larger. These findings may explain (*a*) the relatively narrow particle size distributions observed in certain emulsions and flocculated suspensions and (*b*) the limited flocculation and coalescence behavior observed in certain instances.

THERE ARE many situations involving suspensions and emulsions where with time the particles or droplets of the dispersed phase simultaneously increase in size and narrow in their relative size distributions, and then later became quite stable kinetically. If the dispersed phase is soluble (or miscible) enough in the solvent, then the phenomenon may be accounted for by molecular diffusion (1) or Ostwald ripening. However, there are many examples in the literature (2–5) where the changes appear to occur primarily through particle–particle aggregation or droplet–droplet coalescence.

The authors have recently observed that urea-

Received April 14, 1967, from the College of Pharmacy, University of Michigan, Ann Arbor, MI 48104
Accepted for publication October 3, 1967.
This investigation was supported by fellowship 5-F1-GM-24,039 from the Institute of General Medical Sciences, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

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denatured ovalbumin aggregates precipitated in buffered saline near the isoelectric point are often made up of rather uniform particles of around 1 μ diameter (2). Sherman (3) found that emulsions pass through an initial phase of rapid coalescence followed by a second period of slow coalescence, and then the emulsions became relatively stable. All of his emulsions showed a relatively narrow size distribution in the later stages. Wiley (4) and Gillespie (5) have also studied the problem of limited coalescence when droplets in oil-in-water emulsions are stabilized by finely divided particles.

Although the effects of particle size on the stability of colloids have been recognized, quantitation has been limited to the case involving the potential energy barriers for particles of equal sizes (6) or for particles of different sizes in which the mean size is used (3). While the equations for the repulsive potential energy between two unequal spheres have already been derived, an expression for the attractive potential energy between two unequal spheres with retardation factors has not been considered. The present authors are not aware of quantitative computations involving preferential collision of differing particle sizes in the presence of a repulsive force. It is the purpose of this paper to examine the theory as it relates to preferential and limited aggregation (or coalescence). As will be seen, some of the theoretical conclusions consistently describe many of the findings of investigators in the field.

THEORY

Rate of Preferential Aggregation or Coalescence—Let us assume that there are equal concentrations of spherical particles of three sizes, that is, spheres S_1, S_2, and S_3 of radii a_1, a_2, and a_3, respectively, where $a_1 < a_2 < a_3$. The dispersion is in an electrolyte solution and the particles are allowed to aggregate (coalesce) at 25°. The following question is asked: what are the probabilities of S_1 colliding with S_1, S_2, or S_3, of S_2 with S_2 or S_3, and of S_3 with S_3? By means of theory let us determine the initial rates of aggregation or coalescence for the various size pairs.

It will be assumed following the Derjaguin-Verwey-Overbeek concepts, the interparticle interaction results from electrical repulsion and the London-van der Waals attraction. According to the theories of Smoluchowski and Fuchs (7), the rate of collision between spherical particles of sizes i and j is given by:

$$G_{ij} = \frac{4\pi D_{ij} R_{ij} N_i N_j}{W_{ij}} \qquad \text{(Eq. 1)}$$

where G is the initial rate, D is the Stokes-Einstein diffusion coefficient, R is the distance between centers of the spheres, N is the concentration of particles, and W is the stability factor, which will be later discussed in detail. We may also write:

$$D_{ij}R_{ij} = \frac{kT}{6\pi\mu}\left(\frac{1}{a_i} + \frac{1}{a_j}\right)(a_i + a_j) \qquad \text{(Eq. 2)}$$

where k is the Boltzman constant, T is the absolute temperature, and μ is the viscosity of the suspending medium. Then the initial rate is:

$$G_{ij} = \frac{2kT}{3\mu W_{ij}}\left(\frac{1}{a_i} + \frac{1}{a_j}\right)(a_i + a_j)N_iN_j \qquad \text{(Eq. 3)}$$

The factor W_{ij} accounts for the energy barrier to aggregation and is given by:

$$W_{ij} = 2\int_2^\infty \exp.\left(\frac{V_{T(i,j)}}{kT}\right)\frac{ds}{s^2} \qquad \text{(Eq. 4)}$$

where $s = R/\bar{a}$ and $\bar{a} = (a_i + a_j)/2$. The W_{ij} usually ranges from $1 \lesssim W < \infty$ and takes into account the double-layer potential, the concentration of electrolytes, London-van der Waal dispersion forces, and the particle sizes of the two spheres.

Potential Energy of Interaction—In Eq. 4 the $V_{T(i,j)}$ is the total potential energy of interaction between two particles and is given by the sum of the repulsive energy, $V_{R(i,j)}$, and the attraction energy, $V_{A(i,j)}$; thus:

$$V_{T(i,j)} = V_{R(i,j)} + V_{A(i,j)} \qquad \text{(Eq. 5)}$$

The repulsive energy between interacting spheres of sizes i and j can be calculated by the approximate expression derived by Hogg, Healy, and Fuerstenau (8):

$$V_{R(i,j)} = \frac{\epsilon a_i a_j \psi_0^2}{(a_i + a_j)} \ln\left[1 + \exp.(-KH)\right] \qquad \text{(Eq. 6)}$$

where ϵ is the dielectric constant of the medium, ψ_0 is the surface potential, K is the reciprocal of the Debye-Hückel thickness of the double layer, and H is the shortest distance between the surfaces of the spheres. The equation is valid for Ka_i and $Ka_j \gg 1$.

The attraction energy of particles is more difficult to determine because the force between particles is considerably reduced by the retardation between atoms. Since the retardation effect is a function of distance, a single expression for the attraction energy is not available. When $a_i, a_j \gg H$ and $H \lesssim 150°\ A$, the attraction interaction energy can be calculated by the expression:

$$V_{A(i,j)} = -\frac{Aa_ia_j}{6(a_i + a_j)H}\left(\frac{\lambda}{\lambda + 11.116H}\right) \qquad \text{(Eq. 7)}$$

where A is the Hamaker constant, and λ is the characteristic wavelength of the atoms.

For $H \gtrsim 150°\ A$,

$$V_{A(i,j)} = \frac{Aa_ia_j}{(a_i + a_j)}\left[\frac{-2.45\lambda}{60\pi H^2} + \frac{2.17\lambda^2}{360\pi^2H^3} - \frac{0.59\lambda^3}{1680\pi^3H^4}\right] \qquad \text{(Eq. 8)}$$

One should refer to the *Appendix* for the discussion of the retardation factor and the derivation of Eqs. 7 and 8.

CALCULATIONS

Computations employing Eqs. 3–8 were carried out for a wide range of conditions with the aid of the IBM 7090 digital computer. ψ_0 values from 0 to

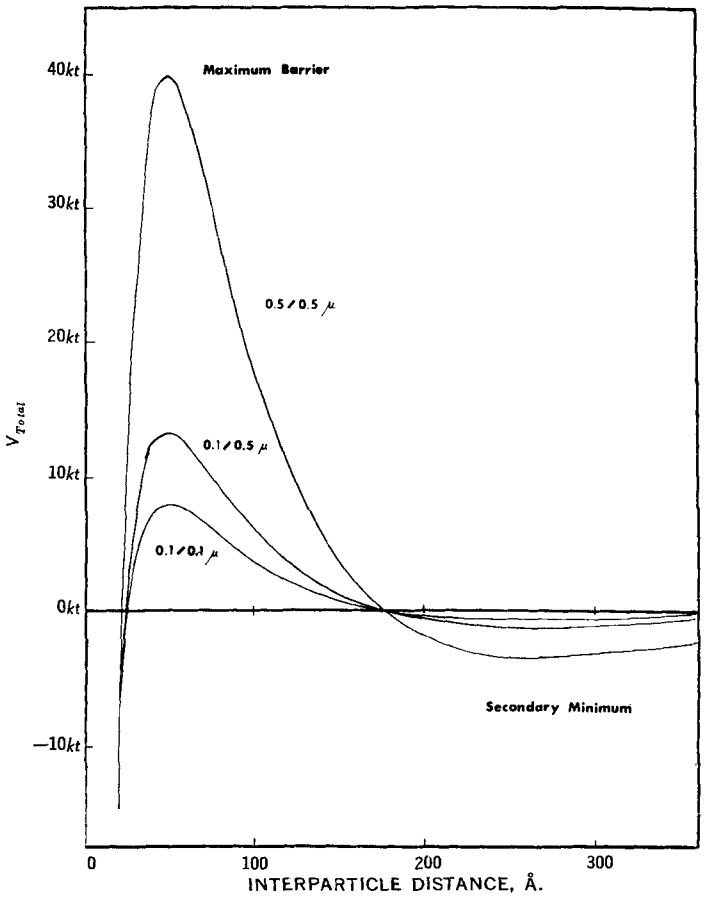

Fig. 1—Influence of particle sizes on the total potential energy of interaction. A = 5×10^{-13} *erg,* ψ_0 = *25 mv., and* K = 2×10^6 *cm.*$^{-1}$.

100 mv. and various radius combinations were taken at different K. Because the Hamaker constant is estimated (9, 10) to be somewhere in the neighborhood of 1 to 5 $\times$ 10^{-13} erg for most organic substances in water, calculations were made using the two values, 1 $\times$ 10^{-13} and 5 $\times$ 10^{-13}. The characteristic wavelength, λ, was taken to be 10^{-5} cm. Some plots of V_T *versus* H are shown in Figs. 1–3.

Table I presents some of the significant results. Calculated values of $V_{T\max.}$, the maximum in the V_T *versus* H curves, are shown. The rate constant, G', is defined as:

$$G' = \frac{G_{ij}}{N_i N_j} \qquad \text{(Eq. 9)}$$

DISCUSSION

Where K is large, *i.e.*, $K \gtrsim 0.4$ to 2 $\times$ 10^7, or when $\psi_0 \simeq 0$, it is seen (Table I) that, for essentially all particle sizes in the range of 0.1 to 5.0 μ radius, the rates of aggregation approach the Smoluchowski rates. Thus, in 1% NaCl solutions, maximum rates may be expected. Figure 4 shows the maximum limiting rate constants under these conditions for which W_{ij} equals unity.[1] The well-known preferential aggregation relationship due to Muller (11) can be seen here. However, under such "rapid" aggregation conditions, the rate constant for a 0.1 μ–1.0 μ pair is only about three times greater than that for particles of the same size.

Let us now direct our attention to the region of kinetic stability where the rates may be regarded as neither very rapid nor very slow. In this intermediate stability region we note a dramatic change in importance of particle size. This can be seen in Table I and in Fig. 5. In Fig. 5 it can be seen how electrolyte concentration may markedly increase the preference for the aggregation (or coalescence) of small particles with each other or with large particles. Thus, when $K \simeq 2$ to 4 $\times$ 10^6 for $\psi_0 \simeq 25$ mv., it can be seen (Table I or Fig. 5) that the rate of aggregation (or coalescence) of 0.1-μ particles with itself or larger particles may be 10 to 30 orders of magnitude greater than that for two 0.5-μ particles. Comparing Fig. 5 with Fig. 6, we note that the rates for various particle sizes are highly sensitive to the Hamaker constant taken as 1 $\times$ 10^{-13} and 5 $\times$ 10^{-13} erg.

The above conclusions depend upon the assumption that other contributions to particle–particle repulsion are absent. Therefore, in the case of emulsion coalescence, strongly adsorbed barrier-forming interfacial films are assumed to be absent. In practice one would expect that when surface coverage of ionic emulsifiers is low, say about 5 to 10% of maximum coverage, the repulsion between two oil droplets in an aqueous medium would be

[1] Actually the limiting W_{ij} values may be somewhat less than unity when all repulsive barriers are removed because V_T will be negative (see Eq. 4).

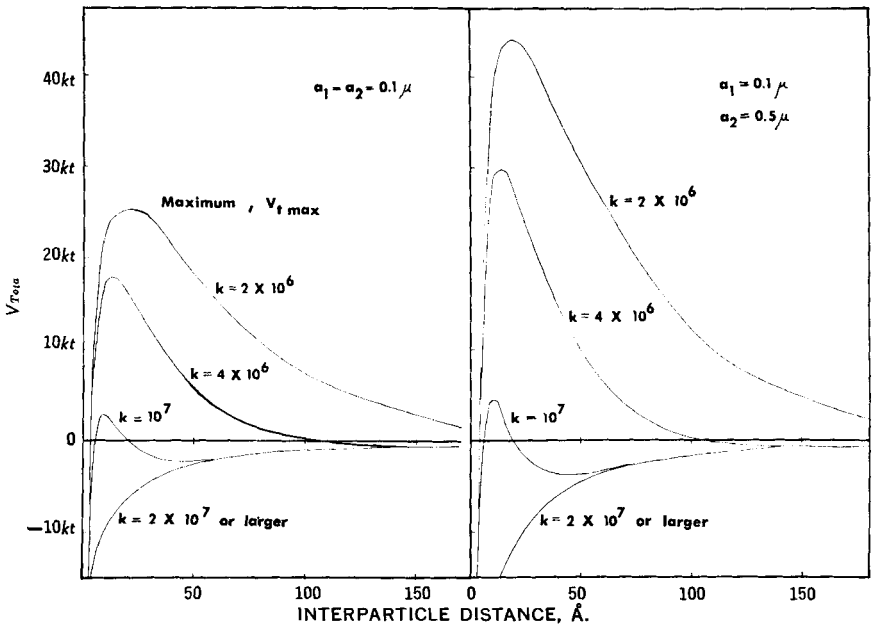

Fig. 2—Influence of electrolyte concentration on the total potential energy of interaction for two particle sizes. A = 1×10^{-13} *erg and* ψ_0 = 25 *mv.*

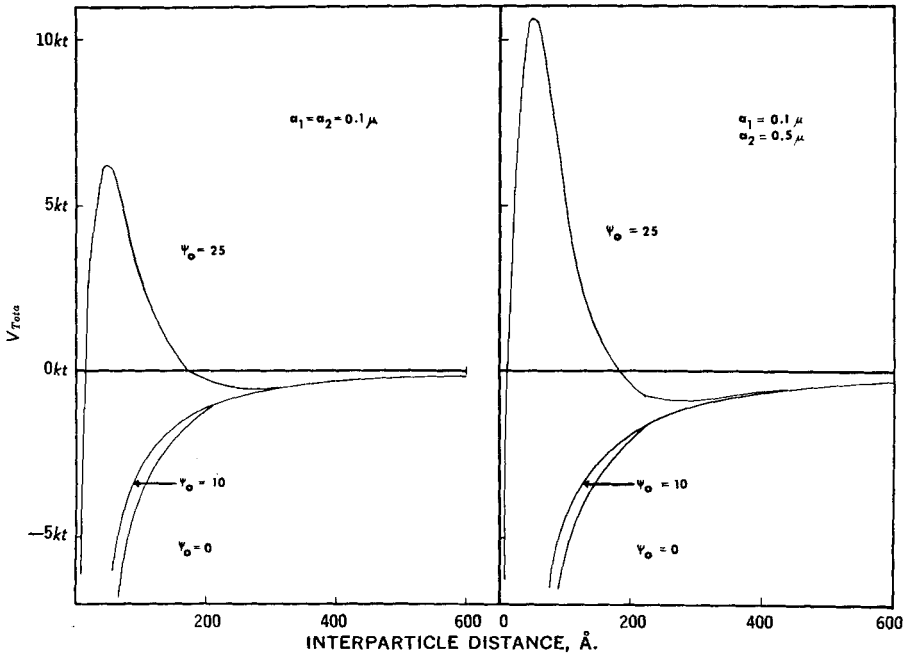

Fig. 3—Influence of surface potential on the total potential energy of interaction for two particle sizes. A = 5×10^{-13} *erg,* ψ_0 = 25 *mv., and* K = 2×10^6 *cm.*$^{-1}$.

primarily electrical. These findings are consistent with the observations (3) that there is frequently an initial period of rapid coalescence with freshly prepared emulsions. Usually this is explained on the basis of the lack of sufficient stabilizer to provide a complete monolayer for the droplets. However, this explanation alone does not account for the relatively narrow size distributions that often result. The absence of small particles indicates that they preferentially disappear rapidly.

It is proposed that in a freshly prepared emulsion the shortage of surfactant prevents the formation of complete interfacial adsorbed films. Consequently, the rupture or displacement of the adsorbed molecules may not be rate determining in coalescence. Then if ψ_0 is low ($\lesssim$25 mv.) or if K is large, the rapid and selective disappearance of small droplets through coalescence would occur. The surviving larger droplets coalesce at a much slower rate as W_{ij} is extremely sensitive to particle size. Thus, a relatively narrow distribution would result if Brownian motion alone is inducing the coalescence.

TABLE I—SUMMARY OF DATA ON THE INFLUENCE OF ELECTROLYTE CONCENTRATION (K), SURFACE POTENTIAL (ψ_0), AND THE HAMAKER CONSTANT (A) ON THE MAXIMUM POTENTIAL INTERACTION ENERGY OF THE BARRIER ($V_{TMAX.}$) AND THE RATE CONSTANT FOR AGGREGATION (G')

ψ_0, mv.	K, cm.$^{-1}$	Radii 0.1 and 0.1 μ: G'	Radii 0.1 and 0.1 μ: $V_{Tmax.}/kT$[a]	Radii 0.1 and 0.5 μ: G'	Radii 0.1 and 0.5 μ: $V_{Tmax.}/kT$[a]	Radii 0.1 and 1.0 μ: G'	Radii 0.1 and 1.0 μ: $V_{Tmax.}/kT$[a]	Radii 0.5 and 0.5 μ: G'	Radii 0.5 and 0.5 μ: $V_{Tmax.}/kT$[a]
				A erg, 1×10^{-13}					
0	...	11.2×10^{-12}	~0	20.2×10^{-12}	~0	33.9×10^{-12}	~0	11.2×10^{-12}	~0
10	2×10^{4}	5.2×10^{-12}	0.77	5.6×10^{-12}	1.28	8.4×10^{-12}	1.40	2.4×10^{-13}	3.84
	$\geq 4 \times 10^{4}$	11.2×10^{-12}	~0	20.2×10^{-12}	~0	33.9×10^{-12}	~0	11.2×10^{-12}	~0
25	2×10^{4}	3.0×10^{-24}	25.73	5.8×10^{-28}	42.88	3.6×10^{-29}	46.78	1.1×10^{-95}	129.63
	4×10^{4}	3.4×10^{-17}	17.43	1.2×10^{-21}	29.05	3.6×10^{-22}	31.69	6.3×10^{-47}	87.15
	1×10^{5}	2.4×10^{-13}	2.56	2.8×10^{-13}	4.26	3.1×10^{-13}	4.65	3.1×10^{-18}	12.79
	$\geq 2 \times 10^{5}$	8.6×10^{-13}	~0	20.2×10^{-12}	~0	33.9×10^{-12}	~0	11.2×10^{-12}	~0
				A erg, 5×10^{-13}					
0	...	11.2×10^{-12}	~0	20.2×10^{-12}	~0	33.9×10^{-12}	~0	11.2×10^{-12}	~0
10	$\geq 2 \times 10^{5}$	11.2×10^{-12}	~0	20.2×10^{-12}	~0	33.9×10^{-12}	~0	11.2×10^{-12}	
25	$\geq 2 \times 10^{5}$	19.4×10^{-14}	7.75	6.0×10^{-15}	12.91	5.7×10^{-15}	14.08	3.4×10^{-26}	38.73
	$\geq 4 \times 10^{5}$	11.2×10^{-12}	~0	20.2×10^{-12}	~0	33.9×10^{-12}	~0	11.2×10^{-12}	~0

[a] Note that when $V_{Tmax./kT} < 0$, little repulsive barrier exists, hence aggregation is dependent on London-van der Waal attraction.

Fig. 4—Probability of rapid aggregation between particles of different sizes when the stability factor W_{ij} *equals unity.*

Gillespie (5) has discussed limited coalescence of oil-in-water emulsions stabilized by finely divided solids. He proposed a theory based on the rate of accretion of the solid stabilizer by the oil droplets. Furthermore, he found that there was an optimum concentration range for an ionic surfactant additive that produced the most uniform stable drops. This concentration range of the ionic surfactant appeared to produce a moderate or small electrical repulsive barrier. These findings are consistent with the theoretical results.

While the findings are only tentative, the precipitation of denatured proteins (2) near the isoelectric point appears to involve the formation of relatively uniform primary particles of around 1 μ diameter. Such narrow distributions are consistent with the above theory that selective aggregation of small particles with large ones take place. The extreme situation where only single denatured protein molecules deposit upon the larger particles would lead to the formation of relatively compact spherical particles of relatively narrow size ranges.

The authors believe that the results presented provide a much better understanding of the role of particle size in the mechanisms of aggregation and coalescence. It provides a possible scientific basis for tailor-making dispersion formulations of controlled size and size distributions.

APPENDIX

Attractive Energy between Unequal Spheres and the Effect of Retardation—Hamaker (12) was the first to describe the potential energy of attraction between unequal spheres, $V_{A(S_1/S_2)}$, of similar constitution. For $a_1, a_2 \gg H$, the limiting expression is:

$$V_{A(S_1/S_2)} = -\frac{A a_1 a_2}{6(a_1 + a_2)H} \qquad \text{(Eq. 1}a\text{)}$$

where Hamaker's constant $A = \pi^2 q^2 \beta$, and q is the number of atoms per cm.^{3}, β is the London constant, a_1, a_2, and H are defined as before.

Schenkel and Kitchener (10) made a thorough analysis of the retardation effect. Using the equations for $f(p)$ as a check that are presented in several references (10, 13), they derived empirical expressions that gave the best fit and that would give an analytic solution for interaction energies with modifications for the retardation effect. Accordingly,

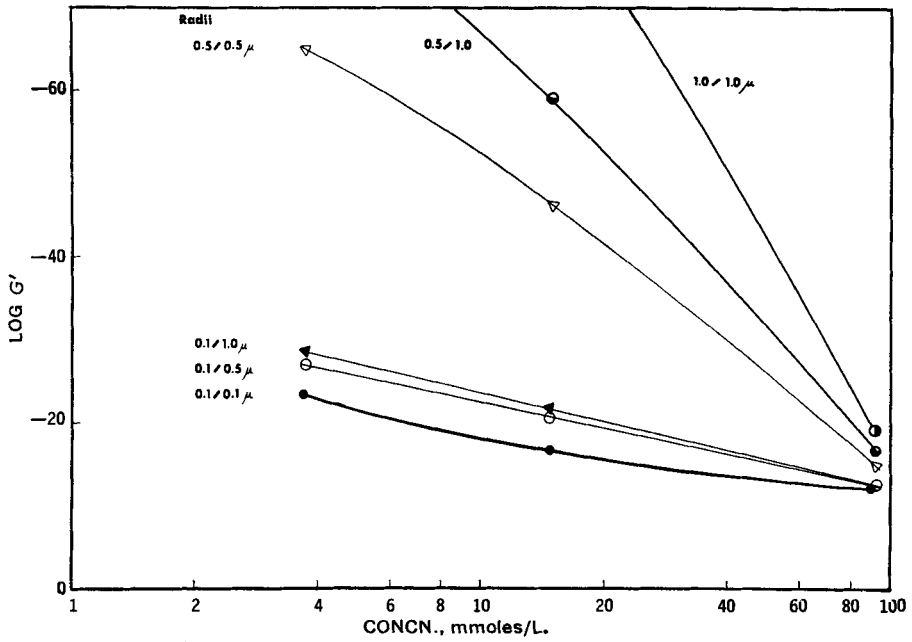

Fig. 5—Rate constant (G′) *as a function of concentration of 1-1 electrolyte for various particle sizes.* A = 1×10^{-13} *erg and* $\psi_0 = 25$ *mv.*

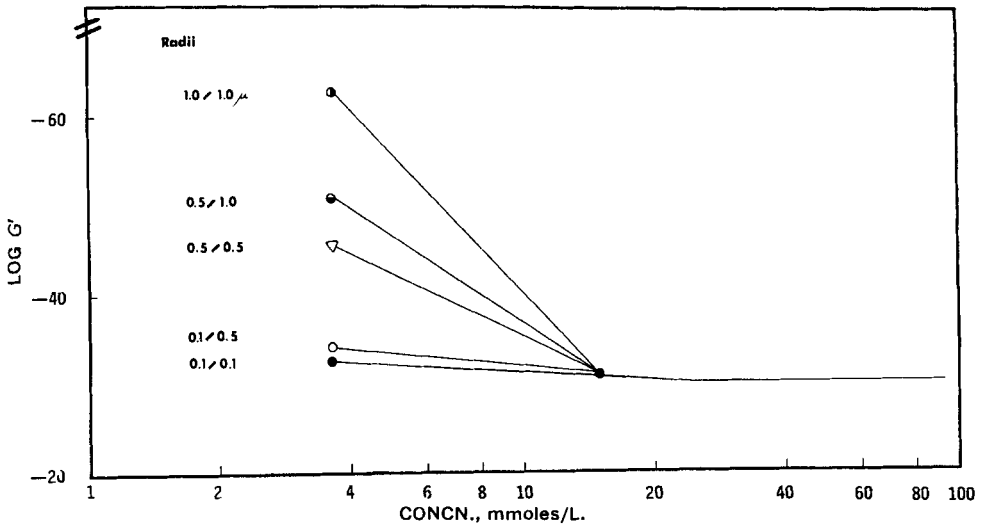

Fig. 6—Rate constant (G′) *as a function of concentration of 1-1 electrolyte for various particle sizes.* A = 5×10^{-13} *erg and* $\psi_0 = 25$ *mv.*

for the range of $0.5 < p < \infty$,

$$f(p) \sim \frac{2.45}{p} - \frac{2.17}{p^2} + \frac{0.59}{p^3} \quad \text{(Eq. 2}a\text{)}$$

For comparatively large distances, Casimir and Polder (14) showed that the London energy between atoms decays very rapidly due to retardation effects. Consequently, the London interaction energy of a pair of atoms of r distance apart,

$$V_{A(\text{London})} = -\frac{\beta}{r^6} \quad \text{(Eq. 3}a\text{)}$$

must be corrected so that

$$V'_{A(\text{at./at.})} = V_{A(\text{London})} f(p) \quad \text{(Eq. 4}a\text{)}$$

where $p = 2\pi r/\lambda$, and λ is the characteristic wavelength of the atoms and β is the London constant. Equation 4a may be rewritten:

$$V'_{A(\text{at./at.})} = -\frac{2.45\lambda\beta}{2\pi r^7} + \frac{2.17\lambda^2\beta}{(2\pi)^2 r^8} - \frac{0.59\lambda^3\beta}{(2\pi)^3 r^9} \quad \text{(Eq. 5}a\text{)}$$

In deriving the expressions for the retarded interaction energies for equal spheres, Schenkel and Kitchener used Derjaguin's method (15, 16) for calculating the interaction of spheres. It was pointed out that the algebra was simpler than for Hamaker's method (12) and that the results obtained were the same by both methods, provided it is only the limiting expressions that are required. In a similar manner, the method is applied to the interaction between unequal spheres.

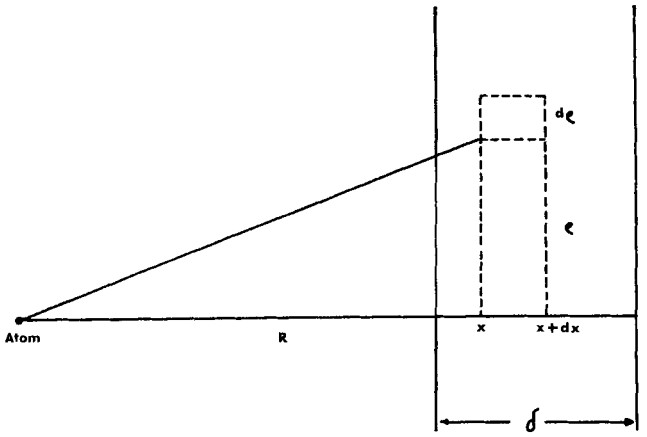

Fig. 7—Geometry of the London-van der Waal interaction between an atom and an infinitely large plate of thickness δ. The plate is built up of infinitesimal rings of diameter ρ, cross-section dρdx at a distance (R + x) from the atom (17).

Applying Eq. 4*a* to the geometry in Fig. 7, the interaction energy between an atom and all of the atoms in an infinitely large flat plate, $V'_{A(\text{at./}p)}$, may be described by the superposition of their retarded attractive energies, *i.e.*,

$$V'_{A(\text{at./}p)} = V''_A + V'''_A + V''''_A \quad \text{(Eq. 6}a\text{)}$$

where

$$V''_A = -\int_0^\delta\int_0^\infty \frac{2\pi q\phi\rho d\rho dx}{[(R+x)^2+\rho^2]^{7/2}} \quad \text{(Eq. 7}a\text{)}$$

$$V'''_A = \int_0^\delta\int_0^\infty \frac{2\pi q\theta\rho d\rho dx}{[(R+x)^2+\rho^2]^4} \quad \text{(Eq. 8}a\text{)}$$

$$V''''_A = -\int_0^\delta\int_0^\infty \frac{2\pi qZ\rho d\rho dx}{[(R+x)^2+\rho^2]^{9/2}} \quad \text{(Eq. 9}a\text{)}$$

and $\phi = 2.45\lambda\beta/2\pi$; $\theta = 2.17\lambda^2\beta/(2\pi)^2$; and $Z = 0.59\lambda^3\beta/(2\pi)^3$. Also, the Hamaker constant $A = \pi^2q^2\beta$. It follows that the interaction between two flat plates, $V'_{A(p/p)}$, is

$$V'_{A(p/p)} = \int_R^\infty qV'_{A(\text{at./}p)}dR \quad \text{(Eq. 10}a\text{)}$$

If the surface of two spheres is thought to be built up of parallel pairs of infinitesimally small rings with radius h (8), the interaction of unequal spheres, $V'_{A(S_1/S_2)}$, is expressed by:

$$V'_{A(S_1/S_2)} = 2\pi\left(\frac{a_1a_2}{a_1+a_2}\right)\int_H^\infty V'_{A(p/p)}dH \quad \text{(Eq. 11}a\text{)}$$

The edge effects are neglected. Finally, the limiting expression for the interaction energy for unequal spheres is:

$$V'_{A(S_1/S_2)} = \frac{Aa_1a_2}{a_1+a_2}\left[-\frac{2.45\lambda}{60\pi H^2}+\frac{2.17\lambda^2}{360\pi^2H^3}-\frac{0.59\lambda^3}{1680\pi^3H^4}\right] \quad \text{(Eq. 12}a\text{)}$$

(for $H \ll a_1, a_2$; $0.5 < p_0 < \infty$).

Since $p_0 = 2\pi H/\lambda$ and λ is usually taken as 10^{-5} cm., Eq. 12*a* should not be used for approximately $H < 150°$ *A*. It is noteworthy that the equation reduces to the identical expression[2] obtained by Schenkel and Kitchener for interaction of equal spheres, *i.e.*, $a_1 = a_2$.

For the range below $p_0 = 0.5$, the retardation effect is still significant until the interparticle distance is comparatively very small, in which case Eq. 1*a* will apply. Based on extrapolation of the attractive potential energy curve, an empirical expression that represents the region $0 < p_0 < 2$, approximately $H < 300°$ *A*, is given by Eq. 7.

[2] See equation (x) in the appendix of *Reference 10.*

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Keyphrases

Heterodispersed systems
Coalescence, aggregation—small particles
Electrical barrier, effect—coalescence, aggregation
Energy of interaction potential—small particles
Spheres, unequal—attractive energy, retardation

Synthesis of Tropine-Labeled Atropine III

Synthesis of *N*-Methyl-^{14}C-Tropine and *N*-Methyl-^{14}C-Atropine

By GILBERT C. SCHMIDT, THOMAS E. ELING, and JOHN M. McOWEN

Using a previously described modification of the Robinson condensation, *N*-methyl-^{14}C-tropine, specific gravity 1 mc./mmole, was synthesized from methylamine-^{14}C-HCl in 65 percent of theoretical yield. Subsequent esterification with unlabeled tropic acid gave *N*-methyl-^{14}C-atropine, specific activity 1 mc./mmole, in 45 percent of theoretical yield, based on methylamine. Synthesis of these compounds validates previous studies and firmly establishes a general pathway for labeling the carbon skeleton of tropine with radioactive carbon.

THE HIGH toxicity of atropine dictates that minute doses be administered to test animals and, thereby, limits studies of atropine metabolism. Exclusive use of separations and purifications to trace and locate metabolites is precluded. Selective, isotopic labeling of the alkaloid is essential. Studies of intermediary animal metabolism, plant biogenesis, the mechanisms of pharmacological actions, and pathways that permit certain microorganisms to utilize atropine as a sole carbon and nitrogen source (1–3) depend on the availability of suitably labeled compounds.

An interest in these problems and the associated need for unavailable compounds stimulated a study of methods for selectively labeling tropine and the tropine moiety of atropine. A general, microsynthetic pathway was established (4) and a prototype method for the synthesis of labeled tropine from labeled arabinose was developed (5). The present communication describes the synthesis of *N*-methyl-^{14}C-tropine and *N*-methyl-^{14}C-atropine from labeled methylamine HCl, confirms for the first time Werner's synthesis of these compounds, and establishes that the previously proposed pathways are suitable for use with radioactive carbon.

EXPERIMENTAL

Microsynthetic methods for tropine and atropine are described in detail in the first two papers of this series (4, 5) and were modified only slightly for the synthesis of *N*-methyl-^{14}C-tropine and *N*-methyl-^{14}C-atropine.

Preparation of O-Acetyltropic Acid—Acetyltropic acid was prepared from 395 mg. tropic acid and 0.36 ml. acetyl chloride in the manner previously described (4).[1] The product was dried over $CaCl_2$ in a vacuum desiccator and was converted to *O*-acetyltropic acid chloride at the time of esterification with tropine.[2]

Preparation of Raney Nickel and Check of Activity—Type W-7 Raney Nickel[3] was prepared from 10 Gm. of alloy by the method of Augustine (6).[4] Although the catalyst is active for at least 2 weeks,[5] catalysts used in radiosyntheses were checked for activity on the evening prior to reduction of labeled tropanone.[6]

Using the apparatus and experimental conditions employed in radiosyntheses, 100 mg. unlabeled tropanone was reduced overnight. During early stages of the reduction, hydrogen uptake was checked, and on the following morning a 10-μl. sample of the reaction mixture was spotted on filter paper, air dried, and checked for completness of reduction by spraying with 2,4-dinitrophenylhydrazine solution (7). Tropine was also isolated as the picrate and the melting point of the picrate determined.[7] If reduction was complete, the catalyst was used for the reduction of labeled tropanone.

***N*-Methyl-^{14}C-tropine HCl from *N*-Methyl-^{14}C-Tropanone**—Labeled tropanone, synthesized from the stated quantities of reactants and weighing approximately 100 mg. when dried for 12 hr., was reduced over W-7 Raney nickel by the general method of Van de Kamp (8).[8]

Tropanone was dissolved in 2 ml. absolute ethanol and, while stirring slowly,[9] the system was flushed three times with ultra-pure hydrogen.[10] The gas buret was filled with hydrogen, the system closed, and reduction continued with constant stirring, at room temperature, until the rate of gas uptake

Received July 5, 1967, from The Children's Hospital, Division of Pharmacology, Departments of Pediatrics and Biological Chemistry, University of Alabama Medical Center, Birmingham, AL 35233

Accepted for publication October 19, 1967.

Abstracted, in part, from a thesis submitted by Thomas E. Eling to the Graduate School, University of Alabama, Department of Biological Chemistry, in partial fulfillment of Doctor of Philosophy degree requirements.

This investigation was supported by grant GM-13844 from the National Institutes of Health and by grant AFOSR 87-65,66 from the U. S. Dept. of the Air Force.

Presented in part, to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

[1] M.p. 87–88°, yield 468 mg.

[2] The product must be thoroughly dry. Storage for 4 days under previously described conditions usually is adequate and storage for 3 months causes no decrease in atropine yields.

[3] Type W-2 catalyst was used in earlier work. Reduction is more rapid and less difficulties are encountered with the W-7 catalyst.

[4] When prepared as described and stored under 250 ml. ethanol, the suspension contains 28 mg. catalyst per ml.

[5] Stored in the refrigerator in bottles filled with absolute ethanol.

[6] A check of catalyst activity under experimental conditions circumvents many difficulties and prevents losses of radioactive intermediates. Traces of impurities of hydrogen quickly poison the catalyst. The gas must be purchased in highly purified form, or it must be purified before use.

[7] Yields should be practically quantitative. M.p. picrate 295°.

[8] Van de Kamp did not indicate the grade of Raney nickel used. Although W-2 catalyst was used in earlier work, the W-7 grade was more satisfactory.

[9] A glass-coated magnetic stirring bar was used.

[10] Ultra-pure hydrogen, E. H. Sargent and Co. Use of ultra-pure hydrogen is virtually mandatory. Although commercial hydrogen of ordinary purity may be purified by passing through concentrated H_2SO_4, results are still erratic.

asymptotically approached zero. A 2-μl. aliquot of the reaction mixture was spotted on filter paper, air dried, and tested with 2,4-dinitrophenylhydrazine solution. If a negative test was not obtained,[11] the reaction mixture was filtered through a fine-porosity filter and the catalyst washed with approximately 10 ml. absolute ethanol. Filtrate and washings were concentrated to approximately 2 ml.[12] and the reduction repeated with fresh catalyst. Finally, the catalyst was removed by filtration and all solvent was removed by use of the flash evaporator. The product was stored in a desiccator, without vacuum, over NaOH pellets,[13] until converted to the hydrochloride.

After suitable storage, *N*-methyl-^{14}C-tropine[14] was converted to the hydrochloride, in the exact manner described previously (4). The hydrochloride[15] was stored in the 10-ml. reaction vessel, in a vacuum desiccator, over NaOH pellets, until used for esterification.

Synthesis of *N*-Methyl-^{14}C-Atropine from O-Acetyltropic Acid and *N*-Methyl-^{14}C-Tropine HCl—To the *O*-acetyltropic acid chloride obtained from 150-mg. *O*-acetyltropic acid (4)[16] was added the thoroughly dried *N*-methyl-^{14}C-tropine HCl produced from the quantities of reactants used in the Robinson condensation.[17] The reactants were esterified to form *O*-acetyl-*N*-methyl-^{14}C-atropine, the acetylated compound was hydrolyzed to the alkaloidal free base, and *N*-methyl-^{14}C-atropine finally isolated, exactly as described for the unlabeled alkaloid (4). The product was dried immediately in a vacuum desiccator over $CaCl_2$. The dry product was dissolved in 75 ml. anhydrous petroleum ether, the solution was concentrated to approximately 10 ml., and labeled atropine precipitated by cooling in the deep freezer. Petroleum ether was decanted from the crystals,[18] the product recrystallized from petroleum ether, then dried in a desiccator over $CaCl_2$.[19]

Synthesis of *N*-Methyl-^{14}C-Tropanone—Labeled tropanone was prepared from methylamine-^{14}C-HCl, succindialdehyde, and acetone dicarboxylic acid, according to previously described procedures (4, 5). The earlier papers should be consulted for essential experimental details.

Acetone dicarboxylic acid was prepared from 1-Gm. citric acid, collected after the final cooling to −8°, and immediately used in the Robinson condensation. During collection of acetone dicarboxylic acid, succindialdehyde was prepared. To 5 μl. of concentrated HCl and 5 ml. water, contained in a 10-ml. beaker, was added 0.34 ml. of 2,5-diethoxytetrahydrofuran. The mixture was hydrolyzed by stirring, at room temperature for 10 min. The resulting dialdehyde[20] was used immediately in the Robinson condensation.[21]

To the solution of succindialdehyde was added 40% of the moist acetone dicarboxylic acid from 1 Gm. of citric acid. Saturated, aqueous Na_2HPO_4 was added to adjust the pH to 4.5–5.0, using a glass electrode. The buffered mixture was transferred to a previously assembled, 250-ml., conical, side-arm flask and the system purged with nitrogen for 10 min. To the buffered mixture was added 5 ml. water, containing 33.8 mg. of methylamine-^{14}C-HCl[22] and 33.8 mg. unlabeled methylamine HCl. The volume was adjusted to 50 ml. and the system quickly evacuated to remove most of the gas phase.[23] The system was closed and the mixture allowed to stand at room temperature for 12 hr. The slight evacuation was repeated and, after standing at room temperature for an additional 12 hr., the solution was saturated with Na_2CO_3.

Tropanone was extracted with 100-ml. portions of petroleum ether, exactly as previously described (4), except that extraction was continued until significant amounts of radioactivity no longer were removed. The petroleum ether extracts were dried over Na_2SO_4, transferred to a 250-ml. sublimation flask, and the solvent removed, *in vacuo*, on the flash evaporator, at 40°. Tropanone, which remained in the flask as a brown oil, was dried *in situ*, in a desiccator, over $CaCl_2$ and NaOH, overnight. The crude product was purified by repeated sublimation at 40° and 1×10^{-4} mm. Hg. After each sublimation, the product was washed from the cold finger with small amounts of absolute ethanol. Sublimation was repeated until the washings no longer contained significant amounts of radioactivity. The combined, alcoholic solutions of *N*-methyl-^{14}C-tropanone were evaporated to dryness in the flash evaporator, in a previously tared 25-ml. flask. The product[24] crystallized quickly on cooling, was dried in a desiccator for 12 hr., then reduced to tropine.

RESULTS AND DISCUSSION

The synthesis of tropine, labeled with ^{14}C in the carbon skeleton, and of atropine labeled in the tropine moiety, has remained an unsolved problem. For synthesis of the heterocycle, the Robinson condensation (9, 10) is inherently versatile and has been proven adaptable for microsynthesis (4, 11). In a similar way, the Wolffenstein and Mamlock esterification has been adapted for microsynthesis (4, 11, and 12) and has been proven adequate for the

[11] Disappearance of the 2,4-dinitrophenylhydrazine color was a more reliable index of complete reduction than was hydrogen uptake. In no instance was a negative color accompanied by unexpectedly low tropine yields. At times, theoretical or greater than theoretical hydrogen uptake was accompanied by a positive 2,4-dinitrophenylhydraline test. Repetition of the reduction gave a negative color and the expected tropine yields.

[12] Losses may occur by volatilization. Evaporation of the solvent must be done carefully.

[13] The product must be dry before it is converted to the hydrochloride, yet drying must not be unduly prolonged because losses occur by volatilization. Crystallization usually occurs within 12 hr. and storage for an additional 24 hr. usually is adequate for drying. A maximum storage of 2 days, without vacuum, is recommended.

[14] Yield 93.8 mg., specific activity 1 mc./mmole, m.p. 63–65°.

[15] Yield 119 mg., specific activity 1 mc./mmole, m.p. 280° with decomposition.

[16] Requirements for freshly prepared acyl halide, removal of all traces of thionyl chloride, and exclusion of moisture must be re-emphasized.

[17] Labeled tropine HCl from the stated amounts of methylamine HCl, acetone dicarboxylic acid and succindialdehyde weighed 115 mg., specific activity 1 mc./mmole, m.p. 280°, when corrected for tropanone contamination.

[18] At this point, the product usually contains small amounts of tropine. In no instance did tropine represent more than 8% of total counts, equivalent to 4% of product weight.

[19] Yield 130 mg. (45% based on methylamine HCl), specific activity 1 mc./mmole, m.p. 114–116°, after recrystallization from petroleum ether.

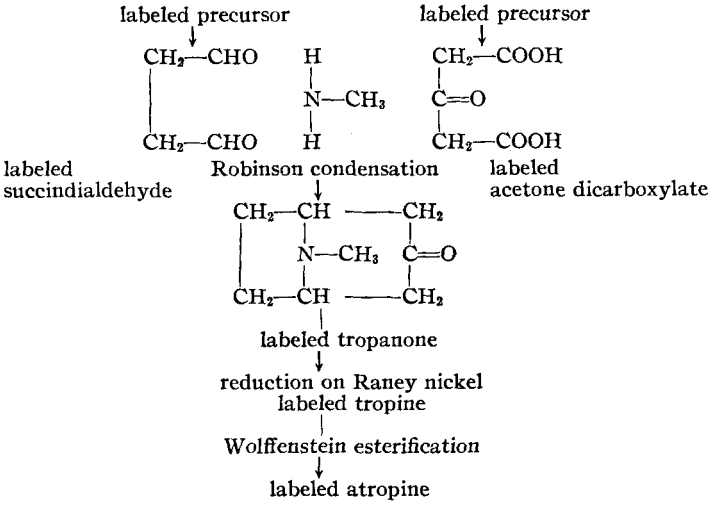
[20] This quantity of diethoxytetrahydrofuran produces 2 mmole (173 mg.) of succindialdehyde.

[21] Ideally, collection of acetone dicarboxylic acid and hydrolysis of diethoxytetrahydrofuran should be completed at the same time.

[22] Mallinckrodt Nuclear (formerly Nuclear Research Chemicals), Orlando, Florida. Specific activity 2 mc./mmole.

[23] Flask evacuated to the point at which the liquid in it bubbles slightly but does not boil vigorously.

[24] Yield 94.3 mg., needles, m.p. 41–43°, specific activity 1 mc./mmole.



Scheme I

esterification of tropine and tropic acid. It is clear, from the reaction sequence (Scheme I), that labeled precursors of succindialdehyde or acetone dicarboxylic acid become logical instruments for labeling the carbon skeleton of tropine.

Several studies with unlabeled compounds support the feasibility of this scheme, but the use of labeled intermediates has been limited and published findings remain unconfirmed. For convenience of discussion, the esterification and condensation steps may be considered as separate entities.

After a single esterification, Werner (11) obtained 70% of theoretical yields for the esterification of *N*-methyl-^{14}C-tropine with unlabeled tropic acid. This is in agreement with yields obtained in the microesterification of unlabeled intermediates (4), but other observations indicate a potential lack of reproducibility. Fodor (13) also esterified *N*-methyl-^{14}C-tropine with unlabeled tropic acid. In this instance, yields were "slightly less than 60%" and the author did not comment on the extent of contamination. Werner obtained similar yields for the esterification of unlabeled tropine with labeled tropic acid, and reported that two esterifications were necessary. These discrepancies indicated a potential lack of reproducibility, possibly associated with the use of ^{14}C-labeled intermediates. To clarify this point and to establish for future studies reproducibility of the esterification, the authors synthesized *N*-methyl-^{14}C-atropine from *N*-methyl-^{14}C-tropine and unlabeled tropic acid. The results indicate that the use of labeled tropine has little or no effect on the esterification. It is clear, from the data in Table I, that synthesis of the labeled heterocycle is the major obstacle encountered in the synthesis of tropine-labeled atropine.

Excluding biosynthetic methods, syntheses of ^{14}C-labeled tropine and correspondingly labeled atropine have been confined to labeling the methyl group attached to the endo nitrogen bridge. Two general approaches have been through the Robinson condensation, employed by the authors and by Werner, and through methylation of nortropine or norhyoscyamine, employed by Fodor (13). Comparative yields are shown in Table I. Fodor's approach does not permit labeling of other positions n the tropine moiety and yields are prohibitively low. The authors' findings with unlabeled intermediates, and Werner's findings with methylamine-^{14}C implied that use of the Robinson condensation would be a far more efficient approach. The data shown in Table I confirm, for the first time, Werner's synthesis of *N*-methyl-^{14}C-tropine and *N*-methyl-^{14}C-atropine. The near identity of results obtained with labeled and unlabeled intermediates indicates no isotope effect on either the condensation or the esterification. These observations firmly establish the Robinson condensation as an instrument for labeling tropine and thereby open the way for introducing radioactive carbon into other ring positions.

Identity of the products was established by melting points, and by paper[25] and thin-layer[26] chromatography in a variety of solvent systems. Paper and thin-layer chromatograms of *N*-methyl-^{14}C-tropine demonstrated traces of an impurity, which represented less than 3% of the total radioactivity and chromatographed as tropanone in all solvent systems used. This impurity disappeared in subsequent steps and no attempt was made to further purify the tropine[27] or more positively identify the impurity. On precipitation from petroleum ether, *N*-methyl-^{14}C-atropine contained an impurity that chromatographed as tropine[28] in all solvent systems used. The impurity represented 7.6% of total radioactivity, equivalent to 3.8% of product weight, and disappeared after a single recrystallization from petroleum ether. Representative paper and thin-layer chromatograms of the final, recrystallized product are shown in Fig. 1.

[25] Paper chromatograms by the ascending technique, Whatman No. 1 paper, using butanol–acetic acid–water (344:56:130). Spots were located by cutting inch-wide strips into 1-cm. segments and counting individually in a Packard Tri-carb liquid scintillation counter. Counting done in toluene, using POP and dimethyl POPOP.

[26] Thin-layer chromatograms on Eastman Silica Gel G sheets, using chloroform, diethylamine, acetone, 25% aqueous ammonia (50:10:35:5). Strips were cut and counted as described for paper chromatograms. For both paper and thin-layer chromatograms, samples were adjusted with HCl to pH 4.5 before application to the chromatograms.

[27] If tropine were the desired end product, tropanone could be removed by repeating the reduction or by fractional sublimation of the impure tropine.

[28] Preliminary studies with labeled tropine have shown that it is not metabolized by mice. Thus the product can be used after a single crystallization from petroleum ether, the trace of tropine serving as an internal standard for locating tropine on chromatograms.

TABLE I—COMPARISON OF OBSERVED AND REPORTED YIELDS[a] FOR THE SYNTHESIS OF *N*-METHYL-^{14}C-TROPINE AND *N*-METHYL-^{14}C-ATROPINE

Ref.	Tropanone		Tropine[c]		Atropine[d]	
	Yield	S.A.[b]	Yield	S.A.[b]	Yield	S.A.[b]
Schmidt *et al.*[e] (4)	70%	...	69%	...	48%	...
This communication	65%	1	65%	1	45%	1
Werner *et al.* (11)	70%	5	70%	5	49%	5
Fodor *et al.*[f] (13)	...	...	44%	0.22	25%	0.21

[a] All yields based on methylamine, except where indicated otherwise. [b] Specific activity is millicuries per millimole. [c] Tropine yields for this communication are corrected for 3% tropanone impurity. [d] Atropine yields after recrystallization from petroleum ether to remove approximately 4% tropine impurity. [e] Microsynthesis using unlabeled intermediates. [f] Fodor did not synthesize tropanone, but methylated tropine with methyliodide-^{14}C.

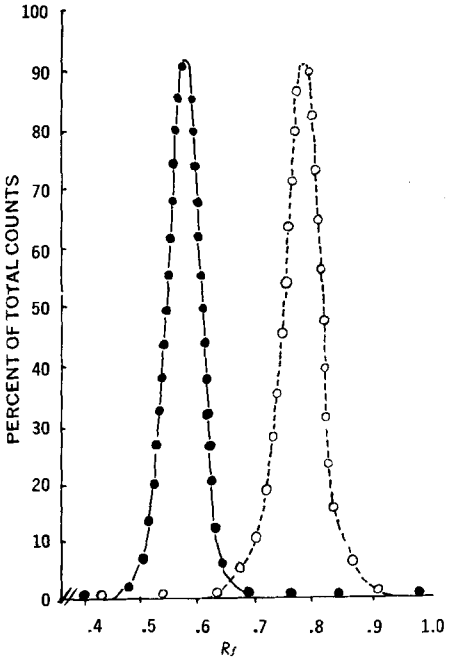


Fig. 1—Representative paper and thin-layer chromatograms of N-methyl-^{14}C-atropine after recrystallization ——●——●——, *thin layer:* ---O---O---, *paper.*

CONCLUSIONS

Using a previously described, micro adaptation of the Robinson condensation, *N*-methyl-^{14}C-tropine was synthesized from methylamine-^{14}C-HCl in 65% of theoretical yield. The product contained less than 3% of an impurity that chromatographed as unreduced tropanone. The impurity was lost in subsequent steps and no attempt was made to further purify the product or more positively identify the impurity.

Labeled tropine was converted to *N*-methyl-^{14}C-atropine in 45% of theoretical yield, based on methylamine ^{14}C-HCl. When crystallized from petroleum ether, the product contained approximately 4% tropine, representing approximately 8% of the total radioactivity. The impurity was removed by a single recrystallization from petroleum ether.

Werner's synthesis of *N*-methyl-^{14}C-tropine and *N*-methyl-^{14}C-atropine is confirmed for the first time. A comparison of present findings, Werner's data, and previous observations with unlabeled Robinson intermediates indicates that no unusual difficulties are encountered in the use of labeled compounds. Accordingly, a general pathway for labeling the carbon skeleton of tropine is firmly established. Labeled succindialdehyde or labeled acetone dicarboxylic acid become logical precursors of labeled tropine. As indicated previously (5), the use of commercially available pentoses-^{14}C would permit labeling of the ring carbons that arise from succindialdehyde. No other method for labeling the carbon skeleton of tropine has been published.

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Keyphrases

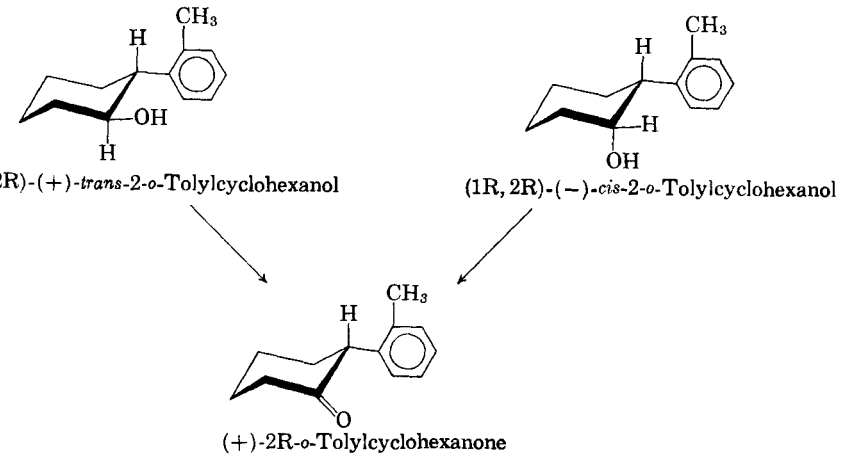
Atropine—tropine labeled
N-Methyl-^{14}C-tropine—synthesis
N-Methyl-^{14}C-atropine—synthesis
Paper chromatography—identity
TLC—identity

Absolute Configuration of *trans*- and *cis*-2-*o*-Tolylcyclohexanols

By DONALD R. GALPIN* and ALAIN C. HUITRIC

Optically active *trans*- and *cis*-2-*o*-tolylcyclohexanols were obtained by basic hydrolysis of their (−)-menthoxyacetate esters previously prepared and separated. Oxidation of (1S, 2R)-(+)-*trans*- (I) and (1R,2R)-(−)-*cis*-2-*o*-tolylcyclohexanol (II) yielded the same ketone, 2R-(+)-*o*-tolylcyclohexanone (III). The absolute configuration of III was established from ORD and CD measurements. This also established the absolute configuration of I and II. I and II have the same absolute configuration about C-2, but the signs of their Cotton effect in the aromatic region are opposite.

THE ABSOLUTE CONFIGURATION of an optically active ketone is available from optical rotatory dispersion (ORD) and/or circular dichroism (CD) studies to determine the sign of the Cotton effect associated with the carbonyl chromophore (1). If the conformation or significant conformations are known or can be predicted, then an application of the octant rule (2) or some of its extensions (3) will provide the absolute configuration. This rule is limited almost exclusively to ketones,[1] but since optically active secondary alcohols can be oxidized to their corresponding carbonyl compounds with retention of optical activity (4), their absolute configuration can be obtained by this method.



Recently the benzene ring has received attention as a chromophore for ORD and CD studies (5–7). The relatively scant information available concerning the anomalous dispersion of the long wavelength transition of this chromophore has precluded the formulation of a definite rule for the correlation of absolute configuration with the sign of the Cotton effect. As with the carbonyl group the Cotton effect associated with the benzene ring is conformationally dependent (5–7) and since most of the studies involving this chromophore have been conducted on compounds with aliphatic side chains of which there are several rotameric forms, the observed ORD and CD data result from a composite of the various conformers present. Brewster and Buta (5) attempted to circumvent this problem by examining rigid 1-substituted indans and based upon their observations suggested that an octant rule might also be applicable to the benzene ring. Because *trans*- and *cis*-2-*o*-tolylcyclohexanol have been shown by NMR to exist very predominantly in the chair conformation with the tolyl group in equatorial orientation (8a), these compounds appeared to provide excellent systems for the investigation of the Cotton effect in the 260 mμ region of the aromatic ring and the influence of a β axial and equatorial hydroxyl group on the optical activity of this transition.

Received April 7, 1967, from the College of Pharmacy, University of Washington, Seattle, WA 98105
Accepted for publication October 25, 1967.
Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This investigation was supported in part by research grant MH 11942-01 from the National Institute of Mental Health, U. S. Public Health Service, Bethesda, Md.
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[1] An octant rule for azides has recently been formulated; see Djerassi, C., Moscowitz, A., Ponsold, K., and Steiner, G., *J. Am. Chem. Soc.*, **89**, 347(1967).

We wish to report on the absolute configuration of (+)-*trans*- (I) and (−)-*cis*-2-*o*-tolylcyclohexanol (II) obtained from ORD and CD measurements on (+)-2-*o*-tolylcyclohexanone (III), the oxidation product of these alcohols, and the anomalous dispersion of the aromatic ring in these compounds.

RESULTS AND DISCUSSION

The resolution of racemic I and racemic II was accomplished through their (−)-menthoxyacetate esters (9). Racemic I and II have been reported previously and some of their pharmacological properties have also been reported (8b, c). The diastereomeric esters of I were separated by fractional recrystallization and column chromatography over neutral alumina and those of II exclusively by column chromatography over this same adsorbent.[2] The progress of both separations was followed by NMR (9). The individual esters were hydrolyzed in an aqueous ethanol solution of 10% sodium hydroxide to obtain the enantiomers.

The chromic acid oxidation (4) of I and II in a two-phase system of ether and water yielded III in both instances. Although I was optically pure and II was approximately 85% one enantiomer (about 70% optical purity), the ketone III isolated from I and II had comparable rotations. The oxidation method is reported to result in little enolization of optical centers adjacent to the carbonyl group (4) but a small degree of racemization through enolization cannot be ruled out. In the oxidation of racemic *trans*-2-*o*-tolylcyclohexanol by this method on a larger scale we obtained about 10% of the keto acid ring opening product (10). This indicates that the rate of oxidation of the enolized form is faster than the rate of racemization, since racemization, if it occurred, was very slight during the oxidation of the optically active alcohols. The progress of oxidation of the optically active alcohols was followed by gas chromatography. Gas chromatography analysis of the purified ketone III obtained from I and II showed only one volatile component. Compounds I, II, and III have different emergence times on the column employed.

The ORD data for the compounds of this study are recorded under *Experimental*. All curves were determined in methanol on two separate samples. On one of each of these samples the determination was repeated on the solution which had been stored for 24–48 hr. The curves were reproducible to within 2% over the region of interest and no change was noted in the curves of the samples which had been stored.

Figure 1 shows the ORD and CD[3] curves for III.

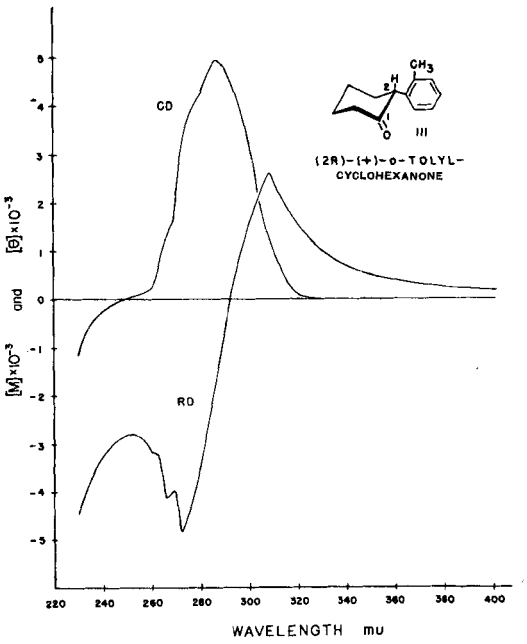
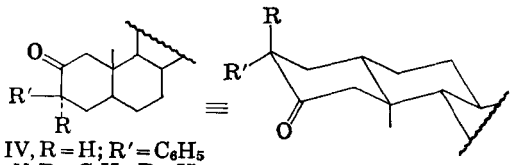


Fig. 1—Rotatory dispersion and circular dichroism curves for 2R-(+)-o-tolylcyclohexanone in menthanol; [M] is molecular rotation and [θ] is molecular ellipticity.

The Cotton effect associated with the carbonyl group is positive as well as the overlapping bands of the aromatic nucleus which appear as shoulders on the CD curve at 274 and 266 mμ. The sign of the Cotton effect of the carbonyl absorption is in agreement with the absolute configuration depicted based upon the octant rule (2) and its extensions (3).



IV, R = H; R′ = C_6H_5
V, R = C_6H_5; R = H

This assignment is supported by the work of Cookson and Hudec (11) who have shown that for the isomeric 3-phenylcholestan-2-ones, the 3-β-isomer IV (phenyl group equatorial) has a molecular amplitude[4] of somewhat less than +53, in which the presence of the aromatic ring gives a negative contribution to the amplitude of the Cotton effect when compared to the unsubstituted cholestan-2-one which has a molecular amplitude of +122 (12). Conversely, in the 3-α-isomer V, which has a molecular amplitude of +303,[5] the axial aromatic ring gives a large positive contribution to the Cotton effect as a result of homoconjugation of the benzene and carbonyl π electrons (3, 11).

The NMR spectrum of III (13) is consistent with the conformation shown in which the tolyl group is equatorially oriented and rules out any large populations of twist or axial conformers. Since the Cotton

[2] Pure (+)-*trans*-2-*o*-tolylcyclohexyl (−)-menthoxyacetate has $[\alpha]_D^{27}$ −32.5° (c 10, chloroform); pure (−)-*trans*-2-*o*-tolylcyclohexyl (−)-menthoxyacetate has $[\alpha]_D^{28}$ −67.9° (c 10, chloroform). The most pure sample of (−)-*cis*-2-*o*-tolylcyclohexyl (−)-menthoxyacetate isolated has $[\alpha]_D^{28}$ −143.5° (c 10, chloroform) and was judged to be at least 95% pure on the basis of its NMR spectrum; the sample of this ester from which II was obtained had $[\alpha]_D^{25}$ −123° (c, 0.1026 methanol) and was considered approximately 85% pure on the basis of its optical rotation and NMR spectrum (9).

[3] The CD curves were also obtained in methanol. The authors are deeply indebted to Dr. William F. Trager who made all the CD determinations reported in this paper while he was at the Chelsea College of Science and Technology, London, England.

[4] The trough was not quite reached in this determination. Moscowitz *et al.* (3) report a value of +45 in dioxane.

[5] Moscowitz *et al.* (3) show a value of +375.

effect of III is positive, it must have the configuration corresponding to the mirror image of the equatorially substituted 3-β-phenylsteroid IV (3). For an independent assignment of the absolute configuration of III based on chemical evidence see Huitric and Newell (14).

The influence of the *o*-tolyl methyl group on the carbonyl Cotton effect in compound II is unknown and difficult to predict. Examination of Dreiding molecular models indicates that rotation of the equatorially oriented tolyl group causes the position of the methyl group to alternate between back and front upper- and lower-left octants, but the weighted time-average positions are uncertain. There is also some question regarding the applicability of the octant rule to front-octant perturbations (15). It would appear that the predominant effect of the *ortho* methyl group is through its influence on the preferred conformation of the molecule. For example, it will decrease contribution from a chair conformation with the aromatic group in axial orientation and will also affect the rotation of the phenyl ring. It is interesting to note that in the other chair conformation of III (aromatic group axial) the phenyl ring is located in the lower-right back octant. Any contribution from this conformation would therefore also contribute to the positive Cotton effect and would also cause an enhancement of the molecular amplitude (3, 11).

The ORD and CD curves for I and II appear in Figs. 2 and 3, respectively. In both instances there is a weak multiple Cotton effect associated with aromatic absorption in the 260 mμ region. The strength of the Cotton effect is best obtained from a comparison of the CD curves. If the background curve in the ORD is steeply rising, it may obscure the Cotton effect (6, 16). Although both compounds have the same absolute configuration about the carbon atom bearing the tolyl group, the signs of their Cotton effects, measured in methanol, are opposite. The CD curve of I was determined on its enantiomer $[\alpha]_D^{26}$ −70.6° (c 10, chloroform)[6] and the mirror image is reproduced.[7] The CD curve of II, which was determined to 225 mμ, exhibits another negative transition beginning to appear at 227 mμ; the CD curve for I was measured only to 230 mμ.

In the 1-substituted indan series Brewster and Buta (5) concluded that a large Cotton effect in the 250–270 mμ region appears to be associated with the presence of a group capable of direct perturbation of the π electron cloud of the ring by hydrogen bonding or by electrostatic effects. In our systems intramolecular hydrogen bonding with the π electron cloud should be solvent dependent. The ORD curve of I measured in isooctane[8] showed a smoothing off in the region of 260–270 mμ. Our system lacks the conformational rigidity of the indans and our data on the aromatic chromophore are not yet sufficient to draw conclusions. Solvent effects will be studied more thoroughly from CD measurements and the authors are in the process of studying the *p*-tolyl isomers and the 2,6-dimethylphenyl analogs.

[6] The extremely weak pen displacements in the CD determinations of these alcohols did not permit the elucidation of fine structure. Consequently, the shape of these curves is only approximate and the sign and magnitude of the absorption are the more significant information.

[7] The ORD curve was determined on both enantiomers and the curve of (−)-I was the exact mirror image of that of (+)-I and agreed in absolute values of rotation to within 2% over the entire spectrum.

[8] The ORD curve in isooctane was determined by Todd D. Cochran.

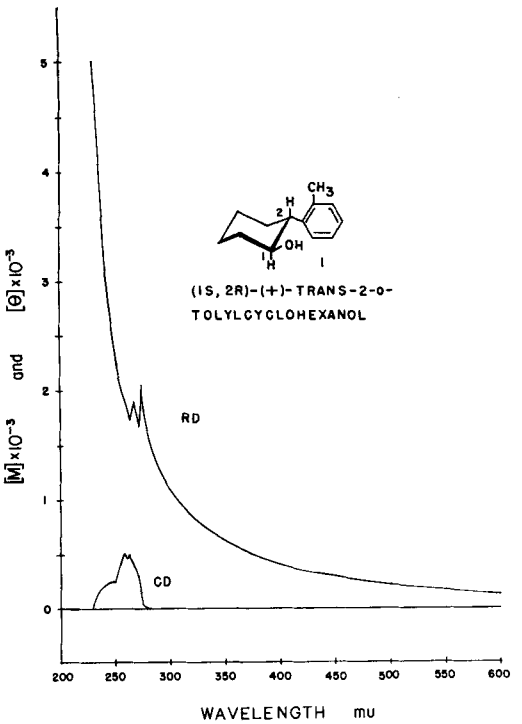


Fig. 2—Rotatory dispersion and circular dichroism curves for (1S,2R)-(+)-trans-2-o-tolylcyclohexanol in methanol; [M] *is molecular rotation and* [θ] *is molecular ellipticity.*

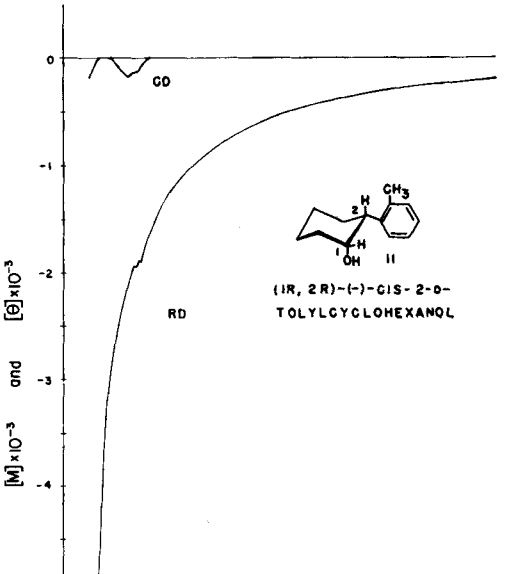


Fig. 3—Rotatory dispersion and circular dichroism curves for (1R,2R)-(−)-cis-2-o-tolylcyclohexanol in methanol; [M] *is molecular rotation and* [θ] *is molecular ellipticity.*

EXPERIMENTAL

(1S,2R)-(+)-trans-2-o-Tolylcyclohexanol (I)—A mixture of 4.0 Gm. of (+)-*trans*-2-*o*-tolylcyclohexyl (−)-menthoxyacetate (9) and 100 ml. of a 10% solution of sodium hydroxide in 70% ethanol in water was refluxed for 2.5 hr., cooled, and diluted with 100 ml. of water. The mixture was extracted four times with 50-ml. portions of ether and the combined ether extracts were washed twice with 25-ml. portions of 5% sodium hydroxide, once with 5% hydrochloric acid, then with water until the washings were neutral, and dried over anhydrous calcium sulfate. Filtration, solvent removal, and distillation yielded 1.70 Gm. of I, b.p. 81–82° at 0.20 mm.; $[\alpha]_D^{26}$ +70.6° (c 10 chloroform). Analysis of this alcohol by VPC on a Carbowax 20 M column at 190° showed a single volatile component present. The IR spectrum on sodium chloride plates was essentially the same as racemic I, exhibiting minor variations in the fingerprint region; RD in methanol (c 0.1096) showed $[M]_{600}$ +132°, $[M]_{589}$ +141°, $[M]_{500}$ +222°, $[M]_{400}$ +399°, $[M]_{300}$ +1084°, $[M]_{273}$ +2056° (peak), $[M]_{270}$ +1671° (trough), $[M]_{266}$ +1900° (peak), $[M]_{263}$ +1730° (trough), $[M]_{230}$ +4999°.

The levorotatory enantiomer was obtained by the same method, $[\alpha]_D^{26}$ −71.1° (c 10, chloroform); the ORD curve was the exact mirror image of that of (+)-I.

Acidification of the combined basic solutions and workup yielded after distillation 1.82 Gm. of (−)-menthoxyacetic acid, b.p. 108–110° at 0.4 mm.; $[\alpha]_D^{26}$ −94.7° (c 10, chloroform). Reported: b.p. 134–137° at 2 mm. (17); $[\alpha]_D^{25}$ −91.5° (c 2, 95% ethanol) (18).

(1R,2R)-(−)-cis-2-o-Tolylcyclohexanol (II)—A 2.1-Gm. sample of the *cis* esters ($[\alpha]_D^{25}$ −123° [c 0.1026, methanol]), approximately 85% (−)-*cis*-2-*o*-tolylcyclohexyl (−)-menthoxyacetate (9), was hydrolyzed as described for the isolation of I. Following workup the product was distilled to give 0.85 Gm. of II, b.p. 76–78° at 0.18 mm.; $[\alpha]_D^{25}$ −102° (c 0.1280, methanol). Analysis of this alcohol by VPC on a Carbowax 20 M column at 190° showed it to be free of volatile contaminants. The IR spectrum on sodium chloride plates was identical to racemic II. The reported b.p. of racemic II is 76–78° at 0.15–0.17 mm. (8). On the basis of the specific rotation and NMR spectrum (9) of the starting ester the compound is estimated to be about 85% one enantiomer (about 70% optical purity). RD in methanol (c 0.1280) showed $[M]_{600}$ −188°, $[M]_{589}$ −195°, $[M]_{500}$ −300°, $[M]_{400}$ −530°, $[M]_{300}$ −1231°, $[M]_{272}$ −1896° (trough), $[M]_{270}$ −1876° (peak), $[M]_{267-265}$ −1944° (shoulder), $[M]_{250}$ −2556°, $[M]_{230}$ −5932°.

2R-(+)-o-Tolylcyclohexanone (III)—This ketone was obtained by the oxidation of either I or II by the method of Brown and Garg (4). The oxidation of I is given. To a stirred solution of 0.905 Gm. (4.76 mmoles) of I in 10 ml. of ether in a 50-ml. flask, fitted with a reflux condenser, dropping funnel, and a magnetic stirrer, a solution of 0.905 Gm. (3.17 mmoles.) of sodium dichromate dihydrate and 0.72 ml. of 96% sulfuric acid in 10 ml. of water was added over a period of 30 min. The progress of the reaction was followed by VPC on a Carbowax 20 M column at 190°. When the reaction was complete, the ether layer was separated and the aqueous phase extracted several times with small portions of ether. The combined ether solutions were washed twice with 10-ml. portions of 5% sodium bicarbonate followed by two water washings and dried by passage through anhydrous sodium sulfate. The solvent was removed and the residue recrystallized once from *n*-hexane to give a first crop of 0.500 Gm. of colorless crystals, m.p. 72–73.5° (Fisher-Johns); $[\alpha]_D^{25}$ +11.80° (c 1.000, methanol). The IR spectrum (potassium bromide disk) was essentially identical to that of racemic III, exhibiting only minor variations in the fingerprint region. Analysis of this ketone by VPC showed a single peak with the same emergence time as racemic III; RD in methanol (c 0.0996) showed $[M]_{600}$ +20.7°, $[M]_{589}$ +22.2°, $[M]_{500}$ +50.6°, $[M]_{400}$ +183°, $[M]_{350}$ +502°, $[M]_{308}$ +2598° (peak), $[M]_{272}$ −4824° (trough), $[M]_{269}$ −3978° (peak), $[M]_{266}$ −4138° (trough), $[M]_{263}$ −3209°, $[M]_{255}$ −2839°, $[M]_{250}$ −2812°, $[M]_{230}$ −4442°.

The oxidation of II by this method yielded III, m.p. 73.5–74.5° (Fisher-Johns), mixed m.p. with III obtained from I 73–74.5°; $[\alpha]_D^{25}$ +11.00° (c 1.000, methanol). Analysis of this ketone by VPC showed one component present. The m.p. of racemic III is 55.5–56.5° (19).

The ORD curves reported in this paper were obtained on a Cary 60 recording spectropolarimeter using a 250-w. xenon lamp as the light source at 25° in a 1.0-cm. cell and employing a fixed slit width of 0.3 mm. from 600 mμ to 450 mμ and the manufacturer's preprogrammed slit width from 450 mμ to 230 mμ. The specific rotations quoted at the sodium D line (589 mμ) in methanol were also determined on this instrument employing the same fixed slit width and cell length as for the ORD curves.

All specific rotations determined in chloroform were performed on a Rudolph polarimeter using a sodium lamp and a 1-dcm. tube. Rotations were taken at room temperature.

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Keyphrases

2-o-Tolylcyclohexanols, *trans* and *cis*
Optical rotatory dispersion—absolute configuration
Circular dichroism—absolute configuration
Column chromatography—separation
NMR spectrometry—identity
Specific rotation—identity
IR spectrophotometry—structure
GLC—analysis

Specific Assay Methods for Droperidol and Fentanyl Citrate in a Pharmaceutical Combination

By CASIMIR A. JANICKI, RONALD J. BRENNER, and BARBARA E. SCHWARTZ

In acid solution, droperidol, 1-{1-[3-(*p*-fluorobenzoyl)propyl]-1,2,3,6-tetrahydro-4-pyridyl}-2-benzimidazolinone, is decomposed to form 4′-fluoro-4-(4-oxopiperidino)butyrophenone and 2-benzimidazolinone. Fentanyl, *N*-(1-phenethyl-4-piperidyl)propionanilide, is decomposed in acid solution to form 4-anilino-1-(2-phenylethyl)piperidine. A procedure to assay droperidol in the presence of its hydrolysis products, fentanyl, and its hydrolysis product is given. Droperidol is analyzed by its UV absorption after being separated from interfering products. A procedure to assay fentanyl in the presence of its hydrolysis products, droperidol, and its hydrolysis products is given. Droperidol and 4-anilino-1-(2-phenylethyl)-piperidine are separated from fentanyl through the formation of a reineckate derivative and fentanyl is subsequently determined by a methyl orange procedure. Data are presented to show the accuracy and precision of the methods.

DROPERIDOL, 1 - {1 - [3 - (*p* - fluorobenzoyl)-propyl]-1,2,3,6 - tetrahydro - 4 - pyridyl} - 2-benzimidazolinone, was first synthesized by Janssen *et al.* and has been shown to be a sedative or tranquilizer (1, 2). Fentanyl citrate [*N*-(1-phenethyl-4-piperidyl) propionanilide dihydrogen citrate] was also synthesized by Janssen *et al.*, and has been shown to be a potent narcotic analgesic (2). It has been reported to be 100 times more potent on a weight basis than morphine as an analgesic (3).

A combination of droperidol and fentanyl citrate in solution has been employed for neuroleptanalgesia or as an adjunct to nitrous oxide for general anesthesia in man (4). It has also been successfully employed in veterinary medicine as an analgesic tranquilizer[1] for use in surgery in dogs (5).

The specific assays for each active ingredient in the presence of the other was essential to study the stability of the various combinations prepared in these laboratories. The usual ratio of droperidol to fentanyl in solution was 50:1. The fentanyl content varied from 20 mcg./ml. to the concentration used in the veterinary product 400 mcg./ml.

Fentanyl, because of its low concentration and its low ratio with respect to droperidol, presented a formidable analytical problem. For the assay of fentanyl to be specific, fentanyl had to be separated not only from its own hydrolysis product, but also from a large amount of droperidol and hydrolysis products of droperidol.

The proposed assay methods for droperidol and fentanyl, alone or in combination, are specific for the intact drug.

EXPERIMENTAL

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Fentanyl (IV), prepared from its citrate salt, was refluxed overnight in 1 *M* hydrochloric acid and was found to undergo complete hydrolysis. One

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The authors thank Dr. Janis Plostnieks, Chemical Research Department, for the hydrolysis studies of droperidol and fentanyl.

[1] Marketed as Innovar-Vet by McNeil Laboratories, Inc., Fort Washington, Pa.

Keyphrases

2-o-Tolylcyclohexanols, *trans* and *cis*
Optical rotatory dispersion—absolute configuration
Circular dichroism—absolute configuration
Column chromatography—separation
NMR spectrometry—identity
Specific rotation—identity
IR spectrophotometry—structure
GLC—analysis

Specific Assay Methods for Droperidol and Fentanyl Citrate in a Pharmaceutical Combination

By CASIMIR A. JANICKI, RONALD J. BRENNER, and BARBARA E. SCHWARTZ

In acid solution, droperidol, 1-{1-[3-(*p*-fluorobenzoyl)propyl]-1,2,3,6-tetrahydro-4-pyridyl}-2-benzimidazolinone, is decomposed to form 4′-fluoro-4-(4-oxopiperidino)butyrophenone and 2-benzimidazolinone. Fentanyl, *N*-(1-phenethyl-4-piperidyl)propionanilide, is decomposed in acid solution to form 4-anilino-1-(2-phenylethyl)piperidine. A procedure to assay droperidol in the presence of its hydrolysis products, fentanyl, and its hydrolysis product is given. Droperidol is analyzed by its UV absorption after being separated from interfering products. A procedure to assay fentanyl in the presence of its hydrolysis products, droperidol, and its hydrolysis products is given. Droperidol and 4-anilino-1-(2-phenylethyl)-piperidine are separated from fentanyl through the formation of a reineckate derivative and fentanyl is subsequently determined by a methyl orange procedure. Data are presented to show the accuracy and precision of the methods.

DROPERIDOL, 1 - {1 - [3 - (*p* - fluorobenzoyl)-propyl]-1,2,3,6 - tetrahydro - 4 - pyridyl} - 2-benzimidazolinone, was first synthesized by Janssen *et al.* and has been shown to be a sedative or tranquilizer (1, 2). Fentanyl citrate [*N*-(1-phenethyl-4-piperidyl) propionanilide dihydrogen citrate] was also synthesized by Janssen *et al.*, and has been shown to be a potent narcotic analgesic (2). It has been reported to be 100 times more potent on a weight basis than morphine as an analgesic (3).

A combination of droperidol and fentanyl citrate in solution has been employed for neuroleptanalgesia or as an adjunct to nitrous oxide for general anesthesia in man (4). It has also been successfully employed in veterinary medicine as an analgesic tranquilizer[1] for use in surgery in dogs (5).

The specific assays for each active ingredient in the presence of the other was essential to study the stability of the various combinations prepared in these laboratories. The usual ratio of droperidol to fentanyl in solution was 50:1. The fentanyl content varied from 20 mcg./ml. to the concentration used in the veterinary product 400 mcg./ml.

Fentanyl, because of its low concentration and its low ratio with respect to droperidol, presented a formidable analytical problem. For the assay of fentanyl to be specific, fentanyl had to be separated not only from its own hydrolysis product, but also from a large amount of droperidol and hydrolysis products of droperidol.

The proposed assay methods for droperidol and fentanyl, alone or in combination, are specific for the intact drug.

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Hydrolysis Studies—Droperidol (I) was refluxed overnight in 1 *M* hydrochloric acid and was found to undergo complete hydrolysis. Two major products were isolated, 4′-fluoro-4-(4-oxopiperidino)butyrophenone (II) and 2-benzimidazolinone (III) (Scheme I).

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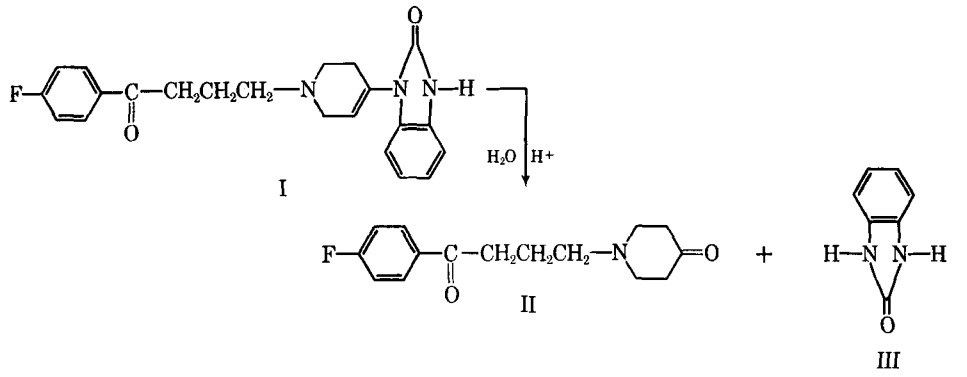
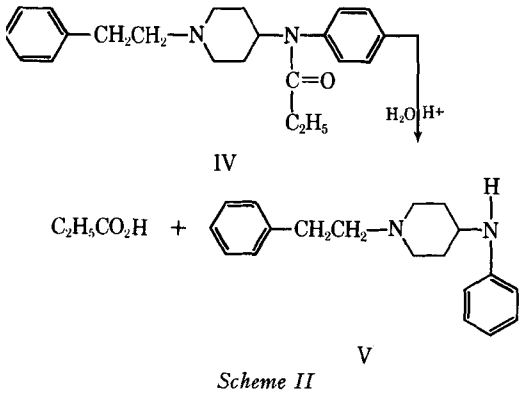


Scheme I



Scheme II

major product was isolated, 4-anilino-1-(2-phenylethyl)piperidine (V) (Scheme II).

A monoglyme solution of fentanyl was recovered unchanged after refluxing in 2 *M* sodium hydroxide overnight.

Method Development—*Droperidol Assay*—Droperidol exhibits a strong ultraviolet absorption spectrum and an assay method was chosen to utilize this property. The two major hydrolysis products have ultraviolet absorption spectra which would interfere in a direct ultraviolet assay for droperidol. The ultraviolet absorption spectra of droperidol ($\epsilon_{max.}$ 276 mμ = 7100; $\epsilon_{max.}$ 247 mμ = 14,500), II ($\epsilon_{max.}$ 248 mμ = 12,500), and III ($\epsilon_{max.}$ 276 mμ = 6,800) are given in Fig. 1.

Fentanyl ($\epsilon_{max.}$ 258 mμ = 465) and its hydrolysis product (V) ($\epsilon_{max.}$ 257 mμ = 440) have ultraviolet absorption spectra which are of low intensity. The spectra are presented in Fig. 2. Unless they were present in solution at very high concentrations, fentanyl and its hydrolysis product would not be expected to interfere in an ultraviolet assay method for droperidol.

A reaction, which is characteristic of aldehydes and some ketones, is the addition of sodium bisulfite to form water-soluble bisulfite addition products. One of the hydrolysis products of droperidol (II) quantitatively forms such an addition product very readily. The addition of bisulfite would be expected to add first to the piperidino carbonyl group. Sodium bisulfite may also react with droperidol under the proper conditions. When mixtures of droperidol and II were extracted with chloroform from 0.3% aqueous sodium bisulfite solution buffered at pH 6, droperidol was quantitatively found in the chloroform layer, while II was

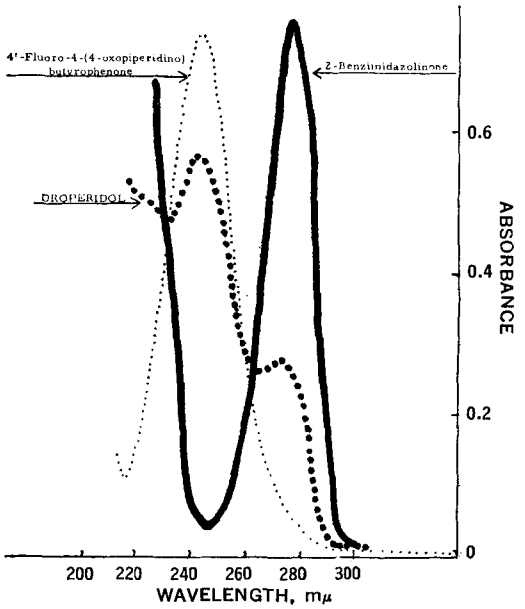


Fig. 1—The UV absorption spectra of droperidol (0.0147 mg./ml.), 4'-fluoro-4-(4-oxopiperidone)butyrophenone (0.0155 mg./ml.), 2-benzimidazolinone (0.0151 mg./ml.) in 0.1 M hydrochloric acid, 1-cm. cells.

quantitatively found in the aqueous layer. The concentration of sodium bisulfite is critical. Increasing the sodium bisulfite concentration from 0.3 to 1.2% resulted in losses of droperidol in the chloroform layer. At a concentration of 0.9% sodium bisulfite, there was a 3% loss of droperidol and a 40% loss at 1.2%. No losses of droperidol were encountered using the conditions described in the proposed assay. Lowering the pH resulted in incomplete extraction of droperidol into the chloroform. The 2-benzimidazolinone is unaffected by sodium bisulfite and is found essentially in the pH 6 layer having a partition coefficient of 0.15 with chloroform.

The droperidol in the chloroform layer was now separated from II and about 96% of III. Droperidol was quantitatively extracted from chloroform into citric acid. The III present in the chloroform was also extracted into the citric acid. Since III has no ultraviolet absorption at 246 mμ, the absorbance at 246 mμ is entirely due to the droperidol (Fig. 1). Mixtures of droperidol and III were assayed for droperidol by the use of the proposed assay. No

TABLE I—ASSAY OF DROPERIDOL IN PRESENCE OF 2-BENZIMIDAZOLINONE

Droperidol, mg.	2-Benzimidazolinone, mg.	Droperidol Found, mg.
2.00	0	2.00
1.80	0.07	1.79
1.60	0.14	1.58
1.40	0.21	1.37
1.00	0.35	0.98
0.00	0.70	0.00

TABLE II—THIN-LAYER CHROMATOGRAPHIC[a] R_f VALUES FOR COMPOUNDS

Compd.	R_f
Droperidol	0.28
4′-Fluoro-4-(4-oxopiperidino)-butyrophenone	0.47
2-Benzimidazolinone	0.22
Fentanyl	0.71
4-Anilino-1-(2-phenylethyl)piperidine	0.40

[a] Silica Gel GF, 5% v/v methanol in chloroform, no paperwick in tank.

interference was found from the hydrolysis product as indicated from the data presented in Table I.

The fate of each component of known mixtures of droperidol and its two hydrolysis products was followed by a thin-layer chromotographic method. Samples spotted on a fluorescent silica plate[2] were developed once, in a tank without a paper wick, *with 5% v/v methanol in chloroform. After air-drying* the plate, droperidol and its two hydrolysis products could be detected under short wavelength UV light. The spots appeared dark against a fluorescent background. However, when mixtures of all three compounds were chromotographed only 2 spots appeared under short wavelength UV light. The droperidol spot overlapped the III spot. When the plate was sprayed with a 0.2% alcoholic solution of dichlorofluorescein, dried at 60° for 15 min., and viewed under long wavelength UV light, only the spot of III could be observed. Under short wavelength UV light, two spots appeared, the II spot and the droperidol–III combined spot. With the dichlorofluorescein spray, as little as 1 mcg. of III could be detected in large amounts (100 mcg.) of droperidol. The thin-layer study confirmed that II remained completely in the aqueous bisulfite solution, while droperidol could not be detected in any significant amounts in the aqueous layer.

The R_f values obtained for droperidol and its hydrolysis products and for fentanyl and its hydrolysis product are given in Table II.

In quantitative TLC work it was necessary to prepare the fluorescent silica plates in the laboratory, using Silica Gel GF_{254}.[3] Impurities in the commercial precoated plates made this necessary.

When the samples were in acidic media it was necessary to obtain the free bases for the TLC study. The aqueous solution was made neutral by the addition of 0.1 *N* sodium hydroxide and evaporated to dryness without the aid of heat. The residue was taken up in methanol or acetone and streaked onto a fluorescent silica plate and developed as previously described. An alternate procedure that can be used is to make the aqueous solution slightly alkaline with 0.1 *N* sodium hydroxide and extract with chloroform. The chloroform solution is then streaked onto the fluorescent silica plate and developed as previously described.

Fentanyl Assay—An ultraviolet assay method for fentanyl was not suitable because of its low $\epsilon_{max.}$ value and its usually low concentrations found in pharmaceutical dosage forms. The ultraviolet absorption spectra for fentanyl and its major hydrolysis product are given in Fig. 2. Fentanyl, because of the basic nitrogen in the piperidine ring, was found to form a chloroform-soluble methyl orange complex. The hydrolysis product of fentanyl also forms a chloroform-soluble methyl orange complex. However, V forms a chloroform-insoluble reineckate in sulfuric acid, while fentanyl under the same conditions is quantitatively extracted in chloroform. The thin-layer method described previously, confirmed that V was not extracted into the chloroform with fentanyl.

Droperidol also forms an insoluble reineckate precipitate in sulfuric acid. Therefore, the use of Reinecke salt quantitatively removes both droperidol and the hydrolysis product of fentanyl. On silica thin-layer plates, no trace of droperidol could be found in the chloroform layer. II also forms an insoluble reineckate under the same conditions while III does not. III does not form a chloroform-soluble methyl orange complex, and therefore does not interfere in the proposed fentanyl assay.

The use of 2% methanol in chloroform was used instead of pure chloroform as a precaution to prevent any possible adsorption of fentanyl by the glassware. The concentration of fentanyl in the chloroform solution in the proposed assay is low, being 0.004 mg./ml.

PROPOSED METHODS

Reagents—Hydrochloric acid, 1 *M;* citric acid, 0.1 *M;* sulfuric acid, 20% v/v methanol in chloroform; sodium bisulfite solution, 1%, prepare fresh daily; methyl orange (Fisher Scientific Co.), aqueous solution; sodium phosphate solution,

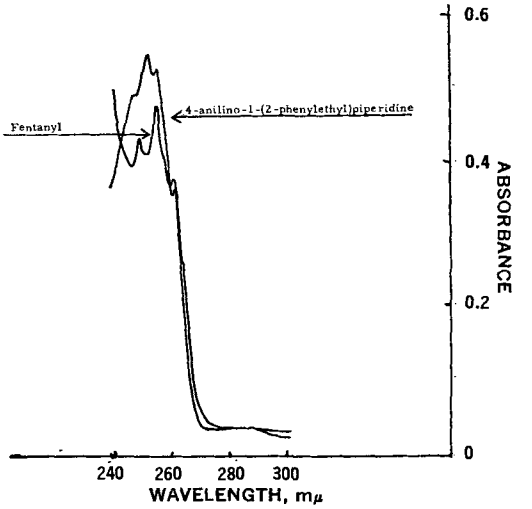


Fig. 2—The UV absorption spectra of fentanyl (0.342 mg./ml.) and 4-anilino-1-(2-phenylethyl)piperidine (0.348 mg./ml.) in 0.1 M hydrochloric acid, 1-cm. cells.

[2] Uniplate, precoated thin-layer chromatography plates from Analtech, Inc., Wilmington, Del.
[3] Brinkmann Instruments, Westbury, N. Y.

0.2 *M;* pH 5 buffer and pH 6 buffer, adjust the 0.2 *M* sodium phosphate solution to pH with 1 *M* sodium hydroxide; lactic acid USP; analytical reagent grades of chloroform, ether, anhydrous methanol.

One percent Reinecke solution, 1 Gm. of Reinecke salt (ammonium tetrathiocyanodiammonochromate, Fisher Scientific Co.) added to 100-ml. water shaker for 15 min., and filtered; prepare this solution immediately before use.

Apparatus—A Beckman DK-2A recording spectrophotometer and a Burrell wrist-action shaker were used. Boston round flint bottles, 30 ml., 60 ml., and 120 ml., fitted with plastic caps having a conical polyethylene liner were obtained from Erno Products Co., Philadelphia, Pa.

Assay for Droperidol—Dilute the sample of droperidol, or the sample of droperidol in combination with fentanyl, with water to obtain a 0.15 mg./ml. solution of droperidol. Pipet 15 ml. of the diluted sample into a 60-ml. bottle containing 10 ml. of the pH 6 buffer, 10 ml. of a 1% sodium bisulfite solution, and exactly 15 ml. of chloroform. Shake for 15 min. on a mechanical shaker, centrifuge, aspirate, and discard the aqueous layer. Pipet 5 ml. of the clear chloroform layer to a 120-ml. bottle containing exactly 50 ml. of 0.1 *M* citric acid. Shake for 30 min. on a mechanical shaker. Centrifuge for a couple of minutes and decant the clear citric acid solution to a clean container. Record the ultraviolet absorption spectrum from 300 to 230 mμ *versus* 0.1 *M* citric acid in 1-cm. cells.

An 0.15 mg./ml. solution of droperidol standard in chloroform is prepared, and taken through the proposed assay. The calculation is then:

$$\frac{\text{absorbance at 246 m}\mu \text{ for the sample}}{\text{absorbance at 246 m}\mu \text{ for the std.}} \times 100 = \%\ \text{droperidol}$$

If the sample contains a preservative such as methyl- or propylparaben, the UV spectrum of droperidol in 0.1 *M* citric acid will be distorted. The following step will remove the preservatives from citric acid solution. The droperidol standard should also be taken through this step.

Extract 20 ml. of the citric acid solution of droperidol with two 70-ml. portions of ether. Discard the ether extracts and record the UV spectrum of the sample as directed.

Assay for Fentanyl—Dilute the fentanyl sample or sample of fentanyl in combination with droperidol with water to obtain a 0.02 mg./ml. solution of fentanyl. Pipet 5 ml. of the diluted solution (rinsing the pipet with the solution once before the sample to be analyzed is taken) into a 60-ml. bottle containing exactly 2 ml. of 20% sulfuric acid. Add exactly 25 ml. of the 2% methanol in chloroform solution and 10 ml. of the Reinecke solution to the sample. Shake the bottle for 15 min. on a mechanical shaker. Do not centrifuge the sample but aspirate and discard the aqueous layer. Filter the chloroform layer through a cotton pledget to remove any traces of the precipitate.

Pipet 15 ml. of the chloroform layer to a 60-ml. bottle containing 10 ml. of pH 5 buffer. Add 1 ml. of the methyl orange reagent and shake the bottle on a mechanical shaker for 15 min. Centrifuge, aspirate, and discard the aqueous layer. Pipet 10 ml. of the chloroform layer to a bottle containing exactly 10 ml. of 1 *M* hydrochloric acid. Shake for 5 min. on a mechanical shaker, centrifuge, and carefully decant the acid layer. Determine the absorbance of the acid layer in 1-cm. cells at the maximum near 505 mμ.

A standard is prepared by dissolving fentanyl citrate in water and diluting it with water to obtain a 0.020 mg./ml. solution expressed as the free base. If the sample contains droperidol, the standard should contain the same amount of droperidol as present in the sample. Droperidol is insoluble in water. It may be solubilized by adding a few drops of lactic acid and heating on a steam bath until dissolved. Upon solution, add hot water and dilute to volume with water after the solution has reached room temperature.

Table III—Precision of the Proposed Assay Method Calculated as the Standard Deviation

Sample	No. of Assays	Droperidol Assay, %	Fentanyl Assay, %
Droperidol	16	1.53	...
Droperidol and fentanyl	118	2.40	3.98
Fentanyl	34	...	2.87

RESULTS AND CONCLUSIONS

The precision of the proposed assays was tested on actual samples that were being tested for their chemical-physical stability. The standard deviations calculated are those which would be normally expected in the laboratory. The standard deviations are presented in Table III.

The proposed assays are stability assays. Each hydrolysis product of droperidol, fentanyl, and the hydrolysis product of fentanyl were taken through the droperidol assay and found not to interfere in the assay. Droperidol, both hydrolysis products of droperidol, and the hydrolysis product of fentanyl were taken through the fentanyl assay and found not to interfere in the assay. The fate of the compounds was followed by a thin-layer chromatographic study of each phase of each extraction procedure.

The assay for droperidol does not appear to have any critical steps. The sodium bisulfite solution should be made fresh daily.

The assay for fentanyl, once started, should be carried to completion without undue delay. Colorimetric reading variation is high when the samples are left in contact with the Reinecke solution for longer than 1 hr. The fentanyl–methyl orange complex in the chloroform decomposes slowly on standing. This does not present a major problem since the chloroform solution is next extracted with 1 *M* hydrochloric acid. The red colored acid solution is stable for days.

If the ratio of droperidol to fentanyl in solution is varied from 50:1 to 25:1; no problems are encountered in either the droperidol or fentanyl assays. However, if the ratio of droperidol to fentanyl is lowered to 15:1, high fentanyl results are encountered. The high results are due to droperidol. From 1 to 3% of the droperidol is not completely precipitated by the Reinecke solution and is extracted into chloroform along with the fentanyl. At this point it is possible to do a TLC separation of fentanyl from droperidol using the TLC method previously described. The fentanyl band is scraped

off and extracted with chloroform and the chloroform solution treated with methyl orange as described. However, it was found that simply by adding sufficient droperidol (in lactic acid solution) to the sample during dilution to raise the ratio of droperidol to fentanyl to 50:1 in the final solution (0.02 mg./ml. fentanyl) complete precipitation of the droperidol was achieved.

The reineckate precipitate of droperidol can be used as a quantitative measure of droperidol. Once 4′-fluoro-4-(4-oxopiperidino)butyrophenone is removed by the addition of sodium bisulfite as directed in the proposed assay for droperidol, the chloroform solution of droperidol can be treated with the Reinecke solution and sulfuric acid. The droperidol-Reinecke precipitate is collected, dissolved in acetone, and the absorbance of the red colored solution read at about 525 mμ. The interference of 4-anilino-1-(2-phenylethyl)piperidine in the Reinecke precipitation of droperidol is small for the droperidol–fentanyl pharmaceutical preparations. The fentanyl would have to be completely hydrolyzed to affect the droperidol reineckate precipitate by 2%.

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Keyphrases

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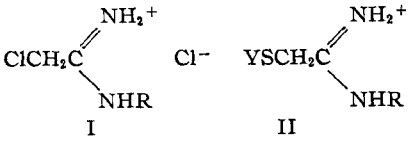
Fentanyl citrate, droperidol—hydrolysis products
Chloroform extraction—droperidol
UV spectrophotometry—analysis
Methanol–chloroform extraction—fentanyl citrate
Colorimetric analysis—methyl orange reagent
TLC—identity

Synthesis of α-Mercaptoacetamidinium Chlorides *via* the Corresponding Phosphorothioates

By TERRY T. CONWAY, ABOO SHOEB, and LUDWIG BAUER

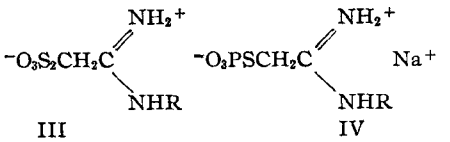
A facile synthesis of α-mercaptoacetamidinium chlorides is described. The procedure consists of treating α-chloroacetamidinium chlorides with trisodium phosphorothioate that formed the corresponding *S*-alkyl phosphorothioates. The latter were not isolated, but were hydrolyzed in the reaction medium by hot dilute acid to furnish the required products.

THE SYNTHESIS of α-mercapto amidines and derivatives as potential antiradiation agents has been under study in this laboratory for some time (1–3). The general approach consisted of treating an α-chloroacetamidinium chloride, I, with a sulfur bearing nucleophile, YS^-, to obtain a substituted acetamidinium cation, II. These



I II

reactions were successful since the substitution reaction of the halo group of the free α-chloroacetamidine was considerably faster than any reaction involving nucleophilic attack at the sp^2 amidine carbon. It was particularly important to exclude the latter type of reaction in an aqueous basic medium since displacement of the halo group by YS^- could easily be accompanied by hydrolysis of the amidine.[1] It was found that a relatively weakly basic, but excellent nucleophile like thiosulfate ion reacted quickly with I to form a series of α-amidinium Bunte salts, III. Due to the relatively easy preparation of



III IV

compounds of type III, it was of interest to explore the reaction of I with phosphorothioate ion, $^-O_3PS^-$, in the endeavor to synthesize a series of α-amidinium phosphorothioates, shown as the sodium salt, IV. It was intended to test a

Received September 15, 1967, from the Department of Chemistry, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60680
Accepted for publication November 15, 1967.
Taken in part from the Doctoral dissertation of T. T. Conway, University of Illinois at the Medical Center, Chicago, Ill., June 1967.
The generous support of this work by the U. S. Army Medical Research and Development Command (research contract, DA-49-193-MD-2047) is gratefully acknowledged.

[1] The basic hydrolysis of amidines is a well-documented reaction. [For a recent reference see DeWolfe, R. D., and Keefe, J. R., *J. Org. Chem.*, **27**, 493(1962).]

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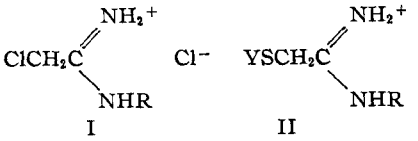
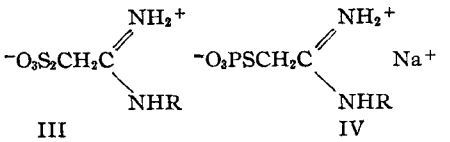


I II

reactions were successful since the substitution reaction of the halo group of the free α-chloroacetamidine was considerably faster than any reaction involving nucleophilic attack at the sp^2 amidine carbon. It was particularly important to exclude the latter type of reaction in an aqueous basic medium since displacement of the halo group by YS^- could easily be accompanied by hydrolysis of the amidine.[1] It was found that a relatively weakly basic, but excellent nucleophile like thiosulfate ion reacted quickly with I to form a series of α-amidinium Bunte salts, III. Due to the relatively easy preparation of



III IV

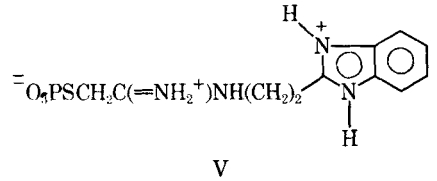
compounds of type III, it was of interest to explore the reaction of I with phosphorothioate ion, $^-O_3PS^-$, in the endeavor to synthesize a series of α-amidinium phosphorothioates, shown as the sodium salt, IV. It was intended to test a

Received September 15, 1967, from the Department of Chemistry, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60680
Accepted for publication November 15, 1967.
Taken in part from the Doctoral dissertation of T. T. Conway, University of Illinois at the Medical Center, Chicago, Ill., June 1967.
The generous support of this work by the U. S. Army Medical Research and Development Command (research contract, DA-49-193-MD-2047) is gratefully acknowledged.

[1] The basic hydrolysis of amidines is a well-documented reaction. [For a recent reference see DeWolfe, R. D., and Keefe, J. R., *J. Org. Chem.*, **27**, 493(1962).]

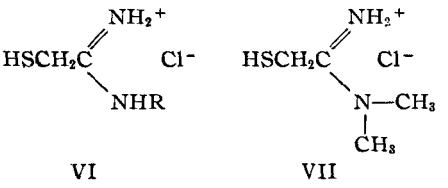
number of these salts, IV, as potential antiradiation agents since a number of analogous *S*-[β-aminoalkyl] phosphorothioates had been shown frequently to afford good protective action against ionizing radiation in mice (4, 5).

The reaction of a number of chloroacetamidinium chlorides with trisodium phosphorothioate in water was followed initially by the change in the NMR spectrum. For example, the proton magnetic resonance spectrum[2] of α-chloroacetamidinium chloride (I, R = H) in water showed a singlet for the CH_2 proton at δ 4.53 which disappeared quickly on the addition of trisodium phosphorothioate. There arose a doublet at δ 3.66 which was assigned to the CH_2—S—P resonance, coupling of the protons over three bonds with phosphorus being observed (J = 13.5 c.p.s.).[3] Addition of methanol to such an aqueous solution furnished the salt (as the trihydrate), IV (R = H). Analogous reactions of I (R = CH_3, $CH_2C_6H_5$, *cyclo*-C_6H_{11}) with Na_3SPO_3 invariably gave aqueous solutions whose NMR spectra showed a doublet (J = 13.5 c.p.s.) in the vicinity of δ 3.70 ± 0.1. Attempts to isolate these phosphorothioates by the addition of alcohols or *N,N*-dimethylformamide to the aqueous solution yielded solids which tenaciously clung to polar solvents. These solids were found to possess varying degrees of stability as was evident by their change in physical appearance and their NMR spectra.[4] One notable exception was the isolation of the relatively stable zwitterion, V. Apparently the basic β-(2-benzimidazole) ethyl group neutralized the second negative charge and V could be kept for months.



V

It was observed, however, that these phosphorothioates, IV, were hydrolyzed readily by hot dilute mineral acids to the corresponding thiols, VI. As a matter of expediency, it was found that the isolation of the intermediate phosphorothioates, IV, was not essential, but these could be hydrolyzed *in situ* and this proved to be a general method for the ready conversion of I to VI. It was possible to synthesize a number of derivatives of VI, where R was representative alkyl and aralkyl groups starting



VI VII

with the corresponding crystalline α-chloroacetamidinium chlorides, I, and without the isolation of IV. These solid α-mercaptoacetamidinium chlorides were stable in air. The only exception in the authors' experience was *N*-methyl-α-mercaptoacetamidinium chloride (VI, R = CH_3), which decomposed quickly. This particular salt began to smell badly of hydrogen sulfide, commenced to liquify within 24 hr. (*in vacuo* at 25°), and its NMR spectrum changed drastically. By contrast, the corresponding *N,N*-dimethyl analog, VII, was quite stable. This salt showed, as expected, two different N—CH_3 signals in the NMR. This restricted rotation about the CN bond is in keeping with that observed in other *N,N*-dimethyl amidinium salts (6).

An extension of this type of synthesis towards *N,N'*-disubstituted α-mercaptoacetamidinium chlorides led into the preparation of imidazole derivatives. For example, 2-(chloromethyl)-imidazoline was readily converted to 2-(mercaptomethyl)imidazoline by this method. An aromatic benzimidazole analog was prepared as shown in Scheme I. This involved originally the preparation of 1-methyl-2-(chloromethyl)-benzimidazole from the reaction of methyl chloroacetimidate (prepared *in situ* from the addition of methanol to chloroacetonitrile) (7) with *N*-methyl-*o*-phenylenediamine hydrochloride.[5] The chloro derivative was then readily converted to the corresponding mercaptan.

Attempts were made to utilize α-amidinium thiosulfates (Bunte salts), III, as suitable precursors to VI. On a preparative scale, the acid hydrolysis of the thiolsulfate group[6] in III was not nearly as satisfactory as that of the

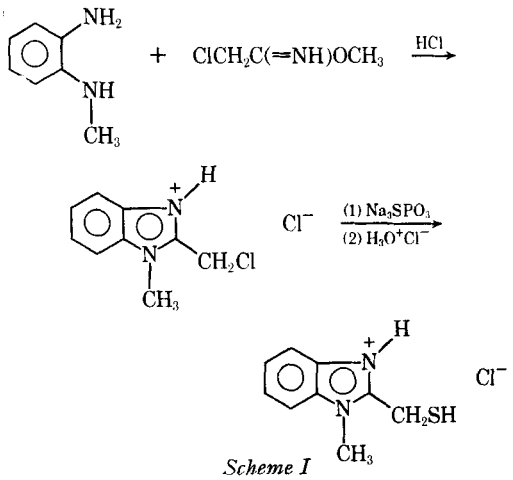
[2] The nuclear magnetic resonance (NMR) spectra were determined at 60 Mc. by means of a Varian A-60 spectrometer and chemical shifts are recorded in parts per million (δ) downfield from such internal standards as tetramethylsilane (TMS) in organic solvents and sodium 3-(trimethylsilyl)-propanesulfonate (TPS) in aqueous or D_2O solutions. Multiplicities are denoted as follows: singlet, s; doublet, d. Assignment of the signals is also based on correct integral information.

[3] Long-range coupling over three bonds, $J_{P\text{-}S\text{-}CH_2}$, has been reported for a number of similar systems: in a number of mercaptophosphazenes, J = 18.1 ± 0.5 c.p.s. [Boden, N., Emsley, J. W., Feeney, J., and Sutcliffe, L. H., *Chem. Ind.*, (London), 1909 (1962)]; in *S*-alkylphosphorothioates, J = 15 c.p.s. [Oswald, A. A., Griesbaum, K., and Hudson, B. E., Jr., *J. Org. Chem.*, **28**, 1262(1963)]; J = 12.7 – 17.5 c.p.s. [Mueller, W. H., Rubin, R. M., and Butler, P. E., *ibid.*, **31**, 3537(1966); Mueller, W. H., and Oswald, A. A., *ibid.*, **32**, 1730(1967)].

[4] Attempts to crystallize these salts with solvent of crystallization or to dry them *in vacuo* at 5° were unsuccessful in most instances and purification resulted in partial decomposition. Most of the *S*-(aminoalkyl)phosphorothioates were isolated with varying proportions of solvent of crystallization (see *References 4* and *5*).

[5] The synthesis of benzimidazoles from *o*-phenylenediamines and imidates was recently reinvestigated [see deSelms, R. C., *J. Org. Chem.*, **27**, 2163(1962)].

[6] For a recent study on the acid-catalyzed hydrolysis of Bunte salts, see Kice, J. L., Anderson, J. M. and Pawlowski, N. E., *J. Am. Chem. Soc.*, **88**, 5245(1966).



Scheme I

phosphorothioate moiety in IV to give VI. The acid hydrolysis of several α-amidinium thiolsulfates, III, was investigated in detail. For example, brief exposure of III (R = benzyl) to hot dilute hydrochloric acid yielded VI (R = benzyl) in poor yield. Prolonged acid treatment (even under nitrogen) produced predominantly the corresponding disulfides, $[-SCH_2C(=NH_2^+)NHCH_2C_6H_5\ Cl^-]_2$, VIII. Presumably, these disulfides are formed by the oxidation of the corresponding mercaptans, although the possibility of displacement of HSO_3^- in III by RSH of VI does exist.[7] This point was not investigated further.

In connection with the general study of nucleophilic displacement reactions of I, *N*-benzyl-α-chloroacetamidinium chloride was treated with sodium sulfite. There was formed the corresponding α-amidinium sulfonate, $^-O_3SCH_2C(=NH_2^+)HNCH_2C_6H_5$, IX. To prove that indeed this substitution reaction involved the formation of a C-S bond, the disulfide, VIII, was oxidized to give IX. The infrared spectrum of IX clearly showed it to be a zwitterion since the characteristic amidinium bands showed up, $-C(=N^+<$ stretching frequency in the vicinity of 1700 and the $>C=NH_2^+$ deformation mode around 1650 cm.$^{-1}$. These bands were also found in III and IV.

EXPERIMENTAL[8]

N-Cyclopentylchloroacetamidinium Chloride—The procedure developed previously (1–3) was used for this and related syntheses. Chloroacetonitrile (7.55 Gm., 0.1 mole) was added slowly to a stirred ice-cold solution of sodium (0.23 Gm., 0.01 Gm.-atom) in methanol (75 ml.). After 1 hr., cyclopentylammonium chloride (13.38 Gm., 0.11 mole) was then added and the mixture stirred for 1 hr. longer. The mixture was filtered and solvent removed at 20–30 mm. and at the lowest temperature. The purple residue was triturated with dry ether and the resultant solid (11.26 Gm., 55%) recrystallized from 2-propanol–ether, m.p. 142.5–143.5° dec.

Anal.—Calcd. for $C_7H_{14}Cl_2N_2$: C, 42.67; H, 7.16; N, 14.22. Found: C, 42.73; H, 7.53; N, 14.00.

N-Cyclohexylchloroacetamidinium Chloride—This salt was prepared in 80% yield in an analogous fashion, using cyclohexylammonium chloride, and recrystallized from 2-propanol, m.p. 181–182.5° dec.

Anal.—Calcd for $C_8H_{16}Cl_2N_2$: C, 45.40; H, 7.62; N, 13.24. Found: C, 45.55; H, 7.83; N, 12.95.

N - [β - (2 - Benzimidazolium)ethyl]chloroacetamidinium Dichloride—When 2-(β-aminoethyl)-benzimidazole dihydrochloride (8) was used as the ammonium salt, the title product commenced to precipitate, was filtered, and an additional quantity obtained when solvents were concentrated. The salt was obtained in 82% yield and was recrystallized from aqueous ethanol, m.p. 244–246°.

Anal.—Calcd. for $C_{11}H_{15}Cl_3N_2$: C, 42.65; H, 4.84; N, 18.09. Found: C, 42.50; H, 4.98; N, 17.98.

1-Methyl-2-(chloromethyl)benzimidazolium Chloride—This salt was obtained in 51% yield when *N*-methyl-*o*-phenylenediamine hydrochloride (Eastman Organic Chemicals) was substituted in the synthesis described above, m.p. 209° (from 2-propanol).

Anal.—Calcd. for $C_9H_{10}Cl_2N_2$: C, 49.77; H, 4.60; N, 12.90. Found: C, 49.49; H, 4.55; N, 12.97.

Sodium S-(Carboxamidiniummethyl)phosphorothioate Trihydrate—α-Chloroacetamidinium chloride (1) (2.58 Gm., 0.02 mole) was added to a solution of trisodium phosphorothioate[9] (3.6 Gm., 0.02 mole) in water (30 ml.) and the resultant solution stirred at 25° for 10 min. A small precipitate was filtered off and the filtrate diluted with methanol (50 ml.), and the mixture chilled for 10 min. The product (3.25 Gm., 66%) was collected, washed copiously with dry ether (two 40-ml. portions), and dried *in vacuo* (over H_2SO_4). On heating in an m.p. tube, it turned yellow and decomposed above 90°.

Anal.—Calcd. for $C_2H_6N_2NaO_3PS\cdot 3H_2O$: C, 9.75; H, 4.91; N, 11.38; S, 13.02. Found: C, 9.75; H, 5.02; N, 11.12; S, 13.11.

Sodium S-[N-(Benzyl)carboxamidiniummethyl]-phosphorothioate Hydrate—*N*-Benzyl-α-chloroacetamidinium chloride (1) (4.38 Gm., 0.02 mole) was added to a stirred aqueous solution of trisodium phosphorothioate (3.6 Gm., 0.02 mole in 35 ml.). After 10 min. at 25°, the mixture was filtered to remove a trace of insoluble material and the filtrate diluted with 2-propanol (50 ml.). The product was filtered, washed with 2-propanol. It was recrystallized by dissolving it in cold water

[7] In their review on Bunte salts, B. Milligan, and J. M. Swan [*Rev. Pure Appl. Chem.*, 12, 72(1962); *J. Chem. Soc.*, 6008 (1963)] state that the reaction of mercaptans with Bunte salts to give disulfides is favored at a high pH. Other relevant literature has been summarized by H. Distler [*Angew. Chem.*, 79, 520(1967)].

[8] All melting and boiling points are uncorrected. Infrared spectra were all determined in Nujol mull (unless otherwise specified) by means of the Perkin-Elmer model 337 spectrophotometer. Microanalyses were performed by Micro-Tech Laboratories, Skokie, Ill. and Dr. Kurt Eder, Geneva, Switzerland. Most of the nitrogen analyses were obtained by Mr. Leo Horner in this department, using a Coleman model D-29.

[9] This salt was prepared from thiophosphoryl chloride (supplied by the Stauffer Chemical Co.) and sodium hydroxide solution at 60°. This modification of S. Akerfeldt's procedure [*Acta Chem. Scand.*, 14, 1980(1960)] was essential and was also reported by J. R. Piper, and T. P. Johnston, [*J. Org. Chem.*, 32, 1261(1967)].

and adding 2-propanol. It was dried at 5° at 20 mm. over concentrated H_2SO_4 until its NMR spectrum showed the absence of signals due to 2-propanol. In H_2O, the salt showed signals due to CH_2S at δ 3.75 (d, J_{CH_2SP} = 13.5 c.p.s.), CH_2N at δ 4.58 (d, J = 6.3 c.p.s.), and C_6H_5 at δ 7.54 (s).

Anal.—Calcd for $C_9H_{12}N_2NaO_3PS \cdot H_2O$: C, 36.01; H, 4.70; N, 9.33. Found: C, 36.46; H, 5.23; N, 8.83.

S - {N - [β - (2 - Benzimidazolium)ethyl]carboxamidiniummethyl}phosphorothioate, V—*N*-[β-(2-Benzimidazolium)ethyl]chloroacetamidinium dichloride (0.3 Gm., 0.001 mole) was added to an aqueous solution of trisodium phosphorothioate (0.18 Gm., 0.001 mole in 10 ml.) and the solution stirred at 25° for 10 min. The product (0.21 Gm., 70%) was collected, washed with hot water, boiling ethanol; m.p. 168–169°. It was dried *in vacuo* at 25° prior to analysis.

Anal.—Calcd. for $C_{11}H_{15}N_4O_3PS \cdot {}^1/_2H_2O$: C, 40.86; H, 4.95; N, 17.33; S, 9.90. Found: C, 41.19; H, 4.97; N, 17.29; S, 9.89.

α-Mercaptoacetamidinium Chloride—All stages of this and cognate experiments were conducted under a blanket of nitrogen. To an aqueous solution of trisodium phosphorothioate (3.66 Gm., 0.02 mole in 25 ml.) was added α-chloroacetamidinium chloride (2.58 Gm., 0.02 mole). The solution was stirred at ambient temperature until the test for phosphorothioate ion was negative [black precipitate with $AgNO_3$ (9), about 3–5 min.]. After 10 min., 6 *N* hydrochloric acid (20 ml.) was added and the solution heated at 90° for 10 min. and then evaporated *in vacuo*. The residue was extracted with 2-propanol (20 ml.) and filtered from inorganic salts. Addition of dry ether (150 ml.) yielded the salt (1.9 Gm., 75%), m.p. 120–122° dec.; infrared absorptions, 2540 (νSH), 1700 (νC=N), 1650 cm.$^{-1}$ (δ C=NH_2^+); NMR signal (D_2O) at δ 3.57 (s) for CH_2S.

Anal.—Calcd. for $C_2H_7ClN_2S$: C, 18.97; H, 5.57; N, 22.13; S, 25.32. Found: C, 18.97; H, 5.55; N, 21.99; S, 25.21.

This procedure was used to convert a number of crystalline α-chloroacetamidinium chlorides to the corresponding thiols.

N,N-Dimethyl-α-mercaptoacetamidinium chloride was prepared in this manner (64%); m.p. 160°; infrared, 2450 (νSH), 1690 (νC=N^+) and 1650 cm.$^{-1}$ (δ C=NH_2^+); NMR, δ (D_2O) 3.65, (s, CH_2S), 3.18, 3.26 (two s, NCH_3).

Anal.—Calcd. for $C_4H_{11}ClN_2S$: C, 31.06; H, 7.12; N, 18.12; S, 20.71. Found: C, 31.15; H, 7.10; N, 18.03; S, 20.64.

N-Cyclopentyl-α-mercaptoacetamidinium chloride was synthesized in this way (72%), m.p. 146–147.5° dec.

Anal.—Calcd. for $C_7H_{15}ClN_2S$: C, 43.17; H, 7.76; N, 14.39; S, 16.47. Found: C, 43.16; H, 7.78; N, 14.59; S, 16.39.

N-Cyclohexyl-α-mercaptoacetamidinium chloride was prepared (44%) as above, m.p. 163–165° dec.

Anal.—Calcd. for $C_8H_{17}ClN_2S$: C, 46.02; H, 8.21; N, 13.42; S, 15.36. Found: C, 45.77; H, 8.15; N, 13.36; S, 15.30.

N-Benzyl-α-mercaptoacetamidinium chloride was made from the corresponding α-chloro compound (1), in 44% yield, m.p. 115–117° dec.; infrared, 2570 (γSH), 1700 (νC=N^+), 1650 cm.$^{-1}$ (δ C=NH_2^+); NMR, δ (D_2O) 3.65 (s, CH_2S), 4.60 (s, CH_2N), 7.46 (s, C_6H_5).

Anal.—Calcd for $C_9H_{13}ClN_2S$: C, 49.87; H, 6.04; N, 12.93; S, 14.80. Found: C, 50.26; H, 5.88; N, 12.91; S, 14.80.

N-(2-Picolyl)-α-mercaptoacetamidinium chloride hydrochloride was produced from the chloro precursor (3) in 63% yield, m.p. 196–197°.

Anal.—Calcd. for $C_8H_{13}Cl_2N_3S$: C, 37.79; H, 5.11; N, 16.53; S, 12.60. Found: C, 37.55; H, 5.15; N, 16.44; S, 12.70.

N - (6 - Methyl - 2 - picolyl) - α - mercaptoacetamidinium chloride hydrochloride was prepared from the corresponding chloro derivative (3) in 48% yield, m.p. 179–181°.

Anal.—Calcd. for $C_9H_{15}Cl_2N_3S$: C, 40.30; H, 5.64; N, 15.64; S, 11.96. Found: C, 40.22; H, 5.53; N, 15.39; S, 11.79.

N - [β - (2 - Pyridyl)ethyl] - α - mercaptoacetamidinium chloride hydrochloride was obtained from the corresponding chloro compound (3) (60%), m.p. 180°.

Anal.—Calcd. for $C_9H_{15}Cl_2N_3S$: C, 40.30; H, 5.60; N, 15.67; S, 11.94. Found: C, 40.15; H, 5.63; N, 15.34; S, 11.91.

2-(Mercaptomethyl)imidazolinium chloride was synthesized in analogous fashion from 2-(chloromethyl)-imidazoline (3) (60%), m.p. 129–130°.

Anal.—Calcd. for $C_4H_9ClN_2S$: C, 31.47; H, 5.90; N, 18.35; S, 20.98. Found: C, 31.60; H, 6.17; N, 18.04; S, 20.40.

1 - Methyl - 2 - (mercaptomethyl) - benzimidazolium chloride hydrate was prepared (in 84% yield) from 1-methyl-2-(chloromethyl)benzimidazolium chloride as described above, m.p. 127°.

Anal.—Calcd. for $C_9H_{11}ClN_2S \cdot H_2O$: C, 46.45; H, 5.59; N, 12.04; S, 13.76; Cl, 15.26. Found: C, 46.53; H, 5.76; N, 12.14; S, 13.80; Cl, 15.30.

N - [β - (2 - Benzimidazolium)ethyl] - α - mercaptoacetamidinium Dichloride—*S*-{*N*-[β-(2-Benzimidazolium)ethyl]carboxamidiniummethyl}phosphorothioate (1.3 Gm., 0.004 mole, prepared above) was heated with 6 *N* HCl (10 ml.) on the steam bath for 10 min. (nitrogen atmosphere). Solvents were removed *in vacuo* to yield a solid (0.8 Gm., 66%), which was recrystallized from a mixture of ethanol–ether to give colorless crystals, m.p. 215–216°.

Anal.—Calcd. for $C_{11}H_{16}Cl_2N_4S$; C, 43.50; H, 5.21; N, 18.23; S, 10.42. Found: C, 43.07; H, 5.13; N, 18.08; S, 10.28.

N-Benzyl-α-mercaptoacetamidinium Chloride from the Hydrolysis of III (R = $CH_2C_6H_5$)—A solution of S-[*N*-(benzyl)-carboxamidinomethane]thiosulfuric acid (1.16 Gm., 0.004 mole) in 6 *N* hydrochloric acid (15 ml.) was heated at 96° for 1.5 hr. (N_2 atmosphere). After removing solvents *in vacuo*, the yellow oily residue crystallized and was recrystallized from ethanol–ether. It weighed 0.2 Gm., (24% yield), m.p. 115–116°, and was identical to the sample made *via* the phosphorothioate.

(N-Benzylcarboxamidinium)methyl Disulfide Dichloride—A solution of S-[*N*-(benzyl)carboxamidinomethane]thiosulfuric acid (1) (2.64 Gm., 0.01 mole) in 6 *N* hydrochloric acid (50 ml.) was heated at 96° for 15 hr. On cooling, the oil which separated solidified and was crystallized from water (1.6 Gm., 75%, m.p. 207–208.5° dec; infrared, 1680 (νC=N^+), 1635 cm.$^{-1}$ (δ C=NH_2^+); NMR in D_2O, δ 3.83 (s, CH_2S) 4.60 (s, CH_2N) 7.48 (s, C_6H_5), in (DMSO-d_6), δ 3.96 (s, CH_2S) 4.72 (d, CH_2N, J_{CH_2-NH} = 5 c.p.s.), 7.49 (s, C_6H_5), 9.95, 9.55, and 10.79 (broad s, NH).

Anal.—Calcd. for $C_{18}H_{24}Cl_2N_4S_2$: C, 50.11; H, 5.61; N, 12.91; S, 14.86. Found: C, 50.43; H, 5.86; N, 12.72; S, 15.21.

(Carboxamidinium)methyl Disulfide Sulfate—A solution of α-(carboxamidino)methanethiolsulfuric acid (1) (10.0 Gm., 0.059 mole) in 6 *N* hydrochloric acid (250 ml.) was heated at 96° for 17 hr. The reaction mixture was evaporated *in vacuo* and the yellow gummy residue recrystallized from 50% aqueous alcohol (4.5 Gm., 55%), m.p. 225–227° dec.; infrared,† 1700 cm.$^{-1}$ (ν C=N$^+$); NMR (D_2O), δ 3.82 (s, CH_2S–S–).

Anal.—Calcd. for $C_4H_{12}N_4O_4S_3$: C, 17.38; H, 4.38; N, 20.27; S, 34.81. Found: C, 17.62; H, 4.50; N, 20.00; S, 35.01.

(N-Benzylcarboxamidino)methanesulfonic Acid—(*a*) To an aqueous solution of *N*-benzyl-α-chloroacetamidinium chloride (1) (5 Gm., 0.019 mole) in 30 ml. was added an aqueous sodium sulfite solution (2.42 Gm. 0.019 mole in 22 ml.) and the mixture heated at the reflux for 0.5 hr. The crystals (1.2 Gm., 37%) which separated were recrystallized from methanol–ether to give the product (0.20 Gm., 47%), m.p. 245–246°; infrared, 1690 (ν C=N$^+$), 1645 cm.$^{-1}$ (δ C=NH_2^+); NMR (D_2O), δ 4.10 (s, CH_2S), 4.60 (*s*, CH_2N), 7.45 (s, C_6H_5).

Anal.—Calcd. for $C_9H_{12}N_2O_3S$: C, 47.35; H, 5.30; N, 12.27; S, 14.05. Found: C, 47.25; H, 5.52; N, 12.27; S, 13.90.

(*b*) A solution of (*N*-benzylcarboxamidinium)-methyl disulfide dichloride (0.40 Gm.), 30% hydrogen peroxide solution (5 ml.), and 6 *N* hydrochloric acid (10 ml.) were heated at 96° (17 hr.). Solvents were removed *in vacuo* and the residue crystallized and found to be identical to that described in *a*.

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Keyphrases

α-mercaptoacetamidinium chlorides—synthesis
S-alkyl phosphorothioates—intermediate products
IR spectrophotometry—structure
NMR spectrometry

Drug Standards

Selective Determination of Tolbutamide in Pharmaceutical Dosage Forms by Reaction with Ninhydrin

By K. K. KAISTHA and W. N. FRENCH

A colorimetric method is described for the quantitative determination of tolbutamide in pharmaceutical dosage forms. The procedure is based on the interaction of *n*-butylamine, liberated *in situ* from tolbutamide, and ninhydrin to form a blue complex which can be quantitated spectrophotometrically at 585 mμ. No color is produced by *p*-toluenesulfonamide or dibutylurea, two possible decomposition products of tolbutamide. Urea and its acyl derivatives, carbamates, amidines, guanidine, and common tablet excipients do not undergo the reaction. A modified procedure is described for the removal of *n*-butylamine, a major hydrolytic product of tolbutamide, from samples of the drug that have undergone decomposition. Results of the application of the assay procedure to a stability study of tolbutamide tablets are presented.

ALTHOUGH ULTRAVIOLET SPECTROPHOTOMETRIC analysis, as described in the USP XVII (1), is commonly used for tolbutamide preparations, this method is not satisfactory if ultraviolet-absorbing degradation products such as *p*-toluenesulfonamide or impurities such as *p*-toluenesulfonylurea are present in the sample. Similarly, a procedure involving total nitrogen determination, as described in the BP 1963 (2),

Received May 19, 1967, from the Research Laboratories, Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada.
Accepted for publication October 26, 1967.
Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
The authors thank Dr. Leo Levi for his interest and encouragement.

Anal.—Calcd. for $C_{18}H_{24}Cl_2N_4S_2$: C, 50.11; H, 5.61; N, 12.91; S, 14.86. Found: C, 50.43; H, 5.86; N, 12.72; S, 15.21.

(Carboxamidinium)methyl Disulfide Sulfate—A solution of α-(carboxamidino)methanethiolsulfuric acid (1) (10.0 Gm., 0.059 mole) in 6 *N* hydrochloric acid (250 ml.) was heated at 96° for 17 hr. The reaction mixture was evaporated *in vacuo* and the yellow gummy residue recrystallized from 50% aqueous alcohol (4.5 Gm., 55%), m.p. 225–227° dec.; infrared,† 1700 cm.$^{-1}$ (ν C=N$^+$); NMR (D_2O), δ 3.82 (s, CH_2S–S–).

Anal.—Calcd. for $C_4H_{12}N_4O_4S_3$: C, 17.38; H, 4.38; N, 20.27; S, 34.81. Found: C, 17.62; H, 4.50; N, 20.00; S, 35.01.

(N-Benzylcarboxamidino)methanesulfonic Acid—(*a*) To an aqueous solution of *N*-benzyl-α-chloroacetamidinium chloride (1) (5 Gm., 0.019 mole) in 30 ml. was added an aqueous sodium sulfite solution (2.42 Gm. 0.019 mole in 22 ml.) and the mixture heated at the reflux for 0.5 hr. The crystals (1.2 Gm., 37%) which separated were recrystallized from methanol–ether to give the product (0.20 Gm., 47%), m.p. 245–246°; infrared, 1690 (ν C=N$^+$), 1645 cm.$^{-1}$ (δ C=NH_2^+); NMR (D_2O), δ 4.10 (s, CH_2S), 4.60 (*s*, CH_2N), 7.45 (s, C_6H_5).

Anal.—Calcd. for $C_9H_{12}N_2O_3S$: C, 47.35; H, 5.30; N, 12.27; S, 14.05. Found: C, 47.25; H, 5.52; N, 12.27; S, 13.90.

(*b*) A solution of (*N*-benzylcarboxamidinium)-methyl disulfide dichloride (0.40 Gm.), 30% hydrogen peroxide solution (5 ml.), and 6 *N* hydrochloric acid (10 ml.) were heated at 96° (17 hr.). Solvents were removed *in vacuo* and the residue crystallized and found to be identical to that described in *a*.

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Keyphrases

α-mercaptoacetamidinium chlorides—synthesis
S-alkyl phosphorothioates—intermediate products
IR spectrophotometry—structure
NMR spectrometry

Drug Standards

Selective Determination of Tolbutamide in Pharmaceutical Dosage Forms by Reaction with Ninhydrin

By K. K. KAISTHA and W. N. FRENCH

A colorimetric method is described for the quantitative determination of tolbutamide in pharmaceutical dosage forms. The procedure is based on the interaction of *n*-butylamine, liberated *in situ* from tolbutamide, and ninhydrin to form a blue complex which can be quantitated spectrophotometrically at 585 mμ. No color is produced by *p*-toluenesulfonamide or dibutylurea, two possible decomposition products of tolbutamide. Urea and its acyl derivatives, carbamates, amidines, guanidine, and common tablet excipients do not undergo the reaction. A modified procedure is described for the removal of *n*-butylamine, a major hydrolytic product of tolbutamide, from samples of the drug that have undergone decomposition. Results of the application of the assay procedure to a stability study of tolbutamide tablets are presented.

ALTHOUGH ULTRAVIOLET SPECTROPHOTOMETRIC analysis, as described in the USP XVII (1), is commonly used for tolbutamide preparations, this method is not satisfactory if ultraviolet-absorbing degradation products such as *p*-toluenesulfonamide or impurities such as *p*-toluenesulfonylurea are present in the sample. Similarly, a procedure involving total nitrogen determination, as described in the BP 1963 (2),

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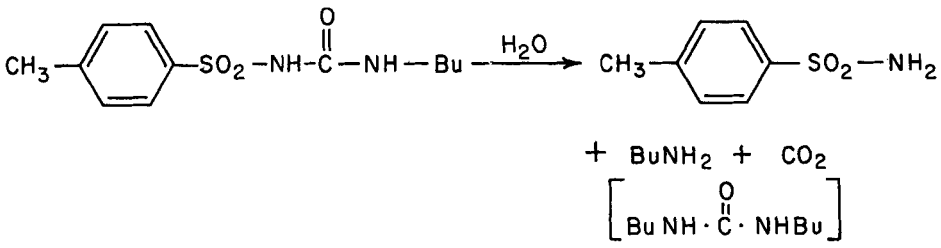

Scheme I

is unsatisfactory if nitrogen-containing contaminants are present. Spingler and Kaiser (3) determined tolbutamide in serum by recording the absorption at 228 mμ, while Spingler (4) reported a colorimetric procedure based on the treatment of an amyl acetate extract of serum with 1-fluoro-2,4-dinitrobenzene followed by measurement of the absorbance at 380 mμ. The latter technique was adopted by Wiseman and co-workers for the estimation of a number of sulfamylureas and their metabolites in plasma and urine (5).

It was observed that a blue color was formed when an ethanolic solution of tolbutamide was heated with ninhydrin. The color was not formed with *p*-toluenesulfonamide or dibutylurea and ninhydrin, but was generated with *n*-butylamine. On the basis of this reaction, a method wherein the *n*-butylamine is generated *in situ* from tolbutamide and subsequently reacts with ninhydrin is presented.

The main hydrolytic decomposition of tolbutamide occurs as shown in Scheme I. Therefore, this method may be applied to decomposed formulations for the selective determination of tolbutamide by prior removal of any *n*-butylamine present.

EXPERIMENTAL

Reagents—Ninhydrin solutions: (*a*) 0.3% ninhydrin (reagent grade) in 80% ethanol. (*b*) 0.3% ninhydrin (reagent grade) in absolute ethanol.

Standard Reference Solution—Tolbutamide USP reference standard (200 mg.) in absolute ethanol (200 ml.).

Assay Procedure for Tolbutamide in the Absence of *n*-Butylamine—Procedure I

Procedure—Twenty tablets were weighed and finely powdered. An accurately weighed aliquot containing about 200 mg. of tolbutamide was quantitatively transferred to a 200-ml volumetric flask with the aid of absolute ethanol. The mixture was made to volume with absolute ethanol, shaken for 10 min., and filtered. A 25-ml. aliquot of the filtrate was heated under reflux in a 100-ml. flask fitted with a water-cooled condenser by immersing the flask up to the level of its contents in a stirred paraffin oil bath maintained at 96–100°. A small boiling chip was added to promote continuous and uniform boiling. After 90 min., the solution was cooled, transferred quantitatively into a 50-ml. volumetric flask, and made to volume with absolute ethanol.

A 2-ml. aliquot of the latter solution was pipeted into a 50-ml. flask and diluted with 3 ml. of the 80% ethanolic ninhydrin solution and 35 ml. of absolute ethanol. The solution was heated under reflux for 4 hr. as described above, cooled, then transferred quantitatively to a 50-ml. volumetric flask, and made to volume with absolute ethanol. The absorbance of the sample solution and that of tolbutamide standard carried simultaneously through the assay procedure were measured at 585 mμ against absolute ethanol.[1]

Calculations are as follows:

$$\text{mg. tolbutamide per tablet} = \frac{A_U}{A_S} \times \frac{W_S \times W_O}{W_U}$$

where:

A_U = absorbance of sample solution
A_S = absorbance of standard reference solution
W_S = weight of standard tolbutamide (mg.)
W_U = weight of sample (mg.)
W_O = average weight per tablet (mg.)

Procedure for Partially Degraded Tolbutamide Formulations—Procedure II

Total Unchanged Tolbutamide (Part A)—An aliquot of sample originally containing the equivalent of 200 mg. of tolbutamide was placed in a 200-ml. volumetric flask and chloroform added to volume. The mixture was shaken for 10 min., filtered, and a 25-ml. aliquot of filtrate transferred to a 60-ml. separator. The chloroform layer was extracted with 20 ml. of 0.1 *N* hydrochloric acid, and the aqueous extract in turn washed with 4 × 10-ml. portions of chloroform. The combined chloroform extracts were taken to dryness by means of a rotary film evaporator and the residue dissolved in 25 ml. of absolute ethanol. The color was developed as described in the assay procedure for tolbutamide (*Procedure I*) beginning at "A 25-ml. aliquot. . .was heated under reflux. . . ."

n-Butylamine (Part B)—The aqueous extract obtained as described under Part A was washed with 5 ml. of benzene (discarded), made alkaline with 5 ml. of 30% NaOH, and extracted successively with 9 ml. and three 5-ml. portions of benzene. The combined benzene extracts were diluted to 25 ml. with benzene in a volumetric flask, mixed, and passed through a pledget of glass wool.

A 5-ml. aliquot of the filtrate was transferred to a 50-ml. flask, 3 ml. of ethanolic ninhydrin solution

[1] This blank gives the same absorbance as a true "reagent blank."

(0.3% in absolute ethanol), and 25 ml. of absolute ethanol were added, and the color developed by heating under reflux for 3.5 hr. The reaction solution was cooled, diluted with absolute ethanol to 50 ml. in a volumetric flask, and its absorbance determined at 585 mμ. A standard solution of *n*-butylamine in benzene (0.1 mg./ml. ≡ 1.37×10^{-3} mmole/ml.) was analyzed concurrently for comparison purposes. The content of *n*-butylamine in the sample was calculated from the respective absorbance values.

Free Tolbutamide (Part C)—A 25-ml. aliquot of the chloroform filtrate obtained as described in Part A was extracted with three 15-ml. portions of water. The combined aqueous extracts were then extracted once with 15 ml. of chloroform, and the combined chloroform solutions evaporated to dryness by means of a rotary film evaporator. The residue was dissolved in 25 ml. of absolute ethanol, and the color developed as described under the assay procedure for tolbutamide (Procedure I) starting at "A 25-ml. aliquot ... was heated under reflux ..." The amount of free tolbutamide was calculated from the absorbance values of standard and sample.

Tolbutamide—n-Butylamine Salt (Part D)—The combined aqueous extracts obtained as described in Part C were acidified with 10 ml. of *N* HCl, and extracted with three 15-ml. portions of chloroform. The combined chloroform extracts were evaporated to dryness by means of a rotary film evaporator, and the residue dissolved in 25 ml. of absolute ethanol. The color was developed as described under the assay procedure for tolbutamide (Procedure I) starting at the words "A 25-ml. aliquot ... was heated under reflux ..." Ten-milliliter aliquots of this refluxed solution which had been diluted to 50 ml. with absolute ethanol were used for the color development instead of 2 ml. The amount of tolbutamide forming a salt with *n*-butylamine was calculated from the absorbance values of standard and sample.

p-Toluenesulfonamide (Part E)—A 25-ml. aliquot of the chloroform filtrate isolated as described in Part A was assayed according to the USP XVII procedure for tolbutamide tablets, and the apparent amount of tolbutamide calculated. The weight of *p*-toluenesulfonamide was calculated by subtracting the weight of tolbutamide per tablet as determined by the colorimetric method (Part A) from the apparent weight of tolbutamide per tablet as determined by the USP assay procedure.

Thin-Layer Chromatography—Silica Gel GF and layer thickness of 250 μ were used throughout. For the separation and detection of *p*-toluenesulfonamide and/or *n*-butylamine in tolbutamide preparations, a solvent system of benzene–acetone–methanol–acetic acid (70:20:5:5) (6) was used. R_f values were 0.60, 0.05, and 0.70, respectively. Examination under short-wave ultraviolet light enabled the detection of the aromatic compounds. Spraying with 0.5% ethanolic ninhydrin solution followed by heating at 80–100° for 10–15 min. caused the formation of orange to pink spots for *n*-butylamine and tolbutamide (limiting sensitivities of 2 and 10 mcg., respectively). *p*-Toluenesulfonamide was visualized with a spray of 3% $KMnO_4$ in H_2SO_4 (limiting sensitivity of 6 mcg.). Alternatively, chlorine treatment followed by benzidine reagent (7) gave blue spots for *p*-toluenesulfonamide (limiting sensitivity of 5 mcg.). Semiquantitative thin-layer chromatographic estimation was accomplished by comparing spot areas of unknown and standard.

RESULTS AND DISCUSSION

The blue color formed during the assay of tolbutamide arises from the interaction of *n*-butylamine and ninhydrin. Although *n*-butylamine is formed readily from tolbutamide in boiling ethanol, the ethanolysis proceeds only partially when no reagent such as ninhydrin is present to remove *n*-butylamine from the equilibrium. Thus, when ethanolic tolbutamide solutions (1 Gm./100 ml.) were refluxed for 1 or 40 hr., the reaction was found to have proceeded to the extent of about 10% only, as indicated by semiquantitative thin-layer chromatography for the amount of released *n*-butylamine. Further evidence that *n*-butylamine was the species interacting with ninhydrin was furnished by isolation of *n*-butylamine as its hydrochloride salt from a refluxed ethanolic solution of tolbutamide, by its absence in the solution after complete color development with ninhydrin (as determined by thin-layer chromatography), and by the fact that similar absorbance curves (Fig. 1) were obtained for the final solution when either tolbutamide or *n*-butylamine was used in the assay procedure. The absorption maximum near 585 mμ was independent of the ninhydrin concentration in the reaction solution while the maximum near 410 mμ was affected by ninhydrin concentration.

Although it was observed that the colorimetric reaction went to completion on heating tolbutamide in absolute ethanol in the presence of ninhydrin (as indicated by the absence of tolbutamide by thin-layer chromatography), the color formed was not sufficiently reproducible for quantitative analysis. Only by refluxing a solution of tolbutamide in ethanol for 90 min. followed by dilution and subsequent reaction of an aliquot with ninhydrin were accuracy and reproducibility achieved.

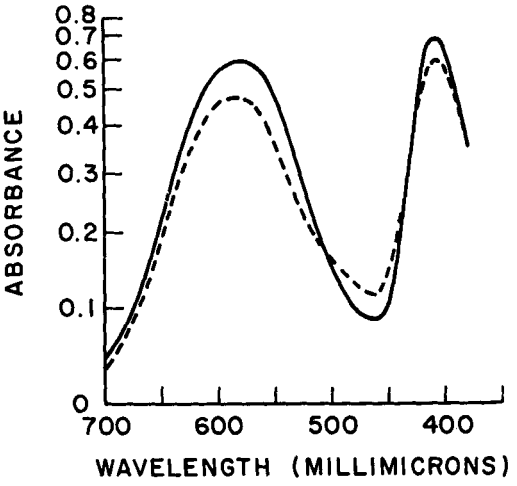

Fig. 1—Absorption spectra of final solutions. Key: ——, tolbutamide and ninhydrin after 4 hr. (concentration equivalent to 7.39×10^{-5} mmoles of tolbutamide per ml.); —, n-butylamine and ninhydrin after 3.5 hr. (concentration equivalent to 5.48×10^{-5} mmoles of n-butylamine per ml.).

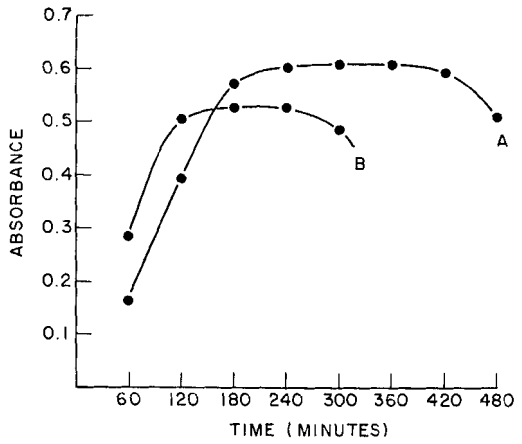

Fig. 2—Effect of time on color formation. Curve A, *tolbutamide concentration equivalent to 7.39 × 10^{-5} mmoles/ml. after dilution to final volume; Curve* B, *n-butylamine concentration equivalent to 6.08 × 10^{-5} mmoles/ml. after dilution to final volume.*

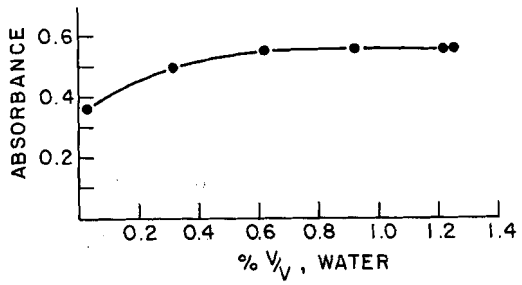

Fig. 3—Effect of water content on color formation after 4 hr. refluxing of the reaction solution (concentration equivalent to 20 mcg. of tolbutamide per ml. after dilution to final volume).

Variables examined to obtain optimum color development and reproducibility were: (*a*) Ninhydrin concentration—Satisfactory color development occurred using a molar ratio of 13.5:1 of ninhydrin to tolbutamide, although molar ratios as high as 22.8:1 did not adversely affect the reaction. On the other hand, a molar ratio of 4.6:1 was insufficient for maximum color development. (*b*) Reaction time—A minimum reflux time of 90 min. for tolbutamide in ethanol prior to color development was found to be an essential requirement to obtain reproducible results. Longer reflux times affected neither color intensity nor rate of color formation. Maximum color development in the ninhydrin reaction occurred within 4–6 hr. for tolbutamide and 3–4 hr. for *n*-butylamine alone (Fig. 2). (*c*) Water content of solvent—Variations in color intensity occurred with the use of "absolute" ethanol. The presence of 0.6 to 1.2% water (v/v) in the final reaction solution resulted in more reproducible results as well as maximum color development (Fig. 3). A higher water content did not adversely affect the amount of color formed, but did cause more nonreproducibility than a water content of 0.6–1.2%. It is essential, however, to use absolute ethanol in the initial reflux stage to achieve reproducible results. (*d*) Temperature of reaction—Reproducible color development occurred only when the reaction solution was maintained under continuous but gentle solvent reflux. This was achieved by immersing the flasks containing the reaction solutions in a stirred paraffin oil bath kept at 96–100°.

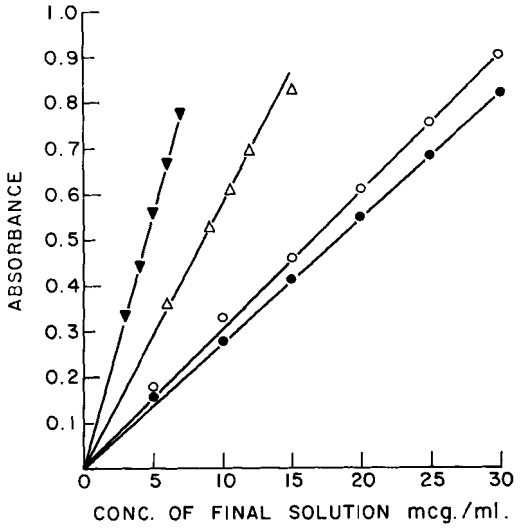

Fig. 4—Relationship between concentration and absorbance. Key: ●, *reaction solution of tolbutamide at 585 mμ;* ○, *at 410 mμ;* △, *reaction solution of* n-*butylamine in absolute ethanol;* ▼, *reaction solution of* n-*butylamine using benzene (5 ml.) with absolute ethanol (28 ml.).*

As shown in Fig. 4, a linear relationship exists between concentration and absorbance for both tolbutamide and *n*-butylamine throughout the region most practical for absorbance measurement. A final reaction solution containing the equivalent of 20 mcg./ml. of tolbutamide or 5.4 mcg./ml. of *n*-butylamine (7.38 × 10^{-5} mmole/ml.) gave an absorbance value of about 0.58. The color intensity of *n*-butylamine in ethanol–benzene was less than that obtained in ethanol alone. In all cases, the color developed was stable for at least 1 hr. with the absorbance decreasing 1–2% after 3 hr.

Results obtained by applying assay Procedure I to six products containing tolbutamide are shown in Table I. Comparison of the experimental data with

Table I—Analysis of Tolbutamide Tablets

Product	Ninhydrin Method: % of Label Claim, Av.	S.D.	No. of Detns.	USP Method: % of Label Claim, Av.	S.D.	No. of Detns.
A	100.9	1.34	10[a]	100.6	1.80	5
B	102.9	0.90	10[a]	103.3	0.70	4
C	103.0	0.13	4	103.5	...	2
D	101.4	0.65	4	101.8	...	2
E	100.4	0.57	4	100.3	...	2
F	99.1	0.32	4	100.9	...	2

[a] Performed on three separate aliquots of 20 tablets (4, 3, and 3 determinations on each aliquot, respectively).

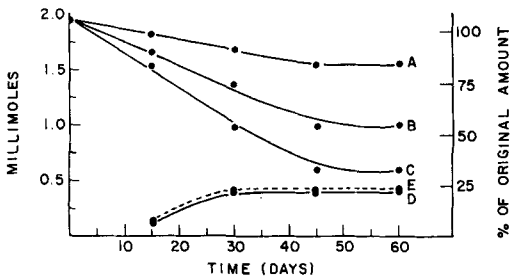

Fig. 5—Decomposition of tolbutamide tablets at 70° and 75% humidity. A, assay values by USP XVII method; B, total tolbutamide; C, free tolbutamide; D, tolbutamide (combined with n-butylamine); E, n-butylamine (combined with tolbutamide).

dilute hydrochloric acid, and subsequent color development of the remaining tolbutamide. The amount of *n*-butylamine in the aqueous phase then could be determined by extraction (after making alkaline with NaOH) with benzene, followed by ninhydrin color development. The *n*-butylamine generated during the hydrolytic decomposition of tolbutamide in the powdered tablets formed a salt with unchanged tolbutamide, and this salt could be readily extracted into water from a chloroform extract. Analysis of these aqueous solutions gave curves D and E of Fig. 5 while the chloroform-soluble tolbutamide, which had not formed a salt with *n*-butylamine, yielded curve C.

Curve A shows the assay values obtained by the USP XVII procedure. Although *p*-toluenesulfonamide was carried through this assay procedure, a

TABLE II—AMOUNT OF *p*-TOLUENESULFONAMIDE IN DECOMPOSED TABLETS

Decomposition Period, Days[a]	Assay Values for Tolbutamide[b] (%) USP Method	Ninhydrin Method	% Error[c] in USP Method	*p*-Toluenesulfonamide[d] (%) Photometric[e]	TLC
0	103.29	102.88	0.39	...	...
15	97.93	90.13	8.65	7.80	8
30	90.94	73.98	22.92	16.96	14
45	84.57	53.04	59.44	31.53	30
60	84.35	53.05	59.0	31.30	30

[a] 70° and 75% relative humidity. [b] Calculated relative to the label claim. [c] % Error = $\frac{\%\ \text{USP} - \%\ \text{Ninhydrin}}{\%\ \text{Ninhydrin}} \times 100$. [d] % by weight relative to the labelled amount of tolbutamide. [e] As determined by the difference between the USP assay values and the ninhydrin procedure assay values.

those obtained by the USP XVII method shows a good correlation between the two, with comparable precision for products A and B.

Although the direct colorimetric procedure (Procedure I) is applicable to dosage forms of tolbutamide containing degradation products such as *p*-toluenesulfonamide or impurities such as *p*-toluenesulfonylurea, it is not applicable to those containing *n*-butylamine. Therefore, for stability studies, or for the analysis of decomposed samples, the procedure was modified to allow for the separation of this interfering amine prior to color development. For decomposed products, the presence of *n*-butylamine may be readily determined by thin-layer chromatography (sensitivity of 2 mcg.) and the appropriate procedure followed.

The specificity of the modified procedure (Procedure II) was demonstrated by its application to tolbutamide tablets which had been subjected to hydrolytic conditions. Weighed aliquots of powdered tablets were stored in individual open vials in a chamber maintained at a temperature of 70° and a relative humidity of about 75% (by means of a saturated sodium chloride solution), and analyzed at various time intervals by assay Procedure II. Results are shown in Fig. 5. As determined by thin-layer chromatography, the decomposition products were *p*-toluenesulfonamide and *n*-butylamine. Dibutylurea, which has been reported as a decomposition product (8), was not detected in the present study.

Since only *n*-butylamine and tolbutamide were found to form colored products with ninhydrin under the reaction conditions, the amount of total unchanged tolbutamide (curve B of Fig. 5) could be readily determined by removal of *n*-butylamine from the chloroform extract of the tablet powder into

small decrease in assay values with length of hydrolytic exposure was observed because this compound's molecular extinction coefficient is lower than that of tolbutamide. Of interest, however, is the fact that both compounds have the same absorptivity value. Therefore, the difference in assay values obtained by the USP procedure and the ninhydrin method is equivalent to the weight of *p*-toluenesulfonamide present in the sample. Since the determination involves a difference between two relatively large numbers, each of which may have significant inherent errors, assay values must be treated with caution if the content of *p*-toluenesulfonamide in a tolbutamide sample is relatively low.

A comparison of the results obtained for tolbutamide by the USP procedure and the ninhydrin method is shown in Table II along with the percent error in results obtained by the USP method. The amount of *p*-toluenesulfonamide, deduced from the difference between these assay values, is also depicted along with the amounts ascertained by thin-layer chromatography as a check on the overall procedure.

Under the conditions described, the ninhydrin method was found to be specific for tolbutamide and did not give a color with many related compounds such as carbamates, urea, barbiturates, amidines, guanidine, and excipients commonly used in tablet formulations.

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Keyphrases

Tolbutamide dosage forms—analysis
Degradation products—tolbutamide
TLC—separation
Colorimetric analysis
Ninhydrin, *n*-butylamine—color formation

Determination of Microgram Quantities of Belladonna Alkaloids in Neomycin-Kaolin-Pectin Suspension

By ALFRED BRACEY and GEORGE SELZER

A method combining liquid–liquid extraction and column chromatography has been developed to determine microgram quantities of belladonna alkaloids in kaolin-pectin suspensions. The alkaloids are first separated from the suspension by several ethanol-ether extractions. Interfering substances, such as methylparaben and propylparaben, are eliminated by extracting the alkaloids with chloroform from a basic solution. The alkaloids are further purified by column chromatography. The chloroform extract is passed through an acid siliceous earth column, which retains the alkaloids. The column is made basic with ammonium hydroxide, and the alkaloids are eluted with chloroform. The isolated alkaloids are nitrated, then reduced to the aromatic amine, and finally diazotized and coupled with the Bratton-Marshall reagent for color formation. Recoveries of atropine sulfate added to blank kaolin-pectin suspensions ranged from 92.9 to 99.4 percent.

THE BELLADONNA ALKALOIDS, atropine sulfate, hyoscyamine sulfate, and scopolamine hydrobromide, are often formulated with kaolin and pectin in aqueous suspensions for treatment of intestinal disorders. These preparations may also contain neomycin. The suspensions combine the adsorbent and detoxifying effects of kaolin (which functions in the stomach and small intestines) and pectin (which functions in the large intestines) with the antispasmodic effect of the belladonna alkaloids to help control hypermotility and hypersecretion in the gastrointestinal tract.

Methods for assaying belladonna alkaloids in multicomponent formulations include Koch, Levine, and Zenker's method (1) for belladonna alkaloids in combination with phenobarbital and Woodson's procedure (2) for determining atropine sulfate and hyoscyamine in the presence of methylene blue. However, no procedure has been reported that involves the assay of microgram quantities of belladonna alkaloids in suspension with strong adsorbents such as kaolin.

This paper presents a method of extracting the alkaloids from kaolin–pectin suspensions (3) and determining their total concentration, using the color development procedure of Koch, Levine, and Zenker (1).

Since atropine sulfate and hyoscyamine sulfate are optical isomers, and are not differentiated by this assay procedure, they are considered analytically equivalent. The third alkaloid component of the preparation is scopolamine hydrobromide, which constitutes only 5% of the total alkaloid dose. This compound has been demonstrated to be equivalent to atropine sulfate in the assay procedure except for a small difference in the colorimetric absorptivity, which is made insignificant by its low concentration. Therefore, the belladonna alkaloids are expressed as the equivalent of atropine sulfate.

ASSAY PROCEDURE

Reagents—The following reagents are used: absolute ethanol; ethyl ether; fuming nitric acid (90%); concentrated hydrochloric acid; ammonium

Received August 4, 1967, from the Division of Antibiotics and Insulin Certification, Bureau of Science, Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Washington, DC 20204
Accepted for publication October 10, 1967.

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Keyphrases

Tolbutamide dosage forms—analysis
Degradation products—tolbutamide
TLC—separation
Colorimetric analysis
Ninhydrin, *n*-butylamine—color formation

Determination of Microgram Quantities of Belladonna Alkaloids in Neomycin-Kaolin-Pectin Suspension

By ALFRED BRACEY and GEORGE SELZER

A method combining liquid–liquid extraction and column chromatography has been developed to determine microgram quantities of belladonna alkaloids in kaolin-pectin suspensions. The alkaloids are first separated from the suspension by several ethanol-ether extractions. Interfering substances, such as methylparaben and propylparaben, are eliminated by extracting the alkaloids with chloroform from a basic solution. The alkaloids are further purified by column chromatography. The chloroform extract is passed through an acid siliceous earth column, which retains the alkaloids. The column is made basic with ammonium hydroxide, and the alkaloids are eluted with chloroform. The isolated alkaloids are nitrated, then reduced to the aromatic amine, and finally diazotized and coupled with the Bratton-Marshall reagent for color formation. Recoveries of atropine sulfate added to blank kaolin-pectin suspensions ranged from 92.9 to 99.4 percent.

THE BELLADONNA ALKALOIDS, atropine sulfate, hyoscyamine sulfate, and scopolamine hydrobromide, are often formulated with kaolin and pectin in aqueous suspensions for treatment of intestinal disorders. These preparations may also contain neomycin. The suspensions combine the adsorbent and detoxifying effects of kaolin (which functions in the stomach and small intestines) and pectin (which functions in the large intestines) with the antispasmodic effect of the belladonna alkaloids to help control hypermotility and hypersecretion in the gastrointestinal tract.

Methods for assaying belladonna alkaloids in multicomponent formulations include Koch, Levine, and Zenker's method (1) for belladonna alkaloids in combination with phenobarbital and Woodson's procedure (2) for determining atropine sulfate and hyoscyamine in the presence of methylene blue. However, no procedure has been reported that involves the assay of microgram quantities of belladonna alkaloids in suspension with strong adsorbents such as kaolin.

This paper presents a method of extracting the alkaloids from kaolin–pectin suspensions (3) and determining their total concentration, using the color development procedure of Koch, Levine, and Zenker (1).

Since atropine sulfate and hyoscyamine sulfate are optical isomers, and are not differentiated by this assay procedure, they are considered analytically equivalent. The third alkaloid component of the preparation is scopolamine hydrobromide, which constitutes only 5% of the total alkaloid dose. This compound has been demonstrated to be equivalent to atropine sulfate in the assay procedure except for a small difference in the colorimetric absorptivity, which is made insignificant by its low concentration. Therefore, the belladonna alkaloids are expressed as the equivalent of atropine sulfate.

ASSAY PROCEDURE

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hydroxide; chloroform distilled from a mixture of chloroform and 1 *N* sulfuric acid (4:1); 20% sodium hydroxide solution; 0.2 *N* sulfuric acid; 5% sodium nitrite solution; 5% ammonium sulfamate solution; 0.1% *N*-(1-naphthyl)-ethylene diamine dihydrochloride (prepared fresh before use); solid sodium hydrosulfite.

Standard Solution—Atropine sulfate, prepared by stepwise dilution as an aqueous solution containing 0.100 mg./ml. of USP atropine sulfate reference standard.

Apparatus—(*a*) An acid trap column is prepared by placing a small wad of glass wool at the juncture of the barrel and the stem of a chromatographic tube about 150 mm. long and 20 mm. in diameter. The column was packed with 5.0 Gm. of chromatographic siliceous earth[1] thoroughly mixed with 4.0 ml. of 0.2 *N* sulfuric acid, transferred to the column in several portions, and packed firmly with a tamping rod after each addition. (*b*) Filtering tube consisting of a wad of glass wool at the juncture of the barrel and the stem of a tube about 250 mm. long and 40 mm. in diameter. (*c*) Colorimeter or spectrophotometer

Extraction of Alkaloids—Place a 60-ml. aliquot of the suspension containing about 250 mcg. of alkaloids into a 500-ml. glass-stoppered conical flask. Add 100 ml. of ethanol and 4.0 ml of hydrochloric acid. Stopper tightly and place on a mechanical shaker for 30 min. Add 240 ml. of ether and shake vigorously. Add 10 ml. of ammonium hydroxide, swirl gently, and then shake well. Allow the suspended material to settle. Decant the supernatant through the filtering tube, collecting the filtrate in an 800-ml. beaker. Extract the sample a second time by adding 10.0 ml. of water and 50 ml. of ethanol to the flask. Stopper and shake well. Add 120 ml. of ether and shake. Add 2.0 ml. of ammonium hydroxide, swirl gently, and then shake vigorously. Allow the suspended material to settle and pour the supernatant through the filtering tube, combining the filtrates. Add 50 ml. of ethanol and 120 ml. of ether; then shake vigorously. Add 1.0 ml. of ammonium hydroxide, swirl gently, then shake, and allow suspended material to settle. Pour the supernatant into the filtering tube, followed by as much of the residue as possible. Finally, rinse the flask with 25 ml. of ether and pour it into the filtering tube. After the tube drains, acidify the combined filtrate with hydrochloric acid until it is acid to pH paper and evaporate it to about 50 ml. on a warm steam bath under a gentle stream of air.

Transfer the aqueous residue quantitatively to a 125-ml. separator. Adjust the solution to about pH 9 with 20% sodium hydroxide and immediately extract with one 50-ml. portion and three 30-ml. portions of chloroform. Pass each extract through the acid trap column. Add 2.0 ml. of ammonium hydroxide to the column, followed by 125 ml. of chloroform, and collect the alkaloid eluate in a 250-ml. beaker. Evaporate the eluate to about 30 ml. and transfer it quantitatively to a 50-ml. graduated glass-stoppered centrifuge tube. Add about four drops of hydrochloric acid to the tube, stopper, and shake. Evaporate to dryness by placing the tube in a hot water bath under a gentle stream of air.

[1] Celite 545, Johns-Manville Corp., New York, N. Y.

A procedural blank, consisting of 60 ml. of water, and a procedural standard, consisting of 200 mcg. of atropine sulfate in 60 ml. of water, are treated as samples throughout the assay.

Color Development—Add 1.0 ml. of fuming nitric acid to each tube containing the chloroform residue and to a reagent blank tube. Stopper each tube loosely, place on a steam bath with test tube clamps, and heat vigorously for 30 min. Remove the tubes from the steam bath and to each one add 10 ml. of water, 2.0 ml. of ammonium hydroxide, and about 50 mg. of sodium hydrosulfite. Shake each tube gently to dissolve the sodium hydrosulfite and allow to stand at room temperature for 20 min. Add 5.0 ml. of 5% sodium nitrite and 2.0 ml. of hydrochloric acid. Shake each tube gently and place in an ice-water bath for 30 min. Slowly add 10.0 ml. of 5% ammonium sulfamate in 5-ml. portions, and shake the tube gently after each addition to dispel the nitrous oxide fumes. Add 4.0 ml. of freshly prepared 0.1% solution of *N*-(1-naphthyl)-ethylenediamine dihydrochloride. Allow 30 min. for color development before diluting to the 50.0-ml. mark with water. Determine the absorbance at 550 mμ against the reagent blank. Correct the absorbance of the standard and sample for that of the procedural blank.

RESULTS

The recovery of atropine sulfate added to blank preparations containing all the ingredients except the alkaloids ranged from 92.9 to 99.4%, as shown in Table I. The amount of atropine sulfate added was approximately equivalent to the belladonna alkaloid concentration in commercial preparations. As shown in Table II, the assay results for commercially available preparations ranged from 90.3 to 106.5% of label claim. These batches represent formulations ranging in date of production from recent to those

TABLE I—RECOVERY OF ATROPINE SULFATE FROM KAOLIN-PECTIN SUSPENSIONS

Sample	Mfr.	Atropine Sulfate Added, mg./30 ml.	Atropine Sulfate Recovered, mg./30 ml.	% Recovery
1	I	0.1000	0.0985	98.5
2	I	0.1000	0.0929	92.9
3	I	0.1000	0.0940	94.0
4	II	0.1000	0.0994	99.4
5	II	0.1000	0.0971	97.5

TABLE II—DETERMINATION OF BELLADONNA ALKALOIDS IN COMMERCIAL SAMPLES[a] LABEL CLAIM 0.1296 mg./30 ml.[b]

Mfr.	Sample[c]	Amt. Found, mg./30 ml.	% Label Claim
I	1	0.1320	101.8
I	2	0.1380	106.5
I	3	0.1290	99.5
I	4	0.1170	90.3
II	1	0.1170	90.3
II	2	0.1170	90.3
II	3	0.1190	93.0
II	4	0.1190	93.0

[a] The active ingredient formulation of these products is as follows: atropine, sulfate 0.0192 mg./30 ml.; hyoscyamine sulfate, 0.1037 mg./30 ml.; scopolamine hydrobromide, 0.0065 mg./30 ml. [b] Weight of belladonna alkaloid expressed as the equivalent weight of atropine sulfate. [c] Aqueous belladonna alkaloid, neomycin, kaolin-pectin suspensions.

TABLE III—DETERMINATION OF INTERFERENCE OF BLANK FORMULATION AS ATROPINE SULFATE EQUIVALENT

Blank[a]	Mfr.	Apparent Atropine Sulfate, mg./30 ml.	% Interference[b]
1	I	0.0016	1.2
2	II	0.0054	4.1

[a] The commercial blank preparation contains all the ingredients of belladonna alkaloids neomycin suspension except the alkaloids. [b] The percent of interference is relative to the 0.1296 mg./30 ml. label claim of alkaloids in commercial preparation.

nearing expiration date. In Table III is shown the interference of a blank preparation from each manufacturer in terms of apparent atropine sulfate. By the method presented, this interference was found to be 1.2 and 4.1%.

DISCUSSION

The liquid extraction column chromatography (4) method described here permits the isolation and quantitative determination of belladonna alkaloids in kaolin-pectin suspensions. Conventional extraction methods were ineffective because of the strong adsorptive properties of kaolin and the strong tendency of this type of preparation to form an unmanageable emulsion with organic solvents. Therefore, the first phase of the assay consists of a desorption procedure (3), in which ethanol, water, and ether are used to remove all of the soluble ingredients from the suspension. After evaporation of most of the organic portion of the extract, the aqueous portion is adjusted to pH 9 with 20% sodium hydroxide and the alkaloids are extracted with chloroform, leaving behind most of the methyl and propylparaben preservatives. If tropic acid is present most of it will also be retained by the aqueous phase. Tropic acid is one of the principal degradation products of the belladonna alkaloids and the portion of the molecule responsible for the color reaction. The alkaloids are further purified by column chromatography. The column retains the alkaloids while traces of methylparaben, propylparaben, and tropic acid are washed through. Then the purified alkaloids are eluted and determined colorimetrically by the method of Koch, Levine, and Zenker (1).

An aqueous kaolin suspension containing only kaolin and atropine sulfate at concentrations equivalent to that of commercial preparations was centrifuged to remove the kaolin and a portion of the supernatant assayed according to the color development procedure. Duplicate determinations yielded recoveries of 17.2 and 17.5% (or 82.8 and 82.5% adsorption). The recovery for a similar preparation extracted by the proposed procedure and assayed colorimetrically was 99.7%. One of the problems encountered in developing this method was the relatively high absorbance of the procedural blank. This high blank was found to be caused by impurities in the chloroform. Attempts were made to purify the chloroform by filtering it through columns containing either silica gel or aluminum oxide, without satisfactory improvement. Reagent grade chloroform gave a procedural blank with an absorbance of about 28% of that from a 200 mcg. atropine sulfate standard. The column-purified chloroform gave procedural blanks with absorbances of about 10% of the same standard. However, when the chloroform was purified according to the distillation method of Koch, Levine, and Zenker (1), the procedural blank had an absorbance of about 5% of that from a 200-mcg. atropine sulfate standard.

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Keyphrases

Belladonna alkaloids, neomycin, kaolin, pectin suspension
Microanalysis—belladonna alkaloids
Liquid–liquid extraction—ethanol–ether
Column chromatography—separation
Colorimetric analysis

Determination of Phenylephrine Hydrochloride in Combination with Other Drugs

By CARL PONDER

Phenylephrine is retained on an acetic acid–NaCl–diatomaceous earth column, while other amino compounds and drug excipients are eluted with chloroform acidified with acetic acid. Phenylephrine is eluted with ethyl ether and is determined by ultraviolet spectrophotometry.

THE USP XVII general analytical procedure for organic bases is a liquid–liquid extraction in which the free bases partition into the organic phase and the salts into the aqueous phase. The phenylephrine base deviates from this solubility rule by partitioning predominately into the aqueous phase. This characteristic makes phenylephrine difficult to isolate from colors, flavors, and other extraneous substances.

Phenylephrine possesses both a secondary amino group and a hydroxyphenyl nucleus. Several analytical methods for phenylephrine based on the action of these functional groups have been published. Auerbach (1) coupled the hydroxyphenyl group with diazotized *p*-nitroaniline and determined the resulting colored compound photometrically at 495 mμ. Kelly and Auerbach (2) developed a method for phenylephrine based on reactions with both functional groups. Nitrogen bases were isolated by ion exchange and color was developed with those bases containing a phenolic group. Koshy and Mitchener (3) published a colorimetric method using 4-aminoantipyrine as the color-developing reagent. This method also depends upon the presence of the hydroxyphenyl nucleus. Each of these methods has one or more of the limitations inherent to colorimetric determinations. An obvious objection is that phenylephrine is often formulated with other ingredients that contain a common functional group.

Recently published methods are based on ultraviolet spectroscopy, which is more specific than colorimetric measurements, although the phenylephrine must first be separated from all interfering substances. Clark and Rosenberg (4) eluted nonphenolic amines from a sodium borate–diatomaceous earth column with chloroform. Phenylephrine remained on the column until acetylated. It was then removed with chloroform, saponified with alcoholic potassium hydroxide, and determined spectrophotometrically. Hyatt (5) used essentially the same procedure for the analysis of phenylephrine. Smith (6) used a sodium hydroxide–diatomaceous earth column to separate phenylephrine from other amines. After the free amines were eluted, phenylephrine was removed from the column with ethanol and determined spectrophotometrically at 290 mμ.

The present paper describes chromatographic column procedures which permit phenylephrine to be isolated from a wide range of compounds and determined by ultraviolet spectrophotometry. These procedures are based upon the "salting out" of phenolic compounds from an aqueous medium by sodium chloride and the selective elution of organic salts with different solvents from a chromatographic column. The principle evolved from the author's discovery (7) that a sodium hydroxide–ether liquid–liquid partitioning of phenolic compounds such as diethylstilbestrol can be reversed by saturating the basic solution with sodium chloride. Subsequent work has shown that phenylephrine behaves in a similar manner when the sodium hydroxide is omitted and the volume of the aqueous phase is limited.

The first procedure presented separated phenylephrine from other amino compounds. Ammonium hydroxide is added to the sample aliquot to liberate phenylephrine from the chloride, and the hydroxide is neutralized with an excess of acetic acid. Codeine, dextromethorphan, phenylpropanolamine, and most antihistamines can then be eluted from the column with chloroform or ether, but phenylephrine can be eluted only with ether.

Aspirin, magnesium hydroxide, and phenylephrine hydrochloride combinations require a different separation method. Traces of salicylic acid in aspirin interfere with the extraction of phenylephrine. Experimental recoveries of phenylephrine in the presence of salicylic acid averaged only 50% by the first procedure. This difficulty can be avoided by extracting aspirin and salicylic acid from a hydrochloric acid solution with ether before the ammonium hydroxide–acetic acid neutralization phase of the procedure.

Received August 11, 1967, from the U. S. Department of Health, Education, and Welfare, Food and Drug Administration, Atlanta District Laboratory, Atlanta, GA 30309
Accepted for publication October 20, 1967.

TABLE I—RECOVERIES OF MIXED STANDARDS

Mixture	mg. Added	% Recovery
Phenylephrine hydrochloride	1	97.8
Chlorpheniramine maleate	1	100.0
Dextromethorphan hydrobromide	5	100.0
Phenylephrine hydrochloride	1	98.0
Pheniramine maleate	2	97.8
Pyrilamine maleate	2	98.2
Phenylephrine hydrochloride	1	98.3
Doxylamine succinate	3	100.0
Phenylephrine hydrochloride	1	99.0
Tripelennamine citrate	2	100.0
Phenylephrine hydrochloride	1	97.6
Phenyltoloxamine dihydrogen citrate	2	98.9
Dextromethorphan hydrobromide	2	99.2
Phenylephrine hydrochloride	1	98.7
Phenylpropanolamine hydrochloride	2	100.0
Phenylephrine hydrochloride	1	98.6
Aspirin	81	97.5
Magnesium hydroxide	31	...
Phenylephrine hydrochloride	1	97.6
Aspirin	125	...
Chlorpheniramine maleate	1	100.0

The mixed antihistamine standards in Table I were assayed according to the methods of Levine (8, 9).

EXPERIMENTAL

Apparatus—A recording spectrophotometer with matched 1-cm. glass-stoppered silica cells was used for ultraviolet assays.

Chromatographic Column—Pack a pledget of fine glass wool in the base of a 25 × 300-mm. column. Overlay this support with a mixture of 1 Gm. of diatomaceous earth[1] and 2 Gm. of 100-mesh sodium chloride.

Tamping Rod—Use a disk of stainless steel, aluminum, or glass, with a diameter slightly less than that of the column, and with a handle 15 in. long.

Reagents—*Sodium Chloride*—Finely powdered to pass 100-mesh sieve.

Standard Phenylephrine Hydrochloride—Accurately weigh a suitable quantity of USP phenylephrine hydrochloride reference standard and dissolve in 0.1 *N* hydrohloric acid. Dilute quantitatively stepwise with 0.1 *N* hydrochloric acid to obtain a solution containing 40 mcg./ml.

Procedure—*Tablets Containing Phenylephrine Hydrochloride, Antihistamines, and Dextromethorphan Hydrobromide*—Weigh and finely powder 20 tablets. Weigh accurately a portion of the powder equivalent to 1 mg. of phenylephrine hydrochloride and transfer to a 250-ml. beaker. Wet the powder with 1 ml. of water. Add one drop of concentrated ammonium hydroxide, mix thoroughly, and then neutralize with three drops of glacial acetic acid. Add 3 Gm. of powdered sodium chloride and mix until the salt-liquid mixture forms a thick paste. Let sit for approximately 10 min. and then knead 3 Gm. of diatomaceous earth into the mixture. When all ingredients are uniformly dispersed, transfer quantitatively to the column containing the mixture of 1 Gm. of diatomaceous earth and 2 Gm. of 100-mesh sodium chloride. Gently tap the column, then tamp with the rod, using firm pressure to compress into a uniform mass. Scrub the beaker with 1 Gm. of dry diatomaceous earth, follow with a pledget of glass wool, and tamp both into the column. Measure 150 ml. of redistilled, water-saturated chloroform containing 3 drops of acetic acid into the beaker. Pass this solvent mixture through the column to elute chloroform-soluble acetate salts. Collect the eluate in a beaker and save for antihistamine and dextromethorphan assay. Elute phenylephrine from the column with 300 ml. of redistilled, water-saturated ether ino a 500-ml. separator. When elution is complete, immediately extract the phenylephrine from the ether with 10-, 10-, and 5-ml. portions of 0.1 *N* hydrochloric acid. Combine the extracts in a 25-ml. volumetric flask. Make to volume with acid and mix. With 0.1 *N* hydrochloric acid saturated with ether in the blank cell, scan the solution from 350 to 225 mμ. Determine the absorbance at 273 mμ. Calculate the quantity in milligrams of phenylephrine hydrochloride in the portion of the tablet taken by the formula $25C(A_U/A_S)$, in which C is the exact concentration of the reference solution in mg./ml.; A_U is the absorbance of the sample solution; and A_S is the absorbance of the reference standard solution.

Syrups Containing Phenylephrine Hydrochloride, Antihistamines, Codeine, Dextromethorphan Hydrobromide, and Phenylpropanolamine Hydrochloride—Measure a quantity of syrup equivalent to 1 mg. of phenylephrine hydrochloride into a 250-ml. beaker. Evaporate to approximately 1 ml. on a steam bath at 80–100° under a gentle air current. Add 1 drop of concentrated ammonium hydroxide, mix thoroughly, and then neutralize with 3 drops of glacial acetic acid. Determine phenylephrine hydrochloride by the method for tablets above, beginning with "Add 3 Gm. of powdered sodium chloride...."

Tablets Containing Phenylephrine Hydrochloride, Aspirin, and Antihistamines, or Phenylephrine Hydrochloride, Aspirin, and Magnesium Hydroxide—Weigh accurately a portion of the finely powdered tablets equivalent to about 2.5 mg. of phenylephrine hydrochloride and transfer to a 60-ml. separator containing 10 ml. of 1.0 *N* hydrochloric acid. Shake vigorously for 5 min. Add 25 ml. of ether. Shake 1 min. and drain the acid phase into a second separator containing 25 ml. of ether. Shake and repeat this procedure with a third and fourth 25-ml. ether extraction in a 60-ml. separator. Filter the acid phase through a small, loose pledget of glass wool into a 25-ml. volumetric flask. Extract each of the four ether extractions successively with 10- and 5-ml. portions of 1 *N* hydrochloric acid and combine the extracts in a 25-ml. volumetric flask. Dilute to volume with acid, mix, and pipet 10 ml. of the acid solution into a 250-ml. beaker. Immediately evaporate to dryness on a steam bath at 60–70° under a gentle air current. When acid odor is no longer perceptible, add 1 ml. of water and 1 drop of concentrated ammonium hydroxide. Mix and add 3 drops of acetic acid. Proceed as directed in the assay for phenylephrine hydro-

[1] Celite 545, Johns Manville Corp., New York, N. Y.

TABLE II—ANALYSIS OF COMMERCIAL PRODUCTS

Dosage Form	Declared Composition	mg.	Determined	% of Labeled Amount Found
		Per Tablet		
Tablets, timed release	Phenylephrine hydrochloride	10	Phenylephrine	96.9
	Four antihistamines	70		
		Per Tablet		
Tablets, multilayer	Phenylephrine hydrochloride	10	Phenylephrine	98.8
	Aspirin			
	Antihistamine mixture			
		Per Tablet		
Tablets, multilayer	Phenylephrine hydrochloride	1.25	Phenylephrine	96.7
	Aspirin	81		
	Magnesium hydroxide	31		
	Flavor and color material			
		Per 5 ml.		
Syrup	Phenylephrine hydrochloride	10	Phenylephrine	100.0
	Codeine phosphate	10	Codeine	97.2
	Pheniramine maleate	12.5	Pheniramine	92.8
	Plus aromatics			
		Per 5 ml.		
Syrup	Phenylephrine hydrochloride	5	Phenylephrine	97.7
	Chlorpheniramine maleate	2	Chlorpheniramine	96.7
	Dextromethorphan hydrobromide	10	Dextromethorphan	94.0
	Plus aromatics			
		Per 5 ml.		
Syrup	Phenylephrine hydrochloride	6	Phenylephrine	102.0
	Dextromethorphan hydrobromide	15		
	Pyrilamine maleate	4		
	Phenindamine tartrate	3		
	Chlorpheniramine maleate	1.0		
	Plus aromatics			
		%		
Nasal spray	Phenylephrine hydrochloride	0.25	Phenylephrine	101
	Phenylpropanolamine hydrochloride	0.30		
	Chlorobutanol	0.15		
	Sodium bisulfite	0.03		

chloride and antihistamines beginning with "Add 3 Gm. of powdered sodium chloride..." The formula for the calculation of phenylephrine hydrochloride gives the quantity in the 10-ml. aliquot taken for the assay.

RESULTS AND DISCUSSION

The procedure for amino compounds has been applied to the determination of phenylephrine in tablets and syrup preparations, including those containing antihistamine mixtures, codeine, dextromethorphan hydrobromide, and aromatics. The phenylephrine–aspirin procedure has been applied to commercial and simulated tablet preparations which also contained either antihistamine, magnesium hydroxide, bioflavonoids, or a mixture of these.

Table I shows recoveries of phenylephrine hydrochloride and of the added components in simulated preparations. The added components include examples of each antihistamine structural type and those specific antihistamines most often found in commercial preparations.

The analyses of some commercial products are given in Table II. These analyses are in good agreement with their labeled declarations. The precision of these analyses, the quantitative recoveries obtained with standard simulated samples (Table I), and the purity of the isolated material, based on the ultraviolet spectra, establish the validity of the procedures reported.

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Keyphrases

Phenylephrine dosage forms—analysis
Analysis—phenylephrine in drug mixtures
Column chromatography—separation
UV spectrophotometry—analysis

Gas Chromatographic Assay for α-Tocopheryl Acetate in Multivitamin Products

By P. B. BOWMAN and W. E. WEST

A gas chromatographic procedure for α-tocopheryl acetate in soft elastic capsules, liquid formulations, and compressed tablets is described. The procedure compares favorably with the NF and USP dipyridyl procedures with respect to speed (10 *versus* 5 per day per analyst), precision (1.2 percent coefficient of variation *versus* 1.5 percent), and specificity. Unlike the dipyridyl procedures, the α-tocopheryl acetate is separated completely from other tocopherols.

THE NEED for a more precise, specific, and rapid assay for α-tocopheryl acetate (vitamin E) led to the development of a gas chromatographic procedure that met these requirements. The procedure is applicable to soft elastic capsules, liquid formulations, and coated compressed tablets.

Considerable work has been done on the gas chromatography of the tocopherols (3–7), but it has been concerned primarily with the separation of the various tocopherols and their determination in natural products. In 1966, at the 80th annual meeting of the Association of Official Analytical Chemists, Pillsbury *et al.* described a gas chromatographic procedure for vitamin E in pharmaceuticals (8, 11). Since the chromatography has been studied in the above references, this paper will be concerned with the procedural aspects of the assay.

EXPERIMENTAL

Instrument—An F & M model 402 gas chromatograph[1] equipped with a flame-ionization detector and U-shaped glass columns, 2 M. long × 3 mm. i.d., was used for this work. The columns were packed with 3% SE-30 on 100/120 mesh Gas Chrom Q.[2] The carrier gas was helium, at a flow rate of approximately 50 ml./min. and hydrogen and air flows were adjusted for maximum flame response. The column oven was operated isothermally at 235°, the flash heater at 275°, and the detector at 300°. The overall sensitivity of the instrument was 64 × 10^{-11} amp. full scale. The recorder was equipped with a model 227 Disc chart integrator[3] that was used for all peak area measurements.

Reagents and Chemicals—Chloroform, USP, anhydrous sodium sulfate, A.R., anhydrous diethyl-ether (no further purification required), 3-(*p*-methoxyphenyl) - 2,4,5 - triphenylcyclopentadieneone,[4] *d*-α-tocopheryl acetate, NF type 6-100,[5] and dry *d*-α-tocopheryl acetate types 667 and 333-S.[6]

The internal standard solution was 3-(*p*-methoxyphenyl)-2,4,5-triphenylcyclopentadieneone, approximately 1 mg./ml. in chloroform. This material was selected on the basis of its retention time (1.6 relative to α-tocopheryl acetate) and thermal stability.

The reference solution was prepared by dilution of a stock solution of the working standard of *d*-α-tocopheryl acetate in chloroform to a final concentration of approximately 1 mg./ml. An aliquot of this solution was evaporated to dryness then the residue dissolved in an equal volume of internal standard solution.

The surfactant solution used in the assay of coated compressed tablets was prepared according to the procedure of Ames and Tinkler (9).

Sample Preparation—Soft elastic capsules were prepared for assay by processing for 5 sec. with 50.0 ml. of internal standard solution at high speed in a blender. (The blender was equipped with a screw-cap jar and Teflon gaskets.) Enough capsules were processed to give a final concentration of α-tocopheryl acetate of approximately 1 mg./ml. The sample for chromatography was filtered by withdrawal through a fluted filter paper.

Liquid formulations were assayed by mixing a volume of the formulation containing 10 mg. of α-tocopheryl acetate with 5 ml. of surfactant solution in a 30-ml. glass-stoppered centrifuge tube, and shaking for 10 min. with 10.0 ml. of internal standard solution. The mixture was centrifuged if necessary, to break any emulsions formed. A portion of the chloroform layer was withdrawn for chromatography.

The assay of coated compressed tablets required the following preparation prior to chromatography: A number of tablets (color coating first removed by water washing) equivalent to approximately 50 mg. of α-tocopheryl acetate was introduced into a 125-ml. glass-stoppered conical flask with five 5-mm. glass beads and 25 ml. of surfactant solution. The mixture was shaken on a mechanical shaker until the tablets were completely disintegrated (1–3 hr.). If desired, the samples can be allowed to soak overnight before shaking. Ten grams (1 teaspoon) of granular anhydrous sodium sulfate and exactly 50.0 ml. of internal standard solution were added to the flask. The flask was stoppered tightly and shaken again for 10 min. on the mechanical shaker. A portion of the liquid was transferred to a 30-ml. glass-stoppered centrifuge tube, centrifuged, and a portion of the chloroform

Received September 11, 1967, from the Control Research and Development Department, The Upjohn Co., Kalamazoo, MI 49001

Accepted for publication October 24, 1967.

The authors wish to thank Dr. M. F. Grostic for obtaining the mass spectra, J. S. Flint for running the NF and USP assays, and Dr. R. W. Lehman for his helpful suggestions.

[1] F & M Scientific Corporation, Avondale, Pa.

[2] Applied Science Labs, State College, Pa.

[3] Disc Instruments, Inc., Santa Ana, Calif.

[4] Aldrich Chemical Co., No. M-2450, Milwaukee, Wis.

[5] Working standard, assayed according to NF XII, ceric sulfate titration, used as 983 mg./Gm. Distillation Products Industries, Rochester, N. Y.

[6] Type 667 potency = 667 IU/Gm. and 333-S = 333 IU/Gm., Distillation Products Industries, Rochester, N. Y., used in coated compressed tablets.

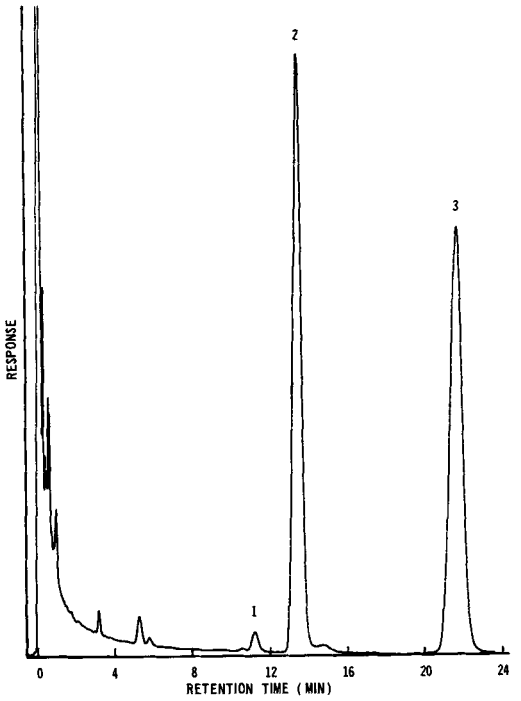

Fig. 1—Chromatogram of α-tocopheryl acetate from a lot of coated compressed tablets. Key: 1, impurity in internal standard; 2, α-tocopheryl acetate; 3, internal standard. The slopes of the integrator trace before and after the α-tocopheryl acetate were determined at 12.5 and 15.5 min., respectively. The peak width was taken at 1.6 min.

layer withdrawn and filtered through a fluted filter paper. The filtrate was then used for chromatography.

The volume of solution injected was adjusted to give nearly full-scale response for the α-tocopheryl acetate peak; this required between 1–2 mcg. of α-tocopheryl acetate. It was found necessary to saturate the column with one or two injections of reference solution at the beginning of each day's run. At least two additional injections of reference solution were made before any samples were run. Reference solution was then injected at intervals, usually after every third sample.

Calculations—The potency of α-tocopheryl acetate in the product was calculated according to the following formula:

$$\text{potency} = \text{Ru} \cdot \frac{\text{Cr}}{\text{Rr}} \cdot \text{X}$$

where

Ru = area of α-tocopheryl acetate peak in sample/area of internal standard peak
Rr = area of α-tocopheryl acetate peak in reference/area of internal standard peak
Cr = concentration of α-tocopheryl acetate in mg./ml in the reference solution
X = dilution factor

RESULTS AND DISCUSSION

Chromatography—An actual chromatogram of a sample of a production lot of coated compressed tablets is shown in Fig. 1. The small peak after the α-tocopheryl acetate peak was not identified. This peak was not considered in the integration; since it was present in the working standard as well as the products. In some cases a base line correction was required, particularly in the soft capsule formulations in which the injected oil bled from the column at an appreciable rate. This correction was done with the drift corrector No. 1290 supplied with the integrator, according to the manufacturer's directions.

Earlier work on the tocopherols (3–7) suggested a number of column packings that were applicable to these compounds and several of these were examined. However, the desired specificity was obtained only with SE-30 on 100/120 mesh Gas Chrom Q. It was found in our laboratories that

TABLE I—RECOVERY OF α-TOCOPHERYL ACETATE FROM VARIOUS MULTIVITAMIN PREPARATIONS

No.	Added, mg.	Found, mg.	% Recovery
	Soft Elastic Capsule[a]		
1	37.35	37.23	99.7
2	37.35	37.42	100.1
3	37.35	37.38	100.1
4	37.35	37.80	101.2
5	37.35	38.22	102.3
	Mean Recovery		100.7
	S.D.		1.1
	Liquid Formulation[b]		
1	8.76	8.73	99.6
2	8.76	8.97	102.4
3	8.76	8.70	99.3
4	8.76	9.00	102.7
5	8.76	8.76	102.3
6	8.76	8.70	99.3
	Mean Recovery		100.9
	S.D.		1.6
	Coated Compressed Tablets		
Formulation 1[c]			
1	55.50	55.76	100.5
2	55.63	55.39	99.6
3	56.53	56.83	100.5
4	56.15	55.23	98.4
5	55.22	55.92	101.3
6	56.20	57.36	102.1
	Mean Recovery		100.4
	S.D.		1.1
Formulation 2[d]			
1	38.78	38.90	100.3
2	37.30	37.41	100.3
3	39.29	39.34	100.1
	Mean Recovery		100.2
	S.D.		0.1
Formulation 3[c]			
1	38.72	37.98	99.6
2	37.51	38.56	102.7
3	38.28	38.33	100.1
	Mean Recovery		100.8
	S.D.		1.3
Formulation 4[c]			
1	112.15	112.90	100.7
2	112.24	111.81	99.6
3	119.50	122.50	102.5
	Mean Recovery		100.9
	S.D.		1.5

[a] *d*-α-Tocopheryl acetate NF; [b] *dl*-α-tocopheryl acetate; [c] *d*-α-tocopheryl acetate type 667; [d] *d*-α-tocopheryl acetate type 333-S.

TABLE II—COMPARISON OF THE GAS CHROMATOGRAPHIC AND NF XII ASSAYS FOR α-TOCOPHERYL ACETATE IN MULTIVITAMIN PREPARATIONS

Sample	Formulation	Gas Chromatographic	NF XII	Theory
1	SEC[a]	7.47 mg./cap.	7.43 mg./cap.	7.65 mg./cap.
2	SEC	7.58	7.32	
3	SEC	7.46	7.43	
4	SEC	7.63	7.32	
5	SEC	7.46	7.35	
6	SEC	7.48	7.30	
7	SEC	7.53	7.30	
8	Liquid[b]	8.65 mg./ml.	8.58 mg./ml.	10.10 mg./ml.
9	Liquid	9.06	8.61	
10	Liquid	9.39	8.85	
11	CCT[c]	10.97 mg./tab.	10.59 mg./tab.	11.03 mg./tab.
12	CCT	10.94	10.88	
13	CCT	10.70	10.73	
14	CCT	11.30	10.51	
15	CCT	11.39	10.88	
16	CCT	11.43	11.03	
17	CCT	11.12	11.01	
18	CCT	7.93	7.04	7.35
19	CCT	7.73	6.99	7.35
20	CCT	23.70	21.47	22.00

[a] Soft elastic capsule. [b] Liquid formulation. [c] Coated compressed tablet.

there was considerable variation in the performance of different lots of this packing, even among the pretested lots; hence some selectivity may be required to find a satisfactory lot of the material. The best indications of a good lot of support were found to be column efficiency (> 700 theoretical plates per foot for α-tocopheryl acetate)[7] and retention time (12–14 min. at the prescribed temperature and flow rate).

Mass spectral and infrared examination of α-tocopheryl acetate eluted from the column indicated that no decomposition occurred during chromatography. The infrared spectrum of the chromatographed material was compared with that of a sample of the same material that had not been chromatographed. No significant differences were visible. The mass spectrum of the chromatographed material showed the molecular ion at *m/e* 472 and major peaks at *m/e* 430, 207, and 165. These peaks are consistent with the structure of the molecule.

Although no concentration limits were established, the chromatographic response was linear over the range from 0.1 to 4 mcg. of α-tocopheryl acetate which bracketed the concentration ranges of the assay procedure.

Recovery—Recovery of α-tocopheryl acetate was determined to be quantitative (> 99%) for each of the three formulations. For soft elastic capsules, recovery was determined by replicate assays of the same fomulation without α-tocopheryl acetate but using chloroform that contained both internal standard and the theoretical quantity of α-tocopheryl acetate. These results are listed in Table I. Although this was not the ideal procedure for a recovery study, it proved to be a practical approach. For the liquid formulation, a similar procedure was followed; the organic phase again contained the internal standard and the α-tocopheryl acetate. The results are shown in Table I. In this case, it was impossible to dissolve the α-tocopheryl acetate in the blank formulation.

Recovery from coated compressed tablets was determined by assaying tablets without α-tocopheryl acetate, but with the label amount of the appropriate dry form weighed into the flask before addition of the surfactant solution. These results are in Table I. Recovery of the α-tocopheryl acetate from the dry material alone was also determined and found to be quantitative.

Precision—Assay precision was determined for each type of formulation by replicate assay of a single lot of the material. In each case, the coefficient of variation (percent standard deviation) was less than 1.2%. According to Lehman (10), the interlab coefficient of variation was 1.52% in a collaborative study of the dipyridyl method (1, 2). The latter procedure suffers from the lack of a recognized standard for α-tocopherol, thus requiring the use of a somewhat arbitrary absorptivity and careful timing of the reaction before measuring the absorbance.

Comparison of the Gas Chromatographic and Dipyridyl Procedures—The results of the gas chromatographic procedure on several lots of each type of formulation are listed in Table II. For comparison, the results obtained by the NF procedure and the label potency are listed.

It should be noted that the gas chromatographic results are consistently higher than those of the NF procedure. The USP procedure gave results similar to those obtained by gas chromatography. The NF method requires a column chromatographic separation to eliminate the interference of vitamin A, whereas this is accomplished by a hydrogenation step in the USP procedure. The low results obtained by the NF procedure can probably be attributed to loss of α-tocopherol during the column chromatography step.

An experienced analyst in our laboratory was able to complete three assays for α-tocopheryl acetate in a working day using the NF XII procedure. Using the USP hydrogenation, five assays could be completed. The gas chromatographic procedure permits the assay of at least 10 samples a day. An analyst operating two chromatographs could double this daily output and still have sufficient time to prepare samples and perform the calculation of results.

[7] Theoretical plates per foot $= \left(\frac{\text{retention time}}{\text{width at half height}}\right)^2 \frac{5.5}{\text{column length}}$

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Keyphrases

Vitamin (multiple) dosage forms
α-Tocopheryl acetate—analysis
GLC—analysis
Mass spectrometry—identity

Determination of Terpin Hydrate by Gas-Liquid Chromatography

By ERNEST J. KUBIAK

A gas chromatographic procedure has been developed for the quantitative determination of terpin hydrate. The procedure may be applied to any of the NF dosage forms containing this compound. The terpin hydrate is extracted with chloroform from a saturated sodium chloride solution and chromatographed as intact terpin on a hydrogenated castor oil column. Degradation products of terpin hydrate, other terpenes, and formulation excipients do not interfere. The method is specific, rapid, and accurate. A coefficient of variation of less than 1 percent was determined from replicate analyses.

METHODS FOR THE ANALYSIS of terpin hydrate, which have been proposed and described in the literature, include precipitation with mercury salts (1, 2), esterification with 3,5-dinitrobenzoyl chloride (3), and spectrophotometric determinations (4, 5). These methods lack specificity. They do not distinguish terpin hydrate from its decomposition products, and are not satisfactory for many formulations.

A method for the determination of terpin hydrate is presented in "Official Methods of Analysis"(6). In this procedure terpin hydrate is converted by acid dehydration to a mixture of terpenes which is reacted with molybdo-phosphotungstic acid to produce a blue reduction product which is measured spectrophotometrically. Any mixture of terpenes, treated similarly, will result in comparable color formulations.

The gas chromatographic (GLC) procedure presented in this paper is specific for the intact terpin molecule. Degradation products, other terpenes, and formulation excipients do not interfere with the quantitation of terpin hydrate by the procedure described.

Received September 13, 1967, from the Control Research and Development Department, The Upjohn Co., Kalamazoo, MI 49001
Accepted for publication October 19, 1967.
After this manuscript was submitted, a paper by L. Kurlansik, C. Damon, and E. F. Salim was published on the gas chromatographic determination of terpin hydrate (7).

METHOD

Reagents—(*a*) *Internal Standard Solution*—Prepare a chloroform solution containing 5.5 mg. of 3-tert-butylphenol per ml. of chloroform. (*b*) *Reference Preparation*—Dry terpin hydrate NF ($C_{10}H_{20}O_2 \cdot H_2O$) at 60° in vacuum at 10 mm. mercury for 3 hr. Determine any residual moisture in the terpin hydrate reference material by Karl Fischer, and use on the completely anhydrous basis for preparation of the reference solution. Transfer an accurately weighed portion of the anhydrous terpin ($C_{10}H_{20}O_2$), approximately 158 mg., into a 200-ml. volumetric flask. Add exactly 25.0 ml. of the internal standard solution and dilute to volume with chloroform.

Sample Preparation—Transfer an accurately measured portion of the formulation containing approximately 170 mg. of terpin hydrate NF ($C_{10}H_{20}O_2 \cdot H_2O$) into a 250-ml. separator. Add about 80 ml. of saturated sodium chloride solution and mix. Extract the terpin hydrate with two successive 50-ml. portions of chloroform. Collect the chloroform extracts in a 200-ml. volumetric flask, add exactly 25.0 ml. of the internal standard preparation, and dilute to volume with chloroform.

Chromatography—Chromatograph aliquots of the sample and reference preparation. Under typical circumstances the following instrument conditions were found to be satisfactory.

Chromatograph—F & M 402 with flame ionization detector. Column—Glass 3 mm. i.d. × 1.2 meters, 5% hydrogenated castor oil[1] on 80–100 mesh

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Diatoport-S, operated isothermally at 140°. Sample volume—8 μl. Carrier gas—Helium at about 60 ml./min. Attenuation—10 × 64.

Calculations—Calculate the quantity, in mg., of $C_{10}H_{20}O_2 \cdot H_2O$ in each ml. of the formulation taken by the formula:

$$\frac{R_1}{R_2} \times \frac{C}{D} \times F$$

in which,

R_1 = Sample terpin peak area / Internal standard peak area

R_2 = Reference terpin peak area / Internal standard peak area

C = Weight in milligrams of anhydrous terpin ($C_{10}H_{20}O_2$) in the reference preparation

D = The milliliters of the formulation in the sample preparation

F = 1.1045 = the factor for converting anhydrous terpin ($C_{10}H_{20}O_2$) to terpin hydrate NF ($C_{10}H_{20}O_2 \cdot H_2O$)

RESULTS AND DISCUSSION

In the proposed procedure, time-consuming distillation and the conversion of terpin to a mixture of terpenes by acid dehydration are avoided. Terpin hydrate is quantitatively extracted with chloroform from a saturated salt solution; a partition coefficient of $K > 100$ was determined for this system.

Based on replicate determinations, the procedure described gave an average recovery of 100.8% with a coefficient of variation of 0.75% (Table I). Three NF dosage forms manufactured by various pharmaceutical companies, were assayed for terpin hydrate (Table II). Except for one lot which showed evidence of decomposition, the results were in good agreement with the label requirements.

The procedure is specific for anhydrous terpin. The infrared spectra of a GLC fraction showed terpin hydrate chromatographed with a loss of water but without rearrangement or decomposition.

The accuracy of the procedure is dependent on the careful preparation of the reference anhydrous terpin. Terpin hydrate sublimes at 60° in vacuum with the possibility of incomplete dehydration. Therefore, the dried terpin reference material should be corrected for residual moisture, as determined by the Karl Fischer procedure, and used on the anhydrous basis for preparation of the reference solution.

The ratio of the areas of terpin to the internal standard, *3-tert*-butylphenol, *versus* the amount of terpin hydrate in the sample is linear for injected quantities between 0–75 mcg. of terpin hydrate.

Anticipated acid dehydration products and closely

Table I—Replicate Analyses of Terpin Hydrate Elixir NF

Terpin Hydrate, mg./ml.		
17.27	Theory	= 17.00
17.10	$\bar{x}$	= 17.14
17.20	σ	= 0.13
16.89	Coefficient of variation	= 0.75
17.16		%
17.15		
17.19		

Table II—Determination of Terpin Hydrate in NF Dosage Forms

Type of Formulation	Lot	Terpin Hydrate, mg/ml. Found	% Label[a] Found
Terpin hydrate elixir NF	1	16.93	99.6
	2	16.95	99.7
	3	17.27	101.6
	4	17.10	100.6
Terpin hydrate and codeine elixir NF	5	16.99	99.9
	6	17.11	100.6
	7	17.14	100.8
	8	16.88	99.3
	9	17.27	101.6
Terpin hydrate and dextromethorphan hydrobromide elixir NF	10	17.13	100.8
	11	17.07	100.4
	12	17.24	101.4
	13	17.07	100.4
	14	15.87[b]	93.4
	14	15.97[b]	93.9

[a] All dosage forms labeled 17.00 mg./ml. [b] Degradation products noted in chromatogram.

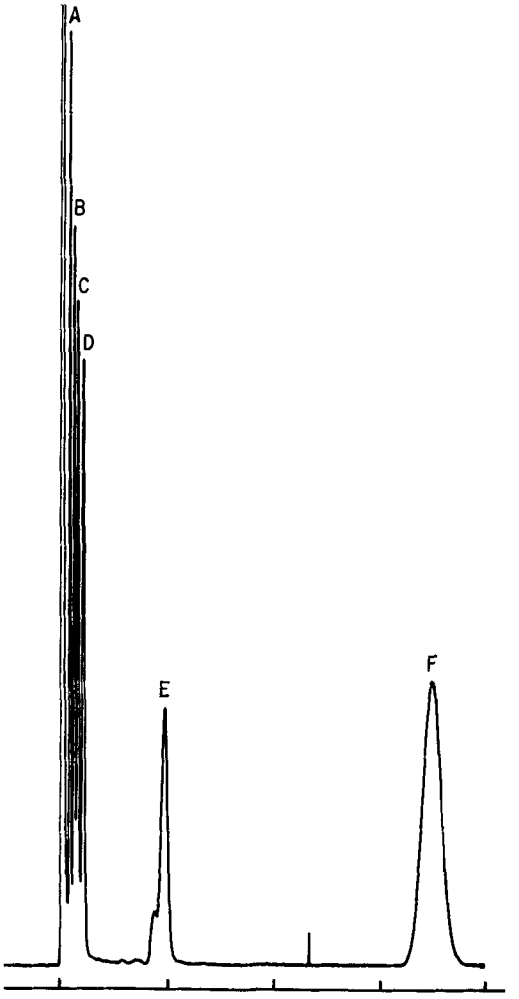

Fig. 1—Chromatogram of a terpene mixture. A = α-Pinene, B = β-Pinene, C = α-Terpinene and Limonene, D = γ-Terpinene, E = α-Terpineol, and F = Terpin.

TABLE III—RELATIVE RETENTION TIMES OF SOME TERPENES

		R.R.T.[a]
A	α-Pinene	0.11
B	β-Pinene	0.14
C	α-Terpinene and Limonene	0.18 0.19
D	γ-Terpinene	0.23
E	α-Terpineol	1.00
F	Terpin	3.89

[a] Retention time relative to terpineol.

associated compounds are completely separated (Fig. 1), having retention times much less than that of terpin (Table III). Figure 2 is a chromatogram showing the separation of terpin and the internal standard, 3-tert-butylphenol.

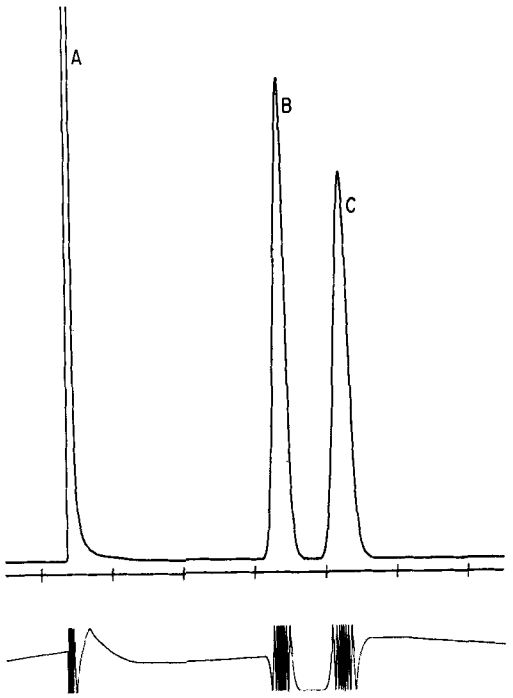

Fig. 2—Typical chromatogram. A = Solvent, B = Terpin, C = 3-tert-Butylphenol.

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Keyphrases

Terpin hydrate formulations—analysis
GLC—analysis
IR spectrophotometry—structure
Terpenes—relative retention times

Observations Concerning a Gas Chromatography Study of Resorcinol Monoacetate

By LEON KURLANSIK and EDWARD F. SALIM*

Initial investigations to develop a gas chromatographic assay for resorcinol monoacetate indicated that commercial material was not a single substance but a three-component mixture. A resorcinol monoacetate standard has been prepared and studies conducted at elevated temperatures to evaluate the conversion of resorcinol monoacetate to a mixture which includes resorcinol and resorcinol diacetate. Thermodynamic values have been calculated from experimental data. Characterization of the composition of commercial resorcinol monoacetate has been demonstrated by gas chromatography.

THE SYNTHESIS of resorcinol monoacetate (RMA) was first reported in 1899 (1). In 1931 Chattaway (2) prepared the compound by reacting resorcinol and acetic anhydride in sodium hydroxide solution and published physical constants for the material. Israelstam and Simpson (3) produced RMA by a modification of Chattaway's method and by the reaction of

Received July 7, 1967, from the Drug Standards Laboratory, American Pharmaceutical Association Foundation, Washington, DC 20037
Accepted for publication October 19, 1967.
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TABLE III—RELATIVE RETENTION TIMES OF SOME TERPENES

		R.R.T.[a]
A	α-Pinene	0.11
B	β-Pinene	0.14
C	α-Terpinene and Limonene	0.18 0.19
D	γ-Terpinene	0.23
E	α-Terpineol	1.00
F	Terpin	3.89

[a] Retention time relative to terpineol.

associated compounds are completely separated (Fig. 1), having retention times much less than that of terpin (Table III). Figure 2 is a chromatogram showing the separation of terpin and the internal standard, 3-tert-butylphenol.

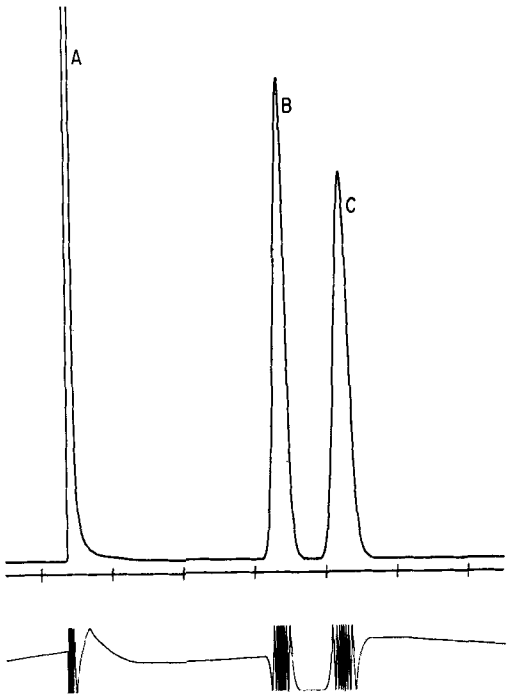

Fig. 2—Typical chromatogram. A = Solvent, B = Terpin, C = 3-tert-Butylphenol.

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Keyphrases

Terpin hydrate formulations—analysis
GLC—analysis
IR spectrophotometry—structure
Terpenes—relative retention times

Observations Concerning a Gas Chromatography Study of Resorcinol Monoacetate

By LEON KURLANSIK and EDWARD F. SALIM*

Initial investigations to develop a gas chromatographic assay for resorcinol monoacetate indicated that commercial material was not a single substance but a three-component mixture. A resorcinol monoacetate standard has been prepared and studies conducted at elevated temperatures to evaluate the conversion of resorcinol monoacetate to a mixture which includes resorcinol and resorcinol diacetate. Thermodynamic values have been calculated from experimental data. Characterization of the composition of commercial resorcinol monoacetate has been demonstrated by gas chromatography.

THE SYNTHESIS of resorcinol monoacetate (RMA) was first reported in 1899 (1). In 1931 Chattaway (2) prepared the compound by reacting resorcinol and acetic anhydride in sodium hydroxide solution and published physical constants for the material. Israelstam and Simpson (3) produced RMA by a modification of Chattaway's method and by the reaction of

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resorcinol diacetate (RDA) and disodium resorcinate. Recently, investigators have synthesized the monoacetate by acetylation of resorcinol with ketene (4, 5).

A survey of the literature indicated an almost total absence of methods of analysis for RMA. Brud and Daniewski (6) have effected a thin-layer chromatographic separation of resorcinol, RMA, and RDA on the product of RMA synthesis and quantitatively determined each component. It was felt that gas chromatography could be employed for determination of RMA but initial chromatograms of commercial RMA were characterized by the presence of three components which persisted throughout variations in chromatographic conditions. An early report (1) which indicated that RMA could be prepared from resorcinol and RDA and the analysis of Brud and Daniewski (6) offered the suggestion that resorcinol monoacetate is not a pure compound but rather an equilibrium mixture consisting of resorcinol and its mono- and diacetates. The following study has been undertaken in support of this theory.

EXPERIMENTAL

Standards—*Resorcinol*—USP grade material was recrystallized twice from 1,1,1-trichloroethane, m.p. 110.5–111.0°.

Resorcinol Monoacetate—Prepared according to the method of Chattaway (2). The RMA so obtained was not chemically pure when examined by GLC and further purification was necessitated. About 30 Gm. of the product was transferred to a separator containing 25 ml. of ethylene glycol. After mixing, 45 ml. of water was added, and the contents shaken and extracted with 25 ml. of chloroform. The chloroform wash was discarded. To the separator was added 50 ml. of water and the solution extracted with two 25-ml. portions of chloroform. Evaporation of the combined chloroform extracts *in vacuo* resulted in a slightly yellow, viscous liquid; density 1.234; n_D^{20} 1.5349 [lit. (4) 1.5328]. The product was observed to yield only one peak chromatographically.

Resorcinol Diacetate—Matheson, Coleman and Bell RDA was purified by alternate washings with cold 5% sodium hydroxide solution and water until the material was chromatographically pure; density 1.185; n_D^{20} 1.5029 [lit. (4) 1.5034].

Equilibrium Studies—About 5 ml. of resorcinol monoacetate standard was placed in a test tube and heated in an oil bath at the specified temperature. At varying time intervals, a 0.1-ml. aliquot was diluted to 25.0 ml. with a mixture of chloroform–methanol (20:1). A 4-μl. portion of this solution was injected into an F & M model 810 dual-column gas chromatograph equipped with a dual-flame ionization detector and a Minneapolis Honeywell recorder. The columns were stainless steel (6 ft. × 1/8 in.) packed with 10% SE-30 on Diatoport S, 60–80 mesh (prepared by F & M Scientific Company). Chromatographic conditions: column temperature, 105°; injection port temperature, 110°; detector temperature, 110°; helium flow, 83 ml./min.; hydrogen, 66 ml./min.; air, 450 ml./min.

RESULTS AND DISCUSSION

The appearance of three distinct peaks by gas chromatographic injection of commercial RMA dissolved in chloroform indicated impure material or degradation of the compound on the column. RMA was subjected to thin-layer chromatography by application on a silica gel strip (Eastman Chromagram, type K301R2) and development of the chromatogram in a solvent system consisting of chloroform–hexane–acetone (50:75:10). Visualization with iodine vapor disclosed the presence of three spots that were identified as the suspected resorcinol, RMA, and RDA by R_f values corresponding to reference compounds chromatogrammed on the same thin-layer strip. This confirmed the composition of commercial RMA to be a mixture but it was not certain that the samples tested were simply below expected quality.

Equilibrium studies were conducted whereby RMA standard was exposed to constant heat at selected temperatures to note the production of GLC peaks corresponding to resorcinol and the diacetate and a simultaneous reduction in the height (in actuality the area) of the original single RMA peak. Experimentation at each temperature was continued to a point at which the reduction of the RMA peak was no longer significant and it was assumed that equilibrium had been attained. Equilibrium concentrations of resorcinol, RMA, and RDA at experimental temperatures are recorded in Table I. Quantitative determination of each constituent observed chromatographically was based on a comparison of peak area to a calibration curve prepared for each standard using the same instrument parameters. Data obtained from these investigations were used to calculate thermodynamic values included in Table II. Substantiation of experimental equilibria has been obtained by a graphical representation of log K *versus* reciprocal

Table I—Equilibrium Between Resorcinol, Resorcinol Monoacetate, and Resorcinol Diacetate at Various Temperatures

Temp., °C.	Concn., moles/L. Resorcinol	RMA	RDA
150	2.14	5.51	0.83
190	2.67	5.26	0.72
	2.67	5.26	0.72
240	2.85	4.94	0.86
264	3.04	4.73	0.93
283	3.63	4.48	0.80
	3.51	4.53	0.81

Table II—Thermodynamic Values Calculated from Equilibrium Studies on Resorcinol Monoacetate

Temp., °C.	$K_c \times 10^2$	ΔH_R, cal./mole	ΔF, cal./mole	ΔS, cal./deg./mole
150	23.5	3069	1218	4.37
190	30.9	3069	1085	4.29
240	40.6	3069	918	4.19
264	50.8	3069	724	4.37
283	56.3	3069	636	4.38

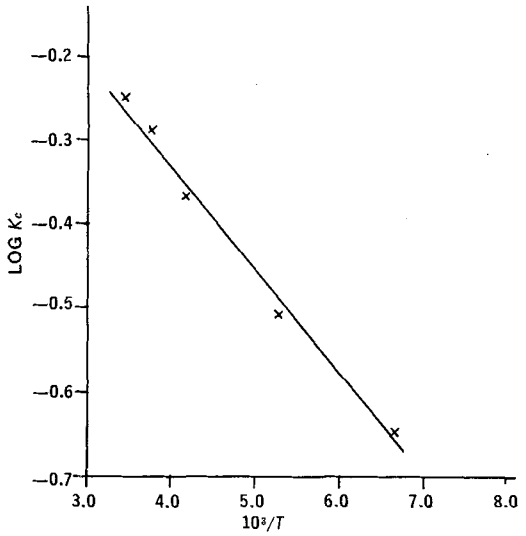

Fig. 1—Effect of temperature on equilibrium constants of RMA.

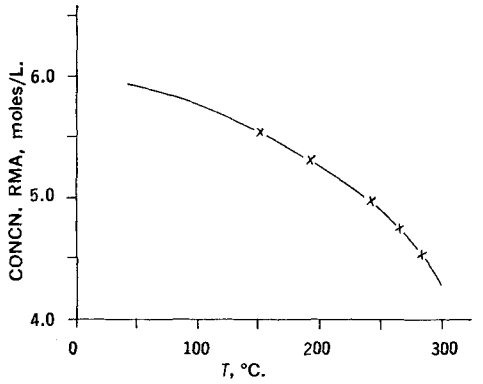

Fig. 2—Influence of temperature on equilibrium concentrations of RMA.

temperature to yield a linear relationship as shown in Fig. 1 and by the converse reaction of resorcinol and RDA to form the monoacetate at selected temperatures. The influence of temperature on equilibrium concentrations of RMA is plotted in Fig. 2.

Commercially available RMA has been characterized as a mixture whose composition can vary depending on the method of manufacture, purification, and age. Nevertheless, analysis can be achieved by gas chromatography to accurately determine the percentage content of each major constituent. An accurately weighed quantity of RMA is diluted to volume with chloroform–methanol (20:1) and a sample injected into the instrument. A typical resultant chromatogram is shown in Fig. 3. The percentage of RMA is calculated by correlation of the peak area to the calibration curve prepared for use in the equilibrium studies. In a similar manner is is possible to obtain the quantities of resorcinol and RDA. Total analyses of three commercial samples have been investigated and the recoveries summarized in Table III.

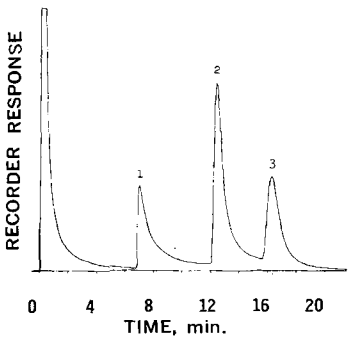

Fig. 3—Chromatogram of a chloroform–methanol solution of commercial RMA on a 10% SE-30 column. Key: 1, resorcinol; 2, RMA; 3, RDA.

TABLE III—COMPOSITION OF COMMERCIAL RESORCINOL MONOACETATE SAMPLES ANALYZED BY GAS CHROMATOGRAPHY

	Recoveries, %		
	Resorcinol	RMA	RDA
Sample A	19.5	61.7	18.9
	19.6	61.8	18.6
Sample B[a]	19.1	60.9	20.0
	18.6	61.8	19.6
Sample C[a]	20.2	63.7	16.1
	20.3	64.1	15.7

[a] NF material.

Quantitative analysis of RMA has been attempted by formation of trimethylsilyl (TMS) ethers by the method of Brobst and Lott (7) and other conventional procedures. A GLC chromatogram of the reaction mixture would represent TMS derivatives of resorcinol and RMA and unreacted RDA. Inspection of chromatograms prepared subsequent to etherification indicated the presence of two peaks instead of the expected three. Supporting investigations on standard materials have shown that the TMS ethers of resorcinol and RMA are eluted simultaneously as a single peak and that this approach is not feasible as a specific assay for RMA in the presence of resorcinol.

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Keyphrases

Resorcinol monoacetate composition
Resorcinol, resorcinol mono- and diacetate combination—confirmed
Equilibrium studies
TLC—separation
GLC—analysis

Granulation of Griseofulvin

By C. F. HARWOOD and N. PILPEL

Griseofulvin, in fine and coarse grades, has been bowl granulated using binder solutions. The granules have been assessed for strength and flow characteristics. A mechanism of granule growth has been suggested, which satisfactorily explains the experimental results.

GRISEOFULVIN tablets are normally prepared from granules containing griseofulvin powder. Granulation facilitates handling, prevents segregation in the formulated mix, and eliminates the problem of dust. Methods of granulation include slugging, wet granulation, spray drying (1, 2), bowl and dish granulation (3, 4). In bowl granulation, the powder is rotated in a tablet coating pan and is lightly sprayed with binder solution which causes it to aggregate into granules. Dish granulation is similar, but involves the use of a low walled dish which is tilted to the horizontal. This is a continuous process for obtaining uniform granules. Both techniques yield a smooth, rounded product, and theories concerning them have been developed by Newitt and Conway-Jones (5), Capes and Dankwerts (6), and by Rumpf (7).

The purpose of this investigation has been to granulate two grades of powdered griseofulvin in order to study the effect of the starting powder on such properties of the granules as their shape, density, and flow characteristics. No excipients were used, but the pharmaceutical grade of polyvinylpyrrolidone,[1] was employed in various concentrations as a binder and a study made of its effects on the strengths of the resulting granules.

EXPERIMENTAL

Materials—Two closely sized pharmaceutical grades of griseofulvin were used, the fine grade (USP microfine grade) having an average particle size of 4 μ, as measured on the Fischer subsieve sizer, and the coarse grade an average particle size of 10 μ. The actual size data, obtained by Coulter counter measurements (8), are given in Table I.

Water and aqueous solutions of polyvinylpyrrolidone (PVP), in 5, 10, 15, and 20% concentrations by weight, were used as the binding fluids.

Granulation Method—Four hundred grams of powder was placed in a 16-in. copper tablet coating pan, mounted on a Manesty Varispeed motor, which was rotated at 30 r.p.m. The binding liquid was sprayed tangentially onto the powder from a Burgess electric spray, type VS 625, in 2-sec. periods every 10 sec. for approximately 15 min. The granules formed without the provision of flights and quickly attained a limiting size. Rotation was continued for a further 15 min., while warm air was blown into the bowl, and the granules were finally dried in shallow trays for 12 hr. at 70°.

Batches were also prepared using a dish granulator of 60-cm. diameter, with 5-cm. walls, tilted at 45° and rotated at 15 r.p.m. In both methods the coarse powder yielded rounded granules but the fine material produced only irregular granules, when viewed under a low power microscope, even when all the operating variables were altered. In further attempts to produce rounded granules from the fine powder, alcohol–water and acetone–water solutions and methylcellulose solution in water were also used as binding fluids; all, however, were unsuccessful.

Measurements on the Granules—*Sieving*—The granules were separated into size fractions by sieving for 10 min. on a vibration sieve. Surface fines were removed by resieving 30-Gm. portions for 1 min. on the Alpine airjet sieve. Microscopic examination confirmed that the granule fractions were uniform in size and free from fines.

PVP Content—The PVP contents of the granules were measured by extracting a known weight with boiling water, filtering, evaporating the filtrate to dryness, and weighing the extracted PVP. Blank tests were conducted using griseofulvin powder and PVP powder alone.

Granule Strength—The breaking strengths of individual granules were measured by raising single granules on a flat plate by means of a lab jack, just into contact with a second flat plate fixed to the underside of a chemical balance pan (Fig. 1). Lead shot was poured steadily onto the balance pan until the granule broke. The weight of the lead shot was then taken as the breaking load. Typical results are recorded in Table II.

Granule Porosity—This is given by the expression:

$$\text{porosity} = \frac{\text{void vol.}}{\text{total vol.}} \times 100$$

where the void volume = $(1/\rho_G - 1/\rho_\rho)$. The granule density, ρ_G, was determined from the weight

Received July 24, 1967, from the Department of Pharmacy, Chelsea College of Science and Technology, University of London, London, England.

Accepted for publication October 2, 1967.

The authors are indebted to J. Cockton, A. G. Fishburn, Dr. D. M. Moulden, and M. J. Thorton, Imperial Chemical Industries, Pharmaceuticals Division, for advice and helpful discussions and to the directors of I.C.I. Ltd. for financial support.

[1] Plasdone.

TABLE I—PARTICLE SIZE DISTRIBUTION OF GRISEOFULVIN[a]

Fine Grade						
Particle size, μ	<1	1–2	2–3	3–5	5–10	>10
Wt. Percentage	3	31	30	29	8	9
Coarse Grade						
Particle Size, μ	2–5	5–10	10–20	20–30	30–60	>60
Wt. Percentage	10	15	35	20	15	5

[a] Abstracted from *Reference 8*.

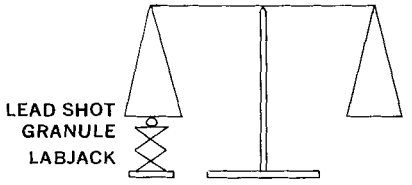

Fig. 1—Granule strength testing apparatus.

TABLE II—BREAKING STRENGTH OF 6–8 MESH GRANULES CONTAINING PVP

PVP Content, %				
0	0.6	1.0	1.6	3.6
Breaking Load, Gm.				
5	65	180	270	450
15	60	230	420	660
20	80	280	400	570
10	75	215	260	640
5	70	320	350	590

and volume of about 200 granules and the powder density, ρ_p, was determined by the British Standards specific gravity bottle method.

Bulk and Tapped Density—Bulk and tapped density values were measured in the Heywood apparatus, using the method described in British Standards 1440.

Granule Flow—The flow of granules in each size fraction was measured from a vertical copper tube of internal diameter 3.8 cm., fitted with a flat base plate with circular orifices of 0.605, 0.707, 0.900, 1.130, 1.330, and 1.650 cm. diameter (9).

RESULTS

The histograms of the granules formed from the fine and from the coarse grades of griseofulvin are shown in Fig. 2. It is seen that the 20% PVP binder solution leads to a slight increase in granule size, compared to water as a binder. As the two liquids have practically the same surface tensions, 64 and 72 dynes/cm., respectively, the effect is probably due to their different viscosities, 40 cps. and 1 cps., respectively. The surface tension force holding two particles together is enhanced if the liquid between the particles has a high viscosity; this is because the liquid has a resistance to deformation, which would result from separation. Thus the "suction" pressure increases with the rate of particle separation and a bond, once formed, resists separation (10).

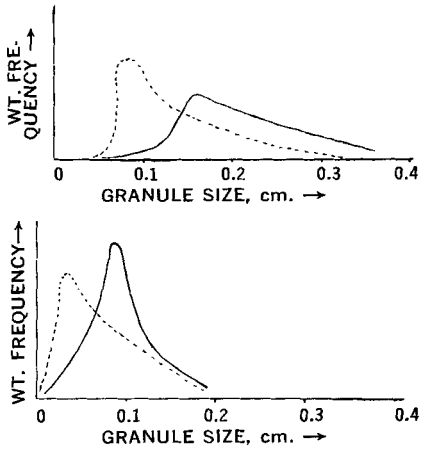

Fig. 2—Histograms. Top, fine grade, yielding irregular granules. Bottom, coarse grade, yielding rounded granules. Key: ——, granulated with 20% PVP solution; - - -, granulated with water.

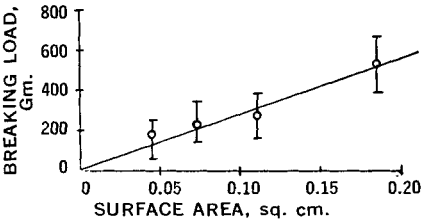

Fig. 3—Increase in granule strength with surface area.

Table III shows that the PVP content of the different size fractions varies through a particular granule batch. During granulation, segregation of sizes occurs so that particular fractions may be favorably presented to the binder spray, and this would account for the variation.

Figure 3 shows that a definite relation exists between granule strength and granule size (*i.e.*, surface area) and that within limits the relation, granule strength = $k \times$ surface area, is applicable. Figure 4 indicates that the granule strength in a particular size group depends on the PVP content. In both cases, considerable scatter of results occurs and this was observed also by Rumpf (7) when measuring the tensile strength of moist granules.

Average porosity values are given in Table IV for the two grades of powder and for the rounded granules containing PVP. These results may be

TABLE III—VARIATION IN PVP CONTENT WITH GRANULE SIZE

B.S. Sieve Size	6–8	8–10	10–12	12–16	16–22
μ Size	2800–2000	2000–1680	1680–1400	1400–1000	1000–710
% PVP	3.6	3.1	2.9	3.9	4.2

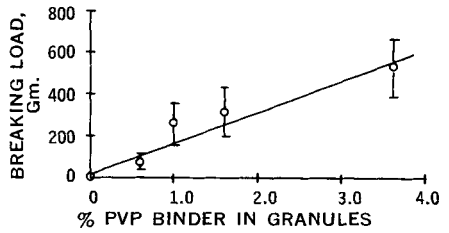

Fig. 4—Variation in breaking load with PVP content for 6–8 mesh granules.

TABLE IV—POROSITY OF GRISEOFULVIN POWDER AND GRANULES

Sample	Bulk Powder Coarse	Bulk Powder Fine	Rounded Granule Size B.S.S. 8–10	Rounded Granule Size B.S.S. 10–12	Rounded Granule Size B.S.S. 12–16
Porosity	80	74	43	42	30

TABLE V—POROSITY OF SPHERES IN IDEAL PACKINGS

Three-Dimensional Arrangement of Spheres			
90°	60°	60°	60°
90°	90°	60°	60°
90°	90°	116°34′	90°
Porosity, %			
47.6	39.5	30.2	25.9

compared with those quoted by Ono and Taneya (11) for hard, ideal spheres in loosely packed and tightly packed states, Table V. The fine and coarse griseofulvin powders have a high porosity, indicating bridging by the cohesive forces: these forces are overcome by the granulation process and a tighter state of packing is achieved.

Bulk and tapped density figures are plotted in Fig. 5, which shows that, because of their geometry, rounded granules pack together more closely than irregular granules. Typical flow rates for the two types of granules through a particular sized orifice are plotted in Fig. 6. The flow rate of rounded granules is seen to be considerably higher than of irregular ones.

DISCUSSION

Some understanding of the growth of granules during granulation can be gained by considering the centrifugal forces acting on two coalescing granules. The values given in Table VI are calculated for equal sized granules by assuming that one of them is thrown around the other with a speed equal to that of the peripheral speed of the drum.

The centrifugal force at the point of coalescence would have to be balanced by the number of pendular bonds acting between individual particles. The value of the pendular bonds can be calculated from (12):

$$F = \frac{2\pi r T}{1 + \tan \theta}$$

where F is the bond strength in dynes, r is the radius of the particles, T is the surface tension of the binder solution, and θ is related to the quantity of binding liquid forming the bond. The values of the pendular bond strengths, calculated for different particle sizes, are given in Table VII, taking θ as 14°.

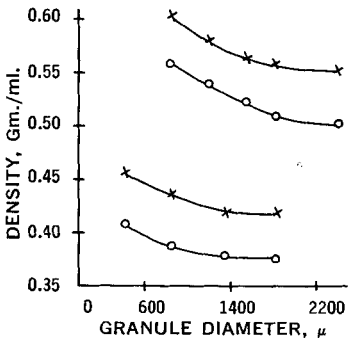

Fig. 5—Bulk and tapped densities against granule diameter. Key: ×, tapped density; O, bulk density. Top, rounded granules; bottom, irregular granules.

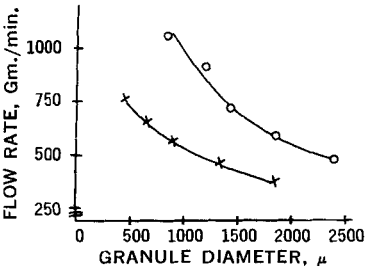

Fig. 6—Granule diameter against flow rate through 1.330-cm. orifice. Key: O, rounded; ×, irregular.

TABLE VI—CENTRIFUGAL FORCE FOR DIFFERENT GRANULE DIAMETERS

Granule Diam., cm.			
0.05	0.1	0.5	1.0
Centrifugal Force, dynes			
7.24	28.9	724	2890

TABLE VII—PENDULAR BOND STRENGTHS AT DIFFERENT PARTICLE SIZES

Particle Size, μ			
4	10	50	200
Bond Strength, dynes			
6.4×10^{-2}	1.6×10^{-1}	8.0×10^{-1}	3.2

The limit of growth by coalescence is probably about 0.5 cm., since, above this size, the centrifugal force becomes very large. However, growth can continue by a secondary process in which small granules are crushed by larger ones (6); the fragments then join the larger granule by coalescence. The limit of growth probably occurs when the crushing effect becomes so frequent that granules are prevented from reforming.

In the case of griseofulvin, in fine and coarse grades, the limit of growth is quickly reached, due to the weak pendular bonds formed at small particle sizes. Thus the granule size at which crushing occurs is never reached.

In the dry state, the strength of a granule is due to the cohesive forces in the griseofulvin and, to a larger extent, to the solid bridges formed from the evaporated binder. PVP in the solid state is an

extremely brittle substance and the large strength variation (Figs. 3 and 4) is consistent with the theory of brittle solids proposed by Griffith (13). Dollimore and Gregg (14) have shown that the ratio of the theoretical strength, R_m, to the practical strength, R, is given by:

$$\frac{R_m}{R} = \left(\frac{\pi c}{a}\right)^{\frac{1}{2}}$$

where a is the range of the attractive forces, and $2c$ is the length of a crack in the brittle solid. Taking $a \approx 3 \times 10^{-8}$ cm., and $c \approx 1 \times 10^{-4}$ cm., then a crack of only 2 μ in the binder would cause a 100-fold reduction of strength. The strength of the granules would also be expected to vary due to irregularities in their shape and the presence of localized powder or binder voids.

CONCLUSION

Griseofulvin can be granulated by the bowl method and the strength of the granules formed has been shown to be dependent on both their size and on their PVP content.

It appears to be more difficult to prepare smooth, rounded granules with good flow and storage characteristics from the fine grade than from the coarse grade, although the former is preferred for therapeutic reasons.

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Keyphrases

Griseofulvin
Granulation, griseofulvin—bowl, dish methods
Polyvinylpyrrolidone solution—binder
Granule strength, porosity, sizing, flow rate
Density, bulk, tapped—granules
Centrifugal force—granule diameter
Pendular bond strength—particle size

"Crushing-Strength" of Compressed Tablets I

Comparison of Testers

By DAVID B. BROOK and KEITH MARSHALL

By means of a piezo-electric force transducer the actual compressional load exerted at certain scale readings by four types of commercially available "crushing-strength" instruments has been determined. The results indicate that variations in crushing-strength values between instruments are due in part to inaccuracies in instrument scale values, zero errors and varying methods of applying the load. Calibration is therefore necessary for accurate measurements using one instrument or when comparing results from more than one tester.

THE MECHANICAL strength of medicinal tablets is an important property of this form of drug presentation and plays a significant role in development and control procedures. It has been described by various terms including "fracture resistance" (1), "friability" (2), "hardness" (3), "bending strength" (4), and "crushing-strength" (5). Measurement of the latter is probably the most widely used technique and may be precisely defined as "that compressional force which, when applied diametrically to a tablet, just fractures it." In most cases the tablet is placed upon a fixed anvil, and the force is transmitted to it by means of a moving plunger.

Crushing-strength is widely employed in commercial production as a control procedure and has been compared by several authors with other properties of the tablet (5–9). Many individual instruments for crushing-strength determinations have been described (3, 9–12) and comparisons between commercially available testers made (3, 11, 13).

In the present investigation a comparative study, against an absolute standard has been made on four commercially available instruments, "Strong-Cobb," "Monsanto," "Pfizer," and "Erweka" hardness testers.

Received August 21, 1967, from the Industrial Pharmacy Unit, University of Bradford, Bradford England.
Accepted for publication October 23, 1967.

extremely brittle substance and the large strength variation (Figs. 3 and 4) is consistent with the theory of brittle solids proposed by Griffith (13). Dollimore and Gregg (14) have shown that the ratio of the theoretical strength, R_m, to the practical strength, R, is given by:

$$\frac{R_m}{R} = \left(\frac{\pi c}{a}\right)^{\frac{1}{2}}$$

where a is the range of the attractive forces, and $2c$ is the length of a crack in the brittle solid. Taking $a \approx 3 \times 10^{-8}$ cm., and $c \approx 1 \times 10^{-4}$ cm., then a crack of only 2 μ in the binder would cause a 100-fold reduction of strength. The strength of the granules would also be expected to vary due to irregularities in their shape and the presence of localized powder or binder voids.

CONCLUSION

Griseofulvin can be granulated by the bowl method and the strength of the granules formed has been shown to be dependent on both their size and on their PVP content.

It appears to be more difficult to prepare smooth, rounded granules with good flow and storage characteristics from the fine grade than from the coarse grade, although the former is preferred for therapeutic reasons.

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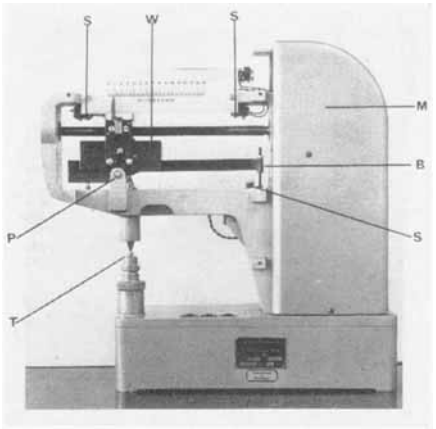

Fig. 1—The Erweka tablet hardness tester, type TBT. (Cover and attached zero light removed.) Key: B, beam; M, motor housing; P, beam pivot; S, microswitch; T, tablet; W, sliding weight.

The first three of these testers are adequately described elsewhere (3, 11, 12). In the "Erweka" tablet hardness tester (type TBT) the compressional force is applied by a loaded beam. The loading is produced by a sliding weight moved along the beam by an electric motor, which is stopped automatically, immediately the tablet fractures (see Fig. 1). The initial zero position is indicated by a red warning light. Hence, errors associated with a nonuniform rate of loading and varying zero adjustment are minimized. A recent report (14) using this instrument suggests that a more realistic test is to apply the force axially to the tablet, a procedure suggested by other authors (1) for the Strong-Cobb hardness tester.

In fact almost all the published work on the crushing-strength of tablets refers at some point, to discrepancies between readings obtained by different techniques. The work described endeavors to correlate results and explain the observed variations in crushing-strength values.

EXPERIMENTAL

Calibration of Force Transducer—In order to ascertain the absolute load transmitted to a tablet by the four instruments at certain scale readings, a small load washer was substituted for the tablet. The transducer employed (Kistler, type 901A) was based on the piezo-electric effects of a quartz crystal. Compressional loads on these cause a re-orientation of electrons within the crystal, giving rise to an electrostatic charge on it. The transducer was coupled to a piezo-charge amplifier (Kistler, type 568) which precisely converted this charge into a voltage output signal which was registered on a digital voltmeter (Weyfringe, type 4NP).

Although conversion data were supplied with the transducer, it was felt desirable to calibrate the instrument accurately over the intended range of loading. This was carried out by two methods: (*a*) by placing known weights at fixed distances from the fulcrum of a simple, freely pivoted beam (illustrated diagrammatically in Fig. 2) which rested on one of the transducer's sensitive surfaces, and (*b*) using an accurate compression/tension testing machine (Instron, floor model TT-C).

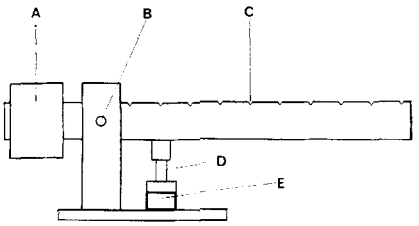

Fig. 2—Diagram of simple beam used to calibrate the load transducer. Key: A, counterbalance weight; B, beam pivot; C, beam graduated in exact 2-cm. divisions; D, plunger fixed to beam and resting on transducer surface; E, load transducer.

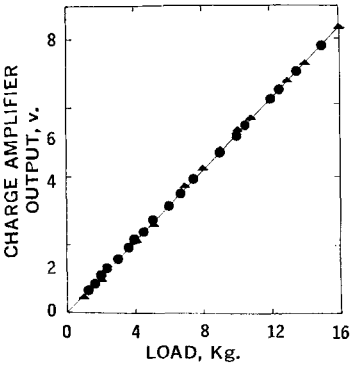

Fig. 3—Calibration curve for load transducer. Key: ●, reading obtained by loaded beam; ▲, reading obtained on Instron testing machine.

The results summarized in Fig. 3 indicated that the relationship between compressional load and voltage output was substantially linear over the range tested; *i.e.*, compressional load (Kg.) was equal to 1.925 × voltage output. This relationship was used in the subsequent experiments.

Calibration of Instrument Scales—The four testers were then taken in turn and with the load transducer in place of a tablet, loads were applied to fixed scale intervals, the resulting voltage being noted. Each series of readings was repeated to give a total of 25 at each level of loading. The arithmetic mean of these was then converted into Kg. using the above relationship. The values obtained are plotted against instrument scale readings in Fig. 4. Calibration values were only obtained up to actual loads of about 15 Kg. since in normal use the testers are unlikely to be operated beyond this point.

The Monsanto hardness tester is probably the most well known and it was therefore decided to compare five instruments of this type to ascertain the individual variation. In addition to the tester in the authors' laboratory, three new testers were donated by the manufacturers and a well used instrument was obtained from an industrial source. The results of calibration tests similar to those described above on these testers are given in Table I and Fig. 5.

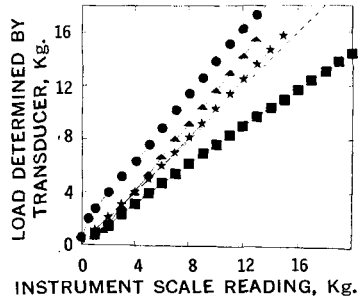

Fig. 4—Relationship between instrument scale reading and actual load being exerted as determined by load transducer. Key: ●, Erweka tablet hardness tester; ▲, Pfizer tablet hardness tester; ★, Monsanto tablet hardness tester; ■, Strong-Cobb tablet hardness tester. Dotted line is theoretical ideal of slope one.

TABLE I—RESULTS OF CALIBRATION BY LOAD TRANSDUCER OF FIVE MONSANTO TABLET HARDNESS TESTERS

Monsanto Scale Reading (Kg.)	Load in Kg. Determined by Transducer				
	A[a]	B	C	D	E
1	1.100	0.960	1.009	1.125	0.820
2	2.263	1.974	2.000	2.086	1.528
3	3.050	2.983	3.157	2.981	2.599
4	4.220	4.140	4.299	3.976	3.451
5	5.370	5.370	5.493	4.997	4.624
6	6.720	6.623	6.680	5.966	5.518
7	7.890	7.853	7.918	7.024	6.527
8	8.970	8.950	9.087	8.171	7.530
9	10.150	10.193	10.221	9.200	8.511
10	11.362	11.461	11.440	10.222	9.507
11	12.625	12.734	12.671	11.422	10.519
12	13.902	13.950	13.943	12.530	11.693
13	15.180	15.117	15.250	13.595	12.796
14				14.634	13.799
15				15.705	14.696
16					15.797

[a] Testers A, B, and C were new. Tester D had been used in the authors' laboratory for 3 years and tester E was a well used instrument obtained from an industrial source.

DISCUSSION

The results shown in Figs. 4 and 5 indicate discrepancies between the load as registered on the instrument scales and the actual load as determined by load transducer. The graphs approximate to straight lines over most of the range studied, and it is therefore convenient to utilize the slopes S (which were determined graphically) of these plots as a means of comparison with the ideal line of slope one. Since all lines should ideally pass through the origin, S is the correction factor necessary to adjust instrument scale readings so that they lie on the theoretical line.

Strong-Cobb—This tester registers the air pressure required to crush the tablet and has a scale graduated in Kg./sq. in. Comparison with other results is therefore only possible if these readings are converted to compressional load in Kg. The manufacturer of the instrument specifies a conversion factor based upon piston dimensions of 0.780. In this investigation the value of S was found to be 0.728 and Table II gaves the values of instrument scale readings corrected by this factor.

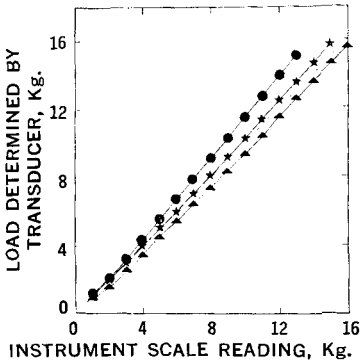

Fig. 5—Relationship between instrument scale reading and actual load being exerted as determined by load transducer for 3 Monsanto testers. Key: ●, Monsanto A (new); ★, Monsanto D (authors); ▲, Monsanto E (well used). Dotted line is theoretical ideal of slope one.

TABLE II—COMPARISON OF CORRECTED SCALE VALUES FOR CRUSHING STRENGTH AND ACTUAL LOAD EXERTED BY STRONG-COBB AND ERWEKA TABLET HARDNESS TESTERS

Strong-Cobb		Erweka	
Actual Load (Kg.)[a]	Corrected Scale Reading (Kg.)[b]	Actual Load (Kg.)[a]	New Scale Reading (Kg.)
0	0		
0.650	0.728	0.493	0.500
1.508	1.456	2.033	2.000
2.369	2.184	2.689	2.700
3.147	2.912	3.918	4.000
3.919	3.640	5.154	5.200
4.722	4.368	6.271	6.250
5.438	5.096	7.515	7.500
6.201	5.824	8.728	8.750
6.905	6.552	10.090	10.000
7.616	7.280	11.314	11.300
8.311	8.008	12.503	12.500
9.003	8.736	13.742	13.750
9.702	9.464	15.108	15.000
10.395	10.192	16.048	16.000
10.910	10.920	17.260	17.250
11.748	11.648		
12.499	12.376		
13.095	13.104		
13.800	13.832		
14.536	14.560		

[a] Actual loads determined by load transducer. [b] Scale reading × 0.728.

Monsanto—The results for these testers appear to indicate that the springs applying the load become fatigued with prolonged use, *i.e.*, S values falling, although when new they exert loads which are consistent but in excess of the scale reading.

McCallum, Buchter, and Albrecht compared the Monsanto and Strong-Cobb hardness testers and calculated a conversion factor of 0.625 for Monsanto to Strong-Cobb units (3). The results reported here gave a factor of 0.627 (new Monsanto) and 0.697 (well used).

Pfizer—This tester was found to exert loads in excess of the scale readings (S value 1.260), and since the instrument was comparatively new it is probable that, as with new Monsanto testers, the spring does not match the scale graduations initially. In addition it was noted that at loads below 3.0 Kg. the load was less than the scale reading, probably due to frictional losses.

TABLE III—DIFFERENCES IN TABLET HARDNESS TESTER CONSTRUCTION WHICH MAY CONTRIBUTE TO VARIATION IN CRUSHING-STRENGTH VALUES BETWEEN INSTRUMENTS

Loading Mechanism	Monsanto Coil Spring	Strong-Cobb Hydraulic Pressure	Pfizer Coil Spring	Erweka Loaded Beam
Scale length/Kg.	0.3 cm.	0.79 cm.	0.88 cm.	0.935 cm.
Scale graduated in:	1.0 Kg.	0.728 Kg.[a]	0.2 Kg.	0.25 Kg.

[a] One scale unit is equal to 0.728 Kg.

Erweka—Examination of this tester revealed a zero error because at the moment the zero warning light became activated the instrument was already exerting a load of about 0.5 Kg. Furthermore, as soon as the sliding weight began to move along the beam there was a sudden increase in load to about 1.4 Kg. Subsequent increase in loads of one scale division resulted in an increase in true load of 1.211 Kg.

The initial errors were traced to an incorrect zero adjustment and frictional losses in the pivot. Utilizing the principle of moments it was possible to calculate the theoretical load from the known scale dimensions (0.935 cm./division) and the magnitude of the sliding weight (1.925 Kg.). The distance of the plunger from the fulcrum was accurately measured as 1.425 cm. From these readings a value of 1.262 Kg./scale division was obtained. The difference between this and the observed reading (1.211 Kg./scale division) was thought to be due to frictional effects.

From these experiments a true zero position was attained by adjustment of the zero light trip switch position and a new scale was constructed. These modifications were entirely successful in producing true response to actual loads (see Table II).

Krowczynski (13) compared the Monsanto and Erweka hardness testers and found that the results from the two instruments differed widely. This author calculated a conversion factor of 1.6 for Erweka to Monsanto units. In the present investigation it was impossible to determine such a simple factor because a large constant zero error in the Erweka tester results in a factor which increases as the crushing-strength values decrease. This may well account for the difficulty reported by Krowczynski in determining his conversion factor.

Other Differences in the Testers—Any comparison of these four types of testers must take into account the additional factors due to the inherent differences in design summarized in Table III.

For example the variations in scale length for 1-Kg. units of loading are significant enough to introduce considerable errors in estimation of the load. The different methods of applying the load might also be expected, as is shown here, to give rise to differing values of crushing-strength for different instruments.

Since the initial load applied to grip a tablet is, in three types of instruments, subject to operator variation this must also lead to discrepancies. In the Monsanto and Pfizer hardness testers it was found that with extreme care the initial load required to grip the transducer could be as little as 0.1 Kg., but unless the operator exercized this care, loads of 0.3 to 0.5 Kg. were more likely. In the Strong-Cobb hardness tester it appeared that the setting for zero was less critical. Although the pressure gauge needle was sluggish in leaving the zero mark, the load applied at a scale reading of 1 unit was reasonably constant irrespective of the initial "gripping load." Before adjustment of the Erweka hardness tester the average zero load applied to a tablet, when the zero light was activated, was found to be 0.5 Kg. Any slight overshooting of the zero position resulted in initial loads up to 1.3 Kg. which of course were not accounted for in the subsequent crushing strength indicated on the instrument scale. Even after adjustment any overshooting still applies large initial loads.

Finally, it may be noted that all instruments were less accurate at crushing-strengths below about 2.0 Kg. and it is doubtful if values below this figure have any significance.

CONCLUSIONS

Variations in crushing-strength values obtained from different types of tester have been shown to be due in part to inaccuracies in instrument scale values, zero errors, and varying methods of applying the load. Calibration is therefore necessary where a high degree of accuracy is desired or when comparing results from different types of testers. The physical dimensions and shape of the tablet as well as other physical properties must also contribute to the property of crushing-strength. The results reported here are now being used to study these additional factors.

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Keyphrases

Tablets, compressed—crushing-strength
Hardness testers—comparison
Monsanto hardness testers, new, used—variation
Force transducer—scale calibration
Load, initial gripping—hardness tester accuracy

Dissolution Apparatus with Multiple Testing Stations

By R. A. CASTELLO, G. JELLINEK, J. M. KONIECZNY, K. C. KWAN, and R. O. TOBERMAN

An apparatus in which 20 dissolution tests may be conducted simultaneously is described. A single set of controls maintains the test conditions in all 20 dissolution vessels. Provisions have been made to sample automatically and simultaneously from each vessel at predetermined time intervals. Results obtained for the dissolution of experimental potassium chloride tablets using this apparatus are in good agreement with those obtained when tablets were tested individually.

IT HAS BECOME increasingly evident that the rate and extent of the dissolution of drug compounds from solid dosage forms such as capsules or tablets are important criteria for optimum absorption and therapeutic effect. For example, Levy *et al.* (1) have used the dissolution rate of prednisone to differentiate between therapeutically active and inactive tablet formulations. Moore *et al.* (2) demonstrated the value of dissolution tests in evaluating various formulations of nalidixic acid. Under some circumstances, it has been shown (3) that the dissolution data for a given formulation may be injected directly into a relatively complex pharmacokinetic model for the prediction of biological disposition of a drug.

In the course of the development of a dosage form, the examination of dissolution properties may be helpful in various ways. Dissolution may be used to study the effects of formulation and process changes, to evaluate the reproducibility within a batch as well as between batches, and to verify the physical quality of a product. When the dissolution characteristics correlate with the absorption or clinical effectiveness of the dosage form, it may be desirable to include the dissolution test as a regular quality control requirement for that product. The apparatus to be described was designed with this need in view and particularly to accommodate those situations where a large number of individual dissolution tests may be required for proper statistical assessment of the dosage form.

Specifically, this communication describes a mechanical unit in which 20 individual dissolution tests may be conducted simultaneously. A master panel controls the rate of agitation, the position of the agitators, the duration of the dissolution test period, the frequency of sampling, and also provides for the automatic withdrawal of samples at predetermined times. The dissolution vessels are immersed in a constant temperature water bath and are held in fixed positions relative to the stirring mechanism.

Received April 28, 1967, from the Department of Pharmaceutical Research and Development, Merck Sharp & Dohme Research Laboratories, West Point, PA 19486 and the Engineering Development & Standards Department, Merck & Co., Inc., Rahway, NJ 07065

Accepted for publication November 14, 1967.

Presented to the Drug Standards, Analysis and Control Section, APHA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

APPARATUS

The apparatus (Fig. 1) consists of four parts: (*a*) a stand and raising mechanism; (*b*) an agitator support and d.c. motor drive; (*c*) electric controls; and (*d*) a sampling device.

The agitators are raised and lowered by a vertical screw which is driven by a 1/4-hp. Boston Ratiomotor with a 10 to 1 ratio. Upper and lower limit switches which are mounted on the stand allow for maximum vertical travel of 16 in. The electrical equipment is mounted on a sliding tray in the lower part of the stand. All operating controls are mounted on a control panel on the right-hand side of the stand.

The 20 agitators are driven by a single Bodine 1/4-hp. d.c. gear motor with a ratio of 10 to 1. The extended armature shaft is connected by a flexible coupling to a tachometer. The extension on the agitator support is equipped with a knurled adjusting and lock screw that operates the lower limit switch and controls the agitator heights. Agitators operate only with the agitator support at the lower limit of travel.

All operational functions, such as raising and lowering, fast and slow agitation speeds, timing and signals, are accomplished through Allen-Bradley relays and Eagle "Cycl-Flex" timers. Using elec-

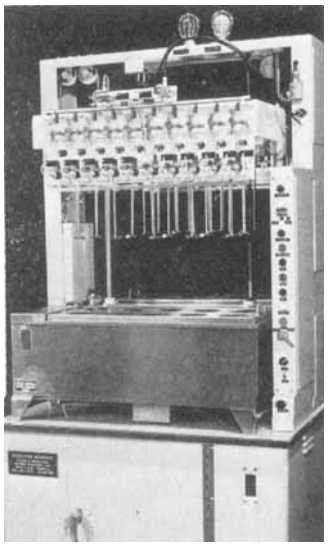

Fig. 1—Dissolution apparatus.

trical interlocks, only one function can be operated at any one time. The speed of the agitators is controlled by a d.c. motor control. Two ranges of speed, slow and fast, provide maximum rates of agitation of 100 and 250 r.p.m., respectively. Speed control is accomplished by balancing the output of the tachometer against a variable voltage obtained from zener diodes by adjusting a potentiometer. The agitator speed can be read directly in r.p.m. on a meter. Speed is maintained to ±2% regardless of line voltage between 105–130 v.

All the necessary push-button switches are mounted on the panel (Fig. 2). Briefly their functions are as follows: (*a*) The main selector switch energizes the whole unit. (*b*) A wing lever selector switch selects the mode of operation—"manual" or "automatic." (*c*) The "sample" push button is used to take samples at any time when the wing lever switch is in the "manual" position. (*d*) The "agitator" push button operates agitators manually while depressed and is active only on "manual" operation. (*e*) The "down" push button is used to lower the carrier while depressed and is active on "manual" operation only. (*f*) The "up" push button raises the carrier while depressed but does not have to be held and is operational only in the "manual" mode. (*g*) "Start cycle" push button starts the automatic cycle if the wing lever switch is in the "automatic" position.

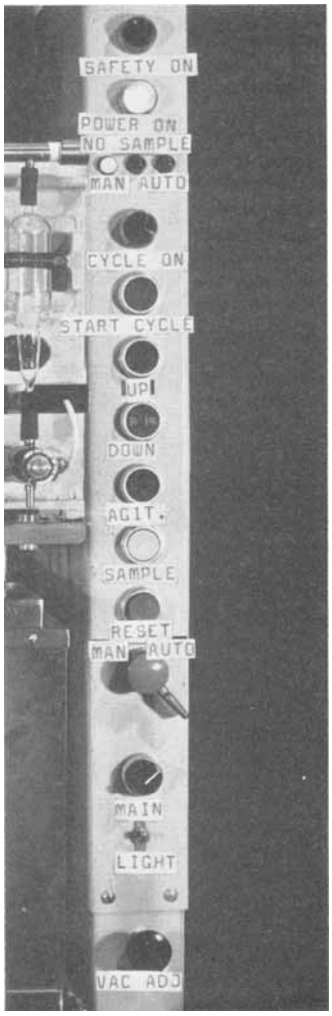

Fig. 2—Operational panel of dissolution apparatus.

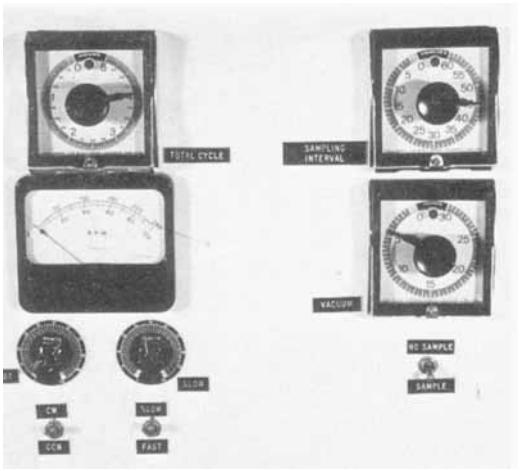

Fig. 3—Preset controls of dissolution apparatus.

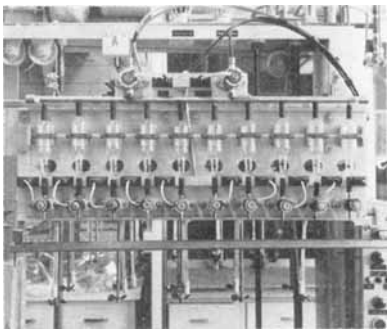

Fig. 4—Sampling device.

Preset controls are all mounted on the front panel of the electrical equipment tray (Fig. 3). A total cycle timer controls the length of agitation during the automatic cycle. A sample interval timer energizes the sampling device to take samples at set intervals and may be bypassed at the operator's discretion. The vacuum timer activates a vacuum pump for a set period of time and controls the level of fill in the sampling bulb. A toggle switch controls the direction of motion of the agitators.

The sampling device (Fig. 4) will take a volumetrically controlled sample from each of the 20 dissolution flasks simultaneously and transfer all samples to corresponding sample cups. This is accomplished in the following manner: when the vacuum timer is energized the vacuum pump starts and two vacuum valves open. A vacuum is thus created in a manifold and in all 20 sample bulbs (Fig. 5). Liquid from the flasks is transferred through the suction tubes adjacent to each agitator into the sample bulbs. By setting the vacuum timer or the vacuum adjust valve, the volume of fill can be regulated. When taking samples it is only necessary to let the liquid rise slightly above the narrow portion of the sample bulb. When the vacuum timer stops, the two

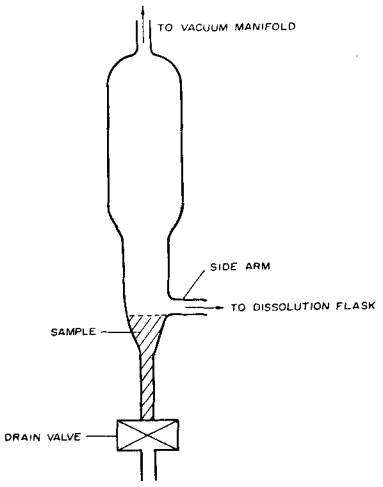

Fig. 5—Schematic drawing of sample bulb.

vacuum valves close and the vacuum pump stops. At the same time, two vent valves open to allow the excess sample fluid to drain back into the flasks leaving only the desired quantity of sample in the bulbs. After a delay of 5 to 10 sec., the 20 drain valves open and the liquid empties from each bulb into 20 sample cups. Suction tubes may be back flushed with air prior to sampling to avoid dead volume in the line; or, alternatively, up to three samples may be flushed through the sampling device prior to actual taking of the analytical specimen to avoid dilution effects. The sample size is fixed by the location of the horizonal extension of the sample bulb.[1]

APPLICATION

Use has been made of the apparatus described to monitor the dissolution behavior of several new dosage forms, but especially an experimental, slow-releasing potassium chloride tablet formulation. Such a tablet was selected for study because it enabled us to use an automated assay procedure where the potassium concentration was measured by flame photometry. For this particular preparation, 20 individual tablets were tested in the apparatus simultaneously. The agitators (2 in., three-blade impellers, A. H. Thomas, 9240-K) were individually adjusted to a height of 3 cm. from the bottom of each of 20 dissolutions flasks (reaction flask, Corning 6947). The flasks were individually centered with respect to the agitator shaft and then fixed in this position. Each flask contained 750 ml. of 0.01 *M* sodium phosphate buffer at pH 7.2 and maintained at 37° in a constant temperature water bath. The rate of agitation was adjusted to 180 r.p.m. in the counterclockwise direction, when viewed from above. Samples were withdrawn automatically at the beginning of the dissolution cycle and at 30-min. intervals during the dissolution test for a total of 4 hr. The sample bulbs were designed to deliver 1.5 ml. of sample into the sample cups for assay. The concentration of potassium in solution in the cups, and consequently in the dissolution flasks at each time was determined by flame photometry. The rates of dissolution measured with this apparatus were compared with those obtained when 20 individual tablets were tested one at a time and consecutively in a single dissolution test apparatus which involved timed sampling by pipet. Dissolution results on samples withdrawn from a single batch of the experimental potassium chloride tablets by both methods illustrate good agreement (Table I) and validate the use of the automated apparatus for large-scale dissolution testing.

[1] Built by Mr. P. J. Dockery, Glass Blowing Shop, Merck Sharp & Dohme Research Laboratories.

TABLE I—DISSOLUTION OF INDIVIDUAL EXPERIMENTAL POTASSIUM CHLORIDE TABLETS *FROM A SINGLE BATCH*

	% Dissolved, min.							
	30	60	90	120	150	180	210	240
	20 Tablet Apparatus							
	3	2	8	13	20	28	36	47
	0	1	6	12	20	29	37	46
	1	1	8	12	21	31	40	51
	1	1	6	11	18	26	35	43
	1	1	7	14	22	31	41	51
	1	1	6	12	20	28	37	46
	1	0	6	12	20	29	39	50
	1	1	6	12	19	27	36	46
	1	0	3	7	13	19	26	34
	1	1	6	11	19	27	36	46
	1	0	4	9	16	23	32	42
	1	2	9	16	26	37	49	60
	1	2	8	15	24	35	45	56
	1	4	10	18	26	37	46	57
	1	2	7	13	20	29	38	48
	2	5	11	19	29	40	51	60
	1	1	5	9	16	24	33	43
	2	3	8	16	26	26	37	56
	1	0	4	8	15	22	31	40
	1	3	8	15	25	35	45	54
Av.	1	2	7	13	21	30	39	49
	20 Individual Determinations							
	1	6	13	20	29	37	46	54
	0	4	10	17	25	32	41	49
	0	3	9	15	23	30	39	47
	0	3	9	15	23	30	39	47
	0	4	11	17	26	33	42	50
	0	6	14	22	33	42	53	62
	0	3	8	14	21	28	37	44
	0	7	15	24	35	44	57	67
	1	4	10	17	25	32	41	49
	0	4	10	17	24	32	41	48
	0	4	9	14	23	31	40	48
	0	2	8	14	21	29	38	46
	0	4	11	18	26	26	44	53
	0	6	15	23	33	44	55	64
	0	4	10	17	24	33	42	40
	0	5	11	18	25	35	44	52
	0	5	12	19	28	37	47	56
	0	5	12	20	27	37	48	56
	0	2	7	12	18	25	33	40
	0	3	9	16	23	32	42	50
Av.	0	4	11	17	26	33	43	52

SUMMARY

An apparatus capable of performing multiple dissolution tests simultaneously under controlled conditions is described.

This apparatus has the following features: (*a*) 20 individual tests may be performed simultaneously. (*b*) The degree of agitation may be maintained at constant rates up to 250 r.p.m. (*c*) Samples may be withdrawn automatically at any predetermined frequency within a specified length of time. (*d*)

Relative positions of stirrers and dissolution vessels may be adjusted at will. (*e*) Size of samples taken is adjustable.

The dissolution behavior of an experimental potassium chloride tablet was used to illustrate the application of this apparatus in comparison with a more laborious single dissolution flask procedure.

REFERENCES

(1) Levy, G., Hall, N. A., and Nelson, E., *Am. J. Hosp. Pharm.*, **21**, 402(1964).

(2) Moore, W. E., Portmann, G. A., Stander, H., and McChesney, E. W., *J. Pharm. Sci.*, **54**, 36(1965).

(3) Wiegand, R. G., and Taylor, R. G., *Biochem. Pharmacol.*, **3**, 256(1960).

Keyphrases

Dissolution apparatus—multiple testing
Apparatus, dissolution—photographs
Operating instructions—dissolution apparatus

Development and Evaluation of a Sampling Device for the Analysis of Pharmaceutical Aerosols

By SIDNEY P. TUESLEY, JOHN J. SCIARRA, and ANTHONY J. MONTE-BOVI

Various devices and techniques were studied in an attempt to obtain suitable samples of material from aerosol products. Generally accepted sampling procedures cannot be used since the aerosol product contains propellants that are extremely volatile. Several of these methods were investigated and used as the basis for assaying various aerosol products. A sampling device was developed and evaluated. The sampling device was designed in a manner that made available a sample of aerosol product that could then be assayed directly in the chamber. Openings were fitted with specially designed valves that allow for transfer of the contents without loss of volatile propellant or active ingredients. Various samples of aerosol products containing local anesthetics, steroids, and amines, were assayed by this method and found to give acceptable results. In all cases, the amount of active ingredient contained in each product could be accurately determined. The device makes possible a technique applicable to the analysis of most pharmaceutical aerosols. This method, which can be carried out with ease in a relatively short period of time, was shown to produce accurate results when used in the manner described.

THE ANALYSIS of pharmaceutical aerosols presents many unique problems to the analytical chemist. Generally encountered analytical techniques such as extractions and titrations cannot be performed without modification of the intact aerosol product. Since one or more of the components of an aerosol product may be extremely volatile, it is difficult to obtain a representative sample. The vapor pressure of the system may vary from 15 psig to about 40 psig, depending upon the nature and amount of propellant and other solvents that may be present.

All aerosol products consist of a volatile and a nonvolatile portion. The nonvolatile portion, generally referred to as the product concentrate, consists of the active ingredients dissolved, suspended, or emulsified in various solvents and other ingredients. While this concentrate may contain volatile solvents such as ethyl alcohol, acetone, *etc.*, the vapor pressure of these solvents at room temperature is considerably less than the more volatile propellants.

The propellant may consist of a compressed gas such as nitrogen, carbon dioxide, or nitrous oxide or, more commonly, of a liquified gas of the fluorocarbon type. Hydrocarbons such as propane, butane, and isobutane have not been used for pharmaceuticals at the present time. Dichlorodifluoromethane (propellant 12), dichlorotetrafluoroethane (propellant 114), and trichloromonofluoromethane (propellant 11) are generally used for this purpose. The positive pressure within the container indicates the need for a sampling procedure by which active ingredients can be determined from a known amount of aerosol product. The form in which the active ingredients are found will vary accord-

Received April 28, 1967, from the Graduate Division, College of Pharmacy, St. John's University, Jamaica, NY 11432

Accepted for publication October 27, 1967.

Abstracted in part from a thesis submitted by Sidney P. Tuesley to the Graduate Division, College of Pharmacy, St. John's University, Jamaica, N. Y., in partial fulfillment for the Master of Science degree requirements, June 1967.

Presented to the Drug Standards, Analysis, and Control Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

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ing to the intended use of the product. This can be as a fine mist, wet spray, or foam.

Various attempts have been made to overcome the difficulties caused by the presence of the propellant. Different methods have been employed for the sampling of pharmaceutical aerosols. One commonly used method allows the propellant to escape, leaving behind the concentrate that consists of active ingredients and other nonvolatile agents used in the formulation. The solution may then be assayed and the percent active ingredient(s) in the aerosol formulation determined. This method of sampling, which has been used in a solubility study of different medicinal agents in fluorocarbon propellants by Tinney (1), involved the transfer of a filtered aliquot of a saturated solution of medicinal agent in the fluorocarbon propellant to a tared glass aerosol container. A specially designed transfer valve and actuator was used to effect the transfer. The dip tube, attached to the sample bottle, was removed from the transfer valve so that the propellant could be removed as a vapor. The total weight of the bottle containing the filtered saturated solution was determined. The propellant vapor was then removed from the sampling bottle. Following removal of the propellant, the weight of the container and valve was determined and the solubility of the medicinal agent calculated by the "weight by difference" method. This method is limited to those propellants and solvents that can be removed by vaporization at room temperature.

Young *et al.* (2) developed a method which was applicable to the assay of an aerosol containing epinephrine bitartrate and similar agents. The gross weight of the aerosol package was first determined and then the valve closure was punctured. A hypodermic needle was inserted through the valve closure that then allowed the propellant to escape through the opening of the needle. At this point, the valve could be safely removed. The remaining suspension was then assayed spectrophotometrically. Knowing the weight of the empty container and valve, the amount of active ingredient was determined. In order to determine uniformity of dosage released from a metered valve, Young *et al.* (2) employed a somewhat different method. This method consisted of inverting an aerosol package of epinephrine bitartrate or isoproterenol sulfate over a 250-ml. glass-stoppered graduated cylinder containing 200 ml. of 0.1 *N* acetic acid. A metered dose of medication was released into the cylinder and the cylinder was immediately stoppered and shaken. An aliquot of the acid solution containing the active ingredient was assayed spectrophotometrically.

Another method that was developed for this purpose utilized an "artificial respiratory system" (2). This system was designed to measure the delivery of the active ingredient dispensed through an oral adaptor. This procedure consisted of discharging the aerosol through the oral adaptor into a funnel attached to a glass tube which was immersed into a gas washing bottle containing distilled water. A vacuum was drawn on the system. This procedure essentially simulated conditions closely related to inhalation of the medication by a patient. The solution was then subjected to spectrophotometric assay.

Sampling techniques were developed by Lawless, Sciarra, and Monte-Bovi (3) and by Rifino, Monte-Bovi, and Sciarra (4) for the analysis of various medicinal amines and volatile oils, respectively, in aerosol formulations. Their sampling technique consisted of weighing the intact aerosol package and then bubbling a given volume of the aerosol into a suitable solvent and reweighing the container. The resulting solution, brought to the desired volume, was analyzed using gas-liquid chromatography. From this, the amount of active ingredients present in the aerosol package could be determined. The method whereby an aerosol product was bubbled through a solvent had previously been developed for the analysis of moisture in aerosols by the Fischer titration method (5)

This study was concerned with the development and testing of various devices and techniques which could routinely be applied to the analysis of various pharmaceutical aerosols. The applicability of the method to different types of aerosol packages, the speed with which the sample can be obtained, and the accuracy with which the active ingredients can be removed from the pressurized package were major considerations in the development of the sampling procedures. Various commercially available pharmaceutical aerosols were assayed, using the sampling techniques developed during this study. In this manner the application and suitability of the methods could be determined.

Several materials were investigated in order to determine the suitability of the material for the sampling device. Extruded stainless steel tubing, together with Plexiglas, was fabricated into a chamber. However, difficulty was encountered in securing an efficient seal between the metal and Plexiglas. Several openings had to be drilled, tapped, and fitted with stainless steel bolts. When the device was used, the Plexiglas, under the strain of the bolts, developed cracks. Based

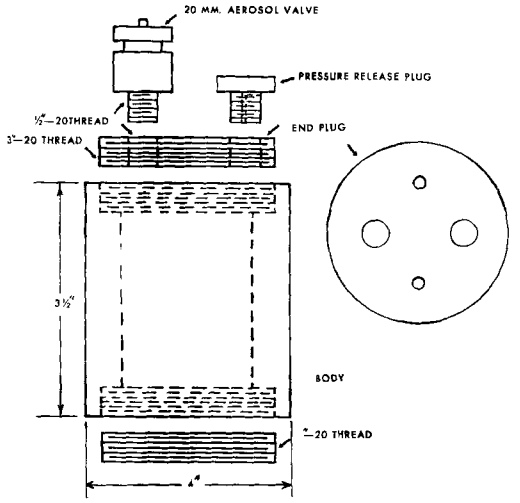

Fig. 1—Sampling chamber for aerosol products.

glass membrane and a 25-ml. buret. This was specially designed so as to allow potentiometric titrations directly within the cylinder.

Assay Procedures—The sampling device was washed with distilled water and dried with acetone prior to and following each assay. An accurately weighed sample of benzocaine NF, about 0.5 Gm., dried over phosphorus pentoxide for 3 hr., was placed into the open sampling device and 30 ml. of glacial acetic acid was added. A Teflon-coated magnetic stirring bar was placed into the container, which served to produce efficient stirring of the solution throughout the titration. The top cover of the device was screwed into place. The combination electrode was placed into one of the openings located in the top of the sampling container so that the tip of the electrode was immersed in the glacial acetic acid solution of benzocaine. A 25-ml. buret was introduced into the second opening. The electrode was connected to a Beckman Zeromatic pH meter, which had been set at the 1400-mv. range and adjusted to 0 mv. at the start of each titration. The benzocaine solution was then titrated with 0.1 *N* perchloric acid in glacial acetic acid. The equivalence point was determined potentiometrically and the milliliter of titrant represented at this point was obtained from a first derivative plot of $dmv./dml.$ *versus* ml. of titrant added. The procedure was repeated several times and these results are shown in Table I.

TABLE I—ASSAY OF BENZOCAINE NF IN DIFFERENT SOLVENT SYSTEMS

	Benzocaine in Glacial Acetic Acid		Benzocaine in Ethanol and Propylene Glycol		Benzocaine Aerosol		
	Determination No.						
	1	2	1	2	1	2	3
Weight of sample, Gm.	0.4419	0.4037	3.9698	4.0395	4.3827	6.4826	4.7262
Benzocaine in sample, Gm.	0.4419	0.4037	0.4054	0.4417	0.2799	0.4090	0.2982
0.1 *N* perchloric acid, ml.	26.8	24.2	24.7	26.75	16.6	25.2	18.0
Benzocaine found, Gm.	0.4437	0.3997	0.4080	0.4419	0.2792	0.4163	0.2973
Recovery, %	100.18	99.02	100.6	100.04	99.76	101.8	99.7

upon these preliminary results, Teflon was selected as the material of choice for construction of the chamber.

EXPERIMENTAL

Construction of the Sampling Device—This sampling device was constructed from a 3-in. piece of Fluoroflex "T" tubing having an internal diameter of 2.5 in. and external diameter of 3.5 in. The top and bottom of the sampling container were constructed from Fluoroflex "T" sheeting, 0.5 in. in thickness. The inside walls of the tube were threaded 0.5 in. down on each end. Both the top and bottom plates for the container were cut and threaded to match the threads of the sampling tube. The ends were than screwed into the cylinder and tightened, making a pressure-tight seal. Teflon tape was used on the threads to ensure an efficient seal. A piece of Fluoroflex "T" was machined to the same configuration as the neck and mouth of a standard uncoated glass aerosol bottle and fitted with a 20-mm. aerosol valve.[1] This assembly was then fitted into one of the openings on the top cover as shown in Fig. 1. The openings in the top plate were large enough to accommodate a Corning semimicro combination electrode with triple-purpose

[1] The valves used in this study are referred to as the "male-female" valves and were of the type used on various "mother-daughter" aerosol packages. A female-type valve was attached to the sampling device. Other standard aerosol valves can also be used provided a section of tubing or transfer actuator is attached to the valve stem.

Solutions of benzocaine NF were then prepared in a solvent mixture of 55% by weight of ethanol USP and 45% by weight of propylene glycol USP. These solutions were then placed into the sampling cylinder and assayed in a manner similar to the above described method. These results are also shown in Table I.

Having determined the suitability of the sampling device as a titration vessel, a sample aerosol product was prepared. This aerosol contained 6.3088 Gm. benzocaine NF, dissolved in a mixture of 55% by weight of ethanol USP and 45% by weight of propylene glycol USP. A sufficient amount of solvent was added to bring the total weight to 50 Gm. Propellant, consisting of a mixture of propellant 12/114 (40:60) was added to produce a total weight of 100 Gm. This formulation was packaged into a glass aerosol container and fitted with a female-type aerosol valve.

Thirty milliliters of glacial acetic acid was placed into the open sampling device and the top cover sealed into place. The two openings in the top cover were sealed with the two plugs, one containing the aerosol valve and the other the pressure release plug. The entire unit was then weighed and chilled

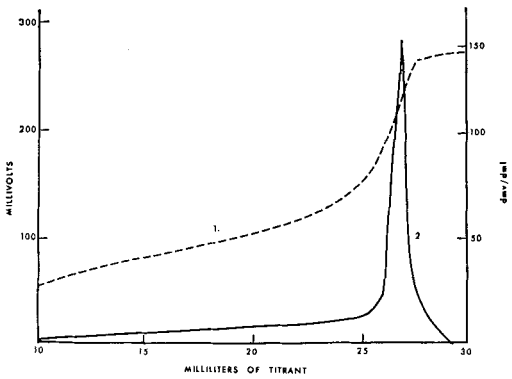

Fig. 2—Titration curve for benzocaine aerosol using 0.1 N perchloric acid as titrant. Key: 1, standard titration curve; 2, first derivative plot.

by placing it into a freezer or dry ice chest for about 10–15 min. A sample of benzocaine aerosol was then injected into the sampling chamber by inverting the sampling device over the upright aerosol container and fitting the valve stem of one valve into the valve opening located on the other container. By pushing the sampling device down, both valves will open allowing the product from the container to flow into the sampling device due to the difference in pressure between the two containers.[2] For the purpose of this study the standard type "male-female" or "mother-daughter" valves were used since these valves allow for transfer of contents from one aerosol container to another without the use of adaptors. Since most commercially available pharmaceutical aerosols do not make use of this valve, an adaptor must be used.

About 5.0 Gm. of aerosol product was allowed to flow into the sampling device. The actual amount acquired is dependent upon the nature of the active ingredient(s) and the type of assay used. This must be determined on an individual basis. The sampling device is than allowed to return to room temperature, dried of any moisture which may have condensed on the surface, and reweighed. The difference between the two weighings indicates the weight of sample.

The propellant was removed from the container by turning the pressure release plug one turn. The aerosol valve and pressure release plug are removed from the top cover and the openings fitted with the electrode and buret as previously described. The remaining solution was then titrated with 0.1 *N* perchloric acid and the equivalence point determined potentiometrically, and the milliliter of perchloric acid was obtained from a first derivative plot as illustrated in Fig. 2. From this result the amount of benzocaine in the formulation was calculated. These results are also indicated in Table I.

Various commercially available pharmaceutical aerosols were assayed using this sampling device and the appropriate assay. The aerosols utilized in this study are indicated in Table II. These products were selected on the basis of being representative of various types of aerosols requiring different analytical procedures for assay.

[2] This procedure is applicable for those aerosols containing a dip tube. Where no dip tube is present, the positions of the sampling device and aerosol container are inverted so that a liquid sample is obtained. Aerosols used in the upright position contain a dip tube attached to the valve housing while those used in the inverted position do not have a dip tube. Certain aerosols containing a dip tube and special valve can be used in either position.

Table II—Representative Pharmaceutical Aerosol Products

Active Ingredient	Concn.	Amount/ Dose, mg.
Epinephrine bitartrate[a]	7.0 mg./ml.	0.3
Ergotamine tartrate[a]	9.0 mg./ml.	0.36
Isoproterenol HCl[b]	2.5 mg./ml.	0.125
Prednisolone[c]	0.332 mg./Gm.	Non-metered
Cyclomethycaine HCl[b]	2.645 mg./Gm.	Non-metered
Pramoxine HCl[c]	10.0 mg./Gm.	Non-metered
Epinephrine HCl[a]	8.3 mg./ml.	0.3
Tetracaine[b]	10.0 mg./Gm.	0.5

[a] Suspended in vehicle of propellent with dispersing agents. [b] Solution in vehicle of propellant, ethanol, and other agents. [c] Solution in vehicle of propellant and other agents.

These products were separated into two groups; those that could be assayed by a potentiometric technique and those that utilize a spectrophotometric method. All of these aerosols contained a valve fitted with a stem. In order to effectively transfer material to the sampling container, a small piece of polyethylene tubing was fitted over the stem and attached to the sampling device. A transfer actuator could also be used.

Potentiometric Method—The aerosol product was sampled by determining the tare weight of the sealed sampling device containing 20 ml. of glacial acetic acid and fitted with the valves previously described. After chilling the device a sample of product was injected into the chamber. The weight of sample was determined by allowing the contents to reach room temperature and then reweighing the container. Where the aerosol product was fitted with a metered valve, the number of doses injected was recorded. In this manner the quantity of active ingredient dispensed each time the valve was depressed could be determined. The propellant was allowed to escape through the pressure release valve. The valves were removed from the top and replaced with the buret and electrode. The active ingredient was then assayed by titrating the nonaqueous solution with 0.01 *N* perchloric acid in glacial acetic acid as previously described.

Aerosol products containing tetracaine, epinephrine bitartrate, epinephrine hydrochloride, pramoxine hydrochloride, and cyclomethycaine hydrochloride were assayed by this method and the results are shown in Tables III and IV.

Spectrophotometric Method—Essentially the same method as described in the previous section was developed for the aerosols assayed spectrophotometrically. In most cases, part of the solvent used in the assay was added to the sampling device which was then weighed. The aerosol sample was injected into the tared and chilled container in a manner similar to that previously described and the weight of sample determined by reweighing the container after the contents attained room temperature. The propellant was allowed to escape and the contents were transferred to a volumetric flask. The sampling container was rinsed with solvent until all of the active ingredient was removed. The rinsings were added to the volumetric flask, necessary reagents added, and the solution was then

TABLE III—ASSAY OF METERED AEROSOLS BY POTENTIOMETRIC TITRATION

Active Ingredient →	Tetracaine		Epinephrine Bitartrate		Epinephrine HCl	
	Determination No.					
	1	2	1	2	1	2
No. of sprays	30	20	30	30	30	30
Amt. of active ingredient, mg.[a]	15.0	10.0	9.0	9.0	9.0	9.0
0.01 *N* perchloric acid, ml.	5.65	3.77	2.61	2.63	4.18	4.14
Amt. found, mg.	14.9	9.95	8.96	9.04	9.18	9.10
Recovery, %	99.4	99.53	99.62	100.4	102.0	101.0
Amt./dose, mg.	0.497	0.498	0.299	0.301	0.306	0.303

[a] Calculated on basis of labeled amount.

TABLE IV—ASSAY OF NONMETERED AEROSOLS BY POTENTIOMETRIC TITRATION

Active Ingredient →	Cyclomethycaine HCl		Pramoxine HCl	
	Determination No.			
	1	2	1	2
Wt. of sample, Gm.	10.6140	14.1880	3.0865	3.6942
Amt. of active ingredient, mg.[a]	28.0	37.47	30.81	36.94
0.01 *N* perchloric acid, ml.	7.085	9.45	9.325	14.2
Amt. found, mg.	28.04	37.42	30.71	36.95
Recovery, %	100.13	99.87	99.67	100.02

[a] Calculated on basis of labeled amount.

TABLE V—SPECTROPHOTOMETRIC ASSAY OF SEVERAL METERED PHARMACEUTICAL AEROSOLS

Active Ingredient →	Ergotamine Tartrate			Isoproterenol HCl		
	Determination No.					
	1	2	3	1	2	3
No. of sprays	10	10	10	40	40	40
Amt. of active ingredient, mg.[a]	3.6	3.6	3.6	5.0	5.0	5.0
Absorbance of active ingredient, A_U	0.129	0.127	0.130	0.59	0.585	0.598
Absorbance of standard, A_S	0.192	0.192	0.192	0.596	0.596	0.596
Amt. found, mg.	3.599	3.543	3.627	4.95	4.91	5.02
Recovery, %	99.98	98.42	100.75	99.16	98.15	100.34
Amt./dose, mg.	0.360	0.354	0.363	0.124	0.123	0.125

[a] Calculated on basis of labeled amount per dose.

brought to the desired volume. Aliquots of the solutions were then assayed using an appropriate spectrophotometer.

Isoproterenol Hydrochloride Aerosol—This aerosol was assayed using a modification of the official method of assay (6). A Beckman DU spectrophotometer was used and the absorbance of the solution at 279 mμ was determined. The absorbance (A_U) was then compared to the absorbance (A_S) of USP isoproterenol hydrochloride reference standard and the amount of isoproterenol hydrochloride in the sample calculated. These results are shown in Table V.

Ergotamine Tartrate Aerosol—Approximately 25 ml. of tartaric acid solution (1 in 100) was added to the sampling chamber that was then sealed, weighed, and chilled. The desired number of doses of ergotamine tartrate aerosol was added, the solution mixed and reweighed after having come to room temperature. This was then treated in a manner similar to the procedure followed for isoproterenol hydrochloride aerosol, and the absorbance (A_U) at 550 mμ was determined, using a Bausch and Lomb Spectronic 20, according to the USP method of assay (7). This result was then compared to the absorbance (A_S) of a standard solution of USP ergonovine maleate reference standard. The amount of ergotamine tartrate in the aerosol sample was calculated using the conversion factor of 1.488 which is the ratio of one-half the molecular weight of ergotamine tartrate to the molecular weight of ergonovine maleate. Table V also indicates these results.

Prednisolone Aerosol—About 30 ml. of ethanol USP was added to the sampling chamber. The chamber was sealed, weighed, and chilled. A sample of prednisolone aerosol was injected and the chamber was allowed to come to room temperature and reweighed. This was then treated in a manner similar to the isoproterenol hydrochloride aerosol and the absorbance (A_U) at 525 mμ was determined using a Bausch and Lomb Spectronic 20 according to the USP method of assay (8). This result was then compared to the absorbance (A_S) of USP prednisolone reference standard, and the amount of prednisolone in the aerosol was calculated. These results are shown in Table VI.

TABLE VI—SPECTROPHOTOMETRIC ASSAY OF NONMETERED PHARMACEUTICAL AEROSOLS CONTAINING PREDNISOLONE

Active Ingredient	Prednisolone Determination Number 1	2	3
Sample weight, Gm.	3.2604	3.5874	4.2604
Amt. of active ingredient, mg.[a]	10.83	11.19	14.14
Absorbance of active ingredient, A_U	0.452	0.497	0.595
Absorbance of standard, A_S	0.545	0.545	0.545
Amt. found, mg.	10.79	11.87	14.20
Recovery, %	99.68	99.67	100.39

[a] Calculated on basis of labeled amount.

DISCUSSION

The results obtained in this study indicate the applicability of the sampling chamber to the routine

sampling and analysis of several different types of pharmaceutical aerosols. Since the container can be easily closed to make a pressure-tight seal, there is practically no loss of product concentrate or propellant. The transfer from original container to sampling device can be achieved rapidly and accurately.

The sampling device was constructed from Fluoroflex "T" in order to provide a chamber that would be compatible with almost all medicinal materials, solvents, and reagents used in various assay procedures. This material allows for close tolerances and is strong enough to withstand the pressure of the intact aerosol samples when injected into the chamber. The sampling chamber was designed so that it can be easily disassembled for cleaning while the adaptor for the aerosol valve and the pressure relief valve were designed for easy removal so that the sample could be removed or titrated directly in the chamber.

This system was found to be especially applicable to those materials which are assayed by a potentiometric titration, thus eliminating the need to transfer material from the chamber. The value of the chamber and this type of analysis becomes of increasing importance when small amounts of active ingredients are involved. Benzocaine was chosen as the material to test the sampling device since it is found in many aerosol formulations and is suitable for a nonaqueous potentiometric titration. The end points of the titrations for this material were determined potentiometrically since the use of visual indicators were not possible due to the opaque nature of the Fluoroflex "T." However, the chamber can be redesigned to provide a window for titrations utilizing a visual indicator for the end point. The possibility of interference in the titrations due to the presence of dispersing agents, suspending agents, or other adjuncts in the formulation was considered. The benzocaine aerosol consisting of benzocaine in propylene glycol and ethyl alcohol was accurately prepared, with and without the propellant, and titrated. No interference in the titration was observed as indicated by the results.

For the analysis of commercially available aerosol products, the labeled quantity of the medicinal agent present was compared to the quantity found upon analysis of the sample. In all cases, the labeled quantity of drug and the amount of drug found upon analysis varied by no more than 1%; that is, recovery was 99% or better.

Spectrophotometric analysis was performed on various aerosols so that the accuracy of the chamber could be determined for those products requiring a transfer of active ingredients. As can be noted from the results obtained, over 99% of material was recovered. These results indicated the suitability of the sampling chamber for assays requiring transfer of materials from the sampling chamber to another container such as a volumetric flask.

SUMMARY

The sampling chamber proved to provide a simple, fast, and accurate method for the sampling of a variety of aerosol products. No limitations were found in the device with the aerosol products assayed. Those products selected were chosen on the basis of the type of assay which could be used and that they represent different types of aerosols. In addition to allowing for efficient sampling, the chamber was found to be quite suitable as a titration vessel, thus eliminating the need of transferring the material after removal of the propellant to another vessel. All of the aerosol products assayed by this method gave acceptable results. Further study will involve the use of this chamber for solubility determinations of medicinals in fluorocarbon propellants, analysis of water-based aerosols, and for use with aerosol foams.

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(8) *Ibid.*, 1965, pp. 88, 501.

Keyphrases

Aerosols—pharmaceutical
Sampling device—aerosol analysis
Potentiometric analysis
UV spectrophotometry—analysis
Colorimetric analysis

Apparatus for Solution Kinetics

By J. THURO CARSTENSEN

An apparatus is described which allows for rapid convenient kinetic studies and overcomes several disadvantages associated with other kinetic study arrangements. The thermal degradation of bromothymol blue in alkaline aqueous solution has been used as a trial system to demonstrate advantages and precautions associated with the apparatus.

IT IS A GENERAL PRACTICE in the pharmaceutical industry to conduct accelerated kinetic studies by placing finished containers (ampuls, bottles) in constant temperature ovens and removing samples at appropriate times and assaying them (discarding the unused sample portions). The problem of an initial lag period in which the temperature increases from room temperature to the desired temperature is always problematic as is temperature variation within the oven itself. The fact that solutions in different containers are used at the various sampling times introduces further uncertainty into the kinetic study. When temperatures above 60° are used, short sampling intervals (2–6 hr.) are frequently necessary and under such circumstances the lag-time error becomes large and although compensation methods (equilibrium-time-temperature-equivalent) for this have been described by Eriksen (1), a straightforward method of studying a reaction which is initially at the desired temperature would be desirable. It would furthermore be advantageous to be able to sample from the same bulk at all sampling intervals.

The use of a good thermostatically controlled water (or oil) bath for this purpose can overcome the lag-time problem but is cumbersome and expensive. Agitation is difficult to achieve and sampling usually requires opening the system to the atmosphere, so that oxygen-sensitive reactions may require further precautions.

A simple and more direct means of achieving constant temperature, avoiding lag time, and minimizing air exposure and interactions with oxygen results from the apparatus setup described in the following.

APPARATUS

A schematic layout of the apparatus is shown in Figs. 1 and 2. A 500-ml. round-bottom flask with thermometer joint and equipped with a 24/40 condenser joint (G) is modified by the side arm sampling arrangement ABCD and a sample introduction well (E) with a solid stopcock (F). The stopcocks A, B, C, and D are all solid. The round-bottom flask is attached to a condenser,

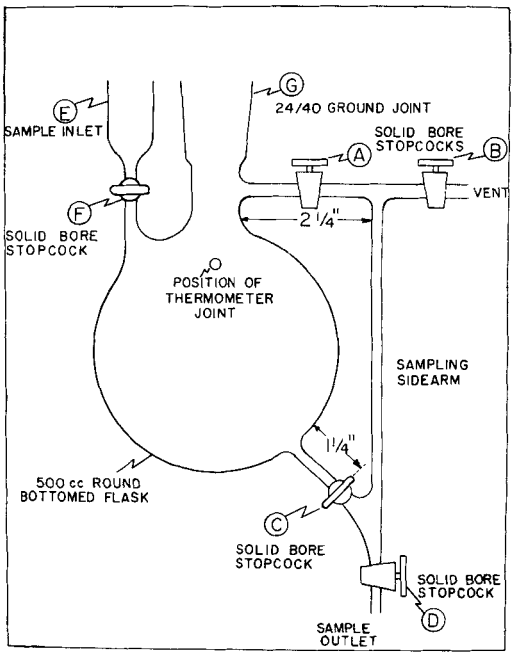


Fig. 1—Reaction flask used in apparatus arrangement showing stopcocks, joints, and sample introduction well.

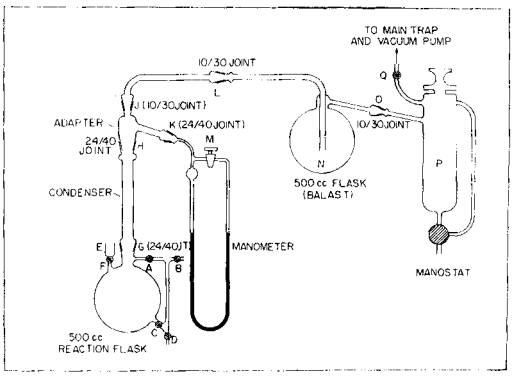


Fig. 2—Apparatus arrangement showing reaction flask, manometer, ballast, and manostat. Cross-hatched circles represent stopcocks.

which in turn is attached at (H) to an adapter with two 24/40 joints and one (top) 10/30 joint. The 24/40 side arm is attached to a direct reading manometer. The top joint (J) is connected *via* a 5-mm. tube equipped with a 10/30 ground joint inlet (L) and outlet (O). This latter is connected to a cartesian manostat[1] which in turn is connected

Received October 4, 1967, from the Extension Services in Pharmacy, University of Wisconsin, Madison, WI 53706
Accepted for publication November 8, 1967.
The author thanks Mr. Joseph A. Ranucci of Hoffmann-LaRoche, Nutley, N. J., for valuable suggestions.

[1] Manostat Corporation, New York, Manostat No. 7, dimensions: 15 in. high, 2½ in. wide at widest joint.

to a vacuum pump. If desired, a trap may be inserted between manostat and vacuum pump.

The principle and operation of the manostat is described elsewhere (2). By means of the diver the pressure of the system may be adjusted to a desired pressure.

OPERATION

The system is first run empty with the vacuum stopcock (M) open and stopcocks A, B, C, D, and E closed at full vacuum.[2] After the right side arm of the manometer has been evacuated in this fashion, stopcock M is closed and air let into the system by opening E.

The system is now operated by introducing 300 ml. of water (or other liquid)[3] *without boiling stones* or other boiling aids into the reaction flask with stopcocks A, B, C, D, and E closed. If a study at $t°$ is planned, the vacuum pump is started and the manostat operation fixed when a pressure, equivalent to the vapor pressure of water (or other liquid) at $t°$, is reached. A heating mantle[4] with the side opening is then attached to the reaction flask, so that both the bottom and the lower side arm down to stopcock C is covered. The rheostat connected to the heating mantle is adjusted to allow the water (or other liquid) to boil gently and the temperature and pressure checked periodically to ensure that the temperatures and pressure are constant. Since most studies, to which the apparatus applies, are conducted at temperatures between 60 and 100°, the temperature gradient dT/dp is very small (or conversely, dp/dT is large) so small fluctuations in pressure result only in very small fluctuations in temperature.

It usually takes less than 0.5 hr. for the temperature to stabilize. Once the temperature is stable, a concentrated solution of the substance(s) whose degradations are to be followed, are placed in the sample introduction well (E) and sucked into the system by opening and closing stopcock F, and (if desired) washing with a small amount of water. An initial sample is then taken by opening stopcock A, effecting equilibration of the pressure in the sampling side arm with the pressure in the system. Opening stopcock C will then allow the liquid to flow into the side arm. Closing stopcocks A and C and opening B restores atmospheric pressure to the sampling side arm. Opening D allows the sample to be collected. Samples are than collected at predetermined intervals in the same fashion, and assayed by suitable means.

EXAMPLES AND DISCUSSION

A kinetic study of the degradation of bromothymol blue at a concentration of 0.00134% in 0.1 NaOH was conducted by introducing 300 ml. of 0.1 NaOH into the reaction flask, and obtaining constant temperature. The 466 mg. of dibromothymolsulfonphthalein[5] was then dissolved in ethanol and the volume adjusted to 100 ml. with ethanol. The absorptivity (a) at 619 mμ in 0.1 N NaOH is 60.5. One milliliter of the ethanolic solution was then diluted to 10 ml. with 0.1 N NaOH and introduced *via* the sample introduction well, and the first sample taken 2 min. afterwards; the absorbance (A) was then determined at 619 mμ. In this manner, a true initial assay is not achieved. However, to demonstrate that this is not a particular drawback, a study at 90° was performed so that the first sample was not taken until 1 hr. after the start of the degradation study. The ensuing plot of $\ln_e (A)$ *versus* time yields good linearity and extrapolates to an absorbance at zero time, which equals the theoretical figure, calculated from the amounts added and the absorptivity cited above.

The Naperian logarithms of the first-order rate constants obtained in this fashion were plotted against reciprocal absolute temperature, and linearity is quite obvious, as seen from Fig. 3.

The study at 70° was conducted for a total of 45 hr. with the temperature fluctuating between 70 and 70.5°. Twelve samples were removed during this period. The fluctuation in the first 8-hr. period was about 0.3°.

The apparatus, hence, works well, but the following phenomena were observed and serve as good operating practices. The tighter the system is, the better the pressure control. Every time a sample is taken, a small amount of air is introduced. This causes a slight, transient temperature rise. Stopcock C would occasionally freeze up on prolonged runs, preventing the sample from flowing into the sampling side arm. The run need not be discontinued in such a case. By opening stopcock D (stopcocks A and B being closed) and then carefully opening stopcock C, a small amount of air is allowed to enter the reaction flask and the grease will dislodge. The usefulness of the apparatus is probably limited to studies of less than 48-hr. operation, and temperatures should be selected accordingly. It is, however, in these short interval studies, that initial lag time errors are cumbersome to account for, when conventional methods are used, and the method should be viewed as an adjunct to, not a replacement for, common practices.

Figure 4 shows kinetic data obtained in this latter fashion at lower temperatures and longer storage periods. In this case, sample solutions were deaerated by bubbling through nitrogen, and storing the solutions in sealed ampuls (with nitrogen in the head space) in constant temperature ovens (±0.5°). The correlation appears to be good. Finally the results from a study in closed containers in a 37° constant temperature bath is also shown in Fig. 4 and falls in line with the remaining points.

It will be noted that the apparatus described here results in solution kinetics in the absence of dissolved oxygen. This was demonstrated by checking the oxygen content after 0.5 hr. of operation (*i.e.*, when the temperature had stabilized, but prior to sample addition). Oxygen content was determined by an oxygen analyzer[6] and found to be 7.3 p.p.m. before operation and 1.7 p.p.m. after 0.5 hr. of operation.

A loss of solvent may be expected during operation, based on the temperature of the cooling water in the condenser. Using 5-mm. tubing beyond the condenser keeps this loss low. A 48-hr. run at 90° of 350 ml. water gave a total loss (by weight) of

[2] It is advantageous to cool the trap during this operation, but the trap is not needed for subsequent operations.

[3] The type of liquid (water, buffer solution, alkali, organic solvent, *etc.*) is dictated by the kinetic study in question.

[4] Glass-Col Ser. No. 553607, Scientific Glass Co. 1-1-1860-3.

[5] Eastman Kodak Co., Rochester, New York.

[6] Beckman Oxygen Sensor, Bulletin 70139, Beckman Instruments, Inc., Fullerton, California.

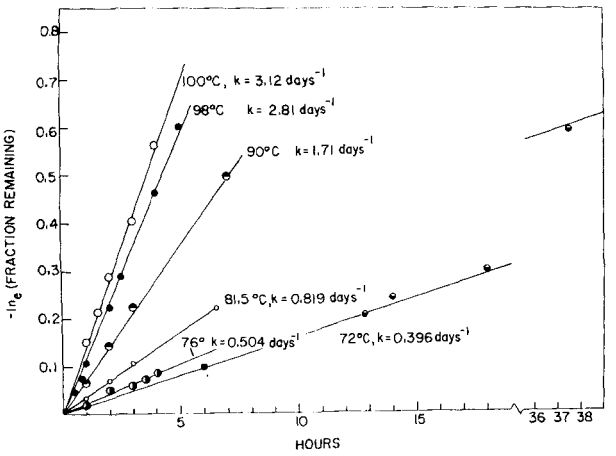


Fig. 3—Results from degradation of bromothymol blue in 0.1 NaOH at various temperatures. The run at 100° was performed at atmospheric pressure, i.e., system disconnected at joint H.

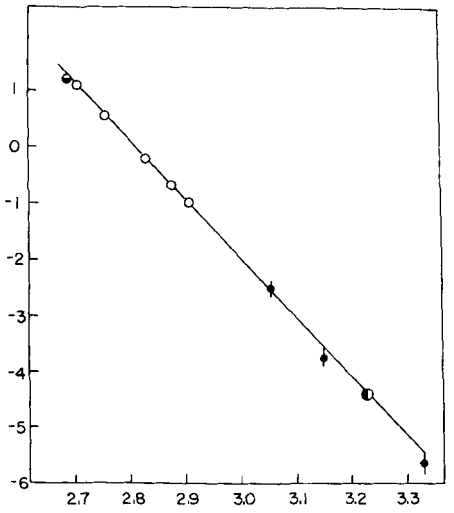


Fig. 4—Arrhenius plot of bromothymol blue degradation in 0.1 NaOH. Ordinate is the natural logarithm of the Naparian rate constant; abscissa is 1,000 × reciprocal temperature (°K⁻¹). Key: ●, runs in stationary ampuls; ◓ ◐, run in constant temperature bath; ○, runs in apparatus described here.

about 1–2 Gm. This aspect hence contributes less than a 1% error on long runs and can be considered negligible on short runs.

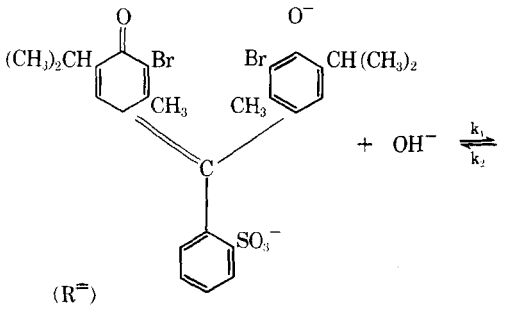
Although the operation of the manostat may be performed with the bleed valve slightly open, it appears best for stable operation, to keep it closed at all times after the required pressure has been reached.

A final point, which is also no shortcoming, is that in starting the manostat in operation at the desired pressure, the pressure attained usually differs slightly from the pressure aimed for. Letting the pressure drop slowly prior to setting the manostat can be accomplished by suitable operation of stopcock Q, and will allow fixing the temperature at the desired point fairly accurately. The point is not critical since one might, at worst, be conducting a study at, say 72°, rather then 70°, and so on, and it is not difficult to obtain six suitably separated temperatures in six separate runs, and this, after all, is the only intent of the kinetic study.

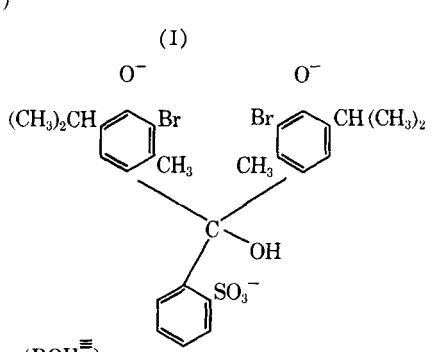
Although the mechanism of breakdown of bromothymol blue in alkaline solutions is not the primary question here, a few notes on the subject are in order. Sager *et al.*(3)[3] have reported on the alkaline degradation of bromothymol blue and find the following rate expression:

$$\frac{d(R^-)}{dt} = 0.028 \cdot 10^{-3}(R^-)(OH^-) - 0.0016 \cdot 10^{-3}(ROH^{\equiv})$$

in units of M^{-1} min.$^{-1}$, at 25.° R^- and $ROH^{\equiv}$ have the structures I and II.



(I)



(II)

At a hydroxyl ion concentration of 0.1 M, therefore, the rate constant should be 2.8×10^{-6} min.$^{-1}$ or 3.98×10^{-3} days^{-1}. The data in Fig. 4 imply a room temperature rate constant of anti-ln$_e$ (−5.45) $= 4.3 \times 10^{-3}$ days^{-1}, *i.e.*, in good agreement with the data of Sager *et al.*

SUMMARY

An apparatus is described which permits kinetic studies in solution. Bromothymol blue degradation

in alkaline solution was checked with this system and gave results in accordance with literature values.

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Keyphrases

Solution kinetics apparatus
Schematic diagram—solution kinetics apparatus
Bromothymol blue—alkaline, aqueous degradation

Effect of Size on Other Physical Properties of Granules and Their Corresponding Tablets

By ALAN M. MARKS* and JOHN J. SCIARRA

Investigations were conducted to determine the relationship and effect of granule size upon the physical properties of the granule and the tablets produced from these granules. These properties were evaluated in the areas of spacial capacity, mobility, and physical stability. Additional studies were conducted which involved the manufacture of tablets and the subsequent correlation of the results of the physical testing of these tablets to the corresponding granule size. The physical properties of the granule were found to be interrelated and for the most part, related to the granule size. In the range of the granule sizes studied in this experiment, no direct relationship was found between granule size and granule volume. Both the degree of granule friability and flowability were found to increase as the size of the granule decreased. As the granule size became smaller, the weight variation of the tablets was found to decrease. No defined or observable relationship was found to exist between the granule size and the hardness of the corresponding tablet. The effect of granule size upon tablet disintegration was not distinguishable under the conditions of this study and probably was due to the variability and limitations of the disintegration apparatus used for this determination.

VARIOUS METHODS useful for the measurement of several physical properties of granules have been indicated in the literature. Arambulo and Deardorff (1) conducted a study which showed the relationship between granule size and the resulting tablet weight. They noted that as the granule size was reduced, the average tablet weight increased, presumably due to the decrease in void space. Large granules were found to contain a greater percentage of intergranular void space as compared to smaller granules and the resulting die fill, containing a smaller volume of material, produced tablets of a lighter weight. Arambulo, Fu, and Deardorff (2) noted the effect of granule size upon the weight variation of compressed tablets. As the granule size decreased, the weight variation decreased, passing through a minimum at 400–800 μ and then increased. They also noted that larger granules were found to yield greater weight variation. This effect was presumed to be caused by variation in the proportion of voids, which they attributed in part to the varying amount of breakage of granules and the resultant removal of the powder by the movement of the feed shoe of the single-press tablet machine.

Forlano and Chavkin (3) reported a definite relationship among granule size, disintegration time, and degree of capping. Using a sodium bicarbonate granulation, tablets compressed with granules in the 8–40-mesh range exhibited a rise in disintegration time and a decrease in capping. Tablets compressed using granules of 60 mesh or smaller exhibited a decreased disintegration time and an increase in capping. It was found that the optimum granule size of the granulation they used was found in the range of 16–60 mesh. Augsburger and Shangraw (4) described a method of measuring the fluidity of semifluid powders. They felt that the measurement of tablet weight would not only give comparable fluidity values but would also have practical importance from a production standpoint.

Fonner, Banker, and Swarbrick (5) tested

Received April 11, 1967, from the Graduate Division, College of Pharmacy, St. John's University, Jamaica, NY 11432

Accepted for publication October 13, 1967.

Abstracted in part from a thesis submitted by Alan M. Marks to the Graduate Division, College of Pharmacy, St. John's University, Jamaica, N.Y. in partial fulfillment of the Master of Science degree requirements, January 1967.

Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

The authors would like to acknowledge the assistance of Leonard Kaplan, Head of Pharmaceutical Development at Walker Labs., for his aid in developing the statistical analysis of the data. They would also like to thank Walker Labs. for the use of their laboratory facilities for portions of this study.

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in alkaline solution was checked with this system and gave results in accordance with literature values.

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Effect of Size on Other Physical Properties of Granules and Their Corresponding Tablets

By ALAN M. MARKS* and JOHN J. SCIARRA

Investigations were conducted to determine the relationship and effect of granule size upon the physical properties of the granule and the tablets produced from these granules. These properties were evaluated in the areas of spacial capacity, mobility, and physical stability. Additional studies were conducted which involved the manufacture of tablets and the subsequent correlation of the results of the physical testing of these tablets to the corresponding granule size. The physical properties of the granule were found to be interrelated and for the most part, related to the granule size. In the range of the granule sizes studied in this experiment, no direct relationship was found between granule size and granule volume. Both the degree of granule friability and flowability were found to increase as the size of the granule decreased. As the granule size became smaller, the weight variation of the tablets was found to decrease. No defined or observable relationship was found to exist between the granule size and the hardness of the corresponding tablet. The effect of granule size upon tablet disintegration was not distinguishable under the conditions of this study and probably was due to the variability and limitations of the disintegration apparatus used for this determination.

VARIOUS METHODS useful for the measurement of several physical properties of granules have been indicated in the literature. Arambulo and Deardorff (1) conducted a study which showed the relationship between granule size and the resulting tablet weight. They noted that as the granule size was reduced, the average tablet weight increased, presumably due to the decrease in void space. Large granules were found to contain a greater percentage of intergranular void space as compared to smaller granules and the resulting die fill, containing a smaller volume of material, produced tablets of a lighter weight. Arambulo, Fu, and Deardorff (2) noted the effect of granule size upon the weight variation of compressed tablets. As the granule size decreased, the weight variation decreased, passing through a minimum at 400–800 μ and then increased. They also noted that larger granules were found to yield greater weight variation. This effect was presumed to be caused by variation in the proportion of voids, which they attributed in part to the varying amount of breakage of granules and the resultant removal of the powder by the movement of the feed shoe of the single-press tablet machine.

Forlano and Chavkin (3) reported a definite relationship among granule size, disintegration time, and degree of capping. Using a sodium bicarbonate granulation, tablets compressed with granules in the 8–40-mesh range exhibited a rise in disintegration time and a decrease in capping. Tablets compressed using granules of 60 mesh or smaller exhibited a decreased disintegration time and an increase in capping. It was found that the optimum granule size of the granulation they used was found in the range of 16–60 mesh. Augsburger and Shangraw (4) described a method of measuring the fluidity of semifluid powders. They felt that the measurement of tablet weight would not only give comparable fluidity values but would also have practical importance from a production standpoint.

Fonner, Banker, and Swarbrick (5) tested

Received April 11, 1967, from the Graduate Division, College of Pharmacy, St. John's University, Jamaica, NY 11432

Accepted for publication October 13, 1967.

Abstracted in part from a thesis submitted by Alan M. Marks to the Graduate Division, College of Pharmacy, St. John's University, Jamaica, N.Y. in partial fulfillment of the Master of Science degree requirements, January 1967.

Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

The authors would like to acknowledge the assistance of Leonard Kaplan, Head of Pharmaceutical Development at Walker Labs., for his aid in developing the statistical analysis of the data. They would also like to thank Walker Labs. for the use of their laboratory facilities for portions of this study.

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granular pharmaceutical solids which they prepared by five different granulation methods. Each granulation was evaluated for its physical properties. The "particulate hardness" test was of special interest. This test, based on the size of the sample, involved shaking the granules in a closed container for a constant period of time and then measuring the percentage of material retained on a 30-mesh screen. These workers found that the hardness index increased as the density of the granule increased. In order to determine the bulk density, these investigators used a 30-Gm. sample of 20/30-mesh material. The material was placed in a 100-ml. cylindrical graduate and the graduate was dropped from a height of 0.75 in. onto a hard wooden surface. This was repeated 20 times. The results of this study showed that dissimilar shapes have a great effect upon the bulk density of a material. Jones and Pilpel (6) measured the flow of granular magnesium oxide through a circular orifice. The apparatus used for the flow rate determination consisted of a vertical copper tube, with an internal diameter of 3.83 cm., fitted with a base plate. A shutter and sliding aluminum sheet were fitted to the tube. Flow rates (in Gm./min.) were determined in triplicate by collecting and weighing the powder that escaped from the tube in fixed time intervals ranging from 5–60 sec. Shutter manipulation and incipient failure of the powder column were found to affect the flow rate in the initial stages.

This study is concerned with the relationship of granule size to selected properties of the granule and the tablets prepared from these granules. Specifically, bulk volume, friability, flowability, and die fill weight will be determined and evaluated for each of the granule sizes used. A knowledge of the effect of these properties upon the production of the finished tablet will be of value in the development of tablet formulation. The effect of granule size upon the weight variation of tablets produced under production conditions as well as tablet hardness and disintegration will also be of value. The weight variation may exceed limits if the granulation contains an excess of large granules and may laminate if the particles are too fine. A specification relating to the range of granule size could be maintained as a guide and control during the manufacture of the tablet. Several of the above properties can be related to the ability of the granules to withstand the abuse of normal handling during the processing of tablets. The effect of these variables upon tablet properties were investigated and related by correlating the physical properties of the tablet to the corresponding granule size.

EXPERIMENTAL

Three different formulations were introduced into this study. Three batches of each formulation were prepared in order to have sufficient data to subject to statistical analysis.

Preparation of Granules—Formulation I is a placebo granulation, and its formula is shown in Table I. Formulation II is a sulfadiazine granulation and was chosen so that the effect of an insoluble drug constituting a major portion of the tablet weight could be determined. The formula is also shown in Table I. Formulation III is a granulation containing a soluble drug, sodium saccharin, and is present as a minor portion of the total tablet weight. The formula is also shown in Table I.

The formulations used in this study are basically the same except that formulations II and III contain an active ingredient which replaces a portion equal to half its weight each of dicalcium phosphate dihydrate USP and lactose USP. Each granulation was prepared in the same manner. The weighed ingredients were passed through a No. 30 screen and mixed in a Hobart-type mixer. Methylcellulose, 15 cps., was used as the binding agent and added dry with the powders. The powders were granulated by using a mixture of isopropanol and water. The material was spread onto trays and dried at 32° overnight in an oven with an air flow. The dried material was passed through an 8-mesh screen fitted onto an oscillating granulator. This produced granule sizes of not larger than 8 mesh and a great variety of smaller sizes, including powder of 60 mesh and finer.

In order to control the amount of moisture present in each batch, a moisture determination was made using the Cenco moisture balance. It was found that the moisture level for each batch was 1.4 ± 0.1%. By keeping the moisture at approximately the same level in each batch, the effect of moisture

Table I—Tablet Formulations

Ingredients	I Placebo (mg.)	II Sulfadiazine (mg.)	III Sodium Saccharin (mg.)
Sulfadiazine USP	...	100.0	...
Sodium saccharin USP	...	...	30.0
Dicalcium phosphate dihydrate USP	100.0	50.0	85.0
Lactose USP	100.0	50.0	85.0
Starch USP	31.0	31.0	31.0
Methylcellulose, 15 cps.	12.0	12.0	12.0
Total wt. per tablet	243.0	243.0	243.0

Table II—Classification of Granule Size Groups

Screen Size (NBS No.)	Opening, (μ)	Particle Size (Screen Size)	Particle Size (Av. μ)
8	2380	8/12	2030
12	1680	12/16	1435
16	1190	16/20	1015
20	841	20/30	718
30	595	30/60	423
60	250		

TABLE III—SPECIFIC GRANULATION BULK VOLUME[a]

Granulation	8/12 Mesh			12/16 Mesh			16/20 Mesh			20/30 Mesh			30/60 Mesh		
	A[b]	B[c]	C[d]	A	B	C	A	B	C	A	B	C	A	B	C
Placebo	92–102	95.9	1.91	93–100	96.3	1.92	95–104	98.9	1.99	96–106	99.3	1.98	90–96	93.8	1.87
Sulfadiazine	95–100	97.9	1.96	95–100	97.8	1.95	96–101	98.8	1.97	97–103	99.8	1.99	94–99	96.3	1.92
Sodium saccharin	76–78	77.1	1.55	79–82	81.0	1.62	82–84	82.9	1.65	82–86	83.2	1.66	77–80	78.7	1.57

[a] Values are expressed in milliliters (columns A and B). [b] A, range. [c] B, mean. [d] C, specific bulk volume.

upon the properties evaluated would be the same for each of the granule sizes studied.

The separation of the granule sizes was carried out through use of the Ro Tap testing sieve shaker and USP standard mesh wire screens, 8 in. in diameter. Table II indicates the screens which were used and their position in the Ro Tap sieve shaker. The particles are identified by the number of the smallest screen that they will pass through and by the number of the largest screen they will be retained upon. Depth to the wire cloth and the distance between cloths in adjacent nested series was 2 in. The screens were placed into the apparatus in the order shown in Table II. The screens rotated in a circular motion and a hammer pounded a cork mounted on a cover plate over the screens. The apparatus was operated for a period of 2 min., resulting in the separation of the granulation into the various mesh sizes. Further agitation for periods of 3–4 min. did not appreciably change the results, indicating that complete separation occurred within 2 min. Each group of granules was collected and placed into a plastic bag. The bag was sealed and stored until the granules were ready for testing.

Specific Granulation Bulk Volume—The specific bulk volume of the granulation indicates the volume/unit weight of the solid, the volume of the intraparticle pores, and the void volume or volume of the interparticle spaces. Specific bulk volume is the reciprocal of bulk density.

Fifty grams of each granulation was weighed on a balance. The granulation was then poured slowly through a funnel into a 250-ml. graduated cylinder. The graduate was tapped lightly 10 times on a towel placed on a laboratory work bench and the volume measured. This procedure was repeated three times for each individual group of granules from each of the three different formulations. This involved three granulations of each formulation, and five granule size groupings for a total of 135 readings.

TABLE IV—ANALYSIS OF VARIANCE[a]

Source of Variation	df	SS	MS	F Test
Between treatments	4	41.7	10.4	0.108
Within treatments	10	963.3	96.3	
Total	14	1005		

[a] At the 95% level of significance the tabulated $F(4,10) = 3.48$. The calculated $F = 0.108$. Therefore, the treatment effect (mesh size) is not significant.

The specific granulation bulk volume was calculated by dividing the volume by the weight of the sample. These results are shown in Table III. An analysis of variance to determine the significance of the effect of granule size upon the specific granulation bulk volume is shown in Table IV.

Granule Friability—Granule friability is related to the strength of the granule and its ability to withstand abuse during normal handling. The Roche friabilator (7) was used for this determination. The apparatus was set to rotate 25 times a min. for a 4-min. period. Ten grams of granulation was weighed and placed in the friabilator. At the end of 4 min., all of the material was placed onto a 60-mesh screen and placed into the Ro Tap apparatus. The Ro Tap testing sieve shaker was operated for 15 sec. The material remaining on the screen was weighed and the results are shown in Tables V and VI. Figure 1 indicates the friability of each of the granule sizes studied.

Granule Flowability—Granule flowability is a measure of the rate of flow of granules through an orifice. In order to measure this properly, a special

TABLE VI—ANALYSIS OF VARIANCE[a]

Source	df	SS	MS	F Test
Between treatments	4	50.98	12.7	5.08
Within treatments	10	25.47	2.5	
Total	14	76.45		

[a] At the 95% level of significance the tabulated $F(4,10) = 3.48$. The calculated $F = 5.08$. Therefore, the treatment effect (mesh size) is statistically significant at the 95% level.

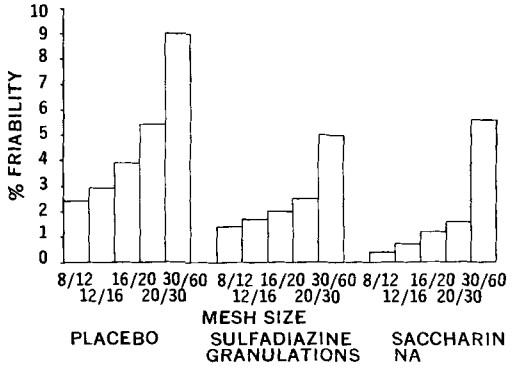

Fig. 1—Granule friability.

TABLE V—RETENTION OF GRANULES ON 60-MESH SCREEN[a]

Granulation	8/12 Mesh Range	8/12 Mesh Mean	12/16 Mesh Range	12/16 Mesh Mean	16/20 Mesh Range	16/20 Mesh Mean	20/30 Mesh Range	20/30 Mesh Mean	30/60 Mesh Range	30/60 Mesh Mean
Placebo	97.1–98.3	97.6	96.7–97.5	97.1	95.5–96.6	96.1	92.8–96.5	94.6	88.7–95.3	91.0
Sulfadiazine	98.4–98.8	98.6	98.1–98.6	98.3	97.9–98.2	98.0	97.1–97.7	97.5	94.1–95.1	95.0
Sodium saccharin	99.4–99.7	99.6	98.7–99.4	99.3	98.0–99.4	98.8	98.1–98.8	98.4	93.3–95.6	94.4

[a] Values are expressed in terms of percent remaining on 60-mesh screen.

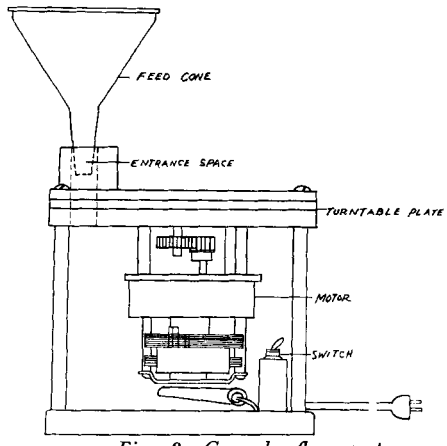

Fig. 2—Granule flow meter.

space which allows for free passage of granules through the opening and prevents the stoppage of granules in the tip of the cone. The disk rotates slowly at a constant rate, and as the opening in the disk aligns with the opening in the frame, the granules are allowed to flow through the opening. The flow of granules stops as soon as the opening in the disk is past the opening in the frame. A tared container was placed beneath the opening and the weight of granules collected during the time period determined. This procedure was repeated three times for each of the granule sizes. These results are shown in Tables VII and VIII.

Granule Size Versus Die Fill Weight—An attempt was made to determine the relationship of the granule size, in several fixed die situations, to the die fill weight. Magnesium stearate USP, in a concentration of 0.25%, was added to each batch of granules as a lubricant. A minimum amount of lubricant was used so as not to significantly influence the results. The bottom punch was lowered to a specified depth and set in place. The following settings were used:

	Diameter of Die, mm.	Depth of Fill, mm.	Tablets/ Min.
A	7	10	60
B	7	15	60
C	7	15	40
D	14	15	60

Approximately 250–300 tablets were produced from each of the granulations using a Stokes single-punch tablet press, model F, and 7-mm. and 14-mm. diameter flat-face punches. Each tablet was weighed on an analytical balance. The percent change in weight from the 8/12-size granule value was calculated. These results are shown in Table IX and

TABLE VII—GRANULE FLOWABILITY[a]

Granulation	8/12 Mesh Range	8/12 Mesh Mean	12/16 Mesh Range	12/16 Mesh Mean	16/20 Mesh Range	16/20 Mesh Mean	20/30 Mesh Range	20/30 Mesh Mean	30/60 Mesh Range	30/60 Mesh Mean
Placebo	7.675–14.045	10.3	20.380–27.116	23.8	30.949–33.486	32.1	38.110–40.068	39.1	48.378–52.066	50.4
Sulfadiazine	11.563–16.475	13.5	21.880–24.405	23.9	30.095–32.523	31.7	39.203–42.850	41.3	46.793–48.763	47.8
Sodium saccharin	11.815–19.113	14.7	24.262–29.601	26.7	34.312–37.332	36.1	45.807–47.049	46.3	58.810–59.686	59.2

[a] Values are expressed in Gm. of granulation.

TABLE VIII—ANALYSIS OF VARIANCE[a]

Source	*df*	*SS*	*MS*	*F* Test
Between treatments	4	2808.1	702.0	55.6
Within treatments	10	126.3	12.6	
Total	14	2934.4		

[a] At the 99% level of significance the tabular $F(4,10) = 5.99$. The calculated $F = 55.6$. Therefore, the treatment effect (mesh size) is statistically highly significant.

piece of apparatus was designed and made. This consisted of a circular disk mounted onto a frame as shown in Fig. 2. The disk contains an opening, 1.25 cm. × 0.75 cm., and is allowed to rotate at 2 r.p.m. by means of an electric motor. The frame is also fitted with an aluminum feed cone. In operation, the granules are placed into the feed cone through which they enter into a wider cylindrical

TABLE IX—GRANULE SIZE *versus* DIE FILL WEIGHT

Mesh Size	Placebo 8/12	Placebo 12/16	Placebo 16/20	Placebo 20/30	Placebo 30/60	Sulfadiazine 8/12	Sulfadiazine 12/16	Sulfadiazine 16/20	Sulfadiazine 20/30	Sulfadiazine 30/60	Sodium Saccharin 8/12	Sodium Saccharin 12/16	Sodium Saccharin 16/20	Sodium Saccharin 20/30	Sodium Saccharin 30/60
A (7 mm. Diameter, 10 mm. Depth, 60 Tablets/min.)															
Mean wt., mg.	161	170	174	176	192	160	172	173	175	186	207	208	209	219	231
% Change	...	5.5	8.0	9.3	19.2	...	7.5	8.1	9.3	16.2	...	0.5	1.0	5.8	11.6
B (7 mm. Diameter, 15 mm. Depth, 60 Tablets/min.)															
Mean wt., mg.	237	256	262	263	281	224	252	254	260	272	297	304	313	322	338
% Change	...	8.0	10.5	10.9	18.5	...	12.5	13.4	16.0	21.4	...	2.3	5.3	8.4	13.8
C (7 mm. Diameter, 15 mm. Depth, 40 Tablets/min.)															
Mean wt., mg.	242	256	260	262	285	241	254	257	263	276	303	310	319	322	340
% Change	...	5.8	7.4	8.6	17.7	...	5.3	6.6	9.1	14.5	...	3.3	5.2	6.2	12.2
D (14 mm. Diameter, 15 mm. Depth, 60 Tablets/min.)															
Mean wt., mg.	1059	1081	1084	1099	1163	1062	1081	1083	1096	1128	1359	1322	1308	1355	1411
% Change	...	2.0	2.3	3.8	9.8	...	1.7	1.9	3.2	6.2	...	−2.7	−3.7	−0.3	3.8

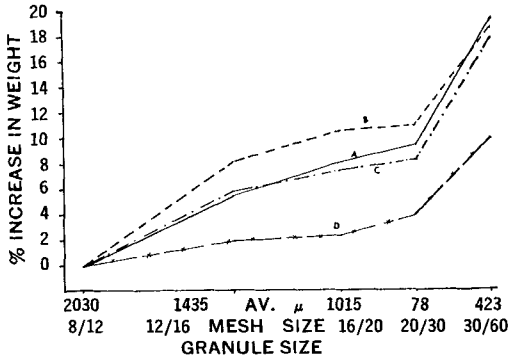

Fig. 3—Granule size versus *die fill (placebo granulation). Key:* A,——*7 mm. diameter, 10 mm. depth, 60 tabs./min.*; B,—*7 mm. diameter, 15 mm. depth, 60 tabs./min.*; C,—·—*7 mm. diameter, 15 mm. depth, 40 tabs./min.*; D,-*-14 mm. diameter, 15 mm. depth, 60 tabs./min.*

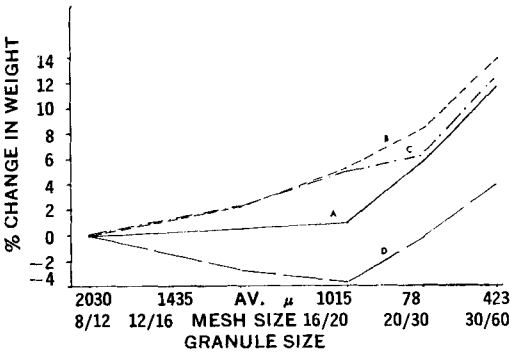

Fig. 5—Granule size versus *die fill (sodium saccharin granulation). Key:* A,——*7 mm. diameter, 10 mm. depth, 60 tabs./min.*; B,—*7 mm. diameter, 15 mm. depth, 60 tabs./min.*; C,—·—*7 mm. diameter, 15 mm. depth, 40 tabs./min.*; D,- -*14 mm. diameter, 15 mm. depth, 60 tabs./min.*

TABLE X—ANALYSIS OF VARIANCE[a] GRANULE SIZE *versus* DIE FILL WEIGHT

Source	*df*	*SS*	*MS*	*F* Test	*F*(4,10) (95% Level)
		A			
Between treatments	4	404	101	7.4	3.48[b]
Within treatments	10	135	13.5		
Total	14	539			
		B			
Between treatments	4	208.68	52.	1.2	3.48[d]
Within treatments	10	445.38	44.5		
Total	14	654.06			
		C			
Between treatments	4	350.6	87.6	30.3	3.48[c]
Within treatments	10	29.3	2.9		
Total	14	379.9			
		D			
Between treatments	4	94	23.5	3.9	3.48[b]
Within treatments	10	64	6		
Total	14	158			

[a] Percent change in weight used for analysis. See Table IX. [b] Significant at 95% level. [c] Highly significant. [d] Not significant.

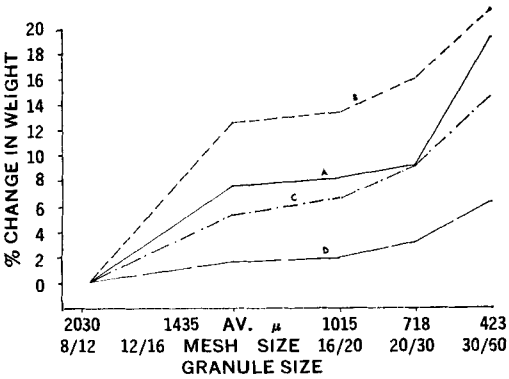

Fig. 4—Granule size versus *die fill (sulfadiazine granulation). Key:* A,——*7 mm. diameter, 10 mm. depth, 60 tabs./min.*; B,—*7 mm. diameter, 15 mm. depth, 60 tabs./min.*; C,—·—*7 mm. diameter, 15 mm. depth, 40 tabs./min.*; D,- -*14 mm. diameter, 15 mm. depth, 60 tabs./min.*

Figs. 3–5. Table X indicates the statistical treatment of these results.

Tablet Weight Variation—For this study it was necessary to adjust the tablet press to produce tablets of a specified weight and thickness. Five mg. of starch and 2 mg. of magnesium stearate were added to the granules to ensure proper lubrication and disintegration. The primary adjustment of the tablet machine was set at a weight of 250 ± 5 mg. and 0.135 ± 0.005-in. thickness. A Stokes single-punch tablet press, model F, and $^{7}/_{16}$-in. flat-face, bevel edge punches were used. The first 10 and the last 10 tablets produced were discarded to prevent weight variation due to initial flow resistance, insufficient weight of granules in the hopper, and excess fines. About 2000 tablets were produced and dusted to remove excess powder. Twenty tablets were weighed individually on an analytical balance. The mean weight, standard deviation, and coefficient of variation were calculated. Results are shown in Table XI and represent the average of 20 tablets for each of three determinations.

Tablet Hardness—Tablets from the tablet weight variation test meeting the specifications of 250 ± 5 mg. and 0.135 ± 0.005 in. were selected from each granule size group. The hardness of the tablet was measured using the Strong Cobb hardness tester fitted with an air pressure attachment. The mean for each group was calculated and the results recorded in Table XII and the analysis of variance in Table XIII.

Granule Size Versus Tablet Disintegration—The

TABLE XI—GRANULE SIZE *versus* TABLET WEIGHT VARIATION[a]

Mesh Size	8/12	12/16	16/20	20/30	30/60
		Placebo			
Mean, mg.	250	256	255	247	264
Range, mg.	229	248	234	233	253
	262	273	274	265	277
S.D.	9.6	7.5	10.3	9.1	5.3
Coefficient of variation, %	3.8	3.0	4.0	3.7	2.0
		Sulfadiazine			
Mean, mg.	245	262	247	250	251
Range, mg.	232	250	231	238	241
	265	275	261	255	254
S.D.	9.4	5.9	5.9	4.5	3.5
Coefficient of variation, %	3.8	2.3	2.4	1.8	1.3
		Sodium Saccharin			
Mean, mg.	252	257	248	249	251
Range, mg.	237	246	234	236	243
	274	271	256	254	255
S.D.	10.0	6.5	6.0	4.2	3.5
Coefficient of variation, %	4.0	2.5	2.4	1.7	1.4

[a] The treatment effect of mesh size is highly significant.

TABLE XII—GRANULE SIZE *versus* TABLET HARDNESS[a]

Mesh Size	8/12	12/16	16/20	20/30	30/60
		Placebo			
Mean	15.9	13.7	13.5	14.3	13.5
Range	11–22	11–18.5	10–17.5	10.5–17	11–19
		Sulfadiazine			
Mean	18.4	18.8	18.8	21.1	22.9
Range	15–21.5	14–24.5	13–25	17–24.5	17.5–27
		Sodium Saccharin			
Mean	15.7	16.9	15.1	14.5	13.9
Range	12–22.5	13.5–21.5	11.5–19	12–16	10–19

[a] Values shown are in Strong Cobb hardness units.

standard USP XVII disintegration method (8) for uncoated tablets was utilized. This method provides for the disintegration of six tablets in water maintained at 37°. Disks were used during this study. This method did not prove satisfactory due to the error or deviation introduced by the disks. It was reported by Kaplan and Kish (9) that many tablets will either adhere to the rising and falling disks, or adhere to the bottom stainless steel wire cloth. The result may be a flattening out of the tablet core caused by the constant action of the falling disk on the same point of the tablet surface.

The above procedure was modified by the removal of the disks from the disintegration apparatus. The disintegration time of the individual groups varied and overlapped. No definite relationship or trend due to the granule size could be noted. In this case, the error introduced by the disk was eliminated, however, the difference in the specifications of the tablets which were not greatly defined in this case possibly introduced another variable.

TABLE XIII—ANALYSIS OF VARIANCE[a]

Source	*df*	*SS*	*MS*	*F* Test
Between treatments	4	1.74	0.435	0.039
Within treatments	10	110.78	11.080	
Total	14	112.52		

[a] Based on Strong Cobb hardness units. At the 95% level of significance, the tabulated $F(4,10) = 3.48$. Calculated $F = 0.039$. Therefore, the treatment effect (mesh size) is not significant at the 95% level.

In a stricter selection of tablets having the specifications of 245–250 mg. weight and 0.134–0.136 in. thickness, a trend was discernable by one set of results but repudiated by another set, leading to an inconclusive set of data. The trend noted in one case was toward a longer disintegration time as the particles became smaller up to 20/30 mesh and then a shorter disintegration time at 30/60 mesh.

DISCUSSION OF RESULTS

The true effect of granule size upon bulk volume may have been disguised by other contributing factors such as granule shape, rate and direction of flow, positioning of the granules in relationship to each other and to the cylinder, powdering due to the impact and handling of the granules, and experimental error. The erratic results which were obtained may be due to: (*a*) The particle sizes chosen for this study may not have been sufficiently different from one another to show an effect upon the bulk volume. Theoretically, the void space should increase as the granule size is increased, thereby increasing bulk volume; (*b*) the rate and direction of flow of the granules into the cylinder may have been such so as to position the granules in a manner where bulk volume would not change appreciably with the size of the granule.

The degree of friability was found to be inversely proportional to the granule size. A definite reproducible trend in the results is observed. These results were verified by numerous readings and a statistical analysis of variance. The formula treat-

ments or the effect of the mesh size was found to be statistically significant at the 95% level of confidence. As the size of the granule becomes smaller, there is an increase in friability as shown by the greater loss of weight. This weight loss is due to material which has been detached from the granule due to the abuse taken in the apparatus and is comprised of powder 60 mesh and finer. It should be noted that the granules remaining on the screen may be of different mesh size than at the start of the test, but are still in the effective granule size range for tableting. The hardness of the particles is an important factor when considering friability. If the granule is hard, only surface particles will be lost when one particle rubs against another while with soft granules the entire granule will break into smaller particles. It is possible to determine the relative hardness of the granules, subjecting them to the friability test. In most cases, soft granules will lose considerably more weight than hard granules.

Flowability of granules was also found to be affected by the size of the granules studied. As the granule size was decreased, the flow rate of the granules through an orifice was found to be greater. These results were found to be highly significant statistically at the 99% level of confidence. A definite reproducible trend was demonstrated indicating that the size of the granule is related to the flow rate. If the size of the granules is further reduced, other factors such as bridging may interfere with its flow. However, results cannot be projected to all granule sizes. They apply only to the range of granule sizes actually studied in this investigation. Further experimentation is necessary to determine the flow rate of other granule sizes. The flow rate of the granulation and thus the granule size is significant and related to the efficiency of the die fill. This effect would be compounded by irregular shaped dies and greater die fills. The rate at which a tablet press may be operated is partially dependent upon the flow rate of the granulation. In the case of the single-punch tablet press, where there is no overfill of the dies as is found in rotary tablet presses, the flow rate of the granulation becomes more important. Where flow rates cannot be adjusted to produce satisfactory results, then the tablet press must be operated at a slower speed, or in the case of rotary presses, may require the use of force feed units.

A relationship between the granule size and the die fill weight has been noted. As the granule size is reduced the fill weight is increased. This has been observed in all four of the conditions studied. In the case utilizing setting *D*, "14 mm. diameter, 15 mm. depth, 60 tablets/min.," two readings did not follow this trend and may be due to experimental error. These results were tabulated based on a percentage change in weight and have been shown to be statistically significant at a 95% level and higher in all cases except one. The condition found not to be statistically significant when treated in this manner was setting *B*, "7 mm. diameter, 15 mm. depth, 60 tablets/min." Although it was not found to be statistically significant, the change in weight as a result of change in granule size is an important consideration in pharmaceutical production. From the results, the following was noted: (*a*) as the granule size decreases, the weight of granules required to fill the die increases; (*b*) as the depth of fill is increased, the effect of the granule size upon the die fill becomes greater; (*c*) as the speed of the tablet press used in this study is increased, the effect of the granule size becomes less; (*d*) as the diameter of the die is increased (greater fill space), the effect of the granule size is decreased and there is less of a weight difference between the sizes.

In addition to several physical properties of the granule, other factors will also influence the filling of the die in a tablet press. The diameter of the die, the depth of fill, and the speed of the machine are closely related to this property. It has been noted that as the granule size is reduced, the rate of flow of the granules is increased. While no significant difference in bulk volume due to the granule size was found, it has been suggested that the positioning of the granules as they land in the die is an important factor. Thus, the smaller granules flow through the orifice faster and are able to more efficiently fill the die as compared to larger granules. The greater friability of the smaller granules yields an increase in fines and aids in filling of the void spaces.

Experiments previously described in this study have shown that the granule size contributes to the physical properties of the granule. Granule flowability, friability, and die fill weight were shown to be influenced by the granule size. Thus, on the basis of these factors, it would be expected that the granule size would affect the tablet weight variation. In this study, the results showed an interrupted trend in weight variation due to the granule size. Upon close observation, however, a definite and statistically significant trend due to granule size was noted. Eliminating the intermediate groups of 12/16- and 20/30-mesh size from the evaluation, a definite relationship between the granule size and tablet weight variation can be noted. Due to the relative small difference in size between the neighboring groups, it is possible that these results are due to experimental error or that the size difference was not great enough to affect the weight variation. Comparing the 8/12-mesh, 16/20-mesh, and 30/60-mesh groups, the relationship was found statistically to be highly significant. As the granule size becomes smaller the weight variation was reduced. Further studies are indicated to fully substantiate this observation.

No defined or observable relationship between the granule size and the hardness of the corresponding tablet was noted in this study. It was further derived statistically that the treatment effect (mesh size) was not significant at the 95% level of significance. While no trend was noted in the results, there was an observed difference in hardness between the groups. Since granules interlock during the compression stage of tablet manufacture and result in added binding strength, the size of the granule may be related to the degree of interlocking which may take place and hence affect the hardness of the tablet.

It was not possible to obtain significant results in regard to the effect of granule size upon disintegration time. This was probably due to the limitations of the disintegration apparatus as well as the fact that there are other factors which have a greater effect upon the disintegration time.

The evaluation, knowledge, and use of granule

properties is important to the formulation, production, and quality control of tablets. The quality of tablets and the rate of productivity have been found to be influenced by the size of the granule. Automation requires standardization of the granule size for reproducibility. The testing of the granule by means of the granule flow meter and the modified friabilator may prove to be useful methods for evaluating several of the physical properties of granules.

CONCLUSIONS

(*a*) The effect upon the granule volume by the granule size was not clearly defined. (*b*) The degree of granule friability was found to decrease as the size of the granule increased. As the size of the granule becomes smaller, there is a greater loss of weight due to the friability of the granule. (*c*) Granule flowability was found to be inversely proportional to the granule size of the granule sizes tested. As the granule size becomes smaller, the flow of the granules through an orifice was found to be greater. (*d*) As the granule size decreases, the weight of the die fill increases. As the depth of fill is increased, the effect of the granule size upon the die fill is increased. As the speed of the tablet machine is increased, the effect of the granule size upon the die fill is decreased. As the diameter of the die is increased (greater fill space), the effect of the granule size is decreased and there is a smaller weight of fill difference between the sizes. (*e*) As the granule size becomes smaller, the tablet weight variation was found to decrease. This was found to exist especially in the 8/12, 16/20, and 30/60 groups. (*f*) No defined or observable relationship between the granule size and the hardness of the corresponding tablet was noted under the conditions of this test. However, difference in hardness between the groups was observed. (*g*) Due to the variability and limitations of accuracy of the tablet disintegration apparatus, no effect was noted in regard to granule size and disintegration time.

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Keyphrases

Granule size—effect, physical properties
Tablets, physical properties—granule effect
Friability—granule size
Flowability—granule size
Die weight fill—granule size
Weight, tablets—granule size

Electrostatic Characteristics of Pharmaceutical Solids and Packaging Materials I

Design of Testing Equipment and Preliminary Findings

By LEON LACHMAN and SONG-LING LIN

The design and operating principles of an apparatus capable of measuring the inherent static charge on materials, as well as being able to induce a positive or negative electrostatic potential, are described. The dual polarity high voltage power supply unit of this instrument can apply up to 10,000 v. to the materials under study. The voltage accumulation or decay of the sample being evaluated in the modified Faraday cage of the apparatus can be read on the ammeter of the electrostatic voltage sensing pistol or displayed on an X-Y recorder from the sensing pistol. Measurements are reported on the relative static electrification tendencies of sodium chloride, stearic acid, sulfisomidine, and iodochlorhydroxyquin when exposed to negative and positive potentials of 6,000 v.

THE ACCUMULATION of electrical charge on solid and solid-liquid systems by the process of static electrification is one of the earliest physical phenomena known. However, because of the complexity of the problem and mechanisms involved and an incomplete understanding of solid-state reactions, this phenomenon has only within the past 20 years received considerable

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Accepted for publication October 23, 1967.
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attention. Consequently, the problem associated with electrostatics of solids is still not completely understood, and this has, for the most part, not permitted the characterization of the static electrification behavior of a given material quantitatively.

Static electrification may be defined to encompass all processes which produce segregation of positive and negative electrical charges by mechanical actions operating through contact, impact, or friction between two surfaces, or by rupture or separation of solid and liquid surfaces. It is evident from this description that common pharmaceutical processing procedures, such as mixing, micronizing, milling, sieving, compressing, spray drying and congealing, pan coating, and packaging can induce static electrification. The forces of attraction and repulsion that exist between charged surfaces can cause difficulties in the efficient operation of processing equipment, as well as fire and explosion hazards, and affect the quality of the pharmaceutical product.

For example, in the preparation of tablets there is an accumulation of electrostatic charges resulting from the blending, granulating, and compression processes. In solids blending, electrostatic charges developed on the surface of the particles is one of the principal factors for the phenomenon of unblending. This unmixing effect can cause drugs to be nonuniformly distributed in the mixture resulting in unit doses of variable drug content.

The contact, impact, and friction encountered by the surfaces of the granules as they are fed from the hopper, revolve around the turret and are compressed contribute to the overall charge development on the finished tablets. With the advent of high speed tablet presses and forced-flow powder feeding devices, frictional electrification has become aggravated. The charge on the tablet surface can cause tablets to adhere to the feed frame and "back-up" resulting in serious damage to the tableting equipment. In compression coating the charge developed on the core tablets could cause serious core dislocation problems by adhering to the transfer mechanism and not falling into the center of the bed of coating granulation in a flat position.

Although it has been generally recognized that static electrification can be a serious problem in the processing and handling of pharmaceutical solids, only a minimal number of reports appear in the literature on this subject (1–7). These reports presented limited information, and the data were given essentially in a qualitative manner.

It became evident that before more useful information could be gathered relative to the electrostatic characteristics of various pharmaceutical solids and packaging materials, a more informative method of measuring and evaluating (*a*) the inherent polarity and magnitude of the charge on the material, (*b*) the tendency of the material to take on a negative and positive potential, and (*c*) the decay rate of the charge from the material, must be developed. It is only by having such a reliable instrument available that it will be possible to acquire the fundamental knowledge regarding the electrostatic behavior of these materials to permit a scientific approach toward alleviating static electrification problems in pharmaceutical systems.

Such an instrument was designed and fabricated and is presently being used in these laboratories. Because of its unique capabilities, it should be of considerable utility to the pharmaceutical research scientist concerned with product and process development and packaging materials research. In this report, full details will be given relative to the design and operating characteristics of the electrostatic tester. In addition, certain preliminary findings will be presented on its use with several pharmaceutical solids.

ELECTROSTATIC TESTING INSTRUMENT

The photograph in Fig. 1 shows the electrostatic tester, X-Y recorder, and humidifying chamber. The humidifying chamber is at the right of the photograph and X-Y recorder on the left. In the center of the photograph is located the electrostatic tester that is composed of three parts: a dual polarity high voltage power supply unit, a modified Faraday cage, and an electrostatic voltage sensing pistol. A simple schematic diagram given by Fig. 2 describes the composite photograph in Fig. 1.

In order to more clearly describe the function of the various components shown in Fig. 1, the more important ones will now be described individually. The high voltage power supply component of the electrostatic tester is capable of inducing a positive or negative voltage up to 10,000 v. on the material

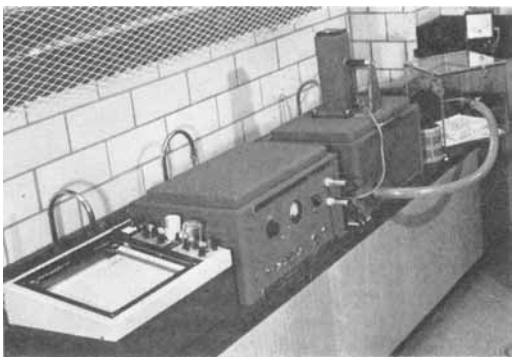

Fig. 1—Composite photograph of electrostatic tester, X-Y recorder, and humidity chambers.

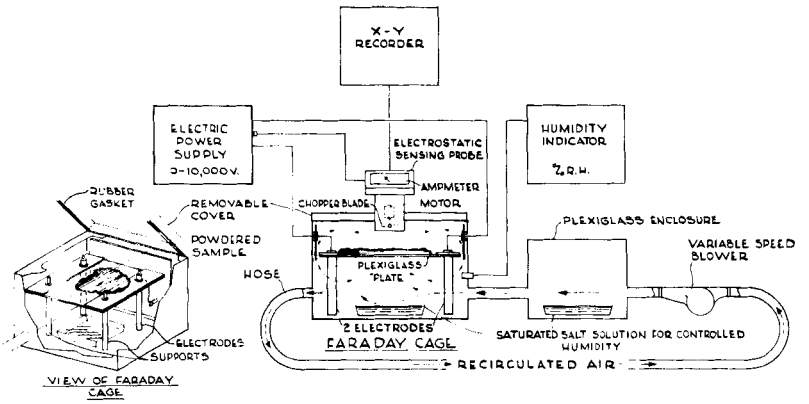

Fig. 2—Schematic diagram depicting the several components of the electrostatic tester and auxiliary equipment.

to be tested in the modified Faraday cage. This power-supply unit also serves to charge the battery system in the electrostatic voltage sensing pistol. The electrical circuitry and components of the power supply are given in Fig. 3, as can be seen from the diagram, it serves a dual function in providing the low voltages to charge the three batteries in the electrostatic voltage sensing pistol, as well as the high voltages for use in inducing a positive or negative potential on the materials in the modified Faraday cage. In one instance, the 110-v. a.c. current supply is converted by 12.6 and 36.0 v. center tap transformers and then full wave rectified to provide $+7.5 \pm 13.5$-v. d. c. for the three batteries. The 2,000 μF capacitors and the chokes smooth out the waveform and remove any ripples from the d.c. supply. The additional resistors and Zener diodes in the circuit provide the stabilized voltages to the batteries. The 110-v. a.c. current supply also feeds the high voltage supply which is a sealed solid state unit having a variac to control the output voltage over a range from 0–10,000 v. The contactor circuitry for the high voltage power supply consists of relay K-2 and conventional start and stop circuitry. The circuitry also contains a safety relay circuit K-1 that is adjusted by a preset resistor to cut out the high voltage power supply in the event the amperage goes beyond 0.0006 amp. The negative or positive on the center tap of the power supply can be grounded in order to achieve a positive or negative, as well as a floating, applied voltage.

The modified Faraday cage, shown at the far left of Fig. 1, where electrostatic voltages are supplied to the sample undergoing evaluation, consists of four Lucite posts which are used to support the sample plate in position. Two of these posts serve as electrodes as is evidenced by the brass collars on them. The plate containing the sample for measurement is made of Plexiglas and is $^1/_{16}$ in. thick, 17 in. long, and 9 in. wide. A 2-in. strip of stainless steel is bonded to each end of the plate and

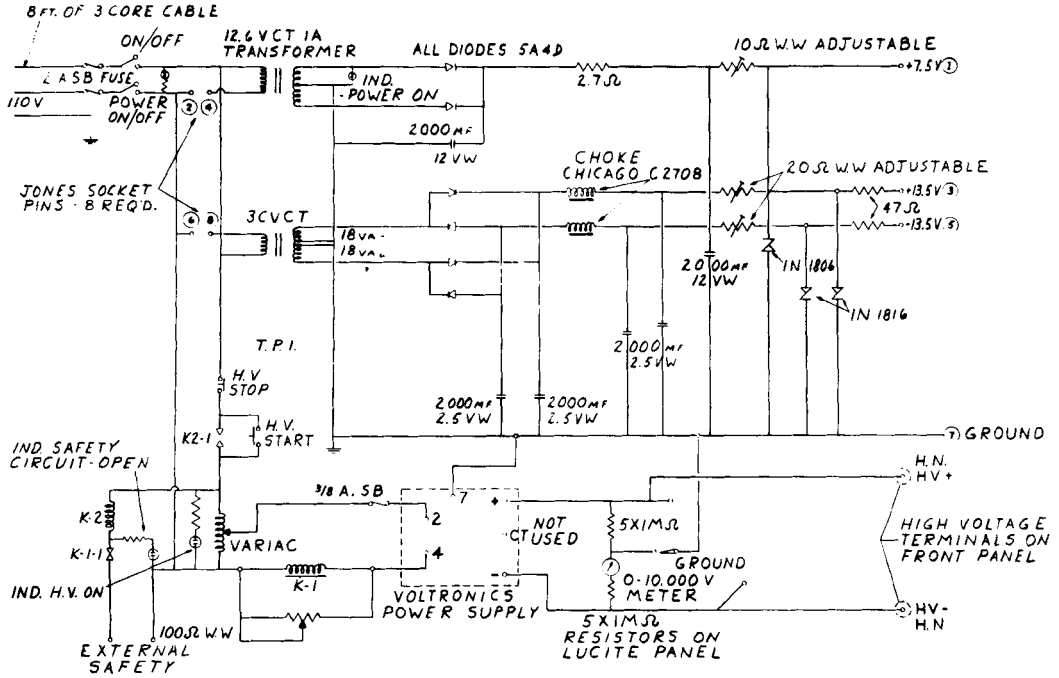

Fig. 3—Composite circuit diagram for power supply to electrodes of modified Faraday cage and batteries of electrostatic sensing probe.

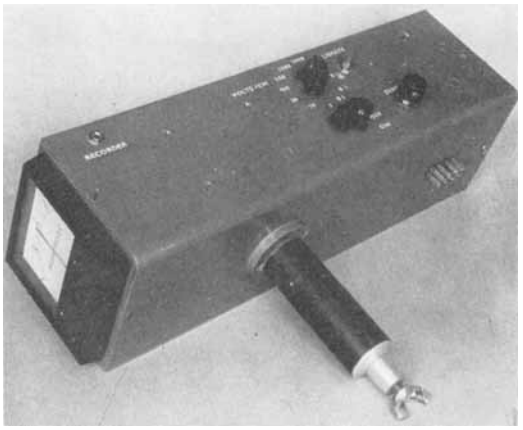

Fig. 4—Electrostatic voltage sensing probe.

the holes in the plate that fit on the Lucite posts go through these steel sections making contact with the electrodes. At the bottom of this cage is located a pan containing a saturated salt solution for humidity control, as well as humidity sensing probe. On two sides of the cabinet housing the Faraday cage, there are openings for connecting the tubing from the humidifying cabinet. The metal cabinet housing the Faraday cage is grounded.

The electrostatic voltage sensing pistol shown in Fig. 4 is detachable from the top of the cabinet housing the Faraday cage for use separately or within the cage. The meter indicator on the sensing pistol is used to standardize the sensor, as well as read out the negative or positive electrostatic voltage on the sample. A jack is located at the side of the sensor near the read out meter which is used for attaching the cable from the X-Y recorder. The recording jack will give an output of 100 mv. for full-scale deflection of the meter and has an output impedance of 100 ohms. For maximum stability, the sensing pistol should be connected to the power supply unit through the Jones plug at the side of the pistol. The pistol will, however, operate on the internal battery system for periods up to 2 hr. Rechargeable nickel-cadmium batteries are used to power the amplifier and chopper motor. The first three positions B_1, B_2, and B_3, shown on the range switch on the side of the pistol are provided for battery checking purposes. A fully charged battery system will read full scale on the positive or negative sides of the meter for battery polarity. The reading ranges for the sensing probe are calibrated in v./cm. Calibration is achieved with the sample plate in place and inducing a positive or negative potential across the plate from the high voltage supply unit. When the pistol is positioned in the holder and cavity at the top of the metal cabinet housing the Faraday cage, its sensing element is 20 cm. from the charged plate. If the plate is then charged to 6,000 v. and with the range switch on the pistol set at 300 v./cm., the pistol is calibrated so that it reads full scale.

A detailed schematic drawing of the electrical circuitry and components of the pistol is shown in Fig. 5. The power supply to the pistol enters through the Jones plug connection to the power supply unit. The probe consists of a 1-cm. metal disk insulated to greater than 10^{14} ohms by a Teflon insulator. The disk is rigidly mounted to assure stability of measurement and the lead to the amplifier is shielded by a grounded conductor so that it does not pick up any stray electrical signals. Between the disk and the source of electrostatic potential is a rotating shutter which is in close proximity to the disk. The disk will see alternately the source of potential and the shutter, and since the shutter is true ground, it will cause the disk to be grounded. Simultaneously with the shutter being opposite the disk, a photocell is exposed to light by another blade of the same shutter. The photocell has an impedance of 10^{10} ohms dark and 10^{6} ohms when exposed to a low level of light. Consequently, the disk is virtually grounded when the shutter is in front of it and has a high impedance when exposed to static charge. The photocell is exposed to low level illumination from the lamp, to give it optimum sensitivity and overcome any failure to return to high resistance during the dark period. A d.c. permanent magnet shielded motor is used to drive the chopper to prevent any electrical noise from developing which could interfere with the charge measurement. The 0.01 μF capacitor on the motor circuitry is used to eliminate any electrical noise from the motor brushes which could interfere with the charge measurement.

The output of the sensing system is a series of half wave pulses whose polarity is opposite that of the electrostatic charge. The potential is amplified

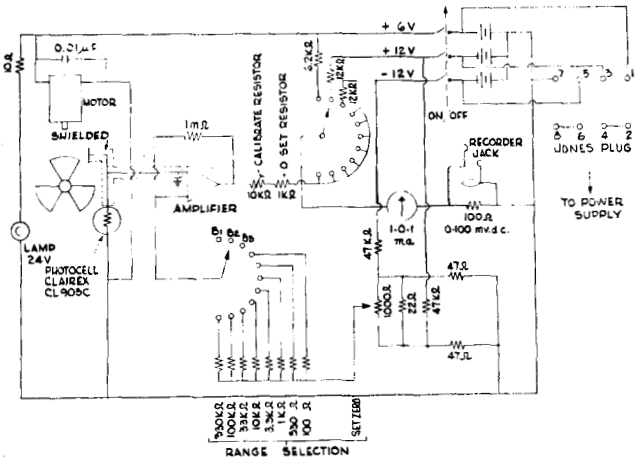

Fig. 5—Schematic drawing of the electrostatic sensing probe circuitry and auxiliary components of the probe.

and integrated by a differential operational amplifier whose gain is controlled by a feedback network consisting of a 1 megohm resistor and a resistor selected by the range selector switch. The output of the amplifier is indicated by a 1–0–1 milliammeter in series with a 10,000-ohm preset variable resistor that is used for calibration. The output could also be taken from the recorder jack as a d.c. signal of 0–100 mv. corresponding to 0–1 ma. on the meter. Consequently, the d.c. restored signal can be read out on the meter or recorded on the X-Y recorder. The ratio of the feedback and series resistors determines the gain of the amplifier and the sensitivity of the instrument. The sensitivity can be varied over the range of 1 v./cm. to 10,000 v./cm. by switching the feedback resistor. Zero adjustment of the sensing pistol is achieved by applying a ±12 mv. offset potential to the feedback system. The zero control consists of a 10-turn helical potentiometer of 1,000 ohm resistance.

The humidifying chamber constructed from Plexiglas employs a saturated salt solution in a tray at the bottom of the chamber for providing the particular humidity condition desired for the test. The chamber also has an in-line blower entering about 1 in. above the pan containing the saturated salt solution, whose speed is regulated by a rheostat. There are two reinforced plastic tubes 2 in. in diameter connected to the Faraday cage from the humidifying chamber for permitting the circulation of the humidified air during electrostatic tests.

EXPERIMENTAL

Material—Sodium chloride USP; stearic acid USP; iodochlorhydroxyquin[1] USP; sulfisomidine[2]; anhydrous ethanol.

Saturated sodium dichromate (Matheson, Coleman & Bell reagent crystal) aqueous solution to give 35% relative humidity.

Saturated sodium sulfate (Baker analyzed grade) aqueous solution to give 75% relative humidity.

Equipment—Electrostatic tester described earlier in the text; Model HR-96 X-Y recorder, Houston Instrument Corp., Houston, Tex. Hygrometer model HID 20-100 with model HS 1-6 sensor, Amlab, Inc., Rahway, N. J. Humidifying chamber for sample conditioning on Plexiglas plate prior to electrostatic measurements. Humidity cabinet for preconditioning sample plates.

Electrostatic Charge Measurement—Before performing any measurements, the unit is allowed to warm up for at least 5 min. with the 110-v. a.c. current to the power supply, sensing probe, blower in the humidity cabinet, and the recorder turned on. The Plexiglas plate which will act as the sample holder is rinsed with double distilled water, dipped into a Teflon-coated tray containing absolute ethanol, wiped gently with tissue paper saturated with absolute ethanol, and then allowed to dry under ambient conditions. The plate is conditioned at a prescribed humidity for 12 hr. before use in the Faraday cage of the electrostatic testing unit.

The electrostatic voltage sensing pistol positioned on the Faraday cage is zero calibrated by placing the palm of one hand at the sensing end of the probe and the dial reading is adjusted to zero with the zero control knob on the probe. With the selector switch of the power supply unit set at either negative or positive ground, a piece of aluminum foil is placed over the Plexiglas plate in the Faraday cage extending from one end covering the electrode and stopping short at least 1.5 in. before the 2-in. stainless steel strip begins on the other end of the Plexiglas. This arrangement will induce a positive or negative potential on the material to be tested. The lid to the Faraday cage housing the electrostatic voltage sensing pistol is closed tightly over the cage. The humidity in the Faraday cage is now allowed to reach its desired value and 10 min. after the humidity has equilibrated to the prescribed value, 6,000 v. of positive or negative potential is applied to the aluminum foil covered Plexiglas plate. With the sensing pistol set at 300 v./cm., the dial reading on the indicator of the pistol is set to +1 ma. with the calibration knob on the sensing pistol. This corresponds to the full-scale deflection of the indicating needle on the dial. The X-Y recorder is adjusted so that the abscissa reads 6,000 v./10 cm. and the ordinate reads 12 cm./min. The applied voltage is then changed to 3,000 v. and the meter reading should be half scale (0.5 ma.) which is indicated on the recorder as 5 cm. above the origin on the abscissa. The applied voltage is then grounded and the meter should read zero (0.00 ma.) which corresponds to the origin on the axis of the abscissa on the X-Y recorder.

After completing the calibration of the electrostatic voltage sensing pistol and adjustment of the axis of the X-Y recorder, the aluminum foil is removed from the Plexiglas plate. The powder sample (150 Gm.) to be measured is spread gently on the plate to cover the same area as did the aluminum foil. The lid on the Faraday cage is secured and the humidity allowed to come up to the desired level. Ten minutes after the humidity has reached its equilibrium level, a preset voltage, which in this study was 6,000 v., is applied and the charge accumulation is recorded on the X-Y recorder. When the charge has reached its equilibrium value, the sample plate is grounded and the decay characteristics of the powder are recorded.

RESULTS AND DISCUSSION

In order to demonstrate the utility of the electrostatic tester to rate pharmaceutical powders as to their static electrification properties, a good conductor (sodium chloride), a poor conductor (stearic acid), and two powdered drugs were chosen.

Since the static electrification characteristics of solids are influenced by temperature (8–9) and humidity (10–12), the studies performed in this investigation were carried out at a temperature between 22–23° and a relative humidity of 34 ±1%. Both a positive and negative electrostatic potential of 6,000 v. were applied to the samples, and their tendencies to accept and dissipate the applied charge were displayed on an X-Y recorder. The rates of charge accumulation and decay were calculated for each of the substances evaluated.

The plots in Figs. 6–8 represent the charge and decay curves for sodium chloride, stearic acid, and sulfisomidine, Since the curve for iodochlorhydroxyquin and sulfisomidine closely resembled that shown for stearic acid in Fig. 7, it was not

[1] Vioform, Ciba.
[2] Elkosin, Ciba.

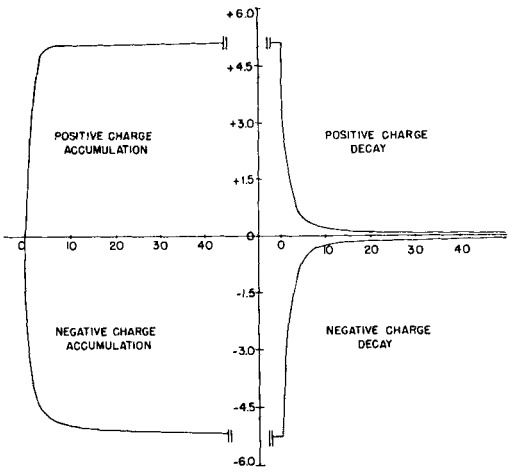

Fig. 6—Charge accumulation and decay cycles for sodium chloride at positive and negative applied potentials of 6,000 v. Abscissa is time in sec.; ordinate is voltage in Kv.

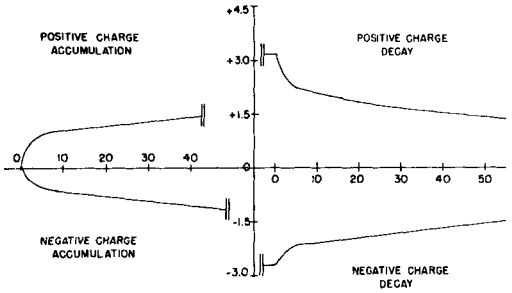

Fig. 7—Charge accumulation and decay cycles for stearic acid at positive and negative applied potentials for 6,000 v. Abscissa is time in sec.; ordinate is voltage in Kv.

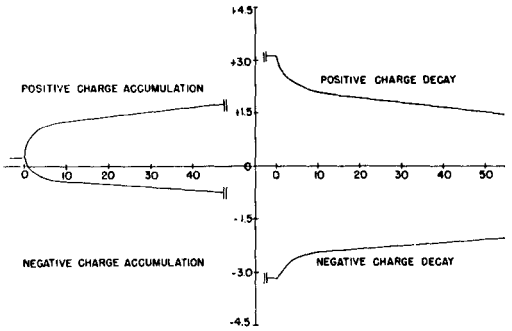

Fig. 8—Charge accumulation and decay cycles for sulfisomidine at positive and negative applied potentials for 6,000 v. Abscissa is time in sec.; ordinate is voltage in Kv.

presented. It can be seen from Fig. 6 that sodium chloride is a good conductor since the charge accumulation and decay under both positive and negative applied potential takes place readily as demonstrated by an accumulation of approximately 90% of the applied voltage within 5 sec. While in the case of stearic acid which is a poor conductor, the charge accumulation and decay is considerably slower as depicted in Fig. 7 since even after 60 sec. only about 50% of the applied potential has accumulated on the material. This is further evident

TABLE I—EQUILIBRIUM POTENTIAL ON POWDERS AT APPLIED POTENTIAL OF 6,000 V.

Materials	Polarity of Applied Potential +	−
Sodium chloride	85.0%	90.0%
Stearic acid	55.0%	45.0%
Sulfisomidine	60.0%	48.0%
Iodochlorhydroxyquin	50.2%	50.3%

by the data in Table I which summarizes the percent applied potential that has been accumulated on the powder material at equilibrium (t^{∞}).

The data in this table were calculated in accordance with the following equation:

$$\% = \frac{\text{limiting potential at } t^{\infty}}{\text{applied potential}} \times 100$$

The equilibrium time, t^{∞}, is taken as the time at which the voltage on the sample remained essentially at a steady state for 0.5 hr.

It is evident from the data in this table that the percent saturation at equilibrium of the applied potential was approximately twice as great for sodium chloride as for stearic acid regardless of the polarity of the applied charge. This finding is in agreement with the concept that charge flows faster in a good conductor (sodium chloride) as compared to a poor conductor (stearic acid). Equilibrium conditions of about the same magnitude are obtained for the two drugs, sulfisomidine and iodochlorhydroxyquin at both negative and positive applied potentials and are approximately equivalent to stearic acid which is a relatively poor conductor.

For sodium chloride, stearic acid, and iodochlorhydroxyquin, there exists no measurable static charge on the surface of these materials in the steady state. However, as is evident from Fig. 8, sulfisomidine differs from the other three powders in that it shows an initial inherent positive potential of approximately 240 v.

Since it was the main purpose of this initial report to describe the design and operation of the quantitative electrostatic tester, the experimental data obtained with the four powders were treated in accordance with first-order kinetics to obtain charge accumulation and decay rates. It should be understood that although the data were treated in this simple fashion and obtained relative charge accumulation and decay rates, the overall mechanism of electrification is considerably more complex, and detailed treatment of the overall process of electrification of powders will be the substance of a separate report.

The charge accumulation and decay data plotted according to first-order kinetics for positive and negative applied voltage for a poor conductor, stearic acid, are given in Fig. 9 and for the negative applied potential for a good conductor, sodium chloride, are given in Fig. 10. It can be seen that for each material and for both charge accumulation and decay at positive and negative applied voltage, there are two slopes, the first one being steeper than the second one in each case. It is felt that the initial slope is due to the inherent tendencies of the materials to be polarized by the applied electrostatic field and the second slope represents the saturation of the surface of the material with the applied potential, as well as loss of charge from the material due to radiation and conduction to the

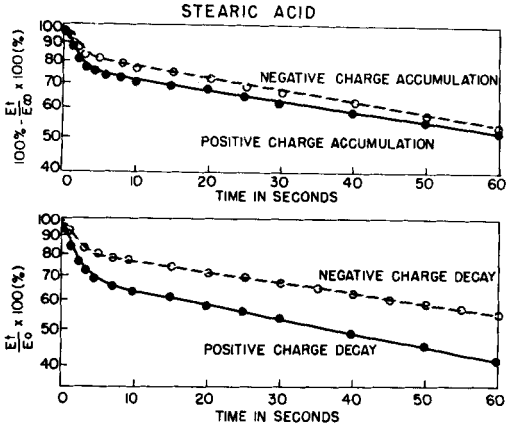

Fig. 9—First-order plot of charge accumulation and decay data for stearic acid at a positive (——) and negative (---) applied potential of 6,000 v.

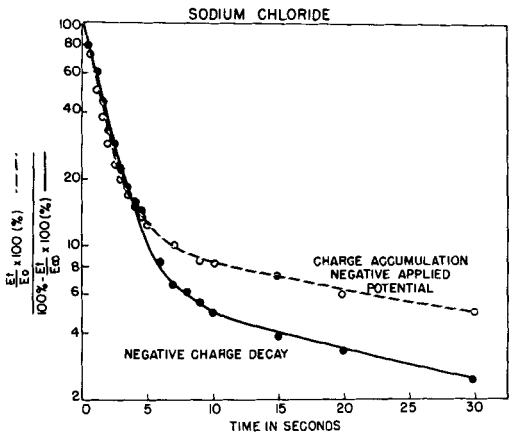

Fig. 10—First-order plot of charge accumulation and decay data for sodium chloride at an applied negative potential of 6,000 v.

TABLE II—ACCUMULATION AND DECAY OF NEGATIVE AND POSITIVE ELECTRIFICATION AT APPLIED POTENTIAL OF 6,000 V.

Materials	Charge	Accumulation Rate, sec.$^{-1}$ k_1	Accumulation Rate, sec.$^{-1}$ k_2	Decay Rate, sec.$^{-1}$ k_1	Decay Rate, sec.$^{-1}$ k_2
Sodium chloride	+	8.04×10^{-1}	1.77×10^{-1}	5.52×10^{-1}	1.62×10^{-1}
	−	5.36×10^{-1}	2.70×10^{-2}	4.90×10^{-1}	3.30×10^{-2}
Stearic acid	+	8.92×10^{-2}	5.90×10^{-3}	1.06×10^{-1}	8.35×10^{-3}
	−	7.90×10^{-2}	6.37×10^{-3}	5.36×10^{-2}	6.96×10^{-3}
Sulfisomidine	+	1.12×10^{-1}	8.01×10^{-3}	8.72×10^{-2}	9.96×10^{-3}
	−	7.59×10^{-2}	3.07×10^{-3}	7.67×10^{-2}	3.58×10^{-3}
Iodochlorhydroxyquin	+	1.12×10^{-1}	4.68×10^{-3}	5.82×10^{-2}	5.41×10^{-3}
	−	5.29×10^{-2}	3.60×10^{-3}	7.60×10^{-2}	9.62×10^{-3}

surrounding air. The rate constants for these plots are summarized in Table II. It is evident from these data that for the four powders the initial rate for either charge accumulation or decay is greater than the second rate. The rate of charge accumulation or decay is generally greater for the positive voltage than for the negative voltage.

These preliminary findings indicate that it is possible to rate the static electrification tendencies of powdered materials by the use of the electrostatic tester and treatment of the data in accordance with first-order kinetic principles. In subsequent publications of this series, the relationship between static and dynamic charge development of solids will be explored.

SUMMARY AND CONCLUSIONS

The design and operational characteristics of an electrostatic tester were presented. This equipment consists of (*a*) a power supply unit capable of inducing up to ±10,000 v. on materials to determine the tendencies of these materials to acquire and leak off electrostatic charges, (*b*) a modified Faraday cage to induce the applied electrostatic potential on the material, and (*c*) an electrostatic voltage sensing pistol capable of reading out the voltage on the sample or displaying the voltage on an X-Y recorder. The utility of this equipment to rate pharmaceutical powdered materials as to their tendencies of acquiring static electrification has been demonstrated.

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Keyphrases

Pharmaceutical solids—electrostatic characteristics
Electrostatic tester—design, operation
Schematic diagram—electrostatic tester
Static electrification—positive, negative, applied

Cholinergic Receptor I. Cholinomimetic Activities of Some Analogs of *cis*-2-Methyl-4-dimethylaminomethyl-1,3-dioxolane Methiodide

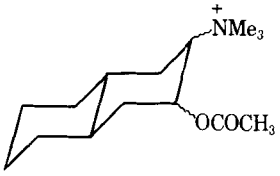
By M. MAY and D. J. TRIGGLE

The cholinomimetic activities of acetylcholine, *cis*-2-methyl-4-dimethylaminomethyl-1,3-dioxolane methiodide, *N*-methyl-6,8-dioxa-3-azabicyclo[3.2.1]octane methiodide, and *N*-methyl-7,9-dioxa-3-azabicyclo[4.2.1]nonane methiodide have been compared and an interpretation of their relative activities in terms of a conformation of receptor-bound cholinomimetic molecules has been put forward. *N*-Methyl-7,9-dioxa-3-azabicyclo[4.2.1]nonane methiodide has been shown to have some acetylcholine releasing activity.

A FUNDAMENTAL assumption in the analysis of structure–activity relationships is that mutual complementarity exists between the drug and receptor. With this assumption, analysis of structure–activity relationships should yield information relevant to an understanding of the binding sites of the receptor. However, with flexible molecules there is no reason to assume that the conformation of lowest free energy in the solution or crystalline state is necessarily that which is involved in binding at the receptor surface. In the absence of evidence concerning the conformation of receptor-bound molecules, analysis of structure–activity relationships in terms of the relative geometry of binding sites becomes equivocal.

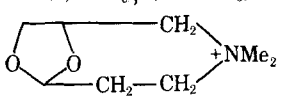
A partial solution to this problem may be obtained through the use of conformationally rigid analogs of active molecules where the distances between potentially important binding groups are fixed. A number of workers have used this approach in attempts to define more precisely the structure of the cholinergic receptor (1, 2). Most recently, Smissman, LaPidus, and their co-workers (3) have studied the four isomeric 2-acetoxy-3-trimethylammonium-*trans*-decalins (I*a*,*b*,*c*,*d*), which contain the basic acetylcholine structure, and found them to have negligible muscarinic activity. However, the observed inactivity of these compounds may well be due, not to a failure to reproduce the geometry of receptor-bound acetylcholine, but to the incorporation of additional potential binding groups—specifically the large hydrocarbon skeleton—a feature which has been repeatedly demonstrated to depress cholinomimetic activity and convert agonists to antagonists (4, 5).

This latter point is considered to be of substantial importance. Accordingly, in this approach to the design of semirigid and rigid analogs of acetylcholine the authors have endeavored to meet the requirements of minimum conformational flexibility without the incorporation of additional potential binding groups. In this paper are reported the synthesis

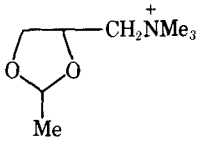


II

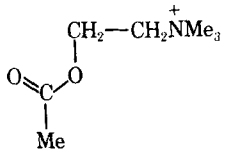
I*a* (*a*, NMe_3; *a*, $OCOCH_3$)
b (*a*, NMe_3; *e*, $OCOCH_3$)
c (*e*, NMe_3; *e*, $OCOCH_3$)
d (*e*, NMe_3; *a*, $OCOCH_3$)



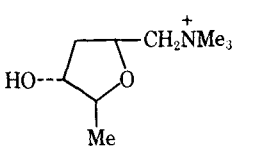
III



IV



V



VI

and cholinomimetic activity of two bicyclic compounds (II and III) which are related to *cis*-2-methyl - 4 - dimethylaminomethyl - 1,3 - dioxolane methiodide (IV) whose high muscarinic activity was first reported by Triggle and Belleau (6). *N*-Methyl - 6,8 - dioxa - 3 - azabicyclo[3.2.1]octane methiodide (II) was first reported by Fourneau and Chantalou (7) but adequate comparative pharmacological data were not presented. The close structural resemblance of IV to both acetylcholine (V) and muscarine (VI) is of considerable interest and has been noted earlier by several workers (6, 8–10).

EXPERIMENTAL

Chemistry

Melting points were determined on a Thomas-Kofler hot stage and are corrected. GLC analyses were carried out with a Varian Aerograph (model A-90-P). Analyses are by Dr. A. E. Bernhardt, Mulheim, Ruhr, West Germany.

Received June 29, 1967, from the Department of Biochemical Pharmacology, School of Pharmacy and the Theoretical Biology Center, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication September 26, 1967.

This work was supported by grant GM 11603 from the U.S. Public Health Service, Bethesda, Md.

cis - 2 - Methyl - 4 - dimethylaminomethyl - 1,3-dioxolane Methiodide (IV)—This was prepared by the method of Triggle and Belleau (6) and had m.p. 144° [lit. (6) m.p. 143–144°].

N - Methyl - 6, 8 - dioxa - 3 - azabicyclo[3.2.1]-octane Methiodide (II)—This was prepared by the method of Fourneau and Chantalou (7). *cis,-trans* - 2 - Bromomethyl - 4 - chloromethyl - 1,3-dioxolane was prepared from bromoacetaldehyde dimethyl acetal and glycerol α-monochlorhydrin (7) and was shown by GLC to consist of a 3:2 mixture of the *cis,trans* isomers. *cis,trans*-2-Bromomethyl - 4 - chloromethyl - 1, 3 - dioxolane (10.2 Gm., 0.05 mole) and methylamine (7.8 Gm., 0.25 mole) in benzene (100 ml.) were heated at 100° for 12 hr. On cooling, the mixture was filtered from methylamine hydrohalides and fractionally distilled to give *N*-methyl-6,8-dioxa-3-azabicyclo[3.2.1]octane (3.5 Gm., 87% based on *cis*-content of *cis,-trans* - 2 - bromomethyl - 4 - chloromethyl - 1,3-dioxolane) with b.p. 75°/30 mm.

Anal.—Calcd. for $C_6H_{11}NO_2$: C, 55.78; H, 8.57; N, 10.84. Found: C, 55.81; H, 8.72; N, 10.76.

N-Methyl-6,8-dioxa-3-azabicyclo[3.2.1]octane was quaternized by treatment with excess methyl iodide in ether to give II in 95% yield [MeOH–EtOH] with m.p. 252° [lit. (7) m.p. 250° dec.].

Anal.—Calcd. for $C_7H_{14}INO_2$: C, 31.12; H, 4.85; I, 46.97; N, 5.18. Found: C, 30.81; H, 5.15; I, 46.79; N, 5.00.

N - Methyl - 7,9 - dioxa - 3 - azabicyclo[4.2.1]-nonane Methiodide (III)—This was prepared by a method similar to that described for II. *cis,trans*-2-Chloroethyl-4-chloromethyl-1,3-dioxolane was prepared from β-chloro-propionaldehyde trimer (55.2 Gm., 0.2 mole, from Shell Development Corp.) and glycerol α-monochlorhydrin (22.2 Gm., 0.2 mole) by refluxing in benzene (300 ml.) in the presence of *p*-toluenesulfonic acid (0.2 Gm.) with continuous removal of water. Fractional distillation gave the product in 85% yield with b.p. 80–85°/0.2 mm., which was shown by GLC to consist of a 3:2 mixture of *cis,trans* isomers.

Anal.—Calcd. for $C_6H_{10}Cl_2O_2$: C, 38.93; H, 5.44; Cl, 38.31. Found: C, 38.81; H, 5.41; Cl, 38.21.

cis,trans - 2 - Chloroethyl - 4 - chloromethyl - 1,3-dioxolane (9.2 Gm., 0.05 mole) and methylamine (7.8 Gm., 0.25 mole) in benzene (100 ml.) were heated at 100° for 16 hr. On cooling the mixture was filtered from methylamine hydrochloride and fractionally distilled to give *N*-methyl-7,9-dioxa-3-azabicyclo[4.2.1]nonane (3.8 Gm., 91% based on presumed *cis*-content of *cis,trans*-2-chloroethyl-4-chloromethyl-1,3-dioxolane) with b.p. 85–87°/25 mm.

Anal.—Calcd. for $C_7H_{13}NO_2$; C, 58.73; H, 9.15; N, 9.78. Found: C, 58.51; H, 9.01; N, 9.71.

The *N* - methyl - 7,9 - dioxa - 3 - azabicyclo[4.2.1]-nonane was quaternized with methyl iodide to give III in 91% yield with m.p. 244–246° [MeOH–EtOH].

Anal.—Calcd. for $C_8H_{16}INO_2$: C, 33.71; H, 5.65; I, 44.51; N, 4.91. Found: C, 33.78; H, 5.64; I, 44.38; N, 4.74.

PHARMACOLOGY

Muscarinic activities of the compounds were determined using the rat jejunum. Male rats (250 Gm.) were decapitated, bled, and a length of jejunum removed and washed through with Tyrode's solution. Short lengths (3 cm.) were then suspended in oxygenated Tyrode solution in 20-ml. muscle baths with the temperature maintained at 37°. The tissues were allowed to relax for 1 hr. and cumulative dose-response curves for the compounds II, III, and IV were obtained. Dose-response curves for acetylcholine were determined simultaneously using jejunum segments from the same animal. Compounds were added in saline to give the expressed final bath concentration and isometric contractions were recorded on a Grass polygraph (model 5.D).

Nicotinic activities were determined using the frog rectus abdominus preparation. The isolated muscle was suspended in oxygenated Ringer's solution at room temperature in 20-ml. muscle baths and sensitized with eserine ($3 \times 10^{-5}M$, final bath concentration) for 20 min. Dose-response curves were determined and isometric contractions recorded with a Grass polygraph.

Acetylcholine release from the rat jejunum was determined essentially according to the method of Schaumann (11). (See also *Reference 12.*) Jejunum from three freshly decapitated rats were washed through with Tyrode's solution and cut into segments of 1.5 cm. length. To one portion (5 Gm.) suspended in oxygenated eserinized ($3 \times 10^{-5}M$) Tyrode's maintained at 35° (20 ml.) was added III to give a final concentration of 10^{-4} Gm./ml. and at 15, 30, and 45 min. additional equal amounts of III were added to give a final bath concentration of 4×10^{-4} Gm./ml. Another portion (5 Gm.) of jejunum served as a control and was treated identically save that Tyrode's was added instead of III. The incubation mixtures were then centrifuged and the supernatant fluid was assayed for acetylcholine-like activity on the rectus abdominus preparation described above.

RESULTS

Muscarinic and Nicotinic Activities—The pharmacological activities are given in Table I. The results confirm the observation of Triggle and Belleau (6) who found *cis*-2-methyl-4-dimethylaminomethyl-1,3-dioxolane methiodide (IV) to be as potent as acetylcholine in the guinea pig ileum preparation: IV is found to be only slightly less potent than

Table I—Muscarinic and Nicotinic Activities

Compd.	Rat Jejunum, ED_{50} Gm./20 ml.	Relative *M* Potency	Frog Rectus, ED_{50} Gm./20 ml.	Relative *M* Potency
Acetylcholine	$2.2 \pm 1.8 \times 10^{-7}$	1.0	$3 \pm 0.5 \times 10^{-6}$	1.0
II	Inactive at 10^{-3}	...	Produced 8% response at 10^{-4}	...
III	$2.7 \pm 4.7 \times 10^{-5}$	0.012[a]	Inactive at 5×10^{-4}	...
IV	$4.0 \pm 3.2 \times 10^{-7}$	0.75	$9 \pm 1.0 \times 10^{-5}$	0.049

[a] Produced only 75% of the maximum response (ACh = 100).

acetylcholine on the rat jejunum preparation and to have only one-twentieth of the nicotinic activity of acetylcholine on the frog rectus preparation. The bicyclic analogs, II and III, of IV were found to have negligible activities at both muscarinic and nicotinic receptors.

Acetylcholine Releasing Activities—Reproducible dose-response curves could not be obtained with III on the rat jejunum preparation. The maximum response elicited decreased with repetition of the dose-response curve until after 4–5 such determinations the response remained steady at 45–55% of the original maximum response. This behavior was in marked contrast to acetylcholine and IV which gave 6–8 highly reproducible dose-response curves over a 60–90 min. period. It was found that III was able to release acetylcholine from rat jejunum. As determined by bioassay on the frog rectus abdominus preparation, III (4×10^{-4} Gm./ml.) liberated 4.2 ± 2.4 mcg./Gm. tissue/hr. compared to a spontaneous release of 1.5 ± 1.08 mcg./Gm. tissue/hr. (P value, 0.05–0.02).

DISCUSSION

The finding that the bicyclic analogs II and III of IV have negligible activities and that, in fact, II is apparently devoid of muscarinic activity is of some interest. This finding clearly suggests that IV, and by analogy acetylcholine, acetyl-β-methylcholine, and muscarine, do not adopt, when bound at the muscarinic receptor, any of the limiting conformations represented by II and III. Whether this is related to the inactivity of II and III remains to be established through the synthesis of further bicyclic analogs of this type (14).

The complete inactivity of II at the muscarinic receptor also relates to the proposed modes of binding of muscarine and muscarone at cholinergic receptors. Waser (9) has suggested that, in order to account for the relative loss and inversion of optical specificity of muscarone relative to muscarine, D-muscarone is bound at the receptors in a conformation in which the trimethylammonium group is symmetrically disposed over the ether and carbonyl oxygen functions; the carbonyl group is assumed to be able to bind equivalently to the receptor site which normally binds the ether oxygen of L-muscarine. According to Waser's argument, II should be extremely active, since in one of its limiting conformations the quaternary ammonium group is centered symmetrically over the two electronically equivalent ether oxygens (the latter feature is not really true in Waser's proposed conformation for D-muscarone). Its complete inactivity reinforces the earlier arguments of Belleau and Puranen (15), based on the relative stereospecificity of L- and D-*cis*-2-methyl-4-dimethylaminomethyl-1,3-dioxolane methiodide, concerning the difficulties with Waser's interpretation of the binding of D-muscarone at the muscarinic receptor and provides further support for their suggestion that the carbonyl group in muscarone binds to an accessory nucleophilic site at the receptor thus introducing a binding characteristic that is not available for muscarine.

The remaining point of interest in connection with these compounds concerns the acetylcholine releasing activity of III. This activity, while relatively feeble, may be related to the steady decrease in maximum response obtained with III on repeated administration and raises again the question that certain cholinomimetic compounds may act, at least in part, by releasing acetylcholine from peripheral storage sites (16–18).

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Keyphrases

Cholinergic receptor
cis-2-Methyl-4-dimethylaminomethyl-1,3-dioxolane methiodide analogs—synthesis
Cholinomimetic activity
Muscarinic activity
Nicotinic activity
GLC—analysis

Multiregressional Analysis of the Griffin HLB Numbers for Polyoxyethylene Polyoxypropylene Surfactants

By WERNER LOWENTHAL

Using data from 32 polyoxypropylene polyoxyethylene surfactants and the technique of multiregressional analysis, an equation was determined. This equation describes the relationship between Griffin HLB number, percent polyoxyethylene in the total molecule, and the typical molecular weight of the polyoxypropylene. From the equation, given any two variables, the third variable can be readily determined with good accuracy for this series of polymeric surfactants.

POLYOXYETHYLENE POLYOXYPROPYLENE[1] surfactants are block polymers prepared by adding ethylene oxide to a polyoxypropylene chain. This produces a series of compounds that have the following formula: $HO(CH_2CH_2O)_a(CHCH_3CH_2O)_b(CH_2CH_2O)_cH$, where a and c are the number of ethylene oxide units and b is the number of propylene oxide units. Since the chain lengths can be varied, surfactants having different properties may be prepared. The propylene oxide chain is hydrophobic and the ethylene oxide chains are hydrophilic.

RESULTS AND DISCUSSION

A method called the Pluronic Grid which characterizes the available surfactants in this group has been published (1). This grid is actually a graphical plot of percent polyoxyethylene in the total molecule on one axis, and the typical molecular weight of the polyoxypropylene hydrophobic base on the other axis (1). At the points of intersection of the two variables on this grid, the different surfactants available are noted. The Griffin HLB number (2) for 32 of these surfactants is also available. Using these three sets of data, it is possible to calculate a multiregressional analysis to determine the relationships between the molecular weight of the hydrophobic group, the percent of hydrophilic groups in the total molecule, interactions between these two variables, and the HLB number.

The initial equation for which the constants and coefficients were determined using the RPC 4000 computer[2] was written as:

$$Y = b_0 + b_1X_1 + b_{11}X_1^2 + b_2X_2 + b_{22}X_2^2 + b_{12}X_1X_2 \quad \text{(Eq. 1)}$$

where Y is the HLB number, X_1 is the typical molecular weight of the hydrophobic group, X_2 is the percent polyethylene oxide in the total molecule, b_0 is a constant, and the other b values are coefficients. The constant and coefficients were calculated, and t tests (3) showed that only the coefficients for X_1, X_1^2, and X_2 were statistically significant. Equation 1 then reduced to:

$$Y = 3.01 - 0.00231X_1 + 1.94 \times 10^{-7}X_1^2 + 0.371X_2 \quad \text{(Eq. 2)}$$

A more detailed grid was drawn from HLB values obtained by selecting values of X_1 between 950 and 4000 and values of X_2 between 10 and 80, substituting these numbers into Eq. 2, and solving for the HLB values. The HLB numbers for each corresponding set of X_1 and X_2 values were then plotted on rectangular coordinate paper and lines drawn connecting equal HLB numbers. The topography of this grid showed that, in the range of values used, a flat plain existed inclining from high HLB numbers at high percent hydrophilic groups and low molecular weight hydrophobic group, to low HLB numbers at low percent hydrophilic groups and high molecular weight hydrophobic group.

TABLE I—CALCULATED HLB NUMBERS USING EQ. 2

Surfactant	Given HLB No.	Calcd. HLB No.
L31	4.5	4.7
L35	18.5	19.5
F38	30.5	30.7
L42	8.0	7.9
L43	12	11.6
L44	16	15.4
L61	3	3.3
L62	7	7.0
L63	11	10.7
L64	15	14.4
P65	17	18.1
F68	29	29.2
L72	6.5	6.5
P75	16.5	17.6
F77	24.5	25.1
L81	2	2.5
P84	14	13.6
P85	16	17.3
F87	24	24.8
F88	28	28.5
L92	5.5	5.5
P94	13.5	13.0
F98	27.5	27.8
L101	1	1.3
P103	9	8.7
P104	13	12.4
P105	15	16.1
F108	27	27.2
L121	0.5	0.6
L122	4	4.3
P123	8	8.0
F127	22	22.8

Table I shows the values calculated with Eq. 2 and the published values. The numbers in column one are the commercial names of the polyoxyethylene polyoxypropylene surfactants.

The average difference between the given and calculated HLB numbers is 0.45 units, which is probably within the error in determining HLB numbers.

CONCLUSIONS

The solution of a multiregression equation showed that the Griffin HLB numbers are directly related to the molecular weight of the hydrophobic group and percent of hydrophilic groups in the total molecule,

Received July 18, 1967, from the Department of Pharmacy, School of Pharmacy, Medical College of Virginia, Richmond, VA 23219

Accepted for publication September 11, 1967.

This investigation was supported in part by grant 5PO-700016-05 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

The author thanks the Department of Biometry for their help.

[1] Marketed as Pluronic by Wyandotte Chemicals, Wyandotte, Mich.

[2] General Precision, Inc.

in polyoxypropylene polyoxyethylene surfactants. The HLB numbers can be calculated from the derived equation which closely agree with the observed values for this series of surfactants. Conversely, part of the molecular structure of the surfactant can be determined, *e.g.*, the molecular weight of the hydrophobic group or the percent hydrophilic groups in the total molecule, when the HLB number and one of the other variables of a particular surfactant of this series are given

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Keyphrases

Polyoxyethylene polyoxypropylene surfactants
Griffin HLB numbers—multiregressional analysis
Hydrophobic group, M.W.—HLB relation
Hydrophilic group, percent—HLB relation

Cactus Alkaloids IV. Macromerine from *Coryphantha runyonii*

By L. E. BELOW, A. Y. LEUNG, J. L. MCLAUGHLIN*, and A. G. PAUL

Macromerine, a β-phenethylamine alkaloid previously isolated from *Coryphantha macromeris*, has been isolated from *Coryphantha runyonii*

HODGKINS *et al.* (1) have recently isolated a new β-phenethylamine, macromerine, from the cactus, *Coryphantha macromeris* (Engelm.) Lemaire. Physiological tests demonstrated that macromerine has potential hallucinogenic and sympatholytic activities. From physical data, spectra, and elemental analysis they proposed that macromerine is *l*-α-3,4-dimethoxyphenyl-β-dimethylaminoethanol, and they have confirmed this structure by synthesis of the racemic mixture.

Working independently in this laboratory using thin-layer chromatography (TLC) several unknown alkaloids were detected in a related species of cactus, *Coryphantha runyonii* Britton and Rose (2). Since the alkaloids detected produced TLC patterns different from those of previously known cactus alkaloids, isolation attempts were initiated.

EXPERIMENTAL

Using large Soxhlet extractors, alkaloids were extracted with ethanol from 1.8 Kg. of dried and pulverized plant material.[1] Nonphenolic alkaloids were isolated from the ethanolic residue using the procedures and ion-exchange column technique of purification method No. 2 (3). The total nonphenolic alkaloid fraction was chromatographed over 560 Gm. of activated alumina in a 40 × 150 cm. column using chloroform as the developer. Eluant fractions of 15–20 ml. each were collected and analyzed by TLC. Those fractions richest in concentrations of the major alkaloid were combined. The crystalline residue obtained (1.314 Gm.; yield 0.07%) was recrystallized several times from ethyl ether (m.p. 65–65.5°; picrate m.p. 159°; optical rotation: $[\alpha]_D^{26} = -54.2$, c = 0.0120 Gm./ml. in methanol). The isolated alkaloid appeared as a single spot upon TLC analysis in several solvent systems, indicating its homogeneity.

Permanganate oxidation (4) of the alkaloid produced a crystalline derivative which was identified as 3,4-dimethoxybenzoic acid by melting point, mixed melting point, and IR spectral comparison. Elemental analysis[2] of the alkaloid indicated an empirical formula of $C_{12}H_{19}NO_3$.

Calcd. for C, 63.98; H, 8.50; N, 6.22; mol. wt. 225.29. Found: C, 65.02; H, 8.39; N, 6.29.

The UV spectrum (0.016 mg./ml. methanol) showed a $\lambda_{max.}$ at 202 mμ and smaller peaks at 230 mμ and 278 mμ, indicating typical benzene ring absorption. The IR spectrum in KBr revealed a broad band at 3120 $cm.^{-1}$ (hydroxyl). NMR spectra in $CDCl_3$ showed peaks at 6.99 δ and 6.89 δ, indicative of three aromatic hydrogens, and a doublet of doublets centered at 4.67 δ, indicative of a benzylic hydrogen adjacent to two nonequivalent methylene protons (5). An alcohol hydrogen (3.96 δ) showed shifts with varying concentrations and disappeared with D_2O exchange. A doublet centered at 3.90 δ indicated two methoxyl groups, a doublet centered at 2.48 δ indicated a methylene group, and a singlet at 2.35 δ indicated two methyl groups attached to nitrogen.

Analysis by high resolution mass spectrometry[3] showed a weak molecular ion peak at m/e = 225.1362, in agreement with the proposed empirical formula. Prominent peaks appeared at m/e = 208, 207, 192, 180, 167, 166, 165, 164, and 151, postulated structures of which are compatible with the expected fragmentation pattern of macromerine.

The UV, IR, and NMR spectra of the alkaloid and the spectra of natural *l*-macromerine from *C. macromeris* as well as synthetic *d,l*-macromerine are essentially identical. In addition the melting point of *l*-macromerine (m.p. 65–65.5°) was not depressed by admixture (mixed m.p. 65–65.5°) with the alkaloid isolated from *C. runyonii*.[4]

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Accepted for publication September 26, 1967
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[1] Obtained from Davis Cactus Garden, Kerrville, Tex., and D. E. Wiley, Corpus Christi, Tex. Identification confirmed by Dr. E. U. Clover, Botany Department, University of Michigan.
[2] Spang Microanaytical Lab., Ann Arbor, Mich.
[3] The authors are indebted to Dr. A. Brossi and Dr. F. Vane, Hoffmann-LaRoche, Inc., for the mass spectral data.
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Following detection of unknown alkaloids in the cactus *C. runyonii*, one of the alkaloids was isolated in crystalline form by employing ion-exchange and adsorption column chromatography. Attempts at characterization of the unknown alkaloid resulted in spectral and chemical data which are in accord with *l*-α-3,4-dimethoxyphenyl-β-dimethylaminoethanol. This compound has recently been isolated by other workers from a related cactus, *C. macromeris*, and has been given the common name *l*-macromerine. Comparison of spectral and chemical data revealed that the unknown alkaloid from *C. runyonii* is identical to *l*-macromerine from *C. macromeris*. Thus, the occurrence of macromerine is not restricted to the latter species.

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Keyphrases

Alkaloids, cactus—*Coryphantha runyonii*
Macromerine—isolation
Column chromatography—separation
TLC—analysis
IR spectrophotometry—structure
UV spectrophotometry—structure
NMR spectrometry
Mass spectrometry

Deoxyalloxazines (Benzopteridines) II. Methylation of 2,4-Diamino-6,7-dimethylbenzo(*g*)pteridine

By S. L. MUKHERJEE, Z. F. CHMIELEWICZ, and T. J. BARDOS*

Methylation of 2,4-diamino-6,7-dimethylbenzo(*g*)pteridine (I) with excess methyl iodide in boiling cellosolve gave a red methyl-methiodide derivative (II) which, on treatment with hot aqueous sodium carbonate solution was converted to the N_1-methyl derivative (III), as shown by hydrolysis of the product to the corresponding dioxo-compound (IV) and its further degradation to the quinoxaline derivative (V). Reaction of I with methyl iodide in nitromethane solution afforded a quaternary methiodide derivative (VI) which, through a series of reactions with cold, concentrated acid and base reagents, was converted to III. On several occasions, the latter was obtained in what appeared to be a different, unstable tautomeric form. Hydrolysis of the corresponding intermediate methiodide (VI) or, of the hydrochloride salt (VII), with acid or alkali, gave IV; thus the methylation of I in nitromethane, as in cellosolve, appears to have occurred exclusively in the N_1-position of the pyrimidine ring.

THE SYNTHESIS of a series of deoxyalloxazines (benzopteridines) was reported several years ago (1). One of these compounds, 2,4-diamino-6,7-dimethylbenzo(*g*)pteridine (2,4-diamino-2,4-deoxylumichrome, I) was found to be a highly active antimetabolite of both folinic acid and riboflavin in various microbiologic test systems (1, 2), and it also inhibited the growth of several transplanted tumors in mice (3, 4). Unfortunately, the very poor solubility and tissue absorption properties of this interesting compound sharply limited its potential therapeutic usefulness. For this reason, the synthesis of more soluble derivatives was attempted.

Substitution in the N_{10}-position of the central ring appeared to be of particular interest because the resulting flavin-type compounds would display greater structural similarity to riboflavin and may prove to possess higher "anti-riboflavin" activities than I. It was also of interest to investigate the possibility of obtaining quaternary derivatives of I which would bear some structural resemblance to the chemotherapeutically effective acridines and phenanthridines.

Methylations of 4-aminopteridine, 2,4-diaminopteridine, and related compounds, with methyl iodide, were reported by Brown and Jacobsen (5, 6) to give the N_1- and/or N_8-methyl substituted derivatives in the form of their hydroiodides or quaternary methiodides (the N_8-position of the pteridine nucleus corresponds to the N_{10}-position in the benzopteridine system). The N_1-substituted derivative of 2,4-diamino-6,7-diphenylpteridine was obtained by Boon and Bratt (7) by methylation of the parent compound with methyl iodide. In a series of papers, Angier (8) reported his studies on the methylations of 2-amino-4-hydroxypteridines with dimethyl sulfate; substitution in the N_1, N_3, and/or N_8 position was shown to occur under the various conditions employed. Preliminary experiments indicated that I could not be methylated with dimethyl sulfate under the conditions employed by Angier, while the use of methyl iodide appeared to be more promising.

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Accepted for publication October, 9, 1967.

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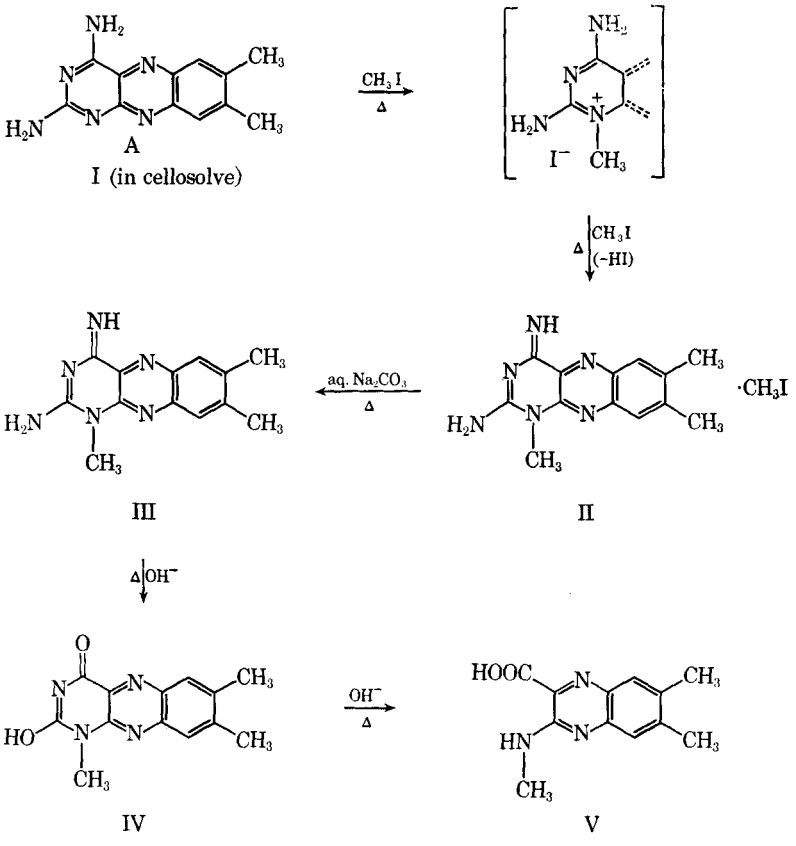
In all of the reactions studied, the very low solubility of I in organic solvents caused considerable difficulties. Boiling ethanol dissolves the compound at less than 0.05 mg./ml. concentration; the resulting yellow, green-fluorescent solution shows absorption maxima at 265, 343, and 420 mμ, almost identical to those reported for the anion of 2-amino-4-hydroxybenzo(*g*)pteridine (9) and the di-anion of lumichrome (1). On the basis of Jones' rule (10), structure A is, therefore, assigned to the tautomeric form of I in ethanol (Scheme I). Somewhat higher solubility, with similar spectrum, was obtained in boiling cellosolve. Treatment of I in cellosolve with excess methyl iodide at reflux temperature resulted in complete dissolution of the starting material; on cooling, an orange-red compound precipitated which, after recrystallization, gave an elemental analysis corresponding to a methyl-methiodide (or, dimethyl-hydroiodide) derivative of I. Subsequent reactions and spectral comparisons suggest that the most probable structure for this compound is the one indicated in formula II.

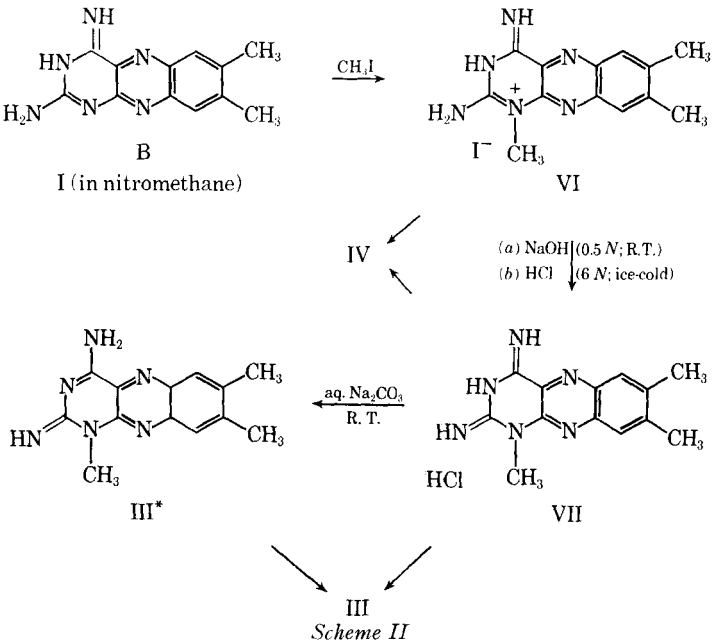
Further treatment of II with an aqueous solution of sodium carbonate at 100° gave the N_1-methyl derivative (III). The position of the methyl group was established by alkaline degradation which yielded 3-methylamino-6,7-dimethylquinoxaline-2-carboxylic acid (V). Milder alkali treatment of III resulted in hydrolysis to the corresponding dioxo-derivative (IV).

Solutions of I in more polar solvents (nitromethane or dimethylsulfoxide) showed no fluorescence, and the second and third absorption bands in the ultraviolet spectra appeared at 360 and 395–400 mμ, respectively (the first absorption band cannot be established because of the absorption of these solvents in the 200–300 mμ region). Structure B (Scheme II) was tentatively assigned to the tautomeric form of I present in these solvents. Reaction of I with excess methyl iodide in boiling nitromethane yielded a water-soluble, red crystalline compound which gave elemental analysis and spectral properties corresponding to a quaternary methyl-iodide (VI) of the starting material. Treatment of this compound with hot sodium carbonate solution, under conditions employed for the conversion of II to III, led to decomposition (evolution of ammonia). However, when the aqueous solution of VI was added at *room temperature* to a solution of 0.5 *N* sodium hydroxide, immediately a red solid precipitated; this could not be purified but could be converted to a crystallizable mono-hydrochloride (VII). The latter was dissolved in water and neutralized with aqueous sodium carbonate to give III.

It should be mentioned that, on several occasions, this series of reactions led to a final product which gave analysis corresponding to a monomethyl derivative of I but appeared to differ from III in appearance, solubility, fluorescence, and ultraviolet spectrum in absolute ethanol. Due to the strong fluorescence and longer-wavelength absorption ($\lambda_{max.}$ 422 mμ) of this compound in absolute ethanol, it was believed, at first, that the methylation in nitromethane occurred at the N_{10}-position; how-



Scheme I



Scheme II

ever, hydrolysis of the corresponding hydrochloride (VII) with either acid or alkali gave a dioxo-compound which was identical with IV (as shown by spectra and thin-layer chromatography). The same dioxo-benzopteridine was obtained by alkaline hydrolysis of VI. It was also found that addition of water to their alcoholic solutions changed the ultraviolet spectra of both III and the "new" monomethyl compound, and the two spectra became identical, both showing in the range above 300 mμ a single absorption maximum (at 360 mμ) and an inflection (at 395 mμ). The spectra of the two compounds in 0.1 *N* HCl were also identical (see under *Experimental*). Moreover, after a sample of the "new" compound was stored in a desiccator for more than a year, all the above-mentioned characteristic differences disappeared, and the sample became indistinguishable from III. It was concluded that the "new" compound represented an unstable tautomeric form of III. Consequently, the methylation of I in nitromethane (as in cellosolve) appears to occur exclusively in the N_1-position of the pyrimidine ring.

These studies indicated that, due to the fused benzene ring, the aromaticity of the pyrazine ring and its resistance to *N*-methylation is greatly enhanced in the benzopteridine system.

EXPERIMENTAL[1]

2 - Amino - 4 imino - 1,6,7 - trimethyl - 1,4 - dihydrobenzo(g)pteridine - methiodide—2,4 - Diamino-6,7-dimethylbenzo(*g*)pteridine (I) (2.0 Gm., 0.0083 mole), was added to a solution of methyl iodide (5.0 ml., 0.1 mole) in 40 ml. of cellosolve, and the reaction mixture was heated at refluxing temperature for 45 min. under vigorous stirring. The resulting clear, deep-red colored solution was concentrated to about one-third of its original volume, then cooled and stored in the refrigerator overnight during which time an orange-red solid precipitated. This was collected by filtration and triturated at room temperature with 125 ml. of methanol; the methanolic extract was filtered from the undissolved solids and concentrated to dryness. The residue of the methanolic filtrate was washed with 60 ml. of acetone. Yield: 1.0 Gm. (30%) of essentially pure product, a portion of which was crystallized from methanolic solution by very slow and careful evaporation of the solvent. Long, orange needles which, however, on repeated crystallization changed into small, red flakes, m.p. > 360°. Similar result was obtained by precipitation of the compound from methanolic solution with slow addition of ether. This process was repeated several times until the UV absorption maxima reached constant values. $\lambda_{max.}^{0.1\ N\ HCl}$ 365 mμ (ε 14,130); 260 mμ (ε 46,770). $\lambda_{max.}^{EtOH}$ 396 mμ (ε 11,090); 360 mμ (ε 12,670); 256 mμ (ε 44,540).

Anal.—Calcd. for $C_{14}H_{17}IN_6$: C, 42.42; H, 4.29; I, 32.07; N, 21.21. Found: C, 42.21; H, 4.15; I, 32.10; N, 21.10.

2 - Amino - 4 - imino - 1,6,7 - trimethyl - 1,4 - dihydrobenzo(g)pteridine (III)—To a boiling, 10% aqueous sodium carbonate solution (25 ml.) was added 170 mg. (0.0043 mole) of the above compound (II), and the mixture was stirred for 15 min. without further heating. A green precipitate was obtained which was allowed to settle at room temperature, filtered, and washed with water followed by acetone. Yield: 100 mg. (92%) of crude III. This was purified by dissolving in boiling methanol and treatment of the hot solution with charcoal (Darco); on cooling, the compound crystallized in the form of yellow micro-needles, no melting point (apparently decomposes above 250°). $\lambda_{max.}^{0.1\ N\ HCl}$ 365 mμ (ε 10,400); 260 mμ (ε 32,760). $\lambda_{max.}^{EtOH}$ 390 mμ (ε

[1] Microanalyses by Galbraith Laboratories, Knoxville, Tenn., and by Dr. S. N. Nagy, Microchemical Laboratory, Massachusetts Institute of Technology, Cambridge, Mass. The melting points were taken on the Kofler block and are uncorrected. The ultraviolet spectra were determined on a Beckman DU spectrophotometer attached to a Gilford model 2000 absorbance indicator. The infrared spectrum was determined on a Perkin-Elmer model 137 Infracord spectrophotometer. The NMR spectrum was determined on a Varian A-60, with tetramethylsilane as external standard.

7,340); 345 mμ (ε 8,100); 258 mμ (ε 32,100).

Anal.—Calcd. for $C_{13}H_{14}N_6$: C, 61.42; H, 5.51; N, 33.07. Found: C, 61.79; H, 5.35; N, 32.87.

2 - Hydroxy - 4 - oxo - 1,6,7 - trimethyl - 1,4 - dihydrobenzo(g)pteridine (IV)—To a boiling solution of 2 - amino - 4 - imino - 1,6,7 - trimethyl - 1,4-dihydrobenzo(*g*)pteridine (III) (100 mg., 0.0004 mole) in 2-methoxyethanol (30 ml.), 1 *N* sodium hydroxide (20 ml.) was added, and the resulting solution was heated for 60 min. on the steam bath (9). The hot, greenish yellow solution was acidified with glacial acetic acid (about 10 ml.) and cooled in the refrigerator overnight. The precipitate obtained was collected by filtration and washed with water until the washings were neutral to litmus paper. The residue (30 mg., 30%) was further purified by dissolving in methanol and treatment with charcoal. Concentration of the solution afforded yellow microcrystals; yield, 20 mg. (20%), m.p. 292–294°. $\lambda_{max.}^{EtOH}$ 392 mμ (ε 8,800); 336 mμ (ε 9,000); 250 mμ (ε 36,500). $\lambda_{max.}^{0.1\ N\ NaOH}$ 400–392 mμ (ε 9,972); 358–332 mμ (ε 8,976); 252 mμ (ε 39,060). Due to sparing solubility of the compound in 0.1 *N* HCl, the cationic spectra of the compound could not be taken.

Anal.—Calcd. for $C_{13}H_{12}N_4O_2$: C, 60.93; H, 4.68; N, 21.87. Found: C, 60.96; H, 4.99; N, 21.75.

3 - Methylamino - 6,7 - dimethylquinoxaline - 2-carboxylic Acid (V)—To a suspension of III (300 mg., 0.0012 mole) in 2-methoxyethanol (30 ml.) was added 50 ml. of 10% aqueous sodium hydroxide. The mixture was refluxed for 52 hr. with stirring. A white amorphous solid appeared which was filtered after cooling. The filtrate was concentrated to one-third of its original volume and then acidified with excess concentrated hydrochloric acid, whereupon a white precipitate was formed. Without separation, the mixture was further concentrated, under reduced pressure to dryness. The residue was then extracted in a Soxhlet apparatus with a chloroform–benzene mixture (3:1 v/v) until no more colored material was eluted by the solvent (about 15 hr.). The yellow solution was evaporated to dryness and the residue was extracted with boiling ether. The hot ether extract was filtered and evaporated to dryness. The residue was dissolved in minimum quantity of methanol, and crystallized at 0°, to yield an orange-colored solid (50 mg., 18%), m.p. 172–175°. Ultraviolet $\lambda_{max.}^{0.1\ N\ HCl}$ 400 (ε 6,650); 340 mμ (ε 8,529); 252 mμ (20,900). $\lambda_{max.}^{EtOH}$ 405mμ (ε 4,650); 315 mμ (ε 4,620); 258 mμ (ε 27,900). Infrared $\nu_{max.}^{KBr}$ (cm.$^{-1}$): 3400(s) (OH, NH); 2920 (m) (CH_3); 2700 (vw); 1720(s) (C=O); 1660, 1620(w) (NH); 1570(s), 1535(sh), 1510(m) (aromatic C=C, C=N); 1460 (m) (C—CH_3); 1400(m) (COOH); 1370(m) (C—CH_3); 1345(s) (C—N); 1290(w) (COOH); 1240(m), 1100(m), 1000(m) (ring-vibrations); 835 (benzene-C—H). NMR (in CF_3COOH): 2.15, 3.10, 7.3, and 7.7 p.p.m. Integrated ratio: 6:3:1:1 (2 benzene-CH_3, 1 N—CH_3, 1 + 1 benzene ring-protons).

Anal.—Calcd. for $C_{12}H_{13}N_3O_2$: C, 62.33; H, 5.63; N, 18.18. Found: C, 62.16; H, 5.89; N, 17.91

2 - Amino - 4 - imino - 1,6,7 - trimethyl - 3,4 - dihydrobenzo(g)pteridinium Iodide (VI)—To nitromethane (75 ml.) was added, under continuous stirring, 2,4-diamino-6,7-dimethyl(*g*)benzopteridine (*a*) (500 mg., 0.00207 mole), and this was followed by dropwise addition of methyl iodide (3.0 ml., 0.059 mole). The mixture was refluxed for 3–4 hr. (during which time its color turned black) and, while still hot, filtered through a diatomaceous earth[2] bed. From the red filtrate the solvent was removed on the flash evaporator, and the residue was taken up in boiling methanol. The hot methanolic solution was treated with charcoal (Darco) and filtered through a diatomaceous earth bed. On concentrating the solution to about 30 ml., red micro-needles separated out; yield, 250 mg. (32%), m.p. 360°. This was further purified by repeated crystallization from the same solvent. $\lambda_{max.}^{0.1\ N\ HCl}$ 460 mμ (ε 2,188); 372 mμ (ε 12,880); 260 mμ (ε 41,690); 220 mμ (ε 35,480). $\lambda_{max.}^{EtOH}$ 460 (ε 4200); 394 mμ (shoulder; ε 11,140); 358 mμ (ε 13,050); 258 mμ (ε 38,200); 219 mμ (ε 45,000).

Anal.—Calcd. for $C_{13}H_{15}IN_6$: C, 40.83; H, 3.92; N, 21.99. Found: C, 40.82; H, 4.09; N, 21.88.

2,4 - Diimino - 6,7 - dimethyl - 1,2,3,4 - tetrahydrobenzo(g)pteridine Hydrochloride (VII)—The quaternary iodide (VI) (450 mg., 0.0012 mole) was dissolved in hot water, and the red solution was added at room temperature to a 0.5 *N* aqueous sodium hydroxide solution (50 ml.) at room temperature. Immediately a red solid precipitated. The reaction mixture was cooled in the refrigerator overnight, then centrifuged, and the clear supernatant was decanted. The residue was washed with one portion of cold water and centrifuged, then added to ice cold 6 *N* HCl (50 ml.); on further cooling in an ice bath, a solid separated out. After filtration, the residue was dissolved in methanol, the solution was charcoaled, and the resulting yellow filtrate was concentrated in the flash evaporator to about 25 ml., whereupon a yellow solid began to separate at room temperature. This was collected by filtration (100 mg., 29%) and further purified by repeating the same process, until an analytically pure sample was obtained.

Anal.—Calcd. for $C_{13}H_{14}N_6 \cdot HCl$: C, 53.70; H, 5.16; N, 28.91. Found: C, 53.42; H, 5.40; N, 28.93.

Conversion of VII to Free Base—Sixty milligrams (0.0002 mole) of VII was dissolved in water (15 ml.), without heating. To the cold aqueous solution, 10% aqueous sodium carbonate solution was added until the solution became neutral to litmus. A red precipitate separated immediately after addition. This was centrifuged, washed thoroughly with water, and then with a minimum quantity of acetone. The crude residue was then dissolved in boiling methanol and treated with charcoal. The yellow filtrate (which showed green fluorescence) was concentrated, until a yellowish-green solid separated. This was further purified by repeated crystallizations from methanol. $\lambda_{max.}^{0.1\ N\ HCl}$ 364 mμ (ε 10,910); 259 mμ (ε 33,400). $\lambda_{max.}^{EtOH}$ 422 mμ (ε 9,612); 355–340 mμ (ε 7,600); 258 mμ (ε 34,370). In some preparations, the 422 mμ absorption of the ethanolic solution was partially or wholly shifted to 390–400 mμ, and the spectrum of the product was similar to the "normal" spectrum of III (see above) in absolute ethanol as well as in 0.1 *N* HCl.

Anal.—Calcd. for $C_{13}H_{14}N_6$: N, 33.07. Found: N, 32.76.

Structure Determination of Compounds Derived from the Reaction in Nitromethane—*Acid Hydrol-*

[2] Celite. Johns-Manville Corp., New York, N. Y.

ysis of VII—The hydrochloride salt (VII) (100 mg., 0.00034 mole) was mixed with 2-methoxyethanol (50 ml.), and concentrated hydrochloric acid (10 ml.) was added. The mixture was refluxed for 36 hr., during which time additional quantities (5 ml.) of concentrated hydrochloric acid were added, after 8 hr. and again after 14 hr. Initially the color of the solution was light brown; on heating for 7–8 hr., some solid separated and the color of the solution turned yellow. On continued heating, the solid dissolved and the solution turned deep brown in color. After evaporation to dryness, the residue was boiled with water for 20 min. and filtered. The residue (35 mg., 40%) was thoroughly washed with acetone and crystallized from ethanol, m.p. 295–296°. $\lambda_{max.}^{EtOH}$ 390 mμ (ε 8,772); 334 mμ (ε 8,772); 250 mμ (ε 36,600).

Anal.—Calcd. for $C_{13}H_{12}N_4O_2$: C, 60.93; H, 4.68; N, 21.87. Found: C, 61.39; H, 5.04; N, 21.97.

Alkaline Hydrolysis of VII—This was conducted in the same manner as described in the preparation of IV from III. $\lambda_{max.}^{EtOH}$ identical with acid hydrolysis product.

Alkaline Hydrolysis of VI—The quaternary methyl iodide (VI) was hydrolyzed, in the same manner, and the product obtained showed the same spectrum as the acid and base hydrolysis products from VII above.

Thin-Layer Chromatography—The above compounds obtained from VII by acid and alkaline hydrolysis, respectively, were compared with IV (obtained by alkaline hydrolysis of III) by simultaneously conducted thin-layer chromatography, using alumina as the adsorbent and dimethylformamide–water (9:1) mixture as the solvent. Each compound gave a single fluorescent spot under ultraviolet light, corresponding to an R_f value of 0.55

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Keyphrases

Deoxyalloxazines (benzopteridines)
2,4-Diamino-6,7-dimethylbenzo(*g*)-pteridine—methylation
UV spectrophotometry—structure
IR spectrophotometry—structure
NMR spectrometry
TLC—UV light fluorescence

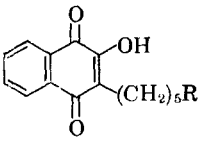
Lawsone Derivatives IV. 3-ω-Substituted Alkyllawsones and Related Compounds

By H. MACHATZKE, W. R. VAUGHAN*, C. L. WARREN, and G. R. WHITE

This paper reports the preparation of two 3-alkyllawsones in which there is a nitrogen mustard function in the ω-position. In addition, a potentially general route to the preparation of 3-ω-aminoalkyllawsones is described. The method involves introduction of an ω-amino group *via* an ω-azido group.

THE OBJECTIVES of this continuing research have been discussed in a previous paper (1), and the purpose of the present paper is to report preparation of lawsones (2-hydroxy-1,4-naphthoquinone) with 3-alkyl substituents terminating in an alkylating function. In addition, the development of a potentially general route to preparation of 3-ω-aminoalkyllawsones has been studied, and various ancillary problems have been delineated.

Two related series of lawsone derivatives have been investigated: one in which the 3-position carries a normal pentyl chain terminating in a functional group (general structural formula I, in which lower case letters refer to known starting materials involved in synthesis); and one in which the 3-position carries a normal decyl chain terminating in a functional group (general structural formula II, with notation as for I).



I

R

Ia —$CO_2C_2H_5$	*IA* —CO_2CH_3 methyl ether
Ib —COCl	*IB* —CH_2OH
Ic macrocyclic lactide derived from *Ib*	*IC* —$CON(CH_2CH_2OH)_2$
	ID —$CON(CH_2CH_2Cl)_2$

Received October 28, 1966, from the Department of Chemistry, University of Michigan, Ann Arbor, MI 48104
Accepted for publication October 18, 1967.
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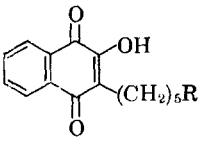

I

R

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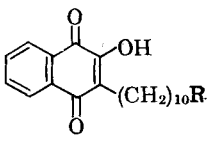

II

R

IIa —OH IIA —OSO_2SCH_3 methyl ether

IIb —Br IIB —$OCON(CH_2CH_2Cl)_2$

IIC —N_3

IID —$NH_2 \cdot HO_2CCH_3$

IIE —$NHCOCH_3$

DISCUSSION

Series I—In this series the preparation of methyl 6-(2-methoxy-1,4-naphthoquinon-3-yl)hexanoate (I*A*) and of 6-(2-hydroxy-1,4-naphthoquinon-3-yl)hexanol are trivial, the former involving boron trifluoride-etherate catalyzed trans-methylation of I*a* (1) followed by treatment with dimethyl sulfate of the sodium salt; and the latter involving lithium aluminohydride reduction of I*a* followed by silver oxide oxidation.

However, the preparation of *N,N*-bis(2-chloroethyl)-6-(2-hydroxy-1,4-naphthoquinon-3-yl)hexanamide (I*D*) from *N,N*-bis(2-hydroxyethyl)-6-(2-hydroxy-1,4-naphthoquinon-3-yl)hexanamide (I*C*) proved singularly difficult. In the first place treatment of I*a*, I*b*, and I*c* with varying amounts of diethanolamine afforded in each case a product resembling that expected, but in no instance could an analytically satisfactory sample be obtained owing to the high polarity and hydrophilic character of the material. The general appearance and physical characteristics of the products obtained from I*a*, I*b*, and I*c* were invariably the same, the reported procedures being typical of many runs of each. And while no absolute proof of structure is available, the fact that the infrared spectra of all products correspond in the significant bands, which in turn are favorably comparable to those in the infrared spectra of 6-(2-hydroxy-1,4-naphthoquinon-3-yl)-hexanamide and I*D*, strongly suggests that one obtained I*C* from any of the reported procedures in comparable yield and purity. Unquestionably, the difficulty in converting I*C* to I*D* was a consequence of the difficulty of obtaining analytically pure I*C*. However, in one instance one of the authors (H. M.) was able to effect the conversion by treating a sample of I*C* with thionyl chloride.

Series II—In this series the preparation of the first two members was a relatively simple matter. But is is interesting to note that intended methanesulfonation of the decanol, II*a* (1) surprisingly resulted in simultaneous *methylation* of the 2-hydroxyl group when the reaction was carried out under very mild conditions (methanesulfonyl chloride and excess pyridine in dry benzene at room temperature), giving 10-(2-methoxy-1,4-naphthoquinon-3-yl)decyl methanesulfonate (II*A*). There can be no question of the structure, since the infrared spectrum lacks hydroxyl absorption, the NMR spectrum shows *O*-methyl as well as *S*-methyl, and II*A* is insoluble in alkali, retaining its color rather than turning red, as do free lawsones.

No problems were encountered in the reaction of the decanol (II*a*) with *N,N*-bis(2-chloroethyl)-carbamoyl chloride (2) in pyridine to give *N,N*-bis(2-chloroethyl)-10-(2-hydroxy-1,4-naphthoquinon-3-yl)decyl carbamate (II*B*).

The remaining compounds in this series constitute the culmination of an exhaustive series of attempts to introduce an amine function bonded to carbon at the terminus of a 3-alkyl chain in lawsone. It was originally intended to use the decyl bromide (II*b*) (3) as a substrate for reaction with diethanolamine or bis(2-chloroethyl)amine; and when these two amines failed to give characterizable products, ammonia was also tried, with equally unsatisfactory results. Certainly these failures are attributable in part to complex additions to the lawsone system, such as are described in the preceding paper in this series.

Confronted with this discouraging experience, the authors turned their attention in the final phases of the study to preparation of 10-(2-hydroxy-1,4-naphthoquinon-3-yl)decylamine, which was actually isolated as its acetate salt (II*D*) and was characterized as such and as its acetyl derivative (II*E*). The availability of II*D* should permit future study of methods for *N*-hydroxyethylation and *N*-chloroethylation of ω-(2-hydroxy-1,4-naphthoquinon-3-yl)alkylamines, especially since there is no *a priori* reason why the authors' method of synthesis for II*D* should not be general.

By contrast with wholly unsuccessful attempts to obtain ω-(2-hydroxy-1,4-naphthoquinon-3-yl)alkylamines by various reduction processes applied to amides, notably by means of complex metal hydrides, the route involving reaction of the decyl bromide II*b* with azide ion, followed by hydrogenation was relatively straightforward. The only problem was in preparation of 10-(2-hydroxy-1,4-naphthoquinon-3-yl)decyl azide (II*C*) which had to be carried out in *N,N*-dimethylformamide using lithium azide instead of the more conventional sodium azide-methanol reaction, which led to recovery of unchanged II*b*. Reduction of II*C* to II*D* was achieved by hydrogenation at 3 Atm. over Adams' catalyst, followed by air oxidation, and the product proved to be an extremely stable acetate salt, which could readily be converted to the acetamide (II*E*).

EXPERIMENTAL[1]

Methyl 6-(2-hydroxy-1,4-naphthoquinon-3-yl)-hexanoate—In an attempt to methylate the free hydroxyl group of I*a*, 5.1 Gm. (16.1 mmoles) of this ethyl ester was dissolved in 100 ml. of methanol, and 12 ml. of boron trifluoride-etherate was added. The mixture was held at 70°, with stirring, overnight and then was held at 75–80° for 24 hr. Evaporation to dryness under reduced pressure left a yellow solid, m.p. 79–83°, and recrystallization from petroleum ether (b.p. 60–75°) raised this to 88–90°. Some 85% of this transesterified material was recovered in all.

Methyl 6-(2-methoxy-1,4-naphthoquinon-3-yl)-hexanoate IA—A sodium salt was prepared by adding 297 mg. of sodium (12.9 mmoles) to a solution of the above compound (3.9 Gm., 12.9 mmoles)

[1] Melting points uncorrected. Kofler block method indicated where used. Microanalyses by Spang Microanalytical Laboratory, Ann Arbor, Mich. Infrared spectra of Nujol mulls obtained with Perkin-Elmer model 21 double beam spectrometer. NMR spectra obtained with Varian A-60 spectrometer, 60 Mc., internal TMS reference. We are indebted to Mr. B. E. Wenzel, Mr. Günter Schütze, and Mr. Frank Parker for preparation of most of these spectra.

in 40 ml. of methanol, and the resulting red solution was evaporated to dryness under reduced pressure.

The red sodium salt was heated to 120–130° and treated, with stirring, with 1.64 Gm. (13 mmoles) of dimethyl sulfate. Some yellowing was noted, and more dimethyl sulfate (1.64 Gm., 13 mmoles) was added. After about 1 hr. all red color had disappeared, leaving a brown-yellow reaction mixture. Dilution with water (10 ml.) and addition of 10 ml. of saturated sodium carbonate solution resulted in production of the previous dark red color. Next 60 ml. of 5% hydrochloric acid and 6 ml. of concentrated hydrochloric acid was added and the mixture was extracted with chloroform. After drying (magnesium sulfate), filtration, and evaporation, there was left an oil which crystallized on being triturated with petroleum ether (b.p. 40–60°). Recrystallization from petroleum ether (b.p. 60–75°) afforded 2.339 Gm. (60%) of starting material (infrared spectrum and m.p.).

The mother liquor was concentrated and cooled to −5°, whereupon yellow needles precipitated, m.p. 43°. Recrystallization from the same solvent raised the m.p. to 47° and finally to a constant 57° (805 mg., with an additional 815 mg., m.p. 55° obtained as a second crop: 40% total). The infrared spectrum showed no hydroxyl absorption.

Anal.—Calcd. for $C_{18}H_{20}O_5$: C, 68.34; H, 6.37. Found: C, 68.46; H, 6.52.

6 - (2 - Hydroxy - 1,4 - naphthoquinon - 3 - yl)-hexanol (IB)—To a vigorously stirred suspension of lithium aluminohydride (LAH) (0.57 Gm., 15 mmoles) in 25 ml. of tetrahydrofuran (THF) (distilled from LAH) was added a solution of 3.16 Gm. (10.0 mmoles) of I*a* (1) in 25 ml. of similarly dried THF. Residue of *Ia* was washed into the reaction vessel with an additional 10 ml. of dry THF, and the brownish-red mixture was refluxed for 4 hr. and then cooled to 0°. Next, 6 ml. of ice water was added followed by 26 ml. of 6 *M* hydrochloric acid. Extraction with three 25-ml. portions of ether followed, and the combined ethereal extracts were vigorously stirred with 3.47 Gm. (15 mmoles) of freshly precipitated silver oxide. The mixture was then dried over anhydrous magnesium sulfate and filtered. Removal of the ether under reduced pressure left 2.73 Gm. of a dark brown solid, m.p. 86–87°, which was purified by digestion with three 200-ml. portions of petroleum ether (b.p. 90–100°) on the steam bath. The extracts were combined and evaporated under reduced pressure leaving a solid which was recrystallized from benzene to give 1.24 Gm. (45%) of *IB*, m.p. 103.5–104.0°. The infrared spectrum is appropriate for *IB:*

Anal.—Calcd. for $C_{16}H_{18}O_4$: C, 70.05; H, 6.61; mol. wt., 274. Found: C, 69.99; H, 674; mol. wt., 265.

An additional 0.30 Gm. (11%) of less pure *IB*, m.p. 98°, was slowly deposited from the mother liquor.

N,N - Bis(2 - hydroxyethyl) - 6 - (2 - hydroxy-1,4-naphthoquinon-3-yl)hexanamide (IC)—(*a*) From ethyl 6-(2-hydroxy-1,4-naphthoquino-3-yl)hexanoate (I*a*) (1). A mixture of 0.64 Gm. of I*a* (2 mmoles) and 0.22 Gm. (2 mmoles) of diethanolamine in 10 ml. of ethanol, with which 6 mg. of sodium had been allowed to react, was heated in a sealed tube for 3 hr. at 160°. The product, a dark red tar, was taken up in 30 ml. of chloroform at 65°, concentrated and then chromatographed on Florosil (10 × 2-in. column). After elution of traces of a pale yellow band and a broad pink band (with chloroform), further eluates obtained with chloroform and then methanol possessed infrared spectra comparable in most respects to that of *IC* obtained from I*c* (below). (*b*) From 6-(2-hydroxy-1,4-naphthoquinon-3-yl)hexanoyl chloride (I*b*). A solution of I*b* (1.06 Gm., 0.35 mmoles) and diethanolamine (4.0 Gm., 38 mmoles) in 10 ml. of toluene was stirred with refluxing for 15 hr. Next 45 ml. of concentrated hydrochloric acid was added, and the lower layer was continuously extracted with ether for 24 hr., the original toluene being part of the extraction medium. Evaporation of the filtered red organic extracts left a deep red solid possessing an infrared spectrum comparable in most respects to that of *IC* obtained from I*c* (below). (*c*) From the macrolactide (I*c*). A mixture of 1.0 Gm. of I*c* and 5 ml. of diethanolamine was heated at 100–150° for 2 hr. and then was allowed to stand overnight at room temperature. Next the solution was filtered, acidified with excess hydrochloric acid, and continuously extracted with ether. The ethereal extract was then diluted with 20 ml. of methanol and dried over anhydrous magnesium sulfate. Filtration and removal of the solvent left a red oil whose infrared spectrum possessed the expected characteristic bands for *IC:* (Nujol) 3400–3160; 1740 (shoulder); 1732–1720; 1672; 1650; 1640; 1600; 1470; 1440; 1372; 1280; 1220; 730 cm.$^{-1}$ In no case could *IC* be obtained sufficiently pure for satisfactory analysis.

N,N - Bis - (2 - chloroethyl) - 6 - (2 - hydroxy - 1,4-naphthoquinon-3-yl)hexanamide (ID)—The crude product from the previous experiment was dissolved in 10 ml. of absolute chloroform and treated with 2 ml. of thionyl chloride at ice-bath temperature with subsequent stirring for 2 hr. at room temperature. Next the excess thionyl chloride was removed by evaporation *in vacuo* followed by two additions and vacuum distillations of benzene. The residual red gum was taken up in chloroform and diluted with dry ether, whereupon yellow crystals separated (0.30 Gm.), m.p. 111–112° (Kofler). The infrared spectrum is consistent for *ID*.

Anal.—Calcd. for $C_{20}H_{23}Cl_2NO_4$: C, 58.20; H, 5.63; Cl, 17.20; N, 3.39. Found: C, 58.33; H, 5.80; Cl, 17.06; N, 3.50.

Numerous attempts to repeat this procedure using I*c* from *a*, *b*, and various modifications of *c* were unsuccessful.

10 - (2 - Methoxy - 1,4 - naphthoquinon - 3 - yl)-decyl methanesulfonate (IIA)—A solution of 2.2 Gm. of II*a* [10-(2-hydroxy-1,4-naphthoquinon-3-yl)-decanol (2)] (6.7 mmoles) and 2.3 Gm. (20 mmoles) of methanesulfonyl chloride in 10 ml. of dry benzene containing 5 ml. of dry pyridine was stirred for 8 hr. at room temperature. The solvent was distilled off and the residue taken up in ether and filtered. The ether solution was washed with dilute hydrochloric acid and water and dried over anhydrous magnesium sulfate. Removal of the ether left a yellow oil which was dried *in vacuo* over phosphorus pentoxide. After 4 days, the oil crystallized and then was recrystallized from acetone–water: (2.0 Gm.), m.p. 57–58° Kofler.

Anal.—Calcd. for $C_{22}H_{30}O_6S$: C, 62.53; H, 7.16; S, 7.59. Found: C, 62.12; H, 7.14; S, 7.51.

The infrared spectrum shows no hydroxyl absorption. Treatment with 5% sodium hydroxide at room temperature resulted in no observable color change, although on prolonged standing (over 30 min.) the compound slowly became dark red. The NMR spectrum (in trifluoroacetic acid, 60 Mc.) shows a 4-proton complex multiplet centered at $\sim\tau = 2$, a multiplet integrating for 8 protons at $\tau = 5.85$–6.30 which may be assigned to CH_3O—, CH_3SO_3—, and —CH_2O—. The next band is a broad unresolved multiplet centered at $\tau = 7.20$ (two protons, —CH_2– adjacent to the quinone nucleus), and finally there is a 16-proton multiplet centered at $\tau = 8.60$ (remaining methylenes).

N,N - Bis(2 - chloroethyl) - 10 - (2 - hydroxo - 1,4-naphthoquinon-3-yl)decyl carbamate (IIB)—A solution of 1.7 Gm. (5 mmoles) of II*a* and 0.95 Gm. (5 mmoles) of *N,N*-bis(2-chloroethyl)carbamyl chloride (2) in 5 ml. of pyridine was stirred at room temperature for 8 hr. during which time pyridine hydrochloride precipitated. Water was then added and the mixture was acidified with hydrochloric acid and extracted with ether, the ethereal extracts being washed with water and dried over anhydrous magnesium sulfate. Evaporation of the ether left a yellow oil which was taken up in methanol–water and deposited yellow crystals on being cooled. These were dried over phosphorus pentoxide: 2.2 Gm., m.p. 57–58° Kofler; recrystallized from methanol, m.p. 60–61° Kofler.

Anal.—Calcd. for $C_{25}H_{33}Cl_2NO_5$: C, 60.24; H, 6.67; N, 2.81. Found: C, 60.59; H, 6.79; N, 2.48. The infrared spectrum shows a broad absorption for hydroxyl.

10 - (3 - Hydroxy - 1,4 - naphthoquinon - 3 - yl)-decyl Azide (IIC)—To a stirred solution of II*b* [10-(2-hydroxy-1,4-naphthoquinon-3-yl)decyl bromide (3)] (1.95 Gm., 5.0 mmoles) in 60 ml. of dry anhydrous magnesium sulfate], distilled *N,N*-dimethylformamide (DMF) there was added lithium azide. This was prepared from 6.5 Gm. (0.1 mmole) of sodium azide and 7.05 Gm. (0.055 mmole) of lithium sulfate monohydrate dissolved in 35 ml. of warm water, followed by slow addition of 95% ethanol (175 ml.). Sodium sulfate was filtered off and evaporation of the filtrate under reduced pressure at 80° afforded a yellowish solid which was dried azeotropically with benzene and then used without further purification (4). The reaction mixture, a red solution, was stirred at 95–100° for 16 hr. (5) and then evaporated under reduced pressure to a dark red gum. To this was added 100 ml. of water followed by 10 ml. (excess) concentrated hydrochloric acid (pH = 2 at end of addition). The mixture was then extracted with ether (100 ml. then five 25-ml. portions). Evaporation of the dried (anhydrous magnesium sulfate), filtered ethereal extracts and recrystallization of the yellow residue from petroleum ether (b.p. 60–75°) afforded 1.57 Gm. (94%) of II*C*, m.p. 80.5°.

Anal.—Calcd. for $C_{20}H_{25}N_3O_3$: C, 67.58; H, 7.09; N, 11.82. Found: C, 67.48; H, 7.11; N, 11.96.

10 - (2 - Hydroxy - 1,4 - naphthoquinon - 3 - yl)-decylamine Acetate Salt (IID)—A 2.02-Gm. (5.70 mmoles) sample of II*C* suspended in 200 ml. of absolute ethanol containing 0.25 Gm. of Adams' catalyst was shaken for 19 hr. under 50 p.s.i. of hydrogen. No pressure drop was observed, but a red solid precipitated. This was filtered off and washed with glacial acetic acid until the washings were colorless. The experiment was repeated, and the combined ethanolic acetic acid filtrates were oxidized by bubbling an air stream through the solution for 3 days, by which time the solvent had evaporated leaving an intense red solid, m.p. 190–195°. This was dried over solid sodium hydroxide: 4.093 Gm. (97.5% $B \cdot HO_2CCH_3$).

The product was dissolved in glacial acetic acid, precipitated by addition of water, and collected by centrifugation to give an intensely crimson product, m.p. 196°, with darkening above 185°.

Anal.—Calcd. for $C_{22}H_{31}NO_5$: C, 71.52; H, 8.43; N, 3.79. Found: C, 71.81; H, 8.09; N, 3.87.

***N* - 10 - (2 - Hydroxy - 1,4 - naphthoquinon - 3-yl)decylacetamide (IIE)**—A 426-mg. sample of I*D* was added to 10 ml. of glacial acetic acid to give an orange solution. Next, 1.50 ml. of acetic anhydride was added and the mixture was stirred at room temperature for 2 hr. during which time a yellow precipitate appeared. The mixture was then held at −5° for 2 hr. during which time the color of the precipitate changed. Filtration afforded a pale primrose solid (*A*) (315 mg., m.p. 146°). This was washed with 10 ml. of glacial acetic acid and the filtrate and washings were diluted with water to give 185 mg. of orange solid (*B*) (m.p. 144°).

Both of the products were recrystallized from ethanol-water, the former affording a pale primrose substance softening >140°, m.p. 150° (*A*) and the latter a yellow solid softening >137°, m.p. 146–147° (*B*), and both analyzed correctly for II*E*.

Anal.—Calcd. for $C_{22}H_{29}NO_4$: C, 71.13; H, 7.87; N, 3.77; mol. wt., 371. Found (*A*): C, 70.51, 70.78; H, 7.84, 7.95; N, 4.00; mol. wt., 367, 371. Found (*B*): C, 70.50, 70.76; H, 7.84, 7.94; N, 3.82; mol. wt., 380, 378.

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Keyphrases

Lawsone derivatives
3-Alkyl 2-hydroxy-1,4-naphthoquinones—synthesis
3-ω-Aminoalkyl 2-hydroxy-1,4-naphthoquinones—synthesis
Column chromatography—separation
IR spectrophotometry—structure
NMR spectrometry

Chromatographic Methods for Analysis of the Metabolites of Acetophenetidin (Phenacetin)

By A. KLUTCH* and M. BORDUN†

A simple, specific means has been developed for routine analysis of *N*-acetyl-*p*-aminophenol and the other possible metabolites of acetophenetidin in the urine of animals given the drug. The analytical procedures fell into two parts: (*a*) the choice of solvent and pH for extraction and (*b*) the most suitable solvent system for thin-layer chromatography and a selective means for visualization of the compound being assayed. Gas chromatographic separations have also been developed for the analysis of the metabolic transformation products of acetophenetidin.

THE METABOLISM of acetophenetidin[1] has been well studied in man and other animals. It has been shown that most of the dose of acetophenetidin can be accounted for as *N*-acetyl-*p*-aminophenol[2] and its conjugates (1–3). Chemical methods have been developed for the analysis of this phenol in the physiological fluids of animals given acetophenetidin (1, 4).

In the course of studies on the metabolism of acetophenetidin in these laboratories, selective chromatographic methods have been developed for the estimation of the various possible transformation products of acetophenetidin present in urine or other biological material. The advantage of these chromatographic methods is their sensitivity and specificity for a compound present in a multicomponent mixture, but they are not as precise as the chemical methods described. The compound to be estimated is first extracted from buffered solution with a suitable solvent and then subjected to analysis by thin-layer chromatography. More recently, gas chromatographic separations have been developed for more accurate quantitation of the metabolites of acetophenetidin.

MATERIALS AND METHODS

Chemicals—Phenetidin as the free base or the hydrochloride salt was obtained from the Aldrich Chemical Co., Inc., Milwaukee, Wis. *p*-Aminophenol as the free base or the hydrochloride salt was obtained from K and K Laboratories, Inc., Plainview, N. Y. *N*-Acetyl-*p*-aminophenol was supplied by Eastman's Distillation Products Industries, Inc., Rochester, N. Y. 2-Hydroxyacetophenetidin was synthesized in these laboratories by Harfenist (5). Acetophenetidin (Burroughs Wellcome & Company, Tuckahoe, N. Y.) and *N*-hydroxyacetophenetidin were used.[3] All the solvents used were of reagent grade.

Animal Experiments—Acetophenetidin was administered orally in gelatin capsules to 2 adult dogs (8.2 Kg. and 10.2 Kg.) at a level of 100 mg./Kg. for 1 day. A human subject *A* received 420 mg. of acetophenetidin for 1 day. Human subject *B* received 1.8 Gm./day of acetophenetidin for 4 days. Human subject *C* received 1.8 Gm./day of *N*-acetyl-*p*-aminophenol for 4 days. Collections of urine were made for 24-hr. periods during the administration of drugs to dogs and human subject *A*. Urine was collected for the first day (the acute sample) and the fourth day (the chronic sample) of drug administration to human subjects *B* and *C*. Urine collected for the 24-hr. period prior to drug administration was used as control urine.

TABLE I—CHROMATOGRAPHIC EXAMINATION OF URINE

Compd.	Average $Rf \times 100$, System[a] 1	2	3	4	Detection[b]
Acetophenetidin	94	30	80	82	a
Phenetidin	94	70	96	95	a, b, e
N-Acetyl-*p*-aminophenol	88	0	14	34	a, b, c, d
2-Hydroxyacetophenetidin	91	15	55	67	a, b, c, d
N-Hydroxyacetophenetidin	18	0	7	20	a, b
p-Aminophenol	84	0	40	16	a, b, c, d, e

[a] Key to systems: 1, the upper phase of a cyclohexane–propanol–water–acetic acid (20:20:20:1) mixture; 2, the upper phase of benzene–methanol–water–acetic acid (20:10:20:1); 3, the lower phase of ethylene dichloride–methanol–water–acetic acid (20:10:20:1); 4, the lower phase of ethylene dichloride–methanol–water–acetic acid (30:5:10:3). [b] Key to detection: a, ultraviolet absorption; b, alkaline $AgNO_3$ (9); c, Pauly reagent (7); d, diazotized-*o*-dianisidine (8); and e, *p*-dimethylaminocinnamaldehyde reagent (7).

Determinations—The samples to be used for the analysis of acetophenetidin and phenetidin excreted by dogs were prepared by extracting 4 ml. of urine buffered with 1 ml. of glycine buffer at pH 9 (6) and 0.1 ml. of 1 *N* NaOH, with 30 ml. of carbon tetrachloride. A 25-ml. portion of the organic solvent was removed, evaporated, and dissolved in a few drops of methanol. For the measurement of acetophenetidin and phenetidin excreted by human subjects, 8 ml. of urine was treated with 2 ml. of the glycine buffer, 0.25 ml. of 1 *N* NaOH, and extracted with 60 ml. of carbon tetrachloride. A 50-ml. portion of solvent was taken for analysis. The extracts were examined chromatographically with system 2 described in Table I. Phenetidin and acetophenetidin were visualized with ultraviolet light.

A 0.5-ml. portion of urine to be analyzed for *N*-acetyl-*p*-aminophenol was buffered with 0.5 ml. of 0.2 *N* acetate buffer at pH 5 (6) and treated with 0.5 ml. of diluted glusulase[4] (Endo Laboratories, Inc., Garden City, N. Y.) for 4 hr. at 37.5°. The solution was extracted with 15 ml. of ethyl acetate and portions varying from 1 ml. to 5 ml. were taken for analysis. The sample size depended on the concentration of the phenol in urine. The best quanti-

Received July 19, 1967, from the Wellcome Research Laboratories, Burroughs Wellcome and Co., Inc., Tuckahoe, NY 10707

Accepted for publication October 12, 1967.

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[1] Phenacetin USP XVII.

[2] Acetaminophen NF XII.

[3] The authors are indebted to Dr. H. C. White, Edgar C. Britton Research Laboratories, Dow Chemical Co., for a supply of *N*-hydroxyacetophenetidin.

[4] To each ml. of glusulase, 1.5 ml. of the acetate buffer was added.

tative results were obtained when the sample applied to a thin-layer plate contained from 10 mcg. to 40 mcg. of phenol (urine highly concentrated with the compound was diluted prior to hydrolysis). After evaporation of the solvent, the residue was dissolved in a few drops of methanol. The *N*-acetyl-*p*-aminophenol was analyzed chromatographically with system 4 described in Table I. The Pauly Reagent (7) was used to visualize the compound.

The analysis for 2-hydroxyacetophenetidin excreted by dogs required hydrolysis of 1 ml. of urine with 1 ml. of the diluted glusulase and 1 ml. of the acetate buffer at pH 5. After hydrolysis, the urine was extracted with 30 ml. of a carbon tetrachloride–methylene chloride (3:1) mixture, and 25 ml. of the solvent mixture was removed. After evaporation of the solvent, the resulting residue was dissolved in a few drops of methanol and examined chromatographically. For the human studies, twice the amounts described above were taken for analysis. The quantification of the hydroxylated acetophenetidin was carried out with system 3 (Table I) and diazotized *o*-dianisidine (8).

To determine the *N*-hydroxyacetophenetidin excreted by human subjects, 4 or 5 ml. of urine was mixed with an equal volume of the acetate buffer and hydrolyzed with 2 ml. of glusulase (nondiluted reagent). The *N*-hydroxy compound was extracted from the hydrolyzed urine with 60 ml. of carbon tetrachloride and a 50-ml. portion of the solvent was taken for analysis. For the hydrolysis of 1 ml. of dog urine, 1 ml. of the acetate buffer and 0.5 ml. of glusulase were added to the urine and 15 ml. of solvent was used to extract the *N*-hydroxyacetophenetidin after hydrolysis. Chromatographic measurements were made on the residue (dissolved in methanol) obtained after evaporation of 10 ml. of solvent, using system 1 (Table I). *N*-Hydroxyacetophenetidin was visualized with ultraviolet light.

Urines obtained prior to drug administration were used as control samples and were treated similarly in all of the above analyses. The reference standards used in the analysis consisted of the appropriate compound added to control urine. Several concentrations, approximating the value of the experimental sample, were made by dissolving the compound in the acetate buffer at pH 5 or glycine buffer at pH 9 and mixing this solution with the urine before hydrolysis or extraction. Standards dissolved in the buffer could be stored at 5° for a reasonable amount of time, but care was taken never to freeze the solutions because of the extremely low solubilities in aqueous solution at the freezing point of most of the above compounds.

Thin-Layer Chromatography—Thin-layer plates were prepared from aluminum oxide GF[5] using techniques previously described (8).

Gas Chromatography—Synthetic mixtures of the above compounds were analyzed on a Barber-Coleman model 10 gas chromatograph equipped with a Sr-90 detector. The inlet pressure was 30 psig for the 6-ft. columns and 25 psig. for the 4-foot columns; the carrier gas was argon. The outlet pressure was atmospheric. The column packings[6] were a mixture of SE-30 (1%)–Carbowax 20M (1%) coated on Anakrom AS, 80–90 mesh, packed in a U-shaped, glass column, 6-ft. in length and 5 mm. i.d., or an SE-30 (0.5%)–Carbowax 20 M (0.5%) mixture on the same support in a column 4-ft. in length and 3.5 mm. i.d.

The instrument was operated isothermally at 180° for the 6-ft. column and at 190° for the 4-ft. column. The detector temperature was always 225° and the temperature of the flash heater was 240°. The samples dissolved in ethyl acetate varied from 0.1 to 0.5 mcg. The retention times are given in Table III.

RESULTS AND DISCUSSION

The analysis for these metabolites in physiological fluids can be accomplished easily using the techniques described. Urines being analyzed for a hydroxylated derivative of acetophenetidin were first subjected to enzymatic hydrolysis to liberate conjugates. All the analyses required the adjustment of the urine to a particular pH value, and a specific organic solvent or solvent mixture was used for extraction. After evaporation of the organic solvent, the residues obtained were chromatographed in one of the solvent systems described in Table I.

The chromatographic systems described appear to be effective in separating several of the reference compounds. However, because of the great differences in the concentrations of the metabolites in the urine and the presence of contaminating substances, a specific system was developed to assay only one particular metabolite. After spraying the developed thin-layer plates, the determinations were made by visual comparison with standards. For precise quantitation, the concentrations of the standards run with each experiment were close to that of the experimental value. These methods have been applied successfully to the analysis of the metabolites of acetophenetidin in the urines of animals treated with the drug or *N*-acetyl-*p*-aminophenol, and the results are given in Table II; the range of the results given in parentheses indicate that this method is not as precise as the methods reported previously, but it is within the usual biochemical variation.

The sensitivities of the methods are given at the bottom of the table. They were not calculated as dose percentages because of the variation in dosages given and urine volumes. The results for the estimation of unchanged acetophenetidin in the urines of animals treated with the drug are in agreement with the values (0.15–3%) reported by Brodie and Axelrod. The method for the estimation of *N*-hydroxyacetophenetidin is the least satisfactory of those described in the experimental section. The amounts usually present were only slightly higher than could be detected with ultraviolet light. The spray reagents tried had high background color.[7] Phenetidin could not be detected in the urines of experimental animals even though a concentration as low as 1 mcg./ml. could be detected in reference samples. Unconjugated *p*-aminophenol is not stable in urine and so could not be estimated by a method which would involve hydrolysis of any conjugates present. The results of the chromatographic estimations confirm *N*-acetyl-*p*-aminophenol as the major metabolite of acetophenetidin and the

[5] Supplied by Brinkmann Instruments Co., Westbury, Long Island, N. Y.

[6] Prepared by Analabs, Inc., Hamden, Conn.

[7] A spray reagent for the visualization of *N*-hydroxylated compounds described by Boyland and Manson (10) appeared since the completion of this work.

TABLE II—THE ESTIMATION OF THE METABOLITES OF ACETOPHENETIDIN BY THIN-LAYER CHROMATOGRAPHY

	Acetophenetidin			*N*-Acetyl-*p*-aminophenol			2-Hydroxyacetophenetidin			*N*-Hydroxyacetophenetidin		
	Urinary mcg./ml.	Concn. Range[a]	Recovery, % of Dose	Urinary mcg./ml.	Concn. Range[a]	Recovery, % of Dose	Urinary mcg./ml.	Concn. Range[a]	Recovery, % of Dose	Urinary mcg./ml.	Concn. Range[a]	Recovery, % of Dose
Human subject *A*	5	4.0–5.6	0.8	350	300–375	73	5	4–7	0.8	0		0
Human subject *B*												
Acute dose	4	3.8–4.1	0.3	500	475–525	62	5	4–7	0.4	10	8–12	0.8
Chronic dose	2	12	0.1	900	850–950	87	20	15–25	1.4	10	6–12	0.7
Human subject *C*[b]												
Acute dose	...	...	...	900	850–950	58	...	...	...	...	...	...
Chronic dose	...	...	...	1200	1100–1230	91.0	...	...	...	...	...	...
Dog 1	16	15–17	0.6	275	250–300	46.0	75	70–80	2.4	10	8–12	0.3
Dog 2	22	20–24	0.9	325	300–350	64	90	85–100	3.4	35	30–40	1.3
Sensitivity of method	0–1 mcg./ml.			Not determined			2–3 mcg./ml.			5 mcg/ml.		

[a] Based on 5 determinations. [b] Treated with *N*-acetyl-*p*-aminophenol.

TABLE III—SEPARATION OF THE METABOLITES BY GAS CHROMATOGRAPHY

	Retention Time, Min.	Column Packing	Length, ft.	Temp.,°C.
Acetophenetidin	13.5	[a]	6	180
	1.2	[b]	4	190
N-Acetyl-*p*-aminophenol	10.0	[b]	4	190
2-Hydroxyacetophenetidin	7.5	[b]	4	190
N-Hydroxyacetophenetidin	13.0	[a]	6	180
	1.1	[b]	4	190
Phenetidin	0.5	[a]	6	180
p-Aminophenol	5.0	[a]	6	180

[a] 1% SE-30, 1% Carbowax 20M. [b] 0.5% SE-30, 5% Carbowax 20 M.

presence of 2-hydroxyacetophenetidin as a minor metabolite (5).

The results are in rough agreement with the earlier studies which employ more time-consuming and less specific methods. There is also present in the urine of animals given acetophenetidin or *N*-acetyl-*p*-aminophenol small amounts of an unknown metabolite with an R_f of 0.35 in solvent system 3[8] which has been reported previously (5).

The analysis of *N*-acetyl-*p*-aminophenol in the urine of animals treated with the tertiary-butyl analog has been reported (11). In this work these separations have been modified and expanded to include acetophenetidin and the other possible metabolites. The separation of acetophenetidin and the related compounds is shown in Table III.

The separation of acetophenetidin from its *N*-hydroxylated derivatives by gas chromatography has not been achieved. Further investigations are continuing in order to find a gas chromatographic means of separating these compounds so that this method can be applied for the analysis of the metabolites of acetophenetidin in the urine of animals given the drug.

[8] It was determined by comparison of the infrared spectrum of this metabolite with the spectrum of 3-hydroxy-acetophenetidin that this compound is not the 3-hydroxylated metabolite.

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Keyphrases

Phenacetin
Metabolites—phenacetin
Urine—phenacetin metabolites, analysis
TLC—analysis
GLC—analysis
IR spectrophotometry—structure

Effect of Mescaline on Nicotinamide Adenine Dinucleotide Synthesis in the Central Nervous System

By GLENN D. APPELT*, NORMAN O. WALKER, and ROBERT G. BROWN

Nicotinamide adenine dinucleotide (NAD) synthesis in mouse brain was demonstrated following the intracerebral injection of nicotinamide (500 mg./Kg.). Animals which received mescaline hemisulfate (100 mg./Kg.) 90 min. prior to nicotinamide were found to have higher levels of brain NAD at the 2 and 3 hr. time intervals.

NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD) levels have been increased in various organs of experimental animals by pretreatment with nicotinamide (1–3). Reserpine was found to prolong these levels in rat liver (4), and a similar effect has been demonstrated utilizing mouse brain (5). The participation of NAD in many dehydrogenase systems is well known (5), and alterations in the synthesis or degradation of this coenzyme could conceivably affect metabolic activity in the organism. Since certain psychotherapeutic drugs such as reserpine and chlorpromazine alter NAD metabolism (4), it appeared of interest to examine the effect of a hallucinogenic drug, such as mescaline, on this parameter.

EXPERIMENTAL

Young adult albino mice of both sexes were used as test animals in this study. Two brains were pooled and used for each NAD assay. The preparation of brain extracts was performed according to the method of Ciotti and Kaplan (7). The animals were stunned by a blow to the base of the skull, decapitated, and the whole brains quickly removed, weighed, and transferred to a Ten Broek tissue grinder. Homogenization was then accomplished with 5 vol. of cold 5% trichloroacetic acid. The total homogenate was centrifuged at 2,000 r.p.m. for 5 min., the supernatant liquid shaken with ether, and aliquot portions of the aqueous layer employed in the NAD assays. NAD determinations were made spectrophotometrically at 340 mμ with a Beckman DB spectrophotometer employing an ethanol–alcohol dehydrogenase system (7).

Animals were divided into two groups. Those of the first group received nicotinamide (500 mg./Kg.) intracerebrally in an adaptation of the method of Haley (8). NAD synthesis was demonstrated in the brain tissue by determination of the coenzyme level at various time intervals following the injection of nicotinamide. Animals of the second group received mescaline hemisulfate (100 mg./Kg.) intraperitoneally 90 min. prior to the intracerebral injection of nicotinamide. Determinations of brain NAD content in these animals were conducted at the same time intervals employed in the study of the control group which received only nicotinamide. The values reported represent the mean of at least five NAD determinations. The standard error of the mean was calculated for each set of NAD values and is designated by the vertical line through each point.

In addition, NAD assays were made 30 and 60 min. following the injection of mescaline hemisulfate prior to the intracerebral injection of nicotinamide. NAD measurements were made after intracerebral injections with normal saline in an effort to detect possible nonspecific elevation due to the experimental technique.

RESULTS AND DISCUSSION

Figure 1 illustrates the synthesis of NAD in mouse brain after the administration of nicotinamide. The value at zero time indicates the mean level of NAD before the intracerebral injection of nicotinamide. The brain NAD was not significantly increased until the fourth hour after the nicotinamide reinforcement. The NAD levels remained elevated at the 5 and 6 hr. intervals and then returned to a value comparable to the zero time determination.

Figure 2 shows the brain NAD elevation in experimental animals that received mescaline hemisulfate intraperitoneally 90 min. prior to the intracerebral injection of nicotinamide. These data indicate a greater increase in brain NAD at the 2 and 3 hr. time intervals than was observed in the animals which received only nicotinamide at zero time.

Table I is a summary of the mean values for brain NAD obtained at the various time intervals. Any significant differences in the mean NAD values of the animals receiving only nicotinamide and those which were injected with mescaline before the nicotinamide administration are denoted.

There was no significant change from control values in the brain NAD levels of animals which had received mescaline hemisulfate 30 and 60 min. prior to the determination. The intracerebral adminstration of normal saline did not alter the brain NAD levels.

Bonasera *et al.* (5) have shown that reserpine given

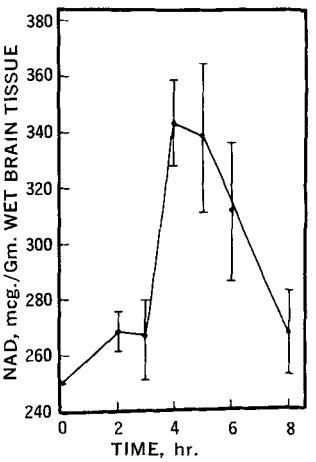

Fig. 1—Synthesis of nicotinamide adenine dinucleotide in mouse brain following intracerebral injection of nicotinamide (500 mg./Kg.) at zero time.

Received July 31, 1967, from the College of Pharmacy, University of Texas, Austin, TX 78712
Accepted for publication October 12, 1967.
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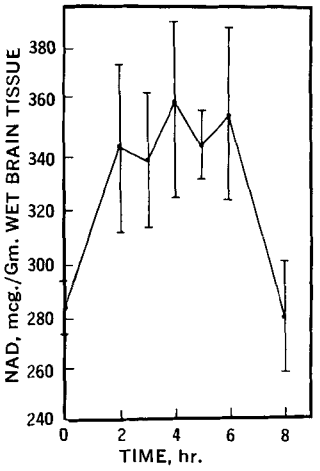

Fig. 2—Synthesis of NAD in mouse brain following intracerebral injection of nicotinamide (500 mg./Kg.) at zero time. Mescaline hemisulfate (100 mg./Kg.) was administered intraperitoneally 90 min. prior to zero time.

enzymatic activities dependent upon its presence. Clark and co-workers have demonstrated that mescaline inhibits the oxidation of pyruvate by brain homogenates; however, no effect was noted on the activity of succinic dehydrogenase (8). Schueler has reported that mescaline does not alter the oxidation of succinate by rat brain minces (9). A considerable amount of evidence indicates that electron transport in the oxidation of pyruvate involves nicotinamide adenine dinucleotide (5). With succinate as a substrate, however, nicotinamide adenine dinucleotide is not required because the electrons enter the transport system at the flavin nucleotide step. It is conceivable that an alteration in NAD metabolism could be a facet in the mechanism whereby mescaline inhibits pyruvate oxidation by brain preparations. This is an important consideration since the major energy source for the brain is glucose. Although the data indicate that mescaline has some effect on NAD synthesis in the central nervous system, it is not possible at this time to correlate any aberration in NAD metabolism with mescaline activity.

TABLE I—NAD LEVELS, mcg./Gm., IN MOUSE BRAIN

Time, hr.	0	2	3	4	5	6	8
Nicotinamide 500 mg./Kg. i.c. at zero time	251 ± 24[a]	269 ± 6	265 ± 13	344 ± 15	338 ± 27	312 ± 24	269 ± 16
Nicotinamide 500 mg./Kg. i.c. at zero time + mescaline hemisulfate 100 mg./Kg. i.p. 90 min. prior to zero time	281 ± 10	342[b] ± 32	336[b] ± 25	358 ± 36	342 ± 12	353 ± 32	278 ± 20

[a] Standard error of the mean. [b] Significantly different from control (nicotinamide 500 mg./Kg. only) $p < 0.05$.

intraperitoneally will delay the peak value of the increase in brain NAD following the administration of nicotinamide intrathecally. In addition, these investigators noted that these values remained elevated for at least 24 hr. after the nicotinamide administration. Earlier, Burton and associates (4) had demonstrated a prolongation of elevated level of liver NAD after nicotinamide administration if animals were pretreated with either chlorpromazine or reserpine.

The NAD values reported in this communication compare favorably with the data available for synthesis of NAD in mouse brain after intrathecal injection of nicotinamide. It is interesting to note that in contrast to the alteration in NAD metabolism effected by reserpine, the apparent changes induced by mescaline indicated an earlier elevation in brain NAD. As in the case of reserpine, the values for the experimental animals receiving mescaline never reached a peak level significantly different from control values of animals receiving only nicotinamide.

Participation of NAD in many dehydrogenase systems is well known and any alteration in the metabolism of this coenzyme could possibly affect

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Keyphrases

Nicotinamide adenine dinucleotide
Brain tissue synthesis—nicotinamide adenine dinucleotide
Mescaline—nicotinamide adenine dinucleotide synthesis
UV spectrophotometry—analysis

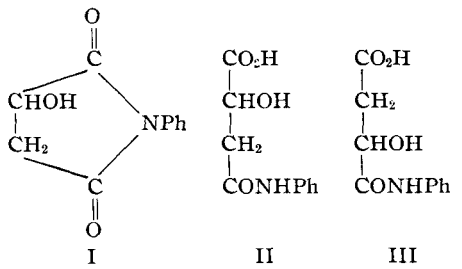
Characterization of Racemic α-Hydroxy- and β-Hydroxysuccinanilic Acids

By ROLF B. PAULSSEN, IAN H. PITMAN, and TAKERU HIGUCHI*

Alkaline hydrolysis of racemic malic acid phenylimide (malanil) yielded two products corresponding to the two possible directions of cleavage. These were shown to be racemic α-hydroxy- and β-hydroxysuccinanilic acid present in the ratio 5:1, the proportion being essentially as expected on the basis of the relative electrophilicities of the respective carbonyl centers.

ALKALINE HYDROLYSIS of DL-malic acid phenylimide (malanil) (I) yielded two isomeric monoanilides of malic acid which had different melting points, ultraviolet spectra, and pKa values. Characterization of these anilides was undertaken so that they could be used as model compounds in an investigation (1) of the anilides formed when aniline was added to a mixture of acetic anhydride in malic acid buffers.

The structures of the anilides were assigned from their pKa values on the basis that the stronger acid would be the one with the hydroxy substituent α to the carboxylic acid function. Thus, the isomer with m.p. 139.5–140.5°; $\lambda_{max.}$ 243 mμ, log ε = 4.06 (in chloroform); pKa = 3.69; was assigned the structure of the α-hydroxy isomer (II), and the isomer with m.p. 149.5–150.5°; $\lambda_{max.}$ = 243 mμ, log ε = 4.12 (in chloroform); pKa = 4.35; was assigned the structure of the β-isomer (III).



I II III

Other examples of the acid-strengthening effect of an α-hydroxy substituent relative to a β-hydroxy substituent include the greater acidity of lactic acid (pKa = 3.86) (2) and ethylmethylglycolic acid (pKa = 3.99) (2) compared to hydracrylic acid (pKa = 4.51) (3) and 2-hydroxy-1-methyl-butyric acid (pKa = 4.65) (2), respectively.

The relative yield of the isomeric hydroxysuccinanilic acids obtained in the alkaline hydrolysis of malanil (I) was [β-hydroxysuccinanilic acid]/[α-hydroxysuccinanilic acid] = 0.2. This is expected as the carbonyl group α to the hydroxy group in malanil is likely to be more electrophilic than the carbonyl group β to the hydroxy group, and thus be more susceptible to attack by hydroxide ions.

Received August 7, 1967, from the School of Pharmacy University of Wisconsin, Madison, WI 53706

Accepted for publication September 20, 1967.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This investigation was supported in part by grant GM-05830 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and the American Chicle Co., Warner-Lambert Pharmaceutical Co., Long Island, N. Y.

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EXPERIMENTAL

DL-Malic Acid Phenylimide (Malanil) (I)—Dry solid anilinium malate, prepared by slowly adding 1 mole of aniline in ethanol to 1 mole of malic acid in ethanol, was heated at 140° for 3 hr. while nitrogen was bubbled through the reaction mixture. The product was recrystallized from toluene and further purified on a silicic acid column. Upon recrystallization from chloroform white crystals were obtained, m.p. 179.5–180°.

Anal.—Calcd. $C_{10}H_9NO_3$: for C, 62.82; H, 4.75; N, 7.33. Found: C, 62.66; H, 4.84; N, 7.48.

α- and β-Hydroxysuccinanilic Acids—Alkaline hydrolysis of malanil yielded two isomeric monoanilides of malic acid which were separated and purified by column partition chromatography. The structures of the two anilides were assigned from measurements of pKa values and the assumption that the stronger acid would be the one with the hydroxy substituent α to the carboxylic function. DL-β-Hydroxysuccinanilic acid (III), m.p. 149.5–150.5° (from toluene), $\lambda_{max.}^{CHCl_3}$ = 243 mμ (log ε = 4.12). pKa = 4.35 ± 0.01 at 25°, determined potentiometrically (4) with [β-hydroxysuccinanilic acid] = 0.01 *M*.

Anal.—Calcd. for $C_{10}H_{11}NO_4$: C, 57.41; H, 5.30; N, 6.70. Found: C, 57.46; H, 5.42; N, 6.82.

DL-α-Hydroxysuccinanilic acid (II), m.p. 139.5–140.5° (from chloroform), $\lambda_{max.}^{CHCl_3}$ = 243 mμ (log ε = 4.06), pKa = 3.69 ± 0.05 at 25° with [α-hydroxysuccinanilic acid] = 0.01 *M*. Found: C, 57.44; H, 5.25; N, 6.60.

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Keyphrases

DL-Malic acid phenylimide—alkaline hydrolysis

α-Hydroxysuccinanilic acid—hydrolysis product

β-Hydroxysuccinanilic acid—hydrolysis product pKa values

UV spectrophotometry—structure

Salivary Levels of Antibiotics from Use of Neomycin-Gramicidin Chewing Troches

By F. WERTALIK and R. BONORDEN*

Previous studies demonstrated that a neomycin-gramicidin chewing troche is effective in reducing the oral bacterial flora. The present study shows that this test preparation is highly efficient in terms of the biologic availability of the preparation's antibiotic components.

POST-TONSILLECTOMY hemorrhage due to secondary bacterial infection remains a serious complication. Systemic penicillin prophylaxis does not seem to be effective in improving the appearance of the surgical wound or in hastening healing (1). Several reported studies with a topical antibacterial chewing troche[1] indicate that this preparation has been used successfully in an attempt to control late post-tonsillectomy hemorrhage by preventing secondary local infection (1–6). However, in none of the latter studies was proof of actual reduction in the bacterial count of the oral cavity specifically sought.

More recently, an investigation (7) was undertaken in normal subjects to determine the effectiveness of this antibiotic chewing troche in reducing the oral bacterial flora. The results showed that the use of the chewing troche reduced by 50% the average bacterial count of the oral cavity. The medicated gum had a prolonged effect as evidenced by significant reduction in the oral bacterial counts three hours after chewing.

The present study was conducted in order to determine if chewing gum troches containing neomycin and gramicidin are in actuality an efficient dosage form for releasing into the saliva, over a relatively sustained period of time, significant levels of the antibiotics.

EXPERIMENTAL

Materials and Method—To determine the biologic availability of the neomycin and gramicidin components of the preparation, saliva samples were collected from five subjects over 3 consecutive 5-min. periods, during which each subject chewed one of the antibiotic gum troches. The gum cud was then removed from the mouth, and an unstimulated saliva sample was collected for a fourth 5-min. period. Antibiotic assays were then performed on each of the four saliva samples and on the gum cud. Neomycin and gramicidin assays were conducted according to the methods described by the Food and Drug Administration (8).

Procedure Used for Collecting Saliva—Five graduated 100-ml. beakers, marked S1, S2, S3, cud, and S4, were set before each subject. At the start of the test, each subject placed an antibiotic gum troche in his mouth, which he then chewed for 15 min. The subjects were asked not to swallow any saliva during the course of the test. Saliva was collected during the first 5-min. period in beaker S1. At the end of the 5-min. period, beaker S1 was removed and the second 5-min. salivary sample was collected in beaker S2. This procedure was repeated for the third 5-min. period. Then the chewing gum cud was removed and placed in the appropriately marked beaker. The subject finally collected his unstimulated saliva for an additional 5 min. in beaker S4. (All subjects later repeated the procedure when fresh salivary samples were required for repeat tests.)

Preparation of Saliva and Gum for Neomycin Assay—Preliminary tests using collected saliva indicated that saliva sample No. 1 contained about 3,000 mcg. of neomycin, saliva sample No. 2 about 200 mcg. of neomycin, saliva samples No. 3 and No. 4 less than 10 mcg., and the cud less than 1 mcg. The cud was prepared for assay by first chilling, then breaking into small pieces, then steaming for 5 hr. in 25 ml. of buffer. The suspension was then blended for 2 min. in a blender, filtered, and the filtrate used in the assay.

Preparation of Saliva and Chewing Gum Cud for Gramicidin Assay—Preliminary tests indicated that saliva sample No. 1 contained abut 150 mcg. of gramicidin, saliva sample No. 2 about 4 mcg., and saliva samples No. 3 and No. 4 and the cud approximately 0.8 mcg. The cud was prepared for assay by first freezing under dry ice. The frozen cud was then ground up and suspended in 20 ml. of alcohol. The suspension was steamed for 2 hr., blended for 2 min. in a blender, and filtered. The filtrate was then assayed.

RESULTS

The results of the neomycin assays of the salivary samples and the gum cuds are given in Table I. The mean volumes of saliva collected are also recorded in this table. An average total of 2.6 mg. of neomycin was recovered for the five subjects from the saliva and cud samples. This amounted to 74% of the claimed neomycin content (3.5 mg.). It is assumed that the unrecovered fraction of the neomycin was adsorbed on the mucosal surface of the oral cavity or was lost by swallowing.

As is apparent from the data in Table I, the greatest amount of neomycin was liberated from the chewing gum vehicle during the first 5 min. of chewing.

The results of the gramicidin assays are given in Table II. Volumes of saliva collected are also shown in the same table.

An average total of 171 mcg. of gramicidin was

Received October 12, 1967, from the Microbiology Laboratory, White Laboratories, Inc., Kenilworth, NJ 07033
Accepted for publication November 15, 1967.
*Foster D. Snell, Inc., a subsidiary of Booz Allen Applied Research, Inc.
[1] Orabiotic chewing troches. Each gum troche contains: neomycin (from the sulfate), 3.5 mg.; gramicidin, 0.25 mg.; and propesin (propyl-*p*-aminobenzoate), 2.0 mg.

TABLE I—AMOUNTS OF NEOMYCIN RECOVERED

Saliva Sample 5-Min. Periods	Mcg. of Neomycin — Subjects RB	CH	BB	VW	AR	Av.
1st	1698	3226	2872	1348	2740	2376.8
2nd[a]	99	298	282	171	218	213.6
3rd[a]	3	7	7	3	3	4.6
4th[a]	3	3	3	3	3	3.0
Total	1803	3534	3164	1525	2964	2598.0
Chewing Gum Cud[a]	0.8	0.5	0.8	0.5	1.0	0.7
Total	1803.8	3534.5	3164.8	1525.5	2965.0	2598.7
Saliva	Volume of Saliva Collected, ml.					
1	12	15	12	10	18	13.4
2	10	12	10	8	15	11.0
3	10	8	10	8	10	9.2
4	5	5	5	5	8	5.6

[a] Approximate values.

TABLE II—AMOUNTS OF GRAMICIDIN RECOVERED

Saliva Sample 5-Min. Periods	Mcg. of Gramicidin — Subjects RB	CH	BB	VW	AR	Av.
1st	146.0	183.0	189.0	130.5	180.0	165.7
2nd	3.3	2.8	2.0	1.6	5.4	3.0
3rd	0.2	1.3	0.2	0.8	0.1	0.5
4th	0.3	0.6	0.2	0.2	0.1	0.3
Total	149.8	187.7	191.4	133.1	185.6	169.5
Chewing Gum Cud[a]	1.5	1.6	1.5	1.7	1.7	1.6
Total	151.3	189.3	192.9	134.8	187.3	171.3
Saliva	Volume of Saliva Collected, ml.					
1	20	15	15	15	20	17
2	15	12	10	10	18	13
3	10	8	8	8	18	10
4	6	6	8	8	10	7

[a] Approximate values.

recovered from the saliva and cud samples. This amounted to 68% of the claimed gramicidin content of the chewing troche (0.25 mg.). It is assumed, as with the neomycin, that the unrecovered fraction of the gramicidin was adsorbed on the tissues of the oral cavity or was lost by swallowing. As shown by the data summarized in Table II, the greatest amount of gramicidin was liberated from the troche during the first 5 min. of chewing.

SUMMARY AND CONCLUSION

The data indicate that the chewing gum vehicle of the test preparation is highly efficient in terms of the biologic availability of the preparation's antibiotic components. The average amounts of neomycin and gramicidin retained in the gum vehicle after 15 min. of chewing were negligible.

Most of each antibiotic component was liberated during the first 5 min. of chewing. The average amount of neomycin liberated after 15 min. of chewing approximated 74% of the labeled neomycin content of the troche. The average amount of gramicidin assayed from the saliva during the same period was approximately 68% of the labeled gramicidin content of the troche.

The unrecovered fractions of the neomycin and gramicidin were undoubtedly swallowed or adsorbed on the oropharyngeal mucosa. Further, the assay data indicate that, particularly during the first 10 min. of chewing, salivary levels of neomycin and gramicidin were maintained, which far surpassed the inhibitory levels required to suppress the growth of the oral flora.

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Keyphrases

Antibiotics—chewing troches
Salivary levels—antibiotics
Neomycin release—chewing troches
Gramicidin release—chewing troches

New Compounds: Synthesis of Some Phosphorus–Nitrogen Compounds for Pharmacological Study II

By A. ABOU-MOUSTAFA and M. KHALIFA

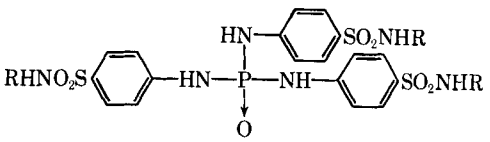
The synthesis of some *N*-substituted derivatives of phosphoric triamide and phosphorodiamidic acid *via* the condensation of phosphorus oxychloride with a number of sulfa drugs is described. Three moles of sulfanilamide, sulfacetamide, sulfapyridine, sulfathiazole, and sulfamerazine condensed with 1 mole of the oxychloride while with sulfisomidine and sulfadiazine 2 moles condensed with the acid chloride. In all the condensation reactions the molecular ratio of the reactants was 2 of the amino compound to 1 of the oxychloride.

IN A PREVIOUS PUBLICATION (1) it was reported that the condensation of phosphorus trichloride with a number of sulfa drugs afforded the *N*-substituted derivatives of phosphorus triamide and phosphorodiamidous acid. In the present investigation the work has been extended to include the phosphoric acid analogs of the previously reported compounds in order that the pharmacological activity of the two series of compounds may be compared. The fact that the phosphoric acid analogs were synthesized by condensing phosphorus oxychloride with the same sulfa compounds offered the opportunity to compare the reactivity of phosphorus trichloride and phosphorus oxychloride in these condensation reactions. With phosphorus trichloride the condensation was complete after 24 hr. except in the case of sulfanilamide where 12 hr. were sufficient. On the other hand, with phosphorus oxychloride a complete reaction was attained after 4 hr. with sulfanilamide and sulfacetamide and after 5 hr. with sulfadiazine, while with the rest of the amino compounds the reaction time never exceeded 12 hr. Moreover, the yields in the condensation reactions in which phosphorus trichloride was used ranged between 20–50%; those in which phosphorus oxychloride was used were much higher, *e.g.*, up to 94% yield was given with sulfapyridine and from 70 to 80% with the rest. In the condensation reactions 8 amino compounds were condensed with each acid chloride. With phosphorus oxychloride six out of the eight condensed in a manner that three molecules of the amino compound condensed with one molecule of the oxychloride. In the case of phosphorus trichloride four out of the eight condensed in this manner. Furthermore, while sulfamerazine failed to condense with phosphorus trichloride, it condensed readily with phosphorus oxychloride affording a 78% yield of the condensation product.

It can be seen from the foregoing that in these condensation reactions phosphorus oxychloride is more reactive than the trichloride.

That the phosphorus atom in the synthesized compounds is linked to the N^4 of the sulfa drug was shown qualitatively by the failure of all the condensation products to diazotize and by their solubility in dilute alkali and their insolubility in dilute mineral acids.

TABLE I—*N*-SUBSTITUTED DERIVATIVES OF PHOSPHORIC TRIAMIDE



Compd.	R	% Yield	Solvent of Crystallization[a]	M.p., °C.[c]	Formula	Anal.,[d] % Calcd.	Found
I	2-Thiazolyl	71	*A*	215–217[b] (260 dec.)	$C_{27}H_{24}N_9O_7PS_6$	N, 15.55	14.74
						S, 23.75	23.71
						P, 3.82	3.28
II	2-Pyridyl	94	*A*	180–183[b] (270 dec.)	$C_{33}H_{30}N_9O_7PS_3$	N, 15.78	14.82
						S, 12.14	13.08
						P, 3.91	3.32
III	4-Methyl-2-pyrimidinyl	78	*A*	186–190[b] (256 dec.)	$C_{33}H_{33}N_{12}O_7PS_3$	N, 20.08	19.39
						S, 11.49	12.49
						P, 3.70	3.24
IV	Acetyl	75	*B*	290 dec.	$C_{24}H_{27}N_6O_{10}PS_3$	N, 12.25	12.77
						S, 14.01	14.94
V	H	73	*A*	200–205[b] (256–260 dec.)	$C_{18}H_{21}N_6O_7PS_3$	N, 14.99	13.64
						S, 17.16	16.64

[a] *A*, aqueous ethanol; *B*, absolute ethanol. [b] Liquid crystal. [c] Melting points were performed by the capillary tube method and are uncorrected. [d] Analyses performed by Janssen Pharmaceutica, Beerse, Belgium.

EXPERIMENTAL

N-Substituted Derivatives of Phosphoric Triamide and Phosphorodiamidic Acid—Compounds I–VII were prepared by the gradual addition of phosphorus oxychloride (0.01 mole) in pyridine–chloro-

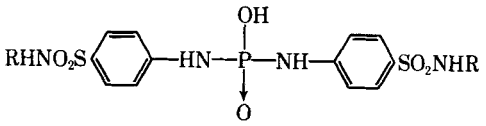
Received May 8, 1967, from the Pharmaceutical Chemistry Department, Faculty of Pharmacy, Riyadh University, Riyadh, Saudi Arabia.

Accepted for publication October 4, 1967.

The authors express their appreciation to Dr. A. A. Khowaiter, Acting Rector of Riyadh University, for research facilities which made this work possible and to Professor H. El Mangowri, Chemistry Department, for his interest in this work.

The products at present are under preliminary screening for possible antineoplastic action or any useful pharmacological activity.

TABLE II—*N*-SUBSTITUTED DERIVATIVES OF PHOSPHORODIAMIDIC ACID



Compd.	R	% Yield	Solvent of Crystallization[a]	M.p.,[b] °C.[c]	Formula	Anal.,[d] % Calcd.	Found
VI	2-Pyrimidinyl	80	*A*	190–193 (270 dec.)	$C_{20}H_{19}N_8O_6PS_2$	N, 19.92 S, 11.41	19.18 12.40
VII	4,6-Dimethyl-2-pyrimidinyl	70	*A*	195–196 (260 dec.)	$C_{24}H_{27}N_8O_6PS_2$	N, 18.11 S, 10.36	17.45 11.56

[a] *A*, aqueous ethanol. [b] Liquid crystal. [c] Melting points were performed by the capillary tube method and are uncorrected. [d] Analyses performed by Janssen Pharmaceutica, Beerse, Belgium.

form (20 ml.) to the sulfa compound (0.02 mole) dissolved in the same solvent (150 ml.). For complete reaction the mixture was refluxed 12 hr. in all except with sulfanilamide, sulfacetamide, and sulfadiazine in which case refluxing for 4 and 5 hr. was sufficient. Then the solvent was distilled *in vacuo*, and the residue suspended in dilute hydrochloric acid, filtered, and washed with water until the washings gave a negative chloride test with silver nitrate T.S. The product after being dried was crystallized from the appropriate solvent. (See Tables I and II.)

N,N',N''-tri-β-Naphthylphosphoric Triamide—This compound was prepared by condensing β-naphthylamine with phosphorus oxychloride according to the above general procedure. The product which was obtained in 75% yield melted at 237–238° after being crystallized from aqueous ethanol.

Anal.—Calcd. for $C_{30}H_{24}N_3OP$: N, 8.88 Found: N, 9.07.

N,N',N'' - tri[N - (2 - Pyrimidinyl) - p - sulfamoylphenyl] Phosphorus Triamide—This compound was prepared by condensing sulfadiazine with phosphorus trichloride adopting the general method reported in the previous paper. The product which was obtained in 20% yield melted at 196–200° (255° dec.) (liquid crystal) (2) after being crystallized from absolute ethanol.

Anal.—Calcd. for $C_{30}H_{27}N_{12}O_6PS_3$: P, 3.98. Found: P, 3.56.

N,N'-di(α-Naphthyl) Phosphorodiamidous Acid and N,N'-di(β-Naphthyl) Phosphorodiamidous Acid—These were prepared by condensing the naphthylamine with phosphorus trichloride adopting the general method reported in the previous paper. The yield in both cases was 30% and the product crystallized from aqueous ethanol. The former compound melted at 228–230°.

Anal.—Calcd. for $C_{20}H_{17}N_2OP$: C, 72.26; H, 5.15; N, 8.40. Found: C, 71.41; H, 5.07; N, 8.61.

The latter compound melted at 240–243°.

Anal.—Calcd. for $C_{20}H_{17}N_2OP$: N, 8.40. Found: N, 8.61.

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Keyphrases

Phosphorus–nitrogen compounds
N-Substituted phosphoric triamide, phosphorodiamidic acid derivatives—synthesis
Condensation—phosphorus oxychloride, sulfa compounds

Anticholinergic Agents Based on Ariens' Dual Receptor Site Theory

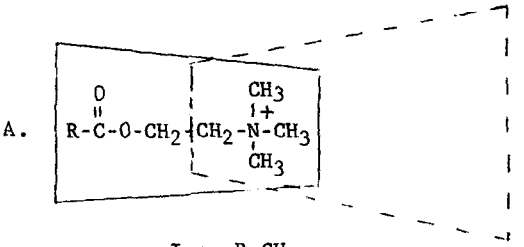
Sir:

Many drugs classed as competitive antagonists seem to bear a close structural resemblance to the corresponding agonist and are presumed to have an affinity for the same receptor. However, it is frequently observed that in many classes of drugs changes in structure of agonists and competitive antagonists do not result in a parallel change in activity. If affinity is the property altered by the structural modification, then classical receptor theory offers no explanation for this. Ariens has suggested that the receptor site for agonists and antagonists in such a case may not be identical (1-3). Thus, competitive antagonists are pictured as occupying a space that overlaps only a portion of the agonist site and gain affinity through attraction to a subsite not utilized by the agonist. This latter portion of the antagonist receptor is referred to as "additional receptor parts."

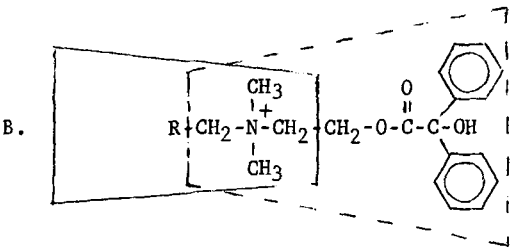
In the case of the cholinergic receptor at postganglionic parasympathetic synapses, an agonist should combine with a certain region of the receptor as shown in Fig. 1A for acetylcholine (Ia) or carbachol (Ib). Classical antagonists such as choline benzilate (IIa) or lachesine (2-dimethylaminoethyl benzilate ethobromide, IIb) are then assumed to combine with a portion of the agonist site and some additional receptor parts (which together make up the antagonist receptor) as shown in Fig. 1B.

The authors became interested in the possibility of preparing compounds such as 2-[(2-acetoxyethyl)methylamino]ethyl benzilate methobromide (IIIa) and 2-[(2-carbamyloxyethyl)-methylamino]ethyl benzilate methochloride (IIIb), which could combine with both the agonist and antagonist receptors as illustrated in Fig. 1C. These two compounds contain a cationic head attached to two chains—one resembling the potent agonists acetylcholine or carbachol and the other resembling the potent antagonist lachesine. If all portions of the molecule can bind to the appropriate portions of the receptor simultaneously, then these compounds should have a greater affinity and, therefore, be more active antagonists than compounds not containing an agonist moiety. The compounds IIIa and IIIb were prepared by alkylation of dimethylaminoethyl benzilate with bromoethyl acetate or chloroethyl carbamate. Compound IIIa has been prepared previously (4).

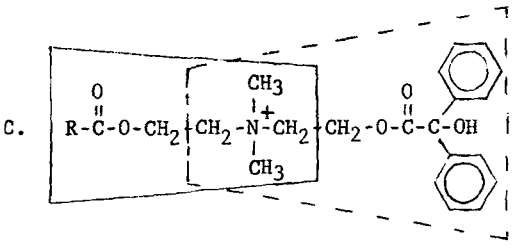
The drugs were evaluated by means of the pA_2 described originally by Schild (5). Segments of isolated ileum from guinea pig were suspended



I a, R=CH_3
b, R=NH_2



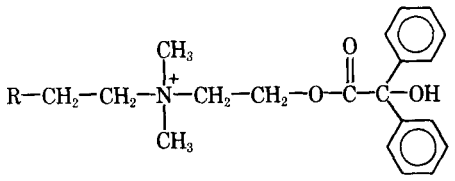
II a, R=H
b, R=CH_3



III a, R=CH_3
b, R=NH_2

Fig. 1—Possible relative position of drugs on the agonist site (solid lines) and antagonist site (dotted lines) of the cholinergic receptor.

TABLE I—AFFINITIES OF LACHESINE AND DERIVATIVES FOR CHOLINERGIC RECEPTORS IN GUINEA PIG ILEUM



Compd.	R	$pA_2 \pm$ S.E.[a] Agonist: Ia	Agonist: Ib
II b	H—	8.71 ± 0.07 (12)[b]	8.70 ± 0.04 (16)
III a	$CH_3-\overset{\overset{O}{\|\|}}{C}-O-$	7.96 ± 0.06 (10)	7.85 ± 0.05 (10)
III b	$H_2N-\overset{\overset{O}{\|\|}}{C}-O-$	7.19 ± 0.07 (8)	6.90 ± 0.10 (7)

[a] S.E. = standard error. [b] Number in parentheses indicates the number of segments of guinea pig ileum used for each determination.

in Tyrode's solution at 25° and aerated with 95% oxygen and 5% carbon dioxide. Tension development was measured by means of FT 0.03 force displacement transducers and recording made on a Grass model 5D polygraph.

Both acetylcholine and carbachol were used as agonists. Four or five dose-response curves were determined on each isolated tissue preparation, and a fresh segment was used for each agonist–antagonist combination. Increasing concentrations of antagonist produced a parallel shift to the right of the dose–response curves of the agonist and did not depress the maximum response. The compounds themselves did not initiate a response in any concentration. Thus, they do behave as true competitive antagonists. The compounds are highly specific for the cholinergic receptors as evidenced by the fact that they do not alter the response of the isolated ileum to potassium.

The compounds IIIa and IIIb were compared with the potent antagonist lachesine which differs only in that it lacks an acetoxy or carbamyloxy group. The pA_2 values were obtained by linear regression analysis according to the method of Steel and Torrie (6). The results are summarized in Table I. There was no significant difference between the pA_2 values obtained with acetylcholine and carbachol. However, differences between antagonists were significant. Lachesine, the model compound for this series, was significantly more effective than either of the derivatives. The addition of an acetoxy group on the ethyl moiety decreased the pA_2 nearly one log unit, and substitution of the carbamyloxy group in the same position further reduced the activity of the derivative.

Clearly the additional acetoxy or carbamyloxy groups attached to lachesine do not lead to the expected increase in affinity for the receptor. A report has appeared recently from Ariens' laboratory involving antihistamine agents based on the same type of agonist–antagonist double molecule (7). These also failed to show any augmented antagonist action.

The present results do not allow any conclusions concerning the general validity of Ariens' dual receptor site theory since several alternative explanations might be advanced for the lack of higher pA_2 values: (*a*) The lower affinity might be only apparent due to a lowered ability of the compounds to reach the site of action. (*b*) The various receptor parts may not be arranged in a linear fashion as depicted in Fig. 1. In such case, the nonbonded interactions within the molecule may hinder the drug from assuming a conformation in which all parts could combine with their respective subsites simultaneously. (*c*) Ariens' dual receptor site theory may not be applicable to the ester-type antagonist used in the present case. Other compounds employing additional cholinergic agonist–antagonist moiety combinations are being studied. Antiadrenergic agents based on a similar approach are also being investigated.

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Received November 16, 1967.
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Naming Organic Compounds. A Programmed Introduction to Organic Chemistry. By JAMES E. BANKS. W. B. Saunders Co., W. Washington Sq., Philadelphia, PA 19105, 1967. viii + 276 pp. 18 × 26 cm. Price $4.50. Paperbound.

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NOTICES

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Received November 16, 1967.
Accepted for publication January 8, 1968.

This investigation was supported by research grant NB 07273:01 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

The authors wish to acknowledge the technical assistance of Miss Tanga Dickerson and Mrs. June Dvorak.

Keyphrases

Anticholinergic agents
Lachesine derivatives—activity
Dual receptor site theory, Ariens—basis for derivative structure

Books

REVIEWS

Matrix Algebra for the Biological Sciences. By S. R. SEARLE. John Wiley & Sons, Inc., 605 Third Ave., New York, NY 10016, 1966. xii + 296 pp. 15 × 23 cm. Price $9.95.

With high school algebra as the only prerequisite, this book shows how to learn matrix algebra, emphasizing its use in biology and statistics. The author points out that his style is informal and that although proofs of the theory are given, they can be omitted if desired in using the text. The mathematical development is illustrated with numerous examples. Matrix algebra up to and including latent roots and vectors and its use in regression analysis are covered. Exercises are included in each chapter.

Staff review

Index Nominum 1966. Swiss Pharmaceutical Society, Zurich, Switzerland 1966. 838 pp. 19 × 25 cm. Price Sw. Fcs. 100.—(about $25.00).

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JOURNAL OF Pharmaceutical Sciences

April 1968 volume 57, number 4

Review Article

Biologically Active Pyrazoles

By RONALD E. ORTH

"No great change occurs in the general nature of the action of a substance if a pyrazole ring be substituted in place of a pyrrole ring" (1).

THREE DECADES and the wisdom of this conclusion have vanished since 1937; however, an awareness of changing "truths" is no more amply illustrated than with the bio-active pyrazoles. Although the impact of pyrazole chemistry on biological systems is uneven, the recognized value of a few pyrazoles for even fewer purposes has characterized the past. A rapid leveling of interest, however, is reflected in recent literature, and a review of pyrazole implications on biological systems quickly identifies nearly all activity as a "happening" of the past decade. Kost and Grandberg listed 799 references (2) in an excellent review of pyrazole chemistry and an earlier compilation may also be of interest (3). Pyrazole NMR and UV spectra were reported by Elguero *et al.* (4, 5), and the nonaromatic pyrazolones and pyrazolidones were reviewed (6). Tautomeric pyrazolones, alone, were of sufficient interest that a review was written (7), and certainly, much excitement was generated by the clinical efficacy of phenylbutazone and its analogs (8). Among the well-known compounds are antipyrine and pyramidon, the analgetic-antipyretic pyrazolones. Knorr (9, 10) first synthesized a pyrazole derived compound in 1883, which led to the discovery of antipyrine (11).

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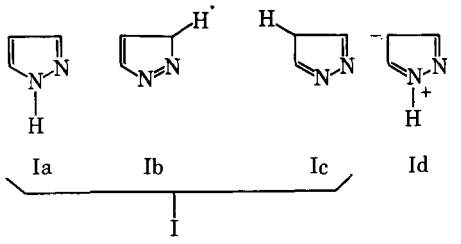
Although the thoroughly investigated 5-pyrazolones and their 3-oxo-isomers react as ketones, the 4-isomers are tautomeric and behave like phenols.

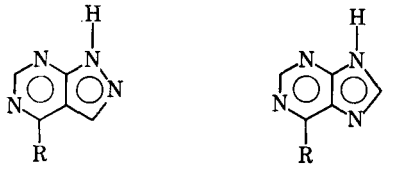
Pyrazole (1) consists of a doubly unsaturated 5-membered ring containing two adjacent N-atoms. Three possible ring-tautomers exist (Ia, Ib, Ic), which have not been separated. The ease of pyrazole-tautomerism may be explained if all the N-H bonds in the associated structures are assumed to be equivalent (12). The polar structure (Id), which contributes much to the resonance hybrid of pyrazole, explains the high degree of electrophilic substitution in the 4-position (13). The presence of the N-H moiety allows pyrazole to ionize as a anion, substitute on this N-atom, and possibly tautomerize. The lone pair of electrons on the secondary N-atom, which is utilized to form an aromatic sextet, is not available for affecting water solubility. The doubly bound N-atom does increase pyrazole solubility over pyrrole because the lone pair of electrons on the N-atom is disengaged and available. Annelation to give indazole decreases water solubility. Pyrazoles with free N-H groups are amphoteric, though more basic than acidic and form associated linear dimers and trimers. Good stability generally characterizes the pyrazoles (14).

The great variety of bio-responses promoted by pyrazoles is illustrated by the insecticidal, anal-



Ia Ib Ic Id

I

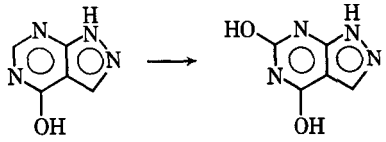


II (when R is H) III (when R is H)
IIa (when R is —OH) IIIa (when R is —SH)
IIb (when R is $-NH_2$)

gesic, sedative, anticonvulsant, antipyretic, and anti-inflammatory activity elicited by only the 1- and 2-dimethylthiocarbamoylpyrazoles (15). The analogous 1-carbamoylpyrazoles effect analgesia, diuresis, antipyresis, sedation, and a bitter taste (16). However, these are selectively toxic medicinal agents, and toxicity is always a concern when these compounds are administered. Hydroxy- and amino-pyrazoles (17) and F.D. & C. yellow No. 5 (an azopyrazole) (18) have been studied and fatty liver was detected following the administration of allopurinol, 4-hydroxypyrazolo[3,4-*d*]pyrimidine (IIa), to four species of warm-blooded animals (19).

BIO-ANTAGONISTIC PYRAZOLES

Pyrazole, pyrazolo[3,4-*d*]pyrimidine (II), and their substitution products include many biologically active agents, which possess enzyme-inhibiting properties of interest. For instance, many xanthine oxidase inhibitors are carcino-



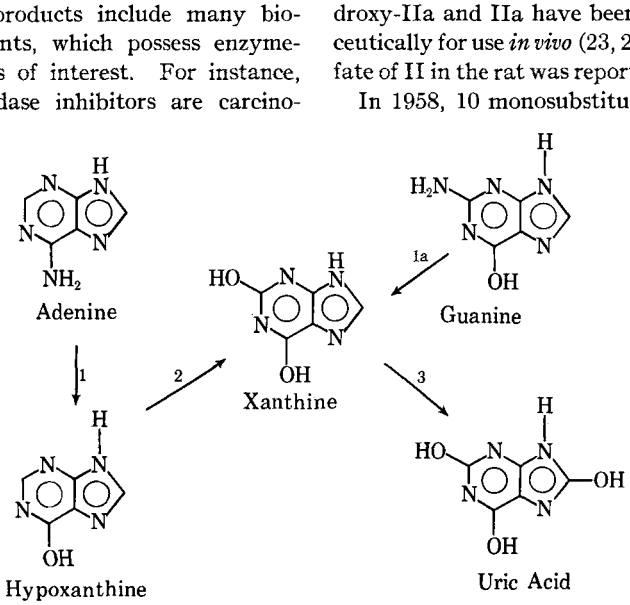
4-Hydroxypyrazolo-[3,4-*d*]pyrimidine (IIa) 4,6-dihydroxypyrazolo-[3,4-*d*]pyrimidine

Scheme II

static or tumor inhibiting, while the effectiveness of pesticides has been correlated with their anticholinesterase activity. Inhibitors of carbonic anhydrase, monoamine oxidase, liver alcohol dehydrogenase, and catalase have been investigated. Some degree of pyrazole activity results from competition with endogenous substances to antagonize an enzyme system. The xanthine oxidase antagonistic pyrazoles, particularly those with antitumor activity, are of great interest. Many of these compounds have been derived from II.

Hypoxanthine and its intestinal metabolites are readily absorbed from the small intestine where xanthine oxidase combines with hypoxanthine, xanthine, and Cu to produce a necessary transport-complex (20). Purine (III) and II-derivatives compete for this common transport process. Although 4-hydroxypyrazolo[3,4-*d*]-pyrimidine (IIa) and 7-hydroxy-IIa strongly inhibited the active transport of uracil across the intestinal wall (Schemes I and II) (21), IIa, uric acid, and adenine form similar transport-complexes with xanthine oxidase. In the chick embryo and chicken, IIa inhibited uric acid formation, but diversion of N to a different end product allowed normal development (22). 6-Hydroxy-IIa and IIa have been developed pharmaceutically for use *in vivo* (23, 24), and the metabolic fate of II in the rat was reported (25).

In 1958, 10 monosubstituted derivatives of II



Scheme I

were prepared and suggested as antimetabolites (26). 6-Hydroxy-IIa and IIa were converted by purinenucleoside phosphorylase to the 1-ribosyl-metabolites and isolated from the urine of treated patients; however, 4-hydroxypyrazolo[3,4-*d*]pyrimidine (IIa) was a better substrate than 6-hydroxy-IIa (27). The sea urchin and other systems have been utilized to investigate the effect of 4-aminopyrazolo[3,4-*d*]pyrimidine (IIb) on purine metabolism (28, 29). Avian glutamine ribosylpyrophosphate 5-phosphate amidotransferase, the first enzyme of purine biosynthesis, was inhibited by IIa-ribonucleotide. A "pseudo-feedback" inhibition of the ribonucleotide may be an important aspect of the *in vivo* biological activity of II derivatives (30).

ANTITUMOR AND CARCINOSTATIC PYRAZOLES

Pyrazole was reported as possessing antitumor activity (31, 32) and 25 courses were given to patients with solid tumors. Bone marrow depression was minimal, while severe thrombocytopenia and hepatotoxic reactions were noted once. Nausea and anorexia were reported, but no objective responses were observed. Pyrazole depressed: adenocarcinoma CA-755 (90–100%); Ehrlich ascites carcinoma (50–60%); and spontaneous mammary tumors in C_3H mice. Crocker sarcoma S-180, leukemia L-1210, and human tumors in conditioned rats, however, did not respond. The i.p. single dose LD_{50} for pyrazole was: 400–600 mg./Kg. for mice and 900 mg./Kg. for rats. Experimentally induced tumors were treated with 4-aminopyrazole (33), and several 1-substituted pyrazoles exhibited significant activity against CA-755 with less effect on S-180. The 1-carboxamide elicited a moderate but consistent action *versus* human tumor HS-1 (34).

The investigation of antitumor activity of II and its substitution products was the most extensive of any for bio-active pyrazoles. The hydrochloride of II showed the greatest activity among 16 pyrimidines tested in 1960.

A xanthine oxidase-inhibiting preliminary screening technique for carcinostatic substances was developed (35) and a large number of II substances were screened *versus* CA-755 and leukemia L-5178 (both were susceptible to inhibition by purine antagonists). Consistent and significant activity was reported using IIb, *N*-alkyl-IIb, *N*-dialkyl-IIb, and their 1-alkyl-derivatives (36). Skipper *et al.* (37), effected antitumor activity with IIb and 1-methyl-IIb. The toxicity of IIb in humans was slight, however, hepatotoxicity and hemorrhage was reported in animals (38). 1-Methyl-IIb and IIb acted on nucleic acid metabolism in ascites carcinoma, but sarcoma S-180 was not sensitive to either material (39, 40).

A Houston team utilized *Neurospora* for screening potential carcinostatic substances and found that 1- and 3-substituted IIb compounds inhibited the growth of this tissue (41). Human HeLa and mouse B-3 adenocarcinoma cells were found to be resistant to IIb (42), but HeLa cell damage was reportedly caused by 13 newer IIb-substances (43). Two of these were more active against *N. crassa* than IIb (44). Active IIb derivatives lower the DNA levels in tumor and liver tissue (45), and 1-methyl-IIb also lowered muscle DNA levels. Interestingly, 12 IIb derivatives gave relief of IIb-inhibition. Since the position of the side chain determines inhibition or relief potential (46), 1-methyl-IIb and IIb were found highly active in recent tumor transplants, while only slightly effective in spontaneous tumors (47). The efficacy of IIb against cultivated human tumor cells, in earlier studies, was greater than for any amino- or 1-substituted-II products (48). Purine synthesis was disrupted in Ehrlich ascites cells by IIb (49), but attempts to establish mutant strains of *N. crassa* with this substance were unsuccessful (50).

Robins (51) reviewed the structure-activity relationship (S-AR) of purines and related compounds (11,300 compounds tested over a 10-year period) against animal tumors (163 references). Similarities of the allowable positions for substitution in II and in purine were most striking. 4-Aminopyrazolo[3,4-*d*]pyrimidine, which is active against CA-755 and numerous other tumors, was the parent compound in that study. Substitution of certain alkyl groups in the 1 position (corresponding to position 9 in purine) provided active compounds, and the presence of a tetrahydropyranyl- or tetrahydrofuryl-group at position 1 also allowed retention of antitumor activity. The 4-alkyl-II derivatives inhibited L-5178 and prolonged L-5178 and L-1210 life span in infected mice. Substitution of alkyl groups on the amino function at position 4 (6 in purine) created less toxic products with superior therapeutic indexes, while substitution at both the 1 and 4 position, simultaneously, usually diminished antitumor activity to a slight degree. 1-Methyl-*N*-*n*-butyl-IIb and *N*-benzyl-IIb were active against Dunnings ascites leukemia, but 1-phenyl-IIb derivatives were inactive. 6-Amino-IIb was active, but the 6-amino moiety (2 in purine) apparently made little difference. The spatial arrangements of II and purine derivatives appear interchangeable, however,

4-mercapto-II and 6-amino-4-mercapto-II, the corresponding analogs of 6-mercaptopurine (IIIa), and 2-amino-IIIa, were without activity *versus* CA-755. An L-1210 azaguanine-resistant strain was found cross-resistant with IIIa, but not with IIb. These results lead to a conclusion that similar steric requirements, alone, were not sufficient to provide antitumor activity. Since IIb cross-resistance did not occur in either case, different mechanisms of action must be involved. Simultaneous administration of IIb with IIIa resulted in potentiation *versus* ascites carcinoma, S-180, and 6C3HED lymphosarcoma. Similar effects were observed with IIb and azaserine or thioguanine (52). This observed potentiation was further evidence favoring separate mechanisms for IIb and IIIa.

The antitumor activity of IIb was probably exerted at the acid-soluble nucleotide level, although it was incorporated readily into nucleic acids of normal and neoplastic tissue. The *de novo* pathway to nucleotide-purines was inhibited, probably by the previously mentioned "pseudofeedback" mechanism. Recent clinical success with *N-n*-propyl-IIb against myeloblastic leukemia has renewed interest in the II tumor inhibitors, and further support for this interest was given by correlation of the pKa values of 4-substituted-II compounds with their antineoplastic activity. Position 5 (1 in purine) may be critical for the adenine-like IIb substances, because a close steric fit was required even at position 3 (7 in purine). 3-Methyl-IIb and the purine analog were devoid of antitumor properties.

Nakajima *et al.* (53), reported correlating the first ionization potential of free doublets of the N-atoms of II and purine and their derivatives with antitumor activity. Electronic structure, chemical reactivity, and the basicity of these agents were related to tumor inhibition (54). Bio-activity was associated with the position of the most basic N-atom and the basic strengths (55). 6-Amino-II was found to be carcinostatic but hepatotoxic, while 5-amino-7-hydroxy-II and a number of trifluoromethyl-derivatives of IIb and *N*-alkyl-IIb were inactive (56–60). Scott and Foye (61) and Sutcliff (62) reported on IIb, which in tumor-bearing mice, decreased formate-^{14}C uptake into polynucleotide purines (DNA, *etc.*) with ^{14}C-accumulation in serine.

The uptake of adenine into the nucleic acids was inhibited by IIb, and nucleic acid synthesis in IIb-sensitive CA-755 and in the less sensitive S-180 ascites was halted for a short period. The S-180 tissue, however, recovers more readily (63), while adenine, given to S-180 ascites-bearing mice prior to IIb, caused a decrease in tumor inhibition (64). 4-Aminopyrazolo[3,4-*d*]pyrimidine was toxic to H. Ep. No. 2 cells, but the "feedback" growth inhibition occurred at much higher concentration and was the mechanism of carcinostasis (65).

Allopurinol (IIa), a powerful xanthine oxidase inhibitor, was found to depress the disappearance rate of ^{32}P-labeled $CrPO_4$ in an attempt to characterize the phagocytic properties of the reticuloendothelial system. Methotrexate, chlorambucil, and thiotepa possessed this facility, while azaserine and busulfan[1] were ineffective (66). A decreased IIIa to thiouric acid oxidation occurred when IIa was given simultaneously to human patients. A severalfold potentiation resulted *versus* CA-755 and in the mouse immune response to sheep erythrocytes. In man, 0.2 to 1.0 Gm. of IIa given daily possessed a higher therapeutic index than other antineoplastic agents (67, 68). When given with IIa or 6-hydroxy-IIa, 6-mercaptopurine was potentiated threefold, while xanthine oxidase oxidation-inhibition of hypoxanthine and xanthine in the liver and kidney gave rise to increased uric acid levels (69). This IIa action was used to test the ability of xanthine to serve as a precursor for nucleic acid purines while being protected from destructive catabolism. The ability of CA-755 (IIIa-resistant)-bearing mice to use xanthine as a precursor was reduced by one-half using IIa (70), and it also inhibited liver and kidney, but not spleen xanthine oxidase in rats (71). Xanthine dehydrogenase-inhibition by IIa was also noted (72).

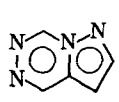
Pyrazolo[3,2-*c*]-*as*-triazines (IV) were more effective S-180-inhibitors in mice and methylcholanthrene-induced tumorstatic agents in rats than IIIa, but the best of the lot were ethyl-4-amino-IV and 6-iodoacetyl-3-methyl-4-methylene-IV (73,74).

1-Methyl-IIb, given to pregnant rats, produced a high number of malformed and dead fetuses (75). The teratogenic nature of IIa, administered with IIIa at subteratogenic levels, was reported (76).

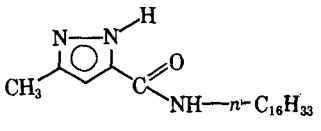
ANTIVIRAL PYRAZOLES

3-Methylpyrazole-5-carboxylic *n*-hexadecylamide (V) demonstrated virucidal activity *versus* the Japanese encephalitis virus (77). Other studies supported that 1-methyl-*N-n*-propyl-IIb, the *N*-isopropyl- isomer, *N*-isopropyl-IIb and IIb suppressed virus plaque formation with *Herpes simplex*, *Vaccinia*, and West Nile (78). Toxicity,

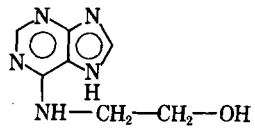
[1] Trademarked as Myleran by Burroughs Wellcome & Co., Tuckahoe, N. Y.



IV



V



VI

but not antiviral activity, decreased when N^6-(2-hydroxyethyl)adenine (VI) was given with IIa (79). In an antiviral formulation (80), one part of the various N^6-(hydroxyalkyl)adenines was combined with 2.5–20 parts of IIa.

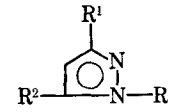
GOUT AND THE PYRAZOLO[3,4-d]-PYRIMIDINES

One of these compounds, allopurinol, is being used in the treatment of patients with gouty conditions. The oxidation of urine xanthine and hypoxanthine was antagonized by the action of IIa on xanthine oxidase (81), which resulted in lower serum and urine uric acid levels (82). Formation of fresh deposits was arrested and mobilization of old deposits occurred, but it was suggested that the best use of IIa was with agents such as colchicine (83, 84). Compound IIa was well tolerated in human patients and was found to be useful in subjects with hyperuricemia who were refractory to uricosuric therapy. Although gouty patients with a low tolerance to other drugs, which lower blood plasma urates, may be helped, no improvement was noted in those with tophaceous gout (85, 86). Further reduction of serum and urine urates and increased urinary hypoxanthines (87) resulted from a sulfinpyrazone and IIa combination. Oxypurinol, 6-hydroxy-IIa, was utilized also in the treatment of gout (88, 90). Toxicity (91), nephropathology (92), human tissue reactions (81), and other pyrazole-gout reports were of interest (93–96).

PYRAZOLES INHIBITING OTHER ENZYME SYSTEMS

One S-A-R report of anti-monoamine oxidase (MAO) 1,3,5-trisubstituted pyrazoles listed several interesting examples (Table I). For instance, transposing of methyl- and the substituted-hydrazide moiety from positions 3 and 5 to 5 and 3, respectively, reduced activity in one series. Activity was found to decrease as R varied, so that when R was aralkyl, separation of the aryl from the hydrazido moiety decreased activity. Benzyl or 2-phenethyl groups produced equal anti-MAO effects, and pyrazole-3,5-dicarboxylic acid hydrazide were ineffective antagonists (97, 98).

TABLE I—MONOAMINE OXIDASE INHIBITORS



Factors Enhancing Inhibition:
- R is benzyl or phenethyl
- R^1 is methyl
- R^2 is benzyl-, β-phenethyl-, or 1-phenethyl-carboxylic acid hydrazide.

Factors That Lower Inhibition or Increase Toxicity:
- R = H
- R^1 = R^2 = CONHNHR
- R^1 and R^2 interchanged
- R^2 alkyl portion > 2 carbon

4-Aminopyrazole *in vitro* and *in vivo* catalase-inhibition was reported, giving the ID *in vitro* as 3.8×10^{-3} *M* (99, 100).

Fundamental biochemical interpretations were derived from the work of Theorell *et al.* (101–103) on liver alcohol dehydrogenase (LADH), and a model of a ternary intermediate in the enzyme reaction LADH-diphosphopyridine nucleotide (DPN)-pyrazole was reported. Pyrazole, the most potent inhibitor of LADH known (1963), adds to the enzyme-coenzyme binary system producing a new complex with a characteristic 290 mμ maximum. The LADH-DPN-pyrazole complex stoichiometry was reported as 1:2:2, respectively. Liberation of one H^+ per complex formed suggested that, following H^+ release, a charged pyrazole carbanion interacted covalently with C-4 of the pyridinium ring of DPN. Binary and ternary complexation increased the stability of LADH toward certain types of inactivation, probably due to conformational changes in the protein molecule. The LADH-NAD-pyrazole and LADH-NAD-4-iodopyrazole complexes have been crystallized to examine "Koshlands Induced Fit Theory." The ternary ligand pyrazoles were found to occupy the active alcoholic binding site and form a bridge between Zn atoms in the LADH and the coenzymic nicotinamide moieties. X-ray crystallography uncovered orthorhombic symmetry for free enzymes and clinic patterns for the ternary complexes. Optical rotatory density data were utilized to calculate Moffitt-Yang plots, and allowance for Cotton effects, introduced by the binding of NAD to the protein, were utilized. The change of Moffitt parameters, b_0, from −100° for the enzyme to −185° in the ternary complex was interpreted as a change of protein conformation due to complex formation.

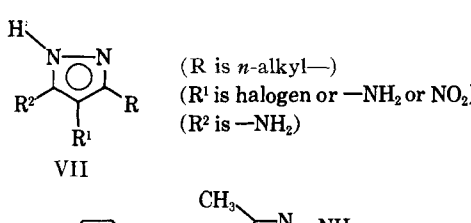
ANTIMICROBIAL PYRAZOLES

The earliest reported anti-infective pyrazole, 5-amino-4-benzeneazo-1-phenyl-3-methyl-

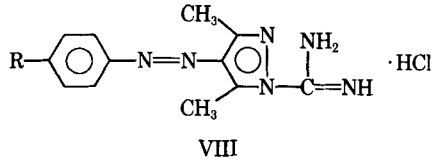
pyrazole, was for use as a urinary antiseptic, but when it was administered orally, albuminuria resulted. A minimum lethal dose (50%) in rabbits was found to be 200 mg./Kg. (104). Grandberg *et al.* (105) tested 96 pyrazoles as effective bactericidal agents, and other investigators reported (106) that 3-nonylpyrazole and its derivatives exhibited bacteriostatic-fungistatic activity. The 3-alkylpyrazoles (VII) reportedly have an extremely specific antibacterial spectrum; *Staphylococcus aureus* and *B. subtilis* were inhibited, but the only sensitive Gram-negative organisms, were *N. gonorrhoeae* and *V. cholerae*. Longer or shorter 3-alkyl chains varied the potency in this series. The *n*-octyl compound possessed the highest surfactant properties (107), while 4-nitro- and 4-halo-3-nonylpyrazoles demonstrated good antimicrobial activity, and the 4-amino-homolog exhibited a marked decrease in efficacy *versus* Gram-positive organisms. Shifting the amino group to the 5 position heightened bacteriostasis, but ring oxidation to the pyrazolone resulted in a loss of activity (108).

1 - Dichloroacetyl - 3 - (*p* - nitrophenyl) - 5 - methylpyrazole inhibited *S. typhosa in vitro* at 500 mcg./ml., but was inactive against several other common organisms (109). 1-Cyclopentyl-*N*-furyl-IIb inhibited the preferential stimulation of the synthesis of DNA, but potentiated kinetin riboside in *D. pneumoniae*. The results indicated a relationship between DNA breakdown products to population changes and DNA synthesis, which illustrated the usefulness of employing a virulent population change system for the study of compounds interfering with DNA synthesis by DNA breakdown products (110).

The adenine isostere 4-aminopyrazolo[3,4-*d*]pyrimidine was effective *versus C. utilis*, *E. coli*, and *B. cereus* (111), inhibiting the growth of *E. coli* and *B. cereus* by about 30%. Adenine or hypoxanthine addition partially relieved the drug-induced inhibition, but a combination of IIb and guanine resulted in potentiated inhibition of cell growth. The formation of 4-amino-5-imidazolecarboxamide, a purine precursor, by an *E. coli* mutant, B-96, was lessened by IIb, resulting in depressed *de novo* purine synthesis. Stimulation of the C-8 guanine, by IIb, to adenosine and ribonucleic acid adenine was observed in *E. coli*, while a depression of the conversion occurred in *B. cereus*. The radio labeling of several purine-containing cell constituents was effected (112). 6-Amino-IIa exhibited good activity *versus B. cereus*, but only slight activity against *E. coli*. Treated *B. cereus* cells elongated, and the RNA content in the cell was reduced, due to the antagonism of an early reaction in purine biosynthesis (113). Three 4-arylazo-1-amidines of 3,5-dimethylpyrazole (DMP) (VIII) were found effective *versus S. aureus* and *S. albus* at $1 \times 10^{-4}M$. They were the 4′-tolyl-, and 4′-chlorophenyl-, and 4-(4′-acetylphenylazo)-3,5-dimethylpyrazolyl-1-amidine aminoguanidine salts, the latter of which possessed good trichomonostatic ($1 \times 10^{-5}M$) and tuberculostatic ($4 \times 10^{-4}M$) activity. Several of these azopyrazoles are also active ascaridostatic agents (114).



VII

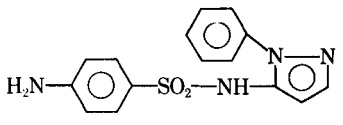


VIII

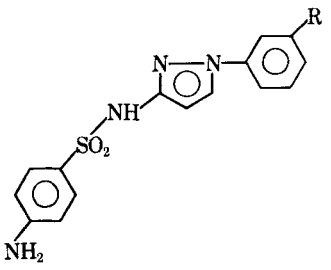
5-Amino-7-methyl-II possessed antitubercular activity and was well absorbed orally in laboratory animals; however, the tuberculostasis produced was less than that shown by streptomycin in the guinea pig and mouse (115, 116). A parallelism was noted between the tuberculostasis and xanthine oxidase inhibition produced by this compound. 4 - (4′ - Acetylphenylazo) - 3,5 - dimethylpyrazolyl - 1 - thiocarbonic acid amide - thiosemicarbazone elicited potent tuberculostatic activity at $1 \times 10^{-4}M$, but unfortunately, the LD_{100} (100 mg./Kg.) in rats was high (114). Certain 4-nitrosopyrazoles were active *in vitro*, but not in the mouse, and some azoxypyrazoles were found to be inactive tuberculostats (118).

Sulfapyrazine, sulfahydantoin, and sulfathiazoline were acclaimed as more promising against *Pneumococcus* than sulfapyrazoles in 1941 (119), however that very year, Jensen introduced 3-sulfanilamidopyrazole as eliciting activity similar to that of sulfathiazole (120). For over 20 years, articles characterizing the chemistry, solubility, microbiology, and toxicity of sulfonamidopyrazoles have been published (121–125).

5-(*p*-Aminobenzenesulfonamido)-1-phenylpyrazole (IX) (sulfaphenazole, orisul), was found to lower an induced rabbit fever, but it is less effective and less toxic than pyramidone for this purpose. Each compound in a series of N^1-aryl-3-pyrazolylsulfanilamides inhibited *S. aureus* at 10 mcg./ml. The *m*-methoxy-homolog was the most active *versus E. coli in vitro* (10 mcg./ml.), and it also exhibits a displacement action of various penicillins bound to blood serum proteins. The decrease in bound penicillin G was 49.5%; peni-



IX



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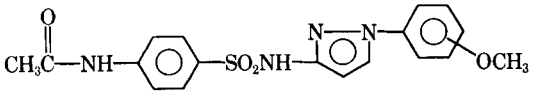
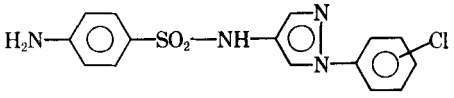
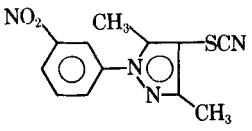


XI



XII



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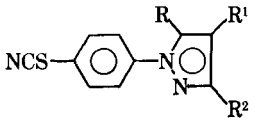
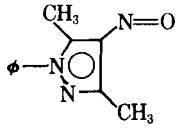


XIV



XV

cillin V, 39.8%; and ancillin, 35.9%. The relative activity of various sulfonamides as penicillin-displacing agents corresponded closely to the known affinity of each for serum proteins. Over 250 chemicals were tested with ^{14}C-labeled penicillins, and the findings showed that replacement of a 3-methoxyl- group by a 3-methyl- substituent increased the duration of activity (X). Active N^1-aryl-3-pyrazolyl-N^4-acetylsulfanilamides (XI) have been prepared, and a number of chloro-derivatives of 1-phenyl-4-sulfanilamidopyrazole (XII) have been found useful. Padeiskaya *et al.* have contributed to sulfonamide chemotherapeutics (126–135).

Seven of eight fungi tested, including *C. militaris*, converted IIb to the riboside, thus, the fungi may utilize IIb as a nutrient (136). More important is the antifungal activity, which was elicited by a number of pyrazole compounds. Two thiocyanato-, eight 4-(*N,N*-disubstituted-thiocarbamoylthio)-, and five 4-alkoxythiocarbonylthiopyrazoles were effective against *Piricularia oryzae*, *Phytophora infestans*, *Colletotrichum lagenarium*, and *Candida albicans*. The thiocyanato derivatives displayed the greatest fungal inhibition, and of these, 1-(*m*-nitrophenyl)-4-thiocyanato-3,5-dimethylpyrazole possessed the greatest fungal inhibitory activity of all compounds tested (XIII) (137). This same Japanese group found high-level activity in 1-(4-thiocyanatophenyl)pyrazoles (XIV) (138). Maximum fungitoxic activity was attained by substitution of an aryl-group in the 1 position of 3,5-dimethyl-4-nitrosopyrazole (XV). Several of these compounds prevent spore germination at a concentration of 0.1 p.p.m. and provide 95% protection against *Alternaria solani* on tomato foliage at 10–60 p.p.m. (139). Zsolnai has found the nitrosopyrazoles effective *versus* fungi which afflict humans (140, 141); pyrazole was one of 165 *N*-compounds studied for fungitoxicity. 4-Nitrosopyrazole toxicity increased with an increase in the bulk of the hydrocarbon side groups, but the mechanism of action was found to be the same, regardless of the group in this series. Increased fungitoxicity was observed as the oil:water partition coefficient increased for these substances, and generally, lipophilic groups, such as aryl- or alkyl-, increased the toxicity. Amino-, nitro-, nitroso, hydroxyl-, and mercapto-group substitution produced a similar response (142, 143). Six mono-, di-, and tri-substituted 4-nitrosopyrazoles (XVI) are active against *P. oryzae* and *Ophiobolus miyabeanus* (144), and nitrosopyrazoles with 1-substituted cyclic groups are active, particularly against pathogenic fungi. It was found that 1-cyclohexyl-3,5-dimethyl-4-nitrosopyrazole exhibited no dermal irritation and possessed low phytotoxicity (145).

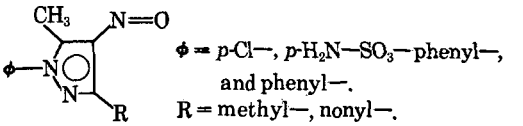
3,5-Dimethyl-4-(4′-chlorophenylazo)pyrazole (XVII), one of 750 chemicals tested, was the most active *versus* 3 selected fungi (146), while a

4-phenylazo-3,5-dimethylpyrazolyl-1-amidine (XVIII) salt was effective *versus T. gypseum*, *E. Kaufman-Wolff*, and *A. quinkeanum* at $2 \times 10^{-4}M$. The 4′-chloro- and 3′-chloro-derivatives (XIX) were effective at $1 \times 10^{-4}M$ against the same fungi. Many of the azopyrazoles were also bacteriostatic, trichomonostatic, and ascaridostatic, but according to Zsolnai, R—N=N—CH— must be present as an intact moiety, in arylazoanti-infective chemotherapeutic agents (114). 3-Nonylpyrazole possessed high antifungal-antibacterial properties (106), and recently, 3-methyl-5-(5-nitro-2-furyl)pyrazole (XX) was introduced for this activity (147). A similar nitrofurylpyrazole was reported in the Netherlands, with claims for effectiveness against a wide spectrum of bacteria and a few fungi (148).

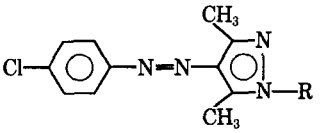
PESTICIDAL PYRAZOLES

Well tolerated 4-aminopyrazoles (XXI) yielded interesting pharmacodynamic spectra; for instance, 1,3,5-trimethyl-4-dimethylaminopyrazole methiodide exhibited a choliomimetic action. Substituting 1-phenyl in place of 1-methyl reversed the action and effected a classical cholinolytic response. Although molecular weight difference was suggested as the underlying factor, the activity of the former substance was attributed to anticholinesterase properties (149). A number of pyrazolyl-carbamates and phosphates, possessing good anticholinesterase activity, were too toxic for use in medicine, but were excellent pesticides. Practical uses of the dimethylcarbamates and dialkylphosphates of 5-hydroxypyrazoles have been studied since 1951 (150–156). 1-Isopropyl-3-methyl-5-pyrazolyl dimethylcarbamate (XXII),[2] and 1-phenyl-3-methyl-5-pyrazolyldimethylcarbamate (XXIII),[3] the 1-phenyl-homolog, were introduced as insecticides in 1952 (157).

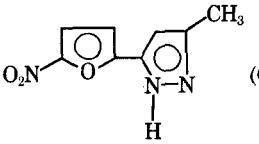
Compound XXII exerted a strong systemic action against aphids (158) and was highly toxic to the honey bee (159), however its selectivity is such that it did not affect the insects natural predators (160). Most organophosphate and carbamate pesticides cause inhibition, followed by recovery, of brain cholinesterase activity in *Musca domestica* (housefly) within 21 hr. Compound XXII was the only pesticide tested that did not allow recovery (161). However, it was a less active systemic insecticide in foliage sprays against the citrus whitefly or gardenia than other pesticides (162). Gaines found XXII more toxic to rats by the dermal route than orally (163). The effects of XXII and XXIII on the



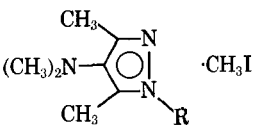
XVI



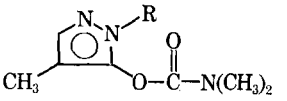
XVII (R = H), XVIII(R = amidino—)
XIX (R = amidino, 3′-Cl–replaces 4′-Cl)



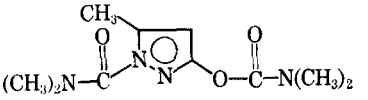
XX



XXI



XXII (R = isopropyl—)
XXIII (R = phenyl)



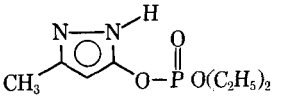
XXIV

stability, toxicity, and reaction mechanisms of human postpartum serum esterase have been reported (164). Aliphatic-esterase activity is inhibited in the housefly by XXII, but resistance to this pyrazole, it was observed, was not related to inhibition of the esterase; mosquito enzymatic activity was not inhibited (165). Many analogs have been reported to have good activity (166, 167). Radio-labeled XXII translocation and persistence in *Theobroma cacao* foliage, and residues in the cacao bean have been studied (168).

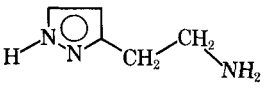
2-Dimethylcarbamoyl-3-methyl-5-pyrazolyl dimethylcarbamate (XXIV),[4] was shown to be toxic to laboratory animals in single oral doses (169, 170), but it was reported that XXIV-^{14}C was detoxified in the cockroach through *N*-methyl hydroxylation. Two out of five of the *N*-hydroxymethyl metabolites have been identified (171). The housefly shows maximum cross-resistance levels between XXII and XXIV (172).

Common commercial aromatic and heterocyclic carbamate pesticides exhibited a tenfold potentiation with piperonyl butoxide, sesoxane, sulfoxide, propyl isome, or sesame oil extracts on the housefly (173). Octachlorodipropyl ether

[2] Isolan.
[3] Pyrolan.
[4] Dimetilan.



XXV XXVI



XXVII

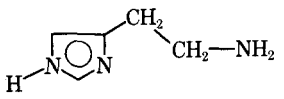
also produced massive potentiation with carbamates on the fly (174), but antagonism, with resultant antidotal protection, was given by atropine and parpanit against all the carbamates tested (175).

Pyrazoxon, containing *0,0*-diethyl-*0*-(3-methylpyrazolyl)-(5)-phosphate (XXV), has become a commercially important foliage spray (176). Phosphoric and thiophosphoric acid derivatives of hydroxy-pyrazolo[1,5-*a*]pyrimidine (XXVI), which were not toxic to warm-blooded animals, acted on insects, red spiders, ticks, nematodes, and fungi (177), and they also exhibited anthelmintic qualities (178). In the Mexican fruitfly, ovaries were affected by IIb sulfate, which reduced oviposition and fertility, acting as a sexual sterilant (179, 180). The screening of various commercial agents against a number of pests has been reported (181).

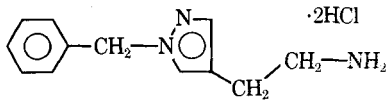
PYRAZOLE HISTAMINE AGONISTS AND ANTAGONISTS

In 1944, Dewar presented histamine-antihistamine models (182), and later, Lee and Jones observed the histamine-like effects produced by several 2-aminoethyl-*N*-heterocyclic compounds. One of these substances 3-(2-aminoethyl)pyrazole, is now known as betazole (XXVII) (183). This isomeric histamine (XXVIII) analog reputedly stimulated gastric acid secretion without producing other histaminic effects (184). Doses of 50 mg. of betazole i.m. elicited a gastric secretory response similar to that for histamine, and like histamine, it was used to study gastric secretions (185). Many clinical studies on betazole HCl have been reported (186–205). Histamine and betazole acted on the same receptors in the guinea pig ileum (206, 207), and additional theoretical histamine-antihistamine models have been reported recently (208, 209).

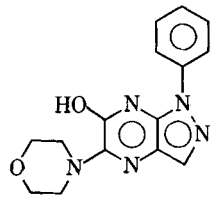
One interesting S-A-R probe indicated that the 1-benzyl-4-(2-aminoethyl)pyrazole·2HCl salt (XXIX) inhibited histamine-stimulated gastric acid secretion. Nevertheless, 4- and 3- (or 5) (2-aminoethyl)pyrazole and bis-2-(3-pyrazolyl)eth-



XXVIII



XXIX

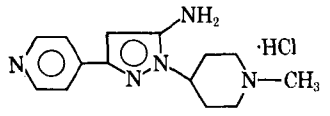


XXX

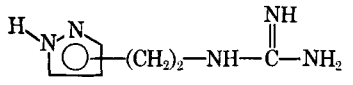
ylamine· 3HCl stimulated acid secretion, even after histamine (210). In one report on the inhibition of histamine, relative to the action of histamine analogs which inhibit histamine methyl transferase, no correlation could be found between antihistaminic activity and enzyme inhibition (211). Alkylamine ethers of 1-phenyl- and 1-benzyl-3-methyl-5-hydroxypyrazole have been prepared as potential antihistaminic substances (212). The 3,5-diphenyl-1-(2-aminoethyl)pyrazole, a diphenyl-substituted analog of betazole, had a procaine-like anesthetic activity and relieved cutaneous pain (213). The allergological significance and toxicity of certain pyrazoles have been reported (214).

DIURETIC, CARDIOVASCULAR, *HYPOCHOLESTEROLEMIC*, AND HYPOGLYCEMIC PYRAZOLES

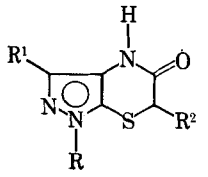
3,5-Dimethylpyrazole (DMP), a true carbonic anhydrase inhibitor with low animal toxicity, given in doses of less than one-half the LD_{50}, only slightly affected blood pressure and respiration (215, 216). Strong K^+ and Cl^- with moderate Na^+ and water excretion patterns were reported (217). It was noted, in a series of DMP relatives, that 3,5-dimethyl-4-bromo- and 1,3,5-trimethyl-4-nitropyrazole caused muscle relaxation and antiedemacious activity (218), while several 3-substituted pyrazolyl-1,2-benzisothiazole-1,1-dioxides showed good hypotensive-diuretic activity. N-Phenethyl-IIb and 1-phenyl-5-morpholino-6-hydroxy-1*H*-pyrazolo-[3,4-*b*]pyrazine (XXX) also produced diuresis. 1-Isopropyl-6-methyl-IIa and 1-isopropyl-6-ethyl-IIa were potent coronary dilators, and 4-mercapto-derivatives of pyrazole and II also elicited strong responses. Ciba-24, 650-Ba suppressed anginose pain, while Ciba-31, 531-Ba, 2-(N-methyl-4-piperidyl)-3-amino-5-(4-



XXXI



XXXII



XXXIII

pyridyl)pyrazole·HCl (XXXI) was a good renal vasodilator. The latter was useful for evaluating renal vascular reactivity to a vasodilating stimulation in various nephropathies. It produced hypotension in conscious dogs and seemed to reduce heart-rate performance in anesthetized unconscious dogs. Pyrazolylguanidines (XXXII) and related substances caused hypotension, sedation, and psychomotor stimulation, and a group of 5-oxo-pyrazothiazines (XXXIII) acted as hypotensive agents (219–224).

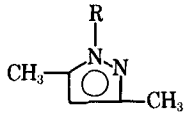
Giachetti *et al.* (225, 226), found that IIb decreased α- and β-globulins, and increased γ-globulins in hepatectomized rats, with a three- to fourfold liver-lipid increase occurring in 24 hr. in mice. Neutral lipids increased 10–16-fold, and the cholesterol level rose by 40%. No significant plasma-phospholipid, free fatty acid, or glucose changes were evident but plasma-triglyceride levels were reduced 40% in 1 hr. 4-Aminopyrazolo[3,4-*d*]pyrimidine (IIb) caused 85% inhibition of orotic-^{14}C acid incorporation into RNA. Hyperlipemia, normally elicited by Triton WR-1339, was inhibited by IIb, which suggested that IIb inhibited the secretion of triglycerides from the liver (227).

Bizzi related the action of DMP to fat and glucose fat, and it was reported that nicotinic acid, salicylic acid, or DMP blocked the mobilization of free fatty acids from adipose tissue. The assertion was made that a new method for preventing or controlling the development of liver triglyceride accumulation is possible. Results indicate that drugs which prevent the release of free fatty acids from adipose tissue could prevent and, in some cases, lower the liver triglyceride accumulation. This was true even if the cause of such an increase of triglyceride was not an increased lipid mobilization. DMP and its metabolite, 3-carboxy-5-methylpyrazole, were effective in lowering the plasma-triglyceride level, with the metabolite proving to be more effective than DMP (228–230). The primary action was on fat metabolism, and the effect on glucose oxidation and blood-sugar depression may be secondary (231). A current review of the effect of drugs on the mobilization of free fatty acid is available (232).

Emphasis on hypoglycemic agents was accelerated by the realization that DMP and its metabolite possessed blood-sugar lowering qualities in addition to hypocholesterolemic properties. DMP increased the glucose oxidation by intact rats and lowered plasma free acids of eviscerate rats, but not their blood-sugar levels. DMP markedly depressed plasma-free fatty acids 15 min. to 3 hr. after administration, and it was assumed that part of the hypoglycemic action may have been due to stimulation of glucose oxidation by the intestines and liver. DMP-^{14}C was utilized in these metabolic fate determinations (233, 234). Vetulani mentioned that an interference with some central mechanism was involved, and species differentiation and diuresis were cited (235). DMP is a "unique tool" when it is used in the study of the carbohydrate-lipid metabolic relationship to aid in understanding normal and abnormal metabolism (associated with diabetes).

Dulin *et al.* (236) have reported on hypoglycemic compositions of 3-carboxy-5-methylpyrazole and closely related 3-substituted analogs. In 1964, they noted that a number of pyrazoles with methyl-groups in both the 3 and 5 positions possessed up to 100 times the hypoglycemic activity of tolbutamide in glucose-primed, intact fasted rats (Table II) (237). DMP was 54 times as active as tolbutamide, suggesting that conversion to the acid metabolite, which has 200 times the activity of tolbutamide, accounted for all of the activity (238, 239). This metabolite had a pronounced effect on plasma free fatty acids and it differs from insulin, sulfonylureas, and biguanide in mechanism of action. Pyrazole-1-carboxami-

TABLE II—HYPOGLYCEMIC PYRAZOLES



R	Hypoglycemic Activity	R	Hypoglycemic Activity
CH_3	4–8	$-CONHNH_2$	16
$CONH_2$	25	$-COC_6H_5$	100
$CONHC_4H_9$	4–8	p-COC_6H_5Cl	25–50
$CONH(CH_3)_2$	30	p-$COC_6H_5NO_2$	25
$CONHC_6H_5$	20–30	Tolbutamide	1

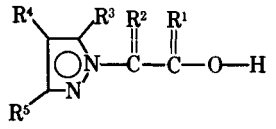
dines (240) and pyrazole-1-ethanol derivatives were reported (XXXIV) (241). The ethanolic substances which are nontoxic acid-addition salts, also affected antiviral and anti-inflammatory responses. 1-Phenylsulfonyl-DMP controlled blood-sugar levels in rats (XXXV) (242) and an excellent hypoglycemic response resulted when approximately 250 mg. of certain sulfanilamidopyrazoles was administered with 500 mg. of tolbutamide (243).

CENTRALLY ACTING PYRAZOLES

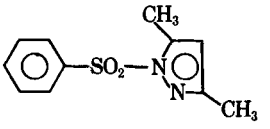
In 1954, a tetra-substituted pyrazole in combination with procaine and a 3,5-dioxopyrazolidine was reported to provoke a strong analgesic-antipyretic response (244). 2-(2-Dimethylaminoethyl)-, 2-alkyl-4,5-DMP homologs, pyrazole, 5-isopropyl-,5-phenyl-, and 4-methyl-5-phenylpyrazoles (XXXVI) caused an analgesic-spasmolytic consequence which paralleled an increase in molecular weight (245). Analgesic 3-hydroxypyrazolecarboxylic acids have been reported (246).

Pyrazole and 3-*n*-alkylpyrazoles (XXXVII) acted as species-selective anticonvulsants in lab animals; optimal convulsion-inhibition occurred with a 4-C side chain in this series (247). Human epileptic seizures were controlled with 4-chloropyrazole or 1 of 3-mono- or 3,4-disubstituted pyrazole·HCl salts (XXXVIII) (248), while a series of 1,4-disubstituted pyrazolecarbinols showed anticonvulsant-muscle relaxant activity (XXXIX) (249).

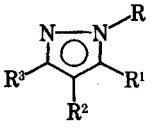
Phemerazole,3-methyl-5-phenylpyrazole (XL), was one of a group of pyrazoles which evoked sharply pronounced sedative, soporific, anticonvulsant, and hypothermic responses. A tenfold species differential was shown in lab animals upon administration of this compound, which inhibited nikethamide, strychnine, and picrotoxin activity in mice and activated camphor. However, when it was given with arecoline and with nicotine, it moderated tremors and convulsions, but increased animal mortality. Phemerazole potentiated the action of diethylstilbestrol on mammary glands (250–254). The anticonvulsant and toxic properties of its 1-allyl- and 1-methyl-4-nitro- analogs (XLI) have been cited. Phemerazole activity has been compared to that of meprobamate on the suppression of a response to an electric stimulus and to nikethamide overdoses (255). It produced no analgesia in rats but did potentiate morphine; however, this combination was more toxic than either substance singly (256). Phemerazole, several of its homologs, and tetrahydroindazole depressed polysynaptic, but not monosynaptic, reflexes. In mice and rats these "mephenesin-type" central relaxants depressed the "turn-over reflex" (257). The distantly related 3-amino-5-(trifluorophenyl)indazole was also a muscle-relaxant analgesic (258). When given in high doses, the majority of aminopyra-



XXXIV

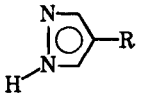


XXXV

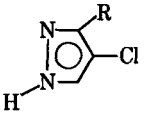


(R = alkyl)
(R′ = H, alkyl)
(R² = H, alkyl)
(R³ = alkyl, phenyl)

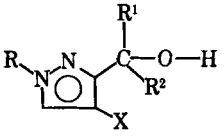
XXXVI



XXXVII

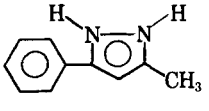


XXXVIII

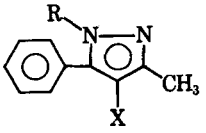


(X = H, halogen)
(R = H, methyl, benzyl)
(R¹ = small alkyl)
(R² = alkyl, *p*-Cl–, and phenyl)

XXXIX

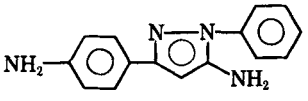


XL

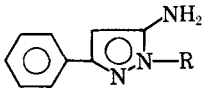


(R = H, methyl, alkyl)
(X = $-NO_2$, halogen)

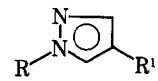
XLI



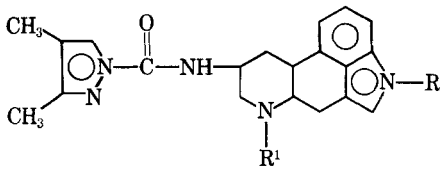
XLII



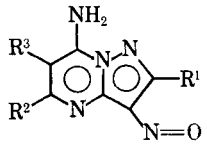
XLIII [R = isopropyl, 1-(2-hydroxyprspyl)]



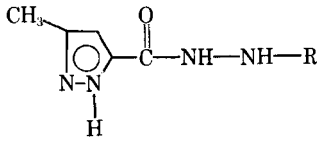
XLIV (R = H; R¹ = ethyl, isopropyl)
XLV (R = phenyl; R¹ = ethyl, isopropyl)



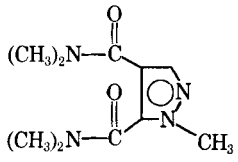
XLVI (R = R¹ = methyl)
XLVII (R = ethyl; R¹ = methyl)



XLVIII



XLIX (R = benzyl)
(R = isopropyl)

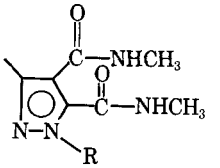


L

zoles depress the CNS; some are capable of potentiating the effect of thiopental, while others eliminate aggression in mice without visibly depressing their behavior. Aminopyrazoles displayed a weak antagonism to the spastic action of pentylenetetrazol[5] and some were classified as tranquilizers, with high activity being attributed to 1-phenyl-3-*p*-amino-phenyl-5-aminopyrazole (XLII). 3-Phenyl-5-aminopyrazole (259) and the 1-isopropyl and 1-(2-hydroxyethyl)-homologs (XLIII) produced distinct muscle relaxation (260). The S-A-R of 13 *N*-substituted 3-amino-4-phenylpyrazoles has been reported, and the parent compound has proved to be the most effective in the series (Table III) (261, 262).

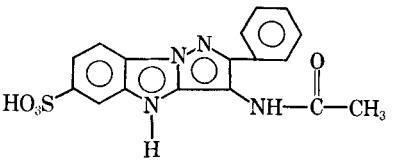
Patients with chronic bronchitis and respiratory failure, who were treated with 2-(*N*-methyl-4-piperidyl)-3-amino-5-(4-pyridyl)pyrazole · HCl (XXXI), showed improvement of some parameters of respiratory mechanics and some indexes of external ventilation. The compound produced bronchospasmolytic effects, but no improvement of internal ventilation was observed (263). Some 4-mono- (XLIV) and 1,4-disubstituted pyrazoles (XLV), which lowered muscle tone, motor activity, and body temperature, antagonized nikethamide and strychnine spasms. The 4-ethyl- and 4-isopropyl- compounds provoked the greatest responses. It was found that the less toxic disubstituted substances were also less active (264). 4-Piperazinoalkylpyrazoles incited a wide range of central responses: central depression, narcosis potentiation, sedation, tranquilization, hypnosis, and narcosis (265). Metoquizine (XLVI) and toquizine (XLVII) were found to be interesting "antiulcer" indolo[3,4-*fg*]quinolinylpyrazoles (266), and 3-nitroso-6-amino-1*H*-pyrazolo[1,5-*a*]pyrimidines (XLVIII) promoted central activity (267).

The clonic convulsions and mortality induced by pentylenetetrazol were promoted by 5-benzyl- and 5-isopropyl-3-methylpyrazole carboxylic acid hydrazides (XLIX), known MAO-inhibitors (268). A 1-methylpyrazole-4,5-diamide (II): prolonged chloral hydrate-induced sleep, antagonized caffeine, intensified the spasmodic effects of corazole, nicotine, and strychnine, stimulated gastric secretion by a central mechanism, was less toxic than the imidazole isomer, and exerted a sedative effect on the cerebral cortex. Several 4,5-



(R = methyl, allyl)

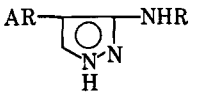
LI



LII

[5] Marketed as Metrazole by Knoll Pharmaceutical Co., Orange, N. J.

TABLE III—MUSCLE RELAXANT 3-AMINO-4-ARYLPYRAZOLES



AR	R	Antistrychnine Activity in Mice: Dose, mg./Kg.	Time, min.	Protection, %	Mortality, %	Preferential Interneuronal Inhibition in Cats: Dose, mg./Kg.	Inhibition, %	Av. Duration, Minimum, min.
Phenyl	H	200	30	144	20	15	70	120 plus
m-Cl-Phenyl	H	150	30	112	..	..	...	...
p-Cl-Phenyl	H	200	30	118	0	..	...	...
3,4-di-Cl-phenyl	H	300	30	175	0	10	12	50
m-Tolyl	H	250	30	155	0	..	...	...
2,3-Xylyl	H	400	30	105	10	..	...	...
3-Thianaphthenyl	H	300	30	115	10	30	100	...
Phenyl	$COOC_2H_5$	350	60	92	0	20	61	188 plus
Phenyl	$CONH_2$	750	30	100	10	20	60	150 plus

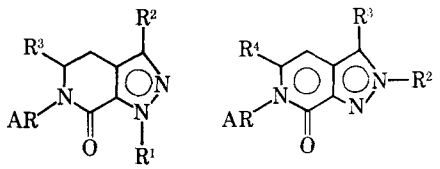
diamides (LI) antagonized caffeine and potentiated amphetamine. The effect of pyrazole dicarboxylic acid on EEG tracings has been reported (269–272). 2-Phenyl-3-acetylaminopyrazolo[1,5-*a*]benzimidazole-6-sulfonic acid (LII) stimulated the nervous system without a growth of spontaneous motricity (273).

Takamizawa *et al.*, who prepared and investigated the pharmacodynamics of a large number of pyrazolo[1,5-*a*]pyrimidines (XXVI), found that 4-substituted homologs possessed antipyretic-antiphlogistic activity. The 7-amino-substances were analgesic, anti-inflammatory, antipyretic, and antiphlogistic, and the 7-carbonyl-amido- and 3-methyl-6-carbethoxy-7-oxo-XXVI products tranquilized, depressed inflammation, and lowered body temperature. The 7-imino-4,7-dihydro-XXVI substances were sedative and antiphlogistic-antipyretic. 2,5-Dimethyl-XXVI was analgetic and antipyretic, and 3-cyanoethyl-2-oxo-5-phenyl-XXVI exhibited anti-inflammatory activity. Also prepared were the 2,3-dialkyl- and 7-formamido-XXVI (274–286). One series of XXVI compounds reduced inflammation (287).

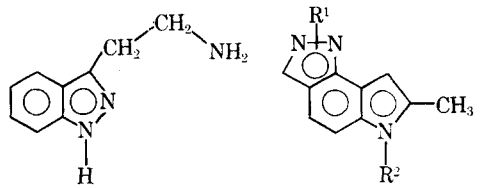
ANTI-INFLAMMATORY AGENTS

Pyrazole demonstrated a reasonable correlation between its antierythemic and antirheumatic activity and this inflammation-inhibiting effect was used in the UV erythema pharmacological assay for anti-inflammatory drugs (288). Activity was apparent in several novel 1-carbamoyl-3-amino-4-nitrilopyrazoles (289). 2*H*- and 1*H*-pyrazolo[3,4-*c*]pyridines (LIII, LIV) reduced inflammation (290) and the indazole (LV) isostere of 5-hydroxytryptamine produced similar responses from isolated smooth muscle preparations (291–293). Several pyrrazoloindazoles (LVI) were analgesic and reduced inflammation (294). Citrates, maleates, and sulfates of 3-dialkylaminoalkyoxyindazoles displayed enhanced activity (295).

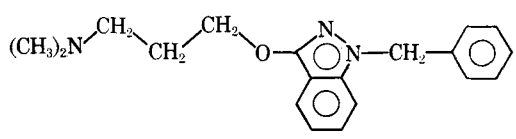
The first therapeutically important indazole was benzydamine, 1-benzyl-3-[3-(dimethylamino)propoxy]1*H*-indazole (LVII). Benzydamine and its salts possessed excellent inflammation-reducing properties, but the hydrochloride salt was the most active of the compounds examined (296). Absorption and elimination studies indicated large quantities of unchanged drug in the blood; 1 mg./Kg., in man, created significant and prolonged blood levels. The drug passed into the



LIII LIV

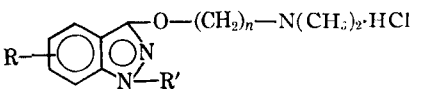


LV LVI



LVII

TABLE IV—BIOLOGICALLY ACTIVE 1-SUBSTITUTED 3-DIMETHYLAMINOALKOXY-1*H*-INDAZOLES[a]

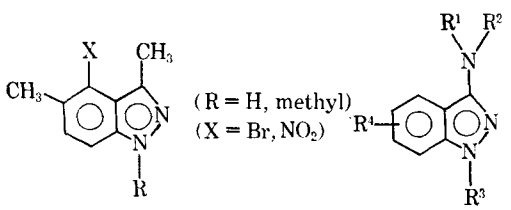


R	R¹	*n*	Analgesia[b]	Anticonvulsants[c]	Antispastic[d]	Anti-inflam.[e]	Local Anesth.[f]	Toxicity[g]
5-NH_2	C_4H_9	3			−			+
5-NH_2	$C_6H_5CH_2$	3					+	+
5-NO_2	C_4H_9	3			−			+
H	CH_3	3	+			+		+
H	C_6H_5	2	+	+		+		
H	C_6H_5	3	+				+	
H	$C_6H_5CH_2$	2	+				+	
H	$C_6H_5CH_2$	3	+	+		+	+	
H	$C_6H_5CH_2CH_2$	2	+	+			+	
6-Cl	$C_6H_5CH_2$	3	+			+		
H	p-$ClC_6H_4CH_2$	2	−			+		
4-Cl	$C_6H_5CH_2$	3	−			+		
5-Cl	C_4H_9	2	−			+		
5-Cl	C_4H_9	3	−	+		+		

[a] These hydrochloride salts produce sedation, muscle relaxation, and motor incoordination at 20–40 mg./Kg. At 80–100 mg./Kg. depression followed by clonic convulsions at near lethal doses occurs. [b] Using the hot plate and phenylquinone tests it is found that the more toxic *n*-2 side chains are least active. ED_{50}'s of 20–40 mg./Kg. are usual. [c] ED_{50}'s 40 mg./Kg. *vs.* strychnine or electroshock induced convulsions. [d] The guinea pig ileum used. A papaverine-like effect produced (0.3 mcg./ml.). [e] ED_{50} 15 mg./Kg. *vs.* plantar edema and induced granuloma. [f] Active at 0.5 mg./ml. *vs.* corneal reflex and tail pinch. [g] Toxicity varies between 120–150 mg./Kg. (LD_{50}) for the series. Compounds with NH_2 and NO_2 in their nucleus are most toxic.

intestinal lumen, in rats, but was not excreted in the feces, suggesting enteric circle. Fifty percent was excreted in the urine of man (297). Liver response, enzyme induction, and resultant increase in liver weight have been cited (298). Benzydamine was compared to codeine and aspirin, using various pain-threshold techniques in mice (299); the local effect and a lack of interference with the adrenals was observed (300). Axerio introduced a new synthesis for benzydamine (301), which was stated to be the most powerful analgetic-anti-inflammatory drug in use (299). Silvestrini has reviewed benzydamine (302).

Low toxicity, reduced inflammation, analgesia, and a decrease of spasms were attributed to 34 homologs of benzydamine (Table IV) (303). Other interesting work on synthetic nonsteroidal inflammation-reducing agents was reported (304), and of these, the 4-bromo- and 4-nitropyrazoles (LVIII) were impressive anti-inflammatory substances (305). The general pharmacology of a few 3-disubstituted-amino-1*H*-indazoles (LIX) was given in another report (306).

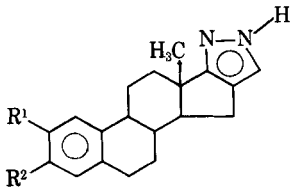


LVIII LIX

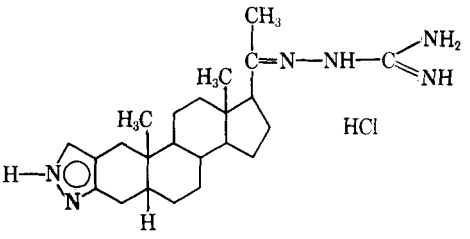
BIOLOGICALLY ACTIVE PYRAZOLOSTEROIDS

Pharmacodynamic interest in steroidal pyrazolo-compounds dates to the 1959 work of Clinton *et al.* (307, 308) with psychopharmacological agents. Hypotensive and hypophyseal-blocking action was observed for a series of estra-1,3,5-(10)-triene[17,16-*c*]pyrazoles (LX) (309) and 20-oxo-4-pregn[3,2-*c*]pyrazole guanylhydrazone · HCl (LXI) (310). Hormonal steroids with pyrazole fused to positions C-2 and C-3 were first reported in the androgen family (311), but some A-aza-steroids (LXII) were found to be inactive androgenic, anabolic, estrogenic, or antiestrogenic substances (312). Rosseels has reviewed LXII (313). Progestation was effected with pyrazolo-[3,2-*c*]-19-nor-4,9-androstadienes (LXIII) (314), and 4-androsteno[3,2-*c*]pyrazoles (LXIV) caused progestational, estrogenic, and lower blood-cholesterol responses (315). Steroidal [17,16-*c*]-pyrazoles reduced cholesterol levels equally as well as estradiol, but their estrogenic potency was only 1/10,000 that of estradiol (316).

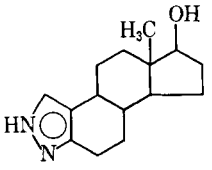
17β-Hydroxy-17α-methylandrostano[3,2-*c*]-pyrazole, stanozolol (LXV), and the 4-eno-analog, when given to human patients, influenced N-retention and electrolyte balance. Chronically ill geriatric mental patients gained weight with stanozolol and 1 to 20 mg. daily for 24–161 days resulted in no hepatotoxicity. Appetites and mental outlook improved without the occurrence of androgenic or other adverse effects. A posi-



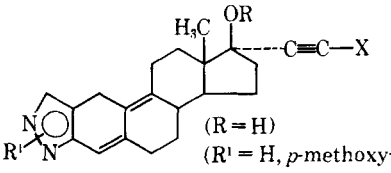
LX



LXI

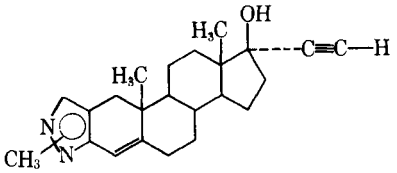


LXII

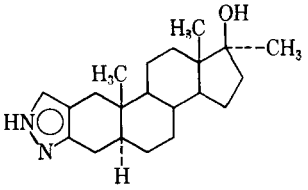


(R = H)
(R^1 = H, *p*-methoxyphenyl)
(X = Br, H)

LXIII



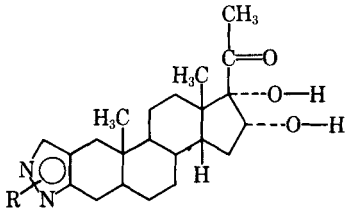
LXIV



LXV

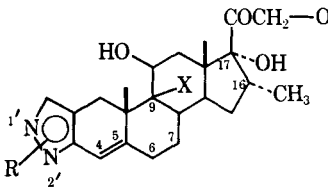
tive N-balance resulted through an unknown mechanism, and calcium metabolism was influenced also. Stanozolol, given orally, was 30 times as potent as methyltestosterone, but had only one-fourth the androgenic activity. This pyrazolosteroid increased hemoglobin and serum-albumin levels with the former being even more dramatically affected than body weight. Serum cholesterol and phospholipid concentration were lowered by stanozolol. Anabolic effects persisted for several weeks following termination of stanozolol therapy. The most serious problems, which were lowering the α-lipoprotein level and the $\alpha:\beta$-lipoprotein ratio, closely paralleled those met with nonpyrazolo anabolic steroids (317–320).

16α,17α-Dihydroxy-2-oxo-4-pregnene[3,2-*c*]pyrazoles (LXVI) were found to be anti-inflammatory compounds. 2′-(*p*-Fluorophenyl)pregn-4-eno[3,2-*c*]pyrazole (LXVII) was the most potent agent in the series (1964) (Table V) and other phenyl-substituted electron-withdrawing groups were found to decrease the activity of the parent phenyl-homolog. One relative was 500 times more active than cortisol for antagonizing granuloma formation and causing adrenal atrophy. Neither this substance, 9α-fluoro-16α-methylcortisol nor LXVII caused Na retention in



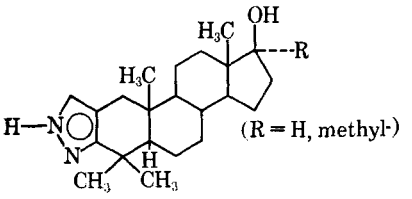
LXVI
LXVII (R = 2′-(*p*-fluorophenyl-))

TABLE V—ANTI-INFLAMMATORY PYRAZOLOSTEROIDS

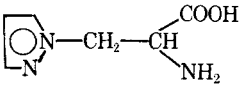


R	R^1	X	Granuloma Inhibition
2′-*p*-C_6H_4F-	H	F	10–12[a,b]
2′-C_6H_5-	Ac	H	60
2′-*p*-C_6H_4F-	Ac	H	100[a,b]
2′-*p*-C_6H_4F-	Ac	F	500[a,b]
1′-C_6H_5-	H	H	2
1′-CH_3-	Ac	H	1.5
2′-CH_3-	Ac	H	5.9
2′-C_6H_5-	Ac	H	1[c,d]
2′*p*-C_6H_4Cl-	Ac	H	15
2′-C_6H_5-	H	H	25[d]
H	H	F	10–12[a]
2′(*o*,*p*-$C_6H_3F_2$)	H	H	115[e]
Cortisol			1

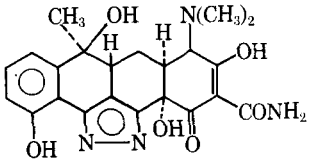
[a] Produces adrenal atrophy. [b] No sodium retention. [c] Saturation of 4–5 bond. [d] A 16 αH replaces the 16α methyl group. [e] The saturated 6–7 bond is replaced to give a delta-6 double bond.



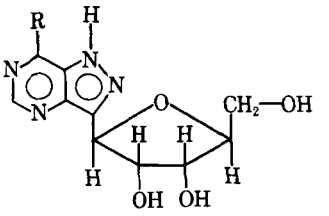
LXVIII



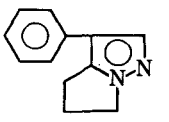
LXIX



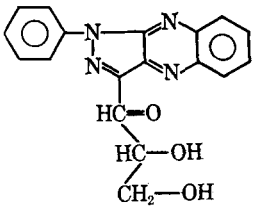
LXX



LXXI (R = NH_2)
LXXII (R = H)
LXXIII (R = NH—NH_2)



LXXIV



LXXV

the rat. While studying the activity of pregn-4-eno and 5α-pregnano-[3,2-*c*]pyrazoles, investigators first made the novel observation that a carbonyl function at C-3 was not required for anti-inflammatory activity. The [3,2-*c*]pyrazole function, unlike the 2-α-methyl-, did not enhance mineralocorticoid activity. These results were intriguing, since the highly potent 2′-phenylpyrazole homolog chemically resembled the biologically inactive isoxazole isostere in its double bond structure; whereas, the nearly inactive 1′-phenylpyrazole homolog, like the natural corticoids, had an exocyclic double bond at C-3 (321–326). Two 17β-hydroxyandrost-5-eno-[3,2-*c*]-pyrazoles (LXVII) were the most effective of 42 androstane derivatives examined as inhibitors of β-hydroxysteroid dehydrogenase. The degree of inhibition was dependent upon the nature of the substituents at positions 2, 3, 4, and 17 of the steroid. Spatial presentation of these groups to the enzyme by the arrangements of double bonds or by their absence also affected inhibition. Estradiol-17β was noncompetitive, while these pyrazolosteroids acted by a competitive mechanism. The 50% inhibitory concentration for estradiol-17β of 5×10^{-5} moles/L. as opposed to 5×10^{-7} moles/L. for LXVIII gave evidence that the pyrazole was more tightly bound to the enzyme (327). Interesting new pyrazolyl- and pyrazolosteroids have been synthesized (328–330).

NATURAL PRODUCTS

Until 1963 pyrazoles of natural origin were unknown but with a little help from Takeshita *et al.* (331), β-(1-pyrazolyl-L-alanine (LXIX) was identified and confirmed. A new enzyme, pyrazolealaninase, which is specific for the L-isomer, has been purified from cells of *Pseudomonas cruciviae* (watermelon juice) grown on the new amino acid. Attempts to activate S-protein at high peptide-protein ratios by replacing the histidine moiety with LXIX or its 3-pyrazolyl homolog have failed. It appears that the ionization behavior of histidine was responsible for the ability of S-peptide to activate S-proteins (332).

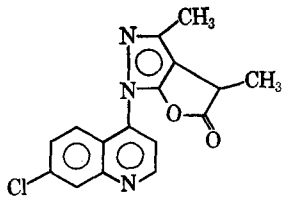
Valcazi *et al.* (333) treated chlortetracycline·HCl with hydrazine in methanol at 20°. The addition product, a conjugated pyrazolotetracycline (LXX), exerted the same action on several organisms as the parent antibiotic. The molecular structure has been established for another natural antimicrobial pyrazolo-compound, formycin, 7-amino-3-(β-D-ribofuranosyl)pyrazolo-[3,4-*d*]pyrimidine (LXXI), and the deaminated product, formycin B (LXXII), also has been identified. 7-Amino- and 7-hydroxypyrazolo-[4,3-*d*] pyrimidine were obtained from LXXI and LXXII, respectively. The semisynthetic hydrazinoformycin (LXXIII) has shown antimicrobial effects (334–337). Withasomnine (LXXIV), isolated from *Withania somnifera* roots, which are used medicinally in India, was structurally proven (338), and 1*H*-pyrazolo[3,4-*b*]quinoxaline "flavones" (LXXV) have been reported (339).

MISCELLANEOUS ACTIVITY

Pyrazole exerted a protective effect on hemin against *in vitro* degradation by ionizing irradiation. A carbamyl-DMP,[6] gave a 50% survival rate at doses of 200 mg./Kg. in mice over a 30-day period (340–342).

DMP stimulated plant growth (343), while 1-aryloxyacylpyrazoles were growth regulators

[6] Dupont-990.



LXXVI

and herbicides (344). 1-Guanyl-DMP·HNO_3, which reacts specifically with the ϵ-amino- groups of lysine (345-347), was utilized for the guanidation of bovine serum albumin, β-lactoglobin, and ovalbumin.

Alkylpyrazoles have been suggested for use in perfumery (348). Pyrazolo[4′,3′:5,6]2-pyrone (LXXVI), a novel annelate, incited no appreciable pharmacodynamic response (349).

CONCLUSIONS

The versatility of action of even the simplest pyrazoles was noteworthy, and pyrazole, itself, was mentioned on several occasions. A dimethylated pyrazole, DMP, and its carboxylic acid metabolite exerted profound effects on diuresis, fat, and glucose metabolism. The simple histamine isomer, betazole, manifested greater specificity on gastric secretions than histamine, while 3-methyl-5-phenylpyrazole, phemerazole, was an excellent central spasmolytic anticonvulsant. The simple pyrazoles, XXII, XXIII, XXIV, and pyrazoxon reportedly are all commercially successful pesticides, and the 1*H*-indazole, benzydamine, has proved to be an excellent nonsteroidal anti-inflammatory agent. Xanthine oxidase inhibitors like allopurinol, used in gout, and its carcinostatic 4-amino analog have been highly effective substances. The androstano-[3,2-*c*]pyrazole, stanozolol, fairly exploded on the scene and has become one of our finest anabolic agents. These drugs were all unknown 10 years ago.

The pyrazoles are exciting entities from which many new biologically active materials have been derived. Unlike the thiophenes (350), there is no slackening of interest in these pi-excessive N-heteroaromatic compounds. Quite to the contrary, the breath-taking pace of discovery of new pyrazolo-ring systems, new substitutions in established nuclei, and the biological responses to these heterocycles and to their modification products has given a momentum to pyrazole researches that has been known to few heterocycles. The widely varied biological activity spectrum is, indeed, unique. Pyrazole has manifested itself, in most instances, as a distinct and accountable portion of any number of diverse chemical structures which effect numerous biological responses. The crest of activity has yet to peak in the vast wave of research in this fertile area of study.

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Keyphrases

Pyrazoles, biologically active—review
Bio-antagonistic pyrazoles
Antitumor, carcinostatic pyrazoles
Antiviral pyrazoles
Pesticidal pyrazoles
Centrally acting pyrazoles
Anti-inflammatory pyrazoles

Use of the Coulter Counter and a Digital Computer for the Evaluation of Stability Ratios in Flocculating Monodisperse Systems

By B. A. MATTHEWS and C. T. RHODES

The combined use of the Coulter counter and a digital computer has been shown to be a powerful tool in evaluating stability ratios of flocculating monodisperse polystyrene lattices. The method involves comparing the experimental and theoretical depletion rates of the singlet species and corrections have been applied for the presence of higher species at the start of the flocculation experiment. The influence of particle size, particle concentration, electrolyte concentration, and surfactant upon suspension stability has been examined.

INCREASING ATTENTION has been focused in recent years upon the interfacial properties of powdered drugs. This interest may be attributed, at least in part, to the growing importance of suspensions as a dosage form. Although a considerable number of useful research and review articles on flocculation in pharmaceutical systems, has been published (1–12), there is still need for further investigation. A number of areas in this field are still in need of further illumination; there is, for example, some controversy as to the exact nature of the role of zeta potential in the flocculation of supracolloidal systems (2–9, 13).

The process of flocculation in a disperse system involves the increase in apparent particle size due to the formation of doublets, triplets, and higher species. Thus, *a priori*, one can consider any experimental technique of use in particle-size measurement, for the study of flocculation kinetics. A number of classical physicochemical methods such as light scattering (14), sedimentation (15, 16), and microscopy (11) can be used for work on flocculation kinetics. However, many of these techniques are of limited utility since they measure the total size of floccules and do not distinguish between the singlet, doublet, triplet, *etc.*, species. The Coulter counter (17, 18) measures particle volume and if the original system is monodisperse, the presence of doublets, triplets, and even quadrupulets can be detected. The Coulter counter technique is capable of measuring several thousand particles per second and once the operator has gained experience in the use of the equipment, data can be accumulated in a remarkably short space of time. However, even with the use of electromechanical or electronic calculators, the conversion of raw counts to singlet and multiplet concentrations is both tedious and time consuming. When such data have to be substituted into the somewhat complex flocculation equations (see later), the calculation process is further extended and the possibility of error greatly increased. In the present paper the combination of a Coulter counter and a digital computer is shown to provide a powerful tool for the study of the interfacial properties of monodisperse supracolloidal suspensions.

Most of the physicochemical studies of flocculation kinetics have been limited to systems containing particles well within the colloidal range, the upper limit of which is about 0.5 μ (19), whereas powdered drugs are almost invariably of supracolloidal dimensions (20, 21). Equations governing colloidal flocculation might well be expected to require modification on application to supracolloidal systems, since sedimentation may occur even in the singlet species. In the present paper a method is presented for studying the flocculation of particles in the size range 0.5–5.0 μ.

THEORY

The kinetics of flocculation in colloidal systems is dependent upon two factors, the Brownian motion

Received August 14, 1967, from The Physical Pharmacy Department, School of Pharmacy, Portsmouth, Hampshire, England.

Accepted for publication November 8, 1967.

The authors thank Miss F. Tringham for technical assistance and Mr. B. R. Martin and Mr. A. N. Richmond of the Department of Mathematics, Portsmouth College of Technology, Portsmouth, Hampshire, England, for helpful discussions on the computer program.

of the particles and the extent of their mutual interaction when in close proximity. The simplest case may be defined as when there is no mutual repulsion between the particles, such that any particle entering the sphere of interaction of another coalesces irreversibly with it. This situation was considered by Von Smoluchowski (22, 23) in his theory of rapid coagulation.

Smoluchowski assumed that at the start of flocculation, N_0 spherical particles of equal size are present per unit volume of liquid and that flocculation is started by a sudden removal of the mutual repulsion of the particles. The number of particulate encounters is determined by considering the process as a diffusion problem.

He showed that the number of aggregates of k single particles at any time, t, N_k, is given by the expression

$$N_k = \frac{N_0(t/T_{1/2})^{k-1}}{(1 + t/T_{1/2})^{k+1}} \qquad \text{(Eq. 1)}$$

$T_{1/2}$ is the coagulation time, defined as the time required to reduce the total number of particles by one-half. The coagulation time may be calculated from the equation

$$T_{1/2} = \frac{1}{4\pi D_i R N_0} \qquad \text{(Eq. 2)}$$

where D_i is the diffusion constant of the particles and R is the distance between the centers of two particles when flocculation occurs. This is, in the simplest case, $2r$, where r is the radius of the primary particles. The derivation of these equations has been given by Overbeek (24).

Equation 1 becomes for the singlet

$$N_1 = \frac{N_0}{(1 + t/T_{1/2})^2} \qquad \text{(Eq. 3)}$$

and for the doublet

$$N_2 = \frac{N_0\, t/T_{1/2}}{(1 + t/T_{1/2})^3} \qquad \text{(Eq. 4)}$$

The total number of particles in the system becomes equal to

$$\sum_{k=1}^{\infty} N_k = \frac{N_0}{1 + t/T_{1/2}} \qquad \text{(Eq. 5)}$$

Figure 1 shows a graph of the number of each species up to triplet as a function of time (taken from *Reference 24*, p. 282).

This theory has been tested experimentally by Turkevich (25) using a gold sol of spheres having a diameter of 400 Å., and good agreement was found with K values up to 6 in the early stages of flocculation.

The Eq. 5 gives no information on the stability of colloids, and although these systems are thermodynamically unstable they can remain, with little apparent change, over long periods of time.

Smoluchowski extended his theory to cover slow flocculation by assuming that only a fraction, α, of the collisions leads to permanent coalescence. The course of coagulation is still governed by Eq. 1, but Eq. 2 now becomes

$$T_{1/2} = \frac{1}{4\pi D_i R N_0 \alpha} \qquad \text{(Eq. 6)}$$

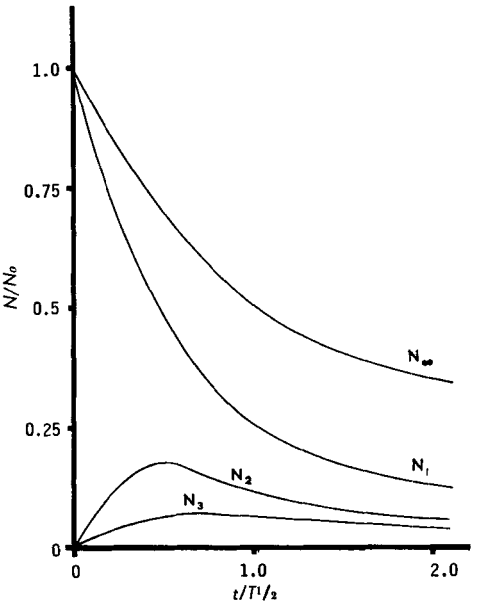

Fig. 1—Theoretical curves for the proportion of various species as a function of time in rapid flocculation (taken from Reference 24).

Smoluchowski assumed that α, which can be called the "collision efficiency," remains constant over the whole period of coagulation but Van Arkel and Kruyt (26, 27) and Tuorila (28) showed that this is not the case and that the rate of coagulation decreases with time. This has been explained by Reerink and Overbeek (29) by the fact that the repulsion between aggregates is larger than that between individual particles.

No theory was available to link the quantity α with such parameters as the zeta potential and electrolyte concentration, and Fuchs (30) modified Smoluchowski's theory to enable collision efficiency to be predicted from a consideration of the energy barrier between the particles.

The total energy of interaction between two particles, V_t, is the sum of the attractive contribution V_a and the repulsive contribution V_r. This potential V_t retards the process for rapid coagulation by a factor W such that

$$W = 2r \int_{2r}^{\infty} \left[\exp.\left(\frac{V_t}{kT}\right)\right] \frac{dS}{S^2} \qquad \text{(Eq. 7)}$$

where r is the particle radius, S is the distance between the particle centers $= 2r$ at coagulation, k is the Boltzmann constant, and T is the absolute temperature.

W denotes the ratio of rapid to slow coagulation, and when V_t is zero for all separations of the particles, $W = 1$ and rapid coagulation occurs according to the Smoluchowski equation. For slow coagulation, Eq. 5 becomes

$$N_k \frac{N_0}{1 + t/T_{1/2}\,(\text{slow})} = \frac{N_0}{1 + t/T_{1/2}\,(\text{fast})\; W} \qquad \text{(Eq. 8)}$$

and the coagulation time becomes

$$T_{1/2} = \left(\frac{1}{4\pi D_1 R N_0}\right) W \qquad \text{(Eq. 9)}$$

From a comparison of Eqs. 6 and 9

$$W = \frac{1}{\alpha} \qquad \text{(Eq. 10)}$$

with α always <1 and W in theory, always >1. W can be <1 (24), and this is explained by the presence of London attraction forces which accelerate coagulation. Equation 7 describes the retardation in flocculation in the initial stages when not many secondary particles have formed, and Overbeek (*Reference 24*, p. 285) has stated that it is not possible to extend the theory to cover collisions between multiple particles. The dependence of W on such factors as particle size, electrolyte concentration, and valency, has been reviewed by Verwey and Overbeek (31), Ottewill and Watanabe (32–36), Somasundaran, Healy, and Fuerstenau (37), and the theory was extended to a dispersion containing two types of particle by Hogg, Healy, and Fuerstenau (38).

Determination of Stability Ratios in Monodisperse Systems—From Eqs. 6 and 9 the collision efficiency α, or stability ratio W, could be determined by simply measuring the change in total concentration of particles with time and recording the $T_{1/2}$. This would, however, not take into account any changes in collision efficiency during the coagulation process and, if coagulation were slow, would require a lengthy experiment. Equations 1, 3, and 4 can be used to determine α or W at any period of time during the reaction provided a method is available to distinguish between the various species in the system. A recent series of papers (39–41) by Higuchi *et al.*, has demonstrated the suitability of the Coulter counter to determine the relative proportions of singlets, doublets, triplets, and sometimes quadrupulets in flocculating monodisperse polystyrene lattices. Study of Fig. 1 shows that the species that undergoes the greatest change in concentration during flocculation is the singlet, and therefore measurement of the concentration of this species is likely to give greatest precision, especially where flocculation is slow, *i.e.*, where α is small and W large, or in systems of low concentration. We have used the Coulter counter to follow the rate of depletion of the singlet species in flocculating monodisperse systems and have used a digital computer to compare this rate with that predicted by the Smoluchowski theory, to evaluate the stability ratio.

Method of Calculation—From Eq. 3 the relative proportion of singlets to the total original concentration at any time t, is equal to

$$\frac{N_1}{N_0} = \frac{1}{(1 + t/T_{1/2})^2} \qquad \text{(Eq. 11)}$$

by rearranging

$$t = \left(\sqrt{\frac{N_0}{N_1}} - 1\right) T_{1/2} \qquad \text{(Eq. 12)}$$

and a graph of t against $\sqrt{(N_0/N_1 - 1)}\, T_{1/2}$ should be a straight line with a slope of 1 if rapid coagulation occurs. When coagulation is slow, the graph has a slope of α or of $1/W$.

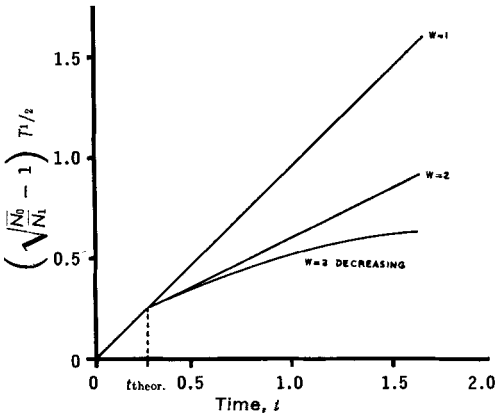

Fig. 2—Theoretical plot of $\left(\sqrt{\frac{N_0}{N_1}} - 1\right) T_{1/2}$ *as a function of time at different stability ratios.*

Since, however, it was not possible to start with 100% singlets, the systems at $t_{exp.} = 0$ always appeared to have already been flocculating for a small time $t_{theor.}$ This value was obtained by substituting the value of N_1/N_0 obtained at $t_{exp.} = 0$, into Eq. 11 and it was added to all subsequent values of $t_{exp.}$ in the flocculation process. This method was considered to be valid since at the beginning of the experiment the relative proportion of singlets, doublets, and tripletsw as always in accord with that which would be predicted from Fig. 1.

Figure 2 shows a graph of t against $(\sqrt{N_0/N_1} - 1)$-$T_{1/2}$ for three systems where $W = 1$, $W = 2$ and where $W = 2$ initially, but falls during the course of the experiment. The graph depicts results obtained from theoretical calculations and illustrates the application of the correction factor $t_{theor.}$ to obtain the true effective zero time.

The coagulation time $T_{1/2}$ was calculated from Eq. 2 (see *Reference 24*, p. 282), which for water at 298°K. becomes equal to

$$T_{1/2} = \frac{2 \times 10^{11}}{N_0 \times 3.6 \times 10^3} \text{ hr.} \qquad \text{(Eq. 13)}$$

In this paper the above approximate equation has been used. However, in studying the flocculation of true colloidal systems when absolute rather than apparent W values can be determined the numerator, under the above conditions, should be stated more precisely as 1.63×10^{11}.

EXPERIMENTAL

A Coulter counter model "B"[1] was employed with 30-μ and 50-μ orifices. The model B is a dual threshold machine, and by setting the upper and lower threshold controls at suitable values, about that which corresponds to the volume of the species to be measured, counts can be obtained directly. Electrolyte, consisting of 1% sodium chloride, filtered through 0.45 μ Millipore[2] filters, was used with the 50-μ orifice and 2% sodium chloride filtered through 0.45 μ and 0.100 μ Millipore filters for the

[1] Manufactured by Coulter Electronics Ltd., Dunstable, Bedfordshire, England.
[2] Marketed by Millipore (U.K.) Ltd., Wembley, Middlesex, England.

30-μ orifice. Background counts, which were always low, were taken on the electrolyte and measurements of the particles were made at concentrations which enabled coincidence to be ignored.

The flocculation was carried out in 150-ml. stoppered flasks which were immersed in a water bath maintained at 25 ± 0.1°.

Materials—Samples of Dow[3] polystyrene lattices of diameter 0.714 μ and 1.305 μ were used, as obtained from the manufacturer.

The standard deviations quoted by the manufacturer for these lattices were 0.0053 μ for the 0.714-μ sample and 0.0158 μ for the 1.305-μ sample. Standard deviations determined from cumulative number/percentage size distribution curves obtained using the Coulter counter were somewhat higher than these values, 0.02 μ and 0.025 μ, respectively. However, the samples are sufficiently monodisperse to enable the vast majority of singlets to be differentiated from higher species, and for the Smoluchowski equation to be employed. Wachtel and La Mer (42) have also noted larger standard deviations of monodisperse polystyrene lattices, measured on the Coulter counter, than those stated by the manufacturers.

Suspensions were diluted in filtered distilled water which had been freshly distilled from an all-glass still and the working level was between 1.0×10^7 and 1.0×10^8 particles/ml. Aluminum chloride hexahydrate, $AlCl_3 \cdot 6H_2O$, was selected as the flocculating electrolyte since it was hoped to extend the work to pharmaceutical suspensions and aluminum was considered the most suitable trivalent metal from the viewpoint of toxicity.

General Method—All glassware used in the flocculation experiments was cleaned with chromic acid and rinsed six times with distilled water and twice with filtered distilled water. All apparatus used for measurement on the Coulter counter was rinsed with filtered electrolyte. Suspensions of the latex particles were prepared at 90% of the required concentration and 90.0 ml. was placed in the reaction flasks and allowed to reach the experiment temperature. Volumes of 2.0 ml. of the suspension were withdrawn and diluted in filtered electrolyte to a suitable working level. A count of singlets, doublets, triplets, and all species above was taken and the results used to determine the original total number of latex particles in the system.

Although ultrasonics and a high speed dispersion mixer were tried, it was not found possible to produce a system of pure singlets to start the reaction. This is in line with the experience of Higuchi *et al.* (39) who took as their starting point 90% singlets, 8% doublets, 1% triplets, and 1% larger aggregates. The method of dilution was standardized as far as possible although variation in the technique was not found to affect the count significantly.

The experiment was initiated by adding 10.0 ml. of the flocculating electrolyte at a concentration of 10 times that required in the reaction mixture and at suitable intervals samples were withdrawn and assayed for the singlet species and the total count. The concentration of singlet species, in the system initially, was calculated from the counts taken at the beginning and the dilution factor.

Experiments were designed to demonstrate the effects of particle size, concentration of particles, concentration of electrolyte, and the presence of surfactant on the stability ratio W.

Computer Program—An Elliot 803 digital computer was programmed in Algol 60 to compute values of W by means of the method outlined above. A print-out of the program is shown in the *Appendix*. Values of the Smoluchowski collision efficiency α were computed also but all results are expressed in terms of W. Mean values of W are given except in a few instances where a pronounced reduction in flocculation rate occurred at the end of the experiment. In these cases initial values of W are used.

RESULTS AND DISCUSSION

Effect of Concentration of Particles on W—The effect of different concentrations of particles on the stability ratio W, at high electrolyte concentrations, was measured using 0.714-μ polystyrene latex at concentrations of 10^7 to 10^8 particles per ml. The concentration of aluminum chloride was 5×10^{-3} M, which is considerably above the critical electrolyte concentration.

The results shown in Fig. 3 indicate a reduction in W with increasing concentration up to a level of about 5×10^7 particles per ml., above which no further decrease occurs. However, in the experiments at the two highest concentrations, it was found that the initial values of N_1/N_0 were very low (0.41 and 0.36, respectively) compared with values of about 0.84 for all of the other systems. This indicates that some spontaneous flocculation, or lack of deflocculation on dilution from the concentrate, occurs at these higher concentrations. While it is known (39) that these polymers contain small amounts of an anionic surfactant which affects stability, the energy barrier is evidently not sufficient to prevent flocculation at this particular concentration. Although values of W on these two systems were, therefore, determined on colloids which were already considerably flocculated, they are in good agreement with the systems at between 4 and 7×10^7 particles per ml. and which were only very slightly flocculated at the start of the experiment. Earlier workers (24, p. 322) with true colloids have reported both increases and decreases in stability with increasing concentration of colloidal

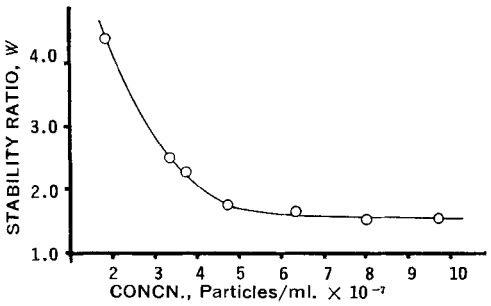

Fig. 3—Influence of particulate concentration on stability ratio.

[3] Kindly supplied by Mr. B. J. Lippie, Dow Chemical Company, Midland, Mich.

[4] This sample when calibrated by means of the 0.714-μ latex and a 1.099-μ latex was found to have a diameter of only 1.20μ but this did not affect the experiments since Coulter threshold settings were calculated from the results of calibration by taking readings at $^3/_2$ and $^1/_2$ of the mean pulse height as recorded on the oscilloscope, as recommended in the instruction manual (43).

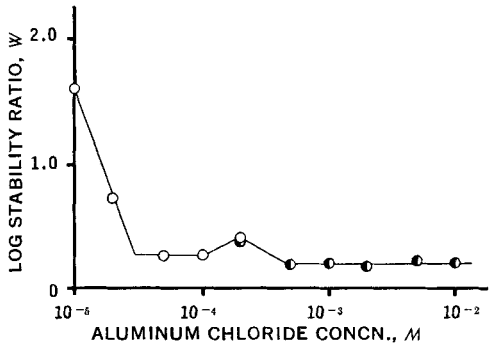

Fig. 4—Stability ratio W *as a function of aluminum chloride concentration for 0.714-μ polystyrene latex. —○— and —◐— are separate experiments.*

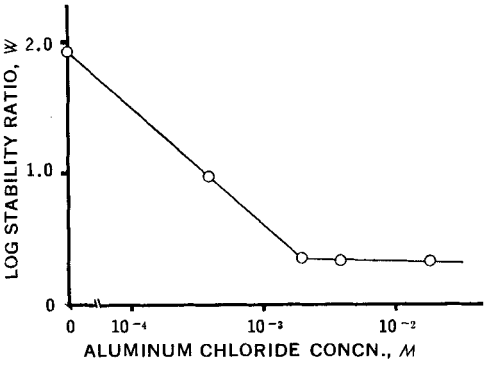

Fig. 5—Stability ratio W *as a function of aluminum chloride concentration for 1.20-μ polystyrene latex.*

particles, but Klompé (24, p. 323) who corrected for exchange of flocculating ions against double-layer ions, reported that for a trivalent electrolyte, higher concentrations of sol required lower concentrations of electrolyte to produce flocculation. Further experiments are required with the lower concentrations of particles to determine whether different concentrations of electrolyte can produce reductions in the value of W. With aluminum salts there is also the possibility of peptization (44) although results on this system suggest that the concentration used in this experiment is higher than the peptizing concentration.

Effect of Electrolyte Concentration on W—After consideration of the results of the above experiment, a concentration of 5×10^7 particles per ml. was selected, and the stability ratio was determined at concentrations of aluminum chloride from 10^{-5} to 10^{-2} M. The results are shown in Fig. 4 (combined results of two experiments).

An initial rapid reduction in W occurs with very low concentrations of electrolyte. If this portion of the graph is extrapolated to log $W = 0$, the critical flocculation concentration is 3.5×10^{-5} M. This is in good agreement with the range 9×10^{-6} to 9×10^{-5} given by Overbeek (24, p. 309) for the flocculation of negatively charged sols by aluminum salts. A small region of partial peptization occurs at 2×10^{-4} M and above this value W reverts to just below its original minimum.

Effect of Particle Size on W—It is of interest that the flocculation rate never reaches the Smoluchowski maximum for rapid coagulation where $W = 1$ and log $W = 0$, in contrast to the experience of earlier workers with conventional colloids (24, p. 32) and of Ottewill and Shaw (45) with monodisperse polystyrene latex particles of diameter 0.06–0.4 μ. The particles used in the above experiments are less than twice the diameter of the largest used by Ottewill and Shaw. The minimum value of W is 1.5 which means that the maximum flocculation rate is 67% of the Smoluchowski rate. This is in good agreement with the results of Higuchi *et al.* (39) who found that the singlet/singlet flocculation rate constant of 1.83-μ polystyrene latex particles approached the Smoluchowski rate to within about a factor of two. Higuchi *et al.* (39) and Swift and Friedlander (46) have suggested that this deviation from theory is due to hydrodynamic interactions when the particles are within one diameter of each other.

The maximum concentration of electrolyte used was 10^{-2} M and providing that peptization does not occur, the energy barrier at such high concentrations will be very low, or nonexistent. Values of W at these concentrations will not be an expression of the energy barrier but of the retardation of coagulation due to these hydrodynamic interactions.

At any concentration of electrolyte

$$W_{exp.} = W_{true} + C \qquad \text{(Eq. 14)}$$

where

$W_{exp.}$ = measured value of stability ratio.
W_{true} = contribution to stability due to energy barrier.
C = additional contribution due to hydrodynamic interaction.

Below a certain critical size, C becomes insignificant and

$$W_{exp.} = W_{true} \qquad \text{(Eq. 15)}$$

This critical size would seem to be somewhere in the region of 0.5 μ which is significantly the upper limit which some authors would place on the colloidal state.

If this explanation is correct one would expect that the minimum values of $W_{exp.}$ would be greater still for particles larger than 0.7 μ.

To test the above theory a similar experiment was performed using the 1.305-μ diameter latex from Dow (Coulter diameter 1.20 μ).

The results (Fig. 5) show that the general rule of increase in stability with increase in particle size stated by Verwey and Overbeek (31, p. 177) is obeyed, the critical concentration having been increased by a factor of about 10^2. The minimum value of $W_{exp.}$ has increased from 1.5 to 2.0, as predicted above. Further experiments are planned on systems in the diameter range 0.5–2.0 μ to investigate this relationship in greater detail.

Effect of Surfactant on W—In order to determine whether adsorbed surfactant was likely to have any effect on the log W/concentration curve an experiment was performed using the same concentration of particles in the presence of 2.5×10^{-5} M ammonium lauryl ether sulfate.[5] This has been shown

[5] Marketed as "Empicol EAB" by Marchon Products Ltd., Whitehaven, Cumberland, England.

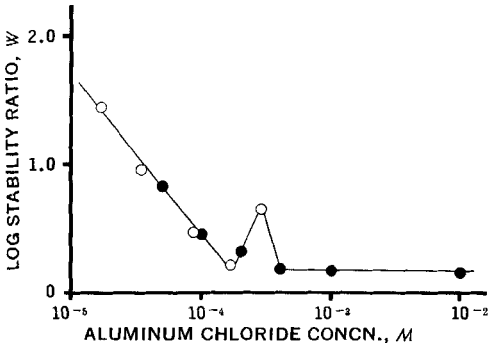

Fig. 6—Stability ratio W, *as a function of aluminum chloride concentration for 0.714-μ polystyrene latex containing 2 × 10^{-5}* M *ammonium lauryl ether sulfate.* —○— *and* —●— *are separate experiments.*

(13) to be compatible with aluminum salts, whereas more commonly used anionic surfactants such as sodium lauryl sulfate and sodium dioctyl sulfosuccinate cause precipitation (6, 13). The results of two experiments are shown in Fig. 6. The graph is similar in form to that obtained in the absence of surfactant, but the latter confers some protective action on the particles at low concentrations of electrolyte. The region of partial peptization occurs at about the same concentration of electrolyte and the ultimate value of $W_{exp.}$ is identical, 1.5. This indicates that the presence of the surfactant has little effect on coagulation at high electrolyte concentrations and is not the cause of the departure from Smoluchowski theory.

SUMMARY

The use of the Coulter counter and a digital computer has been shown to be eminently suitable for evaluating stability ratios in flocculating monodisperse systems, in the particle size range 0.5–2.0 μ. This is above the limits which most workers would set for true colloidal systems and many powdered drugs are likely to have a considerable proportion of particles in this region. Such monodisperse suspensions have been shown to behave in a similar manner to colloidal systems, yet to exhibit important differences particularly in maximum flocculation rates. Further experiments are planned to evaluate more fully the factors which are likely to be of importance in governing the flocculation behavior of pharmaceutical suspensions. Such information is likely to be of great value in rationalizing the behavior of these systems and providing the pharmacist with a sound theoretical basis for formulation

APPENDIX

Sample of Data Input

Program Symbol	Data		Key
X	5		Number of times at which results were taken
B	400		Background count on electrolyte
N	20,662		The initial count less background computed as singlets
M	2		Number of counts at each time
DIL	125.0		The dilution factor
VOL	0.05		The volume counted on the Coulter counter
F	17503	17650	*X* rows of *M* experimental results
	14018	14140	
	11679	11731	
	10380	10349	
	9689	9693	
V (I)	0.0		*X* times in hours when results were taken
	0.50		
	1.00		
	1.50		
	2.00		

Computer Program

Written in Elliot 803 Algol 60

```
COULTER COUNTER DUAL THRESHOLD ALPHA AND STABILITY RATIO'
BEGIN  INTEGER X,B,N,I,P,J,F,M,XM1'
       REAL T,R,W,Z,D,V,SUM,NUM, TAU, PHI, DIL, VOL'
SWITCH S: = L1'
L1: READ X,B,N,M,DIL,VOL'
XM1: = X-1'
T: = (2@8)/(N*DIL*(1/VOL)*3.6)'
BEGIN ARRAY R,W,V(1:X)'
       FOR I: = 1 STEP 1 UNTIL X DO
BEGIN P: = 0' FOR J: = 1 STEP 1 UNTIL M DO
BEGIN READ F' P: = P+F'
END'
       R(I): = ((P/M)-B)/N'
       W(I): = (SQRT(1/R(I))-1)*T'
       Z: = W(1)'
END'
PRINT £ N/NO EXPT N/NO THEO SQNO/N-1T T THEO ?
BEGIN SUM: = 0' TAU: = 0'
       FOR I: = 1 STEP 1 UNTIL X DO
BEGIN READ V(I)'
PRINT FREEPOINT [4], R(I), £ ?, SAMELINE,1/(1+(Z+V(I))/T)**2, £ ?,
```

```
W(I), £ ?, (Z+V(I))'
END'
PRINT £
?'
        FOR I: = 1 STEP 1 UNTIL XM1 DO
BEGIN NUM: = (W(I+1)−W(I))/(V(I+1)−V(I))'PHI: = 1/NUM'SUM: = SUM+NUM'
TAU: = TAU+PHI'
PRINT FREEPOINT (4), NUM, SAMELINE, PHI'
END'
        PRINT £
ALPHA = ?, SAMELINE, FREEPOINT (4), SUM/XM1'
PRINT £
STABILITY RATIO W = ?, SAMELINE, FREEPOINT (4), TAU/XM1' PRINT £
?'
END'
END'
GO TO L1'
END'
```

Sample of Print-out

N/No exp.	N/No theor.	$(\sqrt{No/N} - 1)T$	$T_{theor.}$
0.8073	0.8073	0.1214	0.1214
0.5780	0.4016	0.3390	0.6214
0.4377	0.2395	0.5500	1.1214
0.3425	0.1589	0.7619	1.6214
0.2856	0.1131	0.9365	2.1214

Values of α and W Between Successive Times

α	W
0.4353	2.297
0.4217	2.371
0.4240	2.359
0.3491	2.864

Mean α = 0.4075 Mean Stability Ratio W = 2.473

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Keyphrases

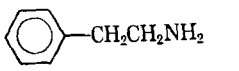
Stability ratios—flocculating monodisperse systems
Particle concentration—stability ratios
Electrolyte concentration—stability ratios
Particle size—stability ratios
Surfactant—stability ratios
Coulter counter—particle counting
Digital computer—calculation, stability ratios

2-(4-Biphenylyl)ethylamines: Potential Cardiovascular and CNS Agents

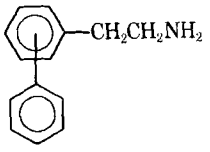
By J. SAM, K. APARAJITHAN, and R. SHAFIK

The syntheses of some 2-(4-biphenylyl)ethylamines are described. The results of preliminary pharmacological tests are reported.

MANY NATURALLY occurring substances which incorporate the phenylethylamine (I) or hydrogenated phenylethylamine moieties in their structures possess interesting biological properties. Ephedrine (1), morphine (2), and bulbocapnine (3) are such examples. The latter two substances also contain the biphenylylethylamine (II) group. Whereas extensive investigations (4–6) have been directed toward the synthesis of phenylethylamines, little effort (7, 8) has been expended in studies of biphenylylethylamines. Consequently, it was of interest to



I



II

determine whether compounds such as II possess central nervous system and cardiovascular properties similar to the phenylethylamines. Moreover it was anticipated that some of the biphenylylethylamines would possess CNS effects similar to morphine and/or bulbocapnine.

The synthetic approaches utilized for the preparation of the biphenylylethylamines are outlined in Scheme I.

The biphenylylethylamines (VIII) were prepared by either the reduction of the corresponding nitriles (VII) or the reduction and hydrolysis of phthalimido-ketones (X). The nitriles were obtained from the corresponding amides (VI).

The biphenylylhydroxyethylamines (XII) were prepared from the chloroacetylbiphenyls (IX). The latter were converted to the phthalimido-ketones (X), reduced with sodium borohydride to the phthalimido-alcohols (XI), and then hydrolyzed to the biphenylylhydroxyethylamines (XII). Alternately, the phthalimido-alcohols were synthesized from the corresponding chloro-alcohols (XIII). The latter also were utilized for the preparation of the *N*-isopropyl derivatives (XIV).

Pharmacological Results[1]—Acute effects after intraperitoneal injection of four compounds (X*b*, IV*a*, V*a*, VI*a*) were observed in albino mice. The LD_{50} values ranged from greater than 1.0 Gm./Kg. for IV*a*, to 261 mg./Kg. for V*a*. No deaths were observed for X*b* or for VI*a* at dosages of 2.1 and 1.0 Gm./Kg., respectively. All of these compounds appeared to produce a mild to moderate sedative effect, but none showed hypnotic or anesthetic levels of CNS depression. Higher (and especially lethal) dose levels of V*a* caused excitation and clonic convulsions. Signs of peripheral vasodilation were noted for compound IV*a* at well below toxic dosages. Signs of pain reflex inhibition led to further examination of IV*a* and V*a* by writhing test in mice. The ED_{50}'s for inhibition of writhing were 300 to 400 mg./Kg. for IV*a* and 112 mg./Kg. for V*a*.

Acute effects after oral administration of VIII*b* were observed in albino mice and rats by several methods. The LD_{50} for VIII*b* was 56 mg./Kg. Toxic doses of VIII*b* caused hyperpnea, muscle weakness, mydriasis, and subconvulsive to clonic convulsive manifestations. At low dosage VIII*b* produced a transient hypotensive effect in anesthetized rats, followed by a slight hypertensive effect. Blood pressure response to standard neurohumoral agents was unaffected by VIII*b*. The latter did not show activity in the hot plate analgesia test, the maximal electroshock anticonvulsant test, or an avoidance-escape behavior test. Compound VIII*b* decreased spontaneous motor activity at all dosage levels, but had only slight ability to increase hexobarbital sleeping time.

EXPERIMENTAL[2]

N-[4-(3′-Chloro-4′-methoxy)biphenylylthioacetyl]morpholine (IVa)—A method similar to that of

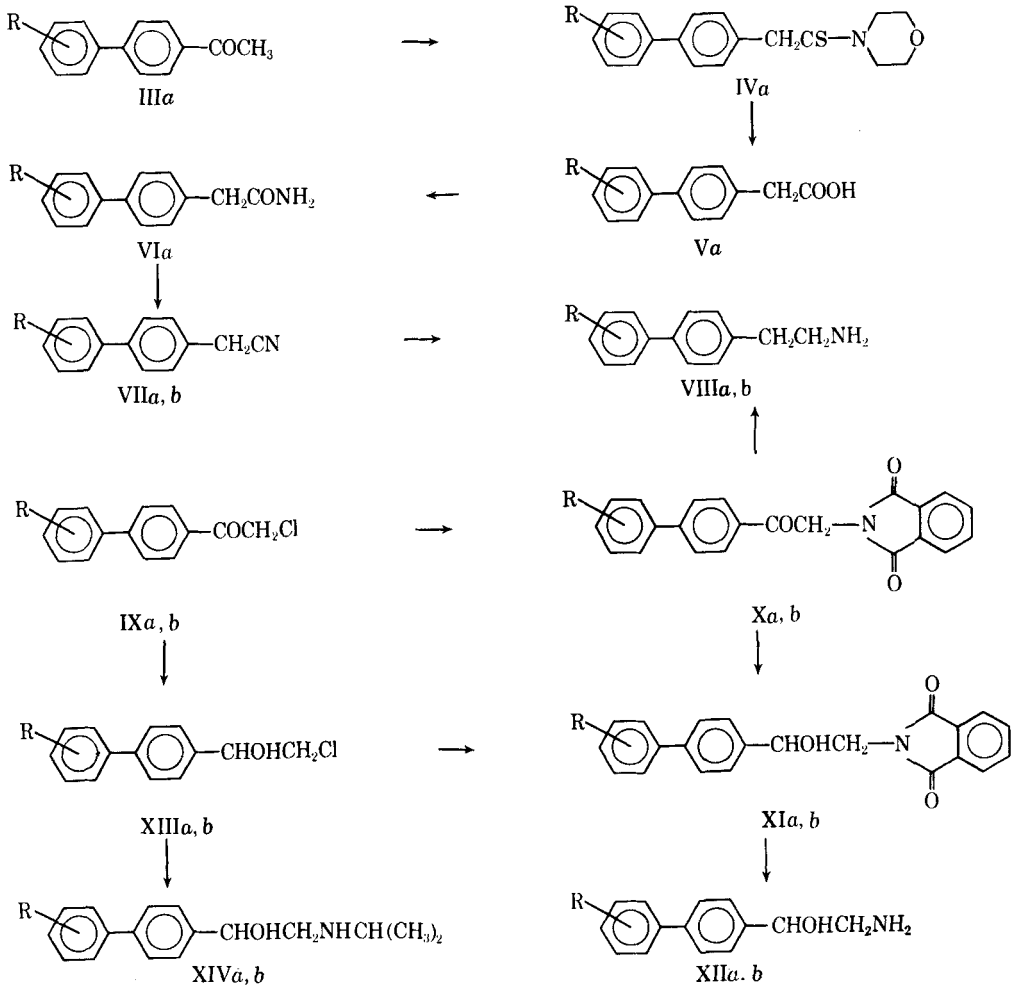
Received August 30, 1967, from the Department of Pharmaceutical Chemistry, The University of Mississippi, University, MS 38677

Accepted for publication October 19, 1967.

This investigation was supported in part by grant MH 05292 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and in part during a tenure of a fellowship (K.A. and R.S.) of the Mississippi Heart Association.

[1] The authors are grateful to Dr. W. Marvin Davis for the pharmacological data of compounds X*b*, IV*a*, V*a*, and VI*a*. Compound No. VIII*b* was evaluated by Hazleton Laboratories, Inc., Falls Church, Va. under contract No. PH 43-63-555 to the Psychopharmacology Service Center.

[2] All melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected. Infrared spectra were determined on a Perkin-Elmer model 137 infracord spectrophotometer using KBr pellets.



Biphenyls (*a* refers to R = 3-chloro-4-methoxy; *b* refers to R = H).

Scheme I

Schwenk and Papa (9) was employed. A mixture of 63.9 Gm. (0.245 mole) of 4-(3-chloro-4-methoxyphenyl)acetophenone (III*a*) (16), 12.5 Gm. (0.4 mole) of sulfur, and 75 ml. of morpholine was heated under reflux for 8 hr. The hot reaction mixture was poured into 300 ml. of methanol and the solid that separated was removed by filtration. The solid was triturated with cold methanol and then recrystallized, $\lambda_{max.}^{KBr}$ 1490, 1260 cm.$^{-1}$.

2-[4-(3′-Chloro-4′-methoxy)biphenylyl]acetic Acid (Va)—A solution consisting of 25.3 Gm. (0.07 mole) of *N*-[4-(3′-chloro-4′-methoxy)biphenylylthioacetyl] morpholine (IV*a*), 16 Gm. of sodium hydroxide, 75 ml. of water, and 110 ml. of ethanol was refluxed for 8 hr. The ethanol was distilled and the residue diluted with water and acidified to pH 2. The solid was removed by filtration, digested with boiling water and recrystallized.

2-[4-(3′-Chloro-4′-methoxy)biphenylyl]acetamide (VIa)—A finely ground mixture of 5.53 Gm. (0.02 mole) of 2-[4-(3′-chloro-4′-methoxy)biphenylyl]acetic acid (V*a*) and 1.52 Gm. (0.02 mole) of thiourea was gradually heated to 200–210° and maintained at this temperature for 1.5 hr. as described by Rehman, Medrano, and Mittal (10). The reaction mixture was cooled, digested with boiling water, and filtered. The brown crystalline solid was triturated with dilute alkali, removed by filtration, and then washed with water. The solid was digested with boiling ethanol, cooled, and removed by filtration; $\lambda_{max.}^{KBr}$ 1640, 1650, 3100, 3280 cm.$^{-1}$.

2-[4-(3′-Chloro-4′-methoxy)biphenylyl]acetonitrile (VIIa)—A mixture of 0.72 Gm. (0.0025 mole) of 2-[4-(3′-chloro-4′-methoxy)biphenylyl] acetamide (VI*a*) and 0.4 Gm. of phosphorus pentoxide was heated at 220–240° in an oil bath for 8 hr. The cooled reaction mixture was extracted with ether. The evaporation of the solvent left 0.15 Gm. (23%) of solid which was recrystallized; $\lambda_{max.}^{KBr}$ 2220 cm.$^{-1}$.

N-(4-Phenylphenacyl)phthalimide (Xa)—The procedure employed was adapted from that described by Sheehan and Bolhofer (11). A solution of 23 Gm. (0.1 mole) of 4-phenylphenacyl chloride in 200 ml. of *N*,*N*-dimethylformamide was treated with 20 Gm. (0.11 mole) of potassium phthalimide. The mixture, after remaining at room temperature for 3 hr., was treated with 500 ml. of water and then extracted with chloroform. The chloroform extract was washed with dilute alkali and water, respectively, and then dried over anhydrous sodium sul-

fate. The solid obtained after distillation of the chloroform solution was washed with ether and recrystallized.

2-(4-Biphenylyl)ethylamine Hydrochloride (VIIIb)—*Method A*—The method described by Nystrom (12) was modified slightly and followed. 4-Biphenylylacetonitrile (13) (7.9 Gm., 0.041 mole) was transferred *via* a Soxhlet extractor into a refluxing mixture of lithium aluminum hydride, anhydrous aluminum chloride, and dry ether (prepared by the rapid addition of a solution of 5.5 Gm. of aluminum chloride in 120 ml. of ether to 2.0 Gm. of lithium aluminum hydride in 80 ml. of ether). The reaction mixture was stirred during and 1 hr. after the transfer of the nitrile into the reaction flask. The reaction mixture, after remaining at room temperature for 16 hr. (during which it was stirred periodically), was treated dropwise with 15 ml. of water and then acidified with 60 ml. of 6 *N* hydrochloric acid. The solid was removed by filtration, washed with ether, and then recrystallized.

Method B—A suspension of 22 Gm. (0.065 mole) of *N*-(4-phenylphenacyl)phthalimide (X*b*) in 350 ml. of methanol was treated with 7.7 ml. of 95% hydrazine and then heated under reflux for 1 hr. The mixture was treated with a methanolic solution of sodium methoxide (prepared from 7.0 Gm. of sodium and 150 ml. of methanol) and then distilled to remove the methanol. Thereafter the mixture was treated with 7.5 ml. of hydrazine and 100 ml. of diethylene glycol. The mixture was refluxed over a free flame for 1 hr. and thereafter the low-boiling material and water were removed by distillation. The reaction mixture was heated to 195–200° and kept at this temperature for 4 hr. The reaction mixture was cooled, diluted with water, and extracted with benzene. The benzene extract was washed with water and dried over anhydrous sodium sulfate. The hydrochloride was prepared in the usual manner and recrystallized. Admixture with the product obtained by Method A showed no depression in the melting point.

4-Phenylstyrene—In the preparation of 2-(4-biphenylyl)ethylamine (Method B) when the heating was continued up to 210–220°, 3.76 Gm. (52%) of a faintly yellow distillate was obtained which solidified. Recrystallization of the solid from methanol gave colorless flakes; m.p. 108–110°, λ_{max}^{KBr} 905, 995, 1625, 2990 cm.$^{-1}$ [lit. (22) m.p. 119°].

Anal.—Calcd. for $C_{14}H_{12}$: C, 93.3; H, 6.7. Found: C, 92.9; H, 6.9.

2-[4-(3′-Chloro-4′-methoxy)biphenylyl]ethylamine (VIIIa)—2-[4-(3′-Chloro-4′-methoxy)biphenylyl]acetonitrile (VII*a*) was reduced with lithium aluminum hydride in the presence of anhydrous aluminum chloride as described for the preparation of 2-(4-biphenylyl)ethylamine (VIII*b*, Method A). The amine was isolated as its sulfate and recrystallized.

α-Chloromethyl-4-biphenylylcarbinol (XIIIb)—A modification of the procedure described by Chaikin and Brown (14) was utilized. To a solution of 4-phenylphenacyl chloride (10 Gm., 0.05 mole) in 125 ml. dioxane were added alternatively and in divided portions 5 Gm. of sodium borohydride and 75 ml. ethanol. The mixture was stirred at room temperature for 18 hr. The solvent was distilled under reduced pressure, the residue was treated with 10% hydrochloric acid, and then exhaustively extracted with chloroform. The extract was washed twice with water and dried over anhydrous sodium sulfate. The solid obtained after removal of chloroform was recrystallized.

N-[2-Hydroxy-2-(4-biphenylyl)ethyl]phthalimide (XIb)—*Method C*—A solution of 1.8 Gm. (0.0077 mole) of α-chloromethyl-4-biphenylylcarbinol (XIII*b*) in 25 ml. of 2-methoxyethanol was treated with 1.5 Gm. of potassium phthalimide and then refluxed for 1 hr. The reaction mixture was cooled and diluted with 150 ml. of water. The solid was removed by filtration, digested with hot methanol, and then recrystallized.

Method D—The procedure described for the preparation of XIII*b* was followed using 7 Gm. (0.02 mole) of *N*-[4-phenylphenacyl]phthalimide, 175 ml. dioxane, 3.5 Gm. of sodium borohydride, and 100 ml. of methanol.

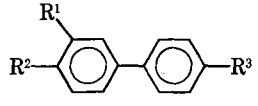
2-Hydroxy-2-(4-biphenylyl)ethylamine Hydrochloride (XIIb)—The procedure employed was adapted from that described by Barber and Wragg (15). A solution of *N*-[2-hydroxy-2-(4-biphenylyl) ethyl]phthalimide (10 Gm., 0.03 mole) in a mixture of 70 ml. chloroform and 70 ml. ethanol was treated with 7 ml. of hydrazine hydrate (50% aqueous solution). The reaction mixture was refluxed for 48 hr. and then left at room temperature for 48 hr. The solvent was distilled under reduced pressure. The residue was stirred for 2 hr. with a mixture of 50 ml. 2 *N* ammonia, 60 ml. of water, and 70 ml. of chloroform. The chloroform layer was separated and extracted with 10% hydrochloric acid. The acid layer was washed twice with chloroform; the solid obtained after distillation of the water under reduced pressure was recrystallized.

α-Isopropylaminomethyl-4-biphenylylcarbinol Hydrochloride (XIVb)—To a solution of α-chloromethyl-4-biphenylylcarbinol (10 Gm., 0.05 mole) in 100 ml. of ethanol was added dropwise a solution containing 1 Gm. of sodium hydroxide, 3 ml. of water, 80 ml. of ethanol, and 15 ml. of isopropylamine. The reaction mixture was stirred at room temperature for 18 hr. The solvent was distilled under reduced pressure and the residue treated with water and exhaustively extracted with ether. The ether extract was washed twice with water and then extracted with 10% hydrochloric acid. The acid layer was concentrated under reduced pressure; the residue was recrystallized.

2-Chloro-4′-(3-chloro-4-methoxy)phenylacetophenone (IXa)—A mixture of 5.5 Gm. (0.025 mole) of 3-chloro-4-methoxybiphenyl (16), 40 ml. of dry carbon disulfide, and 2.4 ml. of chloracetyl chloride was treated with 3.8 Gm. of anhydrous aluminum chloride by a procedure similar to that described by Buu-Hoi, Sy, and Riche (16). The reaction mixture was poured onto crushed ice and the solid that separated was extracted with chloroform. The chloroform extract was washed with an aqueous solution of sodium bicarbonate and thereafter dried over anhydrous magnesium sulfate. The solvent was distilled under reduced pressure and the residual red oil crystallized from ethanol.

N-[4-3′-Chloro-4′-methoxy)phenylphenacyl]-phthalimide (Xa)—The method described by Sheehan and Bolhofer (11) was modified slightly. A solution of 2-chloro-4′-(3-chloro-4-methoxy)phenyl acetophenone (20 Gm., 0.068 mole) in 170 ml. *N,N*-dimethylformamide was treated with 13.6 Gm.

TABLE I—BIPHENYLS



No.	R1	R2	R3	% Yield	M.P.° C. (Recrysn. Solvent)[a]	Molecular Formula	Analysis, % Calcd. C	H	Cl	N	Found C	H	Cl	N
IV*a*	Cl	OCH_3	$CH_2CSNC_4H_8O$[b]	84	152–154 (A)	$C_{19}H_{20}ClNO_2S$[c]	63.0	5.6	9.8	3.9	63.0	5.7	10.0	3.8
V*a*	Cl	OCH_3	CH_2CO_2H	73	167–168 (A)	$C_{15}H_{13}ClO_3$	65.1	4.7	12.8		65.0	4.8	12.8	
VI*a*	Cl	OCH_3	CH_2CONH_2	83	245–247 (D)	$C_{15}H_{14}ClNO_2$	65.3	5.1	12.9	5.1	65.1	5.3	13.0	5.0
VII*a*	Cl	OCH_3	CH_2CN	23	82–83 (M)	$C_{15}H_{12}ClNO$	69.9	4.7	13.8	5.4	69.4	4.8		5.4
VIII*a*	Cl	OCH_3	$(CH_2)_2NH_2$	70	237–240 (W)	$C_{30}H_{34}Cl_2N_2O_6S$[d, e]	58.0	5.5	11.4	4.5	58.0	5.5	11.5	4.6
VIII*b*	H	H	$(CH_2)_2NH_2$	87[f], 13[g]	245 (W)	$C_{14}H_{16}ClN$[h]	71.9	6.9	15.1	6.0	71.9	6.5	15.1	6.0
IX*a*	Cl	OCH_3	$COCH_2Cl$	63	108–110 (E)	$C_{15}H_{12}Cl_2O_2$	61.0	4.1	24.0		60.4	4.3	24.2	
X*a*	Cl	OCH_3	$COCH_2N(CO)_2C_6H_4$[i]	85	239–241 (EA)	$C_{23}H_{16}ClNO_4$	68.1	4.0	8.7	3.5	68.0	4.2	8.6	3.4
X*b*	H	H	$COCH_2N(CO)_2C_6H_4$[i]	31	198–200 (A)	$C_{23}H_{15}NO_3$	77.4	4.4		4.1	77.5	4.4		4.2
XI*a*	Cl	OCH_3	$CHOHCH_2N(CO)_2C_6H_4$[i]	60	186–187 (EA)	$C_{23}H_{18}ClNO_4$	67.7	4.5	8.7	3.4	67.3	4.9	8.9	3.5
XI*b*	H	H	$CHOHCH_2N(CO)_2C_6H_4$[i]	69[j], 13[k]	184.5–185 (AW)	$C_{22}H_{17}NO_3$	77.0	5.0		4.1	76.9	5.6		4.1
XII*a*	Cl	OCH_3	$CHOHCH_2NH_2$	20	180–182 dec. (E)	$C_{16}H_{17}Cl_2NO_2$[h]	57.3	5.5	22.6	4.5	56.8	5.5		4.4
XII*b*	H	H	$CHOHCH_2NH_2$	34	209–211 dec. (E)	$C_{14}H_{16}ClNO$[h]	67.3	6.5	14.2	5.6	7.1	6.6		5.2
XIII*a*	Cl	OCH_3	$CHOHCH_2Cl$	72	92–93 (H)	$C_{15}H_{14}Cl_2O_2$	60.6	4.8	23.9		60.6	4.9	24.1	
XIII*b*	H	H	$CHOHCH_2Cl$	83	78–80 (C)	$C_{14}H_{13}ClO$	72.3	5.6	15.2		72.1	5.4	15.1	
XIV*a*	Cl	OCH_3	$CHOHCH_2NHCH(CH_3)_2$	33	186–188 dec. (E)	$C_{18}H_{23}Cl_2NO_2$[h]	60.7	6.5	19.9	3.9	60.6	6.5	19.7	4.0
XIV*b*	H	H	$CHOHCH_2NHCH(CH_3)_2$	48	175–177 (ER)	$C_{17}H_{22}ClNO$[h]	70.0	7.6	12.2	4.8	69.7	7.7		4.6
XV*a*	Cl	OCH_3	$(CH_2)_2NHCOCH_3$	60	172–174 (MW)	$C_{17}H_{18}ClNO_2$	67.2	6.0	11.7	4.6	67.0	6.2	11.4	4.6
XV*b*	H	H	$(CH_2)_2NHCOCH_3$	90[l]	143–144 (MW)	$C_{16}H_{17}NO$	80.3	7.2		5.9	80.2	7.3		6.0
XVI*a*	Cl	OCH_3	$CH_3CONH(CH_2)_2C_6H_5$	30	144 (E)	$C_{23}H_{22}ClNO_2$	72.7	5.8	9.3	3.7	72.7	5.7	9.2	3.6
XVII*b*	H	H	$(CH_2)_2NHCOCH_2C_6H_5$	49	161–162 (MW)	$C_{22}H_{21}NO$	83.8	6.7		4.4	83.7	6.6		4.6

[a] A = acetic acid; AW = acetic acid–water; C = cyclohexane; D = *N*,*N*-dimethylformamide; E = ethanol; EA = ethyl acetate; ER = ethanol–ether; H = *n*-hexane; M = methanol; MW = methanol–water; W = water. [b] NC_4H_8O = morpholino. [c] Calcd. for S, 8.86; Found, 8.77. [d] Sulfate. [e] Calcd. for S, 5.16; Found, 5.58. [f] Method A. [g] Method B. [h] Hydrochloride. $COCH_2N(CO)_2C_6H_4$ = phthalimido. [j] Method C. [k] Method D. [l] Methods E and F.

(0.073 mole) of potassium phthalimide. The reaction mixture was stirred at room temperature for 18 hr. and then treated with 500 ml. of water. The solid that separated was removed by filtration, washed several times with water, dried, and then recrystallized.

N-{2-Hydroxy-2-[4-(3'-chloro-4'-methoxy)biphenylyl]ethyl}phthalimide (XIa)—The procedure described for the preparation of XIII*b* was followed using 10 Gm. (0.025 mole) of *N*-[4-(3'-chloro-4'-methoxy)phenylphenacyl]phthalimide, 750 ml. of dioxane, 6 Gm. of sodium borohydride, and 250 ml. of methanol.

2-Hydroxy-2-[4-(3'-chloro-4'-methoxy)biphenylyl]ethylamine Hydrochloride (XIIa)—The procedure for the preparation of XII*b* was followed using 1 Gm. (0.002 mole) of *N*-{2-hydroxy-2-[4-(3'-chloro-4'-methoxy)biphenylyl]ethyl}phthalimide, 10 ml. of chloroform, 10 ml. of ethanol, and 1 ml. of hydrazine hydrate (50% aqueous solution).

α-Chloromethyl-4-(3'-chloro-4'-methoxy)biphenylylcarbinol (XIIIa)—The procedure described for the preparation of XIII*b* was followed using 2.95 Gm. (0.01 mole) of 4-(3'-chloro-4'-methoxy)phenylphenacylchloride, 25 ml. of dioxane, 1 Gm. of sodium borohydride, and 15 ml. of methanol.

α-Isopropylaminomethyl-4-(3'-chloro-4'-methoxy)biphenylylcarbinol Hydrochloride (XIVa)—The procedure described for the preparation of XIVb was followed using 4 Gm. (0.013 mole) of α-chloromethyl-4-(3'-chloro-4'-methoxy)biphenylylcarbinol, and 10 ml. of isopropylamine.

N-[2-(4-Biphenylyl)ethyl]acetamide (XVb)—*Method E*—A suspension of 0.1 mole of 2-(4-biphenylyl)ethylamine (VIII*b*) or its hydrochloride in 15% aqueous ethanol, treated with acetic anhydride and sodium acetate, in the usual manner provided the amide.

Method F—A suspension of 19.3 Gm. (0.1 mole) of 4-biphenylylacetonitrile in 50 ml. of freshly distilled acetic anhydride was hydrogenated at 22 p.s.i. with 0.6 Gm. of platinum oxide catalyst according to a modification of the procedure described by Carothers and Jones (17). After 17 hr. the reaction mixture was treated with water; the crystalline solid was collected by filtration and recrystallized.

2-Phenyl-N-(4-biphenylylethyl)acetamide (XVIIb)—A solution of 1.5 Gm. (0.0076 mole) of 2-(4-biphenylyl)ethylamine (VIII*b*) in 5 ml. of pyridine was treated with 2 ml. of phenylacetyl chloride and set aside at room temperature for 25 hr. The amide was isolated in the usual manner and recrystallized.

N-{2-[4-(3'-Chloro-4'-methoxy)biphenylyl]ethyl}acetamide (XVa)—A solution of 0.68 Gm. (0.0026 mole) of 2-[4-(3'-chloro-4'-methoxy)phenylyl]acetonitrile (VII*a*) in 10 ml. of acetic anhydride was hydrogenated at 15 p.s.i. with 0.1 Gm. of platinum oxide catalyst for 12 hr. according to the procedure described for XV*b*, (Method F); $\lambda_{max.}^{KBr}$ 1285, 1545, 1645, 3250 cm.$^{-1}$.

2-[4-3'-Chloro-4'-methoxy)biphenylyl]-N-(phenethyl)acetamide (XVIa)—2-[4-(3'-Chloro-4'-methoxy)biphenylyl]acetyl chloride was prepared in the usual manner from 7.1 Gm. (0.026 mole) of the acid (V*a*) and 7 ml. of redistilled thionyl chloride. A suspension of the acid chloride in 20 ml. of pyridine was treated with 3.2 Gm. of phenethylamine and then allowed to remain at room temperature for 24 hr. The product was isolated in the usual manner.

Pharmacological Tests—Adult male albino mice were utilized for gross symptomologic characterization concurrently with lethality determination. Observations continued for 3–4 hr. after injection and further checks were made daily thereafter for 3 days. Compounds injected intraperitoneally were in aqueous solution or were in acacia suspensions. The one injected intravenously was dissolved in 10% ethanol and the one given orally was suspended in 0.5% methylcellulose. LD_{50} and ED_{50} values were determined using the method and tables of Horn (18).

Blood pressure studies in male albino rats utilized urethan anesthesia, carotid artery cannulation, and recordings *via* a Sanborn pressure transducer.

Analgesia measures used in mice were the benzoquinone writhing test (19) or the hot plate method (20). The standard maximal electroshock testing procedure (21) was utilized. Locomotor activity was recorded in a commercial photocell activity device.

The analytical data for the biphenyls are given in Table I.

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Keyphrases

2-(4-Biphenylyl)ethylamines—synthesis
Pharmacological activity—biphenylethylamines
LD_{50} values—biphenylethylamines
IR spectrophotometry—structure

Some Studies of Flocculation Phenomena in Pharmaceutical Suspensions

By B. A. MATTHEWS and C. T. RHODES

The increasing use of suspensions as a form of pharmaceutical preparation has emphasized the need for further evaluation of the factors controlling the physical stability of these systems. A number of attempts have been made recently to prevent impaction of suspensions by controlled flocculation with polyvalent ions. This paper reports the use of a number of experimental techniques, including microelectrophoresis, the Coulter counter, and standardized stability methods, in studies of the effect of a number of variables upon flocculation and sedimentation, in some systems of pharmaceutical interest.

THE PHYSICAL STABILITY of pharmaceutical suspensions has been the subject of a number of recent research and review articles (1–13). Several workers have found that controlled flocculation is a particularly useful technique for formulating pharmaceutical suspensions, which do not impact on storage (5). In this paper the authors report investigations of flocculation, by polyvalent ions, of an important synthetic drug, griseofulvin, and of a monodisperse model system. Because griseofulvin, like many other modern drugs, is hydrophobic in nature, it was found necessary to include wetting agents. Doscher (14) found that nonionic wetting agents caused changes in flocculation without affecting zeta potential. In this study anionic wetting agents, which have been shown not to precipitate with the flocculating ions, have been selected.

Aluminum chloride was selected as the flocculant since a 3:1 electrolyte was required and aluminum is probably the most suitable trivalent metal with regard to toxicity. Other workers in this field (15) have shown that, above pH 4, hydrolysis products of aluminum ions cause peptization of colloidal systems because of charge reversal. At pH 3 and below, however, the Al^{3+} ion causes flocculation. Experiments were, therefore, restricted to this pH region, although it was realized that a somewhat higher pH value would probably be more suitable for a pharmaceutical formulation.

The effect of a number of variables upon flocculation have been investigated by microelectrophoretic measurement of zeta potential, determination of sedimentation volumes, redispersibility, and stability ratios.

EXPERIMENTAL

Materials—The drug, griseofulvin[1] (16), was chosen for this study because, (*a*) it is available commercially in two particle sizes (17) of mean particle diameter about 4 and 15 μ, (*b*) it has a low water solubility, (*c*) it is stable chemically in aqueous suspension, (*d*) the molecule has no ionic groupings which could react with polyvalent ions of opposing charge, (*e*) the powdered drug is difficult to wet and thus is likely to present problems to the formulator, and (*f*) the drug is often given over long periods of time and a suspension is thus a suitable dosage form, especially for pediatric use.

A total of seven anionic surfactants was screened for possible precipitation with the flocculant by preparing 0.1% solutions of surfactants containing 0.1% aluminum chloride. After storage for 1 week at 25°, solutions of the following surfactants, sodium isopropyl naphthalene sulfonate,[2] sodium dioctyl sulfosuccinate,[3] sodium dodecyl sulfate,[4] and sodium alkyl naphthalene sulfonate[5] showed opalescence. However, solutions of sodium diamyl sulfosuccinate,[6] ammonium lauryl ether sulfate,[7] sodium lauryl ether sulfate,[8] and the sodium salt of sulfated lauryl alcohol ethoxylate[9] remained clear. Further tests for precipitation at high electrolyte concentration and evaluation of wettability with respect to griseofulvin showed that ammonium lauryl ether sulfate was a very suitable surfactant for this study.

The aluminum chloride[10] used in this investigation was reagent grade $AlCl_3 \cdot 6H_2O$. All solutions were prepared with glass-distilled water.

Preparation of Suspensions—The drug was dispersed in solutions of the wetting agent by means of a Silverson[11] mixer/emulsifier. Suspensions were refined for 20 min. and then made up to 90% of the

Received September 22, 1967, from the Physical Pharmacy Department, School of Pharmacy, College of Technology, Portsmouth, Hampshire, England.

Accepted for publication November 15, 1967.

This paper was presented to the Galenic Pharmacy section at the Fédération Internationale Pharmaceutique Conference, Montpellier, France, September 1967.

The authors thank Messrs. P. Harger, R. Rogers, and Miss F. Tringham for technical assistance in this work and the Smith Kline and French Foundation, Welwyn Garden City, England, for financial support.

[1] The authors thank Glaxo Ltd., England, for the gift of generous supplies of griseofulvin.
[2] Aerosol O.S., Cyanamid.
[3] Manoxol O.T., Hardman and Holden.
[4] Sodium lauryl sulfate B.P., Sipons.
[5] Nansa BXS, Marchon.
[6] Aerosol A.Y., Cyanamid.
[7] Empicol EAB, Marchon.
[8] Empicol KSN 27, Marchon.
[9] Empicol ESB3, Marchon.
[10] British Drug Houses, Ltd.
[11] Silverson Ltd., London, England.

final volume. Ninety-milliliter quantities of the suspensions were distributed into 100-ml. graduated cylinders, 10 ml. of the flocculating electrolyte solution at 10 times the required flocculant concentration was added, the cylinders stoppered and shaken. After storage at 25 ± 0.5° for 3 days, the suspensions were examined.

Measurement of Sedimentation Volume—The sedimentation volumes were recorded in terms of the ultimate settled height, H_u, to the original height, H_o, as suggested by Robinson (18) and Ward and Kammermeyer (19).

$$\text{sedimentation volume} = H_u/H_o \quad \text{(Eq. 1)}$$

Measurement of Redispersibility—To standardize evaluation of redispersibility, a machine was constructed which would turn the cylinders through 360° at a constant speed of 20 r.p.m. Experimentation showed that this speed was the optimum for the supernatant liquid to strike the sediment with the maximum force at each revolution. The number of revolutions necessary to restore the suspension to homogeneity was recorded. This design is similar to that described by Lesschafft and De Kay (20), but their apparatus measured redispersibility by shaking the suspensions in a 90° arc not by complete revolution.

Measurement of Electrophoretic Mobility—Electrophoretic mobilities were measured in a flat electrophoresis cell[12] of rectangular cross-section similar to that originally described by Abrahamson (21, 22). The calibration and use of this type of cell has been discussed by Black and Smith (23, 24) and Haines and Martin (6).

The dimensions of the flat portion were 2.11 × 0.51 cm. and the electrodes were zinc/zinc sulfate. A M 2150F2[13] research microscope, fitted with a ×1.5 binocular body and ×17 eyepieces, was used to view the particles. Objectives used were ×10 or ×20 with a long working distance. The uniformity of the fine adjustment of the microscope was checked by recording the thickness of a glass cover slip coated with lycopodium powder over different regions of the fine adjustment. Measurements were made using the most uniform region. Brinton and Lauffer (25) have discussed the need for temperature control and conclude that electrophoretic velocity at constant current is independent of temperature changes within a range of 10°, so that the cell needs to be thermostated only to remove excess heat in order to prevent convection. The flat area of the cell was therefore immersed in a thermostatic bath which was maintained at 25 ± 0.2°, by means of circulated water. The power source was a Shandon[14] Vokam power supply unit type 2541, which could be maintained at constant current. The field strength was determined by measuring the current in the cell by means of a multirange milliameter and a Pye[15] conductance bridge and conductivity cell to record the conductivity of the suspension. The field strength was calculated by substitution into Ohm's law. The position of the stationary layers of the cell was calculated by Komagata's equation (26).

$$a = {}^1/_2 \pm \sqrt{{}^1/_{12} + 32/\pi^5 k} \quad \text{(Eq. 2)}$$

where a is the fractional depth from the top of the cell and k is the cell width/depth ratio.

This was checked by recording the mobility of human erythrocytes in 0.067 M standard phosphate buffer at pH 7.4 at the lower stationary level. Abrahamson (21, p. 257) has reported a standard mobility of $-1.31\,\mu$/sec./v./cm. which is independent of age, race, sex, or blood group. Results obtained on two separate occasions were -1.29 and $-1.31\,\mu$/sec./v./cm. All measurements were subsequently made at this lower stationary level. For each measurement 20 particles were timed (10 in each direction) over a suitable number of squares in the eyepiece graticule. Times were recorded on a stopwatch, accurate to 0.1 sec.

The cell was cleaned regularly with chromic acid and rinsed with distilled water and acetone between measurements. Great care was taken to avoid air bubbles and the cell was filled and emptied by displacement using vacuum.

Visual Examination of Supernatant—The supernatants of the stored suspensions were examined for evidence of opalescence caused by very small discrete particles, which are slow to settle. This is found in deflocculated suspensions, whereas when flocculation occurs, these very small particles are incorporated in the sedimenting flocs and the supernatant is clear.

RESULTS AND DISCUSSION

Calculation of Zeta Potential—From a consideration of the properties of the electrical double layer at the surface of a particle and the laws of liquid flow, the Helmholtz-Smoluchowski equation can be derived

$$\zeta = \frac{4\pi\eta\mu}{D} \quad \text{(Eq. 3)}$$

where η is the viscosity of the suspending medium, μ is the electrophoretic mobility (velocity per unit field), D is the dielectric constant, and ζ is the zeta potential.

The assumptions made in deriving this equation have been listed by Brinton and Lauffer (25, p. 431). The most important of these are: (*a*) The product of the Debye-Hückel constant, k, and the radius of curvature of the particle a, is large. (*b*) The particle surface is nonconducting.

These assumptions are made so that the electric field can be considered parallel to the surface everywhere within the double layer.

Making the assumption that the field is everywhere in the direction of the applied field, Debye and Hückel derived the following equation for spheres which is valid when ka is small:

$$\zeta = \frac{6\pi\eta\mu}{D} \quad \text{(Eq. 4)}$$

Henry (27) has derived an equation valid for any value of ka which can be expressed in the form

$$\zeta = \frac{6\pi\eta\mu}{D} f(ka) \quad \text{(Eq. 5)}$$

When ka is less than 0.1 the 6π factor holds.
When ka is greater than 100 the 4π factor holds.
When ka is between 0.1 and 100 values of $f(ka)$ can be calculated to correct the general equation.

[12] Linskey Brothers, London, England.
[13] Cooke, Troughton and Simons Ltd., York, England.
[14] Shandon Scientific Co. Ltd., London, England.
[15] W. G. Pye and Co., Ltd., Cambridge, England.

Matthews and Rhodes (17) have shown that fine-particle griseofulvin has a mean diameter of 3–4 μ measured on the Coulter counter. Using a particle radius of 1.5-μ values of the factor to be used in the Debye-Hückel equation have been calculated for different concentrations of aluminum chloride. These range from 4π at 10^{-2} *M* and above, to 4.3π at 10^{-5} *M* and these values have been used in calculating zeta potentials. Similar factors were calculated by Nash and Haeger (13) for a 1:1 electrolyte and alternative correction factors for various electrolytes and for different zeta potentials have been given by Overbeek (28).

Flocculation at pH 3.0—The results of flocculation studies at pH 3.0 are shown in Fig. 1 and Table I, the suspension pH had been adjusted to 3.0 by addition of hydrochloric acid.

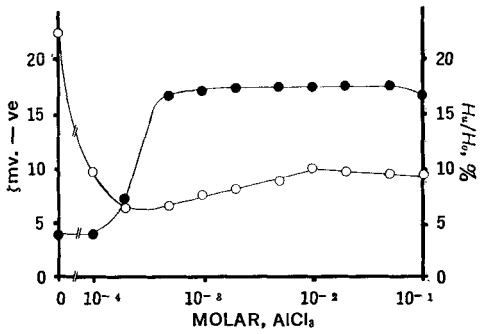

Fig. 1—Flocculation of fine-particle griseofulvin wetted with ammonium lauryl ether sulfate by aluminum ions at pH 3.0. Key: ●, H_u/H_o; ○, *zeta potential.*

From Fig. 1 it may be observed that at this pH value, aluminum ions are very effective in causing flocculation of the suspensions. A concentration of 5×10^{-4} *M* aluminum chloride reduced the zeta potential from −22.7 mv. to −6.2 mv., increased the sedimentation volume from 3.5 to 17.0%, and produced suspensions which redispersed after a very few revolutions in the machine (Table I). Higher concentrations of electrolyte produced little further change in the sedimentation volume, but the measured zeta potential rose slightly.

Flocculation at pH 2.0—The above experiment was repeated in 10^{-2} *N* HCl which gave a constant pH of 2.0 in all systems. The results are given in Table II and Fig. 2.

At this electrolyte concentration, values of *ka* are always greater than 100 and the 4π factor was therefore used in the zeta potential equation. At this pH value, zeta potentials were greater than at 3.0 and higher concentrations of aluminum chloride (10^{-3} *M*) were necessary to achieve maximum flocculation. There was no point of inflection in the zeta potential curve and maximum flocculation occurred at about −15 mv. The above experiment was repeated using the coarse-particle griseofulvin (see also Table II and Fig. 2). In this system there is no sudden increase in sedimentation volume but the concentration of $AlCl_3$ at which the rise begins to occur is the same for both materials (approx. 2×10^{-4} *M*). The progressive rise in sedimentation

TABLE I—FLOCCULATION OF FINE PARTICLE GRISEOFULVIN WETTED WITH AMMONIUM LAURYL ETHER SULFATE BY $AlCl_3$ AT pH 3.0

Concn. of $AlCl_3$, M	Sedimentation Volume H_u/H_o, %	Supernatant	No. Revs. to Redisperse
0	3.5	Opalescent	200+
10^{-4}	3.5	Opalescent	200+
2×10^{-4}	7.0	Clear	20
5×10^{-4}	16.5	Clear	10
10^{-3}	17.0	Clear	5
2×10^{-3}	17.5	Clear	5
5×10^{-3}	17.5	Clear	5
10^{-2}	17.5	Clear	5
2×10^{-2}	17.5	Clear	5
5×10^{-2}	17.5	Clear	5
10^{-1}	16.0	Clear	5

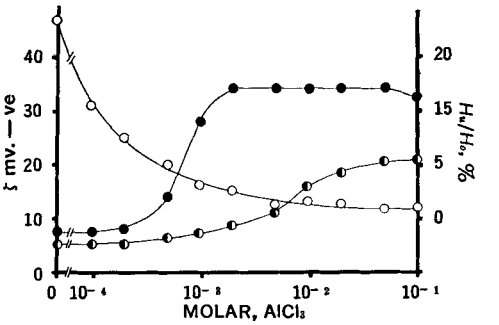

Fig. 2—Flocculation of griseofulvin wetted with ammonium lauryl ether sulfate by aluminum ions at pH 2.0. Key: ●, H_u/H_o *fine-particle griseofulvin;* ◐, H_u/H_o, *coarse-particle griseofulvin;* ○, *zeta potential fine-particle griseofulvin.*

TABLE II—FLOCCULATION OF GRISEOFULVIN WETTED WITH AMMONIUM LAURYL ETHER SULFATE BY $AlCl_3$ AT pH 2.0

Concn. of $AlCl_3$, *M*	Fine Particle			Coarse Particle		
	H_u/H_o, %	Supernatant	No. Turns to Redisperse	H_u/H_o, %	Supernatant	No. Turns to Redisperse
0	3.5	Opalescent	200+	2.5	Opalescent	100+
10^{-4}	3.5	Opalescent	200+	2.5	Opalescent	100+
2×10^{-4}	4.0	Opalescent	50	2.5	Opalescent	100+
5×10^{-4}	7.0	Clear	26	3.0	Opalescent	70
10^{-3}	14.0	Clear	10	3.5	Opalescent	50
2×10^{-3}	17.0	Clear	5	4.2	Opalescent	28
5×10^{-3}	17.0	Clear	5	5.7	Opalescent	15
10^{-2}	17.0	Clear	5	7.0	Opalescent	12
2×10^{-2}	17.0	Clear	5	8.9	Clear	8
5×10^{-2}	17.0	Clear	5	10.0	Clear	5
10^{-1}	16.0	Clear	5	10.3	Clear	5

volume was accompanied by an increase in ease of redispersibility, but again no sudden change occurred. This change in behavior, as the particle size increased, indicated the potential utility of a comparison of flocculation of a colloidal system with the same surfactant and electrolyte combination.

Comparison with a Model Colloid—The kinetics of flocculation in colloidal systems was considered by Von Smoluchowski (29, 30) in his theory of rapid coagulation. Smoluchowski showed that when every collision between particles results in aggregation, then the coagulation time, $T_{1/2}$, or time taken for the total number of particles to fall by one-half is given by the equation:

$$T_{1/2} = \frac{1}{4\pi DRN_0} \qquad \text{(Eq. 6)}$$

where D is the diffusion constant of the particles, R is the distance between the center of two particles when flocculation occurs, and N_0 is the original number of particles.

When there is an energy barrier between the particles the rate of flocculation is reduced, since only a fraction, α, of the collisions is effective. Fuchs (31) modified Smoluchowski's theory to enable the energy barrier to be related to a reduced rate of flocculation and introduced the stability ratio, W, which is given by

$$W = 2r \int_{2r}^{\infty} \left[\exp. \left(\frac{V_t}{kT}\right)\right] \frac{dS}{S^2} \qquad \text{(Eq. 7)}$$

where r is the particle radius, S is the distance between the particle centers $= 2r$ at coagulation, V_t is the total energy of interaction between the particles, k is Boltzmann's constant, and T is the absolute temperature.

Matthews and Rhodes (32) have developed a method involving a digital computer and the Coulter counter to estimate W in flocculating monodisperse systems. The method involves comparing the experimental rate of depletion of the singlet species with that which would be expected from theory. The method has been used to investigate the flocculation of a polystyrene latex polymer in the same system of anionic wetting agent and cationic flocculant as was used for the griseofulvin.

The system selected was a 0.714-μ polystyrene latex[16] which can be considered to be on the borders of colloidal dimensions. The particles are of such low density that sedimentation can be ignored and the polymer is devoid of any ionic groupings on the particle surface and thus is a suitable model for the drug. The particles were present at a concentration of 5×10^7 per ml. Wetting agent, 3×10^{-6} M ammonium ether lauryl sulfate, was added. The pH was standardized as before by the use of 10^{-2} N HCl and the concentration range of aluminum chloride was from 10^{-4} to 10^{-2} M.

The results of the measurements of stability ratio W and zeta potential against log concentration are given in Fig. 3. The graph of log electrolyte concentration against zeta potential is a straight line, similar to that obtained by Ottewill and Watanabe (33) for a silver iodide colloid coated with an anionic surfactant. The graph of log W against log concentration of electrolyte is also characteristic

[16] Kindly supplied by Mr. B. J. Lippie, Dow Chemical Co., Midland, Mich.

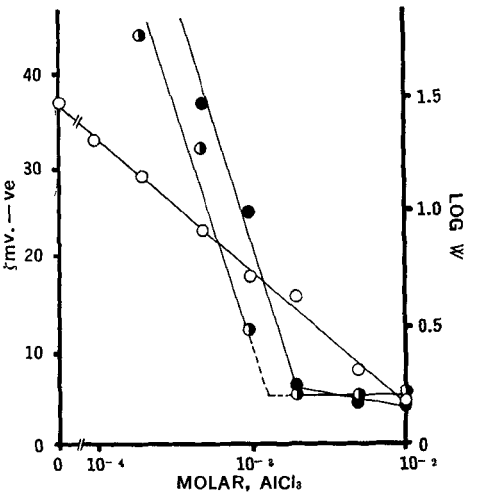

Fig. 3—Flocculation of 0.714-μ latex polystyrene wetted with ammonium lauryl ether sulfate by aluminum ions. Key: ●, log W at pH 2.0; ○, zeta potential at pH 2.0; ◑, log W at pH 3.0.

of a colloid. A rapid decrease in stability occurs at a concentration of about 10^{-3} M $AlCl_3$ although the value of log W does not quite reach zero as it should for a true colloid according to the Smoluchowski equation. This may be due to the presence of the surfactant or to the particle size being just above the upper limits of the colloidal range.

A comparison of the flocculation of this model colloid with that of fine-particle griseofulvin (*cf.* Figs. 2 and 3) under identical conditions reveals that the minimum concentration of electrolyte required to produce maximum flocculation is the same in each system (2×10^{-3} M). The zeta potential at this point in each system is also identical (−15 mv.).

Measurement of stability ratio as a function of aluminum chloride concentration was also made at pH 3 and the results are also given in Fig. 3. The change from pH 2 to 3 results in a displacement of the vertical portion of the graph toward lower concentrations of electrolyte, in the same way as the vertical portion of the sedimentation height/aluminum chloride concentration graph was changed for fine particle griseofulvin (*cf.* Figs. 1 and 2).

This is strong evidence that flocculation in systems with a mean particle size of 3 to 4 μ is essentially similar to that in colloidal systems where van der Waals forces are operative. Kruyt (34) has referred to the theoretical existence of long range London/van der Waals forces in hydrophobic suspensions in the 2–5 μ range, and has stated that flocculation is possible in the secondary minimum that occurs in the energy of interaction curve at about 1000–2000 Å. distance. The particles can, therefore, approach one another to this distance and yield a loosely flocculated structure.

It has been noted by earlier workers (8) and by the present authors that flocculation in pharmaceutical suspensions with polyvalent ions is very rapid, and Wilson and Ecanow (8), in their discussion of the earlier work of Haines and Martin (5), stated that flocculation in colloidal systems was, by contrast, a slow process depending on random collisions due to Brownian motion.

The Eq. 6 for the coagulation time shows that it

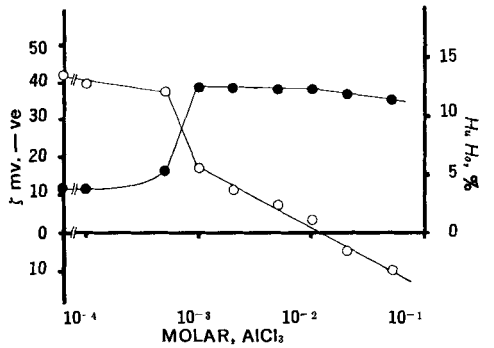

Fig. 4—Flocculation of fine particle griseofulvin wetted with ammonium lauryl ether sulfate by aluminum ions at natural pH. Key: ●, H_u/H_o; ○, zeta potential in 0.1 M sodium acetate.

is dependent on the total number of particles in the system, and an estimate of the number of particles in the 2.5% w/v fine-particle griseofulvin used above was made, by performing a total count with the Coulter counter using the 30-μ orifice. This gave a value for the suspensions of 1.5×10^8 particles per ml. which would have a coagulation time of 20 min. This is quite in accord with the results obtained in the experiments, since sedimentation and heterodispersity are likely to accelerate the process and the Coulter counter is unable to detect the very fine particles of diameter less than 0.5 μ

Measurement of Zeta Potential by Dilution in Electrolyte—Because of the differences in the shape of the zeta potential/electrolyte concentration graphs between systems with different total electrolyte concentrations (*e.g.*, Figs. 1, 2), it was decided to examine the effect of diluting the suspensions in equal parts of a 1:1 electrolyte solution before measurement.

Suspensions of griseofulvin were prepared as before, using the ammonium lauryl ether sulfate wetting agent and different concentrations of aluminum chloride, but at the natural pH. For measurement of zeta potentials the suspensions were diluted with equal parts of 0.1 *M* sodium acetate at pH 5.0. The results are shown in Fig. 4. Good correlation between the zeta potential and sedimentation volume was observed with a rapid reduction in zeta potential and increase in sedimentation volume at 10^{-3} *M* aluminum chloride. Reversal of charge occurred at about 10^{-2} *M*, as has been noted by other workers (15) using aluminum salts with colloids. Slight reduction in the sedimentation volume occurred in these systems with a low positive zeta potential, which may indicate a small degree of deflocculation.

SUMMARY

It has been demonstrated that in suspensions with a mean particle size in the range 3–4 μ, flocculation is essentially similar in character to that in lyophobic colloids, and that such flocculation is accompanied by changes in the zeta potential of the particles.

Suspensions flocculated in this way may be readily redispersed and although the experiments quoted above only lasted 3 days, other experiments still in progress indicate that controlled flocculation in this way can produce suspensions which remain redispersible over long periods of time. Highly flocculated suspensions are somewhat inelegant due to the coarse appearance of the large flocs. However, by controlling the amount of electrolyte, it is possible to produce suspensions with just a sufficient degree of flocculation to prevent impaction. However, these results with the different types of griseofulvin, suggest that some consideration of the particle size is necessary to decide the exact amount of electrolyte necessary. Martin (3) has also remarked that the influence of other ingredients such as flavorings, colorings, and preservatives must be considered.

The work reported in this paper strongly supports the utility of the concept of controlled flocculation in the formulation of pharmaceutical suspensions, although it is felt that further research is required into the variables involved before formulation can be placed on a truly rational, rather than an *ad hoc* basis.

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Keyphrases

Flocculation—suspensions
Surfactants—flocculation
pH effect—flocculation
Electrolyte effect—flocculation
Sedimentation redispersibility—suspensions
Stability ratio—suspensions
Zeta potential—suspensions

Physics of Tablet Compression XV

Thermodynamic and Kinetic Aspects of Adhesion Under Pressure

By ALBERT S. RANKELL and TAKERU HIGUCHI*

A theoretical analysis has been made of the processes by which interparticle adhesion can occur during the compression of tablet granulations in the absence of added fluid adhesives. A reasonable explanation for the development of adhesion in these systems is based on the occurrence of either partial fusion or partial dissolution at the surfaces of the particles induced by energy added during the compression. The possibility that these phenomena do occur gains partial support from an examination of the thermodynamics and kinetics of surface processes that may take place. The Clapeyron equation cannot be applied directly to these processes, but must be modified to take into account the nonuniformity of the pressures exerted on the particles during compression. Another factor which may affect particle adhesion is the production of highly localized frictional heat and slow diffusion of this heat from the particle surfaces into their interior.

THE PROCESSES by which powder particles are made to adhere by compression in a tablet press are not well understood. Pharmaceutical literature concerning the mechanism of interparticle bonding in compressed tablets is not extensive, and most studies applicable to this area are found in the literature of powder metallurgy and of solid friction (1, 2). This report is concerned with theoretical examination of physical processes that may be responsible for compressive bonding of ingredients of pharmaceutical tablets.

In a summary of existing theories of bonding and sintering in powder metallurgy, Goetzel (3, 4) finds three proposed mechanisms to be worthy of extended discussion: (*a*) interatomic forces (surface adhesion, cold-welding, and surface tension), (*b*) liquid surface cements, and (*c*) mechanical interlocking. The liquid surface cement mechanism, which attributes adhesion to the presence of thin films of liquid at the interface between the particles being compressed, has much to recommend it in the analysis of binding in tablets.

Tablet triturates obviously require the presence of liquid at the interparticular interface during their production. The use of solutions of polymeric materials as binding agents for compressed tablets is common knowledge (5, 6) as is the importance of granulation humidity (7, 8). It is possible, however, to compress some materials into tablets without the addition of a binding agent. For such materials, the liquid cement mechanism would indicate that the cementing film is the product of fusion or solution at the particle surface induced by the energy added during the compression.

The reason why substantial melting has not been observed during the compression of commonly tableted materials can be made clear by consideration of a single case. It has been shown that the energy expended in the compression of a 400-mg. sulfathiazole tablet is in the order of 2 calories (9). Assuming that all of the energy expended appears as heat, the resultant temperature rise of the tablet would be only about 5°. This temperature is nowhere near the normal melting point of the material being tableted.

EFFECT OF STRESS DISTRIBUTION ON MELTING POINT

During the compression process, however, conditions at the surfaces of the particles are radically different from those in the particle interior. The load applied by the upper and lower punches is exerted only on particles directly in contact with the punch surfaces. The stresses on the rest of the particles in the mass are the result of particle-to-particle contact. The irregular surface of the particles causes each individual point of contact to have a very small area; thus, the pressures involved are extremely large.

The relative magnitude of these effects is demonstrated by a measurement of the real area of contact between two flat steel plates under pressure (10). It was found that when the surfaces were pressed together under a load equivalent to 4.6 Atm., the true area of contact was only 1/2000 of the apparent macroscopic area. Thus, the pressure at the points of contact was actually 9200 Atm.

Clapeyron Equation—The high pressure at the points of contact have a definite effect on the melting point of the substance being compressed. A relationship between pressure and melting point derived from thermodynamic considerations is the

Received August 11, 1967, from the School of Pharmacy, University of Wisconsin, Madison, WI 53706
Accepted for publication October 13, 1967.
Presented to the Basic Pharmaceutics Section, APhA, Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This investigation was supported in part by grant GM-05830 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., American Chicle Co., Warner-Lambert Pharmaceutical Co., Long Island, N. Y.
Previous paper: Higuchi, T., Shimamoto, T., Eriksen, S. P., and Yashiki, T., *J. Pharm. Sci.*, **54**, 111(1965).
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Clapeyron equation (11):

$$dT/dP = T(V_L - V_S)/\Delta H \quad \text{(Eq. 1)}$$

where: dT/dP is the change in melting point with pressure, T is the absolute temperature, V_L is the volume per mole of the liquid melt, V_S is the volume per mole of the solid, and ΔH is the molar latent heat of fusion.

The latent heat of fusion is positive, thus, the Clapeyron equation predicts that for a solid which expands on melting ($V_L > V_S$), the melting point is increased by raising the pressure, while for a solid which contracts on melting ($V_L < V_S$), the melting point is lowered by increasing the pressure. Except for a few substances, such as water, most solids expand on melting.

Thus, Eq. 1 would indicate that during tablet compression, the rise in melting point with pressure for most substances would make it highly unlikely that fusion could take place. The Clapeyron equation, however, is derived for an ideal process in which the solid is exposed to uniform hydrostatic pressure, and which is thermodynamically reversible. In the case of tablet compression, on the other hand, the process is essentially nonreversible, and the pressure exerted on each particle is far from uniform.

Thermodynamics of Stressed Solids—Thermodynamic relationships for particles being compacted in a tablet press must take into account the stress distributions induced by the point pressures involved. The Clapeyron equation is derived by equating the Gibbs free energies of the two phases of the equilibrium:

$$\text{liquid } (V_L) \rightleftarrows \text{solid } (V_S) \quad \text{(Eq. 2)}$$

At constant temperature and pressure, the equation is of the form:

$$dF_L = dF_S = V_L dP - S_L dT = V_S dP - S_S dT \quad \text{(Eq. 3)}$$

which reduces to Eq. 1. If the system is not under constant pressure, but is subjected to nonhomogeneous stress, the free energy, dF, is a function of the strain, and must be represented in a suitable form, such as that suggested by Li and co-workers (12):

$$dF = \int (\epsilon_i \sigma_i d\epsilon_i) dV - SdT \quad \text{(Eq. 4)}$$

where: σ_i is one of the six components of the stress, and ϵ_i is the corresponding strain component. In such a case, the derivation of an equation corresponding to the Clapeyron equation would be quite involved.

Stressed Beam Analogy—The effect of stress distribution on particle melting points during compression can be more easily illustrated by the following analogy. Consider a crystal which is supported at each end and which is subjected to a point pressure on its upper surface, as shown in Fig. 1. For ease in mathematical treatment, the

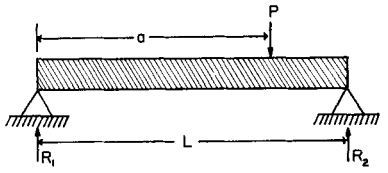

Fig. 1—Point loading on a simply supported beam.

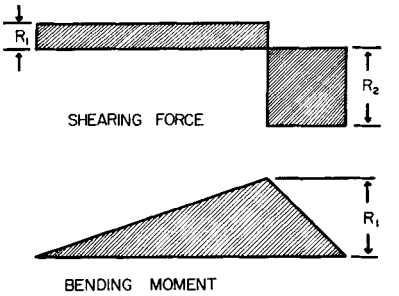

Fig. 2—Shearing force and bending moment diagrams for point loading on a simply supported beam.

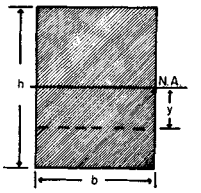

Fig. 3—Cross-sectional view of a beam showing the neutral axis.

crystal can be considered to be a beam with rectangular cross-section. The point pressure p is opposed by the resultant forces R_1 and R_2, producing both shear and bending stresses, as shown in Fig. 2. The shearing force is uniform on either side of the point of application of p, but the bending moment increases to a maximum at the point of application of p.

The bending stress on a cross section of the beam can be shown to be (13):

$$\sigma_n = My/I \quad \text{(Eq. 5)}$$

where: σ_n is the normal stress (force/unit area); M is the bending moment (force × length); y is the distance from the neutral axis of the beam (length); and I is the moment of inertia of the cross-sectional area about the neutral axis (length^3). The moment of inertia for a rectangular cross-section is

$$I = bh^3/12 \quad \text{(Eq. 6)}$$

where: b is the thickness of the beam, and h is the height, as shown in Fig. 3. Thus, the bending stress is

$$\sigma_n = 12(My/bh^3) \quad \text{(Eq. 7)}$$

The range of values for y is

$$-h/2 \leq y \leq +h/2 \quad \text{(Eq. 8)}$$

and the variation of stress over the depth of the beam can be plotted as shown in Fig. 4. It can be seen that there is no stress at the neutral axis, and that the stress is greatest at the surfaces of the beam. The nature of the stress is different on each side of the neutral axis; while the upper surface of the beam is being compressed, the lower surface is under tension, or negative pressure.

Referring once more to the Clapeyron equation (Eq. 1), it may be seen that while the right-hand side of the equation is positive for substances which expand on melting, the left-hand side is negative for those portions of the solid which are under tension. In these areas the melting point will be lowered when point pressure on the opposite side of the crystal is increased. Thus, under the conditions

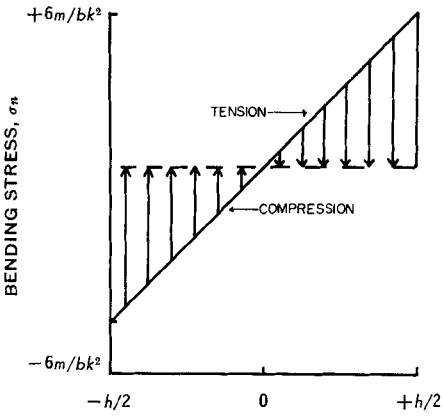

Fig. 4—Stress distribution for point loading on a simply supported beam.

of tablet compression there will be a reduction of melting point on some portion of the surface of each particle, whether the material in question expands or contracts on melting.

Skotnicky Equation—A more recent derivation of the relationship between melting point and pressure is that of Skotnicky (14). The change of equilibrium temperature (melting point) for each phase in the system of Eq. 2 can be shown to be:

$$(\partial T/\partial P)_F = V/S = TV/H \qquad \text{(Eq. 9)}$$

If a pressure P_L acts on the liquid, and a pressure P_S acts on the solid, then the molar latent heat of fusion, ΔH, can be expressed as a function of the change of equilibrium temperature with pressure:

$$\Delta H = H_L - H_S = TV_L(\partial P_L/\partial T)_F - TV_S(\partial P_S/\partial T)_F \qquad \text{(Eq. 10)}$$

For an ideal process, in which the system is exposed to a uniform hydrostatic pressure ($P_L = P_S = P$), Eq. 10 reduces to the usual form of the Clapeyron equation (Eq. 1). However, if a point pressure is exerted on the solid only, and the liquid is subjected to a constant atmospheric pressure P_L, then Eq. 10 reduces to:

$$dT/dP_S = -V_S T/\Delta H \qquad \text{(Eq. 11)}$$

For such a system, the isolated pressure acting locally on the solid always lowers the melting point regardless of the expansion or contraction of the solid on melting.

Skotnicky showed that Eq. 11 agrees qualitatively with the experimentally observed lowering of the melting point of naphthalene under point pressure. This substance, which expands on melting, would be expected to show a rise in melting point with increased pressure by the usual Clapeyron equation.

EFFECT OF STRESS DISTRIBUTION ON SOLUBILITY

The reasoning employed in the analysis of point pressure effects on melting point can also be extended to changes in solubility of solids with pressure. It can be shown that the variation in solubility of a solid with uniform hydrostatic pressure is of the form (15):

$$(\partial \ln x/\partial P)_T = -(V_L - V_S)/RT \qquad \text{(Eq. 12)}$$

where ln x is the natural logarithm of the mole fraction of the solid in saturated solution, and R is the gas law constant. This equation predicts that if the solid expands on melting ($V_L > V_S$), an increase in pressure will diminish its solubility. Conversely, if the solid contracts on melting ($V_L < V_S$), an increase in pressure will increase the solubility.

A consideration of the stress distribution resulting from point pressures under the conditions of tablet compression leads to the conclusion that there will be an increase in the solubility on some portion of the particle surface whether the material in question expands or contracts on melting. The liquid in which solution takes place is usually the film of moisture adsorbed on the particle surface.

The essentially nonadherent nature of most insoluble materials and the ease of compaction of water-soluble substances would seem to indicate that pressure-induced solubilization plays an important role in adhesion of tablet particles. The importance of adsorbed moisture is pointed up by studies on the relationship of tablet compacting properties to granulation moisture content (5, 6). Generally, granulations which are absolutely dry have poor compacting properties (16).

EFFECT OF HEAT TRANSFER KINETICS ON FUSION

A basic requirement for the occurrence of surface fusion, regardless of changes in melting point, is the formation of localized high-temperature areas, or "hot spots," on the particle surface. If the temperature of these hot spots reaches the melting point of the substance being compressed, surface fusion will occur. The temperature reached is a function of the balance between the heat transferred to the surface of the particle and the heat transferred away from the surface by conduction into the interior of the particle.

The heat transferred to the surface of the particle is produced by the conversion of the mechanical energy of compression to thermal energy and is essentially independent of temperature. The rate of heat conduction into the interior of the particle is a function of the specific heat and thermal conductivity of the solid and of the particle geometry. The surface temperature reached cannot be determined exactly because of the time-dependent nature of the heat transfer rates and the complicated geometry of the average particle. However, qualitative estimates can be made by the use of a simplified equation.

The average rate of heat transfer to the particle surface can be estimated by dividing the energy of compression by the total time of compression. The rate of heat transfer into the particle interior can be estimated by neglecting the effects of particle geometry and calculating on the basis of one-dimensional heat transfer into a slab of semi-infinite thickness. A suitable equation for heat transfer under these conditions has been derived by Carslaw and Jaeger (17), and is of the form:

$$\Delta T = (2q/Ak)\,(kt/c\rho\pi)^{0.5} \qquad \text{(Eq. 13)}$$

where: ΔT is the rise in surface temperature, q is the rate of heat transfer to the surface per un t time, A is the surface area, k is the thermal conductivity of the solid, t is the heating time, c is the specific

heat, and ρ is the density of the solid.

The above equation can be applied to the case of the compression of the 400-mg. sulfathiazole tablet mentioned previously. The properties of the granulation particles can be estimated from the published data (7, 18) as: A_t = 1800 cm.²/Gm., k = 0.01 cal. sec.$^{-1}$ cm.$^{-1}$ deg.$^{-1}$, c = 0.75 cal.-Gm.$^{-1}$ deg.$^{-1}$, and ρ = 1.58 Gm.-cm.$^{-3}$. The heating time, t, can be estimated from typical tablet compression times (19) as 0.1 sec., and the heat added is 2 cal. The true area of contact can be represented as the product $f \cdot A_T$, where f is the fraction of area in actual contact, and A_t is the total surface area. Substituting these values into Eq. 13 yields:

$$\Delta T = 0.046/f \qquad \text{(Eq. 14)}$$

Thus, for values of f in the range 10^{-3} to 10^{-4}, the surface temperature of the granules will reach the melting point of commonly tableted organic materials and surface fusion will take place.

The existence of high local surface temperatures produced by the conversion of mechanical energy to thermal energy has been demonstrated by several investigators. Bowden and Ridler (20) measured the temperature of the contact points of dissimilar metals sliding over each other by thermoelectric methods, and found temperatures reaching the melting point of the lower melting metal. In the case of metals with high melting points, transient temperatures of 500–1000° were obtained. Smekal (21) found that when glass was scratched by carborundum, a eutectic melt of $Na_2OSiO_2Al_2O_3$ was produced, indicating that a surface temperature of 700° had been reached.

SUMMARY

The mechanism of interparticle bonding during tablet compression has not been satisfactorily explained as yet, although a number of theories have been advanced. The liquid surface cement theory is compatible with known facts about tableting, although it has not been proven experimentally. The possibility of formation of a liquid film at the particle surface is supported by thermodynamic analysis of the effects of stress distribution on melting point and solubility, and by heat transfer kinetics at the surface.

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Keyphrases

Tablet compression
Particle bonding—tablet compression
Thermodynamics—stressed solids
Stress distribution, solids—solubility
Heat transfer kinetics—fusion

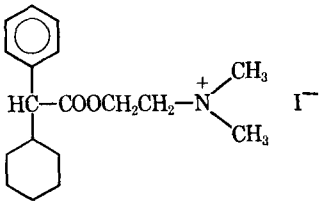
α-Phenyl-α(2,3-cyclohexenyl) Acetic Acid Derivatives as Potential Antispasmodics

By HEINO A. LUTS, J. F. GRATTAN, S. Z. HAIDRI, and W. L. NOBLES*

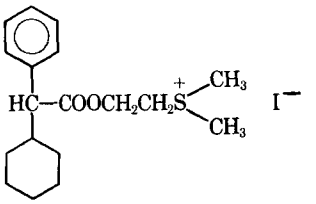
A group of compounds having the (2,3)-cyclohexene moiety has been prepared for pharmacological evaluation. The preparation and biological activities of these compounds are presented.

MANY α-phenyl-α-cyclohexyl acetic acid derivatives have been prepared (1–4) for pharmacological evaluation. They are generally very active antispasmodics, and are often superior to the diphenyl analogs.

Protiva and Exner (5) first reported the active sulfonium compound, thiospasmin, in which the nitrogen of compound I below has been replaced by the sulfur of compound II. This agent demonstrated exceptional antispasmodic activity; it was regarded as being 10 times as active as atropine.



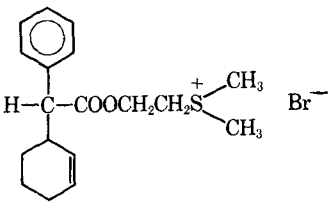
I



II

Thiospasmin has found successful clinical application in Czechoslovakia and in the Union of Soviet Socialist Republics. In radioactive studies Francova and his associates (6) found that orally administered thiospasmin was distributed in the rats in the following order: liver, kidney, lungs, spleen, heart, plasma, muscle, blood cells, skin, and brain. The maximum activity was found after 4 hr. in the gastrointestinal tract.

While proceeding with their studies on the interdependence and effectiveness of substances, Votava and his associates (7) then synthesized α - phenyl - α - cyclohexyloxyacetoxyethyldimethylsulfonium iodide and α-phenyl-α-*n*-propylhydroxyacetoxydimethylsulfonium iodide. Both compounds proved to be superior to thiospasmin in activity and were also less toxic. Since 1953, several structural modifications have been made. In 1956, Neesby and his associates (8) synthesized a series of compounds in which the substituent on the α-carbon atom and the nature of the ionic radical were altered. They found that dimethylsulfonium ethylphenyl α-(2, 3-cyclohexenyl) acetic acid bromide (III) completely voids the xerostomic effect without decreasing the antispasmodic activity.



III

It is noteworthy that in their studies the addition of a hydroxyl group to the α-carbon atom did not increase the activity, which is the generally accepted rule.

To our knowledge, this selectivity does not exhibit itself in any other antispasmodic compound reported to the present. It is speculated that possible hydroxylation takes place in the detoxification process on the 2,3 position of the cyclohexene ring.

In order to explore more extensively the 2,3 cyclohexene analog structures of this type, we have prepared a group of them for pharmacological evaluation (Table I).

MATERIALS AND METHODS

Compound II was made by the standard method of adding *trans*-1,2-dibromocyclohexane to the sodium salt of phenyldiethylmalonate, and the α-phenyl-α-(2,3-cyclohexene) acetic acid (III) was accomplished by hydrolysis and partial decomposition

Received May 22, 1967, from Structure-Activity Research, Inc., College Hill, Oxford, MS 38655, and Smith, Miller and Patch, Inc., New York, NY

Accepted for publication October 18, 1967.

Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

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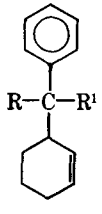
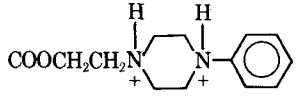
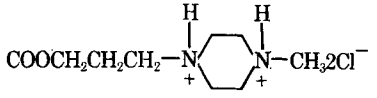
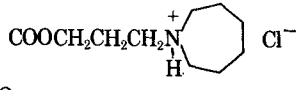
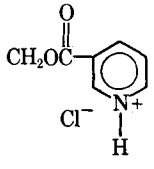
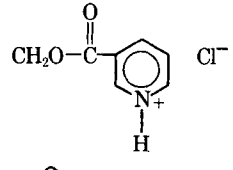
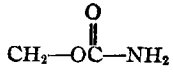
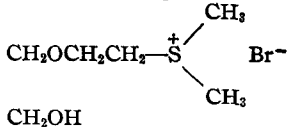
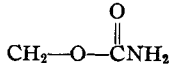
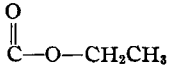
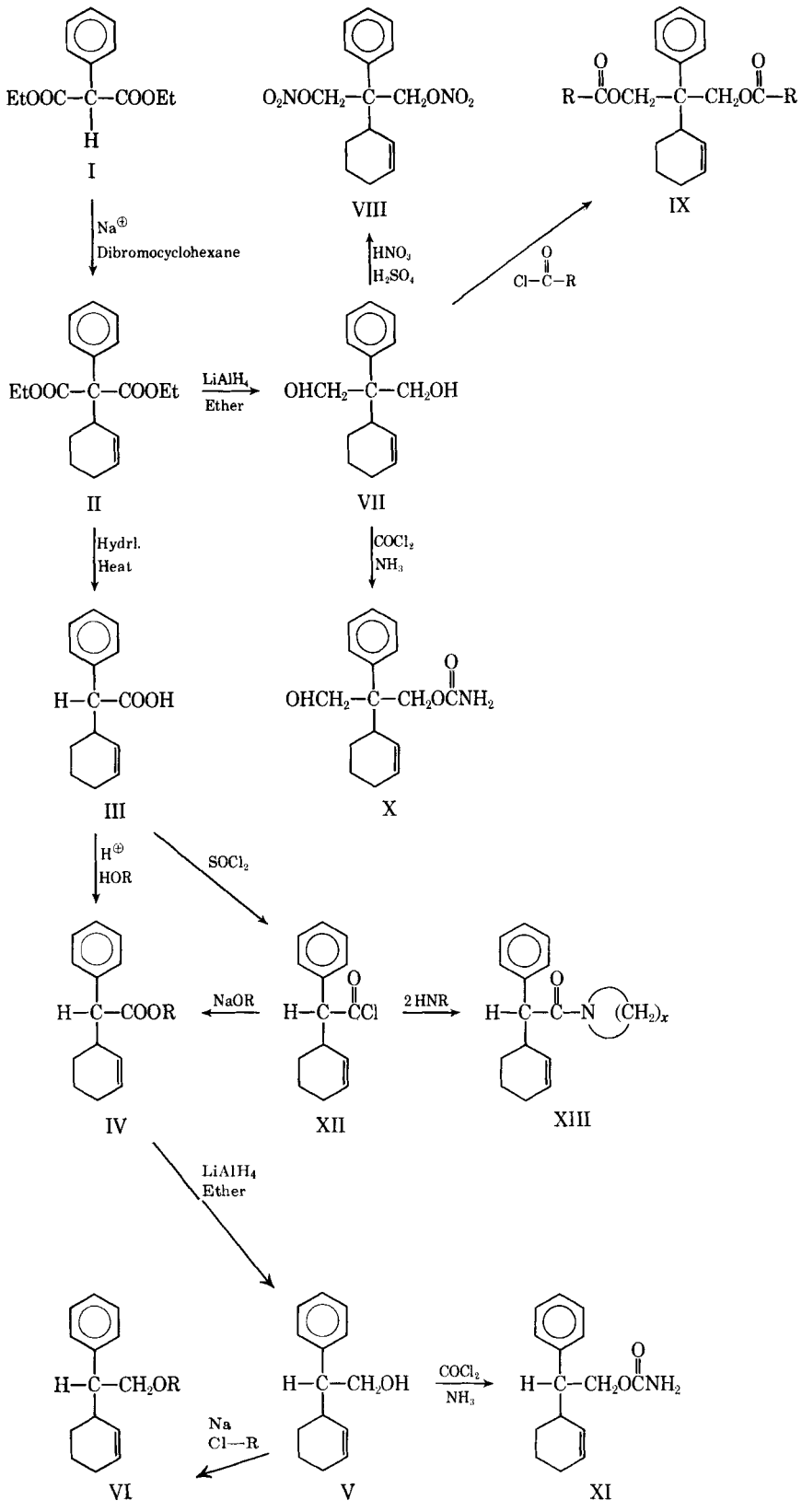
TABLE I—2,3-Cyclohexane Analogs



No.	R	R¹	M.p., °C.	Formula	Calculated C	Calculated H	Calculated N	Found C	Found H	Found N
1	H		179–180	$C_{26}H_{26}N_2O_2Cl$	63.96	5.58	6.18	63.82	5.64	6.15
2	H		222	$C_{22}H_{34}N_2O_2Cl_2$	61.67	8.00	6.67	61.78	8.10	6.59
3	H		160–161	$C_{23}H_{34}NO_2Cl$	61.40	8.76	3.57	61.27	8.72	3.63
4	CH_2ONO_2	CH_2ONO_2	58–159	$C_{15}H_{19}N_2O_4$	55.68	5.92	8.66	55.41	5.78	8.78
5			182–183	$C_{27}H_{30}N_2O_4Cl_2$	56.47	6.27	5.81	56.40	6.30	5.72
6	H		100–101	$C_{15}H_{19}NO_2$	73.84	7.81	5.71	73.96	7.86	5.77
7	H		167–168	$C_{18}H_{27}OSBr$	58.21	7.35	8.9(S)	58.01	7.49	9.20(S)
8	CH_2OH	CH_2OH	75–76	$C_{15}H_{20}O_2$	77.54	8.67	...	77.24	8.60	...
9	CH_2OH		134	$C_{15}H_{23}NO_3$	67.89	8.73	5.27	67.98	8.58	4.99
10	H	CH_2OH	112–117 0.3 mm.[a]	$C_{14}H_{18}O$	83.11	8.96	...	82.82	8.99	...
11	H		117–130 1.2 mm.[a]	$C_{16}H_{20}O_2$	78.65	8.24	...	78.52	8.30	...

[a] Boiling points.


EtOOC–C(H)–COOEt
I
Na⊕
Dibromocyclohexane
EtOOC–C(H)–COOEt
II
LiAlH4
Ether
OHCH2–C(H)–CH2OH
VII
HNO3
H2SO4
O2NOCH2–C(H)–CH2ONO2
VIII
Cl–C(=O)–R
R–COCH2–C(H)–CH2OC–R
IX
Hydrl.
Heat
H–C(H)–COOH
III
COCl2
NH3
OHCH2–C(H)–CH2OCNH2
X
H⊕
HOR
SOCl2
H–C(H)–COOR
IV
NaOR
H–C(H)–C(=O)Cl
XII
2 HNR
H–C(H)–C(=O)–N (CH2)x
XIII
LiAlH4
Ether
H–C(H)–CH2OR
VI
Na
Cl–R
H–C(H)–CH2OH
V
COCl2
NH3
H–C(H)–CH2OCNH2
XI

Scheme I

of the diacid formed. Compound IV was obtained by esterifying the acid; the alcohol V, was prepared by reducing the acid with $LiAlH_4$. The alcohol was then converted to the ether as indicated in the accompanying synthetic scheme. Reducing compound II with $LiAlH_4$ gave compound VII as the main product and the corresponding nitrate (VIII) was accomplished by nitration. Compound IX was made by the addition of 2 moles of the corresponding acid chloride. The 2-hydroxycarbamate (X) was obtained by reaction with phosgene and ammonia. The monocarbamate XI was prepared from compound V by reacting with phosgene and ammonia and compound XII by reacting compound III with thionyl chloride. The acid chloride was then converted to amides (XIII) by reaction with the corresponding amines (Scheme 1).

PROCEDURES

Phenylquinone Writhing Test in Mice—The principle of this test is that phenylquinone, when administered subcutaneously to mice, will induce painful writhing movements. Analgesics will significantly reduce these movements.

Four groups of fasted male mice of the Swiss-Webster strain (body weight 15–17 Gm.) were utilized in this experiment. The drug in question was administered orally to three groups of mice at dose levels of 25, 50, and 100 mg./Kg., respectively, in a volume of 0.2 ml. per mouse. The fourth group which was used as the control received 0.01 ml./Gm./body weight of saline. Five animals per group were employed. Phenylquinone at 20 mg./Kg. was injected subcutaneously to all groups of mice 30 min. after the administration of the tested compound.

Tremorine Test—Five groups of fasted male mice (body weight 15–17 Gm.) were utilized in this experiment. The drug was administered at the standard levels of 25, 50, and 100 mg./Kg. For ready comparison, it was tested with a standard antitremorine drug, in this instance trihexyphenidyl HCl[1] (5 mg./Kg.). Untreated controls were utilized at 0.9% saline. All drugs were prepared in distilled water. All drugs were administered orally 30 min. prior to the tremorine which was administered intraperitoneally.

Hexobarbital-Induced Sleep in Mice—Four groups of fasted male mice of the Swiss-Webster strain (body weight 15–17 Gm.) were utilized. The drug was administered orally at 25, 50, and 100 mg./Kg. concentrations. Hexobarbital was administered intraperitoneally at 100 mg./Kg. 30 min. after the initial administration of the tested drug. The animals were then observed for the time intervals of the onset and duration of sleep. The tested comporids were prepared in distilled water; however, hexobarbital was prepared in 5% sodium hydroxide in the final volume of 9 ml. of water, making a total volume of 10 ml.

Antipyretic Activity—Male rats of the Sprague-Dawley strain (body weight 100–125 Gm.) were utilized. They were given a 20% yeast solution at a volume of 2 ml. per rat, subcutaneously, 24 hr. prior to the administration of the tested compound.

After the yeast treatment, the animals were fasted for 24 hr. and a rectal temperature was taken each hour for 2 successive hr. prior to the administration of the drug (each animal served as its own control). After the drug was given, the rectal temperatures were taken each hour for 4 consecutive hr. to determine whether the tested drugs reduced the body temperature.

Ganglionic-Blocking Action—It has been found through previous experiments that the cat gives the greatest response with respect to this test; therefore, a cat was used. A cat of either sex was anesthetized with diallylbarbituric acid[2] with urethan solution (0.7 ml./Kg.), administered intraperitoneally. The surgery included the exposition of the carotid sheath (the carotid artery and the vagus nerve). Also, included was the exposition of the right femoral vein for the purpose of injection and the left femoral artery for the recording of the blood pressure. There was a unilateral severing of the vagus nerve and an exposition of the superior cervical ganglion, for the purpose of controlling the nictitating membrane. Sustained contraction of the nictitating membrane that disappears after intravenous injection of a ganglion-blocking agent is indicative of preganglionic electrical stimulation.

Hypotensive Activity—This test was conducted on five species of animals—rat, guinea pig, rabbit, cat, and dog.

The rat and the guinea pig were anesthetized with urethan, 1.2 Gm./Kg., administered intraperitoneally. The right sublingual vein was exposed, and cannulated for injection of the drug. The left carotid artery was exposed, and cannulated for recording blood pressure. The blood pressure was measured by means of a mercury manometer and recorded directly on a kymograph. In both species, the pressure was allowed to stabilize approximately 30 min. to 1 hr., depending upon the responses of the individual animals. There was a 30-min. interval between each dose of drug or until the blood pressure returned to the base line.

Heparin was given in the amount of 500 USP units per animal, to prevent coagulation.

In order to test the cholinergic effects of the tested compound, acetylcholine bromide at 2 mcg./Kg. was administered. After the characteristic hypotensive tracing had been obtained, acetylcholine bromide was administered a second time; however, this second injection was preceded by the tested compound.

A slight modification of the above procedures was used to prepare the cat for cardiovascular study. Because of the ready accessibility of the vessels, the left femoral artery and right femoral vein were used to measure blood pressure and to inject the tested drug, respectively.

Terminology—

Phenylquinone writhing test in mice:

good activity	>40% decrease of writhing movements as compared to untreated controls.
slight activity	10–40% decrease of the writhing movements as compared to the untreated controls.

hexobarbital-induced sleep in mice:

good potentiation	>40% increase in sleeping time over the untreated controls.

[1] Marketed as Artane by Lederle Labs, Pearl River, N. Y.

[2] Dial, Ciba Pharmaceutical Products, Inc., Summit, N. J.

slight potentiation	10–40% increase in sleeping time over the untreated controls.

Tremorine test in mice:
Induced Tremors (T), Lacrimation (L), and Salivation (S)

good activity	One or more factors (T, L, S) decreased by 75–100% as compared with untreated controls.
slight activity	One or more factors (T, L, S) decreased by 50–75% as compared with untreated controls.

Preliminary Results—Initial pharmacological screening of some of the compounds in this series has demonstrated the following:

Compound I exhibited a mild depressant effect, slight activity in the phenylquinone test, and slight antipyretic activity. In the hexobarbital-induced sleep test in mice, it demonstrated good potentiation of the barbiturate.

Compound II behaved in the manner of CNS depressant drugs, generally, and also produced tremor in some instances. This compound demonstrated a good sympathomimetic activity, transient hypotensive activity, and slight activity in the phenylquinone test.

Compound III displayed good sympathomimetic activity and produced a mild depressant response. Administration of the drug provoked transient lowering of the blood pressure. It also exhibited a slight analgesic effect with the typical "hot plate" test. Slight activity in the phenylquinone test was noted.

Compound V proved to be active as a sympathomimetic agent and produced a good effect in the phenylquinone writhing test. It decreased hexobarbital-induced sleeping time. It also exhibited a slight antagonism to tremorine-induced tremors and exerted a slight response as an antipyretic agent.

Compound VIII behaved as a mild depressant and exhibited cholinergic activity. It did not have an influence on the response of the organism to epinephrine. The compound also produced a mild hypothermia.

Compound X exhibited depressant qualities and demonstrated anticholinergic activity. It had no effect on body temperature and did not interfere with or potentiate the action of epinephrine. Some data obtained suggest that the compound is possibly a vasodilator. In general, this compound was less active than compound VIII in comparative tests.

Compound XI acted as a mild stimulant. It demonstrated atropine-like action, as well as ganglionic-blocking activity. A slight hyperthermic response was also observed.

EXPERIMENTAL[3]

3-Hexamethyleneiminopropyl α-(2-cyclohexene)-phenylacetate Hydrochloride—To the mixture of 345 mg. (0.015 mole) of sodium in 75 ml. of isopropanol was added 3.24 Gm. (0.15 mole) of α-phenyl-α-cyclohexene acetic acid. The mixture was refluxed for 30 min. and 2.42 Gm. (0.015 mole) 3-chloro-*N*-propylhexamethylimine was added. This mixture was refluxed 4 hr. The salt was removed by filtration and the residue evaporated to dryness. The residue was extracted with 75 ml. of ether and the extract acidified with hydrochloric acid. The crystals thus formed were separated by filtration; yield 3.0 Gm., m.p. 160–161°.

2 - Phenyl - 2 - cyclohexenyl - 1,3 - propanediol—Twenty grams (0.08 mole) of phenylcyclohexenediethylmalonate was dissolved in 250 ml. of anhydrous ether and 8.0 Gm. of $LiAlH_4$ was added in small portions. The reaction mixture was refluxed for 8 hr. and allowed to stand overnight. The excess $LiAlH_4$ was decomposed with water and the ether layer separated. The aqueous residue was extracted twice with 100-ml. portions of ether and the ether layers combined. The ethereal solution was dried over anhydrous calcium sulfate[4]; the ether was then removed to yield a residue; yield 14 Gm., m.p. 75–76°.

Ethyl - α - (2 - cyclohexenyl) - α - phenylacetate—Twenty-five grams (0.115 mole) of α-phenyl-α-cyclohexene acetic acid was refluxed 2 hr. with absolute ethanol in the presence of hydrochloric acid. The ester thus formed distilled in the range 117–130° (1.2 mm.); yield 17 Gm., n_D^{28} 1.519.

2 - (2 - Cyclohexenyl) - 2 - phenylethanol—Seventeen grams (0.08 mole) of the ethyl ester of α-phenyl-α-cyclohexene acetic acid was reduced with 6 Gm. of $LiAlH_4$ in 250 ml. of ether. After refluxing for 6 hr., the excess $LiAlH_4$ was decomposed with a water–ether mixture and the ether layer separated. The residue was extracted twice with 100-ml. portions of ether and the ether layers were combined; the ethereal solution was dried over anhydrous calcium sulfate and the ether was removed. The residual 12 Gm. distilled at 112–117° 0.3–0.35 mm., n_D^{27} 1.5447.

2-Phenyl-2-cyclohexenyl-1,3-propanediol Nicotinate—To 6.96 Gm. (0.03 mole) of 2-phenyl-2-cyclohexenyl-1,3-propanediol, 10.63 Gm. (0.06 mole) of nicotinylchloride hydrochloride was added and the mixture heated at 100° for 20 min.; the mixture was stirred constantly during this period. After cooling, the residue was dissolved in water and made strongly basic with concentrated ammonia water. The solid precipitate was extracted twice with 50-ml. portions of ether. The solution was dried over anhydrous calcium sulfate and then acidified with anhydrous hydrogen chloride. The product thus formed was collected by filtration; yield 16.5 Gm., m.p. 182–183°.

N-Methylpiperazino-N'-propyl α-(2-cyclohexene)-phenylacetate Dihydrochloride—To a solution of 6.5 Gm. (0.03 mole) of α-phenyl-α-cyclohexene acetic acid and 100 ml. of absolute isopropanol, 700 mg. (0.03 mole) of sodium was added. After the salt formation, 5.5 Gm. (0.03 mole) of α-chloropropyl-*N*-methylpiperazine was added and the mixture refluxed for 6 hr. The solvent was removed by distillation and the residue dissolved in 50 ml. of ether. The ether was then treated with anhydrous hydrogen chloride and the product was collected by filtration; yield 7.9 Gm., m.p. 222°.

N-Phenylpiperazinoethyl α - (2 - cyclohexene)-phenylacetate Dihydrochloride—Three grams (0.015 mole) of *N*-α-hydroxy-ethyl-*N*-phenylpiperazine was reacted with 300 mg. (0.015 mole) of sodium in 75

[3] Microanalysis was performed by Mr. G. Roberts, Jr., Florham Park, New Jersey. All melting points are uncorrected.

[4] Drierite, W. A. Hammond Drierite Co., Xenia, Ohio.

ml. of xylene; 3 Gm. (0.015 mole) of α-phenyl-α-cyclohexene acid chloride was added in small portions and the mixture was refluxed for 4 hr. The salt was removed by filtration and the residue distilled to dryness in high vacuum. The residue was dissolved in 60 ml. of ether, the solution decolorized with pulverized carbon and acidified with anhydrous hydrogen chloride. The product thus formed was collected by filtration and dried; yield, 5.0 Gm., m.p. 179–180°.

2 - Phenyl - 2 - cyclohexenyl - 1,3 - propanedinitrate—To 4.6 Gm. (0.02 mole) of 2-phenyl-2-cyclohexenyl-1,3-propanediol was added 20 ml. of 100% nitric acid at 0–5°. (The acid was previously prepared by distilling it over 100% sulfuric acid.) To the reaction mixture was added 20 ml. of 100% sulfuric acid and the mixture was left standing overnight. The solution was then poured into ice; at this point crystals were formed. They were collected by filtration and washed with water. The product was dissolved in methanol and 2% sodium bicarbonate solution added until the crystals separated. The crysals were collected again by filtration and dried; yield 4.1 Gm., m.p. 58–59°.

2 - Cyclohexenyl - 2 - phenylethoxy - 1 - ethylmethylsulfide Methiodide—Six grams (0.03 mole) of 2-(2-cyclohexenyl)-2-phenylethanol was reacted with 690 mg. (0.03 mole) of sodium in xylene. Three and four tenths grams (0.03 mole) of α-chloroethylmethyl sulfide was added over a period of 15 min. and the reaction mixture was refluxed for 12 hr. After filtering off the salt, the xylene was distilled and discarded. To the residue was added 20 ml. of methyl bromide, the mixture was allowed to stand for 2 days and then worked up at room temperature. Ten milliliters of absolute methanol was added and the mixture was refluxed for 2 hr. To this mixture 25 ml. of absolute ether was added; on cooling, the crystallized product formed; yield 3.6 Gm., m.p. 167–168°.

2-Cyclohexenyl-2-phenylethylcarbamate—To a solution of 2.4 Gm. (0.012 mole) of 2-(2-cyclohexenyl)-2-phenylethanol in 25 ml. of benzene was added twice the molar quantity of phosgene in 25 ml. of benzene. This mixture was placed in a pressure bottle and warmed to 50° for 4 hr. The excess phosgene was boiled off and the residue treated with dry ammonia gas. The reaction mixture was reduced to dryness under reduced pressure. The residue was dissolved in alcoholic ammonia and filtered. The clear solution was poured on ice; at this point fine crystals were formed. They were collected and washed with water.

The crystals were recrystallized from an ethanol–water mixture to give a compound, m.p. 100–101°; yield 2.4 Gm.

2-Phenyl-2-cyclohexenyl-1,3-propanediol Carbamate—To a solution of 12.0 Gm. (0.05 mole) of 2-(2-cyclohexenyl)-2-phenylethanol in 50 ml. of benzene, 150 ml. of 12% phosgene in benzene was added. The mixture was placed in a pressure bottle and kept 4 days in sunlight. The volume of the mixture was reduced by one-half and anhydrous ammonia was added. The precipitated crystals were filtered off and discarded. The benzene layer was then concentrated to dryness with the aid of high vacuum; to that residue 50 ml. of concentrated ammonia was added and the mixture was boiled for 1 hr. The solution was cooled and the ammonia was removed by blowing air over it. The crystals were separated and recrystallized from the ethanol–water mixture, m.p. 134°; yield 11.9 Gm.

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Keyphrases

Antispasmodics—potential
α-Phenyl-α(2,3-cyclohexenyl) acetic acid derivatives—synthesis
Pharmacological screening
Microanalysis—identity

Surfactant Effects on Spray-Congealed Formulations of Sulfaethylthiadiazole–Wax

By PHILLIP M. JOHN* and CHARLES H. BECKER

An investigation was undertaken to examine the effects of selected formulation and production variables on the physical characteristics and dissolution patterns of spray-congealed particles of sulfaethylthiadiazole (SETD)–wax. Melted suspensions of SETD in wax formulations contained various concentrations of the lipophilic surfactant, sorbitan monooleate. Particle size was found to correspond to the size of nozzle used; smaller particles were characterized by faster rates of SETD release. Depending upon the concentration employed, sorbitan monooleate may increase or depress the rate of SETD released from the particles in 0.1 *N* HCl; the surfactant consistently promoted faster release rates in alkaline pancreatin solution. A log–log dissolution model was used to predict the release patterns of the products, and the model showed good agreement with the experimental data.

VERY LITTLE WORK of a research nature has been done in the area of spray congealing. Although the technique of spray congealing is used by many industries, the pharmaceutical industry has not accepted this process as quickly as expected. This unit operation (prilling) offers an expedient method of producing a fairly uniformly sized particulate product from a melted wax suspension or solution of a medicament without the difficulties often encountered with a comminution step. Spray-congealed particles may be combined into a tablet granulation, encapsulated, or incorporated into a liquid suspension (1). A variety of suitable matrix materials have been spray congealed to produce essentially tasteless products of iron compounds (2) and water-soluble vitamins (3).

A very complete enumeration of available atomization techniques and equipment has been reported by Marshall (4) in the engineering literature. The particle-size relationships of centrifugal-disk atomizers have been examined by Scott *et al.* (5). Single-fluid pressure nozzles are frequently used to spray congeal drug and lipid material mixtures; Lantz and Robinson (6) used such a nozzle located at the top of a spray-dryer tower. Although less commonly used on a commercial scale, pneumatic atomizing nozzles are particularly useful for producing very fine powders. Such a two-fluid nozzle was used by Cox (7) in a study of the effects of wax matrix, siphon height, and air pressure.

The dissolution rates of spray-congealed products of sulfamethylthiadiazole (SMTD) and hydrogenated castor oil in 0.1 *N* HCl and alkaline pancreatin solution were determined by Robinson, Bondi, and Swintosky (8). They showed that the release in humans of a single 4.0-Gm. dose of the spray-congealed product was extended over a 12-hr. period. In a later work (9), the drug release of a SETD-hydrogenated castor oil spray-congealed product was compared to that of crystalline SETD administered to human subjects; this study showed that approximately 50% of the sustained-release dosage form was available for early absorption, with the balance being absorbed over a prolonged time.

The purpose of the present investigation was to determine the effects of wax matrix material, atomizing nozzle orifice size, and surfactant concentration on the resulting *in vitro* dissolution patterns of spray-congealed products of SETD–wax intended for sustained- or prolonged-release medication. Generalizations concerning the effect of these three treatment variables on rate of production, resulting particle size, density, and porosity were also sought. White wax USP, a synthetic wax-like ester,[1] and a 1:1 combination of these two waxes were used as matrices. These waxes were chosen to obtain a range of plasticity between the pliability of white wax and the brittleness of the synthetic wax-like ester. Three distinct particle-size ranges were obtained by using three sizes of fluid nozzle orifices. The effect of the nonionic lipophilic surfactant sorbitan monooleate[2] was studied over the range of concentrations from 0 to 10% by weight.

EXPERIMENTAL

Throughout this investigation analytical reagent chemicals were used for the assay procedures, and waxes of food grade were employed in the spray-

Received August 31, 1967 from the College of Pharmacy, University of Florida, Gainesville, FL 32601
Accepted for publication December 15, 1967.
Abstracted in part from a dissertation presented by Phillip M. John to the University of Florida, Gainesville, in partial fulfillment of Doctor of Philosophy degree requirements.
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[1] Marketed as Glycowax S-932 by Glyco Chemicals, Inc., New York, N. Y.
[2] Marketed as Span 80 by Atlas Chemical Industries, Inc., Wilmington, Del.

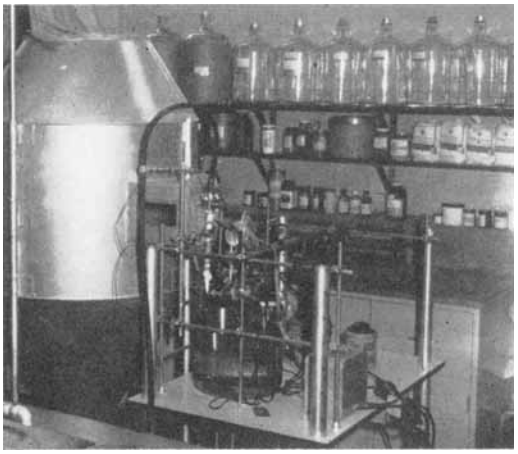

Fig. 1—Collection chamber attached to pneumatic atomizing nozzle of spray-congealing apparatus.

congealed samples. Sulfaethylthiadiazole (SETD) was obtained from American Cyanamid Co.

Manufacture of Spray-Congealed Samples—Ingredients totaling 2,000 Gm. for each formulation were combined and melted in a stainless steel beaker immersed in an 80 ± 0.5° constant-temperature oil bath. Suspension of the SETD was maintained through the use of a Lightnin model F laboratory-type mixer. A Teflon siphon tube wrapped with silicone-treated heater tape brought the melted wax mixture at the desired temperature from the beaker to the spraying nozzle. The 1/4 JCO pneumatic atomizing nozzle[3] was equipped with a specially designed nichrome wire heater and a copper-constantan thermocouple and monitor.[4] The nozzle was maintained at a temperature of 80 ± 3° throughout the spraying operation. Dry air at 60 p.s.i. provided the suction necessary to lift the melted formulation from the beaker through a constant siphon height of 30.5 cm. (12 in.) to the nozzle where atomization took place. The cloud of congealing wax droplets was directed into a galvanized steel collection chamber through the spraying port, and the product was collected from the base of the chamber's lower cone. A vortex was created during spraying operations by having the spraying port located 15 cm. (6 in.) left of center; the swirling flow of air inside the collection chamber helped to prevent the waxy particles from adhering to the walls of the chamber.

The assembled apparatus is shown in Fig. 1. The collection chamber was approximately 274.3 cm. (9 ft.) high. A cloth sleeve joined the atomizing nozzle to the spraying port, a cloth air-escape bag was attached to the upper cone, and a plastic collection bag was joined to the lower cone. The equipment was assembled as shown and preheated to 80° for 20 min. before atomization was begun. After a sufficient amount of product had been collected, the air and heaters were turned off. The product was then removed, weighed, and stored in glass jars. The remaining portion of the 2,000-Gm. formulation melt was allowed to cool in the beaker, then removed, weighed, and stored.

Problem Design—All formulations contained approximately 25% by weight SETD; the exact drug content of each product was determined by subsequent extraction and assay. Three wax bases were chosen that had been shown to release the drug favorably over an extended period of time (10). These bases were: white wax USP, a synthetic wax-like ester, and a combination of equal parts of these two waxes. Four levels of the surfactant sorbitan monooleate were used: 0, 1, 4, and 10% by weight. Each of these 12 different formulations was sprayed once through three fluid nozzle sizes; 0.02(~0.5 mm.), 0.04(~1 mm.), and 0.06(~1.5 mm.), in. diameter. This factorial design led to 36 samples, each with a unique set of the three treatment variables.

Particle-Size Analysis—Microscopic analysis, using a Zeiss filar ocular and a haemocytometer test cell, was selected as the best method of particle-size analysis. Mechanical sieving was not possible since the particles were too fine and too tacky in some instances to flow through the screens. Adequate deflocculation could not be maintained to enable the use of an electronic counter or a sedimentation technique.

Density Determinations—True density data were obtained from measurements made on the congealed portions of each formulation remaining in the beaker after spraying. Apparent bulk densities were determined by a standardized tapping method using a 100-ml. cylindrical graduate. Porosities and specific surfaces for each sample were then calculated from the results of the particle-size analysis and the density determinations.

Assay for SETD Content of Spray-Congealed Products—The test drug was extracted from the product by placing exactly 0.5000 Gm. of the spray-congealed sample in a 40-ml. portion of warm chloroform and washing several times with warm 0.1 *N* HCl, the washings being combined and made to volume in a 250-ml. volumetric flask with distilled water. An aliquot was assayed for SETD content by the Bratton-Marshall (11) colorimetric procedure for sulfonamides. The absorbance of the resulting solutions was determined on a Klett-Summerson colorimeter using a No. 54 filter and compared to a prepared standard curve. Duplicate extractions and assays were conducted on each spray-congealed sample.

Dissolution Studies—The release characteristics of the spray-congealed SETD–wax particles were examined by the method of Robinson and Swintosky (9). For the acid studies, 14 aliquots of the product were weighed and placed in 90-ml. glass vials. Each aliquot contained 0.1200 Gm. SETD, as determined by prior assay, and weighed between 0.4 Gm. and 0.5 Gm. Sixty milliliters of simulated gastric fluid (0.1 *N* HCl, pH 1.1) was added to each vial, and the vials were clamped into the rotating-bottle apparatus described by Souder and Ellenbogen (12). A constant-temperature water bath was used to maintain a temperature of 37 ± 1°, and filtered into 250-ml. conical flasks after 1/4, 1/2, 3/4, 1, 1 1/4, 1 1/2, and 2-hr. intervals. One milliliter of the filtered eluant was transferred to a 100-ml. volumetric flask and made to volume with distilled water. The samples were then assayed for SETD content by the Bratton-Marshall method. Duplicate samples were also eluted in the HCl for extended time periods from 20 to 60 hr. These later points were

[3] Manufactured by Spraying Systems Co., Bellwood, Ill.
[4] Built by the Bioelectronics and Instrument Dept., J. Hillis Miller Health Center, University of Florida.

used to estimate the amount of SETD that would eventually be released if extracted for an infinite period of time.

Similar studies were conducted in simulated intestinal fluid (alkaline pancreatin solution, pH 8.3) with the following differences: Each aliquot contained 0.6000 Gm. SETD and weighed between 2.2 and 2.5 Gm. Two samples were withdrawn from the rotating-bottle device after 1/4, 1/2, 3/4, 1, 2, 3, 4, and 6 hr. had elapsed. Two-tenths milliliter of the filtrate was transferred by pipet to 100-ml. volumetric flasks and made to volume with distilled water. In all other respects, the procedures for the acid and the alkaline dissolution studies were identical.

Mathematical Calculations for Estimating Drug Released—A log–log model first developed by Cox (7) for spray-congealed particles of white wax and the synthetic wax-like ester containing no surfactant showed good agreement with the experimental data of this investigation. It may be written in the following forms:

$$\log (1 - C/C_e) = -c \log (1 + t/d) \quad \text{(Eq. 1)}$$

$$C = C_e [1 - (1 + t/d)^{-c}] \quad \text{(Eq. 2)}$$

where C is the amount of drug released in time t, C_e is the equilibrium solubility, and c and d are parameters specific for a given formulation determined by minimizing the quantity $\Sigma(\hat{C} - C)^2$ utilizing the experimental data of a completed dissolution study. A least-squares solution in terms of $\log (1 - C/C_e)$ (from Eq. 1) would not be the solution of choice in terms of C. Since experimental errors were approximately of equal variance along the dissolution curve, it is best to find the least-squares fit in terms of C, Eq. 2. This second equation is not a linear function of the unknown constants; therefore, there is no explicit formula for the calculations of the least-squares estimates of the parameters. An iterative procedure must be used that will converge to the best-fitting values. A number of such procedures exist; the method used was a modified Gauss-Newton nonlinear least-squares procedure (13).

RESULTS AND DISCUSSION

Production Rates—Of the three treatment variables considered, nozzle orifice size almost entirely determined the rate of production. The average rate for the 0.02-in. nozzle was 3.9 Gm./min., for the 0.04-in. nozzle the rate was 54.0 Gm./min., and for the 0.06-in. nozzle, 127.4 Gm./min. The rate of production of the largest nozzle was approximately of the same order as those reported by Scott and co-workers (5) using a centrifugal-disk atomizer 5 cm. in diameter.

Frequent clogging occurred when the smallest nozzle was used, and almost continuous operation of a built-in nozzle clean-out needle was required to remove obstructions from the fluid nozzle orifice. These obstructions appeared to be congealed wax plugs which formed in the orifice. The 1/4 JCO nozzle operates on an external-mixing principle with the atomizing air passing through a small annular around the fluid nozzle orifice. It was impossible to heat the tip of the fluid nozzle sufficiently to compensate for the cooling effects of the atomizing air. Clogging seldom occurred with the two larger

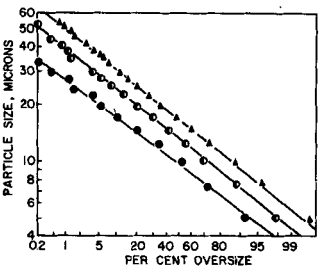

Fig. 2—Particle-size distribution comparison (by number) of spray-congealed products of SETD-white wax atomized through three different nozzles. Key: ●, 0.02-in. nozzle; ◐, 0.04-in. nozzle; ▲, 0.06-in. nozzle.

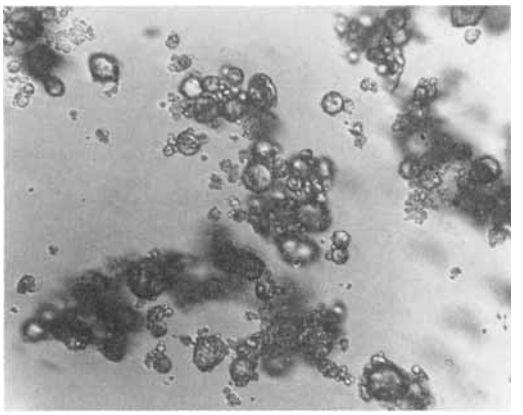

Fig. 3—Spray-congealed particles of SETD–white wax and a synthetic wax-like ester (1:1) containing 4% sorbitan monooleate atomized through a 0.02-in. pneumatic nozzle. Magnification, 210×.

nozzles, and the greater amount of hot wax passing through the nozzle apparently heated the inner surfaces of the nozzle sufficiently to prevent congealing. The use of steam-jacketed nozzles and positive-pressure pumps would likely have eliminated the clogging problems experienced.

Surfactant concentration had no significant effect on the production rate, but the 1:1 combination wax matrices averaged production rates approximately twice those of synthetic wax-like ester matrices.

Particle-Size Analysis—The size of the nozzle used to atomize the melted wax formulations was found to largely influence the volume-surface mean diameter (d_{vs}) of the product; the type of wax matrix used was also found to be significant at the 5% level. The average d_{vs} was approximately linearly related to the nozzle size: 14.18 μ for the 0.02-in. nozzle, 21.23 μ for the 0.04-in., and 29.34 μ for the 0.06-in. nozzle. Figure 2 is a plot of the particle sizes obtained by spraying an identical formulation through three sizes of nozzles used in this investigation. Unusually close compliance with the log-normal distribution was found for each sample. Figures 3 and 4 are photomicrographs of the same formulation atomized through the 0.02-in. and the 0.06-in. nozzles, at the same magnification. The pronounced effect of the nozzle size can be seen in these photographs.

Synthetic wax-like ester formulations produced the smallest particles, and the 1:1 combination

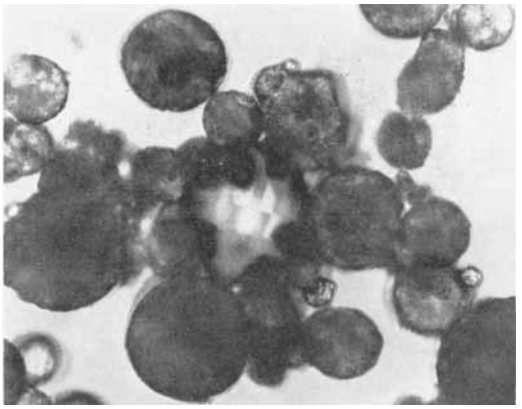

Fig. 4—Spray-congealed particles of SETD–white wax and a synthetic wax-like ester (1:1) containing 4% sorbitan monooleate atomized through a 0.06-in. pneumatic nozzle. Magnification, 210×.

matrix resulted in particles approximately 50% larger. The brittleness of synthetic wax-like ester was thought to be responsible for the small size of the particles since this wax shatters when stressed. The 1:1 combination matrix was more plastic than the synthetic wax-like ester alone and not as tacky as white wax alone. Sorbitan monooleate concentration had no significant effect on particle size.

Density Results—The true density of all formulations was almost the same: 1.063 average. Nozzle size and wax matrix were both important considerations in the bulk densities of the products. Bulk density increased with increasing particle size, as did flowability. White wax had the lowest bulk density as a result of its inherent tacky qualities, and the 1:1 combination had the highest.

Dissolution Studies in 0.1 N HCl—The amount of SETD released during the first 15 min. in 0.1 *N* HCl was taken as a measure of the partially exposed drug on the particles and possibly some free SETD. More drug was released in the first 15-min. period than in any later quarter-hour period, and it was found that all three treatment variables exerted a significant effect on this initial amount of released drug. The specific surface of the product was in part responsible for the amount of drug released in this initial period. Figure 5 shows the effect of the nozzle size on the dissolution patterns of the products; the largest nozzle produced particles with the lowest specific surfaces and lowest rates of dissolution.

White wax formulations released an average of half as much drug as either of the other two matrices within the first 15 min. of exposure to the acid fluid. White wax products tended to agglomerate more than the other matrices, thus presenting less surface to the eluant. Also, white wax, being very pliable, was thought to have insulated the drug particles from the eluting fluid through film formation around each particle. The addition of the brittle synthetic wax-like ester may have so reduced the pliability of white wax that such films were not formed, or were ruptured after formation, with the result that the drug was left exposed on the surface.

Sorbitan monooleate had a very pronounced effect upon the dissolution which occurred in the initial 15-min. period (Fig. 6). From this study, it appears that in concentrations up to 4%, this surfactant softens the particles, promotes wetting, and thus increases the amount of drug released. However, in 35 out of 36 samples prepared, 10% surfactant produced the lowest release. The particles were generally very tacky when this much surfactant was present. If the particles had agglomerated extensively, the total surface area exposed to the fluid would have been considerably reduced. Another possible explanation is that the w/o emulsion-forming tendencies of this lipophilic surfactant (HLB 4.3) may have entrapped the eluant and prevented the dissolved drug from being transported away from the wax particle into the solution.

The initial dissolution rate was obtained from the first derivative of Eq. 2 by setting $C = 0$ when $t = 0$. It was found that comparisons of the initial dissolution rate $(c/d)(C_e)$ closely paralleled the results of the 15-min. analysis. The initial dissolution rates of products atomized through the 0.06-in., 0.04-in., and 0.02-in. nozzles were in the ratios of 1:2:4, respectively. The average initial rate of synthetic wax-like ester formulations was twice that of white wax; the 1:1 combination rate was three times that of the white wax formulations. Increases in the surfactant concentration up to 4% resulted in higher initial rates of dissolution; at 10% concentration, the rate was about one-third that of products containing no surfactant.

There was little change in the effects of nozzle size, wax matrix, and surfactant concentration up

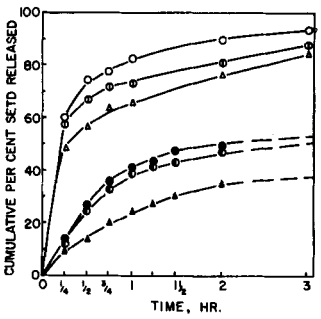

Fig. 5—Dissolution patterns of SETD in 0.1 N hydrochloric acid and alkaline pancreatin solution from spray-congealed particles of SETD–synthetic wax-like ester containing 10% sorbitan monooleate. Key: ○, 0.02-in. nozzle; ◑, 0.04-in. nozzle; △, 0.06-in. nozzle in alkaline pancreatin solution; ●, 0.02-in. nozzle; ◐, 0.04-in. nozzle; ▲, 0.06-in. nozzle in 0.1 N HCl.

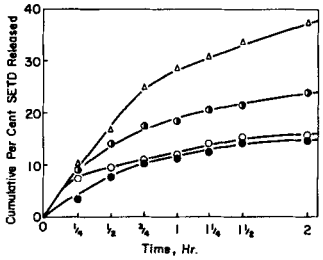

Fig. 6—Effect of sorbitan monooleate concentration on the dissolution patterns of SETD in 0.1 N hydrochloric acid from spray-congealed particles of SETD–white wax atomized through a 0.06-in. pneumatic nozzle. Key: ○, 0%; ◑, 1%; △, 4%; ●, 10% sorbitan monooleate.

to 2 hr. in the acid fluid. After 22 hr., however, it was found that the synthetic wax-like ester formulations had released approximately 50% more drug than had the white wax formulations. As the drug was removed, the wax bead would likely become porous, channeled, and less able to resist the attritional forces of agitation in the vial. White wax, being pliable, would perhaps be more resistant to crumbling when used as a matrix than would the brittle synthetic wax-like ester. After 22 hr., sorbitan monooleate no longer exerted a significant repressant effect upon the total amount of drug dissolved.

Dissolution in Alkaline Pancreatin Solution—An examination of Fig. 5 shows that much more drug was released in a given time period to the alkaline pancreatin eluant than to 0.1 *N* HCl. This increase in dissolution was probably due to partial solubilization and disintegration of the wax particles by the surfactant action of bile and pancreatin, and by the alkalinity of the sodium bicarbonate. When the sample contained sorbitan monooleate, essentially complete solubilization or emulsification of the wax beads was noted after 24 to 48 hr.

After 15 min. in alkaline pancreatin solution, the effects of all three variables could be seen in the amounts of SETD released from the various samples. Products made with the largest (0.06-in.) nozzle released considerably less drug than the smaller particles. White wax showed the highest release, synthetic wax-like ester the lowest, and the 1:1 combination was intermediate. The acids present in white wax are believed to be responsible for the large release observed; synthetic wax-like ester was not as easily disintegrated and solubilized in the alkaline pancreatin solution due to the apparent lack of acids. In the alkaline medium, sorbitan monooleate promoted faster rates of release, and 10% surfactant concentration led to greater releases of drug in 6 hr.

Model Predictions—Figures 7 and 8 are compound log–log plots of $(C_e - C)$ against $(t + d)$. The lines and data points are derived from separate sources and do not represent a least-squares fit of the data in the usual sense. The lines were plotted using the estimated parameters from Eq. 2. Superimposed upon these lines are the data points of the indicated samples. The fit appears to be uniform, and the discrepancies are random. The slopes of the plotted lines $(-c)$ have been assigned no significance since c was determined through adjustment with trial values of C_e and d. Attempts to correlate these three parameters with the treatment variables or with other quantitated properties of the products were not successful.

Higuchi and Hiestand (14) proposed that diffusion-controlled systems released drug from the insoluble matrix according to the square root of time. A relationship to apparent zero- or first-order release patterns was demonstrated by Wagner (15) using previously published dissolution data. Considerable work has been done on idealized systems of solid drug dispersed in solid matrices. Higuchi (16) enumerated several complicating factors which must be considered before any dissolution model can be assumed to apply. He pointed out that break-up or dissolution of the matrix substances would result in erratically changing exposed surface area; also, the possibility of drug on the surface of the particle being released more rapidly than drug imbedded in the matrix must be considered.

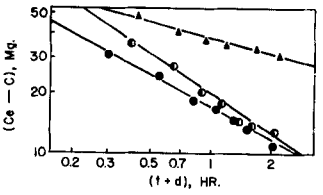

Fig. 7—Experimentally determined dissolution data for SETD in 0.1 N hydrochloric acid plotted on predicted lines for SETD–white wax and synthetic wax-like ester (1:1) formulations containing 1% sorbitan monooleate. Key: ●, 0.02-in. nozzle; ◐, 0.04-in. nozzle; ▲, 0.06-in. nozzle.

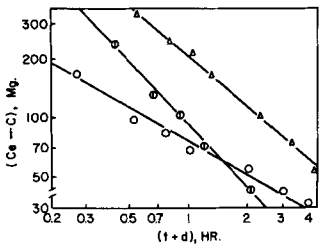

Fig. 8—Experimentally determined dissolution data for SETD in alkaline pancreatin solution plotted on predicted lines for SETD–white wax and synthetic wax-like ester (1:1) formulations containing 10% sorbitan monooleate. Key: ○, 0.02-in. nozzle; ◑, 0.04-in. nozzle; △, 0.06-in. nozzle.

This project dealt with wax particles which clumped, disintegrated, dissolved, and became emulsified; the exposed area was constantly changing. The surfactant concentration of the eluant continuously increased as sorbitan monooleate was extracted along with the drug from the wax particles. The parameters of the log–log model are likely related to the surface area, or to the rate of change of surface area; however, no relationship of this type was discovered during the course of the investigation.

SUMMARY AND CONCLUSIONS

1. Atomization through the use of a pneumatic nozzle and siphon tube was found to provide an expedient method of producing spray-congealed particles of drugs suspended in melted lipid materials.

2. Nozzle size largely determined the rate of production of the spray-congealed products.

3. The resulting particle size of the product was a function of the size of nozzle used for atomization; the particle-size distribution was found to be approximately log-normal.

4. The spray-congealed particles were discretely formed, apparently nonporous spheres; addition of sorbitan monooleate generally impaired flow properties and caused agglomeration.

5. In 0.1 *N* HCl, white wax matrices and large atomizing nozzles produced particles which had the slowest dissolution rates; sorbitan monooleate in concentrations up to 4% increased the solution rate; at 10%, the rate was markedly depressed. There was no noticeable surfactant effect upon the

total amount of drug dissoluted in a 22-hr. period.

6. In alkaline pancreatin solution, white wax matrices had the fastest rates of release, the largest particles had the slowest rates, and sorbitan monooleate consistently promoted faster release rates.

7. Good agreement was obtained between the model predictions and experimentally determined amounts of drug released from a formulation in a particular length of time. Determination of the model parameters adequately characterized the dissolution curve over the time period studied.

8. Sorbitan monooleate, when present in concentrations of 10%, was found to repress the dissolution rate of SETD in 0.1 *N* HCl, and to augment the rate in alkaline pancreatin solution. This behavior could be of use to formulators wishing to insure efficient release of the drug in the intestines after an initial delay.

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Keyphrases

Sulfaethylthiadiazole—wax formulations
Spray-congealed particles
Apparatus, particle preparation—described
Nozzle orifice size—dissolution rate
Sorbitan monooleate—dissolution rate
Waxes—dissolution rate

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By A. N. MASOUD, L. A. SCIUCHETTI, N. R. FARNSWORTH, R. N. BLOMSTER, and W. A. MEER*

***Catharanthus roseus*, treated for 7 weeks with weekly doses of 100 mcg. of gibberellic acid (GA) and harvested biweekly, showed a significant increase in height and in stem dry weight. While GA treatment had a favorable effect on growth and did not affect the rate of flowering, it had an unfavorable effect on the concentration of total alkaloids in the plant organs. At final harvest the concentration of total alkaloids in the leaves was 55 percent of controls; that of the roots, 39 percent. Total alkaloid content per plant was greater than controls at the second harvest, but decreased at the fourth harvest. Detectable quantities of vincaleukoblastine (VLB) were not found in the stems or roots of both treated and untreated groups, but an increase of VLB concentration was noted in the leaves of the treated group. Gibberellic acid treatment had no affect on chlorophyll content, but markedly reduced the petroleum ether and ether extracts of plant organs.**

THE FOLLOWING GENERAL gibberellin effects have been reported in the solanaceous plants producing tropane alkaloids: increased internodal elongation, taller and spindlier plants, increased stem growth, variable effects on total plant growth depending upon the stage of the plant when treated and the concentration of growth hormone applied as well as environmental factors, chlorotic leaves and reduced chlorophyll content, an increased sugar content of the leaf tops, and a reduction in the concentration of alkaloids of the aerial parts (1–6). Many of these effects have been reported for other alkaloid pro-

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Accepted for publication December 27, 1967.
Presented to the American Society of Pharmacognosy meeting, Kingston, Rhode Island, June 1965.
Abstracted in part from a thesis submitted by Asaad N. Masoud to the Graduate School, Oregon State University, Corvallis, in partial fulfillment of the PH.D. degree requirements.
This study was supported in part by a research grant from the Thaw Fund, University of Pittsburgh.
* Meer Corporation, North Bergen, New Jersey.

total amount of drug dissoluted in a 22-hr. period.

6. In alkaline pancreatin solution, white wax matrices had the fastest rates of release, the largest particles had the slowest rates, and sorbitan monooleate consistently promoted faster release rates.

7. Good agreement was obtained between the model predictions and experimentally determined amounts of drug released from a formulation in a particular length of time. Determination of the model parameters adequately characterized the dissolution curve over the time period studied.

8. Sorbitan monooleate, when present in concentrations of 10%, was found to repress the dissolution rate of SETD in 0.1 *N* HCl, and to augment the rate in alkaline pancreatin solution. This behavior could be of use to formulators wishing to insure efficient release of the drug in the intestines after an initial delay.

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ducing plants (1). Markiewicz (7, 8) reported that gibberellin treatment of *Catharanthus roseus* caused an increase in height but reductions in the weight of the aerial parts and the yield of root alkaloids.

The objectives of this research were: (*a*) to determine the effects of gibberellic acid (GA) on the growth of *Catharanthus roseus* (L.) G. Don (*Vinca rosea* L.) when the plant was grown under controlled greenhouse conditions during a 7-week observation period at which time periodic harvests were made; (*b*) to further ascertain the effects of GA on the concentration of alkaloids in the plant organs and the total alkaloid content of the plant; (*c*) to determine the effects of such treatments on the production of the antineoplastic alkaloid, vincaleukoblastine (VLB), during various growth phases of the plant; (*d*) to ascertain the effects of the growth regulator on other metabolic products.

EXPERIMENTAL

Procedure—*Catharanthus roseus* plants employed in this study were grown under greenhouse conditions from seeds obtained from a 1959 crop of mixed pink flowering and white flowering *C. roseus* (L.) G. Don grown in India from stock seed stored at the Department of Pharmacognosy, University of Pittsburgh. Germination was accomplished in flats containing a soil mixture of two parts of loam and one part of sand. About 50 Gm. of complete organic fertilizer[1] was incorporated into the soil of each flat. On July 24, 1964, the larger of the 10-day-old seedlings were transplanted from the flats into paper bands filled with the previously described soil mixture. These paper bands were arranged in flats, 80 per flat and a total of 12 flats were transplanted. One month later the plants were transplanted from the paper bands into individual 1-gal. metal containers that had been filled with the sandy loam mixture. Prior to transplantation approximately 15 Gm. of complete fertilizer had been thoroughly mixed into the soil of each container.

Plants of the pink-flowering variety, recognized by their pink stem were divided into two groups of 120 each. One group, numbered C-1 to C-120, served as the controls; the other, numbered GA-1 to GA-120, was the group treated with GA. These plants, numbered 111 through 120 for each group, were designated for height measurements and each group was further divided into subgroups of 30 plants each so that separate harvests at 2-week intervals could be performed. Plants were labeled according to the aforementioned treatment plan and randomized on two greenhouse benches.

Treatment was instituted on September 11 (zero time). At this time 0.1 ml. of a freshly prepared aqueous solution containing 100 mcg. of GA[2] was applied with a micropipet to the upper leaves of each plant of the GA group. Height measurements were also taken on the same date of the randomly arranged plants numbered 111–120 since these plants would be survivors throughout the whole experimental period. Height, in cm., was taken from the base of the stem at soil level to the apex of the highest leaf. Treatment and height measurements were performed at weekly intervals for 6 subsequent weeks. The 100-mcg. dosage was determined from a preliminary experiment employing dosage levels of 25, 50, and 100 mcg. of GA per group of three plants.

Four harvests were made at 2-week intervals. The first harvest was performed 5 days after the first treatment. Thirty plants were selected per harvest according to the following numbering plan: 1–30, first harvest plants; 31–60, second harvest plants; 61–90, third harvest plants; 91–120, fourth harvest plants. At harvest time each plant was thoroughly washed and excess moisture was removed with a towel. Plants were then divided into leaf, stem, and root portions; fresh weights were taken immediately, the respective plant parts were then dried in a circulating hot-air drier until dry weights were determined. The plant parts were pooled according to harvest and treatment, powdered to a No. 40 powder in a Wiley mill, and stored in air-tight glass containers until used for subsequent analysis.

Alkaloid Analysis—Samples of the pooled powdered material which had previously been dried in an oven at 100° for 24 hr. were assayed for total alkaloids in a manner similar to that of Szasz *et al.* (9). Five grams of the powdered crude drug was thoroughly moistened with 5 ml. of dilute ammonia soln. 58% (w/v), allowed to air dry and transferred, by washing with 75 ml. of chloroform, to a conical flask, stoppered, and allowed to macerate for 24 hr. This material was filtered through a Buchner funnel and the filtrate shaken with 100-ml. portions of 2% (w/v) sulfuric acid until it tested negative for alkaloids with modified Dragendorff's spray reagent. This acid solution was adjusted to pH 10 with dilute ammonia soln. 58% (w/v), and exhaustively extracted with chloroform. The resulting chloroform solution was dried over anhydrous sodium sulfate, filtered and concentrated to dryness, *in vacuo*, utilizing a methanol azotrope to assure complete removal of moisture. This residue was redissolved in chloroform, and adjusted to a volume of 25 ml. Three drops of indicator (0.5% w/v) solution of gentian violet in glacial acetic acid) and 12.5 ml. of glacial acetic acid were added, the mixture continuously stirred, and titrated to a visual green end point with 0.01 *N* perchloric acid in glacial acetic acid. Precautions were observed to assure that atmospheric moisture was not absorbed by the glacial acetic acid prior to its use in the titration procedure. The total alkaloid content was expressed as milligrams of perivine per gram of sample since this alkaloid is representative of the average molecular weight and structural type of alkaloids present in *C. roseus*. It is also the major alkaloid formed in the leaves of the plant (10).

The VLB content was determined from 1-Gm. samples of the pooled powdered plant material by a method devised in our laboratories and described in a recent publication (11).

[1] Organic Morcrop, Chas. Lilly Co., Seattle, Wash. (Analysis: 5% total nitrogen, 3% available phosphate, 2% available potash).

[2] Furnished through the courtesy of Dr. Edwin Alder, Agricultural Research Center, Eli Lilly and Co., Greenfield, Indiana.

Chlorophyll Content—The concentration of chlorophyll *a* and *b* was determined on 1/2-Gm. samples of powdered leaf material by a method previously described (4).

Selective Solvent Extraction—To determine the effects of the treatments on the formation of other types of metabolic products, duplicate 2-Gm. samples of the powdered dried leaves were selectively extracted in a Soxhlet apparatus in sequence with the following solvents: petroleum ether USP, absolute ether A.R., absolute alcohol A.R., and distilled water in a manner previously described (3).

RESULTS

A significant increase in height was induced by the GA treatment (Table I), and the maximal increase (61%) over controls occurred at the end of the second week. Just prior to the terminal harvest the GA-treated plants were 48% taller than controls. Analysis of variance of the height data (Table I) indicated that the height increases of the treated plants were statistically significant at the 1% confidence level. The rate of flowering was not affected by the gibberellin treatment.

A general favorable effect on plant growth was induced as indicated by dry weight data (Table II). Increases of 34, 20, and 14% were found in the total dry weight at the second, third, and fourth harvests, respectively. Stem dry weights were increased 11, 85, 93, and 40% over the controls at the first, second, third, and fourth harvests, respectively. The dry weight of the roots was increased except at the first harvest, but the weight of the leaves was not appreciably affected. Statistical analysis of the dry weight data indicated that there was significant increase in only the stem dry weights of the GA group. This increase was significant at the 5% level in only the second and third harvest plants. The marked increases in root and whole plant dry weights were not statistically significant.

First harvest samples were not assayed for the concentration of total alkaloids because insufficient amounts were available. Alkaloid concentration in the leaves of the controls increased with the age of the plant (Table III) and a reverse trend occurred in the GA group. In general, gibberellin treatment caused a reduction in leaf alkaloids, with the largest (45%) reduction occurring at the fourth harvest. Treated plants showed a lower alkaloid concentration in the stems than controls. In this case, both the treated and untreated groups demonstrated decreased alkaloid concentrations with age. Slight reductions were noted in the concentration of root alkaloids at the second and third harvest, and again the largest decrease (39%) occurred at the fourth harvest. It was observed that GA treatment generally induced a decrease in the concentration of alkaloids in all plant parts. This decrease was more pronounced in physiologically mature plants than in young plants. Alkaloid concentrations tended to increase with maturation in both leaves and roots, then leveled off at the final harvest.

Total alkaloid content in the leaves of treated plants was increased 31% at the second harvest but decreases of 20 and 45% were found at the third and fourth harvests (Table IV). This 31% gain was due both to increased growth and increased alkaloid concentration, while the reductions were due entirely to decreased alkaloid concentration. Total alkaloid content of the stems was increased at all three harvests (Table IV) and the increases were more pronounced at the early growth stages than during the more mature phases. These increases were due primarily to the effect GA had on increasing stem growth. Variable effects were noted on the total alkaloid content of the roots. The 32% decrease at the fourth harvest was due mainly to a significant reduction in the concentration of root alkaloids. The total alkaloid content per plant was increased 35% at the second harvest but decreased 39% at the final harvest, this latter effect was due

TABLE I—HEIGHT DATA OF PLANTS[a] DURING THE EXPERIMENTAL PERIOD

Measurement No. and Date	Controls, cm.	GA-Group, cm.	Control, %	L.S.D.[b] 0.05	L.S.D.[b] 0.01
1, Sept. 11[c]	8.90	9.55	107	...	...
2, Sept. 18	14.25	19.90	140	3.93	5.38
3, Sept. 25	20.20	32.55	161	5.82	7.98
4, Oct. 2	26.55	41.35	156	6.93	9.50
5, Oct. 9	31.25	47.60	152	7.53	10.32
6, Oct. 16	35.75	54.20	152	10.66	14.62
7, Oct. 23	38.65	57.25	148	10.96	15.01

[a] Average per group of 10 plants. [b] The lowest significant difference at the 5% and 1% confidence level calculated from the analysis of variance. [c] This measurement was performed prior to treatment on the date of the first treatment, 5 days prior to the first harvest.

TABLE II—WEIGHTS OF *C. roseus* PLANT PARTS[a]

Treatment and Harvest No.	Total Wt. Fresh, Gm.	Total Wt. Dry, Gm.	Total Wt. Control, Dry Wt., %	Leaves Fresh, Gm.	Leaves Dry Gm.	Leaves Control, Dry Wt., %	Stems Fresh, Gm.	Stems Dry, Gm.	Stems Control, Dry Wt., %	Roots Fresh, Gm.	Roots Dry, Gm.	Roots Control, Dry Wt., %
Control, 1	2.29	0.249	...	1.36	0.166	...	0.34	0.038	...	0.59	0.045	...
GA, 1	2.36	0.251	101	1.36	0.164	97	0.40	0.042	111	0.61	0.045	100
Control, 2	7.71	0.888	...	4.36	0.544	...	1.39	0.187	...	1.95	0.157	...
GA, 2	10.00	1.188	134	4.93	0.627	115	2.51	0.346	185	2.56	0.215	137
Control, 3	17.88	2.298	...	10.01	1.345	...	4.34	0.580	...	3.54	0.373	...
GA, 3	17.98	2.766	120	8.57	1.256	93	6.15	1.122	193	3.26	0.388	104
Control, 4	43.11	6.081	...	24.40	3.171	...	11.42	2.041	...	7.30	0.869	...
GA, 4	42.84	6.951	114	21.90	3.143	99	13.61	2.847	140	7.33	0.961	111

[a] Mean weights per group of 10 plants.

TABLE III—CONCENTRATION OF ALKALOIDS[a] OF *C. roseus* PLANT PARTS[b]

Treatment and Harvest No.	Leaves		Stems		Roots	
	Alkaloids, mg./Gm.	Control, %	Alkaloids, mg./Gm.	Control, %	Alkaloids, mg./Gm.	Control, %
Control, 2	5.3	...	4.2	...	7.7	...
GA, 2	6.1	115	4.1	98	6.7	87
Control, 3	7.0	...	2.9	...	8.7	...
GA, 3	6.0	86	2.4	83	7.9	91
Control, 4	7.3	...	2.0	...	8.6	...
GA, 4	4.0	55	1.7	85	5.3	61

[a] Expressed as perivine. [b] Determined from pooled samples of 30 plants per group.

TABLE IV—TOTAL ALKALOID CONTENT[a] (mg.) OF *C. Roseus*

Treatment and Harvest No.	Per Plant		Leaves		Stems		Roots	
	Total Alkaloids	Control, %	Total Alkaloids	Control, %	Total Alkaloids	Control, %	Total Alkaloids	Control, %
Control, 2	4.9	...	2.9	...	0.8	...	1.2	...
GA, 2	6.6	135	3.8	131	1.4	175	1.4	117
Control, 3	14.3	...	9.4	...	1.7	...	3.2	...
GA, 3	13.3	93	7.5	80	2.7	152	3.1	97
Control, 4	34.7	...	23.1	...	4.1	...	7.5	...
GA, 4	22.5	61	12.6	55	4.8	117	5.1	68

[a] Calculated from dry weight and alkaloid analyses data; per plant = leaves + stems + roots.

TABLE V—CONCENTRATION OF VLB[a] AND TOTAL CONTENT[b] OF LEAVES

Treatment and Harvest No.	VLB Concn.		Total VLB Content	
	mcg./Gm.	Control, %	mcg.	Control, %
Control, 1	30	...	5.0	...
GA, 1	36	120	5.9	118
Control, 2	28	...	15.2	...
GA, 2	31	111	19.4	128
Control, 3	30	...	40.4	...
GA, 3	28	93	35.2	87
Control, 4	19	...	60.2	...
GA, 4	24	126	75.4	125

[a] VLB = vincaleukoblastine. [b] Calculated from dry weight and VLB concentration data.

entirely to the appreciable reductions in the concentrations of alkaloids found in the plant organs.

One-gram samples of the dried plant organs were analyzed for vincaleukoblastine (VLB) by the previously described procedure. The concentration of VLB was less than the minimum detectable concentration of 5 mcg./Gm. in both the stems and roots. It was concluded that in both the treated and control groups grown under the aforementioned greenhouse conditions, these organs either contained no VLB, or, if the alkaloid was present, it occurred in concentrations below the detectable concentration of 5 mcg./Gm. dry weight of plant material. Concentration of VLB generally was higher in the leaves of the treated plants than controls (Table V). In the treated plants the concentration of VLB decreased in the leaves as the plants matured. Even in the controls, the leaf concentration was much higher at the first harvest than the fourth harvest. Since gibberellin treatment did not significantly affect the dry weight of the leaves, the total VLB content of the leaves followed the pattern shown for the VLB concentration.

Due to the small quantities of plant material remaining, only the leaves of the fourth harvest plants were assayed for the concentration of chlorophyll *a* and *b*. The concentration of chlorophyll *a* in the controls was 3.76 mg./Gm. compared with 3.94 mg./Gm. in the treated group. Corresponding figures for chlorophyll *b* were 3.45 and 3.57. The GA treatment had no significant effect on chlorophyll concentration.

Selective solvent extraction was performed on the various organs of the final harvest only (Table VI) and the petroleum ether and absolute ether extracts of all organs were markedly less in the treated group. The absolute ether extract of the stems of the treated group was about one-third of the controls. A slight increase over controls was noted in the alcoholic extract obtained from the leaves of the GA group whereas marked reductions were found in the stems and roots. No significant differences were noted in the aqueous extracts of the organs between treated and nontreated groups.

DISCUSSION AND CONCLUSIONS

A typical gibberellin effect characterized by increased internodal elongation which resulted in taller plants was noted in the treated plants. Analysis of variance of the height data indicated that the increased height of the GA group was

TABLE VI—RESULTS[a] OF SELECTIVE SOLVENT EXTRACTION OF FOURTH HARVEST ORGANS

Treatment	Plant Part	Petroleum Ether	Absolute Ether	Absolute Alcohol	Distilled Water
Control	Leaves	176	23	111	322
Control	Stems	131	30	95	193
Control	Roots	145	15	101	147
GA	Leaves	162	15	124	316
GA	Stems	110	11	60	173
GA	Roots	110	14	86	154

[a] Expressed as mg./Gm. dry wt.

significant at the 1% confidence level. Treatment with GA did not affect the rate of flowering, but growth of the plants, indicated by total dry weight, was favorably affected. As could be expected most of the weight gain was concentrated in the stems. The pattern for stem dry weight (over controls) of 85 and 93% at the second and third harvests, respectively, were statistically significant at the 5% confidence level. In general the dry weight of the leaves was not materially altered while slight gains were noted in root dry weights.

There was a general unfavorable effect of the GA treatment on the concentration of alkaloids in the various plant organs. This is in agreement with other investigations (1–6). Several trends were noted during the experimental period. The concentration of alkaloids in the leaves of the control group increased with the age of the plant while the reverse trend was noted in the GA group. Both treated and untreated groups demonstrated decreased alkaloid concentration in the stems with increasing maturity. Alkaloid concentration tended to increase with maturation in both leaves and roots, then leveled off at the final harvest when all of the plants were in full bloom. In other words, the maximum concentration appeared during the flowering stage. The decreases in alkaloid concentration at the fourth harvest of 45% in the leaves and 39% in the roots were considered significant. Total alkaloid content of the leaves and roots generally followed the pattern exhibited with alkaloid concentration, however, the total alkaloid content of the stems was more directly related to the effect of GA on increasing stem growth. Due to significantly increased stem weight the total alkaloid content of this organ was markedly increased at all three harvests. The increases of 75% at the second harvest and of 52% at the third harvest were considered significant.

Of great interest was the disclosure of the effect of GA on the concentration of VLB and the total VLB content. It was concluded that the stems and roots of both groups either contained none of this alkaloid, or that this alkaloid, if present, was present below the minimum detectable concentration that could be demonstrated by the assay procedure. Treatment with GA generally increased the concentration and total content of VLB in the leaves. The concentration of VLB in the leaves of this group decreased with increased plant maturity. This pattern was the reverse of that noted with the concentration of total leaf alkaloids. Even in the control group the concentration of VLB in the leaves was about two-thirds that of the first harvest at the terminal harvest. These disclosures are in agreement with the observations by Noble (12) who found that extracts of leaves of young *C. roseus* plants showed a greater leukopenic activity than did the leaves of older plants. This was undoubtedly due to a lower concentration of the leukopenic alkaloid VLB in the older leaves. This suggests that VLB accumulates at a higher rate in younger leaves than in more mature ones. The total VLB content of the leaves followed a pattern similar to that of VLB concentration since gibberellin treatment did not markedly affect leaf growth.

The following pertinent observations were made concerning other types of metabolic products. Treatment with GA apparently did not affect the chlorophyll concentration. The synthesis and/or accumulation of lipids and resinous compounds was reduced in all of the organs of the treated groups as indicated by the decreased quantities of petroleum ether and ether extracts obtained by the selective solvent extraction. No significant differences were noted in the alcoholic or aqueous extracts.

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Keyphrases

Catharanthus roseus plants
Gibberellic acid effect—growth, alkaloid content
Vincaleukoblastine content—gibberellic acid
Chlorophyll content—leaves

Mechanism of Hypotensive Action of 2-(2,6-Dichlorophenylamino)-2-imidazoline Hydrochloride (ST-155) in the Cat

By R. D. MAGUS* and J. P. LONG

A study of the mechanism of hypotensive and bradycardic action of ST-155 in the cat revealed that this action is in part related to a centrally mediated bradycardia. Bradycardia and the hypotensive action of ST-155 were antagonized or abolished by hexamethonium, bretylium, decerebration, or by stellate ganglionectomy. The action of ST-155 does not appear to involve veratrine-sensitive pathways inasmuch as denervation of the carotid sinus or excision of the nodose and superior cervical ganglia were without effect on the action of ST-155. ST-155 in the dosage used (8 mcg./Kg.) potentiated the pressor responses to epinephrine and tyramine. It is presumed that reduced central sympathetic transmission to the heart from the cardiac sympathetic nerves is a major mechanism for the hypotension and bradycardia following ST-155 administration.

THE TOLAZOLINE ANALOG 2-(2,6-dichlorophenylamino)-2-imidazoline hydrochloride (ST-155) (Fig. 1) is currently being widely investigated in both animals and man as a possible antihypertensive agent. Studies in animals to date indicate that ST-155 administered intravenously exhibits a dose-dependent (1–100 mg./Kg.) reduction in heart rate and blood pressure in vagotomized dogs, an effect which is preceded by a brief pressor response (1). In addition,

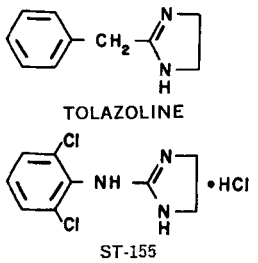

Fig. 1—Structures of ST-155 and tolazoline.

ST-155 has been shown to depress the carotid sinus reflex, reduce cardiac output with little change in peripheral resistance during the hypotensive phase, and exhibit a variety of sympathomimetic actions (*e.g.*, vasoconstriction in the isolated rabbit ear, splenic contraction in cats, increase in contraction amplitude of isolated spontaneously beating guinea pig atrial) (1). With relatively larger doses in rats ST-155 has been shown to increase urine volume and electrolyte excretion although such effects have not been seen in dogs receiving the drug in doses which produced marked hypotensive actions (1).

Clinical studies have thus far shown that ST-155 in microgram dosage is an orally effective antihypertensive agent in man with a low incidence of side effects (2–7) and toxicity (*loc. cit.*, 8). For example, orthostatic hypotension is a minor occurrence with therapeutically effective doses; however, fatigue, sedation, and anticholinergic effects have been repeatedly observed in humans receiving ST-155 (2–7).

To date, the mechanism of hypotensive action of ST-155 has not been elucidated (9). The present experiments were, therefore, undertaken in an attempt to provide a possible mechanism of hypotensive action of ST-155 in the anesthetized cat. The studies appear to indicate that a major component of the drug's hypotensive action is a centrally mediated inhibition of sympathetic cardioaccelerator nerves resulting in bradycardia.

EXPERIMENTAL

Animals—Thirty-five adult female mongrel cats weighing between 1.4 and 3.2 Kg. were used in this study. Anesthesia was performed by intrathoracic administration of sodium pentobarbital (30–35 mg./Kg.). All animals were tracheotomized and artificially respired on a Palmer respirator. Blood pressures were monitored, *via* a heparinized cannula inserted in the femoral artery, by a Statham arterial pressure transducer and recorded on an Offner RS dynograph. Heart rate was monitored with a stopwatch for 15-sec. periods at the intervals indicated on the charts. Injections of test drugs or infusion of additional anesthetic, when required, were made into the contralateral femoral vein either directly or through a cannula. Other operative procedures are described in the text where appropriate.

Received September 14, 1967, from the Department of Pharmacology, University of Iowa College of Medicine, Iowa City, IA 52240

Accepted for publication November 8, 1967.

Supported by grants No. NB-1396 and 5-T01-GM01308, U. S. Public Health Service, Bethesda, Md

The authors are grateful to Drs. W. M. Kirkendall (University of Iowa) and P. A. Kennedy, Jr. (Geigy Pharmaceuticals, Ardsley, N. Y.) for generous supplies of ST-155.

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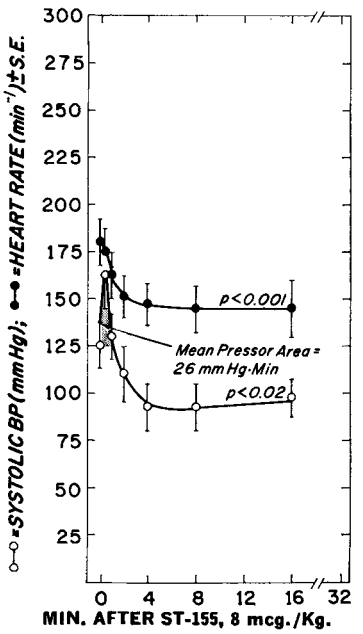

Fig. 2—Blood pressure and heart rate following ST-155 (8 mcg./Kg.) in the anesthetized cat. Key (Figs. 2–8): O, systolic blood pressure; ●, heart rates; S.E. = standard error of the mean; pressor areas are defined as the areas (mm. Hg × time) under the pressor phase of the systolic pressure curve. Statistical comparisons are made between the 0 time and 16-min. point.

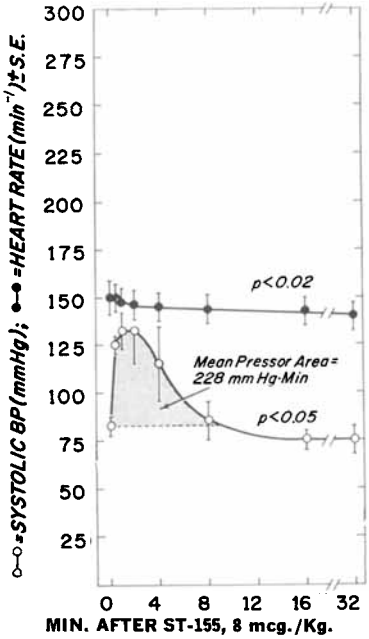

Fig. 3—Effect of hexamethonium pretreatment (10 mg./Kg.) on ST-155 action.

Drugs—ST-155 was administered as the water-soluble hydrochloride prepared in ampul form.[1] Tyramine hydrochloride (Eastman), hexamethonium chloride (Nutritional Biochemicals), and bretylium tosylate (Burroughs Wellcome) were administered in 0.9% saline solution. Epinephrine and isoproterenol were diluted before use from concentrated solutions (Parke-Davis). All dosages are expressed as the salt. Bretylium was administered by slow intravenous infusion; hexamethonium was similarly infused after an initial fraction of the administered dose was given intramuscularly. All other drugs were given by rapid intravenous injection.

In each experiment a total of five cats was used. All data are expressed as mean ± standard error; Student's 2-tailed *t*-test (paired comparison) was employed as described by Steel and Torrie (10).

RESULTS

Hypotensive Action of ST-155 in the Cat—The time course of pressor and depressor responses and bradycardia following 8 mcg./Kg. ST-155 administered intravenously is presented in Fig. 2. This dosage of ST-155 was selected from preliminary experiments as a dose which reduced heart rate and systolic pressure by approximately 20–25% and was used routinely in all subsequent experiments. The response to ST-155 in the cat (vagi intact) shown in Fig. 2 illustrates the initial transient pressor phase (30% increase in systolic blood pressure) concomitant with bradycardia. It will be noted that both the heart rate and arterial blood pressure 16 min. after administration of ST-155 are significantly different from preinjection values.

Effect of Hexamethonium Pretreatment on ST-155 Activity—In order to test the possibility that hypotension and bradycardia from ST-155 were reflexogenic, cats (vagi intact) pretreated with hexamethonium (10 mg./Kg.) were given ST-155 (8 mcg./Kg.) after the base line blood pressure following hexamethonium had become reasonably stable (20–30 min.).

The data in Fig. 3 indicate that hexamethonium pretreatment markedly reduced the bradycardia and hypotensive action of ST-155 and greatly potentiated its initial pressor action. Indeed, there is a ninefold increase in the pressor area (duration × pressure) when a comparison is made with cats not receiving hexamethonium pretreatment (Fig. 3 *versus* Fig. 2). A slight, albeit statistically significant change from pre-ST-155 values in heart rate and systolic blood pressure is seen at 16 min. However, since these changes vary only 7–8% from pre-ST-155 values and may be due to the action of hexamethonium, it may be concluded that bradycardia and hypotension due to the action of ST-155 are associated with a reflex. It would thus seem possible that hexamethonium abolished a depressor reflex which is otherwise triggered by ST-155.

Cardiovascular Response to ST-155 in the Decapitated Cat—That the hypotensive response to ST-155 was centrally mediated was tested by administration of the drug to cats that had a large press clamp placed tightly around the neck centrally to the tracheal cannula. This procedure effectively severed any functional connection of the head to the remainder of the animal. It was seen that such a maneuver abolished any depressor action of ST-155 (8 mcg./Kg.) although a transient bradycardia was seen (Fig. 4). However, no significant difference in both parameters was seen 16 min. after ST-155 administration. Whereas the pressor response to ST-155 showed a 30% increase in systolic pressure as in the intact cat (Fig. 4 *versus* Fig. 2), this action

[1] Catapres, Geigy.

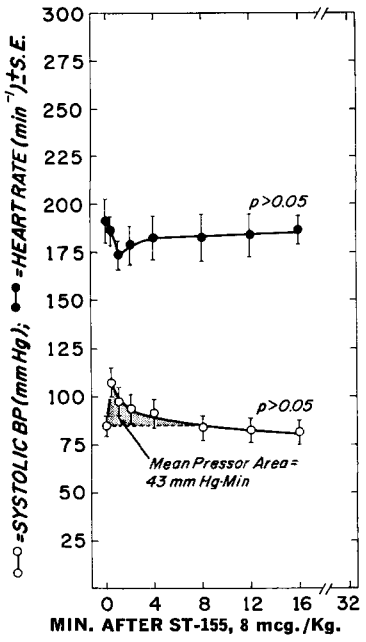

Fig. 4—Response to ST-155 in the headlamped cat.

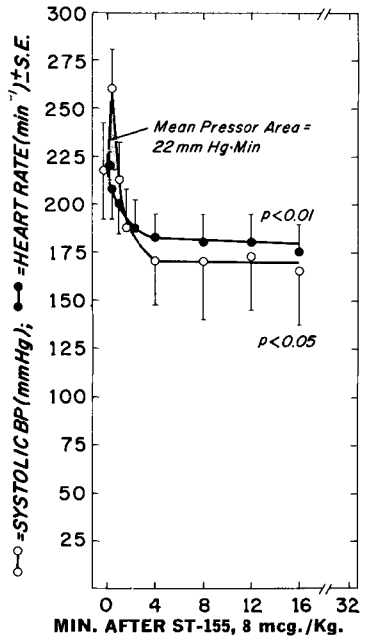

Fig. 5—Response to ST-155 following carotid sinus denervation.

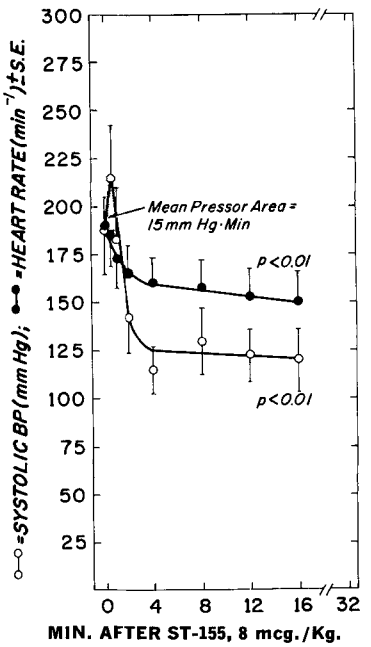

Fig. 6—Response to ST-155 following carotid sinus denervation and superior cervical and nodose ganglionectomy.

was of slightly longer duration in the headless cat (43 mm. Hg min. *versus* 29 mm. Hg min. pressor area). It would therefore appear that bradycardia and hypotension following ST-155 occur through centrally mediated pathways.

Effect of Carotid Sinus Denervation Alone or with Nodose and Superior Cervical Ganglionectomy on ST-155 Action—The possibility that ST-155 was activating reflex hypotension in a manner analogous to that of veratrum alkaloids (11, 12) was examined in the following experiments. Carotid sinuses were denervated bilaterally and painted with 1% lidocaine solution. Such a maneuver did not alter the action of ST-155 (Fig. 5 *versus* Fig. 2), nor did excision of the nodose ganglia and the superior cervical ganglia in addition to carotid sinus denervation (Fig. 6 *versus* Fig. 2). It was apparent, therefore, that the locus of action of ST-155 was unlike veratrum-like agents which appear to act reflexly *via* an action on the baroreceptor buffering mechanism and nodose ganglia (12).

Response to ST-155 After Bretylium Pretreatment or Stellate Ganglionectomy in Vagotomized Cats—It was speculated that a possible mode of action of ST-155 was *via* reflex inhibition of the cardiac sympathetic fibers emanating from the stellate ganglion. Pretreatment of bilaterally vagotomized cats with bretylium (15 mg./Kg.) (on the premise that if sympathetic nerve transmission was already inhibited by this means then ST-155 would not show hypotensive activity), potentiated the pressor action of ST-155 (8 mcg./Kg.), abolished the bradycardia, and markedly reduced the depressor action to a 10% change (at 16 min.) from pre-ST-155 values (Fig. 7). This effect of bretylium was much like that seen earlier with hexamethonium pretreatment (Fig. 3). Furthermore, administration of ST-155 (8 mcg./Kg.) to bilaterally vagotomized cats, in which the stellate ganglia were removed following a midline incision along the sternum, produced only a pressor response (Fig. 8). Again, the pattern of the response resembled that seen in the animals pretreated with hexamethonium (*cf.* Fig. 3).

Effect of ST-155 on Cardiovascular Reactivity to Tyramine, Epinephrine, and Isoproterenol—An investigation of the action of ST-155 was performed in order to examine its action at the adrenergic receptor level. Previous reports (1) indicated that with extremely high doses ST-155 may have adrenergic blocking activity. This would not be unexpected in view of its structural resemblance to tolazoline (Fig. 1). However, it was found that ST-155 in an 8 mcg./Kg. dose appeared to potentiate the pressor actions of epinephrine in both the intact and headless cat and of tyramine in the intact cat; the vasodepressor action of isoproterenol in the intact cat was not affected by ST-155 pretreatment

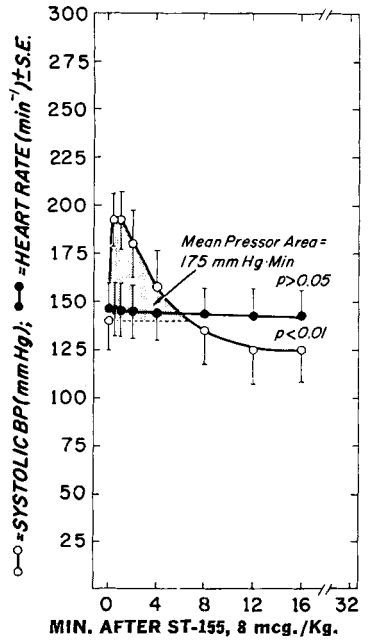

Fig. 7—Effect of bretylium pretreatment (15 mg./Kg.) on ST-155 action.

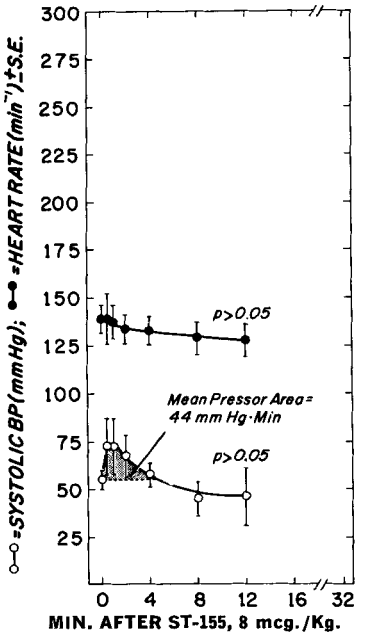

Fig. 8—Response to ST-155 following bilateral vagotomy and stellate ganglionectomy. No measurements were made beyond the 12-min. point.

(Fig. 9). It is apparent, therefore, that adrenergic blockade by ST-155 was not operative in the dosage used in this study.

DISCUSSION

If ST-155 were acting by central inhibition of the cardiac sympathetic fibers, the results obtained in this study would be in agreement with such a mechanism. Inasmuch as only a partial blockade of hypotension was obtained with bretylium and hexamethonium, it is difficult to state unequivocally that this is the mechanism of action. Such conclusions are further complicated by the possibility that hexamethonium and stellate ganglionectomy

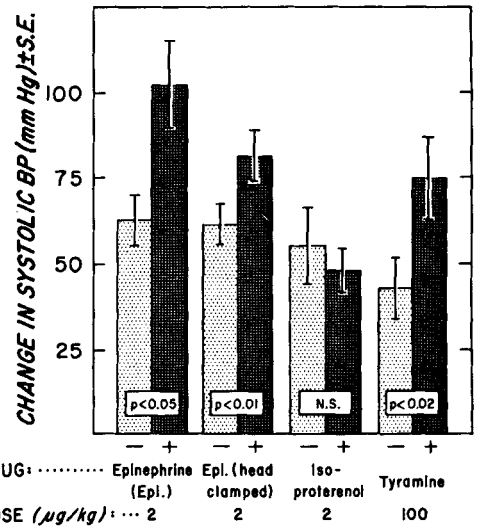

Fig. 9—Effect of pretreatment with ST-155 (8 mcg./Kg., i.v., 20 min.) on pressor responses to epinephrine and tyramine and vasodepressor response to isoproterenol and refer to ST-155.

lowered blood pressure to the extent that it could not be lowered further by ST-155 (Figs. 3 and 8). However, it must be emphasized that the "breakthrough" depressor responses to ST-155 after hexamethonium, *etc.*, were quantitatively minimal compared with the depressor activity seen in normal animals. It may be significant that whenever appreciable hypotension was seen, bradycardia was invariably present. Thus, it may well be that hypotension occurs as a result of a centrally mediated (reflex?) reduction in cardiac output (*cf.* 13). It is of interest to note that ST-155 appears to exert no bradycardia in the isolated spontaneously beating rabbit heart (Langenorff preparation) in doses of up to 100 mcg. (15).

Other studies in animals have shown that ST-155-induced hypotension is abolished by β-adrenergic blockade and by pentolinium (18), which is in agreement with the authors' interpretation of the action of ST-155 after hexamethonium or bretylium. Release of catecholamines by ST-155 would not seem to be involved since no change in cardiac norepinephrine occurs following high doses of ST-155 (1, 14). The sympathomimetic actions of ST-155 are abolished by tolazoline, phenoxybenzamine,[2] or phentolamine, but not by reserpine pretreatment (14, 17). It is thus likely that ST-155 has at least two components of action: an apparently "direct" musculotropic (α-adrenergic) action on blood vessels and nictitating membrane (1, 14, 17) and a centrally mediated bradycardia seemingly involving only the cardiac sympathetics.

The present studies pose a pertinent question of whether these findings can be extrapolated to explain the mechanism of hypotensive action of ST-155 in man. Significant reduction of cardiac output and heart rate was observed in a group of patients with essential hypertension receiving total doses of 150 to 300 mcg. of ST-155 intravenously (3). In these studies no appreciable change in peripheral

[2] Dibenzyline, Smith Kline and French Labs., Philadelphia, Pa.

resistance was noted. There have been reports of hypovolemia (7) and of redistribution of blood (*e.g.*, cutaneous vasconstriction) although these latter effects appear to be due to the vasoconstrictor action of ST-155 (16).

It cannot be stated at present if or how the supersensitization to the pressor actions of epinephrine and tyramine are related to the reflex hypotension or bradycardia. Moreover, that central inhibition of cardiac sympathetic nerve fibers by ST-155 does indeed occur must also remain an unresolved postulate until direct studies are made to test this hypothesis and to correlate this with the hypotensive action of ST-155. However, the authors of a related study which appeared shortly after the present investigation was completed, concluded that ST-155 owed its hypotensive action to a central inhibition of "medullary vasomotor and accelerator centers" (19).

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Cardiovascular activity—ST-155
Central mediation—ST-155 activity

Molecular Sorption on Ion-Exchange Resins: Studies with Coumarin

By R. S. HEGDE, M. J. MEHTA, R. A. BHATT, D. J. PATEL, and S. L. BAFNA

The sorption equilibrium of coumarin on ion-exchange resins of different degree of cross-linking, ionogenic groups, and counter ions in water and aqueous methanols has been studied and the results are discussed.

EARLIER, (1–3) the sorption and separation of coumarins on ion-exchange resins has been reported. This paper covers the study of the sorption equilibrium of coumarin on ion-exchange resins of different degrees of cross-linking, ionogenic groups, and counter ions in water and aqueous methanols at room temperature (~30°).

EXPERIMENTAL

Resins—(*a*) Styrene divinylbenzene copolymer-based sulfonic acid cation-exchange resins[1] of relative degree of cross-linking (% nominal divinylbenzene content) as 4, 8, and 12 (further referred to as X4, X8, and X12) of −100, +200 mesh; the resins were conditioned, regenerated, and moisture content and capacity were estimated (4, 5).

The different ionic forms of the resins were obtained by passing an excess of the salt or hydroxide solution through the resin bed. The resin was then washed, filtered, air dried, moisture content determined, and the capacity of the air-dried form of the resin calculated from the capacity of the oven-dried resin in the hydrogen form.

(*b*) Carboxylic acid cation-exchange resins[2] of relative degree of cross-linking (% nominal divinylbenzene content) X = 2.5, 5, 10, and 15 (further referred to as resin CX 2.5, CX 5, CX 10, and CX 15) of −40, +60 mesh; these are presumably based on acrylic acid divinylbenzene copolymers. The resins were conditioned, regenerated, and the moisture content and capacity were estimated as given before (5). The ionic forms of the resins were obtained by passing an excess of the corresponding hydroxide solution through the resin bed. The resins were then washed free of alkali, filtered, air dried, and the moisture content was determined. The capacity of the resins in different forms was calculated from the capacity of the oven-dried resin in the hydrogen form.

Received September 26, 1967, from the Chemistry Department, M.S. University of Baroda, Baroda, India.
Accepted for publication, December 7, 1967.

[1] Dowex 50W, Dow Chemical Co., Midland, Mich.

[2] Zeo-karb 226, Permutit Co., London, England.

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The different ionic forms of the resins were obtained by passing an excess of the salt or hydroxide solution through the resin bed. The resin was then washed, filtered, air dried, moisture content determined, and the capacity of the air-dried form of the resin calculated from the capacity of the oven-dried resin in the hydrogen form.

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(*c*) IR-200[3] of −20, +60 mesh; this is presumably styrene divinylbenzene copolymer-based sulfonic acid cation-exchange resin of relative degree of cross-linking about 20 but has an expanded structure. The resin was conditioned, regenerated, and moisture content and capacity were determined.

(*d*) IRC-50[4] of −40, +60 mesh; this is presumably methacrylic acid divinylbenzene copolymer-based carboxylic acid cation-exchange resin of relative degree of cross-linking about 8. The resin was conditioned, regenerated, and the moisture content and capacity were determined and the ionic forms prepared as given in (*b*).

(*e*) Styrene divinylbenzene copolymer-based strongly basic anion-exchange resins[5] of relative degrees of cross-linking: 2, 4, and 8 (further referred to as 1X2, 1X4, and 1X8), of −20, +50 mesh; these have presumably trimethyl ammonium groups as ionogenic groups. The resins were conditioned, regenerated, and moisture content was determined. For the estimation of capacity weighed samples (∽0.5 Gm.) of air-dried resin were mixed with 100 ml. of 0.05 *N* hydrochloric acid in well-stoppered flasks with frequent shaking. The next day, the remaining hydrochloric acid was estimated by titrating the aliquots with standard sodium hydroxide solution and the capacity was then calculated. Chloride form of these resins was obtained by passing sodium chloride solution through the resin bed in an excess of, the resin was then washed, filtered, air dried, and the moisture content was determined. The capacity was calculated from an oven-dried capacity of the hydroxyl form of the resins.

(*f*) Styrene divinylbenzene copolymer-based strongly basic anion-exchange resins[6] of relative degrees of cross-linking as 4 and 8 (further referred to as 2X4 and 2X8) of −20, +50 mesh; these presumably have dimethyl methylol ammonium groups as ionogenic groups. The resins were conditioned, regenerated, the moisture content and capacity of the resins were determined, and the chloride forms prepared.

(*g*) Styrene divinylbenzene copolymer-based weakly basic anion-exchange resin G[7] of relative degree of cross-linking about 8 and −14, +52 mesh; the ionogenic groups are presumably diethylamino groups. The resin was conditioned, regenerated, and the moisture content was determined. For the estimation of capacity, a weighed sample (∽1 Gm.) of air-dried resin was mixed with 50 ml., 1 *N* in NaCl and 0.1 *N* in HCl of solution with frequent shaking. The next day, the remaining hydrochloric acid was estimated by titrating the aliquots with standard sodium hydroxide solution and the capacity was then calculated.

Chemicals and Solvents—Chemicals were of A.R. or C.P. grade. Aqueous methanols were prepared by volume; for example, 10% methanol was prepared by making 100 ml. of methanol to 1 L. with distilled water. The dielectric constant of each solvent was measured on Multi-Dekameter-type DK 06 (WTW) using 1.8 m./cs. frequency at room temperature (∽30°).

Procedure—Coumarin solutions of different concentration and of known volume were mixed with weighed amounts of air-dried resins in well-stoppered flasks, with frequent shaking at room temperature (∽30°). After sufficient time, after the equilibrium was reached, the initial and the equilibrium concentrations were determined by ultraviolet absorption (6) with a Beckman model DU spectrophotometer using 10-mm. quartz cells.

[3] Amberlite IR-200, Rohm & Haas Co., Philadelphia, Pa.
[4] Amberlite IRC-50, Rohm & Haas Co., Philadelphia, Pa.
[5] Dowex 1, Dow Chemical Co., Midland, Mich.
[6] Dowex 2, Dow Chemical Co., Midland, Mich.
[7] Deacidite G, Permutit Co., London, England.

RESULTS AND DISCUSSION

The synthetic organic ion-exchange resins (7, 8) consist of an irregular, three-dimensional network of hydrocarbon chains, to which ionogenic groups are attached and the surplus electric charge is balanced by mobile counter ions. The hydrocarbon network is hydrophobic but the ionogenic groups are hydrophilic. Hence, when the resin particle is placed in water, it sorbs water and swells to a limited extent. The amount of water sorbed and the extent of swelling depend on the degree of cross-linking and the counter ions. If the external solvent contains a nonelectrolyte, it is also sorbed to a certain extent and after some time a state of equilibrium is reached. The amount sorbed will depend on several factors (7), which include the London (dispersion) interactions, the dipole–dipole interactions, the relative solubility of the solute in the medium inside and outside the resin particle, the solvent uptake and the volume of the swollen resin particle, the equilibrium concentration of the solute in the external solution, and the temperature. Besides these, there may also be specific interactions in a particular system.

A sorption equilibrium study involves three substances: the resin, the solvent, and the solute. For the resin, the variables may include the matrix structure, the relative degree of cross-linking, the type and the acid or base strength of the ionogenic group, and the charge, shape, size, and hydration of the counter ions. The solvent may be aqueous, mixed, or organic, it may be polar or nonpolar, and have different dielectric constants and solvation of the ionogenic groups and the solute molecules. For the solute, the variables may include the shape, size, polarity, and the relative solubility in the medium inside and outside the resin particle.

The amount of the solute sorbed per unit capacity of the resin at a given temperature will hence be determined by the overall effect and a complex behavior is to be expected; the systematic study of the variables may help toward better understanding of the phenomenon and with this object this study was undertaken.

The sorption equilibrium studies were carried out at room temperature, since the small variation in temperature did not make substantial change. The values of S were calculated from $S = (Co - Ce)/Cr$, where Co and Ce are the initial and equilibrium concentration of solute, gram moles per liter, and Cr is the capacity of air-dried resin (Table I) in equivalents added per liter of solution, and then S was plotted against Ce. The splots were linear. Figure 1 gives a typical example to illustrate this. The slope of such plots is termed as B (4).

Table II gives the values of B for the sorption of coumarin on sulfonic acid cation-exchange resins in hydrogen form in water and aqueous methanols. It is observed that with increase in the % methanol

TABLE I—CAPACITY OF ION-EXCHANGE RESINS

Cation-Exchange Resins		Anion-Exchange Resins	
Resin	Oven-dried Capacity, meq./Gm.	Resin	Oven-dried Capacity, meq./Gm.
X4	5.06	1X2	3.9
X8	4.89	1X4	3.5
X12	4.85	1X8	3.6
IR-200	4.78	...	...
CX 2.5	10.6	2X4	3.5
CX 5	9.77	2X8	2.67
CX 10	8.70	...	...
CX 15	6.84	G	4.0
IRC-50	10.4	...	...

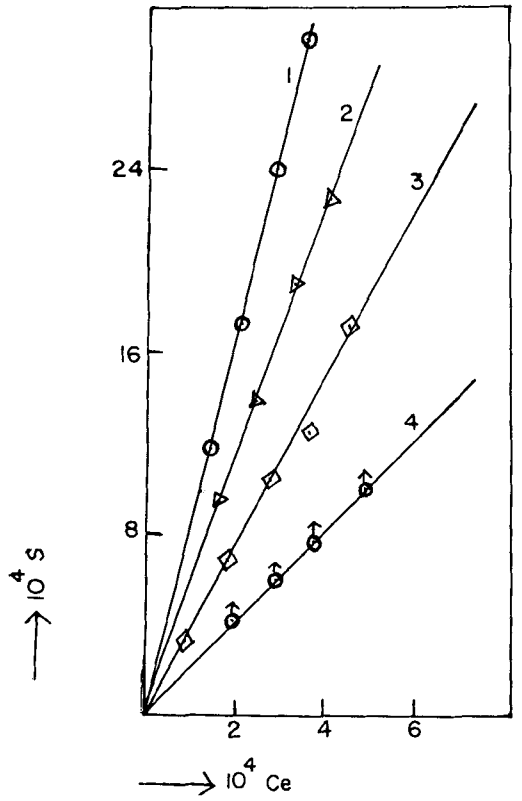

Fig. 1—Plots of S *against* Ce *for the resin X4 in hydrogen form in water* (1), *10% methanol* (2), *19% methanol* (3), *and 30% methanol* (4).

in the external solvent, B decreases. Figure 2 gives the plot of log B against log D (D is the dielectric constant of the external solvent) and the plots are linear; the values of slopes m, and the intercepts I are given in Table II. With the increase in the degree of cross-linking, X and m increase but I and B decrease.

For resin IR-200 (Table II) also, B decreases as the methanol content of the solvent increases, but the values of B are lower than what may be expected for 50W-type resin of $X = 20$. This implies that for sulfonic acid cation-exchange resins, the sorption is relatively less on a resin of expanded structure than that on a gel-type resin.

Table III gives the values of B for sorption of coumarin on the sulfonic acid cation-exchange resins of degree of cross-linking 4, 8, and 12 in H^+, Li^+, Na^+, K^+, and NH_4^+ forms. With the increase in X, for the hydrogen form of the resin, B decreases, but for Li^+, Na^+, K^+, and NH_4^+ forms, B increases. For resin X4, B is in the order $H^+ > Li^+ > Na^+ > K^+ > NH_4^+$, for resin X8 in the order $K^+ \backsim Na^+ > H^+ > Li^+ > NH_4^+$, and for resin X12 in the order $K^+ \backsim Na^+ > Li^+ > H^+ > NH_4^+$. Thus the order for H^+, Li^+, and Na^+ forms for resin X4 is reversed for resin X12. B for NH_4^+ form is always less than that for K^+ and Na^+ forms.

The sorption of coumarin was studied in water on resin X4 in different ionic forms. The values of B (Table IV) may be arranged in the order $H^+ > Fe^{+3} \backsim Al^{+3} > Ca^{+2} > Ni^{+2} \backsim Co^{+2} \backsim Li^+ > Na^+ > Fe^{+2} > Cu^{+2} > K^+ \backsim Sr^{+2} > Ba^{+2} > NH_4^+ > C_5H_5NH^+ > Ce^{+4}$.

Table V gives the values of B for sorption of coumarin on carboxylic acid cation-exchange resins of different X and ionic form. With increase in X, B first increases and then decreases for H^+, Li^+, Na^+, and K^+ forms, but for NH_4^+ form B increases with increase in X; for the ionic forms, B decreases in the order $H^+ > Li^+ > Na^+ > K^+ > NH_4^+$.

TABLE II—SORPTION OF COUMARIN ON SULFONIC ACID CATION-EXCHANGE RESINS IN WATER AND AQUEOUS METHANOLS

Resin	B in Solvent with Methanol % = 0	10	19	30	m	i
X4	8.5	5.8	3.8	2.1	7.6	−13.53
X8	7.3	4.3	2.6	1.2	9.6	−17.42
X12	6.3	3.5	2.0	0.9	10.6	−19.38
IR-200	1.8	0.8	0.41	...	...	...

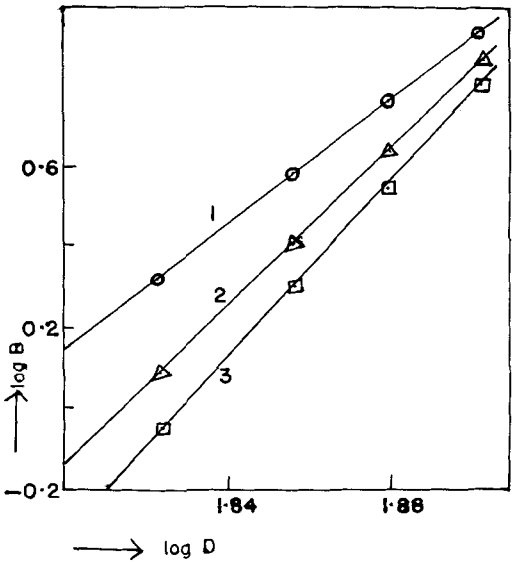

Fig. 2—Plots of log B *against* log D *for the resins X4* (1), *X8* (2), *and X12* (3) *in hydrogen form.*

TABLE III—SORPTION OF COUMARIN ON SULFONIC ACID CATION-EXCHANGE RESINS WITH DIFFERENT COUNTER IONS

Resin	B When Counter Ion is H^+	Li^+	Na^+	K^+	NH_4^+
X4	8.5	6.4	6.2	5.8	5.3
X8	7.3	6.8	7.9	7.9	6.8
X12	6.3	7.6	8.6	8.6	7.8

TABLE IV—SORPTION OF COUMARIN ON SULFONIC ACID CATION-EXCHANGE RESIN X4 WITH DIFFERENT COUNTER IONS (C.I.)

C.I.	B	C.I.	B	C.I.	B	C.I.	B	C.I.	B
H^+	8.5	Li^+	6.4	Ca^{+2}	6.6	Fe^{+2}	6.0	Fe^{+3}	7.4
NH_4^+	5.3	Na^+	6.2	Sr^{+2}	5.8	Cu^{+2}	5.9	Al^{+3}	7.4
$C_5H_5NH^+$	4.8	K^+	5.8	Ba^{+2}	5.1	Co^{+2}	6.4	Ce^{+4}	2.6
						Ni^{+2}	6.4		

TABLE V—SORPTION OF COUMARIN ON CARBOXYLIC ACID CATION-EXCHANGE RESINS WITH DIFFERENT COUNTER IONS

Resin	B when the Counter Ion is H^+	Li^+	Na^+	K^+	NH_4^+
CX 2.5	4.1	2.8	2.5	1.9	0.24
CX 5	9.0	5.9	4.2	3.7	0.71
CX 10	19.5	10.3	8.9	5.4	2.8
CX 15	9.1	9.0	6.9	6.4	5.8
IRC-50	6.7	2.9	1.7	1.3	0.74

For IRC-50, the same order is valid, but the values of B are relatively lower than those expected for carboxylic acid cation-exchange resin type resin of $X \backsim 8$. This implies that the sorption for acrylic and methacrylic acid-based resins is significantly different.

Table VI gives the values of B for the sorption of coumarin on anion-exchange resins in chloride form, with different X and type of ionogenic groups, in water and aqueous methanols. With increase in the methanol content and hence decrease in the dielectric constant of the solvent, B decreases. For 1X2, 1X4, and 1X8 resins, B increases with increase in the degree of cross-linking from 2 to 4 and is practically constant for increase in X for 4 to 8. For 2X4 and 2X8 resins, B increases when X increases from 4 to 8. B for 1X4 is greater than that for 2X4, but for 1X8 is lower than those for 2X8. B for the weak base anion-exchange resin G is greater than that for resin 1X8 but less than that for resin 2X8.

Table VII gives the values of B for sorption of coumarin on strong base anion-exchange resins in the hydroxyl form of different X in water and aqueous methanols. For 1X2, 1X4, and 1X8 resin, B increases with increase in X from 2 to 8 in water and 10% aqueous methanol; for 40% and 70% aqueous methanols, B first increases for increase in X from 2 to 4 and then decreases for increase in X from 4 to 8. For 2X4 and 2X8 resins, B varies in a similar manner for increase in X from 4 to 8. The values of B for 1X2, 1X4, and 1X8 resins are greater than those for the corresponding 2X4 and 2X8 resins of same X.

With increase in % methanol of the solvent and hence decrease in the dielectric constant of the solvent, B for resin 1X2 increases, for resin 1X4 decreases and then increases, and for resin 1X8 decreases. For resins 2X4 and 2X8, B decreases with increase in % methanol of the solvent. For the strong base anion-exchange resins, B for hydroxyl form is considerably greater than that for chloride form. This may be attributed to the strongly alkaline nature of the resins in the hydroxyl form and the relatively greater solubility of coumarin in alkaline medium; this comparison also suggests that in water 1X2, 1X4, and 1X8 resins should be more basic than the corresponding 2X4 and 2X8 resins of the same X.

TABLE VI—SORPTION OF COUMARIN ON ANION-EXCHANGE RESINS IN CHLORIDE FORM IN WATER AND AQUEOUS METHANOLS

Resin	B in Solvent with % Methanol = 0	10	20	30
1X2	9.0	6.1	5.0	2.9
1X4	21.0	12.5	9.2	6.2
1X8	21.0	12.5	9.2	6.2
2X4	13.9	8.7	6.9	4.3
2X8	38.0	21.0	16.7	9.0
G	23	...	...	...

TABLE VII—SORPTION OF COUMARIN ON STRONGLY BASIC ANION-EXCHANGE RESINS IN HYDROXYL FORM IN WATER AND AQUEOUS METHANOLS

Resin	B in Solvent with % Methanol = 0	10	40	70
1X2	24	26	31	75
1X4	97	82	79	117
1X8	121	117	75	69
2X4	68	40	30	23
2X8	106	52	15	9

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Keyphrases

Ion-exchange resins
Coumarin adsorption equilibrium—ion-exchange resins
Ionic resin forms—prepared
Solvent effect adsorption equilibrium
UV spectrophotometry—analysis

Stability of Diosgenin

By G. BLUNDEN and C. T. RHODES

The decomposition of 25α-Δ⁵-spirosten-3β-ol (diosgenin) has been studied by various physical techniques in the solid state, in contact with both 2 *N* hydrochloric acid and 2 *N* sodium carbonate solution, and in solution in both chloroform and benzene. In contact with 2 *N* hydrochloric acid, about 40 percent of the diosgenin was found to decompose within 4 months, whereas in the other conditions, the amount of decomposition was found to be negligible, although decomposition products were detected on thin-layer chromatograms and by ultraviolet spectroscopy.

THE STEROIDAL SAPOGENIN, 25α-Δ⁵-spirosten-3β-ol (diosgenin), is of considerable importance as the main precursor of the pharmacologically active steroids, including the corticosteroids and oral contraceptives. Diosgenin is obtained commercially from the tubers of several species of *Dioscorea* by acid-hydrolysis of the naturally occurring saponins. This treatment with acid has been shown to produce products other than diosgenin. Peal (1) showed that when diosgenin was heated under reflux with ethanolic hydrochloric acid, a certain amount of 25α-spirosten-3,5-diene was formed, the amount varying with the acid concentration, being about 35% when refluxed with 2 *N* hydrochloric acid for 2 hr. Blunden, Hardman, and Morrison (2) found that when diosgenin was refluxed with 2 *N* aqueous hydrochloric acid for 2 hr., the quantity of 25α-spirosten-3,5-diene formed was about 6%. Yamagishi (3) found that the optimum procedure for obtaining diosgenin from the saponin dioscin with the least diene formation was refluxing for 2 hr. with 4 *N* sulfuric acid.

After boiling the *Dioscorea* tuber with acid in the extraction procedure for diosgenin, the acid-insoluble material is collected and washed, to free it from acid. Rothrock, Hammes, and McAleer (4) reported that if the residue were not washed free from acid, the diosgenin yield decreased when the residue was dried. Blunden and Hardman (5) found that if the acid-insoluble residue was stored in an acid condition, the diosgenin content decreased. To overcome this problem, Hershberg and Gould (6) neutralized the acid-insoluble material with a base. A similar technique using sodium carbonate solution was used by Blunden and Hardman (5). However, during the commercial extraction procedure of diosgenin, the process is sometimes continued until the acid-insoluble residue is produced this being exported for the final extraction of diosgenin. In certain cases, the shipped material has been distinctly acidic.

As indicated earlier, diosgenin is known to decompose when stored in contact with hydrochloric acid, but the authors are unaware of any published data concerning the stability of this steroid in the solid state or in solution in common organic solvents. The present investigation was undertaken to study the stability of diosgenin under these conditions over a 4-month period and determine the extent of decomposition compared with the total decomposition of diosgenin stored in contact with 2 *N* hydrochloric acid for the same period.

EXPERIMENTAL

The diosgenin used in this investigation was obtained from Steraloids Ltd. After three recrystallizations from acetone the material had a melting point of 204–205°. Decomposition of the sapogenin was studied over a 4-month period in the following environments: as solutions in chloroform and benzene and as solids, which were untreated or in contact with either 2 *N* hydrochloric acid or 2 *N* sodium carbonate solution. Suitable quantities of solutions or solids were packed in sealed glass ampuls at either 4, 20, or 37°, in light or in the dark. The solid samples and solutions in chloroform and benzene were examined at different time intervals, but only one sample of diosgenin in contact with 2 *N* hydrochloric acid and one sample in contact with 2 *N* sodium carbonate solution were prepared, both of which were stored at 20° in the light and examined after storage for 4 months. All the samples were examined by a number of different methods. (*a*) Optical rotations of diosgenin as solutions in chloroform were measured by a Bellingham and Stanley polarimeter using a cell of 1 ml. capacity and 10 cm. path length. (*b*) Infrared spectra of the diosgenin samples were obtained from Nujol mulls, using a Perkin-Elmer 257 spectrophotometer. (*c*) Nuclear magnetic resonance spectra of solutions of diosgenin in chloroform were determined using a Perkin-Elmer R10 spectrometer, utilizing a computer-assisted technique. (*d*) Thin-layer chromatograms were obtained on air-dried Silica Gel G (Merck) layers, 250 μ thick. Two-way chromatograms were prepared using chloroform-acetone (3:1 v/v) as the developing solvent for the first direction and *n*-hexane–ethyl acetate (3:1 v/v) for the second way. Development in each direction was for 15 cm. from the point of application of the test solution. The compounds were

Received September 25, 1967, from the School of Pharmacy, College of Technology, Portsmouth, Hampshire, England.
Accepted for publication December 20, 1967.
Presented to the Pharmacognosy Section at the 27th Congrés International des Sciencies Pharmaceutiques, Montpellier, France, September 1967.

detected by spraying the plates with a 300% w/v antimony trichloride solution in concentrated hydrochloric acid (7) and heating at 100° for 10 min. (*e*) Ultraviolet spectra of solutions of the sapogenin in chloroform were determined between 250 and 450 mμ using a Unicam SP.800 recording spectrophotometer. (*f*) Melting points were determined on dried material and were uncorrected. (*g*) Quantitative estimations of the diosgenin content of the samples were effected by the densitometric thin-layer chromatographic method of Blunden, Hardman, and Morrison (2). (*h*) Gas–liquid chromatograms were obtained by use of a Perkin-Elmer F11 instrument, stationary phase silicone gum rubber E-301, support A.W.-D.M.C.S. Chromosorb G 80-100 mesh $2^1/_2$: $97^1/_2$. The dual glass columns were maintained at 230°, the injection block temperature was 220°, and the nitrogen flow rate 55 ml. $min.^{-1}$. A standard Perkin-Elmer flame ionization detector was used. Signal amplification was 1×10^2, and sample volume 3 μl. of a 0.4% w/v solution.

RESULTS AND DISCUSSION

Of the techniques used to follow the decomposition of diosgenin, measurement of optical rotation proved of no utility, as no significant change in this property was detected in any sample. This indicates that groups immediately adjacent to the optical center are unaffected by the decomposition process.

The infrared and nuclear magnetic resonance spectra showed marginal changes in some samples, but in general, these techniques seem of little use in examining unresolved decomposition mixtures of diosgenin.

Thin-layer chromatography proved a most useful technique for following decomposition of the steroid. The diosgenin sample that had been stored in contact with 2 *N* hydrochloric acid, when examined by TLC, showed only two antimony trichloride positive spots, one being unchanged diosgenin and the second being a faint spot of high R_f value, which cochromatographed with 25α-spirosten-3,5-diene (Fig. 1). The chromatographic picture differed from that of a sample of diosgenin that had been refluxed with 2 *N* hydrochloric acid for 2 hr., before TLC examination (Fig. 2). These results indicate that the mechanism of decomposition resulting from refluxing diosgenin with acid may be different from that

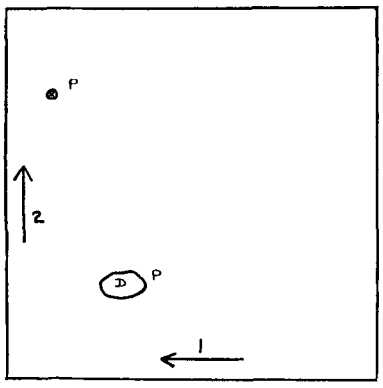

Fig. 1—TLC of diosgenin stored in contact with 2N HCl for 4 months. Solvent system: (1) chloroform–acetone (3:1 v/v; (2) n-hexane–ethyl acetate (3:1 v/v). Key: D, diosgenin; X, 25α-spirosten-3,5-diene; colors produced, p, purple.

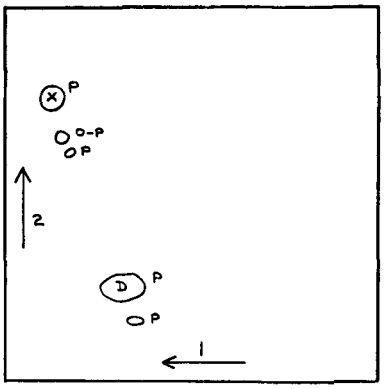

Fig. 2—TLC of diosgenin after 2 hr. reflux with 2 N HCl. Solvent system: (1) chloroform–acetone (3:1 v/v), (2) n-hexane–ethyl acetate (3:1 v/v). Key: D, diosgenin; X, 25α-spirosten-3,5-diene; colors produced, op, orange purple; p, purple.

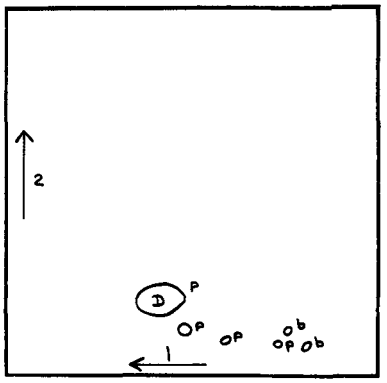

Fig. 3—TLC of diosgenin stored in chloroform solution for 4 months. Solvent system: (1) chloroform–acetone (3:1 v/v), (2) n-hexane–ethyl acetate (3:1 v/v). Key: D, diosgenin; colors of spots, p, purple; b, blue.

operating when the compound is stored in acid at 20°. Moreover, these mechanisms appear to be different from that operating in the other test samples, in which no trace of 25α-spirosten-3,5-diene was detected. Thin-layer chromatograms prepared from diosgenin samples stored in the solid state and as solutions in chloroform and benzene were very similar (Fig. 3).

Further evidence of decomposition was obtained from the ultraviolet spectra. absorbance in the region of 250–350 mμ increasing during the storage period; no peaks were detected in the 350–450 mμ region During the decomposition process the absorbance in the 250 mμ and 285 mμ region increased. The ultraviolet spectrum of diosgenin stored in chloroform is markedly different from that of the drug stored in contact with acid or of pure diosgenin (Fig. 4). This is further evidence of differing decomposition routes. The ultraviolet spectrum of diosgenin with decomposition products is similar to that of 25α-spirosten, 3,5,-diene prepared synthetically. However, measurement in the critical 230–250 mμ region was not possible since the chloroform "cut-off" wavelength is about 245 mμ. The diene was not detected by thin-layer chromatography in diosgenin decomposition samples and thus its extent of formation may be presumed to be very low.

The melting point of decomposition samples de-

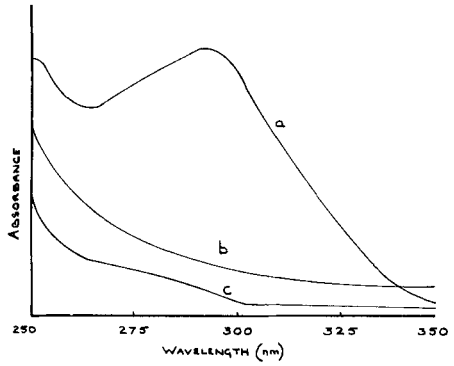

Fig. 4—Ultraviolet spectra of (a) *diosgenin stored in chloroform,* (b) *stored in contact with 2* N *hydrochloric acid, and* (c) *pure diosgenin.*

creased progressively from 204–205° to about 193°, but then remained stationary.

Quantitative TLC studies showed that, at the end of 4 months, the diosgenin had decomposed by an amount that was insignificant in all samples except the one that had been in contact with acid, of which approximately 40% had decomposed (Table I). The quantitative results obtained were subject to an experimental error of ±7% (2). At first sight this result is somewhat surprising, since all samples assayed after 4 months showed several decomposition compounds. However, antimony trichloride solution is a sensitive locating reagent for steroids, able to detect minute quantities of material. From this it was apparent that evaluation of qualitative thin-layer chromatograms of diosgenin for decomposition could be misleading since, even though several decomposition products may be present, the extent of the decomposition may be negligible.

Results obtained from GLC studies also indicated that the extent of decomposition was very low. Thus 4-month-old solid samples of diosgenin, as well as solutions in both chloroform and benzene, showed only two very small signals in addition to the diosgenin peak (Fig. 5). Apparent retention times were diosgenin 29 min. and decomposition peaks 18 and 38 min. Decomposition signals are obviously insignificant in comparison with the unreacted diosgenin.

The effect of temperature and light on the decomposition did not appear important. Qualitatively very similar chromatograms were obtained from all decomposition samples, and no evidence was obtained of photo or thermal effects on the rate of decomposition.

TABLE I—DIOSGENIN DECOMPOSITION AFTER DIFFERENT TIME INTERVALS

Diosgenin Samples	Diosgenin Yield—Percent of Original After 1 month	2 months	3 months	4 months
Solution in benzene[a]	98.9	100.5	98.5	99.1
Solution in chloroform[a]	100.3	100.8	98.7	97.6
Solid[a]	99.5	97.8	100.1	98.5
In contact with 2 *N* HCl[b]	...	...	...	60.0
In contact with 2 *N* Na_2CO_3[b]	...	...	...	101.1

[a] Samples stored in the light at 37°. [b] Samples stored in the light at 20°.

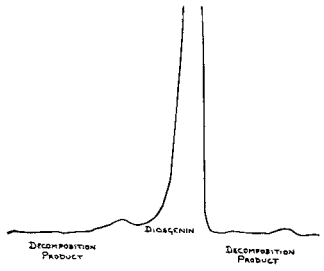

Fig. 5—GLC of diosgenin stored in chloroform for 4 months.

In this study it has been demonstrated that diosgenin rapidly decomposes in contact with hydrochloric acid. During the extraction process of diosgenin from plant sources, it is obvious that after hydrolysis of the naturally occurring saponins to liberate diosgenin, it is imperative to remove the excess acid before processing or transportation of the acid-insoluble residue. The process of neutralizing any residual acid with a base, such as sodium carbonate solution, is a sensible precaution as it does not cause any significant deterioration of diosgenin, even when the two are stored together for 4 months.

When the steroidal constituents of *Dioscorea* species have been studied by paper and TLC, many different spots have been detected. *Dioscorea* species contain both sterols and saponins and as sterols are known to decompose both in the solid state and in solution (8, 9), it will be certain that many of the spots produced on chromatograms of *Dioscorea* extracts will have been produced either during the acid treatment of the plant material to liberate the sapogenins or the subsequent decomposition of the steroidal constituents, in the solid state or in solution (10).

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Keyphrases

Diosgenin stability
Hydrochloric acid—diosgenin solid
Sodium carbonate solution—diosgenin solid
Chloroform—diosgenin solution
Benzene—diosgenin solution
TLC—analysis, decomposition
UV spectrophotometry—analysis, decomposition
GLC—analysis, decomposition

Interaction of 8-Hydroxyquinoline Sulfate with Components Present in a Tuberculin PPD Solution II

Effect of 8-Hydroxyquinoline on Metallic Impurities

By S. LANDI and H. R. HELD

The presence of 8-hydroxyquinoline sulfate (8-HQS) as a preservative in tuberculin purified protein derivative (PPD) solutions leads to the formation of a dark crystalline deposit. In this investigation it has been shown that the deposit was due to chelates resulting from the reaction of metals with 8-hydroxyquinoline (8-HQ) liberated from its sulfate. The metal impurities in tuberculin solution originate mainly from the buffer salts and the PPD. The removal of these metal impurities from the tuberculin PPD did not affect the potency of the preparation. Previous workers have shown the need to maintain the metal content within fairly narrow limits in order to render 8-HQ effective as a preservative. The necessity to keep the metal content within narrow limits in order to render the preservative effective, and to avoid the development of organo-metallic deposits, was considered an important drawback to its usefulness as a preservative.

8-HYDROXYQUINOLINE SULFATE (8-HQS)[1] is added to tuberculin solutions as an antimicrobial agent. These solutions are prepared by adding PPD (10, 50, or 1,000 TU[2]/ml.), polysorbate 80[3] (0.005%), and 8-HQS (0.01%) to a buffered solution of pH 7.38 (1). These solutions are perfectly clear when freshly prepared. However, upon storage at 5° in a 0.5 L. bulk, these solutions showed some turbidity which was accompanied by a dark deposit. Furthermore, after dispensing 1- and 5-ml. volumes of the original clear solutions in vials or ampuls, there was no turbidity, but on careful observation, a few tiny dark particles were present after approximately 6 months' storage at 5° or at 37°.

The authors wished to establish the reason for the appearance of this undesirable deposit and, therefore, attempted to determine which substances took part in its formation, and if the presence of such deposit would affect the biological potency of the tuberculin preparation.

MATERIALS AND METHODS

Reagents—8-Hydroxyquinoline sulfate (8-HQS) (Eastman Organic Chemicals, 1776); 8-hydroxyquinoline (8-HQ) (Fisher Scientific Co., 0-261); 8-hydroxyquinoline zinc salt (8-HQZn) (Eastman Organic Chemicals, 7884); polysorbate 80 (polyoxyethylene 20 sorbitan monooleate); chloroform (Fisher Scientific Co., C-298).

8-Hydroxyquinoline Iron Salt (8-HQFe)—8-HQFe was prepared by dissolving 4.35 Gm. of 8-HQ in 14 L. of distilled water and adding dropwise, while stirring, a solution of 2.70 Gm. $FeCl_3 \cdot 6H_2O$ in 50 ml. H_2O. The black precipitate was filtered on Whatman No. 1 paper and dried in a vacuum desiccator over $CaCl_2$.

8-Hydroxyquinoline Aluminum Salt (8-HQAl) and 8-Hydroxyquinoline Copper Salt (8-HQCu)—These metal chelates were prepared by adding an excess of 8-HQS (10% solution) to 1 L. of a millimolar aqueous solution of $AlCl_3$, respectively, $CuSO_4$ (reagent grade chemicals) and neutralization of the mixture at pH 7–8 by the dropwise addition of concentrated ammonia. The yellowish-green precipitates were filtered on a Millipore filter, washed thoroughly with distilled water, and vacuum dried over $CaCl_2$.

Tuberculin PPD (Trichloroacetic Acid Precipitate) and Tuberculin PPD (Ammonium Sulfate Precipitate)—Both methods of preparation and analysis of the tuberculin PPD preparations were previously carried out (2).

Buffer Solution (pH 7.38)—Isotonic phosphate-buffered solution, pH 7.38 (1.45 Gm. of KH_2PO_4, 7.60 Gm. of Na_2HPO_4, and 4.80 Gm. of NaCl in 1050 ml.). This buffer is also used as diluent for the preparation of tuberculin PPD solution (Mantoux) (1). Distilled water from a metal Barnstead still was used to prepare the buffer solution.

Isolation of a Dark Deposit from Tuberculin PPD Solution (0.01% 8-HQS; 0.005% polysorbate 80)—Five-hundred milliliters of a tuberculin PPD solution (1,000 TU/ml.) which had developed a dark deposit during 9 months' storage at 5°, was filtered through a Millipore TH filter[4] (pore size 0.45 μ).

Received April 5, 1967, from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario, Canada.

Accepted for publication November 13, 1967.

The authors wish to express their thanks to Mr. D. G. Glass for recording the infrared spectra and to Mrs. R. L. McClure for her cooperation in the statistical analyses of data. We also wish to thank Dr. H. D. Sharma, Department of Chemical Engineering, University of Toronto, for carrying out neutron activation analysis on the samples.

[1] Marketed as Chinosol.

[2] The biological potency of tuberculin is expressed in tuberculin units (TU). One TU is biologically equivalent to 0.00002 mg. of the international standard tuberculin PPD.

[3] Marketed as Tween 80 by Atlas Chemical Industries, Wilmington, Del.

[4] Obtained from Millipore Filter Corporation, Bedford, Mass.

The crystalline greenish black deposit was washed with distilled water and dried together with the filter in a vacuum desiccator over $CaCl_2$.

Twelve milliliters of a tuberculin PPD solution (2 mg. PPD/ml.) was prepared and sterilized by filtration. An aliquot of the solution was transferred into a glass-stoppered Pyrex tube and 8-HQS (0.01% final concentration) was added to the remainder of the solution. Both solutions were stored at 5° for 3 weeks. During this time a black deposit (0.3 mg.) had formed in the solution containing 8-HQS. The deposit was removed by filtration of the solution through a Gelman GA-6 filter[5] (0.45 μ) and the filtrate as well as the original solution without 8-HQS were tested for biological potency.

One-milliliter ampuls (capacity 1.5 ml.) filled with 1ml. tuberculin PPD solution (50 TU/ml.) and stored at 5° over a period of 3.5 years contained tiny black specks. The contents of 350 ampuls were filtered through a Gelman GA-6 filter, and the black specks retained on the filter were washed thoroughly with water containing 0.005% polysorbate 80. Filter and deposit were dried in a vacuum desiccator over $CaCl_2$. The weight of the greenish black deposit was 11 mg.

One-milliliter vials (capacity 1.7 ml.) filled with 1 ml. tuberculin PPD solution (50 TU/ml.) and closed with equilibrated[6] white rubber stoppers[7] and sealed with three-piece aluminum caps[8] were stored for 2.5 years at 10°. During storage a greenish dark deposit developed that was isolated by filtering the contents of 770 vials as described above for ampuls. The weight of the greenish black deposit was 11.4 mg.

Ultraviolet Spectrophotometry—The samples were dissolved in chloroform (approximately 4 to 8 mcg./ml.), and the ultraviolet absorption spectrum was determined with a Beckman model DU spectrophotometer using chloroform as a blank.

Infrared Spectrophotometry—KBr pellets of the samples were prepared and the infrared absorption spectra were recorded from 650 to 5,000 $cm.^{-1}$ using a Beckman model IR-4 spectrophotometer fitted with NaCl optics.

Neutron Activation Analysis—The Millipore filter with the dark deposit from tuberculin PPD solution (1,000 TU/ml.) and a Millipore filter used as a blank were sealed in quartz tubes and irradiated with neutrons for 2 days in an atomic reactor. After activation, the filters were taken out of the tubing and examined with a multichannel pulse height γ-ray spectrometer. γ-Ray spectra were taken at intervals, as the activated sample decayed during a 3-month period.

Biological Testing—The tuberculin preparations to be compared were diluted in a buffer solution (pH 7.38, containing 0.005% polysorbate 80) to give the following two concentrations, 0.004 and 0.001 mg./ml. The dose injected intradermally into 20 BCG-sensitized guinea pigs was 0.1 ml. at each of these concentrations as previously described by Landi (1). At 24 hr. after injection the diameter of each reaction zone was measured, and the result was recorded as the sum of two perpendicular diameters in millimeters. The estimate of potency was made by the method of Long, Miles, and Perry (4).

The dark deposit obtained from ampuls and vials was also tested for potency. A solution of the deposit was prepared by dissolving 1 mg. of the deposit in 1 ml. 0.02 *N* HCl and further diluting it to the following two concentrations, 0.004 mg./ml. and 0.001 mg./ml. by the addition of buffer (pH 7.38; 0.005% polysorbate 80); 0.1 ml. of these concentrations and 0.1 ml. of the buffer used to dilute the dark deposit were injected intradermally and at random into 16 BCG-sensitized guinea pigs.

RESULTS AND DISCUSSION

The deposit isolated from tuberculin PPD solutions containing 8-HQS (0.01%) was found to be soluble in chloroform and mineral acids. Microscopic examination of this deposit revealed that it consisted of tiny crystals and crystal fragments (Fig. 1). These crystals gave a bright yellow fluorescence under ultraviolet light (wavelength 360 mμ). In a tuberculin solution some of these crystals were invisible in daylight but their presence was easily revealed under ultraviolet light.

Figure 2 shows the ultraviolet spectra in chloroform of a deposit from tuberculin PPD solution (1,000 TU/ml.; 0.01% 8-HQS; 0.005% polysorbate 80) and for 8-HQFe, 8-HQZn, and 8-HQ. The steep absorption maximum at 260 mμ (Fig. 2, curve A) obtained with the deposit is in close agreement with the maximum at 256 mμ (8-HQFe), 258 mμ (8-HQZn) (Fig. 2, curves B and C, respectively), 260 mμ (8-HQAl), and 263 mμ (8-HQCu), while 8-HQ has a maximum in chloroform at 243 mμ (Fig. 2, curve D). The 8-HQ metal complexes served as controls. Other UV light absorbing substances such as tuberculoprotein with an absorption maximum at approximately 270 mμ and nucleic acid at approximately 260 mμ present in the tuberculin solution could not be considered as part of the deposit. These substances are insoluble in chloroform while the ingredients in the deposit were completely soluble in this organic solvent. The only other UV-absorbing and chloroform-soluble ingredient of tuberculin solution, which could be present in the deposit, was polysorbate 80; however, this nonionic surfactant has a very flat UV absorption maximum at a wavelength of 230 mμ. Therefore, polysorbate 80 certainly could not account for the steep absorption maximum of 260 mμ given by the deposit.

To verify these findings the infrared spectra (Fig. 3) of the following substances were compared: (A) the deposit which formed in a tuberculin PPD solution (1,000 TU/ml.; 0.01% 8-HQS; 0.005% polysorbate 80); (B) 8-HQFe; (C) 8-HQZn; (D) 8-HQ; (E) 8-HQS, (F) polysorbate 80; (G), nucleic acid from filtrate of *M. tuberculosis* var. *hominis*, (H) tuberculin PPD (trichloroacetic acid precipitation); (I) tuberculin PPD (ammonium sulfate precipitation). Figure 3 shows that only the IR spectra of 8-HQFe and 8-HQZn are similar to the IR-spectrum of the deposit. The substances D to I cannot constitute a major component of the deposit because their following distinct peaks are missing in the spectrum of the deposit (wave numbers in $cm.^{-1}$ Fig. 3): 897, 974, 1,900 for 8-HQ; 888, 993, 1,192, 1,400, 1,595, 3,000 for 8-HQS; 945, 1,000–1,200,

[5] Obtained from Gelman Instrument Company, Ann Arbor, Mich.
[6] Equilibrated against buffer solution of pH 7.38 (0.01% 8-HQS final concentration) (3).
[7] Designation V-32-86, obtained from The West Company, Phoenixville, Pa.
[8] Designation 13-31, obtained from The West Company, Phoenixville, Pa.

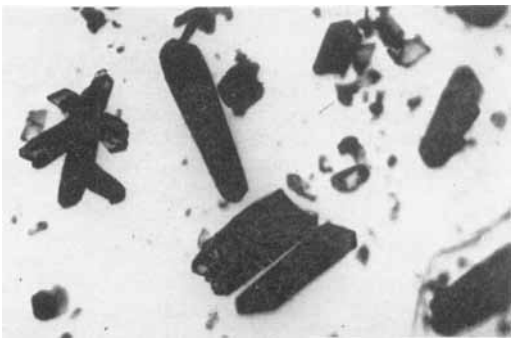

Fig. 1—Greenish black crystals and crystal fragments from ampuls (50 TU/ml.; 0.01% 8-HQS; 0.005% polysorbate 80). Magnification approx. 200×.

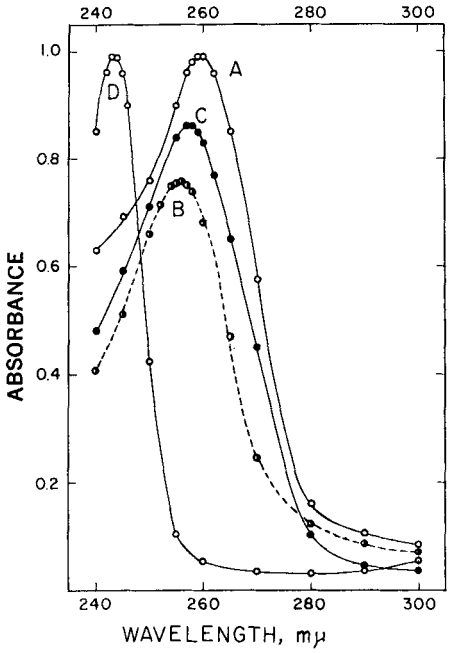

Fig. 2—Ultraviolet absorption spectra in chloroform. Key: A, *dark deposit from tuberculin PPD solution;* B, *8-HQFe;* C, *8-HQZn;* D, *8-HQ.*

1,726, 2,920 for polysorbate 80; 1,000–1,150, 1,200–1,280, 1,580–1,730 for nucleic acid; 1,010–1,130, 1,200–1,260, 1,600–1,730 for tuberculin PPD (trichloroacetic acid precipitation); and 1,520–1,570, 1,600–1,680 for tuberculin PPD (ammonium sulfate precipitation).

The IR spectra (Fig. 3) do not give any clue as to which metals have taken part in the formation of the deposit, since the IR spectra of 8-quinolinolates of different metals are very similar to each other according to Charles *et al.* (5) and Stone (6). Due to the extremely small amount of residue collected on a Millipore filter the deposit was tested for metals on the filter itself by the neutron activation analysis technique. Among the metals which can be determined by this technique only iron, zinc, and sodium were found in appreciable amounts (7.0% Fe, 1.6% Zn, and 0.6% Na), whereas other metals (antimony, cobalt, copper, chromium, gold, lanthanum, manganese) were present in trace amounts only (Table I).

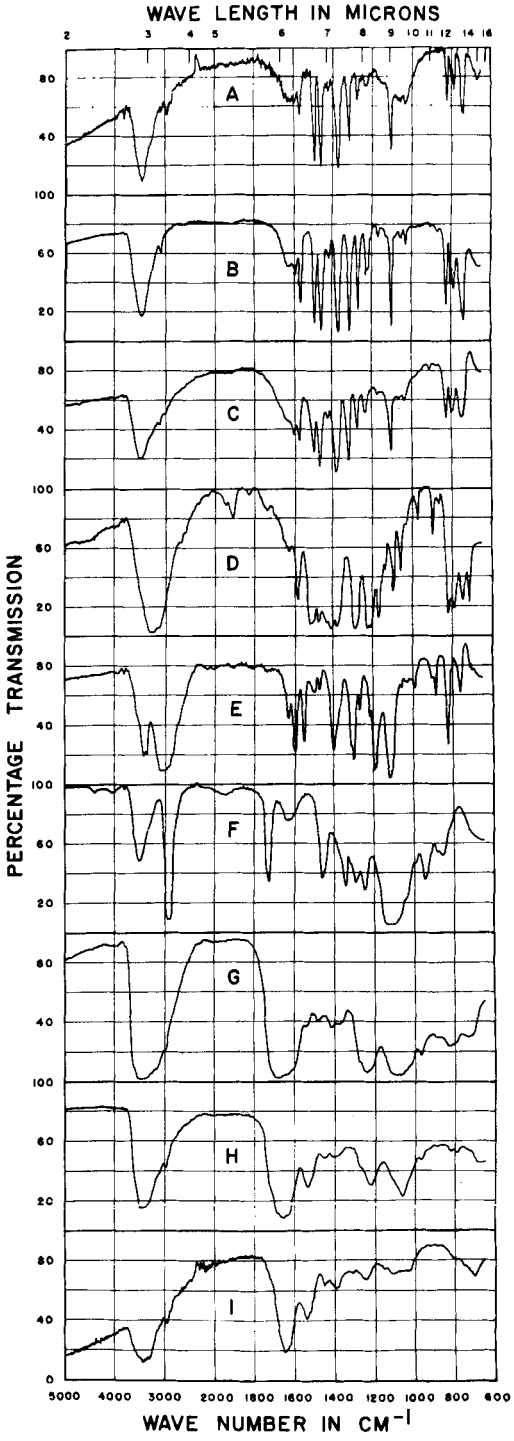

Fig. 3—Infrared absorption spectra of components from tuberculin PPD solution. Key: A, *dark deposit from tuberculin PPD solution;* B, *8-HQFe;* C, *8-HQZn;* D, *8-HQ;* E, *8-HQS;* F, *polysorbate 80;* G, *nucleic acid, prepared from filtrate of* M. tuberculosis *var.* hominis, H, *tuberculin PPD (trichloroacetic acid precipitation);* I, *tuberculin PPD (ammonium sulphate precipitation).*

TABLE I—ACTIVATION ANALYSIS ON A DARK DEPOSIT FROM TUBERCULIN PPD SOLUTION[a]

Metal[b]	Amount, %
Iron	7.03
Zinc	1.60
Sodium	0.62
Copper	0.36
Chromium	5.13×10^{-3}
Antimony	3.36×10^{-4}
Gold	2.97×10^{-4}
Cobalt	1.70×10^{-4}
Scandium	0.58×10^{-4}
Manganese	Trace
Lanthanum	Trace
Bromine	Trace

[a] The deposit had formed from 500 ml. tuberculin PPD solution (1,000 TU/ml.; 0.01% 8-HQS; 0.005% polysorbate 80) during 9 months' storage at 5°. [b] Looked for and not detected: Rb, Cs, Hg, Pt metals (all judged to be less than 1 p.p.m.); Cd, Mo, As, Ag, Ge (all judged to be less than 5 p.p.m.). The activation technique is not sensitive for the detection of Ca, Al, Pb, Si, S, P, I, Ni, Mg, and other light elements.

The above findings strongly suggest that chelation between 8-HQ and the metals present in tuberculin PPD solutions has taken place. In fact it is well known that traces of heavy metals are present in reagent grade chemicals (7, 8) and in the tuberculoprotein (2) used to prepare tuberculin PPD solutions. It is also known that when 8-HQS is added to a buffered solution (pH 7.38) it is dissociated into 8-HQ and H_2SO_4 (3, 9). 8-HQ is one of the best analytical reagents for metals (10–12) since it readily forms chelates which are of extremely low solubility. It is therefore not surprising that chelation could take place in tuberculin PPD solutions, when 8-HQS is employed as a preservative. Therefore, when tuberculin solutions, at pH 7.38, are stored in the bulk form (0.5 L. or more), chelation will at first render the solution slightly turbid and upon further storage a deposit will be formed which will settle at the bottom of the container. Magnusson and Bentzon (13) and Jensen *et al.* (14) reported a similar dark deposit which had formed in tuberculin PPD stock solution containing 8-HQS.

In order to verify to what extent the ingredients present in tuberculin PPD solutions contribute to the formation of the dark deposit, the following experiments were carried out. Solutions of phosphate-buffered saline (pH 7.38) containing 0.005% polysorbate 80 and 0.01% 8-HQS, and other solutions containing PPD in concentrations of 10, 50, and 1,000 TU/ml., respectively, in addition to polysorbate 80 and 8-HQS, were prepared. One liter of each solution was stored at 5° in glass-stoppered volumetric flasks. During a period of 9 months' storage, a deposit appeared in all the solutions. However, in those containing PPD the amount of deposit increased with increasing quantities of PPD. The distilled water used to prepare the buffered solution was only a minor contributor of metals. The distilled water from a metal Barnstead still may contain up to a total of 0.1 p.p.m. heavy metals according to Healy *et al.* (15). To determine if polysorbate contributed to the formation of the deposit, 0.01% 8-HQS was added to a buffered solution (pH 7.38) with 0.005% polysorbate 80 and without polysorbate 80. These two solutions were stored for 9 months at 37°. In both solutions an equal amount of dark deposit was formed indicating that the addition of polysorbate 80 had no noticeable influence on the amount of deposit present in these solutions.

Besides the metals present in the tuberculin PPD solution, a minor metal contribution could be made by the glass of the container and in the case of vials, by the rubber stopper and the metal seal. For instance when tuberculin PPD solutions are dispensed in multiple-dose vials sealed with equilibrated rubber stoppers (equilibrated against 100 p.p.m. 8-HQS) (3), these closures will influence the amount of deposit formed in these tuberculin solutions according to a "push-pull" mechanism engendered by the partition coefficients of the metal chelates between the tuberculin solutions and the rubber closures. As described previously (3), during equilibration of a rubber stopper against a buffered solution (pH 7.38) containing 100 p.p.m. 8-HQS, its base 8-HQ will distribute itself between rubber and aqueous solutions according to its partition coefficient. When 8-HQ enters the rubber, it reacts chemically with the heavy metal ions present in the rubber whereby the metals chelate with 8-HQ. These metal chelates have their own partition coefficients between the organic phase (rubber stopper) and the aqueous phase. Consequently, when rubber stoppers are used the chelates in the stopper and in the solution distribute themselves according to their partition coefficients until an equilibrium is reached. This may contribute to the formation of a deposit which may not necessarily be dark as the color of 8-HQ chelates from different metals varies from nearly colorless to black.

In order to find out if the removal of metals from tuberculin PPD would affect the potency of the preparation, the potency of a tuberculin solution (2 mg. PPD/ml. at pH 7.38) was compared with the potency of the same solution after removal of metal chelates which formed after addition of 8-HQS. It was found that the relative potency of the tuberculin solution after removal of metals and compared with the same tuberculin solution before removal of metals was 1.05 (0.89–1.24).[9] This relative potency is not significantly different from 1.00 and therefore there is no evidence of a loss or of an increase in potency when metal chelates are removed.

Skin tests were also carried out in BCG-sensitized guinea pigs using the dark greenish deposit isolated from vials and ampuls. No tuberculin skin reaction could be detected in the animals indicating that there was no tuberculin PPD present in either deposit.

Formation of an insoluble deposit is undesirable in pharmaceutical solutions and therefore represents a considerable drawback to the use of the preservative 8-HQS in tuberculin PPD solutions. The formation of such deposit could be avoided by (*a*) pretreating a stronger buffer solution (pH 7.38), for instance 10 times its usual strength with 0.01% 8-HQS, then filtering the metal chelates out and diluting the clear filtrate with distilled water containing 0.01% 8-HQS to the required buffer strength to be used to prepare tuberculin PPD solutions; the same treatment could be carried out on a more concentrated (2 mg./ml.) tuberculin PPD solution; (*b*) using closures with low metal content when dispensing tuberculin solutions into vials.

On the other hand, the complete exclusion of metals would not be desirable because 8-HQ is markedly antimicrobial only in the presence of

[9] Figures in brackets represent 95% confidence limits.

traces of certain heavy metals, *e.g.*, copper (Cu^{2+}), iron (Fe^{3+} or Fe^{2+}) while other metals such as cadmium, zinc, nickel, and especially cobalt antagonize the antimicrobial properties of 8-HQ (7, 16, 17).

These incompatibilities render this preservative (8-HQS) cumbersome to use in tuberculin PPD solutions.

SUMMARY

1. It has been shown that tuberculin PPD solutions to which 0.01% 8-HQS had been added as preservative, developed a crystalline deposit when stored for several months.

2. The deposit was found to consist of metal chelates of 8-HQ in which the metals involved were iron, zinc, sodium, and traces of some other metals.

3. The main sources of metals causing precipitation were found to be the reagent grade chemicals constituting the buffer and the PPD used to prepare the tuberculin solutions; a minor but still significant source was the distilled water.

4. The removal of metals from tuberculin PPD caused by the presence of the preservative 8-HQS did not affect the biological potency of the preparation and tuberculin PPD was not present in the deposit.

5. The tendency of the preservative, 8-HQS, to give rise to an organo-metallic deposit in tuberculin PPD solutions, is a considerable drawback. Especially so, since a trace of iron or copper has to be present in order to render this preservative effective.

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Keyphrases

Tuberculin solution—stability
Preservative—tuberculin solution
8-Hydroxyquinoline SO_4—preservative
Metal–8-hydroxyquinoline chelates—activity effect
Biological assay—sensitivity
UV spectrophotometry—identity, chelate
IR spectrophotometry—identity, chelate
Neutron activation analysis—metal identity

Microbiological Evaluation of PCMX Complexes

By M. D. RAY*, K. E. AVIS, and C. C. FLANIGAN, JR.

The minimum inhibitory concentration of *p*-chloro-*m*-xylenol (PCMX) was established against *Bacillus subtilis, Pseudomonas aeruginosa,* and *Aspergillus niger,* using a serial dilution and a pour plate procedure. The inoculum size was standardized by absorbance of light at 450 mμ wavelength. The drug was subsequently complexed with methylcellulose, polyethylene glycol 6000, and polysorbate 80. A minimum inhibitory concentration for the drug against each of the microorganisms was established in the presence of varying concentrations of the macromolecules. The results demonstrated that in most cases the antimicrobial activity of PCMX was reduced in the presence of the macromolecules studied. Since it had been previously established that PCMX interacted with these molecules, it was concluded that the reduction in biological activity was a direct result of the molecular interaction.

PHENOL AND ITS DERIVATIVES have served a major function in antisepsis and disinfection since the introduction of phenol by Lister (1) in aseptic surgery. Although the value of these agents is widely acknowledged, there has been an extensive search in recent years for phenolic derivatives which have an increased spectrum of microbicidal activity with less toxicity to tissue. Considerable interest has been generated in this country and in Europe in the use of *p*-chloro-*m*-

Received August 22, 1967, from the Department of Pharmaceutics College of Pharmacy, University of Tennessee, Memphis, TN 38103
Accepted for publication December 19, 1967.
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traces of certain heavy metals, *e.g.*, copper (Cu^{2+}), iron (Fe^{3+} or Fe^{2+}) while other metals such as cadmium, zinc, nickel, and especially cobalt antagonize the antimicrobial properties of 8-HQ (7, 16, 17).

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1. It has been shown that tuberculin PPD solutions to which 0.01% 8-HQS had been added as preservative, developed a crystalline deposit when stored for several months.

2. The deposit was found to consist of metal chelates of 8-HQ in which the metals involved were iron, zinc, sodium, and traces of some other metals.

3. The main sources of metals causing precipitation were found to be the reagent grade chemicals constituting the buffer and the PPD used to prepare the tuberculin solutions; a minor but still significant source was the distilled water.

4. The removal of metals from tuberculin PPD caused by the presence of the preservative 8-HQS did not affect the biological potency of the preparation and tuberculin PPD was not present in the deposit.

5. The tendency of the preservative, 8-HQS, to give rise to an organo-metallic deposit in tuberculin PPD solutions, is a considerable drawback. Especially so, since a trace of iron or copper has to be present in order to render this preservative effective.

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Keyphrases

Tuberculin solution—stability
Preservative—tuberculin solution
8-Hydroxyquinoline SO_4—preservative
Metal–8-hydroxyquinoline chelates—activity effect
Biological assay—sensitivity
UV spectrophotometry—identity, chelate
IR spectrophotometry—identity, chelate
Neutron activation analysis—metal identity

Microbiological Evaluation of PCMX Complexes

By M. D. RAY*, K. E. AVIS, and C. C. FLANIGAN, JR.

The minimum inhibitory concentration of *p*-chloro-*m*-xylenol (PCMX) was established against *Bacillus subtilis, Pseudomonas aeruginosa,* and *Aspergillus niger,* using a serial dilution and a pour plate procedure. The inoculum size was standardized by absorbance of light at 450 mμ wavelength. The drug was subsequently complexed with methylcellulose, polyethylene glycol 6000, and polysorbate 80. A minimum inhibitory concentration for the drug against each of the microorganisms was established in the presence of varying concentrations of the macromolecules. The results demonstrated that in most cases the antimicrobial activity of PCMX was reduced in the presence of the macromolecules studied. Since it had been previously established that PCMX interacted with these molecules, it was concluded that the reduction in biological activity was a direct result of the molecular interaction.

PHENOL AND ITS DERIVATIVES have served a major function in antisepsis and disinfection since the introduction of phenol by Lister (1) in aseptic surgery. Although the value of these agents is widely acknowledged, there has been an extensive search in recent years for phenolic derivatives which have an increased spectrum of microbicidal activity with less toxicity to tissue. Considerable interest has been generated in this country and in Europe in the use of *p*-chloro-*m*-

Received August 22, 1967, from the Department of Pharmaceutics College of Pharmacy, University of Tennessee, Memphis, TN 38103
Accepted for publication December 19, 1967.
Abstracted from a thesis submitted by Max D. Ray to the Graduate School—Medical Sciences, University of Tennessee, Memphis, in partial fulfillment of Master of Science degree requirements.
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xylenol (PCMX) as an antiseptic in external pharmaceutical products (2–14), as a disinfectant in cleaning solutions (15), and as a preservative for pharmaceutical and nonpharmaceutical commodities (16). Reports have also indicated that PCMX has a very low toxicity to tissue (17–22).

A recent investigation has shown, however, that PCMX reacts with various nonionic macromolecules often present in formulations to form complexes (23). The present investigation was undertaken to explore the effect of such interactions on the biological activity of the agent.

EXPERIMENTAL

Reagents—Recrystallized PCMX,[1] m.p. 115–115.5°; polyethylene glycol 6000[2]; methylcellulose 15 cps., USP[3]; polysorbate 80,[4] and Sabouraud medium.[5]

Microorganisms—*Bacillus subtilis* ATCC 6051, *Pseudomonas aeruginosa* ATCC 10145, and *Aspergillus niger* ATCC 6275.

Apparatus—Beckman spectrophotometer model DB, Bausch and Lomb spectronic 20, Thomas-Hoover melting point apparatus, Precision Scientific Company Thelco oven, constant-temperature water bath.

Procedures

Determination of Cell Count—The microorganisms used in this investigation—*B. subtilis*, *Ps. aeruginosa*, and *A. niger*—were selected because each had a previously reported sensitivity to PCMX and because they represented three general groups of microorganisms, Gram-positive bacteria, Gram-negative bacteria, and fungi, respectively. Sabouraud liquid medium and Sabouraud agar medium were employed as culture media throughout the investigation. The authors felt it advisable to use the same culture media for each organism so that any binding between the PCMX and culture media would be uniform throughout the study. Although, admittedly, bacteria do grow better in other media, Sabouraud media were selected in deference to the more fastidious *A. niger*. It was shown by controls that the bacteria used in this study did grow quite well in Sabouraud liquid medium and Sabouraud agar medium.

A rapid and accurate method for determining cell counts was adopted similar to the light absorption method reported by Guidry and Maier (24). In the author's investigation a loopful of each of the test organisms was transferred at zero time to 50 ml. of sterile liquid medium. Cultures of *B. subtilis* and *Ps. aeruginosa* were incubated at 37°, and the *A. niger* at 27°. After 48 hr. of growth, the suspensions were transferred to a sterile Waring blender bowl and subjected to a dispersion treatment for 30 sec. The dispersed cultures were diluted with a measured volume of nutrient medium, and percent transmittance readings were taken, employing a Bausch and Lomb spectronic 20, at wavelengths of 450 mμ and 500 mμ. Immediately thereafter, a measured aliquot from each dispersed culture was transferred to freshly prepared Sabouraud agar plates and incubated. After 48 hr. the plates were removed and the colonies counted. Several replicate tests were performed in this manner, and a relationship was established between percent transmittance and cell count (see Table I).

Determination of Standard Minimum Inhibitory Concentration (MIC)—An approximate MIC for PCMX against each of the test organisms was determined by inspecting for visible growth of the organisms in a series of concentrations of PCMX in nutrient medium. A standard MIC was subsequently determined by quantitating the growth of each organism in a series of solutions of PCMX having a range which extended above and below the approximate MIC. Replicate tubes of each concentration were inoculated with a measured volume of the standardized microbial suspensions. After incubation for 48 hr., aliquots from each tube were transferred to Sabouraud agar plates. Colony counts were made after a 48-hr. incubation period, comparing this count with the theoretical number that would have been present if all the cells initially introduced into the medium had survived. The effect of PCMX was then evaluated as lethal, inhibitory, or noninhibitory based upon whether the plates showed no growth, growth of fewer colonies than the theoretical cell count, or growth of considerably more colonies than were representative of the cell count of the initial inoculum.

Determination of MIC of Complexation Mixtures—The PCMX and macromolecule were weighed by difference into a dry volumetric flask. Sufficient liquid nutrient medium was added to attain a prescribed volume. The mixtures were transferred to conical flasks, capped with aluminum foil, and sterilized by autoclaving at 121° for 15 min. After cooling, the solutions were placed in a 30° chamber for 48 hr. to permit complexation to occur, after the manner described by Breuninger and Goettsch (23).

An exception was required for the methylcellulose solutions, because the heat of the sterilization process caused the methylcellulose to be partially dehydrated and precipitated from solution. To correct this, the samples containing methylcellulose were refrigerated for 8 hr. to rehydrate the methylcellulose prior to being placed in the 30° chamber.

A series of concentrations of each macromolecule was prepared, and the antimicrobial activity of PCMX at each of these concentrations was determined. The concentrations employed were: polyethylene glycol 6000–0.22, 0.33, 0.44, 0.88, and

TABLE I—CORRELATION BETWEEN PERCENT TRANSMITTANCE AND VIABLE CELL COUNT

Organism	Wavelength, mμ	Percent, T	Viable Cell Count[a]
Aspergillus niger	450	75	2×10^6
Aspergillus niger	500	87	2×10^6
Pseudomonas aeruginosa	450	35	2×10^7
Pseudomonas aeruginosa	500	42	2×10^7
Bacillus subtilis	450	78	1×10^8
Bacillus subtilis	500	86	1×10^8

[a] Determined by multiplying actual plate colony counts by appropriate dilution factors.

[1] Ottasept Extra. Supplied through the courtesy of Ottawa Chemical Company, Toledo, Ohio.
[2] Fisher Scientific Company, Fair Lawn, N. J.
[3] Methocel 15 cps. Supplied through the courtesy of Dow Chemical Company, Midland, Mich.
[4] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.
[5] Baltimore Biological Laboratory, Inc., Baltimore, Md.

1.32%; methylcellulose—0.5, 1.0, and 1.5%; and polysorbate 80—0.5, 1.0, and 1.5%. A known number of cells, as determined by optical density measurement, was added to each complexation mixture and incubated at the appropriate temperature for 48 hr. At the end of the incubation period an aliquot from each sample was transferred to Sabouraud agar plates. The inoculated plates were examined and the colonies counted after 48 hr. of incubation.

Controls were employed to establish the viability of the organisms, the ability of the nutrient to support the growth of each organism, and the ability of PCMX to inhibit growth of each organism in the absence of the macromolecule.

RESULTS AND DISCUSSION

Methylcellulose Complex—In the presence of methylcellulose, PCMX had less inhibitory effect on the growth of *B. subtilis* and *Ps. aeruginosa*, as reflected in an increase in the MIC (Figs. 1 and 2). The MIC for PCMX against *B. subtilis*, in all the concentrations of methylcellulose studied, was found to be 1:15,000 (0.007%). Against *Ps. aeruginosa* the intensity of the effect of methylcellulose varied with the concentration of the complexing agent. When PCMX was equilibrated with 0.5% methyl-

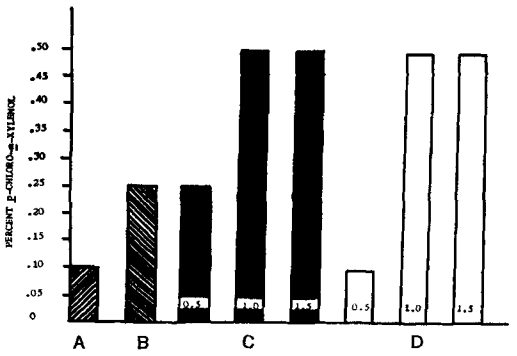

Fig. 1—Effect of complexation on the activity of p-*chloro*-m-*xylenol*—pseudomonas aeruginosa. *Key: A, Minimum inhibitory concentration of unreacted* p-*chloro*-m-*xylenol; B, Minimum inhibitory concentration of* p-*chloro*-m-*xylenol in presence of polyethylene glycol 6000 (0.22-1.32%); C, Minimum inhibitory concentration of* p-*chloro*-m-*xylenol in presence of three concentrations of methylcellulose; D, Minimum inhibitory concentration of* p-*chloro*-m-*xylenol in presence of three concentrations of polysorbate 80.*

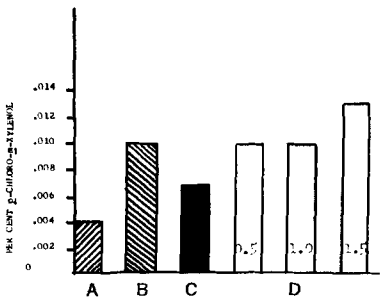

Fig. 2—Effect of complexation on the activity of p-*chloro*-m-*xylenol*—bacillus subtilis. *See Fig. 1 for key.*

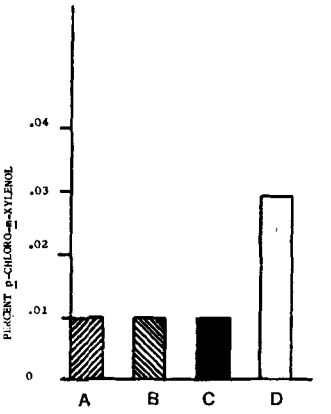

Fig. 3—Effect of complexation on the activity of p-*chloro*-m-*xylenol*—aspergillus niger. *See Fig. 1 for key.*

cellulose, a MIC of 1:400 (0.25%) was observed, as compared with an MIC of 1:1000 (0.1%) in the absence of a complexing agent. With a concentration of 1.0% and 1.5% an MIC of 1:200 (0.5%) was observed.

The activity of PCMX against *A. niger* was apparently unaffected by complexation with methylcellulose (Fig. 3). In all concentrations of methylcellulose studied, an MIC of 1:10,000 (0.01%) was noted, which was the same as the MIC of uncomplexed PCMX against *A. niger*. There was no apparent explanation for this lack of effect, since complexation had occurred in the work of Breuninger and Goettsch (23), although the metabolic effects of the organism on the complex may be a factor.

Through the use of controls it was shown that methylcellulose alone had no stimulatory or inhibitory effect, in the concentration range studied, on any one of the three organisms. Solutions of 0.5, 1.0, and 1.5% methylcellulose in water failed to support the growth of any of the organisms, but did not inhibit the growth in Sabouraud liquid medium. It was concluded therefore that any diminution in the activity of PCMX was a direct result of interaction with methylcellulose.

Polysorbate 80 Complex—The activity of PCMX against all three test organisms was considerably diminished in the presence of polysorbate 80, in the range of concentrations studied. Against *B. subtilis* the MIC of PCMX when complexed with 0.5% and 1.0% polysorbate 80 was found to be 1:10,000 (0.01%), and with 1.5% polysorbate 80 was found to be 1:7,500 (0.015%) (Fig. 2). The MIC for unreacted PCMX against *B. subtilis* was 1:26,000 (0.004%).

There was a uniform reduction in the activity of PCMX against *A. niger* (Fig. 3) at all three concentration levels of polysorbate 80 employed, to an MIC of 1:3300 (0.03%). The MIC of uncomplexed PCMX was 1:10,000 (0.01%).

Complexation of PCMX with 0.5% polysorbate 80 had no effect on the activity of the drug against *Ps. aeruginosa* (Fig. 1), showing an MIC of 1:1000 (0.1%). When equilibrated with 1.0% and 1.5% polysorbate 80, however, PCMX showed a marked reduction in activity, with an MIC of 1:200 (0.5%).

Through the use of suitable controls, it was demonstrated that polysorbate 80 contributed no

stimulatory or inhibitory activity of its own against the three test organisms. Therefore, where polysorbate 80 caused a reduction in the activity of PCMX, it was thought that the reduction was directly attributable to complexation between the agent and the macromolecule, thereby lessening the availability of the drug to exert its antimicrobial activity. Where no inhibition occurred there may have been insufficient polysorbate 80 present.

Polyethylene Glycol 6000 Complex—The activity of PCMX against both *B. subtilis* and *Ps. aeruginosa* was reduced when complexed with polyethylene glycol 6000 (Figs. 1 and 2). The MIC against *Ps. aeruginosa* was found to be 1:400 (0.25%), and against *B. subtilis*, 1:10,000 (0.01%). The MIC for unreacted PCMX against the two organisms was 1:1000 (0.1%) and 1:26,000 (0.004%), respectively.

Polyethylene glycol 6000 did not inhibit the activity of PCMX against *A. niger* in the range of concentrations studied (Fig. 3). This observation, coupled with the fact that methylcellulose had a like effect, suggested the possibility that *A. niger* was capable of disrupting the complex formed with these two macromolecules, thereby restoring the availability of PCMX to exert is antifungal activity, but not the complex formed with polysorbate 80.

Controls similar to those described above for methylcellulose were used to establish the fact that polyethylene glycol 6000 alone had no contributing effect on the growth of the organisms. It was assumed, therefore, that any reduction in the activity of PCMX when complexed with polyethylene glycol 6000 was a direct result of that interaction.

The studies of Breuninger and Goettsch (23) showed that the solubility of PCMX was decreased upon the addition of polyethylene glycol 6000 up to about 0.5%, and that a subsequent increase in the concentration of polyethylene glycol 6000 caused an increase in the solubility of PCMX. In our study, contrary to what might be expected, colony counts of *B. subtilis* and *Ps. aeruginosa* at a polymer concentration of 0.22% were lower than counts at higher concentrations. Polyethylene glycol 6000 had no apparent effect on the activity of PCMX against *A. niger*. Table II shows the effect of polyethylene glycol 6000 on the MIC of PCMX.

Comparison with Equilibrium Dialysis Studies—In the equilibrium dialysis studies of Breuninger and Goettsch, moles of bound PCMX were plotted against moles of unbound PCMX in the presence of 2% methylcellulose and 2% polysorbate 80. The slope for the plot in the presence of methylcellulose was found to be 0.14 and in the presence of polysorbate 80, it was 9.3. This comparison showed that polysorbate 80 had a much greater binding affinity for PCMX than did methylcellulose.

From our data the increase in the required MIC in the presence of the various concentrations of polysorbate 80 and methylcellulose as compared with PCMX alone was calculated. These percentages are shown in Tables III and IV.

A comparison of percent increase in MIC for PCMX–polysorbate 80 complex with PCMX–methylcellulose complex does not reveal a striking parallel with the greater binding affinity that Breuninger and Goettsch found for PCMX-polysorbate 80 complex as compared with PCMX-methylcellulose complex. However, there is a degree of correlation in that the percent increase for PCMX-polysorbate 80 against *B. subtilis* was 150 but only 81 with PCMX-methylcellulose, and against *A. niger* was 200 and 0, respectively. Thus it would appear that with these two organisms the greater binding effect between PCMX and polysorbate 80 resulted in a greater inhibition of activity than with PCMX and methylcellulose.

TABLE II—EFFECT OF POLYETHYLENE GLYCOL 6000 ON THE MINIMUM INHIBITORY CONCENTRATION OF *p*-CHLORO-*m*-XYLENOL

Organism	Percent Polyethylene Glycol 6000	Percent Increase in MIC[a]
Bacillus subtilis	0.22	60
	0.33	60
	0.44	60
	0.88	60
	1.32	60
Pseudomonas aeruginosa	0.22	60
	0.33	60
	0.44	60
	0.88	60
	1.32	60
Aspergillus niger	0.22	0
	0.33	0
	0.44	0
	0.88	0
	1.32	0

[a] MIC = Minimum inhibitory concentration.

TABLE III—EFFECT OF METHYLCELLULOSE ON THE MINIMUM INHIBITORY CONCENTRATION OF *p*-CHLORO-*m*-XYLENOL

Organism	Percent Methylcellulose	Percent Increase in MIC[a]
Bacillus subtilis	0.5	81
	1.0	81
	1.5	81
Pseudomonas aeruginosa	0.5	150
	1.0	400
	1.5	400
Aspergillus niger	0.5	0
	1.0	0
	1.5	0

[a] MIC = Minimum inhibitory concentration.

TABLE IV—EFFECT OF POLYSORBATE 80 ON THE MINIMUM INHIBITORY CONCENTRATION OF *p*-CHLORO-*m*-XYLENOL

Organism	Percent Polysorbate 80	Percent Increase in MIC[a]
Bacillus subtilis	0.5	150
	1.0	150
	1.5	225
Pseudomonas aeruginosa	0.5	0
	1.0	400
	1.5	400
Aspergillus niger	0.5	200
	1.0	200
	1.5	200

[a] MIC = Minimum inhibitory concentration.

SUMMARY AND CONCLUSIONS

In almost all cases the activity of PCMX against three microorganisms, *B. subtilis*, *Ps. aeruginosa*, and *A. niger* was shown to be diminished when allowed to interact with the three macromolecules, polyethylene glycol 6000, methylcellulose, and polysorbate 80, in the concentration ranges studied. Exceptions to this general observation were obtained

with *A. niger* when evaluating the effect of methylcellulose and polyethylene glycol 6000 on PCMX; and the result obtained with *Ps. aeruginosa* in evaluating the effect of polysorbate 80 at a concentration of 0.5% on PCMX. In these instances, no increase was observed. In all cases where a reduction in the antimicrobial activity of PCMX was demonstrated, the reduction was thought to be a direct result of the molecular interaction with the nonionic macromolecule, thereby diminishing the availability of the drug to exert its antimicrobial activity.

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Keyphrases

p-Chloro-*m*-xylenol (PCMX)—microbiological evaluation
Minimum inhibitory concentration—PCMX
Complex formation—PCMX activity
Equilibrium dialysis—PCMX, methylcellulose, polysorbate 80
Spectrophotometry—cell count determinations

Studies on the Effects of Reserpine in Mice as Influenced by its Diluent

By W. VELDKAMP, G. A. JOHNSON, and H. H. KEASLING

The effects of dextroamphetamine sulfate and benzphetamine hydrochloride on locomotor activity in mice pretreated with reserpine varied depending on the reserpine preparations used. Pretreatment with reserpine suspended in aqueous 0.25 percent methylcellulose (pH 5.8) had little effect on the response to dextroamphetamine sulfate but decreased the response to benzphetamine hydrochloride. Pretreatment with a reserpine solution (pH 3.5) significantly enhanced the locomotor response to both compounds at the higher doses tested. The difference in effect produced by the reserpine preparations could not be explained on the basis of differences in brain amine levels at the time locomotor activity was measured since both preparations had depleted brain amines to the same extent. Further studies revealed that variations in pH of the reserpine diluent had a marked effect on brain reserpine levels, on brain amine levels when measured 1 hr. after reserpine administration, and on the rate of onset and intensity of ptosis produced in the mouse.

VAN ROSSUM *et al.* (1) and van Rossum and Hurkmans (2) have reported that reserpine pretreatment in mice has no effect on the locomotor activity response to dextroamphetamine sulfate while the effects of derivatives of amphetamine which have large substituents on the nitrogen atom, such as benzylamphetamine and benzphetamine hydrochloride,[1] are antagonized by pretreatment with reserpine. On the other hand, Smith (3) has reported an enhancement of the effects of dextroamphetamine sulfate on the locomotor activity of mice when pretreated with reserpine.

The present study shows that the effect obtained with dextroamphetamine sulfate and benzphetamine hydrochloride in reserpinized mice will vary depending upon the reserpine preparation used in pretreating the mice. A number of acids such as ascorbic, Burn and Rand, (4), acetic, Fleming and Trendelenberg, (5), and citric, Fleming, (6) have been used to dissolve reser-

Received November 6, 1967, from the Pharmacology Research Laboratories, The Upjohn Co., Kalamazoo, MI 49001
Accepted for publication December 7, 1967.
The authors gratefully acknowledge the technical assistance of Mr. D. M. Devendorf, Miss E. G. Kim, Mr. R. R. Russell, and Mr. H. J. Triezenberg.

[1] Trademarked as Didrex by the Upjohn Co., Kalamazoo, Mich.

with *A. niger* when evaluating the effect of methylcellulose and polyethylene glycol 6000 on PCMX; and the result obtained with *Ps. aeruginosa* in evaluating the effect of polysorbate 80 at a concentration of 0.5% on PCMX. In these instances, no increase was observed. In all cases where a reduction in the antimicrobial activity of PCMX was demonstrated, the reduction was thought to be a direct result of the molecular interaction with the nonionic macromolecule, thereby diminishing the availability of the drug to exert its antimicrobial activity.

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Studies on the Effects of Reserpine in Mice as Influenced by its Diluent

By W. VELDKAMP, G. A. JOHNSON, and H. H. KEASLING

The effects of dextroamphetamine sulfate and benzphetamine hydrochloride on locomotor activity in mice pretreated with reserpine varied depending on the reserpine preparations used. Pretreatment with reserpine suspended in aqueous 0.25 percent methylcellulose (pH 5.8) had little effect on the response to dextroamphetamine sulfate but decreased the response to benzphetamine hydrochloride. Pretreatment with a reserpine solution (pH 3.5) significantly enhanced the locomotor response to both compounds at the higher doses tested. The difference in effect produced by the reserpine preparations could not be explained on the basis of differences in brain amine levels at the time locomotor activity was measured since both preparations had depleted brain amines to the same extent. Further studies revealed that variations in pH of the reserpine diluent had a marked effect on brain reserpine levels, on brain amine levels when measured 1 hr. after reserpine administration, and on the rate of onset and intensity of ptosis produced in the mouse.

VAN ROSSUM *et al.* (1) and van Rossum and Hurkmans (2) have reported that reserpine pretreatment in mice has no effect on the locomotor activity response to dextroamphetamine sulfate while the effects of derivatives of amphetamine which have large substituents on the nitrogen atom, such as benzylamphetamine and benzphetamine hydrochloride,[1] are antagonized by pretreatment with reserpine. On the other hand, Smith (3) has reported an enhancement of the effects of dextroamphetamine sulfate on the locomotor activity of mice when pretreated with reserpine.

The present study shows that the effect obtained with dextroamphetamine sulfate and benzphetamine hydrochloride in reserpinized mice will vary depending upon the reserpine preparation used in pretreating the mice. A number of acids such as ascorbic, Burn and Rand, (4), acetic, Fleming and Trendelenberg, (5), and citric, Fleming, (6) have been used to dissolve reser-

Received November 6, 1967, from the Pharmacology Research Laboratories, The Upjohn Co., Kalamazoo, MI 49001
Accepted for publication December 7, 1967.
The authors gratefully acknowledge the technical assistance of Mr. D. M. Devendorf, Miss E. G. Kim, Mr. R. R. Russell, and Mr. H. J. Triezenberg.

[1] Trademarked as Didrex by the Upjohn Co., Kalamazoo, Mich.

pine in preparation for parenteral administration. In this study the results obtained when mice were pretreated with a reserpine preparation containing citric acid were compared with results from mice pretreated with reserpine suspended in aqueous 0.25% methylcellulose. The following parameters were measured in making these comparisons: locomotor activity after administration of dextroamphetamine sulfate or benzphetamine hydrochloride in the reserpinized mouse and the effects of pH of the reserpine diluent on the onset and intensity of ptosis, on the depletion of biogenic amines, and on brain levels of reserpine.

METHODS

Carworth Farms No. 1 male albino mice weighing between 20 and 25 Gm. were used. Each mouse was used only once.

Measurement of Locomotor Activity—Eight Metro Industries[2] circular actophotometers, each equipped with 6 light beams and 6 photoelectric cells connected to a digital counter, were used. A single mouse was placed in each actophotometer and the number of times the mouse crossed a beam of light was recorded. In determining the locomotor activity effects of dextroamphetamine sulfate and benzphetamine hydrochloride, groups of 8 mice were injected with various doses of the compounds and immediately placed in individual actophotometers. After a 10-min. acclimation period, locomotor activity was recorded for a 30-min. period. Each experiment was carried out using an 8 × 8 Latin square design balanced with respect to treatments, actophotometers and time of day.

Drugs—The reserpine solution used in the motor activity studies contained the following per ml. of solution: 0.25 mg. recrystallized reserpine, 0.20 mg. citric acid anhydrous, 9.0 mg. benzyl alcohol, 15.0 mg. *N,N*-dimethylacetamide, water, q.s., to make 1 ml. This solution had a pH of approximately 3.5. The reserpine suspension contained 0.25 mg. reserpine per ml. of aqueous 0.25% methylcellulose and had a pH of approximately 5.8. In the experiments where the effects of pH were studied, the stock reserpine solution was diluted with 0.25 volume of either distilled water or an 0.25% aqueous methylcellulose solution. The pH was adjusted to the desired pH with 0.1 *N* HCl or 0.5 *M* $NaHCO_3$. The final concentration of reserpine was 0.2 mg./ml. Dextroamphetamine sulfate and benzphetamine hydrochloride were dissolved in saline and the dosages expressed in terms of the salts. All injections were made intraperitoneally.

Reserpine Assay—Two mouse brains were pooled for each determination. Tissues were homogenized and reserpine was extracted by the methods reported by Hess *et al.* (7). The assay of reserpine was modified from that of Hess *et al.* by the omission of selenious acid from the assay mixture. The quantitative determination of reserpine was based upon the fluorescence of reserpine (0.2 mcg.) in duplicate internal standards in split brain homogenates. Fluorescence of reserpine in this assay was linear over the range 0.01–0.2 mcg.

[2] Metro Industries, Mineola, L.I., N.Y.

Biogenic Amine Assays—The extraction procedure of Shore and Olin (8) was employed for the extraction of dopamine (DA), norepinephrine (NE), and serotonin (5-HT) from paired mouse brains. Aliquots of the resulting 4-ml. aqueous extract were removed for the respective analyses with the noted modifications: NE, Shore and Olin (8); DA, Carlsson and Waldeck (9), with reduced volumes to increase the sensitivity and a final acid addition of 5 *N* acetic acid–5 *N* HCl, 2:3; 5-HT, Snyder *et al.* (10), with heating at 60° for 1 hr. All calculations of amine content were based upon tissue-containing internal standards and are expressed as mcg./Gm. wet weight of brain tissue. Results were not corrected for recovery of amines by the extraction procedure.

Ptosis Scoring—Ptosis was graded on a 4-point scale (1 = normal, 4 = complete ptosis) in groups of six mice, 1, 2, 3, and 18 hr. after intraperitoneal administration of reserpine, Rubin *et al.* (11).

RESULTS

Locomotor Activity in Mice Pretreated with Either a Reserpine Solution or a Reserpine Suspension—The effects of dextroamphetamine sulfate and benzphetamine hydrochloride on the 30-min. locomotor activity of mice pretreated once daily for 3 days with either saline or 1 mg./Kg. of reserpine suspension with motor activity run on the fourth day are shown in Fig. 1. Pretreatment with the reserpine suspension decreased the effect of benzphetamine hydrochloride on locomotor activity of both doses tested. Dextroamphetamine sulfate in similarly pretreated mice was less effective at the 0.625 mg./Kg. dose while the 2.5 mg./Kg. dose caused a slight but nonsignificant increase in motor activity compared to the effect in saline pretreated controls.

When the mice were pretreated with the reserpine solution, there was no quantitative difference between the effects of dextroamphetamine sulfate and benzphetamine hydrochloride. Figure 2 shows the results obtained with mice pretreated once daily for 3 days with 1 mg./Kg. of reserpine solution and 30-min. locomotor activity run on the fourth day. At the lower dose both dextroamphetamine sulfate and benzphetamine hydrochloride were less stimulant, while at the higher dose both caused a significantly greater increase in locomotor activity than in the saline pretreated controls.

Brain amine levels after 3 days pretreatment do not explain the difference in results obtained with the two reserpine preparations on motor activity with dextroamphetamine sulfate and benzphetamine hydrochloride. Groups of mice were injected once daily for 3 days with 1 mg./Kg. of either the reserpine solution or suspension and brain amine levels were determined on the fourth day. Results in Table I show that there was no significant difference between the reserpine preparations in their effect on brain amine levels under these conditions.

Effects of pH on Reserpine Activity—Since there was a difference in the pH of the reserpine solution (pH 3.5) and the reserpine suspension (pH 5.8) used in the above locomotor activity studies, the effects of pH on reserpine activity were investigated.

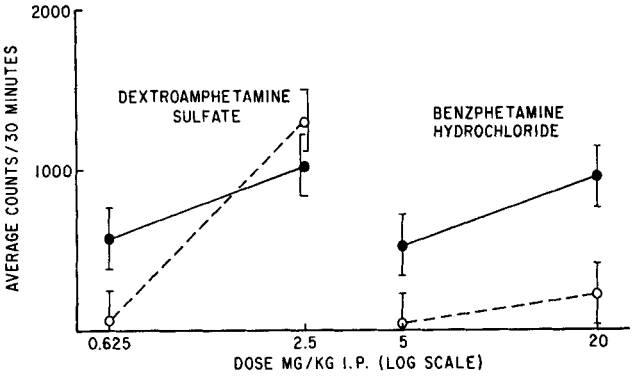

Fig. 1—Effects of reserpine suspension (1 mg./Kg./day, 3 days) on locomotor activity response of mice to dextroamphetamine sulfate and benzphetamine hydrochloride. Key: I, represents ± S.E. of mean; O—O, reserpine suspension pretreatment; ●——●, saline, pretreatment.

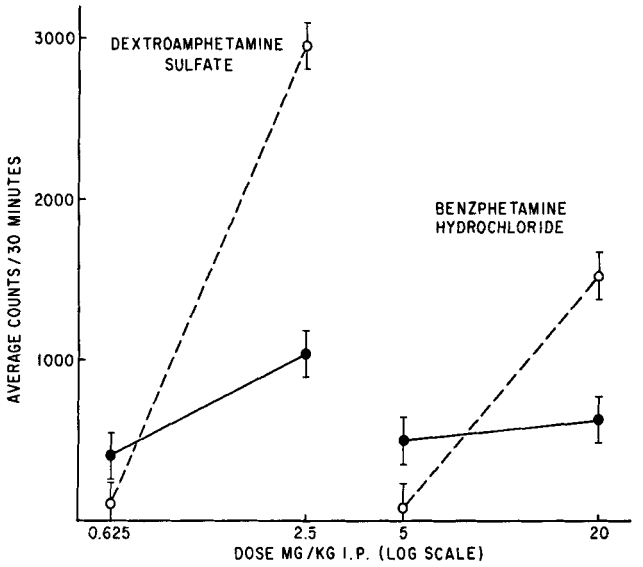

Fig. 2—Effect of reserpine solution (1 mg./Kg./day, 3 days) on locomotor activity response of mice to dextroamphetamine sulfate and benzphetamine hydrochloride. Key: I, represents ± S.E. of mean; O—O, reserpine solution, pretreatment; ●——●, saline, pretreatment.

TABLE I—BRAIN NOREPINEPHRINE AND SEROTONIN LEVELS IN MICE AFTER 1 mg./Kg. RESERPINE ONCE DAILY FOR THREE DAYS

	Norepinephrine			Serotonin		
Preparation	No. of Determinations	Concentration mcg./Gm. ± S.E.M.	% of Control	No. of Determinations	Concentration mcg./Gm. ± S.E.M.	% of Control
Saline control	5	0.50 ± 0.04	100	5	0.80 ± 0.07	100
Reserpine solution	4	0.05 ± 0.01	10	4	0.51 ± 0.04	64
Reserpine suspension	3	0.05 ± 0.01	10	3	0.46 ± 0.06	60

Effect of diluent pH on brain levels of reserpine at various intervals after its intraperitoneal administration is shown in Fig. 3. Maximal reserpine levels were detected with the pH 2.8, 3.5, and 4.5 solutions in the 10–20 min. interval after injection. Reserpine was not detected in the brain following intraperitoneal administration of the pH 5.7 preparation and only trace quantities were found with the pH 2.0 solution. Brain levels of reserpine after intraperitoneal administration of reserpine suspended in aqueous 0.25% methylcellulose at pH 3.7 are less than those obtained after the pH 3.5 reserpine solution.

Variations in diluent pH of the reserpine preparations also influenced the depletion of endogenous brain amines. The effects of diluent pH on the depletion of brain amines during the first hour after intraperitoneal administration of reserpine (5 mg./Kg.) are shown in Table II. The most marked depletion of norepinephrine, dopamine, and serotonin was noted with the reserpine preparation at pH 2.8. No decrease in amine content at 1 hr. was detected with the pH 5.7 and pH 7.0 preparations.

The onset and intensity of ptosis induced by reserpine (2 mg./Kg., i.p.) as influenced by the pH of its diluent is shown in Fig. 4. The reserpine preparation with pH 3.5 caused the greatest degree of ptosis. With all the preparations except the one at pH 7.0 maximum ptosis was detected at the 3-hr. reading. A moderate amount of ptosis was present at 18 hr. with the pH 7.0 preparation.

DISCUSSION

The results presented indicate that the effect of dextroamphetamine sulfate and benzphetamine hydrochloride on locomotor activity in reserpinized mice varies depending on the reserpine preparation

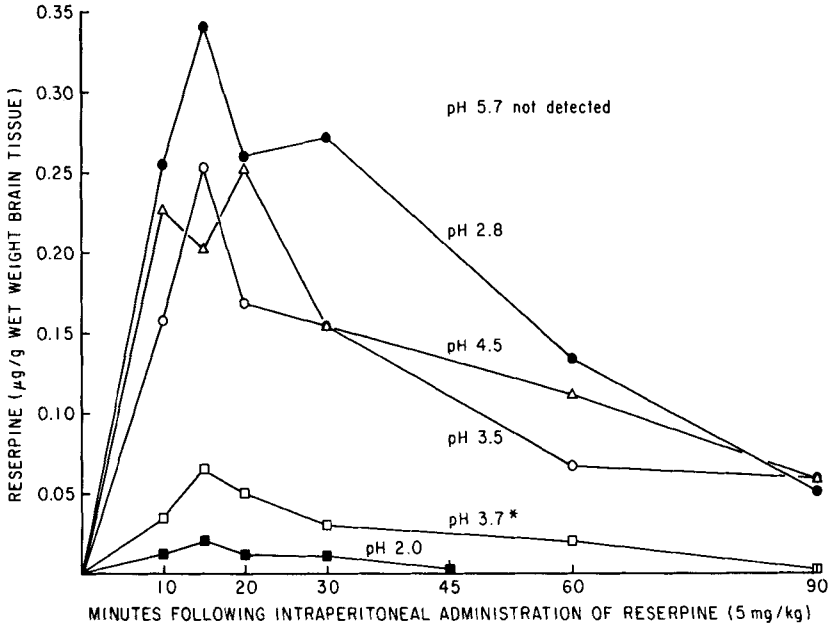

Fig. 3—Effect of diluent pH on brain reserpine content. Stock reserpine solution was diluted with 0.25 volume of distilled water and the pH adjusted as described in Methods. *At pH 5.7 no reserpine was detected. * The reserpine for these results was suspended in 0.25% aqueous methylcellulose and pH adjusted to 3.7.*

TABLE II—EFFECT OF DILUENT pH ON ENDOGENOUS MOUSE BRAIN AMINE LEVELS BY RESERPINE (5 mg./Kg., i.p.) AFTER ONE HOUR[a]

Diluent pH	NE	DA	5-HT
2.0	0.44 ± 0.14[b] (4)[c]	0.46 ± 0.27 (4)	0.69 ± 0.17 (4)
2.8	0.24 ± 0.04 (4)	0.23 ± 0.08 (4)	0.52 ± 0.05 (4)
3.5	0.31 ± 0.03 (4)	0.36 ± 0.14 (4)	0.56 ± 0.05 (4)
4.5	0.37 ± 0.17 (4)	0.51 ± 0.44 (4)	0.57 ± 0.17 (4)
5.7	0.51 ± 0.09 (6)	0.82 ± 0.13 (4)	0.88 ± 0.06 (6)
7.0	0.52 ± 0.13 (6)	0.85 ± 0.17 (4)	0.90 ± 0.07 (6)

[a] Brain amine levels from control animals in these five experiments were: NE, 0.52 ± 0.04 (7); DA, 0.83 ± 0.05 (4); 5-HT, 0.89 ± 0.07 (7). [b] All values are expressed as mg. amine/Gm. wet weight brain tissue ± S.D. [c] Number of determinations.

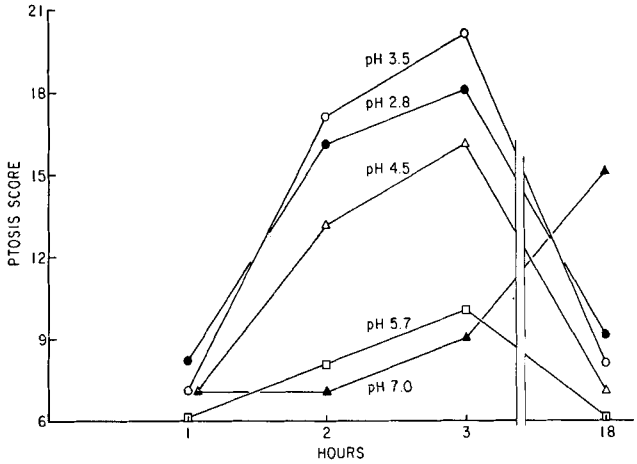

Fig. 4—Effect of diluent pH on reserpine (2 mg./Kg., i.p.) induced ptosis. Composition of the reserpine preparation is the same as described for stock reserpine solution in Fig. 3.

used in pretreating the mice. Pretreatment of mice with a reserpine suspension (pH 5.8) resulted in a decreased motor response to both the low (2.5 mg./Kg.) and high (20 mg./Kg.) dose of benzphetamine hydrochloride. The response to the low (0.625 mg./Kg.) dose of dextroamphetamine sulfate was depressed while there was a slight but non-significant increase at the high (2.5 mg./Kg.) dose. Mice pretreated with the reserpine solution (pH 3.5) had a decreased locomotor activity response to the low dose of both dextroamphetamine sulfate and benzphetamine hydrochloride but the high dose of both compounds caused a significant increase in activity.

The results presented here with dextroamphetamine sulfate in mice pretreated with reserpine solution are in agreement with those reported by Smith (3) who used reserpine solubilized with ascorbic acid to pretreat his mice. The results obtained with both dextroamphetamine sulfate and benzphetamine hydrochloride in mice pretreated with the reserpine solution do not agree, however, with those of van Rossum *et al.* (1, 2) who used acetic acid to solubilize the reserpine (personal communication). Only when the mice were pretreated with a reserpine suspension could the results reported by van Rossum *et al.* be repeated.

Brain amine levels after pretreatment with either the reserpine solution or suspension for 3 days at 1 mg./Kg./day do not explain the differences in results obtained on locomotor activity. These preparations both depleted measurable brain amine levels about the same extent.

The results shown in Fig. 3 indicate that whether the reserpine is in solution or suspension and the pH of the diluent influence the initial rate of reserpine uptake and the level of reserpine attained in the brain. This in turn is thought to influence the rate of decline in the brain amine levels. The pH of the reserpine solution used to pretreat the mice for the locomotor activity studies was 3.5 and therefore gave higher and more rapid reserpine levels in the brain and more rapid decline of brain amine than did the reserpine suspension which had a pH of 5.8. If the reasoning of Stein (12) is correct that reserpine pretreatment may cause supersensitivity to amines in the brain as well as in the periphery, and if the sensitivity is increased with time then the increased locomotor response in the mice pretreated with the reserpine solution may be due to the increased length of time these mice had to develop the supersensitivity.

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Keyphrases

Reserpine activity—diluent effect
Methylcellulose—reserpine activity
Citric acid solution—reserpine activity
Dextroamphetamine SO_4 activity—reserpine–diluent effect
Benzphetamine HCl activity—reserpine–diluent effect
Brain, mouse—analysis
Locomotor activity—analysis

Potassium Absorption—A Comparison of *In Vitro* and *In Vivo* Studies

By A. J. JOUHAR, E. S. GARNETT, and J. S. WALLINGTON

Some ^{42}K was released from a slow release preparation within 15 min. of ingestion and further potassium release continued for approximately 200 min. A similar absorption pattern was found from enteric-coated tablets of potassium chloride although a considerable initial delay occurred before potassium was released from this preparation. The *in vivo* behavior of the slow release preparation and enteric-coated tablets was similar to that seen in static solution experiments but differed considerably from that found in the B.P. disintegration apparatus. There was no evidence that sudden release and absorption of potassium took place from enteric-coated tablets.

LONG CONTINUED ADMINISTRATION of thiazide diuretics reduces exchangeable potassium, and the need for potassium supplements is well recognized (1, 2). These may be given separately or in a compound tablet, such as the film-coated, two-layered slow release preparation.[1] In this preparation crystals of potassium chloride (KCl) are coated with a polymeric material and compressed to form one layer, and the other layer contains bendrofluazide. The KCl layer is intended to dissolve gradually and so release potassium ions (K^+) continuously. Since it has been

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shown radiologically that this preparation passes down the gut (3), localized high concentrations of K^+, which induce gastric irritation and are believed to cause intestinal ulceration (4–6), may be prevented. Such slow release preparations are formulated with the help of *in vitro* control methods which should ensure uniform behavior in the gastrointestinal tract. In this investigation, the authors have compared the results of such *in vitro* experiments with *in vivo* ^{42}K absorption studies in man.

MATERIALS AND METHODS OF MANUFACTURE

Tablets for *in vivo* studies were made containing ^{42}KCl, after it was shown by microscopy that irradiation to give a specific activity at the time of administration of 70–80 μc. per dose did not produce any change in the crystals of KCl. The radiochemical purity of ^{42}KCl was 100% at the time of administration.

The usual production process for the slow release preparation of granulating KCl crystals to form a damp mass was not possible because of difficulty in manipulating 2 ft. 6 in. long hand-operated tongs behind a screen of 1 in. perspex. Instead, a remotely controlled microstirrer was used to mix the ^{42}KCl crystals into a slurry with a granulating solution of coating materials dissolved in excess solvent.

The slurry was dried over an electrically heated mantle at 70° and the solid residue was forced through 8- and 16-mesh sieves with a large glass stopper. Weighed quantities of granules were fed into a hand-operated tablet machine, with a small glass weighing boat designed so that it could be easily picked up with the tongs. After adding inert granules in place of bendrofluazide to form the second layer, the tablet was ejected from the machine. The tablets were film-coated by dipping in warm film-coating solution, and allowing them to dry on a pin bed in a warm air stream.

Enteric-coated tablets of ^{42}KCl were made by directly compressing the weighed crystals in the hand-tablet machine. Each tablet was coated by dipping into cellulose acetate phthalate/vinyl acetate copolymer/solvent solution several times. The slow release tablets and the enteric-coated tablets both contained 630 mg. KCl (8.4 meq. K^+).

Similar quantities of ^{42}KCl were made into solution for the *in vivo* studies. Nonradioactive preparations were used for the *in vitro* work.

In Vitro Studies—(*a*) *Static Solution*—The extent of release of KCl from the tablets was measured at intervals of 20 min. for 7 hr. by the following method: 120 ml. of the double-distilled water or buffered solutions (pH 7.9 and 8.9) at 20° were placed in each of 40 polythene pots. One tablet of the slow release preparation or an enteric-coated KCl tablet was placed in each of two pots at 20-min. intervals for 400 min. Twenty minutes after the last tablets had been placed in the pots, all the tablets were removed. The potassium chloride concentration of the solution remaining was then measured with a Pye conductance bridge, and the rate of release plotted.

(*b*) *Solution in the B.P. Disintegration Apparatus*—Three tablets of the slow release preparation or three enteric-coated KCl tablets were placed in a B.P. disintegration apparatus and agitated according to the B.P. monograph (8). The amounts of KCl released in periods of 5 min. were measured with a Pye conductance bridge.

In Vivo Studies—Male volunteers were fasted overnight and then given a solution of ^{42}KCl, two radioactive slow release tablets, or two enteric-coated ^{42}KCl tablets. Blood samples, each of 20 ml., were taken from an uncuffed arm vein at intervals of 5 to 100 min. for the next 320–540 min.

The plasma from each sample was separated at once and the ^{42}K content of 10-ml. aliquots was measured in a M64 Geiger-Müller liquid counter connected to an EKCO Automatic Scaler Type N530F through an EKCO N558 probe unit with the dead time set at 300 μsec. The background count rate of this system was 12 counts per minute (c.p.m.). The plasma samples were counted for 50 min. or until 3,000 counts had been accumulated (whichever was the shorter). Samples were recorded as containing a significant amount of ^{42}K provided they gave a count rate of at least twice background. The maximum count rate achieved in any one experiment was approximately 50 times background. After subtracting background count and correcting for radioactive decay using a ^{42}K half-life of 12.5 hr., the c.p.m. from each sample were expressed as a percentage of the c.p.m. obtained from that sample containing the maximum radioactivity in each subject.

RESULTS

In Vitro Studies—Figure 1 shows the rate of release of KCl from each of the formulations studied.

After 200 min. in static distilled water, 50% of the available KCl had been released from the slow release preparation and after 420 min. 70% of the KCl had been released.

Because the rate of release of KCl from enteric-coated preparations depends upon pH, tests were carried out in static solutions at various pH levels. Thus at pH 7.9, the rate of release of KCl became maximal after 150 min. and was complete after about 420 min. At pH 7.0 and at pH 8.9 the solution rates were faster than at pH 7.9 and were immediately maximal, slowing after 120 min. The release of KCl was complete after about 420 min.

In the B.P. disintegration apparatus all the potassium was made available from the slow release preparation within 80 min. in acid pepsin solution and within 50 min. in alkaline pancreatin solution.

Release of KCl from the enteric-coated preparation in the B.P. disintegration apparatus in alkaline pancreatin solution was delayed for 10 to 15 min. but was complete at 30 min.

In Vivo Studies—Fig. 2 shows the appearance of $^{42}K^+$ in the plasma after ingestion of a solution ^{42}KCl. In all cases, peak activity occurred within 35 min. Figure 3 shows the appearance of $^{42}K^+$ in the plasma from the radioactive slow release preparation. ^{42}K was detected soon after ingestion, but a peak plasma level was not obtained until about 200 min. Figure 4 shows the appearance of $^{42}K^+$ in the plasma after giving enteric-coated ^{42}KCl; no radioactivity was detected in the plasma for at least 120 min. Thereafter, the pattern of absorption paralleled that obtained from the slow release preparation.

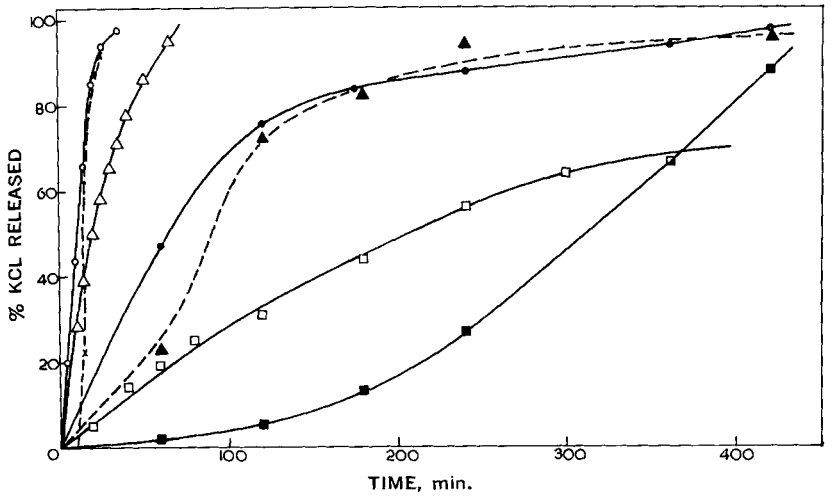

Fig. 1—Release rate in vitro *of KCl from slow release tablets and enteric-coated KCl tablets. B.P. disintegration apparatus held at 37°, and static solutions at 20°. Slow release tablets: ○, B.P. disintegration apparatus—alkaline pancreatin solution; △, B.P. disintegration apparatus—acid pepsin solution; □, static solution at pH 7.0. Enteric-coated KCl: ×, B.P. disintegration apparatus—alkaline pancreatic solution; ▲, static solution at pH 7.0; ■, static solution at pH 7.9; ●, static solution at pH 8.9.*

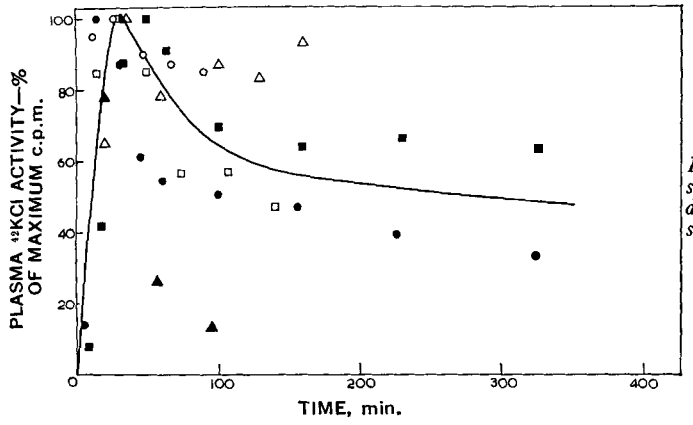

Fig. 2—Plasma ^{42}KCl activity—potassium chloride solution. Mean plot drawn from series of six results. Each symbol represents the results obtained from one human volunteer.

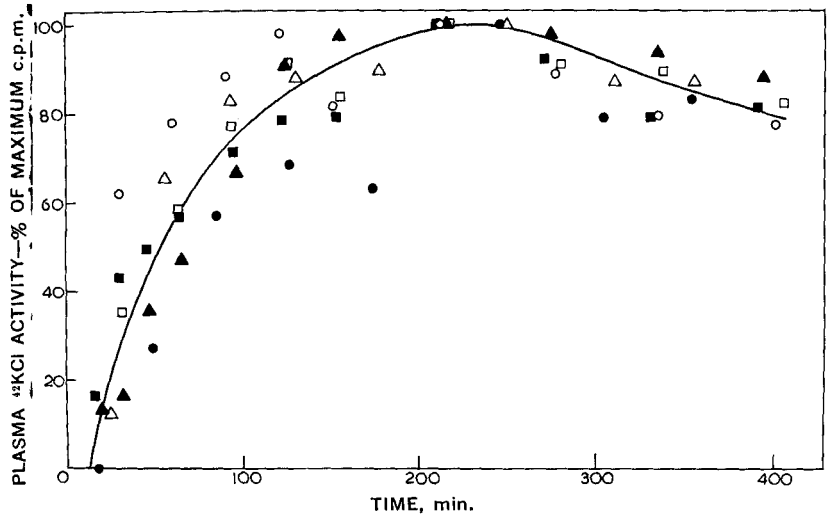

Fig. 3—Plasma ^{42}KCl activity—slow release tablets. Mean plot drawn from series of six results. Each symbol represents the results obtained from one human volunteer.

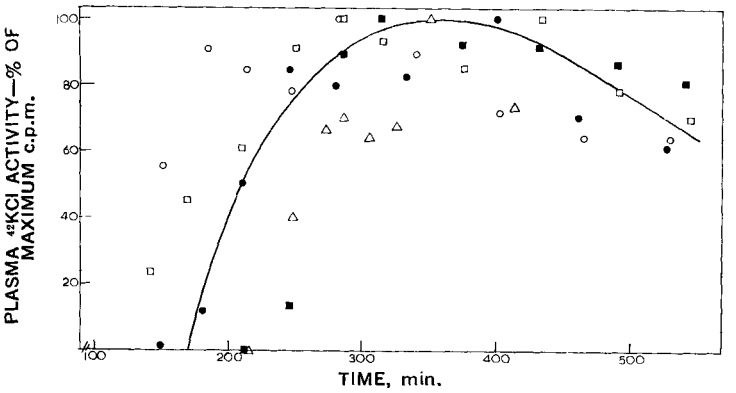

Fig. 4—Plasma ^{42}KCl activity—enteric-coated potassium chloride tablets. Mean plot drawn from series of five results. Each symbol represents the results obtained from one human volunteer.

DISCUSSION

The use of radioactive materials in pharmaceutical formulations presents manufacturing problems as only a few tablets must be specially produced for each investigation. Nevertheless, it has been possible to make radioactive potassium chloride tablets with high specific activity at the time of preparation by using special handling and screening procedures, and with these radioactive preparations it has been possible to study the *in vivo* behavior of a variety of formulations.

The first studies showed that ^{42}K is rapidly absorbed after oral administration of ^{42}KCl in solution. It is assumed that this early absorption occurs in the upper small bowel since activity appeared in the blood within 5 min. of ingestion and peak activity occurred within 30 min. It is unlikely that gastric absorption of ^{42}K contributes significantly to the early uptake curves as Code *et al.* (7) have shown that potassium is absorbed only slowly from the stomach.

When ^{42}KCl was given as the slow release preparation, activity appeared in the plasma within 20 min. indicating that some ^{42}K had been absorbed from the upper small bowel. These results might be expected from the B.P. disintegration experiments which showed that potassium is released early from the slow release preparation both in acid pepsin and alkaline pancreatin solution. They do not, however, give any information about the possible release of ^{42}K from the formulation in the stomach with subsequent absorption from the jejunum.

Following the early appearance of ^{42}K in the plasma after administration of the slow release preparation, the concentration of ^{42}K rose slowly to a maximum at about 220 min. This suggests that ^{42}K was slowly released into the gut, since ^{42}K in solution produced an early peak in plasma activity. The apparent pattern of release is quite different from results obtained in the B.P. disintegration studies in which all of the potassium had been released by 60 min. The pattern accords more nearly to that seen in the static solution experiments in which potassium was released for about 300 min.

The pattern of absorption, once established, from enteric-coated ^{42}KCl tablets was very similar to that seen with the slow release preparation and again, static solution studies seem to give a more realistic measure of tablet breakdown in the intestine. It will be noted however that although the pattern of absorption of ^{42}K from enteric-coated tablets and the slow release preparation is similar, ^{42}K does not appear in the plasma for at least 100 min. after the administration of the enteric-coated preparation. The observed variation in delay probably results from such factors as gastric emptying time and the pH and motility of the small bowel. Thus in one study ^{42}K appeared in the plasma only after 212 min.

It is appreciated that serial measurements of plasma concentration of ^{42}K may not necessarily give a true impression of intestinal absorption since the plasma is only one of at least three compartments between which ^{42}K is distributed. However, since there was no evidence of electrolyte imbalance in any of the subjects, the pattern of ^{42}K redistribution from plasma can be assumed to be qualitatively similar in all of the subjects; thus the pattern of ^{42}K appearance in the plasma reflects uptake from the intestinal tract.

In conclusion, it has been shown that the *in vivo* behavior of the slow release preparation and the enteric-coated tablets is similar to that seen in static solution experiments, and differed considerably from that found in the B.P. disintegration apparatus. Further, there was no evidence for an explos've release of potassium from the enteric-coated tablets.

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Keyphrases

Potassium absorption
In vitro–in vivo absorption compared
^{42}K-Potassium chloride solution and tablets—*in vivo* testing
Liquid scintillation counting—analysis
Potassium chloride tablets—*in vitro* testing
Conductance bridge—analysis
Enteric coating effect—potassium release

Urinary Excretion of Pheniramine and Its *N*-Demethylated Metabolites in Man—Comparison with Chlorpheniramine and Brompheniramine Data

By PETER KABASAKALIAN, MARILYN TAGGART, and EDWARD TOWNLEY

The *N*-demethylated metabolites of pheniramine, *N*-desmonomethylpheniramine and *N*-desdimethylpheniramine, were found in the urine of a male subject using a repetitive dosage schedule. A comparison of the urinary excretion data of pheniramine and its halogenated derivatives indicated the compounds to be metabolized in the following order: pheniramine < chlorpheniramine < brompheniramine. This is inverse to the order of total free and *N*-demethylated drug excreted in the urine.

ALTHOUGH THE URINARY EXCRETION of chlorpheniramine (1, 2) and brompheniramine (3) have been studied, no such study has been made on the urinary excretion of pheniramine.[1]

The present investigation was undertaken to further determine the substituent effect of the halogens in the *p*-halobenzyl group of the pheniramines on urinary excretion.

EXPERIMENTAL[2]

Protocol—A male subject [used in the previous chlorpheniramine study (2)] under a normal diet with no urine pH or fluid intake control was used in this study.

Single Dose Regimen—The subject orally took a single dose of 74 mg. of pheniramine maleate in tablet form (two 37-mg. pheniramine maleate tablets). Urine was collected normally for 6 days and combined into 24-hr. samples.

Multiple Dose Regimen—The same subject orally took 37 mg. of pheniramine maleate in tablet form daily for 21 days. Urine was collected normally for 25 days and combined into 24-hr. samples.

Analytical Methods—A gas chromatographic method previously described (2, 4) was used. The column was operated at 170°. A 2.5-ft. column exhibited 1,500 theoretical plates for pheniramine.

Free pheniramine had a retention time of 7.0 min. Quantitative determinations were made using a pheniramine reference standard. Isolation by thin-layer chromatography on alumina (5) and subsequent mass spectroscopy (6) showed an *m/e* value of 58 for $CH_2{=}N(CH_3)_2$ indicative of a tertiary amine and *m/e* values of 169 and 83.5 which are characteristic of the benzyl-2-pyridyl group. It did not form derivatives either with cyclopentanone or acetone using the procedure of Capella and Horning (7).

Metabolite *X* had a retention time of 13 min. Quantitative determinations *via* the area technique were made using pheniramine as a calibration standard. Isolation by thin-layer chromatography on alumina (5) and mass spectroscopy (6) showed *X* to be a secondary amine with an *m/e* of 44 for $CH_2{=}NH(CH_3)$ and the *m/e* values of 169 and 83.5 for the benzyl-2-pyridyl group. It formed a derivative (retention time > 60 min.) with cyclopentanone (indicative of primary and secondary amines), but none with acetone (indicative of the absence of a primary amine).

Metabolite *Y* had a retention time of 18 min. Quantitative determinations *via* the area technique were made using pheniramine as a calibration standard. Isolation by thin-layer chromatography on alumina (5) failed to separate metabolite *Y* from the fiftyfold excess of metabolite *X*, consequently no mass spectral data could be obtained. It formed derivatives with cyclopentanone (retention time > 60 min.) and with acetone (retention time, 22 min.), indicative of a primary amine.

Lipid-Water Distribution—The procedure of McMahon (8) was used to determine the partition coefficient of pheniramine, chlorpheniramine, and brompheniramine for the heptane and pH 7.4 aqueous phosphate buffer system.

RESULTS AND DISCUSSION

Single Dose Regimen—The urinary excretion of free pheniramine by the subject on the single dose regimen is shown in Table I.

The amount of unchanged pheniramine excreted by the single subject in the first 24 hr. was 16.6% of the ingested dose. The total excretion of free pheniramine (over the 6-day period) was 22.6% of the ingested dose.

TABLE I—URINARY EXCRETION OF FREE PHENIRAMINE BY SUBJECT WHO RECEIVED ONE DOSE OF TWO 37-mg. PHENIRAMINE MALEATE TABLETS

Time After Drug Taken,[a] Days	Combined Volume of 24-Hr. Urine, ml.	pH of 24-Hr. Urine	Pheniramine[b] Excreted in 24-Hr. Urine, mg.
0	...	...	...
1	1420	6.2	12.26
2	1465	6.2	3.17
3	1065	5.9	0.90
4	970	6.0	0.25
5	1080	5.9	0.10
6	760	5.9	0.02

[a] Experiment started at 7:30 p.m. [b] Reported as pheniramine maleate.

Received November 15, 1967, from the Research and Development Division, Schering Corporation, Bloomfield, NJ 07003

Accepted for publication December 19, 1967.

[1] Pheniramine is *dl*-2-[α-[2-(dimethylamino)ethyl]-benzyl] pyridine.

[2] All gas chromatograms were obtained on a F and M model 400 gas chromatograph with a flame ionization detector and a Minneapolis Honeywell recorder. The mass spectra were obtained on a Consolidated Electrodynamics Corporation model CEC-103C mass spectrometer.

Multiple Dose Regimen—The urinary excretion of free pheniramine by the subject on a multiple dose regimen is listed in Table II.

The 24-hr. urinary excretion rate did not form a gradually increasing pattern as in the case of chlorpheniramine (2) and brompheniramine (3), indicating that pheniramine is not building up in the body.

The calculated *average* daily excretion rate (for the first 21 days) of free pheniramine was 9.0 mg., or 24.3% of the repeated 37-mg. pheniramine dose. This compares favorably with the 22.6% which was obtained in the single dose experiment. Large fluctuations (coefficient of variation of 32%) were observed in the daily excretion rate which could not be accounted for by variance in daily urine pH and volume.

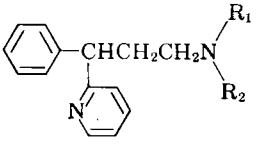
The average daily urine volume was 1,020 ml. (range: 655–1495 ml.) while the average daily urine pH was 6.1 (range: 5.5–6.6).

On termination of the drug intake regimen, the pheniramine excretion rate decreased. From the total amount of free pheniramine excreted after the last dose is taken on day 20 and using the average 9.0-mg. pheniramine excreted per 37-mg. dose, one can calculate the equivalent amount of pheniramine maleate present in the subject immediately after the last dose. This calculation yields a figure of 47 mg. of pheniramine maleate (equivalent to 1.3 tablets, each containing 37 mg. of pheniramine maleate). Since this is well within the daily urinary excretion variance, there is no evidence of drug build-up in the body under this multiple dosage regimen.

Metabolites *X* and *Y*—The metabolites *X* and *Y* were found to be *N*-desmonomethylpheniramine and *N*-desdimethylpheniramine, respectively (Table III).

During the first 21 days an average of 9.3 mg. *N*-desmonomethylpheniramine and 0.17 mg. *N*-desdimethylpheniramine were excreted daily (26.1% and 0.5% of the repeated 37-mg oral dose). Together with the free pheniramine excreted daily, this accounts for 51% of the dose leaving the fate of the remaining 49% still unaccounted for.

TABLE III—STRUCTURAL FORMULA OF PHENIRAMINE AND ITS METABOLITES



Compound	R_1	R_2
Pheniramine	CH_3	CH_3
Metabolite *X*	CH_3	H
Metabolite *Y*	H	H

TABLE II—URINARY EXCRETION OF FREE PHENIRAMINE AND ITS METABOLITES BY SUBJECT WHO DAILY RECEIVED ONE 37-mg. PHENIRAMINE MALEATE TABLET FROM DAY 0 TO DAY 20

Time,[a] Days	Combined Volume[b] of 24-Hr. Urine, ml.	pH of 24-Hr. Urine	Pheniramine[c] Excreted in 24-Hr. Urine, mg.	Metabolite *X*[d] Excreted in 24-Hr. Urine, mg.	Metabolite *Y*[e] Excreted in 24-Hr. Urine, mg.
0	...	...	...	...	...[f]
1	945	5.9	6.8	7.0	0.1
2	1215	6.6	6.1	8.2	0.1
3	1035	6.7	4.9	9.8	0.3
4	975	6.2	8.2	11.2	0.2
5	885	6.2	5.9	9.9	0.2
6	1110	6.2	11.2	11.3	0.2
7	1495	6.1	14.7	11.5	0.2
8	990	6.0	6.5	9.6	0.2
9	1110	6.4	7.7	5.4	0.1
10	655	5.8	11.8	7.6	0.1
11	740	5.6	12.2	8.8	0.1
12	1135	5.9	13.9	11.0	0.2
13	1295	6.2	11.5	11.4	0.2
14	785	6.1	10.6	10.0	0.2
15	1035	6.3	6.4	7.6	0.1
16	835	6.0	10.7	11.4	0.2
17	935	5.5	10.4	7.9	0.1
18	1065	5.9	7.2	7.7	0.1
19	1115	6.0	5.9	9.3	0.2
20	1135	6.1	8.8	10.3	0.2
21	940	5.9	7.4	7.8	0.2
22	720	5.6	2.8	3.5	0.1
23	1045	5.6	0.8	1.3	0.1
24	1270	6.0	0.3	0.4	0.0
25	820	5.5	0.2	0.0	0.0

[a] Underscored days 37 mg. of pheniramine maleate tablet ingested; experiment started at 7:30 p.m. [b] . . ., Sample not collected. [c] Reported as pheniramine maleate. [d] Reported as *N*-desmonomethylpheniramine maleate. [e] Reported as *N*-desdimethylpheniramine maleate. [f] . . ., Assay result not obtained.

TABLE IV—COMPARISON OF URINARY EXCRETION OF PHENIRAMINE, CHLORPHENIRAMINE, AND BROMPHENIRAMINE AND THEIR *N*-DEMETHYLATED METABOLITES IN MAN

Drug	% of Ingested Dose Excreted in Urine			
	Unchanged	*N*-Desmonomethyl-	*N*-Desdimethyl-	Total
Pheniramine	24.3	26.1	0.5	51
Chlorpheniramine	12.6	13.2	5.8	32
Brompheniramine	5.3	6.3	5.8	18

COMPARISON OF BROMPHENIRAMINE AND PHENIRAMINE WITH CHLORPHENIRAMINE

A comparison of the urinary excretion of pheniramine, chlorpheniramine, and brompheniramine and their *N*-demethylated metabolites is given in Table IV. The total urinary excretion of free pheniramine (24.3%) is greater, while that for free brompheniramine (5.3%) is less than that for chlorpheniramine (12.6%). This indicates that these drugs are metabolized (if one assumes that they are all well absorbed and there is no excretion of free drug *via* feces) in the following increasing order: pheniramine (76%), chlorpheniramine (87%), and brompheniramine (95%).

This difference in percentage metabolized may be due to differences in the degree of reabsorption from the kidney tubules by nonionic diffusion (9). Since the base strength of these amines are nearly identical (10), their lipid-water partition coefficient determines the degree of reabsorption. Using the heptane-water (pH 7.4) model, the distribution coefficients of these compounds were experimentally determined and tabulated in Table V. The drug with the highest lipid-water partition coefficient was brompheniramine followed by chlorpheniramine and pheniramine. These data are consistent with the amount of unchanged drug excreted in the urine.

TABLE V—COMPARISON OF LIPID-WATER DISTRIBUTION OF BROMPHENIRAMINE, CHLORPHENIRAMINE, AND PHENIRAMINE

Compd.	Heptane-Water Partition Coefficient[a]
Pheniramine	0.20
Chlorpheniramine	1.71
Brompheniramine	2.23

[a] 25°, water at pH 7.4.

It can be noted from Table IV that the amount of unchanged drug and *N*-desmonomethylated drug excreted are approximately equal for each of the three drugs. The reason for this is not obvious. The total amount of free drug plus *N*-demethylated metabolites excreted in the urine declined in the following order: pheniramine (51%), chlorpheniramine (32%), and brompheniramine (18%).

Urine pH and flow rate have been reported (1) to influence the renal excretion of chlorpheniramine. During the "steady state" period, the daily brompheniramine excretion rate was found to be dependent on the daily (24 hr.) urine pH and urine volume (3). These two variables (assuming a linear relationship) could account for 80% of the fluctuations in the daily free brompheniramine excretion rate. In the present study daily urine pH and urine volume could only account for about 25% of the fluctuations in the daily urinary excretion rate.

Repetitive daily oral dosage regimen appears to cause the build-up of brompheniramine in the body similar to the behavior of chlorpheniramine. However, in the case of pheniramine there is no evidence of drug build-up in the body.

SUMMARY AND CONCLUSIONS

A repetitive dosage regimen has been successfully used to identify two metabolites of pheniramine, *N*-desmonomethylpheniramine and *N*-desdimethylpheniramine, in the urine of a human.

The substitution of hydrogen for chlorine in chlorpheniramine evidently results in a molecule which is less extensively metabolized while the substitution of bromine for the same chlorine results in a molecule which is more extensively metabolized.

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Keyphrases

Pheniramine—urinary excretion
Metabolites, pheniramine—identification
Metabolism—pheniramine, chlorpheniramine, brompheniramine compared
Lipid–water partition coefficients
GLC—analysis

Bubble Size Analysis of High Consistency Aerosol Foams and Its Relationship to Foam Rheology

Effects of Container Emptying, Propellent Type, and Time

By LARRY L. AUGSBURGER and RALPH F. SHANGRAW

A photographic technique for the analysis of bubble size in "aerosol" foams is presented and the relationship between bubble size and foam rheology discussed. Within the same formulation, small bubbles are associated with high consistency. Container emptying gives rise to foams of lower consistency due to decrease in bubble density which more than offsets a decrease in bubble size. Synergistic consistency effects resulting from blends of fluorochlorohydrocarbon propellents are due to bubble size. Flammable propellents give rise to foams of lower consistency and decreased stability.

THE PAST few years have seen the introduction of a number of pharmaceutical and cosmetic foams which utilize the unique properties obtained from pressurized systems. These include contraceptives, hand creams, anogenital antiseptics, and antitussive products. The increased interest in foams as a dosage form makes it imperative that a more detailed investigation of basic foam systems be conducted in order that the formulation, packaging, and stability problems may be approached theoretically as well as empirically.

A review of foam formation and evaluation has been presented by Richman and Shangraw (1). These authors investigated the usefulness of rheological measurements in foam evaluation and found them to be extremely informative and reproducible (2–4). Studies were made on the effect of propellent type, propellent concentration, soap type, soap concentration, and additives on foam consistency and stability. In addition, a detailed study was made of the loss of consistency which occurs on container emptying, and a mathematical model was presented which predicted propellent concentrations at different stages and rates of container emptying.

All changes in foam consistency and stability should be relatable to the surface area of the foam bubbles and surface viscosity. The object of the present investigation was to study the effect of formulation, time, and can emptying variables on the bubble size of foams produced by pressurized systems, and to relate these studies to foam rheology.

Received August 3, 1967, from the Department of Pharmacy, School of Pharmacy, University of Maryland, Baltimore, MD 21201

Accepted for publication December 21, 1967.

Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

Abstracted in part from a thesis submitted by Larry L. Augsburger to the Graduate School, University of Maryland, Baltimore, in partial fulfillment of Doctor of Philosophy degree requirements.

This research was supported by a grant from Stalfort, Inc., Baltimore, Md.

EXPERIMENTAL

Formulation and Preparation—The same basic formulation suggested by Richman and Shangraw (2) was selected for this study because it combines relative simplicity with significant practical importance in regard to a typical high consistency foam such as shaving cream. This basic formulation consists of: soap concentrate 8%, stearic acid 5 parts; coconut oil fatty acids 3 parts, triethanolamine 5 parts; additive q.s.; distilled water q.s. ad. 100%. Total—90%; propellant 12/114 (57:43) 10%.

The soap concentrate was prepared by adding, with constant stirring, an aqueous solution of triethanolamine, which had been heated to 80° to a mixture of the stearic acid and coconut oil fatty acids which had also been heated to 80°. The mixture was held at 80° for 20 min. and then allowed to cool to room temperature with agitation. Any additives to be included were added, and the mixture was brought to the desired final weight by dilution with a suitable quantity of distilled water. Temperature, time, and rate of mixing were reproduced as closely as possible, as they may influence the properties of the resultant products.

The soap preparation was weighed into "six ounce" standard coated Spra-Tainer[1] cans which had previously been purged with a few drops of the liquid propellent and Precision 0.020/0.080 valves (Precision Valve Corporation, Yonkers, N. Y.) were crimped into place. As previous work in our laboratories has shown that the presence or absence of normal dip tubes does not affect foam properties; dip tubes were nevertheless omitted because the inversion of cans facilitates the filling of test vessels. The propellent was pressure filled into the test cans by weight. The finished products were then shaken vigorously and placed in a 40° bath water for 20 min. to test for leakage and can distortions. Although water bathing is routinely carried out in aerosol. production to check for leakage and can distortion,

[1] Spra-Tainer, Crown Can Co., Philadelphia, Pa.

little attention has been paid to the effects of time and temperature on the final product, which may be considerable for some formulations. After removal from the water bath, the cans were allowed to remain at room temperature for 3 days prior to testing. All testing was completed on a given batch of test products within 2 weeks of their preparations so as to minimize any possible aging factor.

Bubble size analysis was carried out utilizing an American Optical series 682 apparatus and a Polaroid camera. Foams were placed in a chamber which was formed by gluing a parallel pair of polyethylene strips ($^5/_{16} \times {}^5/_{16} \times 1$ in.) one-half in. apart onto a standard glass slide and covering with a cover slip. The $^5/_{16}$-in. deep chamber allows excess foam to flow freely out of the open ends, eliminating bubble distortion and at the same time providing greater protection against the heating effects of the illuminating lamp. Illumination was provided by an American Optical No. 735C microscope illuminator provided with heat absorbing glass. Exposure times up to 3 sec. were required through the first 5 min. elapsed time. Thereafter, as the foams aged, exposures of less than 1 sec. were sufficient.

Bubble size analysis was made by comparing bubbles against a scale made from a photograph of an object micrometer at the respective magnification used. Measurements were facilitated by performing the operation under the low power of an American Optical (Spencer) Cycloptic series 58 stereoscopic microscope. Bubbles were classified into 10-μ intervals and the average bubble radius was computed. An attempt was made to draw 100 bubble samples. However, in rare instances, it was necessary to take as few as 75 bubbles because of the limitations of magnification ranges available, field size, and actual bubble size.

Foam Rheology—Foam rheology was carried out in the manner of Richman (2) using the Haake Rotovisco rotational viscometer (Brinkmann Instruments, Inc., Westbury, N. Y.). All foams evaluated were studied using the MV III system, consisting of a smooth surface stainless steel rotor with a radius of 1.520 cm. and a height of 6.0 cm., and a stainless steel cup with a radius of 2.10 cm. Use of the 500 Gm. cm. torsion spring gave a working range in shearing stress of 27–5900 dynes cm.$^{-2}$ through a rate of shear range of 1–210 sec.$^{-1}$. Although this smooth rotor has proven to give excellent results with our experimental foams, some slippage was observed whene valuating commercial foam products of still higher consistency. For this reason the MV II P profiled bob system was used which gives a narrower gap between cup and rotor and has a serrated surface which reduces slippage.

Each pressurized container was shaken 15 sec. before filling the cup. The foam was introduced directly into the cup through a 5-in. extension of 0.25-in. polyethylene tubing attached to a Precision foam actuator No. SP115. All samples were removed with the valve in its maximum open position. In order to fill the cup uniformly, the cup was filled from the bottom up by slowly withdrawing the tube during actuation. All measurements were made at 28° so that they might better correlate with bubble data. Except in stability studies, readings were begun 1 min. after the cup had been filled. In operation, the time spent at each shear rate was limited to that necessary to make a dial reading, approximately 5 sec.

The need for reproducible technique in regard to evaluating any foam system cannot be overstressed. Such points as can shaking, rate of discharge, filling of evaluation vessels, time of setting before measurements are run, and temperature are critical. However, once such techniques have been mastered, reproducible results can be obtained. One other caution might be interjected at this point. If the formulation of a product is such that significant sputtering occurs when the foam is discharged, no meaningful physical measurements can be obtained. Sputtering means loss of entrapped propellent, which leads to an increase in foam density and a decrease in foam consistency. Although the literature is full of such data, it is impossible to interpret it even relatively.

Experiment I—The first experiment was designed to determine if bubble sizes and foam aging could be correlated with foam rheology. At the same time it was hoped to determine how these parameters are affected by the well-known can emptying effect. To accomplish these objectives, rheograms and bubble size measurements were made on foams after 1, 5, 10, 20, 30, 60, 90, and 120 min. of standing at room temperature (28 ± 1°). These determinations were made at 1, 10, 20, 40, 60, and 80 percentages of can emptying. Separate cans were used for each time increment. Can emptying was accomplished by removing approximately 5-Gm. samples at 15-min. intervals. Before removing samples the cans were shaken uniformly for 15 sec. Two formulations were studied. The first formulation was the basic formulation without additives, while the second formulation included 1.5% methylcellulose 400. The methylcellulose had previously been shown to have a marked effect on foam consistency and its effect on foam stability was of interest. Generally speaking, foam consistencies and bubble sizes decreased with increasing can emptying as did bubble rates of decay.

Experiment II—This experiment was designed to elucidate the effect of propellents on consistency and stability. Foams were prepared containing 10% of propellents 12, 114, or 12/114 (57:43) in two formulations: plain soap and plain soap with 10% propylene glycol. In addition, a formulation containing the hydrocarbon propellent A-46 with a vapor volume identical to the 57:43 blend of the fluorochlorohydrocarbons (*i.e.*, at 3.99% concentration) was included. This equivalent concentration was obtained using the equation of Gorman and Hall (5). In order to show the variation in bubble data, replicates of the plain soap formulation were studied. Bubble analysis and rheograms were determined for each product at 5% and 75% can emptyings. It was felt that the choice of 75% rather than 80% can emptying would ensure a higher degree of success in obtaining a sufficiently large and uniform sample. Of the fluorochlorohydrocarbon products, those prepared from plain propellents 12 and 114 showed the largest bubble sizes and the poorest consistencies at 5% can emptying. Products using the propellent blend were superior in consistency and showed the smallest bubbles. The addition of propylene glycol generally enhanced consistency, reduced bubble sizes, and reduced their rate of growth. Propellent A-46

generally produced foams with lower consistencies and less uniformity than those obtained using equivalent amounts of the fluorochlorohydrocarbons.

DISCUSSION

The choice of propellent and propellent concentration for use in an aerosol product has traditionally been governed by empirical evaluation of the initial product, stability, and economics. In most cases it has been difficult to ascertain subtle influences of formulation and packaging components and propellent changes due to the lack of sensitive quantitative tests on the final properties of the foam. Rheological evaluation has proven to be an effective means of determining changes in foam consistency, which is certainly one of the most important properties of foams. Furthermore, changes in foams which occur upon aging are reflected in such measurements. It has also been known that the changes in foam properties which occur during use of the product due to can emptying effects may be monitored by rheological measurements. The discovery of the discrete changes in consistency which can be brought about by relatively small changes in formulation raised the interesting question as to what physical-chemical phenomenon these changes could be attributed. It seemed obvious, and later work has borne this fact out, that changes in consistency can be attributed to changes in surface area or changes in viscosity of the interbubble film, probably as reflected in surface viscosity. Taking these influencing factors one at a time, a comprehensive study of the effect of formulation variables on bubble size was undertaken.

The effect of aging of a plain foam on the average bubble diameter at various stages of can emptying is shown in Fig. 1. The same effects on a formulation including methylcellulose can be seen in Fig. 2. Initial bubble radii for both formulations were in the general region of 10 μ. Bubble growth during the first 20 min. was rapid and essentially exponential. However, after 20 min., average bubble diameter seemed to be directly proportional to time.

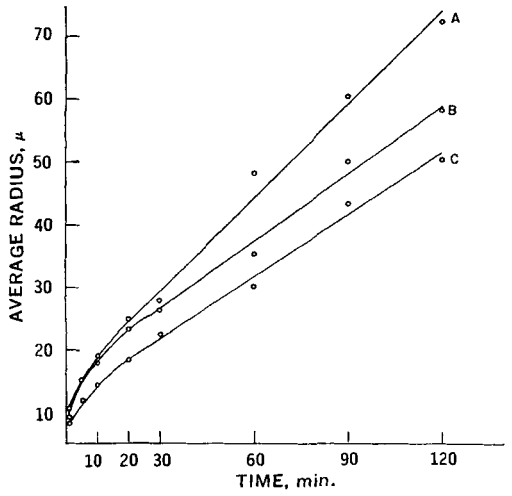

Fig. 1—Bubble growth curves: effect of can emptying (basic soap formulation without additive). Curve A, 10% empty; curve B, 40% empty; curve C, 80% empty.

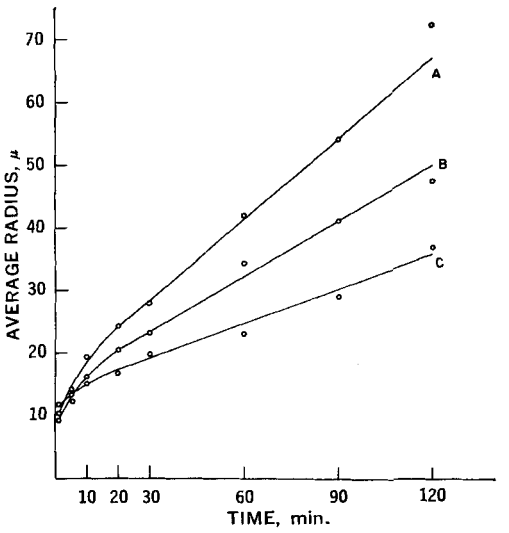

Fig. 2—Bubble growth curve: effect of can emptying (basic soap formulation with 1.5 % w/w methylcellulose 400). Curve A, 10% empty; curve B, 40% empty; curve C, 80% empty.

The initial apparent rapid growth in bubble size can be attributed to the presence of many very small bubbles which would have disappeared after a few minutes. Other factors which may play a role include (*a*) residual vaporiziation of propellent, (*b*) increase in vapor volume as the system equilibrates from cooling effects of propellant vaporization, and (*c*) higher kinetic energy in the initial system and possible greater initial permeability of interbubble films.

One very striking fact can be noted in almost all studies of bubble size *versus* can emptying. The average bubble size decreases as can emptying increases. At the same time the rate of coalescence of bubbles decreases. This means that foams produced at later stages of can emptying are more stable. Smaller bubble size would indicate a larger surface area and thus an increased consistency. However, these results are misleading in regard to the actual consistency of foams, which is known to substantially decrease during container emptying (2). This is due to the fact that although the bubble size decreases, the number of bubbles is also substantially smaller. This lower bubble density results in a much lower surface area which is then reflected in the decreased consistency. The decrease in bubble density helps also to explain the decrease in rate of coalescence of bubbles produced at later stages of emptying. Interbubble films are thicker and diffusion of vapor is thus retarded. These results are consistent with the conclusions of DeVries, who points to the thickness of bubble lamellae as being one of the most important factors in the rate of bubble coalescence (6). The works of DeVries along with that of Clark and Blackman (7) offer an indispensable background for all workers interested in bubble size analyses.

With regard to formulation variables, the plain soap formulation appeared to age more rapidly than the one containing methylcellulose, and this becomes more evident with can emptying. This

can be explained as the result of both a retardation of film drainage and a retardation of drying due to film formation on the surface of the bubbles. As expected, these effects are more significant at later stages of can emptying where the foam is denser.

Foam consistencies decrease with container emptying. Previous works by Richman and Shangraw, and Fong and Shangraw (8) confirmed in this study, show that this decrease in consistency is not directly proportional to can emptying but plateaus in the general region of 40–60% can emptying. It was hoped that bubble size analysis data would show a similar pattern, as it is difficult to envision anything but bubble size playing an important role. However, correlation did not materialize. The plateauing in consistency which occurs in can emptying is thought to be related to the fractionation of propellent blends which occurs as the propellent vaporizes into the headspace. The need for a better understanding of these effects led to the experiment in which the type of propellent was varied.

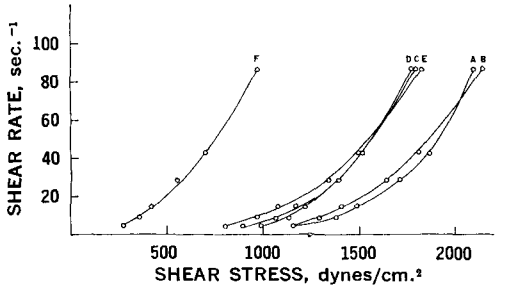

Fig. 3—Pressurized foam flow curves: effect of can emptying on 1 min. rheology (basic soap formulation with 1.5 % w/w methylcellulose 400). Curve A, 1% empty; curve B, 10% empty; curve C, 20% empty; curve D, 40% empty; curve E, 60% empty; curve F, 80% empty.

Bubble analysis can generally be correlated with foam rheology when the formulation remains constant. The effect of can emptying on the methylcellulose formulation may be seen in Fig. 3. As expected, the foam containing the methylcellulose exhibited higher consistencies but the relative drop in consistencies on container emptying remained constant. As initial bubble sizes of the two formulations are very similar, consistency changes are obviously due to viscosity effects, either in the bulk of the liquid, or, more probably, in the surface of the bubbles.

Photomicrographs of a typical shaving cream foam at different times of aging can be seen in Fig. 4. Initially, a shaving cream foam looks more like a gas emulsion since bubbles are essentially spherical. It is only after these high consistency foams have aged for a period of time and the interbubble films have drained, that the classical pentagonal dodecahedrons begin to form.

An interesting insight into the variabilities which can occur in bubble patterns of two high consistency commercial vaginal foams used in contraception is shown in Fig. 5. While one foam appears to be similar to the shaving cream, the other is much more polydisperse and seems to be of poorer quality. The polydisperse foam with thick lamellae shows little change on aging (Fig. 6). The few small bubbles have disappeared as would be expected, but the other bubbles have shown little growth. This photomicrograph points up the basic dynamics of bubble distribution in foams which occurs on aging, *i.e.*, small bubbles become smaller and larger bubbles grow. This is due to the increased curvature of a small bubble which results in higher relative pressure.

The results of the bubble size analysis conducted on foams prepared using different fluorochlorohydrocarbon propellent systems at two stages of can emptying can be found in Tables I and II. Two formulations were employed, one containing

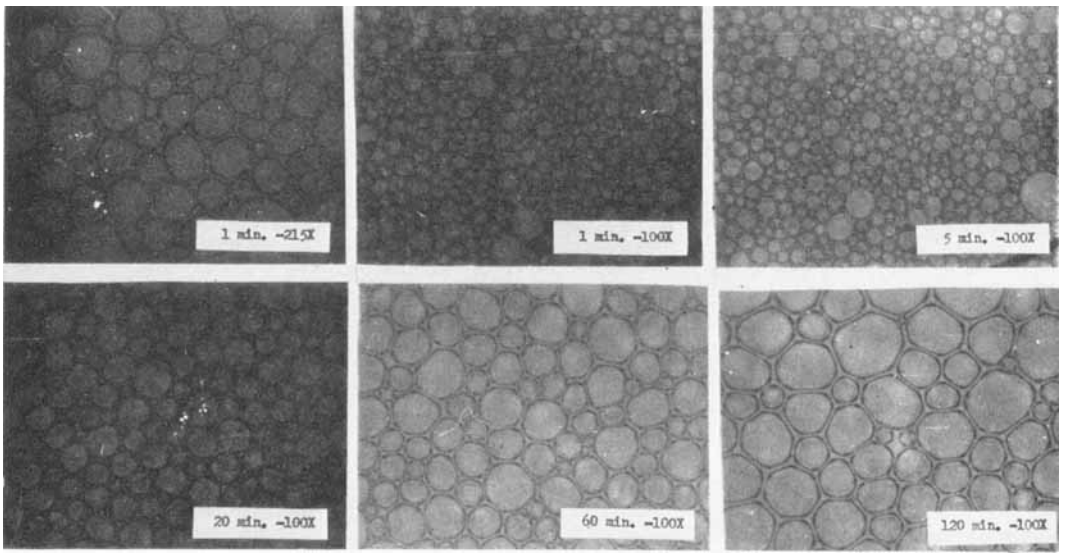

Fig. 4—Photomicrographs illustrating the effect of aging on a typical pressurized shaving foam.

plain soap (with replicate) and one containing 10% propylene glycol. The corresponding flow curves are presented in Fig. 7.

Bubble size data corresponded to consistency curves in all systems, *i.e.*, as bubble size decreased, consistency increased. However, there were some unusual results obtained in regard to bubble sizes produced by each propellent system.

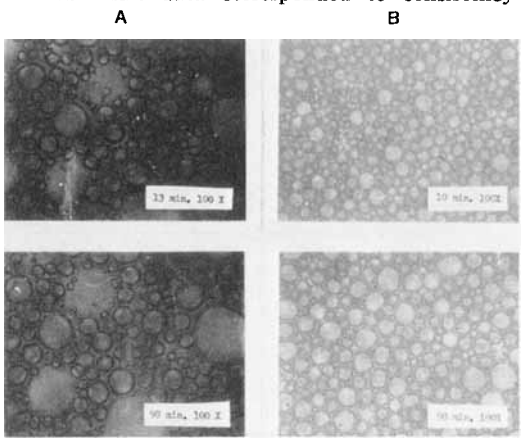

Fig. 5—Photomicrographs comparing commercial pressurized vaginal foams.

Propellent 12 gave smaller bubble sizes and higher consistencies than 114. These consistency data were not in agreement with the preliminary work done by Richman (2), but were reproducible and in line with bubble size measurements. However, the synergistic effect that propellent blends have on foam consistency was apparent in both studies. The reason for this increased consistency can now be seen to be due to the overall decrease in bubble size exhibited by foams prepared from blends. The reason for this decrease in bubble size is not immediately obvious but must be connected to the propensity for solubilized or emulsified molecules of propellent to associate into a single bubble of vapor when pressure is reduced during expulsion of foam.

The changes in bubble size which occur in foam aging can be seen in Fig. 8. Foams containing propellent 12 age extremely rapidly and would be expected to have sharp decreases in consistency.

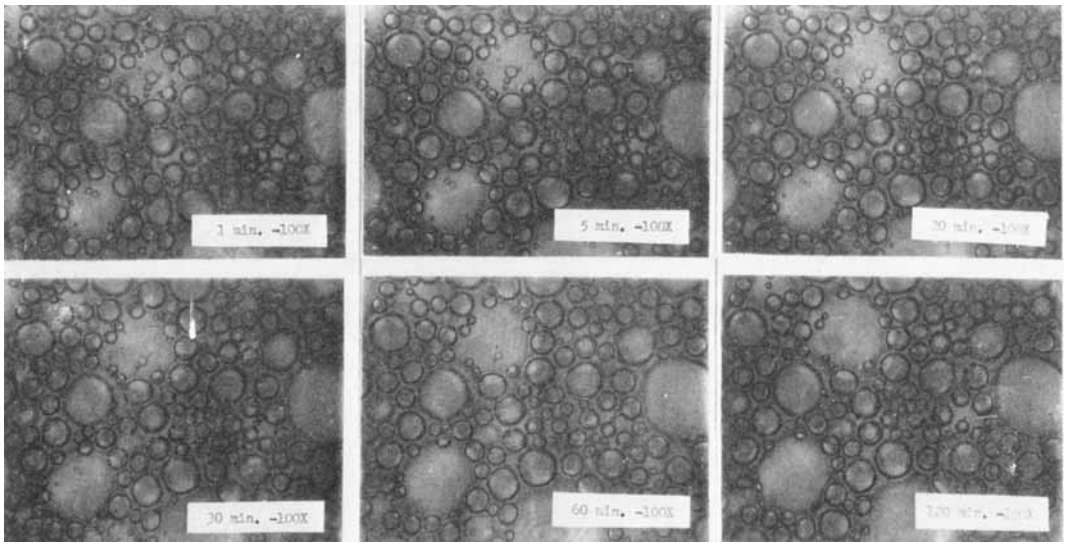

Fig. 6—Photomicrographs illustrating the effect of aging on a commercial pressurized vaginal foam.

TABLE I—EFFECT OF PROPELLENT TYPE ON BUBBLE SIZE OF TYPICAL SHAVING CREAM FOAMS 5% CAN EMPTYING

	Propellent 12 — Radii, μ			Propellent 12/114 (57:43) — Radii, μ			Propellent 114 — Radii, μ		
Time (min.)	Run 1[a]	Run 2[a]	Run 3[b]	Run 1[a]	Run 2[a]	Run 3[b]	Run 1[a]	Run 2[a]	Run 3[b]
1	14.8	15.8	10.5	10.2	9.7	10.6	20.2	21.0	30.2
5	22.4	22.8	15.2	15.3	13.7	12.0	21.1	21.5	31.0
10	27.2	31.6	20.3	21.2	18.2	15.0	21.4	22.7	30.2
20	38.0	37.3	24.8	25.3	22.0	17.1	24.0	24.7	30.6
30	47.0	50.8	31.2	27.1	24.9	21.7	25.1	25.9	31.8
60	63.4	69.4	42.1	36.5	37.3	30.0	28.2	27.7	34.9
90	76.6	83.6	56.0	50.6	51.2	35.0	36.9	36.0	33.9
120	100.2	102.1	64.1	58.0	56.4	43.2	45.2	43.1	34.7
Approx. slopes—linear portions[c] (μ min.$^{-1}$)	0.618	0.625	0.425	0.345	0.344	0.238	0.217	0.182	0.038

[a] Plain soap formulation. [b] Plain soap formulation with 10% propylene glycol. [c] Linear portions taken from 20 to 120 min.

TABLE II—EFFECT OF PROPELLENT TYPE ON BUBBLE SIZE OF TYPICAL SHAVING CREAM FOAMS 75% CAN EMPTYING

Time (min.)	Propellent 12 Radii, μ Run 1[a]	Propellent 12 Radii, μ Run 2[a]	Propellent 12 Radii, μ Run 3[b]	Propellent 12/114 (57:43) Radii, μ Run 1[a]	Propellent 12/114 (57:43) Radii, μ Run 2[a]	Propellent 12/114 (57:43) Radii, μ Run 3[b]	Propellent 114 Radii, μ Run 1[a]	Propellent 114 Radii, μ Run 2[a]	Propellent 114 Radii, μ Run 3[b]
1	10.0	9.4	9.4	7.1	7.6	8.4	6.8	7.5	6.7
5	14.4	15.0	15.4	12.0	10.6	11.9	9.2	9.9	7.7
10	19.5	19.0	17.4	14.2	15.4	16.3	11.9	12.0	9.9
20	26.9	25.4	24.2	19.8	17.5	18.5	13.2	15.1	12.7
30	40.7	40.5	27.1	23.1	21.4	22.5	18.8	17.9	14.7
60	50.8	54.5	37.6	36.9	32.7	29.8	24.4	22.8	18.4
90	72.5	68.1	50.1	48.5	46.0	38.7	29.3	27.8	22.1
120	80.2	84.4	59.4	53.8	55.4	42.3	34.1	35.4	25.9
Approx. slopes— Linear portions[c] (μ min.$^{-1}$)	0.500	0.500	0.357	0.385	0.370	0.285	0.172	0.172	0.125

[a] Plain soap formulation. [b] Plain soap formulation with 10% propylene glycol. [c] Linear portions taken from 20 to 120 min.

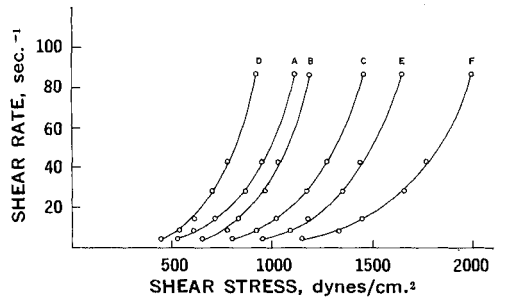

Fig. 7—Pressurized foam flow curves: effect of different nonflammable propellent systems (basic soap formulation with and without 10% w/w propylene glycol—5% can emptying). Key: A, 10% 114–plain soap; B, 10% 12—plain soap, C, 10% 12/114 (57:43)–plain soap, D, 10% 114–10% propylene glycol; E, 10% 12–10% propylene glycol; F, 10% 12/114 (57:43)–10% propylene glycol.

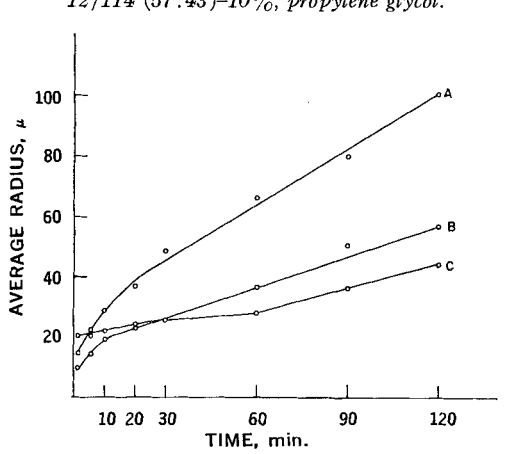

Fig. 8—Bubble growth curves: effect of different nonflammable propellent systems (basic soap formulation without additive—5% can emptying). Key: A, 10% 12; B, 10% 12/114 (57:43); C, 10% 114.

On the other hand, foams prepared from propellent blends and plain propellent 114 have identical bubble sizes after 30 min. All foams containing only propellent 114 age extremely slowly. It seems apparent that propellent 114 does not diffuse as rapidly through the interbubble walls as does propellent 12. The presence of propylene glycol in the formulation gives rise to smaller bubble sizes and slower aging in those foams utilizing propellent 12 or propellent blends. On the other hand, foams produced with propellent 114 have a much larger initial bubble size but show extremely little aging over a 2-hr. period. In all cases, addition of propylene glycol resulted in a decrease in the slopes of the linear portions of the time-radius plots.

One unusual effect is that wherein the propylene glycol formulation actually gives a lower consistency foam than the plain soap when propellent 114 is employed. The effect was so unexpected that additional samples were tested with confirming results. Although the relatively low boiling point and longer time required for vaporization equilibrium to be achieved makes data obtained from propellent 114 less reliable, the effects noted are real and reproducible.

The 75% can emptying data presented in Table II point up the characteristic decrease in bubble size and lower rate of coalescence which occurs in foams produced from the latter part of a pressurized container. This effect is particularly striking in the case of propellent 114 in which the average

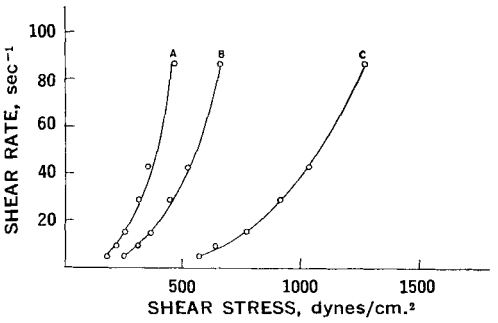

Fig. 9—Pressurized foam flow curves: effect of different nonflammable propellent systems (basic soap formulation with 10% w/w propylene glycol—75% can emptying). See Fig. 8 for Key.

TABLE III—COMPARISON OF PROPELLENT BLEND 12/114 (57:43) AND PROPELLENT A-46 AT MATCHED VAPOR VOLUMES

	Radii, μ							
	5% Can Emptying				75% Can Emptying			
	Propellent 12/114 (57:43)		Propellent A-46		Propellent 12/114 (57:43)		Propellent A-46	
Foam Age, min.	Plain Soap	With Propylene Glycol	Plain Soap	With Propylene Glycol	Plain Soap	With Propylene Glycol	Plain Soap	With Propylene Glycol
1	10.0	10.6	11.6	8.8	7.4	8.4	9.2	8.0
90	50.9	35.0	61.8	48.5	47.3	38.7	48.0	36.6

bubble size is now the smallest of the three propellent systems studied. On this basis, it would be expected that consistency of the 114 foams would not decrease as much as would be expected. The consistency curves shown in Fig. 9 bear out this assumption with the effects in the propylene glycol formulation being particularly striking. The foam obtained at 75% can emptying from propellent 114 in the presence of propylene glycol exhibits a higher consistency than any of the other 114 curves at either level of can emptying. These results have to be relatable to bubble size. Although the reason for this is not clear, the propellent 114 appears to be better emulsified or solubilized in the presence of a large excess of soap. The fact that fractionation of propellents upon can emptying favors the retention of propellent 114 in the concentrate combined with the above effects might serve as a basis for improving foam quality at latter stages of container use. Unfortunately, propellent 114 alone is not energetic enough to serve as the sole propellent in a foam system.

Because it was felt that nonflammable propellents would have a greater application to pharmaceutical foam systems, a great deal of the experimental work was devoted to the investigation of the fluorochlorohydrocarbon propellents. However, the fact remains that economics have resulted in the widespread use of the flammable propellents (isobutane, propane) in cosmetic foams such as shaving creams. A recent analysis of shaving creams of 10 major companies indicates that only two are using nonflammable propellent systems.

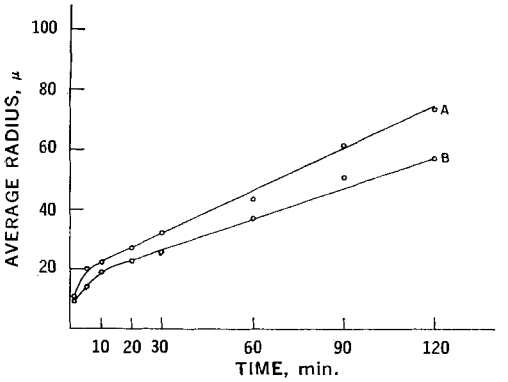

Fig. 10—Bubble growth curves: comparison of flammable and nonflammable propellent systems with matched vapor volumes (basic soap formulation without additive—5% can emptying). Key: A, 3.99% A-46; B, 10% 12/114 (57:43). Slope of linear portion (μmin.$^{-1}$): A, 0.476; B, 0.345.

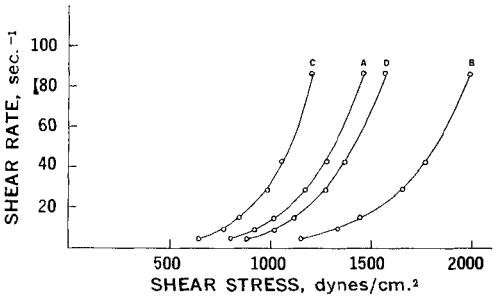

Fig. 11—Pressurized foam flow curves: comparison of flammable and nonflammable propellent systems with matched vapor volumes (5% can emptying). Key: A, plain soap + 10% 12/114 (57:43), B, same as in A + 10% w/w propylene glycol; C, plain soap + 3.99% w/w A-46; D, same as in C + 10% w/w propylene glycol.

A summary of the bubble size data obtained from foams produced from the two propellent systems with matched vapor volumes is shown in Table III. Initial bubble sizes appear to be quite similar but bubble growth is more rapid in the A-46 systems. This increased rate of aging which occurs in the flammable propellent system is illustrated in Fig. 10. It would appear that the flammable propellents diffuse more rapidly through the interbubble walls than do the nonflammable propellents, indicating a slightly lower degree of stability.

The consistency curves shown in Fig. 11 obtained from the two propellent systems indicate the existence of a much greater difference between the foams than was obvious from the bubble data. The A-46 foams gave significantly lower consistencies for both concentrate formulations studied. This decreased consistency in light of fairly equal average bubble diameters can best be explained by looking at a bubble size frequency distribution, as is shown in Fig. 12. The much wider distribution of bubble

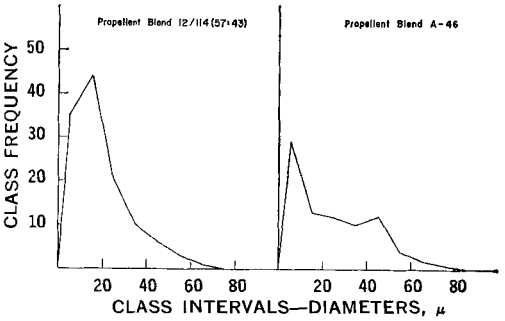

Fig. 12—Initial (1 min.) bubble size frequency distributions: Comparison of flammable and nonflammable propellent systems with matched vapor volumes (basic soap formulation without additive—5% can emptying).

sizes and resultant decrease in total surface area in the A-46 foam helps to explain the lower consistencies. This lack of bubble size homogeneity would seem to indicate that emulsification of A-46 propellent is more difficult. A great deal of work remains to be done on characterization of foams from flammable propellent systems.

SUMMARY AND CONCLUSIONS

(*a*) A photomicrographic technique is described for the determination of the growth of bubbles in aerosol foams which yields a reasonably good degree of reproducibility.

(*b*) Within the same formulation and propellent concentration, smaller bubbles are associated with higher consistencies.

(*c*) As a container is emptied, bubble sizes and growth slopes tend to decrease due to a higher ratio of soap to propellent and thicker interbubble films. Consistency also decreases.

(*d*) The addition of methylcellulose increases foam consistency and decreases the rate of bubble coalescence.

(*e*) Initially, propellent 114 yields larger bubbles than propellent 12. Blends yield smaller bubble sizes than either single propellent. These results are consistent with foam rheology at both 5% and 75% can emptying.

(*f*) The addition of propylene glycol tends to decrease bubble sizes and increase foam consistency except in cases of plain propellent 114 where unusual effects are noted, particularly at 75% can emptying.

(*g*) When vapor volumes are matched, flammable propellents give rise to foams of lower consistency and decreased stability. This is not always obvious when observing average bubble size alone, as bubble sizes are more disperse when A-46 propellents are used.

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Keyphrases

Aerosol foams—bubble size analysis
Bubble size—aerosol rheology
Photomicrographic technique—bubble size determination
Aging foam—bubble diameter effect
Can emptying—bubble size, density
Propellent—foam bubble size

Drug Standards

Determination of Free Salicylic Acid in Buffered Aspirin Tablets

By JOSEPH LEVINE and JOHN D. WEBER

Aspirin and free saliyclic acid are quantitatively released from buffered aspirin tablets, with a negligible degree of hydrolysis of aspirin, by briefly wetting the sample with 98–100 percent formic acid, followed by immediate dilution with chloroform. The salicylic acid is isolated and determined as previously described.

SALICYLIC ACID can be quantitatively separated from aspirin by trapping it as the complex formed with ferric ion on a partition chromatographic column, using ferric chloride (1) or ferric chloride-urea (2) solutions as the immobile phase.

In the application of this procedure to analytical samples, it is essential that the entire amount of aspirin and salicylic acid be dissolved in the mobile phase before the chromatographic treatment, and that the aspirin is not hydrolyzed during the preparation of that solution. These

Received October 5, 1967 from the U. S. Department of Health, Education, and Welfare, Food and Drug Administration, Bureau of Science, Division of Pharmaceutical Sciences, Washington, DC 20204
Accepted for publication December 15, 1967.

sizes and resultant decrease in total surface area in the A-46 foam helps to explain the lower consistencies. This lack of bubble size homogeneity would seem to indicate that emulsification of A-46 propellent is more difficult. A great deal of work remains to be done on characterization of foams from flammable propellent systems.

SUMMARY AND CONCLUSIONS

(*a*) A photomicrographic technique is described for the determination of the growth of bubbles in aerosol foams which yields a reasonably good degree of reproducibility.

(*b*) Within the same formulation and propellent concentration, smaller bubbles are associated with higher consistencies.

(*c*) As a container is emptied, bubble sizes and growth slopes tend to decrease due to a higher ratio of soap to propellent and thicker interbubble films. Consistency also decreases.

(*d*) The addition of methylcellulose increases foam consistency and decreases the rate of bubble coalescence.

(*e*) Initially, propellent 114 yields larger bubbles than propellent 12. Blends yield smaller bubble sizes than either single propellent. These results are consistent with foam rheology at both 5% and 75% can emptying.

(*f*) The addition of propylene glycol tends to decrease bubble sizes and increase foam consistency except in cases of plain propellent 114 where unusual effects are noted, particularly at 75% can emptying.

(*g*) When vapor volumes are matched, flammable propellents give rise to foams of lower consistency and decreased stability. This is not always obvious when observing average bubble size alone, as bubble sizes are more disperse when A-46 propellents are used.

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Keyphrases

Aerosol foams—bubble size analysis
Bubble size—aerosol rheology
Photomicrographic technique—bubble size determination
Aging foam—bubble diameter effect
Can emptying—bubble size, density
Propellent—foam bubble size

Drug Standards

Determination of Free Salicylic Acid in Buffered Aspirin Tablets

By JOSEPH LEVINE and JOHN D. WEBER

Aspirin and free saliyclic acid are quantitatively released from buffered aspirin tablets, with a negligible degree of hydrolysis of aspirin, by briefly wetting the sample with 98–100 percent formic acid, followed by immediate dilution with chloroform. The salicylic acid is isolated and determined as previously described.

SALICYLIC ACID can be quantitatively separated from aspirin by trapping it as the complex formed with ferric ion on a partition chromatographic column, using ferric chloride (1) or ferric chloride-urea (2) solutions as the immobile phase.

In the application of this procedure to analytical samples, it is essential that the entire amount of aspirin and salicylic acid be dissolved in the mobile phase before the chromatographic treatment, and that the aspirin is not hydrolyzed during the preparation of that solution. These

Received October 5, 1967 from the U. S. Department of Health, Education, and Welfare, Food and Drug Administration, Bureau of Science, Division of Pharmaceutical Sciences, Washington, DC 20204
Accepted for publication December 15, 1967.

two requirements are not readily achieved in the case of buffered aspirin tablets (2). Chloroform solutions of aspirin, in the presence of basic materials such as those which comprise the buffering components of the tablets, undergo hydrolysis together with aspirin anhydride formation (1). Boric acid stabilizes the solution, at least with respect to the hydrolysis of aspirin, but does not achieve the necessary release of the aspirin and salicylic acid from the buffer components to permit their complete solution in chloroform.

A preliminary acid treatment of the buffered tablet will release the aspirin and salicylic acid. The acid may, however, concurrently hydrolyze the aspirin. In designing a valid assay procedure, the acid which is used must: (*a*) produce only minimal hydrolysis of aspirin under the conditions of the assay; (*b*) rapidly and completely release aspirin and salicylic acid from the buffer components of the tablet; (*c*) be soluble in chloroform; and (*d*) be readily removed from the chloroform, so that the ferric-salicylic acid complex will not be dissociated during the following step of the analysis.

These requirements are fulfilled by 98–100% formic acid. The common 90% reagent is not suitable because it is immiscible with chloroform. The distribution of formic acid between chloroform and inorganic acids is greatly in favor of the aqueous phase; therefore, the formic acid is removed from the chloroform solution by passage over dilute hydrochloric acid.

In the analysis the powdered sample is thoroughly wetted with the formic acid and then immediately diluted with chloroform to sharply decrease the hydrolytic action of the acid.

The extent of hydrolysis during the period of contact of the sample with the formic acid before dilution was gauged by a direct determination of the rate of the hydrolysis. Although this increase varies somewhat for various brands of buffered aspirin, it lies in the range of 0.01–0.02%/min. The procedure specifies a period of 30–45 sec. for this step; the average time of contact will be shorter since a part of this time is consumed in the wetting of the sample. The total extent of hydrolysis in this step will therefore be in the order of 0.01%.

After dilution, hydrolysis is sharply decreased to an average of only 0.03%/hr., and thus a negligible amount occurs during the 10-min. period specified for dissolving the aspirin and salicylic acid.

The degree of completeness of solution is established by direct examination of the residual solid tablet components. After vigorous treatment of the residue with hydrochloric acid, the spectrum of a chloroform extract indicated the presence of both aspirin and salicylic acid. In view of the hydrolytic effect of the acid treatment, these results indicate that perhaps the major portion of the residual acidic material is aspirin, rather than salicylic acid. In any event, the total amount of the residual material, whatever its original identity, was about 0.02%, calculated as salicylic acid.

EXPERIMENTAL

The ferric chloride-urea reagent, salicylic acid standard, and chromatographic tubes are prepared as previously described (2).

Column A—Pack a plug of cotton about 15 mm. deep in the base of the chromatographic tube to absorb any stationary phase which may bleed off column. Mix 5 Gm. of diatomaceous earth[1] with 3 ml. of 0.05 *N* hydrochloric acid to a uniform mixture, transfer, and pack the mixture in three portions, lightly enough to obtain a 12 ml./min. flow of chloroform.

Column B—Pack a plug of glass wool[2] in the base of the chromatographic tube for support. To prepare the lower state, mix 1 Gm. of diatomaceous earth with 0.5 ml. of 30% phosphoric acid to retain any ferric chloride which may be removed during the elution of larger quantities of salicylic acid (3), as are often found in buffered tablets. Transfer the mixture to the tube and tamp to a uniform mass. For the upper stage, mix 3 Gm. of diatomaceous earth with 2 ml. of ferric chloride-urea solution, transfer to the column directly above the phosphoric acid layer, and tamp. Cover with a pad of cotton about 20 mm. thick to insure uniform distribution of the solvent over the column cross section. Mount column A above column B.

Procedure—(Use water-saturated solvents throughout.) Determine the average weight of a representative number of tablets. Grind to a fine powder rapidly, and immediately weigh an amount equivalent to about 500 mg. of aspirin. Transfer to a 100-ml. volumetric flask and immediately add 5 ml. of 98–100% formic acid. Agitate to wet the sample thoroughly; then within 30–45 sec. add 75 ml. of chloroform. Shake for 10 min., and adjust to volume with chloroform. Filter through a loose plug of glass wool. Pass 20.0 ml. of the sample over the columns, and then wash with three 25-ml. portions of chloroform, allowing each portion to pass through both columns before the successive addition. Discard the eluate and column A, and rinse the tip of column B with a stream of chloroform.

Place as receiver under column B a 100-ml. volumetric flask containing 10 ml. of methanol and 2 drops of hydrochloric acid. Pass a solution of 1 ml. of glacial acetic acid in 10 ml. of ether through the column and follow with 1% acetic acid–chloroform (v/v) to volume.

Concomitantly determine the absorbance of this solution and of the standard at the maximum at about 306 mμ.

[1] Celite, acid-washed, Johns-Manville Corp., New York, N. Y.

[2] Pyrex Filtering Fiber, Corning Glass Co., catalogue No. 3950.

TABLE I—SALICYLIC ACID CONTENT OF COMMERCIAL BUFFERED ASPIRIN TABLETS

Brand	% Salicylic Acid Found
A	0.53
	0.56
	0.57[a]
A[b]	0.51
	0.50
B	1.85
	1.86
C	0.69
	0.70[a]
	0.76[c]
	0.78[c]
D	1.41
	1.45
E	2.66
F	2.82
	2.91
G	0.86
	0.83

[a] Analysis by extrapolation of hydrolysis rate curve. [b] Separate bottle of same brand. [c] Analyzed 1 mo. after bottle was opened.

Determination of Rate of Hydrolysis—Weigh portions of sample equivalent to about 100 mg. of aspirin into individual 150-ml. beakers. Add 1 ml. of 98–100% formic acid to each beaker and agitate to wet the sample thoroughly. Immediately add 2 Gm. of diatomaceous earth to one beaker, mix thoroughly, transfer to column A, and tamp. Pass 25 ml. of chloroform through the columns, recording the interval between the addition of the formic acid and the addition of the chloroform as the period of contact. Continue the analysis as described above. Add the diatomaceous earth to the other samples at proper intervals to provide contact periods of 5, 10, 20, and 30 min.

RESULTS AND DISCUSSION

A number of commercial samples of buffered aspirin tablets purchased from local retailers were analyzed for their salicylic acid content. Results are shown in Table I.

The total extent of hydrolysis of aspirin during an actual assay is shown in the values obtained in the analysis of pure aspirin by the original procedure (2) and by the proposed procedure, using formic acid. The original procedure gave a salicylic acid value of 0.025% and the current procedure 0.036%, an increase of only 0.011%. Although a slightly larger increase may result during the analysis of buffered tablets, since the rate of hydrolysis of aspirin in these tablets is somewhat greater than that for pure aspirin (Fig. 1), it remains at a level close to the precision of the determinative steps (2), and therefore does not constitute a significant source of error. This is substantiated by the fact that the values obtained by extrapolation of the hydrolytic rate series to zero time agreed well with those obtained by the method as proposed.

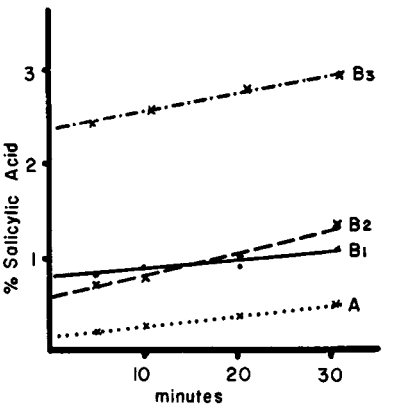

Fig. 1—Rate of hydrolysis of (A) aspirin tablets and (B 1–3) buffered tablets.

Atmospheric humidity can cause an appreciable degree of hydrolysis before the analysis. A freshly opened bottle of buffered tablets gave a value of 0.70% salicylic acid. One month later the salicylic acid content had increased to 0.77%. For this reason, the procedure requires assay immediately after the sample is ground.

The very small amount of salicylic acid found on analysis of residual tablet material demonstrates the effectiveness of the procedure in completely releasing aspirin and salicylic acid from the buffering materials. It has not been resolved whether the free salicylic acid is present as such adsorbed on the buffering components or has been converted to its metallic salts. The formic acid treatment readily recovers salicylic acid from its calcium or magnesium salt, but the aluminum salt is quite refractory to the treatment. Hence, if a metallic salicylate is formed, it must be other than aluminum salicylate.

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Keyphrases

Salicylic acid, free—analysis
Aspirin tablets, buffered—free salicylic acid
Formic acid—preanalysis sample wetting
Chloroform diluent
Hydrolysis rate—aspirin
Column chromatography—separation
UV spectrophotometry—identity

Chemical Determination of the Potency of Thyroid Preparations

By JOSEPH E. MOODY, JR., JOHN R. HOHMANN, and GILBERT B. KAPLAN

Several methods for the chemical analysis of thyroid have been reported in recent years, but none has been adopted by the official compendia. In an attempt to meet the need for a new official chemical assay, a procedure has been devised, and is reported in this paper for the quantitative determination of triiodothyronine (T_3) and thyroxine (T_4). Results for some of the samples tested are compared to the biological assay. The thyroid is hydrolyzed with barium hydroxide and the digest is extracted with *n*-butanol. The iodoamino acids are separated by thin-layer chromatography on cellulose plates and the T_3 and T_4 zones are scraped separately from the plates and ignited in oxygen flasks. The iodine is subsequently released and determined spectrophotometrically in benzene. Commercial thyroid preparations with different biological activities were analyzed and a chemical potency determined for each. The results correlated well with those obtained by biological assay.

THE USP requires that thyroid contain not less than 0.17% and not more than 0.23% of iodine in thyroid combination. This 50-year-old standard does not truly reflect the physiological activity of the drug (1–3). Numerous samples of thyroid meet the official iodine requirement but have little or none of the desired activity.

Many attempts have been made to develop an analytical procedure for thyroid that will more accurately reflect its physiological activity. The complexity of this proteinaceous substance has until recently defeated these efforts. Hydrolysis of thyroid yields a number of iodinated derivatives of tyrosine and thyronine (4–7). However, only four of these, thyroxine, 3,3′,5-triiodothyronine, 3,3′,5′-triiodothyronine, and 3,3′-diiodothyronine, have biological activity. A quantitative method for thyroxine and 3,3′,5-triiodothyronine would suffice to estimate the potency of thyroid since the diiodo compound is present in only trace amounts and the 3,3′,5′-triiodothyronine has negligible activity (8).

Recently several methods have been presented for the determination of thyroxine (T_4) and 3,3′,5-triiodothyronine (T_3) in thyroid (2, 7, 9–14). Most of these procedures use the following scheme: (*a*) hydrolysis of the thyroprotein with sodium hydroxide or barium hydroxide, or by enzymatic action; (*b*) extraction of the released iodoamino acids with *n*-butanol; (*c*) separation of these iodoamino acids by paper, thin-layer or column chromatography, or by gel filtration; (*d*) quantitation of the separated active compounds either by direct determination or by destruction of the compound and determination of the released iodine.

Some of these published methods of thyroid assay have been studied in this laboratory, but the consistent results necessary for routine analysis could not be achieved. Lemieux and Talmage (11) discuss many of the disadvantages and inadequacies of some of the earlier methods. The general scheme of their procedure contains some steps which, with modifications, were adapted to our method. Enzymatic hydrolysis with pronase and alkaline hydrolysis with barium hydroxide were compared in the present study. Devlin and Watanabe (12) reported that hydrolysis with pronase yielded more T_3 and T_4 in a shorter time than hydrolysis with the other enzyme studies. The present data agree with their report with regard to enzymatic hydrolyses, but show that more T_4 is released by hydrolysis with barium hydroxide.

In our procedure the released components of the hydrolyzed thyroprotein are separated by thin-layer chromatography on cellulose plates, with a solution of *p*-toluenesulfonic acid and formic acid as the developing solvent. This procedure releases a significant amount of a component between T_3 and T_4 on the chromatogram which is not usually reported. The compound has been shown to be 3,3′,5′-triiodothyronine.

Received August 21, 1967, from the U. S. Department of Health, Education, and Welfare, Food and Drug Administration, Division of Pharmaceutical Chemistry, Washington, DC 20204

Accepted for publication October 12, 1967.

The authors express deep appreciation to Frieda M. Kunze and Charlotte A. Brunner of the Chromatographic Research Branch of this Laboratory for their assistance and advice in the development of the chromatographic step in this procedure.

The authors also wish to thank Miss Mae E. Biesemeyer and Mrs. Sandra Harvey for their invaluable assistance in the laboratory. Thanks are also expressed to Stanley Srensek, Division of Pharmacology, who performed all of the bioassays.

The areas of the chromatogram containing T_3 and T_4 are scraped from the plate and ignited in an oxygen flask to convert the iodine, after intermediate treatment, to iodate. The final step in the procedure is the spectrophotometric measurement of iodine in benzene.

EXPERIMENTAL

Apparatus—A Cary 14 or Beckman DK recording spectrophotometer was used for these studies. The 10-cm. cylindrical silica cells for the absorption measurements and the Thomas-Ogg oxygen ignitor were purchased from Arthur H. Thomas Co., Philadelphia, Pa. Glass chromatography tanks were 30 × 30 × 10 cm.; the chromatoplates were 20 × 20 cm.

Reagents—All reagents and solutions were prepared from analytical grade chemicals and with water redistilled from an all-glass still. The following reagents were used: $Ba(OH)_2.8H_2O$; 30% formic acid, diluted from 90% analyzed reagent; *n*-butanol; *n*-butanol equilibrated with 0.1 *N* sodium thiosulfate solution; ammoniacal methanol, 5% v/v solution of ammonium hydroxide in methanol; phosphoric acid solution, 260 ml. of 85% H_3PO_4 diluted to 1 L. with water; benzene, "distilled in glass," b.p. 80–81°, Burdick and Jackson Laboratories, Inc., Muskegon, Mich.; bromine water, USP; *p*-toluenesulfonic acid, 40% w/v; NaOH, 2% w/v; freshly prepared potassium iodide, 7.5% w/v; sodium carbonate, half saturated.

To prepare the diazotized sulfanilamide (15), mix 5 ml. of a 1% sulfanilamide solution in 10% v/v hydrochloric acid with 5 ml. of 5% w/v sodium nitrite solution in a 50-ml. glass-stoppered graduated cylinder for 1 min. Add *n*-butanol to 50 ml. and shake for 1 min. Let stand 4 min. and decant the butanol layer to be used as the spraying solution. Prepare just before use.

The cellulose was MN cellulose powder from Macherey Nagel and Co. (distributed by Brinkmann Instrument Co., Great Neck, N. Y.). The gelatin capsules (No. 0 and 00) were obtained from Eli Lilly & Co., Indianapolis, Ind.

To facilitate transferring the cellulose containing the T_3 nd T_4 to capsules, a funnel rack and a capsule holder were devised (Fig. 1).

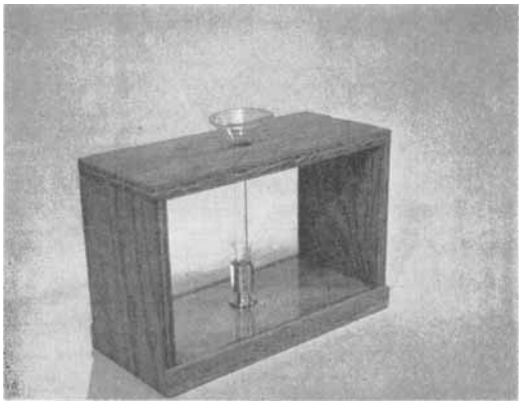

Fig. 1—Device to transfer cellulose from chromatoplates to capsules for oxygen ignition.

Preparation of Samples—*Tablets*—Weigh 20 tablets and grind to a fine powder. Transfer the equivalent of 200 mg. of USP thyroid, accurately weighed, to a 50-ml. centrifuge tube or appropriate test tube to fit a centrifuge and heating block. Add 6 ml. of water, mix well on a vortex mixer, and centrifuge for 15 min. Remove the supernatant with a syringe fitted with a large blunt needle and discard the washings. Repeat with a second 6 ml. of water. Fit the tube with an air condenser at least 10 in. long.

Bulk Powders—Place an accurately weighed sample of thyroid powder equivalent to about 400 mcg. of iodine in a test tube of appropriate size that is fitted with an air condenser.

Total Iodine Determination—Weigh accurately a No. 0 gelatin capsule and a sample of ground tablets or bulk powder equivalent to about 8.4 mg. of USP thyroid. Ignite the capsules as described under *Oxygen Ignition* and continue the procedure from there to completion. Calculate as follows:

$$\% \text{ total iodine} = \frac{(\text{mcg. iodine from standard curve})\ (100)}{(\text{wt. of thyroid in sample in mcg.})\ (6)}$$

Hydrolysis and Extraction—To the tubes containing the accurately weighed samples add 3 Gm. of $Ba(OH)_2.8H_2O$ and 6 ml. of water (16). Mix well and place the air condensers in the tubes. Heat the tubes in an aluminum heating block at 100° or in a boiling water bath for 16–18 hr. Swirl the tubes occasionally to mix the contents. After the hydrolysis, allow the mixture to cool and add about 3.5 ml. of dilute hydrochloric acid (1:1) to a pH of 0.8 or 0.9 as determined with a pH meter. Transfer the acidified solution to a 60-ml. separator. Wash the hydrolysis tube with three 5-ml. portions of water and add the washings to the separator. Extract the hydrolysate solution with 10, 5, and 5 ml. of *n*-butanol previously equilibrated with 0.1 *N* sodium thiosulfate. Wash the combined butanol extracts with 25-ml. portions of butanol-saturated water until the washings are neutral to pH indicator paper. Filter the butanol through a medium sintered-glass funnel into a 50-ml. round-bottom flask. Rinse the separator with three 2-ml. portions of butanol and pass the rinsings through the separator. Add a small quantity of ethanol to the filtered butanol solution to dissolve any droplets of water present. Remove the butanol at 55° under reduced pressure. The dried residue may be stored for at least 1 week in the cold under nitrogen (13).

Chromatography—To prepare five 20 × 20 cm. plates homogenize 15 Gm. of cellulose powder in a fast blender with 90 ml. of water. Spread the cellulose slurry on the plates in a 0.6 mm. (±0.05 mm.) layer, and dry the plates overnight at room temperature. Scratch a line across a plate about 3 cm. from the top. Make two lines, each 6 cm. long, of pin-point dots 0.5 cm. apart, about 1.5 cm. from the bottom of the plate and separated by 3 or 4 cm.

Dissolve the hydrolysate residue in 1.0 ml. of ammoniacal methanol, and transfer as much of the solution as possible to a 1-ml. stoppered flask or tube to reduce evaporation. Spot 200 µl. of the ammoniacal methanol solution uniformly along each of the 6-cm. dotted lines. Scrape a vertical band of

cellulose from top to bottom with a narrow flat-edged spatula on both sides of the spotted areas in parallel lines almost touching the lines of sample application (see Fig. 2). These borders will aid in the development of straight horizontal zones.

Line glass chromatography tanks on one side with filter paper. Pour a mixture of 50 ml. of the 40% *p*-toluenesulfonic acid solution and 50 ml. of the 30% formic acid solution down the paper into the tank. Place the chromatoplate in the developing tank leaning on the unlined side and cover the tank with a glass plate. Allow the chromatograms to develop until the solvent front reaches the line across the top of the plate (about $2^1/_2$ hr). Air dry the plates in a fume hood.

Spray the plate with the freshly prepared diazotized sulfanilamide solution (15). Dry the plate in a current of air for 5 min. and then spray with half-saturated sodium carbonate solution. The zones containing the amino acids will be visible as pink areas on the chromatogram. Allow the plate to air dry in a fume hood.

Place the plate in a chromatogram viewing cabinet and observe with a long-wave ultraviolet light. The pink areas will appear as dark zones (Fig. 3). Mark the T_3 and T_4 zones with a dissecting needle or other sharp instrument. Scrape the marked T_3 and T_4 zones from the plate and place the cellulose in No. 0 or 00 gelatin capsules. Clean the glass in the scraped zones and the instruments used with small pledgets of cotton and add the cotton to the capsule.

Oxygen Ignition—All glassware used from this point on should be extensively rinsed with the redistilled water. Oxygen ignition is carried out in a Thomas-Ogg oxygen igniter. Ignite the capsules in 1-L. flasks containing 30 ml. of 2% sodium hydroxide solution as the scrubbing solvent, and a small strip of black ignition paper as the igniting point. After the flasks are cooled, invert and shake them for 5 min. Quantitatively transfer the solution to well rinsed 250-ml. conical flasks, collecting a total of about 150 ml. from repeated rinsings. Run a reagent blank starting with the addition of 30 ml. of the sodium hydroxide solution to a 250-ml. conical flask and continuing through the assay.

Assay—Slowly add 5 ml. of the dilute phosphoric acid solution to each flask, then 1 ml. of USP bromine water. Do not reverse this order of addition of reagents; otherwise high and irregular blanks and samples may result. Mix by swirling the flasks gently, and add a few boiling chips. Boil the solutions on a hot plate until all the excess bromine is removed as shown with starch-iodide test paper, and until the solutions are between 75 and 100 ml. Do not let the solutions boil down below 75 ml. Wash down the sides of the flasks with a stream of water and boil for an additional 5 min. Cool the solutions to room temperature and transfer to well-rinsed 125-ml. separator. Extract the solutions with 25 ml. of benzene and discard the benzene. Add a second 25-ml. portion of benzene to each separator and 3 ml. of the freshly prepared 75% solution of potassium iodide. Stopper the separators and shake for 1 min. Allow the layers to separate and collect the benzene layer in benzene-rinsed 50-ml. volumetric flasks. Extract the samples with additional 15-ml. and 9-ml. portions of benzene and add the extracts to the volumetric flasks. Bring

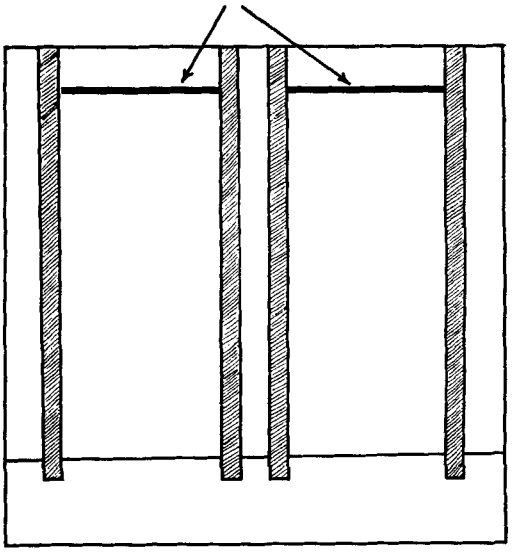

Fig. 2—Diagram of chromatoplate before development. Arrows point to lines of sample application.

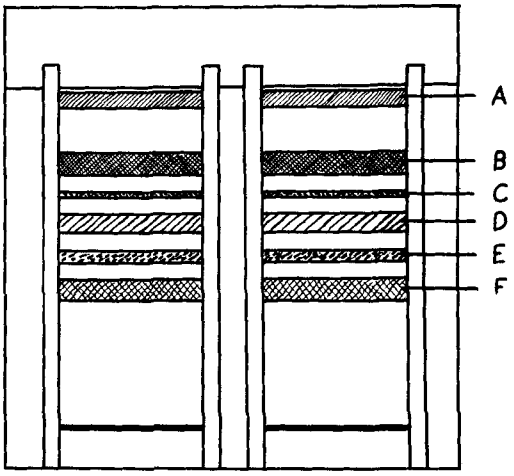

Fig. 3—Diagram of chromatoplate after development. A = solvent front; B = DIT, MIT, T_3; C = iodide; D = T_3; E = T_3-iso; F = T_4.

the solutions to volume with benzene and mix well. Record the absorbance of the benzene solutions at the minimum (400 mμ) and the maximum (295 mμ) in the 10-cm. cylindrical cells with benzene as the reference. Subtract the absorbance at the minimum wavelength from the absorbance at the maximum wavelength. Subtract the difference, if any, in absorbance at the maximum and minimum of the blank from the sample value to give the corrected absorbance. Prepare a standard curve in the same manner for solutions of iodine in benzene of at least 5 different dilutions ranging from about 20 to 100 mcg. in 50 ml. of benzene (Fig. 4).

Calculations—Determine the mcg. of iodine in the 50-ml. benzene solutions from the standard curve, using the corrected absorbances of the sample solutions.

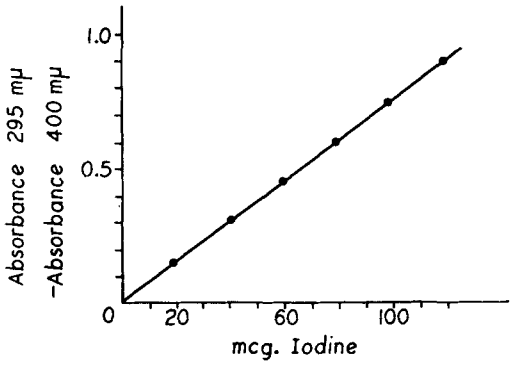

Fig. 4—Standard curve of iodine in 50 ml. benzene.

$$\%\ T_3 \text{ in sample} = \frac{(\text{mcg. of I})\ (1.425)\ (100)}{\text{wt. of sample in mcg.}}$$

$$\%\ T_4 \text{ in sample} = \frac{(\text{mcg. of I})\ (1.275)\ (100)}{\text{wt. of sample in mcg.}}$$

The factors (1.425 and 1.275) in the calculation equations are derived from the percentages of iodine in T_3 and T_4, respectively, as given below. The full equation without using factors is:

$$\%\ T_3 \text{ in sample} = (\text{mcg. of I from std. curve}) \times \frac{(5)}{(6)} \times \frac{(100)}{(58.48)} \times \frac{(100)}{(\text{wt. of sample in mcg.})}$$

where: (5) = multiplication factor due to the use of 200 μl. from 1 ml. of solution for spotting plates;

(6) = oxidation of iodide to iodate and release of iodine with potassium iodide to give six times the iodine in the original iodide; and

$\frac{(100)}{(58.48)}$ = percent factor for iodine in T_3.

$$\frac{(5)}{(6)} \times \frac{(100)}{(58.48)} = 1.425$$

Similarly for T_4: $\frac{(100)}{(65.34)}$ = percent factor for iodine in T_4.

$$\frac{(5)}{(6)} \times \frac{(100)}{(65.34)} = 1.275$$

RESULTS

Table I shows the T_3 and T_4 content of several commercial thyroid powders and tablets as determined by this method. Some of the powders were marketed as USP thyroid and some as triple strength thyroid. The tablets were all labeled as 1-grain USP thyroid tablets.

The total iodine content in thyroid combination agreed with the labeling as to USP and triple strength for all samples tested. Sample A is a USP thyroid powder that was used as the standard.

In Table II, the chemical potency and biological activity are set at 100% for the standard and the other results are compared to it. Assuming T_3 to have five times the antigoitrogenic activity of T_4, a chemical potency was derived for each sample, using the formula $5 \times (\%\ T_3) + \%\ T_4$. These figures were compared with the one calculated for sample A, the standard, to obtain the percent chemical potency. A preliminary biological assay showed that samples B, G, I, J, and K have less activity than the standard. Samples C, D, and F show greater activity. The biological activity was not determined for samples E and H. Sample E is an undiluted porcine thyroid powder purchased directly from the manufacturer. The total organically bound iodine content of this sample is 3.7 times that of the standard sample and the percent chemical potency found is 3.6 times that of the standard. Sample H is a 1-grain tablet from the same manufacturer as sample E and the total organically bound iodine and the chemical potency are, respectively, 1.10 and 1.07 times the corresponding figures for the standard.

Table II shows that the chemical potency gives a good indication of the physiological activity of the samples tested. The biological potency figures are based on a single determination.

A representative sampling of several brands of thyroid tablets was collected from various areas of the United States and the tablets were assayed by this procedure. The results are compared to our standard thyroid powder and are shown in Table III.

The accuracy of the assay procedure was checked with National Bureau of Standards 2-iodobenzoic acid. Recovery results starting with the oxygen ignition step were 101.5% and 99.96%. Recovery results starting with spotting 200 μl. of a 2-iodobenzoic acid solution on a cellulose plate and scraping the area of application from the plate were 101.1% and 100.7%.

TABLE I—T_3 AND T_4 CONTENTS OF SOME COMMERCIAL THYROID POWDERS AND TABLETS

Strength	Sample	No. of Samples	Av. % T_3	Av. Deviation	Av. Deviation from Mean	Av. % T_4	Av. Deviation	Av. Deviation from Mean
USP	A	21	0.016	0.002	0.0004	0.048	0.002	0.0004
USP	B	8	0.015	0.001	0.0004	0.044	0.003	0.001
USP	C	7	0.019	0.0007	0.0003	0.054	0.002	0.0008
USP	D	10	0.022	0.0009	0.0003	0.041	0.002	0.0006
3X	E	17	0.056	0.002	0.0005	0.177	0.007	0.017
3X	F	6	0.061	0.001	0.0004	0.162	0.003	0.001
3X	G	7	0.028	0.002	0.0008	0.046	0.004	0.0015
1-Grain tablet	H	7	0.017	0.003	0.0011	0.052	0.003	0.011
1-Grain tablet	I	8	0.005	0.001	0.0004	0.012	0.001	0.0004
1-Grain tablet	J	4	0.009	0.000	0.000	0.017	0.001	0.0005
1-Grain tablet	K	4	0.008	0.001	0.0005	0.016	0.0003	0.0002

TABLE II—COMPARISON OF THE CHEMICAL POTENCY AND BIOLOGICAL ACTIVITY OF SOME COMMERCIAL THYROID POWDERS AND TABLETS

Sample	% Total Iodine	% T_3	% T_4	5 (% T_3) + % T_4	% Chemical Potency	% Biological Potency
A USP (std.)	0.19	0.016	0.048	0.128	100	100
B USP	0.19	0.015	0.044	0.119	93	85
C USP	0.20	0.019	0.054	0.149	116	164
D USP	0.23	0.022	0.041	0.151	118	132
E 3X	0.7	0.056	0.177	0.457	357	...
F 3X	0.63	0.061	0.162	0.467	122[a]	155
G 3X	0.62	0.028	0.046	0.186	48[a]	30
H 65 mg. (1 gr.)	0.21	0.017	0.052	0.137	107	...
I 65 mg. (1 gr.)	0.20	0.005	0.012	0.037	29	25
J 65 mg. (1 gr.)	0.19	0.009	0.017	0.062	48	44
K 65 mg. (1 gr.)	0.19	0.008	0.016	0.058	45	48

[a] Divided by three for comparison to single strength thyroid.

TABLE III—ANALYSIS OF COMMERCIAL THYROID TABLETS COLLECTED FROM SEVERAL AREAS OF THE UNITED STATES

Product	% Total Iodine	% T_3	% T_4	5 (% T_3) + % T_4	% Chemical Potency
Standard thyroid powder	0.19	0.016	0.048	0.128	100
32 mg. (0.5 gr.) tablets	0.19	0.013	0.053	0.118	92
	0.20	0.018	0.053	0.143	112
	0.20	0.019	0.053	0.148	116
	0.21	0.014	0.052	0.122	95
	0.21	0.009	0.021	0.066	51
65 mg. (1 gr.) tablets	0.20	0.010	0.054	0.104	81
	0.19	0.016	0.037	0.117	92
	0.19	0.019	0.042	0.137	107
	0.24	0.017	0.051	0.136	106
	0.23	0.016	0.047	0.127	99
	0.21	0.016	0.053	0.133	104
130 mg. (2 gr.) tablets	0.19	0.012	0.050	0.110	86
	0.22	0.020	0.042	0.142	111
	0.21	0.019	0.057	0.152	119
	0.19	0.014	0.032	0.102	80
	0.23	0.014	0.046	0.116	91

DISCUSSION

Sample Preparation—The ground tablets are prewashed with water to remove water-soluble tablet ingredients, especially lactose. Lactose, which is present in many tablets, caramelizes during the alkaline hydrolysis, and the resulting dark color obscures the chromatogram. The use of a second wash with chloroform-methanol (1:1) as reported by Lemieux and Talmage (11) was investigated but resulted in considerably lower recoveries of T_3 and T_4.

Hydrolysis—The pronase hydrolysis was an improvement over the earlier reported trypsin-erepsin treatment (13), because a better yield of T_3 and T_4 was obtained and the time required for hydrolysis was considerably reduced. In general, more T_4 was released by the 50% barium hydroxide solution than by pronase, while the quantity of T_3 released was about equal for both procedures. Some samples of thyroid powder yielded very little T_3 or T_4 with pronase hydrolysis, but gave a good yield of these compounds when hydrolyzed with 50% barium hydroxide. Hydrolysis with an 8% solution of $Ba(OH)_2 \cdot 8H_2O$ for 6 hr. was attempted but was less complete and the extracts gave diffused and dirty chromatograms.

Chromatography—Roche *et al.* (17) reported that the 3,3′,5′ isomer of T_3 is a normal constituent of thyroid protein. The majority of systems used in the separation of the components of thyroid digests disregard this isomer. As shown in Fig. 2, this compound is located between T_3 and T_4 on the chromatogram developed by our procedure. T_3, T_3-iso, and T_4 were chromatographed by some paper chromatographic procedures. With formic acid and water (1:5) as the developing solvent, the T_3-iso accompanies the T_3, while with 6 *N* ammonium hydroxide and tertiary amyl alcohol, the T_3-iso accompanies the T_4.

Roche *et al.* (8) compared the antigoitrogenic activity of some of the iodothyronines in propylthiouracil-treated rats to that of thyroxine. DL-3,3′,5′-Triiodothyronine (T_3) was 5–10 times as active as DL-thyroxine and the 3,3′,5′-triiodothyronine (T_3-iso) was only about 5% as active as DL-thyroxine. The extremely low activity of the T_3-iso makes it imperative that this compound be separated from T_3 and T_4 before quantitation.

The cellulose must be removed from the plate on

both sides of the sample line if horizontal zones and good resolution of the components are to be obtained. Without this treatment, the solvent front curves around the ends of the sample line and produces a cone-shaped chromatogram because the developing solvent moves up the plate faster in areas where there is no sample. The plates must be dried thoroughly before spraying to dispel as much formic acid as possible. The color development will occur only when the plate is made basic, and if too much Na_2CO_3 is needed to neutralize the formic acid, some of the color formed is obscured by a coating of sodium carbonate.

Quantitation of T_3 and T_4—The oxygen-ignition procedure has proved to be a direct and accurate method for quantitatively transforming organic iodine to inorganic iodide for subsequent assay. The oxidation of iodide to iodate with bromine does not require the addition of other chemicals to remove excess oxidant as is necessary when potassium permanganate is used.

Some very important precautions must be observed in the procedural operations. The iodide solutions must always be acidified before the bromine is added. If the bromine is added to the alkaline solution, hypobromites, bromates, *etc.*, may be formed (18). High and irregular blanks and samples result from the liberation of iodine if residual amounts of these bromine compounds are present. When the bromine is added to the acidified solutions, these compounds are not formed.

The data in Table II show that the samples marketed as USP all have the necessary amount of iodine in thyroid combination to meet the official requirements. The triple strength samples have a proportional amount of iodine. Many of these samples, though passing the official assay, do not have a corresponding physiological activity. The chemical potency determined for these samples follows the same pattern as the biological activity. The authors believe that this chemical assay procedure can be used to establish standards for ranges of T_3 and T_4 content and ratios of these two compounds that will be meaningful in expressing relative biological activity.

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Keyphrases

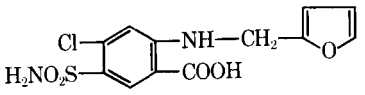
Thyroid preparations—analysis
Thyroxine—quantitative determination
Triiodothyronine—quantitative determination
Hydrolysis and extraction
TLC—separation, identity
Oxygen ignition—iodine release
UV spectrophotometry—iodine analysis

Qualitative and Quantitative Tests for Furosemide

By E. F. SALIM*, A. HAUSSLER†, and J. B. VAUGHAN‡

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

4-CHLORO - *N* - FURFURYL - 5 -SULFAMOYLANTHRANILIC ACID; $C_{12}H_{11}ClN_2O_5S$; mol. wt. 330.75. The structural formula of furosemide may be represented as:



Physical Properties—Furosemide occurs as a white to slightly yellow crystalline, odorless powder. It is practically insoluble in water, sparingly soluble in alcohol, soluble in methanol, very slightly soluble in chloroform, and slightly soluble in ether. It is freely soluble in acetone, in dimethylformamide, and in solutions of alkali hydroxides.

Identity Tests—Dissolve about 5 mg. of furosemide in 10 ml. of methanol. Transfer 1 ml. to a flask, add 10 ml. of 2 *N* hydrochloric acid, and reflux for 15 min. on a steam bath. Cool, add 15 ml. of sodium hydroxide T. S. and 5 ml. of sodium nitrite solution (1 in 1000). Allow the mixture to stand 3 min., add 5 ml. of ammonium sulfamate solution (1 in 200), mix well, and add 5 ml. of *N*-(1-naphthyl)-ethylenediamine dihydrochloride solution (1 in 1000): a red to red-violet color is produced.

A 1 in 200,000 solution of furosemide in methanol exhibits ultraviolet absorbance maxima at about 234 mμ, 274 mμ [absorptivity (*a*) about 66], and 338 mμ and absorbance minima at about 247 and 293 mμ. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.5% dispersion of furosemide in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 2.

Purity Tests—Dry about 1 Gm. of furosemide, accurately weighed, at 105° for 3 hr.: it loses not more than 1% of its weight.

Char about 1 Gm. of furosemide, accurately weighed, in a tared crucible, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.1%.

Determine the heavy metals content of furosemide by the USP heavy metals test, method II: the heavy metals limit for furosemide is 20 p.p.m.

Assay—Transfer about 600 mg. of furosemide, accurately weighed, to a 125-ml. conical flask, and dissolve in 50 ml. of methanol. Add 3 drops of bromothymol blue T. S. and titrate with 0.1 *N* sodium hydroxide to a blue end point. Perform a blank determination, and make any necessary correction. Each milliliter of 0.1 *N* sodium hydroxide is

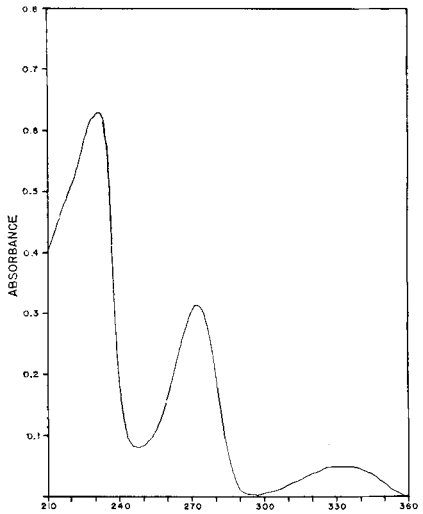


Fig 1—Ultraviolet absorption spectrum of furosemide in methanol (5 mcg./ml.); Beckman model DK-2A spectrophotometer.

Received September 26, 1967, from the *Drug Standards Laboratory (cosponsored by the American Medical Association, American Pharmaceutical Association, and United States Pharmacopeia Convention), of the APhA Foundation, Washington, DC 20037

Accepted for publication December 1, 1967.

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Hoechst Pharmaceutical Co. has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.

The Drug Standards Laboratory gratefully acknowledges the assistance of Miss Hannah Klein.

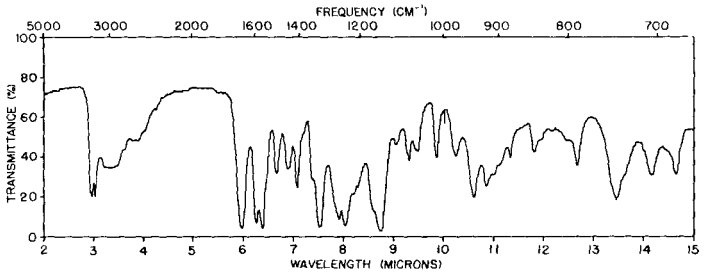

Fig 2—Infrared spectrum of furosemide in potassium bromide disk (0.5%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

equivalent to 33.07 mg. of $C_{12}H_{11}ClN_2O_5S$. The amount of furosemide found is not less than 98% and not more than 101% of the weight of the sample taken.

DOSAGE FORMS OF FUROSEMIDE

Furosemide Tablets—Identity Test—The final solution prepared from the tablet sample in the *Assay* exhibits ultraviolet absorbance maxima and minima at the same wavelengths as the furosemide standard solution.

Assay—Weigh and finely powder not less than 20 furosemide tablets. Weigh accurately a portion of the powder, equivalent to about 200 mg. of furosemide, and transfer to a 100-ml. volumetric flask. Add about 50 ml. of 0.1 *N* sodium hydroxide and shake mechanically for 1 hr. Dilute to volume with the alkaline solution, and mix. Filter the solution, discarding the first portion of filtrate, and transfer 10.0 ml. into a small separator. Add 1 ml. of diluted hydrochloric acid and extract with four 25-ml. portions of a mixture of chloroform–acetone (4:1), filtering each portion through chloroform-washed paper into a suitable beaker. Evaporate the combined extracts on a steam bath in a current of air to dryness. Transfer the residue quantitatively with the aid of about 100 ml. of methanol to a 200-ml. volumetric flask, dilute to volume with methanol, and mix. Dilute 10.0 ml. of this solution to 200.0 ml. with methanol, and mix. Concomitantly determine the absorbance of this solution and of a standard solution of furosemide, in the same medium at a concentration of about 5 mcg./ml., in 1-cm. cells, at the maximum at about 274 mμ, with a suitable spectrophotometer, using methanol as the blank. Calculate the quantity, in milligrams, of $C_{12}H_{11}ClN_2O_5S$ in the portion of tablets taken by the formula $40\ C \times (A_U/A_S)$, in which C is the exact concentration of the standard solution, in mcg./ml., A_U is the absorbance of the sample solution, and A_S is the absorbance of the furosemide standard solution. The amount of furosemide found is not less than 90% and not more than 110% of the labeled amount.

DISCUSSION

USP and NF terminology for solubility, melting range, reagents, *etc.*, has been used wherever feasible

Furosemide[1] is an oral diuretic agent which is useful in the treatment of edema associated with congestive heart failure, cirrhosis of the liver, and renal disease, including the nephrotic syndrome. Chemically, it is distinct from organomercurials, thiazides, and other heterocyclic compounds employed as diuretics.

Identity Tests—Furosemide has been observed to melt between 205° and 207° [lit. (1) m.p. 206°] with decomposition (USP class Ia). Acid hydrolysis of furosemide yields a primary aromatic amine which is diazotized and coupled with Bratton-Marshall reagent to produce a red to red-violet color. This reaction which has been included as an identification test for furosemide has been reported by Hajdu and Haussler as a quantitative determination of the compound (2).

Quantitative Methods—Titration of furosemide with sodium hydroxide using bromothymol blue T.S. gave an average value of 100.1 ± 0.4%.[2] This determination involves neutralization of the carboxylic acid group in the molecule. A solution of furosemide in ethylenediamine titrated with 0.1 *N* sodium methoxide using azo violet indicator exhibits two acidic groups, the carboxylic acid and an acidic hydrogen. The latter is too weak an acid to titrate in aqueous solution but the acidity is promoted in a strongly basic nonaqueous medium so that titration is routine.

Analysis of commercial furosemide tablets (40 mg.) by the spectrophotometric method gave an average value of 99.2 ± 1.0%[2] of the labeled amount of furosemide. The fluorescence of furosemide in 0.01 *N* hydrochloric acid has been utilized as the basis of quantitative measurement by Hajdu and Haussler (2). Concentrations of 0.03–0.5 mcg./ml. of furosemide can be determined at the wavelength of maximum fluorescence at 410 mμ.

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[1] Marketed as Lasix by Hoechst Pharmaceutical Company (formerly Lloyd Brothers, Inc.), Cincinnati, Ohio.

[2] Maximum deviation from the mean value.

Keyphrases

Furosemide—analysis
Identity test, color formation—furosemide
Purity test—furosemide
IR spectrophotometry—identity
Tablets, furosemide—analysis
UV spectrophotometry—analysis, identity

Separation and Determination of Polymyxin B in Formulations

By EUGENE IVASHKIV

A method for the separation and the determination of polymyxin B in formulations is described. It is based on the absorption of the antibiotic on a cation-exchange resin with subsequent elution with methanol–aqueous sodium chloride solution and the reaction of polypeptide with ninhydrin.

NUMEROUS microbiological methods (1, 2, 6–9) have been described for the determination of polymyxin B but little work has been carried out on the chemical assay of the antibiotic. In the fractionation of polymyxins, a spectrophotometric measurement at 259 mμ has been used (4). A gravimetric method has been described for the determination of polymyxin B by precipitation as phosphotungstate (3). The biuret reaction has been suggested for the determination of polymyxin in fermentation broths (5).

In formulations, in addition to polymyxin B, there frequently are substances present which interfere in a spectrophotometric or colorimetric determination of polypeptides. In these cases, it is essential to separate polymyxin B from impurities and other ingredients of a protein character. The proposed method is based on the separation of polymyxin B by means of a cation-exchange resin and the determination of the antibiotic with the ninhydrin reagent.

EXPERIMENTAL

Method

Reagents and Equipment—*Amberlite IRC-50 resin*, pH 7.0, sodium form; *methanolic aqueous sodium chloride solution*, 10% sodium chloride (10 Gm.) is dissolved in methanol–water (1:1); *Polymyxin B standard solution*, polymyxin B equivalent to about 100,000 units is dissolved in 25 ml. water; *Sodium acetate buffer*, pH 5.5 sodium acetate trihydrate (680 Gm.) is dissolved in 0.5 L. of distilled water and to the cooled solution 125 ml. of glacial acetic acid is added. The volume is made up to 1 L. with water. The reagent is stored at 5°; *Ninhydrin reagent*, ninhydrin (0.2 Gm.) and 25 mg. of hydrindantin are dissolved in 12 ml. of methyl cellosolve. Sodium acetate buffer 5.5 (2.5 ml.) is added and the reagent is stored in a low actinic flask. The reagent is prepared daily; *Chromaflex column*, size A-3, made by Kontes, catalog No. K-42082.

Procedure—A sample equivalent to 100,000 units of polymyxin B is weighed in a 100-ml. beaker. About 15 ml. of water is added and the solution is mixed. The solution is filtered through a sintered-glass filter of medium porosity having a diatomaceous earth[1] precoat of a 1/8 in. thickness. The beaker and filter are washed with 10 ml. of water and the wash is combined with the sample. Two columns are packed with wet IRC-50 resin until a height of 12.7 cm. is obtained. Water is withdrawn from the columns to about 0.6 cm. above the resin and the standard and sample solutions are transferred quantitatively to the corresponding columns. The flow of the effluent is regulated to 1 ml./min. After all of the solutions have been passed through the columns, the resin beds are washed with 100 ml. water at the rate of 5 ml./min. Polymyxin B is eluted with methanolic aqueous sodium chloride solution at the rate of 1 ml./min. until 100 ml. of the eluate is obtained.

Some samples may require the following purification: samples and standard solutions (25 ml.) are transferred to suitable flasks and methanol is evaporated *in vacuo* at about 5 mm. Hg and 60°. The remaining aqueous solutions are transferred quantitatively to separators and polymyxin B is extracted with 15 ml. and 2 × 5 ml. of butanol. One milliliter of butanol solution is taken for the ninhydrin reaction.

One milliliter of methanolic sodium chloride solution or 1 ml. of butanol solution (blank), 1 ml. of sample, and 1 ml. of standard eluates are transferred to 17.9 cm. test tubes. To each tube, 1 ml. of ninhydrin reagent is added and the tubes are incubated in a boiling water bath for exactly 15 min. To each tube 15 ml. of ethanol–water (1:1) is added and the solutions are mixed. The absorbance is measured at 570 mμ on a spectrophotometer using 1-cm. cells against the reagent blank.

The polymyxin B content is calculated as follows:

$$\frac{A \times C \times P \times S}{B \times D} = \text{polymyxin B, units/ml.}$$

where:

A = absorbance of sample
B = absorbance of standard
C = weight of standard, mg.
D = weight of sample, Gm.
P = potency of standard, units/mg.
S = specific gravity

DISCUSSION AND RESULTS

In formulations, in addition to polymyxin, other various organic and inorganic substances are usually present. For the quantitative determination of

Received June 6, 1967 from the Squibb Institute for Medical Research, New Brunswick, NJ 08903
Accepted for publication November 30, 1967.
The author is indebted to Dr. D. Isaacson and Miss L. Piltch of the Bioanalytical laboratory of Squibb Institute for Medical Research for the microbiological assays.

[1] Celite, Johns-Manville Products Corp., New York, N.Y.

TABLE I—ASSAY POLYMYXIN B IN EXPERIMENTAL FORMULATION

Run No.	Polymyxin B, units/ml. Method: Biological	Chemical
1	4660	4880
2	4780	4890

polymyxin, it is essential to separate the antibiotic from impurities, especially from those of a proteinaceous nature. In this study, an experimental formulation consisting of neomycin, amphotericin B, polymyxin B, ethylenediamine, and other ingredients was assayed. No chemical methods for the determination of polymyxin B in the presence of neomycin and other amines have been found in the literature. Methods used in the isolation and the purification of polymyxin on a preparative or commercial scale have not been previously applied to analytical procedures.

Polymyxin B and neomycin are adsorbed on cation-exchange resin and their separation by means of ion exchange techniques was investigated. Various solvents were investigated for their efficiency in the elution of polymyxin from the resin. Aqueous hydrochloric acid solutions elute about 80% of the polymyxin, but also about 80% of the neomycin. With methanolic solution of hydrochloric acid, 100% elution of polymyxin and 85% of neomycin is obtained. With ammonium hydroxide only 60% of the polymyxin is eluted and 100% of the neomycin. With the 10% methanol aqueous sodium chloride solution, polymyxin B can be eluted quantitatively from a column while neomycin remains on the resin. With 5% methanol aqueous sodium chloride, only 40% of the polymyxin was eluted.

In the separation of polymyxin B and neomycin in an experimental suspension formulation stored at room temperature for 2 years, an incomplete purification of the polymyxin was obtained. A further purification was attained by the extraction of polymyxin from aqueous solutions with butanol.

Only a limited study has been carried out on the correlation of the proposed chemical assay and the standard microbiological assay. The results of this correlation are shown in Table I.

The correlation of the chemical method with the biological assay was studied by the degradation of polymyxin B in 0.1 *N* NaOH and 0.5 *N* NaOH at room temperature (25°). The samples were withdrawn periodically over 96 hr. and assayed by both the chemical and microbiological methods. About 50% of polymyxin B was degraded in 0.1 *N* NaOH in 24 hr. No further degradation was observed over the period of 72 hr. The degradation of polymyxin B in 0.5 *N* NaOH was rapid. After 24 hr. no polymyxin B activity was found by the microbiological method. About 45% polymyxin was found by the chemical method over the period of 72 hr. It appears that the chemical method does not correlate with the microbiological assay when polymyxin B is degraded in 0.5 *N* NaOH.

When a known amount of polymyxin B was added to the formulation samples, an average recovery of 98% was obtained.

This method is useful as a labeling assay of polymyxin in pharmaceutical formulations. The use of this assay as a stability method has not been explored.

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Keyphrases

Polymyxin B formulations—analysis
Column chromatography—separation
Colorimetric analysis
Ninhydrin reagent

Spectrophotometric Determination of Acetaminophen, Phenylephrine Hydrochloride, Codeine Phosphate, and Pyrilamine Maleate in Tablets or Powder

By FABRIZIO DE FABRIZIO

An ultraviolet spectrophotometric method has been developed for the determination of acetaminophen, phenylephrine hydrochloride, codeine phosphate, and pyrilamine maleate after a partial separation of them by means of column chromatography using alginic acid; codeine phosphate and phenylephrine hydrochloride are both eluted with 0.01 *N* HCl and determined simultaneously while acetaminophen and pyrilamine maleate are determined separately.

NO STANDARD ANALYTICAL PROCEDURE exists for acetaminophen, codeine phosphate, phenylephrine hydrochloride, and pyrilamine maleate in this common pharmaceutical combination[1]; however, various assays were reported for each of the components alone or in other combination (1–4). To achieve the separation of the components, the author employed a chromatography technique using alginic acid 40–100 mesh prepared after the process of Foster and Murfin (5). This cation-exchange medium has been used largely to determine many organic bases, to separate codeine phosphate from aspirin and phenacetin in APC tablets B.P. (6), strychnine from nux vomica (7), and to determine thiamine nicotinamide, and pyridoxine in vitamin B complex tablets (8). In this investigation it was found that acetaminophen was not held by the alginic acid used, as was foreseen in a previous theoretical study of the problem, but passed through the prepared column; codeine phosphate and phenylephrine hydrochloride are both eluted with 0.01 *N* HCl and simultaneously determined at two different wavelengths. Finally, pyrilamine maleate is eluted with 0.1 *N* HCl and determined at 316 mμ.

EXPERIMENTAL

Apparatus—Spectrophotometer, Beckman DB, and 1-cm. square fused silica cells were used. Glass column 35 × 2 cm. with stem (5 cm.) was fitted with a buret key.

Reagents—Cation-exchange resin alginic acid, 40–100 mesh, available from British Drug House (B.D.H.): hydrochloric acid 2 *N* in water; 0.1 *N* hydrochloric acid in water; 0.01 *N* hydrochloric acid in water; 0.01 *N* hydrochloric acid in 80% ethanol in water. Except where otherwise specified all reagents were of B.D.H. analar quality.

Received June 2, 1967, from the Control Pharmaceutical Laboratory, Propan Pharmaceutical Ltd., Germiston, South Africa.
Accepted for publication December 19, 1967.
[1] Marketed as Mepacod.

Standard Solutions—The following solutions were prepared with suitable reference standard: (*a*) acetaminophen, 0.75 mg./250 ml. in 0.1 *N* hydrochloric acid in 80% ethanol in water; (*b*) codeine phosphate, 8.1 mg./250 ml. in 0.01 *N* hydrochloric acid in water; (*c*) phenylephrine hydrochloride, 5 mg./250 ml. in 0.01 *N* hydrochloric acid in water, (*d*) codeine phosphate and phenylephrine hydrochloride in a mixture in 0.01 *N* hydrochloric acid in water; (*e*) pyrilamine maleate, 20 mg./250 ml. in 0.1 *N* hydrochloric acid in water.

Column Preparation—Alginic acid, about 4 Gm. is slurried in water and allowed to soak 4 hr. The slurry is poured into a glass column that has been fitted with glass wool plug and allowed to settle. The column is washed with 2 *N* hydrochloric acid until the absorbance of the eluate (pathlength 1 cm.) is less than 0.005 at 250 mμ and 273 mμ, and then washed with distilled water until the eluate is neutral to litmus solution.

Sample Treatment—Powder 20 tablets and weigh an amount of powder equivalent to one tablet. Place this in a 50-ml. volumetric flask, add water to volume, and mix. Allow to stand for 30 min., shaking occasionally, then centrifuge about 40 ml. of solution. Pipet 20 ml. of the clear solution onto the prepared alginic acid column; place a 1-L. volumetric flask under the column and start collecting the eluate at the rate of 1 ml./min. Then add 200 ml. of water and allow to pass through at the same rate. Adjust the rate to 2 ml./min. and pass more water through the column to the 1-L. mark. Remove and shake. This eluate contains the acetaminophen. Bring the layer of the liquid in the column to just above the glass wool pledget. Elute the column with 0.01 *N* HCl at the rate of 2 ml./min. Discard the first 15 ml. of eluate and collect the rest in a 100-ml. volumetric flask to volume. This solution contains codeine phosphate and phenylephrine hydrochloride. Again allow the level of the liquid in the column to drop to just above the glass wool pledget. Elute the column with 0.1 *N* HCl at the rate of 2 ml./min., collecting the eluate in a 250-ml. volumetric flask. This solution contains pyrilamine maleate.

Determination—Following the base-line technique compare the absorbances of the standard solutions passed through the column with those of the samples. Quantitatively adjust the claimed concentrations of the sample eluates as closely as possible to the corre-

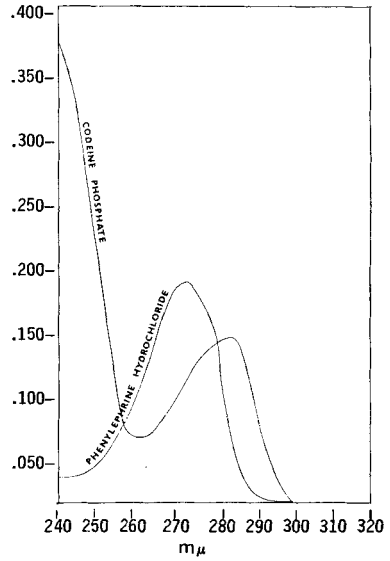

Fig. 1—Spectra of 0.0324 mg./ml. of codeine phosphate and of 0.02 mg./ml. of phenylephrine hydrochloride in 0.01 N HCl.

sponding standard solutions. Calculate the amount of acetaminophen in the sample from the value of the absorptivity of the corresponding standard at 249 mμ. Similarly calculate pyrilamine maleate at 316 mμ and codeine phosphate and phenylephrine hydrochloride at both wavelengths of 250 and 273 mμ.

RESULTS AND DISCUSSION

The first step in the chromatographic procedure was the determination of the "breakthrough" point of each component separately and in a mixture. As previously mentioned, acetaminophen and pyrilamine maleate are separated by the column and their spectrophotometric determination does not present any difficulty. Further study was necessary to choose two different wavelengths where the interferences of codeine phosphate and phenylephrine hydrochloride, contained in the same solution, were as low as possible. In a series of 40 determinations the most favorable system was to be found at the wavelengths of 273 mμ and 250 mμ. However at 250 mμ, the corresponding point lies on the steep part of the curve (see Fig. 1). The error using this wavelength gave a value much lower than the one computed at 248 (the peak), 240, and 245 mμ. The results of 10 determinations of mixtures of codeine phosphate and phenylephrine hydrochloride at 250 and 273 mμ are shown in Table I.

Five mixtures prepared in this laboratory containing acetaminophen, codeine phosphate, phenylephrine hydrochloride, and pyrilamine maleate gave mean values as shown in Table II.

TABLE I—DETERMINATIONS OF MIXTURES OF CODEINE PHOSPHATE AND PHENYLEPHRINE HYDROCHLORIDE

Material	Mean of 10 Determinations, % of Theoretical	S.D. of 10 Determinations, % of Theoretical
Codeine phosphate	98.8	±2.1
Phenylephrine hydrochloride	98.4	±1.0

TABLE II—MEAN VALUES OF FIVE MIXTURES

Material	Taken	Found, % of Amt. Taken
Acetaminophen	150 mg.	99.85
Codeine phosphate	8.1 mg.	98.70
Phenylephrine hydrochloride	5.0 mg.	98.50
Pyrilamine maleate	10.0 mg.	98.97

The total error was about −4 mg. and the mean relative deviation ±2.6%. As can clearly be seen, the proposed method yields results acceptable for routine analysis. In the spectrophotometric determination of pyrilamine maleate, slight differences between the absorbance observed in the standard solution as is and the one passed through the column were taken into account. Finally some water-soluble excipients and dyes, generally used in the manufacture of tablets and powder, pass through the alginic acid column together with acetaminophen; they do not affect the results at 249 mμ as they are in such high dilution.

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Keyphrases

Drug mixtures—analysis
Acetaminophen, phenylephrine HCl, codeine PO_4, pyrilamine maleate—analysis
Cation exchange resin—separation
UV spectrophotometry—analysis

Quantitative Determination of Diethylstilbestrol by Thin-Layer Chromatography

By LOUIS N. JONES, MURRY SEIDMAN, and B. C. SOUTHWORTH

A thin-layer chromatographic procedure has been developed for the analysis of diethylstilbestrol at the 0.01 percent level in a water-dispersible suppository base. Following a preliminary separation on an alumina column, the diethylstilbestrol is isolated on a silica gel chromatoplate. A color is then produced *via* the Folin-Ciocalteu procedure for phenols and the blue spot evaluated densitometrically directly on the chromatoplate. The standard deviation is 3.4 percent. Effects of variations in the operating parameters are discussed and evidence is presented to indicate that only intact diethylstilbestrol is measured.

WHILE BOTH biological and chemical methods are available for the determination of diethylstilbestrol [3,4-bis(*p*-hydroxyphenol)-3-hexene] in pharmaceutical preparations, no rapid assay procedure for this estrogen in combination with nitrofurazone in a water-dispersible suppository base has been reported. Biological procedures (1) require a minimum of 8 days to complete. Nitrofurazone interferes in the official USP XVII procedure (2) for diethylstilbestrol (DES) suppository, and any modification of this procedure would necessitate extensive purification before the irradiation analysis could be used.

Michels *et al.* (3) were able to separate DES from nitrofurazone and the suppository base in sufficiently pure form so that the Folin-Ciocalteu procedure could be used for its determination. Although the total procedure was fairly time consuming, their preliminary separation was rapid and served to separate the DES from the bulk of the suppository base. Lisboa *et al.* (4) have developed a thin-layer chromatographic classification system for a series of estrogens. After separating the DES from the bulk of the suppository base *via* the above-mentioned preliminary separation, Lisboa's system was found to be adequate for the purification and isolation of the DES. Having isolated the DES in pure form, densitometric determination directly on the chromatoplate was found to be feasible.

Lisboa *et al.* used the Folin-Ciocalteu reagent to visualize DES on a chromatoplate. Their techniques were designed for qualitative purposes so that refinements were necessary before a quantitative determination was possible. The stability of the color produced by the Folin-Ciocalteu reagent is very much dependent on the final pH adjustment. This pH adjustment may be obtained by observation of an indicator (thymolphthalein) color change as a base is sprayed on the plate. Alcoholic potassium hydroxide was chosen as this reagent, the base simply for its solubility characteristics; the alcohol for its volatility. After the pH adjustment has been completed, the alcohol evaporates and the color is "fixed." Attempts to use ammonia vapor (5) were not successful in that a wide background variation was noted in the final densitometric determination.

The recommended procedure calls for a sample size calculated to contain 0.5 mg. of DES. After an initial clean-up this 0.5 mg. is contained in 10 ml. of chloroform. The final determination requires four spots of 2 mcg. each for an accurate determination. The ultimate sensitivity of the procedure is about 0.1 mcg. of DES.

EXPERIMENTAL

Apparatus—Photovolt densitometer model 520 A, equipped with an automatic TLC stage, a Varicord recorder No. 42, and an Integraph integrator No. 49.

Reagents—Potassium hydroxide alcoholic solution, 0.5 *N*, V.S.; thymolphthalein solution, 0.1% in alcohol, T.S.; alcohol, 80%, R.

Developing Solvent—Ethyl acetate R., petroleum ether R., glacial acetic acid R., and alcohol R. (72:13.5:10:4.5, respectively).

Folin-Ciocalteu Reagent—Use the procedure according to Folin (6) or use Harleco item 2690.

Preparation of Standard DES Solution—Transfer exactly 100 mg. of USP reference standard diethylstilbestrol to a 200-ml. volumetric flask, dissolve it, and dilute to the mark with chloroform. Further dilute 10.0 ml. of this solution to 100 ml. with chloroform for use in the assay.

Preparation of Chromatoplates—Slurry together Silica Gel G and distilled water in the ratio of 1:2, apply the slurry uniformly to 20 × 20-cm. glass plates to a thickness of 750 μ and allow the plates to air-dry for 30 min. Activate the plates at 105° for 1 hr. and store under desiccation until used. Immediately before use, check the background of the chromatoplate according to the parameters to be used for the final spot measurement. Any variation should not exceed ±0.05 absorbance units.

Received June 5, 1967, from the Quality Control Department, The Norwich Pharmacal Company, Norwich, NY 13815

Accepted for publication November 30, 1967.

Preparation of Developing Chamber—Line the walls of the developing chamber (30.48 cm. × 10.16 cm. × 27.94 cm.) with filter paper. Add 100 ml. of the developing solvent, close the chamber tightly and allow at least 30 min. for complete saturation of the chamber atmosphere. Prepare this tank fresh daily.

Preparation of Column—Place a glass-wool pledget at the bottom of a 1-cm. i.d. chromatographic column and pack it to a height of 10 cm. with basic alumina. Add the adsorbent in small portions with horizontal tapping and gentle bouncing until no further settling occurs (7). Lot to lot variations in adsorptive capacity of the alumina have been noted. The following test is, therefore, recommended. Dissolve about 10 mg. of nitrofurazone in 100 ml. of dehydrated alcohol. Add 10 ml. of this solution to a prepared column. The nitrofurazone should remain at the top of the column in a narrow yellow band. Elute the band with 80% alcohol in water. The band should follow the solvent front. The characteristics of DES are similar to nitrofurazone under these conditions.

Assay Procedure—Melt and mix suppositories to be assayed and weigh accurately sufficient sample to contain 0.50 mg. of DES. Add 10 ml. of chloroform to the sample, warm gently to dissolve, then transfer with the aid of additional chloroform to the alumina column. Wash the column with 100 ml. of chloroform and discard the $CHCl_3$. Elute the DES from the column with 50 ml. of 80% alcohol collecting the eluate in a 150-ml. beaker. Evaporate the eluate to dryness on a steam bath using a stream of air to aid the evaporation. Dissolve the residue in chloroform, transfer it to a 10-ml. volumetric flask, and dilute to volume with chloroform. To an activated chromatoplate apply alternately 40-μl. spots of the reference standard DES solution and the sample solution. Repeat this operation three times (8 spots total). Transfer the plate to the developing chamber, chromatograph 100 mm., remove, and air-dry for 30 min. At a point in line with the 100-mm. mark, place a spot of thymolphthalein solution near each edge of the plate. Dilute the Folin-Ciocalteu reagent 1 to 4 with distilled water just prior to use and spray the plate evenly with about 5 ml. of the diluted reagent, using the spray procedure described by Stahl (8). In a similar manner, spray the plate with the alcoholic potassium hydroxide solution until the thymolphthalein spot remains a permanent blue color. The DES will be visualized as a blue spot (R_f approximately 0.9). Measure the density of the DES in the reference standard and the sample spots with the densitometer. Determine the areas under the recorded curves from the integrator pen traces. The following instrument parameters were used for the Photovolt densitometer: range setting 3; recorder response 4; 610 mμ filter (Photovolt No. 5408), and slit width, 1 × 7 mm.

Calculation—

$$\frac{\Sigma \text{ area of sample spots}}{\Sigma \text{ area of standard spots}} \times \frac{0.05}{\text{sample wt. (Gm.)}} = \% \text{ DES.}$$

RESULTS

Linearity of Results—Chloroform solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 mcg. of DES per 40 μl. of solution were prepared and treated according to *Experimental* starting at the thin-layer chromatographic step. Results are given in Table I.

TABLE I—LINEARITY OF SPOT DENSITY *Versus* CONCENTRATION

Plate	Regression Line	Correlation Coefficient (γ)	Standard Deviation (σ)
1	$y = 1.7 + 49.4x$	0.9990	1.7
2	$y = -0.3 + 41.0x$	0.9995	1.0
3	$y = -2.5 + 45.8x$	0.9997	0.8

The result of a given plate is given in the form $y = a + bx$ where y is the area of a spot in terms of integrator count, a is the y intercept, b is the slope of the line, and x is the quantity of DES in a spot. The remainder of the table is self-explanatory.

Recovery of Standard DES—To test the reproducibility of the above procedure, portions of standard DES equivalent to what would normally be found in a medicated suppository were treated as indicated in *Experimental*. Results are given in Table II.

The average areas of the sample spots and of the standard spots are given in columns $\bar{X}$ and $\bar{Y}$, respectively, as integrator counts. The average recovery was 98.4% with a standard deviation of 3.5%.

The statistical treatment is as follows: the area figures used in the calculations for the sample spots and for the standard spots are each an average of four essentially independent observations. The variance of an average result is:

$$\text{Var}\{\bar{X}\} = \sigma^2/n$$

or

$$\bar{\sigma}^2 = \frac{1}{n^2(n-1)}[n\Sigma X^2 - (\Sigma X)^2]$$

The value of σ^2 is then converted to a percentage coefficient of variance:

$$V^2 = (\sigma^2)\left[\frac{100}{\bar{X}}\right]^2$$

The variance for the result is:

$$V_T^2 = V_{\bar{X}}^2 + V_{\bar{Y}}^2$$

and the standard deviation is the square root of the variance.

Recovery of DES from a Suppository Mixture—A mixture of 0.3% nitrofurazone and 0.0125% DES in a water-dispersible base of glyceryl monolaurate and sorethytan (4) monostearate was prepared to simulate a commercial vaginal suppository.[1] Portions of the mixture were treated as indicated in *Experimental*.

Results are given in Table III. Data are treated as indicated above. The average recovery was 98.7% with a standard deviation of 3.4%.

Another suppository mixture[2] containing 0.2% nitrofurazone, 2% diperodon hydrochloride, and

[1] Furestrol Vaginal Suppository, Eaton Laboratories, Division of The Norwich Pharmacal Company, Norwich, N.Y.

[2] Furacin-E Urethral Inserts, Eaton Laboratories, Division of the Norwich Pharmacal Company, Norwich, N.Y.

TABLE II—RECOVERY OF STANDARD DIETHYLSTILBESTROL

Plate No.	Sample $\bar{X}$	Sample V_X^2	Standard $\bar{Y}$	Standard V_Y^2	Recovery %	Recovery V_T^2	Recovery σ
1	89.0	6.53	89.5	0.94	99.44	7.46	2.7
2	79.0	14.69	80.5	9.91	98.14	24.60	5.0
3	99.0	5.27	100.75	4.00	98.26	9.27	3.0
4	105.5	0.83	106.75	10.58	98.83	11.41	3.4
5	69.75	7.32	70.5	5.87	98.94	13.19	3.6
6	86.0	7.67	85.5	12.89	100.58	20.55	4.5
7	82.5	5.02	85.5	7.87	96.49	12.89	3.6
8	80.0	3.91	83.0	2.18	96.39	6.08	2.5

TABLE III—RECOVERY OF DIETHYLSTILBESTROL FROM A SUPPOSITORY MIXTURE

Plate No.	Sample $\bar{X}$	Sample V_X^2	Standard $\bar{Y}$	Standard V_Y^2	Recovery %	Recovery V_T^2	Recovery σ
1	123.2	1.48	123.2	2.31	100.0	3.78	1.9
2	82.8	1.95	83.4	5.26	99.28	7.22	2.7
3	85.75	8.47	86.25	17.34	99.42	25.81	5.1
4	92.6	5.90	94.6	1.85	97.89	7.76	2.8
5	93.25	7.16	95.0	5.73	98.16	12.89	3.6
6	82.0	1.00	83.25	5.14	98.50	6.13	2.5
7	79.5	17.22	81.5	13.17	97.55	30.40	5.5

TABLE IV—RECOVERY OF DIETHYLSTILBESTROL FROM COMMERCIAL SUPPOSITORIES

Mfr.	Label Claim, mg. DES/ Supp.	DES Added, mg./Supp.	DES Found, mg./Supp.	% of Label Claim Found
1	0.1	0	0.098	98
1	0.25	0	0.25	100
2	0.1	0	0.093	93
2	0.5	0	0.47	94
3	0.1	0	0.075	75
3	0.1	0.1	0.177	177

0.0077% DES in the above base was also studied. The diperodon hydrochloride was eluted in the preliminary chloroform wash and discarded. Otherwise, results were comparable to the above results.

Assay of Commercial Suppositories for DES—The DES present in five different dosage forms (commercially available units) from three different manufacturers was determined by the above procedure. Results are given in Table IV. The validity of the low recovery of DES from manufacturer 3 was confirmed by the addition of a known amount of DES. As an additional check, the insoluble portion of suppository base was extracted with chloroform *via* the Soxhlet procedure for 4 hr. No additional DES was found.

DISCUSSION

Effects of Variations in Column Parameters—It was ascertained (the alumina meeting the above criterion) that 75 ml. of chloroform was sufficient to elute interfering substances from the column. As much as 200 ml. of chloroform can be passed through the column without eluting any DES. The DES can be quantitatively eluted with 20 ml. of 80% alcohol. The DES can be satisfactorily eluted with 75–90% alcohol. Alcohol concentration of less than 70% or more than 95% is unsatisfactory for the elution. The column can handle a threefold increase in sample size without overloading.

Effects of Variations in the TLC Procedure—Using good technique, the only problem area in the TLC procedure is the spray pattern. A uniform spray must be laid down on the plate. The volume of the Folin-Ciocalteu reagent must be held to between 4 and 6 ml. The alkaline spray must also be uniform and close attention must be given to the indicator color change. The spots produced are then stable for about 4 hr.

Intact DES—In order to determine that the DES was not degraded by the assay procedure, DES was eluted from the plate and an infrared spectrum recorded. The spectrum compared favorably with that of standard DES.

Portions of a suppository mass were exposed to ultraviolet radiation from a germicidal lamp. The apparent decomposition approximated a first-order degradation. At 15 cm. from a 17-w. lamp 30% of the DES was decomposed in 10 min. The ketonic product formed was found to have an R_f of 0.7 and did not undergo the Folin-Ciocalteu reaction.

Similar Phenolic Compounds—Compounds with structures similar to DES can also be determined by the above procedure. Dienestrol, hexestrol, and 4,4′-dihydroxystilben will behave the same as DES. If present in admixture an additional separation would be necessary before the determination.

Common suppository base materials such as polyethylene glycol or theobroma oil do not interfere with the above procedure.

CONCLUSION

This method is sufficiently rapid for use as a routine quality control procedure and necessary technical skills can be readily learned. The high degree of selectivity demonstrated also indicates that the method is useful for a stability program. A further use of the method might be as a referee method or as an official procedure for the determination of diethylstilbestrol.

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Keyphrases

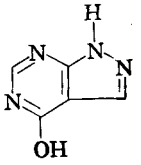
Diethylstilbestrol (DES)—analysis
Nitrofurazone–DES suppositories—analysis
Column chromatography—separation
TLC—identity
Densitometer—analysis

Qualitative and Quantitative Tests for Allopurinol

By EDWARD F. SALIM* and JAMES E. MURPHY†

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

1H-PYRAZOLO [3,4-*d*]-pyrimidin-4-ol; $C_5H_4N_4O$; mol. wt. 136.11. The structural formula of allopurinol may be represented as



Physical Properties—Allopurinol occurs as a white to off-white, practically odorless powder and melts above 300°. It is very slightly soluble in water and in alcohol, and practically insoluble in chloroform and in ether. It is soluble in dimethylformamide and in dilute solutions of alkali hydroxides.

Identity Tests—A 1 in 100,000 solution of allopurinol in 0.1 *N* hydrochloric acid exhibits an ultraviolet absorbance maximum at about 250 mμ [absorptivity (*a*) about 56] and a minimum at about 231 mμ. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.3% dispersion of allopurinol in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 2.

Purity Tests—Dry about 1 Gm. of allopurinol, accurately weighed, in vacuum at 105° for 5 hr.: it loses not more than 1% of its weight.

Dissolve about 10 mg. of allopurinol in 1 ml. of 0.1 *N* sodium hydroxide. Arrange for ascending

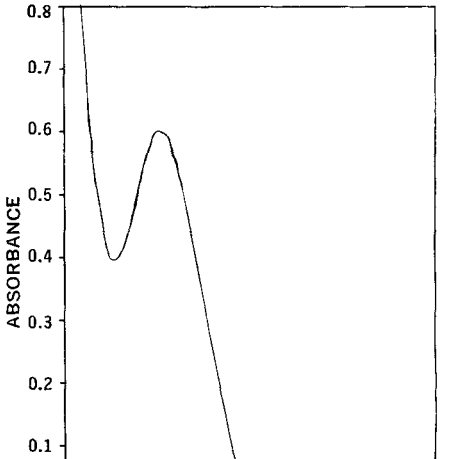


Fig. 1—Ultraviolet absorption spectrum of allopurinol in 0.1 N hydrochloric acid (10 mcg./ml.); Beckman model DK-2A spectrophotometer.

Received October 5, 1967, from the *Drug Standards Laboratory (cosponsored by the American Medical Association, American Pharmaceutical Association, and United States Pharmacopeia Convention), of the APhA Foundation, Washington, DC 20037
Accepted for publication December 12, 1967.
† Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y. 10707.
Burroughs Wellcome & Co. has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.
The authors express their appreciation to C. Wilbur Shank, Burroughs Wellcome & Co., and Hannah Klein, the Drug Standards Laboratory, for valuable contributions to the monograph.

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Keyphrases

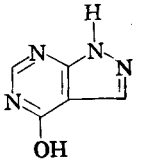
Diethylstilbestrol (DES)—analysis
Nitrofurazone–DES suppositories—analysis
Column chromatography—separation
TLC—identity
Densitometer—analysis

Qualitative and Quantitative Tests for Allopurinol

By EDWARD F. SALIM* and JAMES E. MURPHY†

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

1H-PYRAZOLO [3,4-*d*]-pyrimidin-4-ol; $C_5H_4N_4O$; mol. wt. 136.11. The structural formula of allopurinol may be represented as



Physical Properties—Allopurinol occurs as a white to off-white, practically odorless powder and melts above 300°. It is very slightly soluble in water and in alcohol, and practically insoluble in chloroform and in ether. It is soluble in dimethylformamide and in dilute solutions of alkali hydroxides.

Identity Tests—A 1 in 100,000 solution of allopurinol in 0.1 *N* hydrochloric acid exhibits an ultraviolet absorbance maximum at about 250 mμ [absorptivity (*a*) about 56] and a minimum at about 231 mμ. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.3% dispersion of allopurinol in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 2.

Purity Tests—Dry about 1 Gm. of allopurinol, accurately weighed, in vacuum at 105° for 5 hr.: it loses not more than 1% of its weight.

Dissolve about 10 mg. of allopurinol in 1 ml. of 0.1 *N* sodium hydroxide. Arrange for ascending

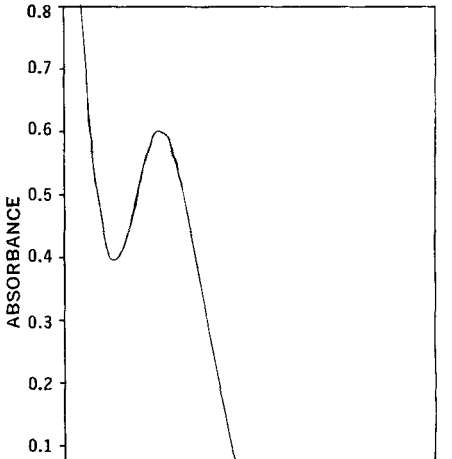


Fig. 1—Ultraviolet absorption spectrum of allopurinol in 0.1 N hydrochloric acid (10 mcg./ml.); Beckman model DK-2A spectrophotometer.

Received October 5, 1967, from the *Drug Standards Laboratory (cosponsored by the American Medical Association, American Pharmaceutical Association, and United States Pharmacopeia Convention), of the APhA Foundation, Washington, DC 20037
Accepted for publication December 12, 1967.
† Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y. 10707.
Burroughs Wellcome & Co. has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.
The authors express their appreciation to C. Wilbur Shank, Burroughs Wellcome & Co., and Hannah Klein, the Drug Standards Laboratory, for valuable contributions to the monograph.

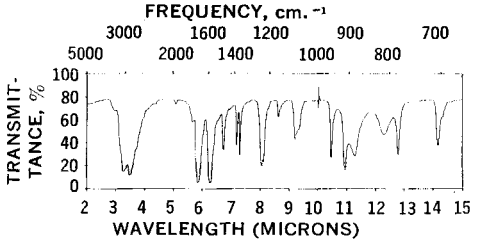


Fig. 2—Infrared spectrum of allopurinol potassium bromide disk (0.3%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

paper chromatography using a strip of paper (Whatman No. 1 filter paper) about 40 cm. in length. Spot 0.005 ml. on the starting line of the chromatographic paper, and suspend the paper in a chromatographic chamber equilibrated with a mobile solvent consisting of ammonium sulfate solution (1 in 20)-isopropanol (95:5). Proceed with the chromatography until the solvent front has advanced about 25 cm., remove the paper, dry, and examine under ultraviolet light: only one spot is visible in the chromatogram.

Assay—Transfer about 300 mg. of allopurinol, accurately weighed, to a 125-ml. conical flask, and dissolve in 60 ml. of dimethylformamide. Add 5 drops of thymol blue T. S., and titrate with 0.1 *N* sodium methoxide, using a magnetic stirrer and taking precautions against absorption of atmospheric carbon dioxide. Perform a blank determination, and make any necessary correction. Each milliliter of 0.1 *N* sodium methoxide is equivalent to 13.61 mg. of $C_5H_4N_4O$. The amount of allopurinol found is not less than 97% and not more than 102%, calculated on the dried basis.

DOSAGE FORMS OF ALLOPURINOL

Allopurinol Tablets—Identity Test—The final solution prepared from the tablet sample in the *Assay* exhibits an ultraviolet maximum and minimum at the same wavelengths as the allopurinol standard solution.

Assay—Weigh and finely powder not less than 20 allopurinol tablets. Weigh accurately a portion of the powder, equivalent to about 100 mg. of allopurinol, and transfer to a 100-ml. volumetric flask. Add 10 ml. of 0.1 *N* sodium hydroxide, shake for about 5 min., dilute to volume with 0.1 *N* hydrochloric acid, and mix. Filter the solution, discarding the first portion of filtrate, and dilute a portion of the clear filtrate quantitatively and stepwise with 0.1 *N* hydrochloric acid to obtain a solution having a concentration of the equivalent of 10 mcg. of allopurinol/ml. Concomitantly determine the absorbance of the final solution and of a standard solution of allopurinol, in the same medium, at a concentration of about 10 mcg./ml., in 1-cm. cells, at the maximum at about 250 mμ, with a suitable spectrophotometer, using 0.1 *N* hydrochloric acid as the blank. Calculate the quantity, in milligrams, of $C_5H_4N_4O$ in the portion of tablets taken by the formula $10\ C \times (A_u/A_s)$, in which C is the exact concentration of the standard solution, in mcg./ml., A_u is the absorbance of the sample solution, and A_s is the absorbance of the allopurinol standard solution. The amount of allopurinol found is not less than 93% and not more than 107% of the labeled amount.

DISCUSSION

USP and NF terminology for solubility, melting range, reagents, *etc.*, has been used wherever feasible.

Allopurinol[1] is an oral uricosuric agent effective in the treatment of hyperuricemia associated with gout and other conditions. Its action differs from that of other uricosuric agents which lower the serum uric acid level by increasing urinary excretion of uric acid. Allopurinol reduces both the serum and urinary uric acid levels by blocking the formation of uric acid.

Identity Tests—An additional identification test for allopurinol is obtained by comparing absorbances of the hydrochloric acid solution at 231 mμ (minimum) and at 250 mμ (maximum). The ratio A_{231}/A_{250} is between 0.5 and 0.62. A ratio of 0.57 was calculated from absorbance data in the ultraviolet absorption identification of allopurinol tablets. A 1 in 100,000 solution of allopurinol in pH 11 buffer solution exhibits an ultraviolet absorbance maximum at about 262 mμ and a minimum at about 235 mμ.

Quantitative Methods—Nonaqueous titration of allopurinol with sodium methoxide using thymol blue T.S. gave an average value of 98.5 ± 0.4%.[2] Spectrophotometric analysis of commercial allopurinol tablets (100 mg.) gave an average value of 100.3 ± 0.6%[2] of the labeled amount.

[1] Marketed as Zyloprim by Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y.

[2] Maximum deviation from the mean value.

Keyphrases

Allopurinol—analysis
IR spectrophotometry—identity
Paper chromatography—purity test
Sodium methoxide titration—analysis
Allopurinol tablets—analysis
UV spectrophotometry—analysis, identity

Determination of DMSO in Solutions and Ointments by NMR

By T. C. KRAM and J. W. TURCZAN

An NMR method for the determination of DMSO in ointments and solutions is presented, using methanol as an internal standard. Samples containing from 20 to 0.05 percent (v/v) DMSO were successfully analyzed with this technique.

DIMETHYL SULFOXIDE (DMSO) is readily analyzed by NMR because it produces a single absorption peak of relatively great intensity and is miscible with a wide variety of solvents. NMR spectroscopy has been used to determine DMSO in a mixture with glycerol and water by measuring total hydrogen in the sample and in that fraction due to DMSO (1). This report discusses the determination of DMSO in solutions and ointments with methanol as an internal standard.

Although volatile liquids are not generally recommended as internal standards, methanol was found to be useful because of the chemical shift of its methyl peak, its low equivalent weight, and its wide miscibility range. Its use simplifies sample preparation and calculation of results for a series of determinations.

Sample and standard were mixed with suitable solvent, NMR spectra were obtained, and the amount of DMSO in the sample was calculated from the integration of the sample and standard peaks of interest (2–4).

Serial dilutions of a mixture of DMSO standard and methanol were similarly analyzed. The use of peak heights (5) as a means of estimation was investigated for solutions too dilute to produce integral presentations of any reasonable reproducibility.

PROCEDURE

Sample Preparation—Liquid—Pipet sample equivalent to 4–5 ml. of DMSO into a 50-ml. glass-stoppered conical flask. If the sample is too viscous to be pipeted accurately, accurately weigh a portion, determine the density of the material, and calculate the volume of the weighed portion ($V = W/d$). Add 5.0 ml. of methanol. Dilute with 10 ml. of water, stopper, and mix thoroughly.

Semi-Solid—Weigh sample equivalent to 4–5 Gm. of DMSO into a 50-ml. glass-stoppered conical flask, add 5.0 ml. of methanol and 15 ml. of water stopper, and mix thoroughly. If material is not soluble in water, use chloroform as diluent.

Received October 5, 1967, from Food and Drug Administration, 850 Third Avenue, Brooklyn, NY 11232
Accepted for publication December 19, 1967.

Transfer approximately 0.4 ml. of solution to an analytical NMR tube. Prepare an NMR spectrum on the Varian A-60, adjusting settings for optimum integration. Each sample is integrated 10 times.

Calculations:

$$\% \text{ DMSO in sample (v/v)} = \frac{A \text{ DMSO}}{A \text{ CH}_3\text{OH}} \times \frac{E \text{ DMSO}}{E \text{ CH}_3\text{OH}} \times \frac{d \text{ CH}_3\text{OH}}{d \text{ DMSO}} \times \frac{V \text{ CH}_3\text{OH}}{V \text{ sample}} \times 100$$

$$\% \text{ DMSO in sample (w/w)} = \frac{A \text{ DMSO}}{A \text{ CH}_3\text{OH}} \times \frac{E \text{ DMSO}}{E \text{ CH}_3\text{OH}} \times \frac{d \text{ CH}_3\text{OH}}{W \text{ sample}} \times V \text{ CH}_3\text{OH} \times 100$$

in which:

A DMSO = area of DMSO peak (2.7 p.p.m. δ)
A CH_3OH = area of methanol CH_3 peak (3.3 p.p.m. δ)
d = density (Gm./ml.)
V = volume (ml.)

$$E \text{ DMSO} = \text{equivalent weight of DMSO} = \frac{78.13}{6} = 13.02$$

$$E \text{ CH}_3\text{OH} = \text{equivalent weight of methanol} = \frac{32.04}{3} = 10.68$$

W sample = weight of sample in Gm.

If a series of determinations is made, the combination of repeated values will greatly simplify the calculations; *e.g.*, in determining % DMSO (v/v) in various solutions, equal volumes of methanol and sample were taken; the densities of methanol and DMSO at room temperature, 74° F., were determined to be 0.7863 and 1.0915, respectively. Combining terms, the calculation becomes:

$$\% \text{ DMSO (v/v)} = 87.83 \times \frac{A \text{ DMSO}}{A \text{ CH}_3\text{OH}}$$

DISCUSSION

A solution containing known amounts of DMSO and methanol in water was prepared. Aliquots were diluted to obtain concentrations ranging from 20% to 0.05% (v/v) DMSO. Table I shows recoveries obtained for concentrations of 2–20% DMSO.

Since integration was not feasible for the more dilute solutions, peak heights of CH_3 (DMSO) and

TABLE I—DMSO DETERMINATION OF STANDARD PREPARATIONS[a]

Solution	DMSO Added, ml.	DMSO Found, ml.	Relative Error, %
1	20.00	20.14	0.7
2	10.00	9.99	0.1
3	5.00	4.94	1.2
4	2.00	1.98	1.0

[a] Equal volumes of DMSO and methanol were diluted to approximately 100 ml. in each case.

TABLE II—EFFECT OF CONCENTRATION OF PEAK HEIGHT RATIO OF DMSO/CH_3OH[a]

Solution	Concn. DMSO, % (v/v)	Mean $\frac{H \text{ DMSO}}{H \text{ } CH_3OH}$	Standard Deviation	Relative Standard Deviation, %
1	20	1.494	0.019	1.3
2	10	1.313	0.009	0.7
3	5	1.226	0.015	1.2
4	2	1.164	0.011	0.9
5	1	1.177	0.016	1.4
6	0.2	1.234	0.040	3.2
7	0.05	1.235	0.066	5.4

[a] H DMSO = height of DMSO peak. H CH_3OH = height of CH_3 peak in methanol. Results are based on 5 scans per solution.

$CH_3(CH_3OH)$ were measured on spectra of the aforementioned solutions, and the ratio of the two peaks was calculated for each spectrum. Table II shows mean ratios and standard deviations for 5 scans of each dilution. Reproducibilities of the mean peak height ratios for concentrations of 2% down to 0.05% were deemed adequate to suggest this procedure for estimating DMSO present as a contaminant in human and veterinary drugs. If any of the usual components of the drug produce an interfering spectrum, they may possibly be kept out of solution by a change of solvent.

As may be noted, there is a marked trend in peak height ratios of DMSO to the methyl in methanol as the concentrations increase from 2% up to 20%. This may be due to differential line broadening effects on the CH_3 peaks of methanol and DMSO resulting from increased viscosity. However, the importance of this phenomenon is only academic, as solutions of greater concentration may be determined satisfactorily by integration.

The DMSO peak is prominent down to a concentration of 0.01%; at this level the signal-to-noise ratio was found to be approximately 7:1 at a spectrum amplitude of 100 and a filter band width of 0.4 Hz.

Water is the solvent of choice; nearly all commercial DMSO preparations analyzed in this laboratory were found to be soluble in it, and its chemical shift (4.6–4.7 p.p.m. δ) is far removed from those of DMSO (2.7 p.p.m. δ) and the methyl peak of methanol (3.3 p.p.m. δ). Certain preparations produced a heavy precipitate upon the addition of water but were soluble in chloroform (7.3 p.p.m. δ). In these cases the addition of a small amount of hydrochloric acid eliminated spin-spin coupling between the methyl and hydroxyl groups of methanol. In either case, the use of a deuterated solvent is unnecessary.

In a series of commercial samples analyzed recently, results were in good agreement with the declared values (Table III).

TABLE III—DMSO CONTENT OF COMMERCIAL PREPARATIONS

Nature of Product	DMSO Declared, %	DMSO Found, %	Found/ Declared, %
Liquid	90 (v/v)	91.3	101.4
Liquid	90 (v/v)	90.9	101.0
Liquid	90 (v/v)	88.5	98.3
Liquid	90 (v/v)	86.1	95.1
Semi-solid	70 (w/w)	68.3	97.6
Semi-solid	70 (w/w)	69.8	99.7
Semi-solid	70 (w/w)	70.5	100.7
Semi-solid	70 (w/w)	69.8	99.7

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Keyphrases

DMSO solutions, ointments—analysis
NMR spectroscopy—identity
Methanol—internal standard in NMR

Comparison of Iron Assays in Multivitamin Products Using an Automated Chemical Procedure and an X-Ray Emission Method

By WILLIAM F. BEYER and KARL G. ZIPPLE

Two methods are described for the determination of iron in multivitamin preparations, formulated with either ferrous sulfate, ferrous fumarate, or ferrous gluconate. In the first procedure, an automated colorimetric procedure, iron is determined with 2,4,6-tripyridyl triazine in a buffered solution. In the second procedure, an X-ray emission spectrometer is used. Samples, analyzed as solutions, are placed in liquid sample cells and counted for 100 sec. Following sample preparation, approximately 20 samples can be assayed per hour with the automated procedure as compared to four (in duplicate) by X-ray emission.

THE DETERMINATION of iron in multivitamin preparations requires the use of assay procedures that are not affected by other minerals, vitamins, or formulation excipients. In achieving this, X-ray emission spectroscopy has given satisfactory results in our laboratories over a long period of time. Requests for large numbers of iron assays in formulation studies and in single tablet or unit dosage assays, however, prompted the use of an automated chemical assay procedure.

This report describes two methods for the determination of iron in multivitamin preparations, formulated with either ferrous sulfate, ferrous fumarate, or ferrous gluconate. In the first procedure, an automated colorimetric procedure, iron is determined with 2,4,6-tripyridyl triazine in a buffered solution essentially as described by Zak *et al.* (1).

In the second procedure, a General Electric X-ray emission spectrometer is used. Samples, analyzed as solutions, are placed in a liquid sample cell and are counted for 100 sec.

METHODS AND MATERIALS

Automated Chemical

Instruments—Automatic analyzer components[1]: Sampler I, proportioning pump I, colorimeter, linear recorder, chart reader, assorted tubing, and glass fittings.

Reagents—Double distilled water used throughout the procedure; hydrochloric acid, A.R.

Acetate buffer, pH 4, 1.0 *M*, 27.22 Gm. of sodium acetate trihydrate is dissolved in approximately 200 ml. of water in a 1,000-ml. flask, 45.79 ml. of glacial acetic acid is added and the solution is made up to volume with water.

2,4,6-Tripyridyl triazine (TPTZ)[2]; 200 mg. of anhydrous TPTZ is dissolved in 0.5 ml. of HCl and transferred to a 1,000-ml. volumetric flask with the aid of 200 ml. of acetate buffer. After dilution to volume with water, the contents are thoroughly mixed and allowed to stand several days with periodic shaking. The solution is filtered before use and stored in light-resistant bottles.

Ascorbic acid, 2% aqueous solution. When refrigerated, it is stable for several days.

Iron Standard—Stock solution; 200 mcg./ml. 1.4045 Gm. of ferrous ammonium sulfate hexahydrate is placed in a 1,000-ml. volumetric flask with 1.0 ml. of HCl, gently mixed, and diluted to volume with water. The standard is stable indefinitely in a tightly closed, glass container.

Working Solutions; The iron stock solution is used to prepare solutions containing 0.75 mcg., 1.0 mcg., 1.25 mcg., and 1.50 mcg. iron/ml. in water. The diluted standards are kept in tightly closed volumetric flasks. The standards appear to be stable indefinitely; however, new standards should be prepared periodically.

Multivitamin Sample Preparations—For single-tablet assays, individual tablets are placed in 10.0 ml. of concentrated HCl and approximately 5 ml. of water. After shaking for approximately 4 hr., or placing on a steam bath for approximately 1 hr., the solutions are diluted to 100.0 ml. with water. An aliquot is transferred to a suitable volumetric flask and diluted with water to a theoretical iron concentration of approximately 1.0 mcg./ml. If

Received October 18, 1967, from the Control Division, The Upjohn Company, Kalamazoo, MI 49001
Accepted for publication December 1, 1967.
Presented in part to the ANACHEM conference, Detroit, Michigan, October 3, 1967.

[1] AutoAnalyzer, Technicon, Ardsley, N.Y.

[2] International Chemical and Nuclear Corporation, City of Industry, Calif.

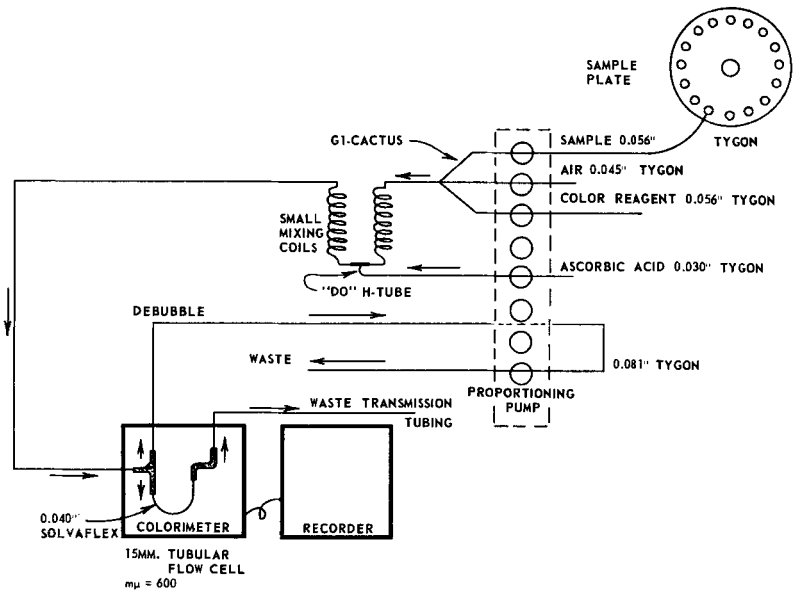

Fig. 1—Manifold-flow diagram for iron (ferrous) with the automatic analyzer.

necessary, the solutions are filtered prior to filling the automatic analyzer cups.

When a specified number of tablets are required in the initial sample preparation, the same ratios of tablet, acid and water are maintained as for single-tablet assays. For example, if a procedure calls for the assay of 10 tablets, they are placed in a 1,000-ml. volumetric flask with 100 ml. of concentrated HCl and 50 ml. of water. Following disintegration of tablets, the volume is adjusted to 1,000 ml. with water and an aliquot is further diluted to the desired concentration of iron.

Procedure—The automatic analyzer is standardized using the manifold-flow system of Fig. 1. As the figure shows, a 15-mm. flow cell is used in the colorimeter and measurements are made at a wavelength of 600 mμ. Standardization is achieved by pumping water through the tubes to establish a 99% transmittance base line, substitution of reagents for water, and adjusting to a new 99% transmittance base line. A known iron solution is continuously sampled to establish initial stability of the automated system. The four levels of iron standards (0.75, 1.0, 1.25, and 1.50 mcg./ml.) are placed in alternate sample cups followed by test preparations. The analysis is set at 40 samples/hr. with a water washout cup between each iron sample, standard as well as test preparation. At regular intervals a 1.0 mcg./ml. iron standard is inserted to compensate for the effect of instrumental or reagent variations.

Calculations—A transmission curve is plotted on the chart reader using recorded peak heights of the iron standards. The mcg./ml. of iron in each test preparation is determined from its corresponding transmittance peak using the chart reader and the iron standard curve. An adjustment factor, determined from standards placed periodically, is applied if necessary. The iron content of each product is obtained by multiplying the mcg./ml. by the proper dilution factors. To express results in terms of the proper ferrous salt (fumarate, gluconate, or sulfate), an additional factor based on the ratio of molecular weights is applied.

X-Ray Emission

Instruments—The General Electric XRD-5 spectrometer, equipped with a lithium fluoride analyzing crystal and a tungsten target X-ray tube operated at 50 kvp. and 45 ma., was used in the study. The flow proportional counter (GE No. SPG4) was operated at 2.100 kv. The spectrometer was equipped with a General Electric large sample holder. General Electric liquid sample cells, closed with 1/4-mil Mylar were used to hold the samples. The liquid sample holder is shown in Fig. 2.

Reagent—Hydrochloric acid, A.R., concentrated and 1:1000.

Iron Standard—A 1.0 mg./ml. iron standard is prepared by dissolving 7.0217 Gm. of ferrous ammonium sulfate hexahydrate in 1,000 ml. of 1:1000 HCl.

Multivitamin Sample Preparation—Sufficient tablets to provide approximately 100 mg. of iron are placed in a 250-ml. volumetric flask with 4 ml. of HCl and 20 ml. of deionized water and left at room temperature for at least 1 hr. or, if convenient, overnight. Another 180 ml. of water is added, the sample is heated on a steam bath for an hour; the solution is then cooled to room temperature and made to volume with water. This solution contains 0.4 to 0.5 mg. of iron per ml.

Procedure—Three separate GE liquid sample cells are used for the following solutions: (*a*) *Blank*, 6.0 ml. of 1:1000 HCl; (*b*) *Sample*, 4.0 ml. of multivitamin test preparation and 2.0 ml. of 1:1000 HCl; and (*c*) *Sample with iron standard*, 4.0 ml. of multivitamin test preparation and 2.0 ml. of iron standard (1.0 mg./ml.). The cells are sealed with 1/4-mil Mylar and the contents are mixed by gentle shaking.

If a thick syrup is to be assayed, a suitable aliquot of the product is transferred with a syringe into two glass-stoppered centrifuge tubes and 1:1000 HCl is added to give an iron concentration of approximately 0.3 mg./ml. An aliquot of iron standard is added to one of the tubes to make the total iron concentration approximately 0.6 mg./ml. An equal volume of 1:1000 HCl is added to the other tube. Six-milliliter aliquots are then pipeted

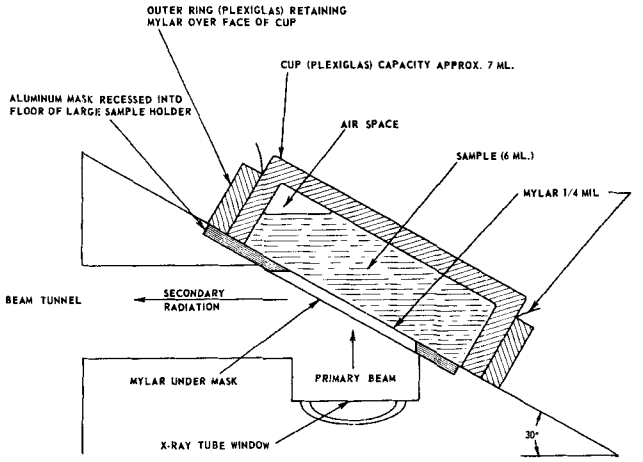

Fig. 2—Liquid sample holder used in X-ray emission analysis.

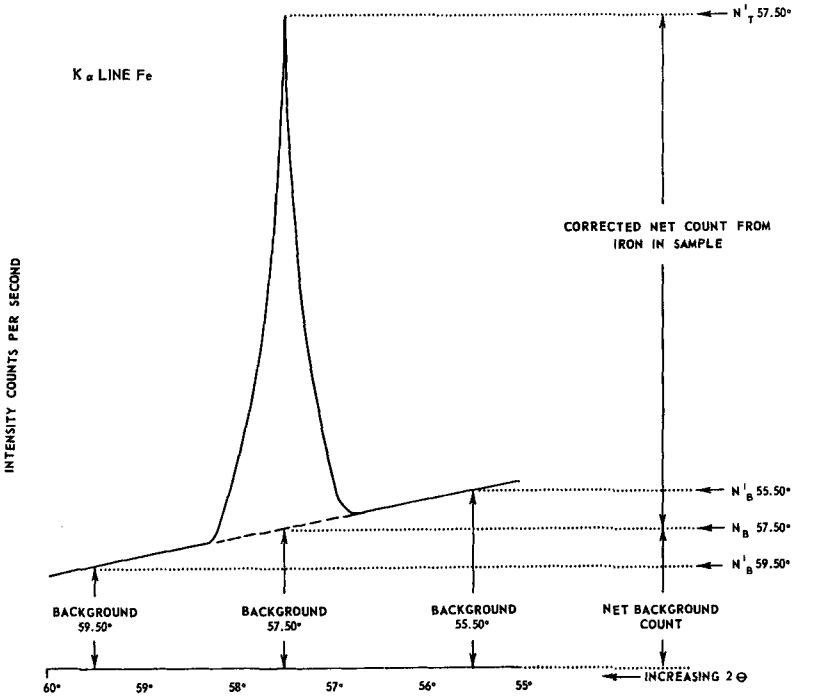

Fig. 3—Spectrum of the analytical line for iron showing background corrections.

into the GE liquid sample cells and sealed with 1/4-mil Mylar.

The cells are gently shaken, inverted, and placed in the large sample holder. Counts are made on each cell for 100 sec. with the spectrometer set for 2θ 55.50° (background), 57.50° (Kα emission line of iron), and 59.50° (background). A representative spectrum of the analytical line for iron showing background corrections is shown in Fig. 3. The average of the background counts taken 2° 2θ each side of the Kα emission line of iron at 2θ = 57.50° is the assumed background at this line and this value is subtracted from the count at 2θ = 57.50° to give the net count due to iron present.

The diluent blank cell gives a net count over background which is a measure of the iron present in the X-ray tube, holder, mask, and cell. This blank is subtracted from the net count for the "sample" and "sample with standard" to give corrected net counts. A schematic of the procedure is shown in Fig. 4. The diluent blank is not counted for each sample; once a day is sufficient.

Calculations—The amount of iron present in the sample aliquot is calculated by applying the straight line-slope equations:

$$\text{Slope } (m) = \frac{\Delta y}{\Delta x} = \frac{\text{corr. net count sample with standard} - \text{corr. net count sample}}{\text{mg. Fe added to sample with standard} - \text{mg. Fe added to sample}}$$

and

$$\text{mg. Fe present in sample aliquot} = \frac{b}{m} = \frac{\text{corr. net count sample}}{m}$$

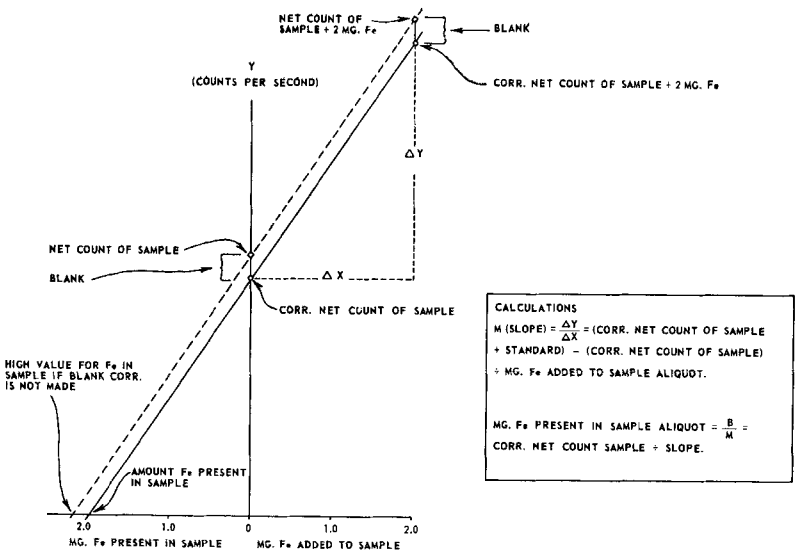

Fig. 4—Calculations used in X-ray emission analysis for iron.

RESULTS AND DISCUSSION

Initial experiments with the automated colorimetric procedure using a standard 10-mm. flow cell and dialysis gave satisfactory results for iron standards; however, product results were extremely variable from day to day. This was thought to be due to variable efficiency of the dialysis membrane or less than optimal sample preparation.

Installation of a 15-mm. tubular flow cell, elimination of dialysis, and the use of initially strongly acid solutions for sample preparation led to a satisfactory assay procedure. Because product excipients caused initial solutions to become basic when using water, the addition of acid was required; however, the volume of acid had to be regulated so that the capacity of the pH 4 acetate buffer was not exceeded.

A linear relationship exists for the log of transmittance and iron concentration in the range of 0.25 mcg. to 2.0 mcg./ml. with the automatic analyzer procedure. The assay has a percent coefficient of variation $\left(\frac{\text{standard deviation}}{\text{mean value}} \times 100\right)$ of approximately 1%.

To test the efficiency of the automated procedure, iron standards in varying amounts, were added to tablet sample preparations containing ferrous sulfate. Results of the study are shown in Table I, an average recovery of 100.3% was obtained, varying from a low of 98.8% to a high of 102.2%.

Table II gives automated assay results of individual multivitamin tablets containing ferrous sulfate. The average iron content was 10.33 mg., with an average tablet weight of 308.8 mg. Ten tablets of the same lot were assayed as a combined sample with an average iron value of 10.50 mg./tab. The average value compares favorably with the value of 10.33 mg./tab. for the single-tablet assay.

X-ray emission analysis (also called X-ray fluorescence analysis) provides a rapid procedure for the determination of iron in pharmaceutical materials. Approximately 25,000 counts are recorded in the 100-sec. period for each mg. of iron present in the cell. Total iron is measured without regard to valence or sample color and there is little interference from other elements. Tedious acid digestion of fluid samples containing sugar is unnecessary. The use of an internal standard for each sample minimizes absorption effects of various excipients. The assay has a coefficient of variation of approximately 1.2%. Four samples can be

Table I—Recovery of Iron (mcg.) from Solutions Prepared from Multivitamin Tablets Using the Automatic Analyzer

Iron from Tablets	Iron Added	Theoretical Amt. Iron Present	Iron Found	Recovery (%)
5.025	4.00	9.025	9.05	99.7
5.100	4.00	9.100	9.30	102.2
5.175	4.00	9.175	9.30	101.4
5.250	4.00	9.250	9.25	100.0
5.250	2.50	7.750	7.75	100.0
5.250	3.75	9.000	9.20	102.2
5.250	5.00	10.250	10.30	100.5
5.250	6.25	11.500	11.40	99.1
5.250	7.50	12.750	12.60	98.8
5.300	4.00	9.300	9.20	98.9
Av. recovery:				100.3%

Table II—Weight and Iron Content of Individual Multivitamin Tablets Assayed with the Automatic Analyzer
(Theory: 10 mg. Iron/Tab.)

Tablet No.	Tablet Wt., mg.	Iron/Tab., mg.
1	299.2	10.05
2	304.2	10.50
3	314.0	10.60
4	305.8	10.20
5	308.0	10.35
6	313.6	10.04
7	308.5	10.05
8	308.5	10.00
9	315.3	11.05
10	310.9	10.50
Averages	308.8	10.33
Coefficients of variation, %	1.60	3.25

TABLE III—WEIGHT AND IRON CONTENT OF INDIVIDUAL MULTIVITAMIN TABLETS ASSAYED WITH X-RAY EMISSION (Theory: 10 mg. Iron/Tab.)

Tablet No.	Tablet Wt., mg.	Iron/Tablet, mg.
11	280.1	10.183
12	278.2	10.207
13	281.6	10.303
14	284.1	10.619
15	274.8	10.547
16	283.2	10.162
17	277.7	10.133
18	277.6	10.141
19	280.4	9.979
20	284.1	10.185
21	279.8	10.690
22	286.0	10.518
23	279.7	10.397
24	277.5	10.234
25	275.7	10.017
26	279.9	10.272
Averages	280.0	10.287
Coefficients of variation, %	1.12	2.04

assayed in duplicate in an hour; however, it is possible to automate the assay with accessories now available commercially.

A large number of single-tablet assays of multivitamin tablets from studies involving formulation and tableting process changes were carried out using X-ray emission. Data from a study of this nature are shown in Table III. The coefficients of variation for both tablet weight and iron content are lower than that found with an earlier lot of tablets assayed by the automated colorimetric procedure (Table II). Sixteen additional tablets from the same lot were individually weighed and assayed on each of 2 days with the X-ray emission method. The coefficients of variation were 0.82 and 1.05% for tablet weight and 1.94 and 2.34% for iron content, respectively. These data indicated that a uniform tablet had been produced, from the standpoint of weight and iron content.

Assay results of pulverized tablets obtained by both procedures are shown in Table IV. Weighed amounts of the ground tablets approximating the average tablet weight were analyzed. More deviation in iron content was found with the X-ray procedure than with the automated colorimetric method; however, the average of the results agree quite favorably. Satisfactory assays with the two

TABLE IV—IRON CONTENT OF SEPARATE WEIGHINGS OF PULVERIZED MULTIVITAMIN TABLETS, 10 mg. IRON/TABLET

Automatic Analyzer		X-Ray Emission	
Sample No.	Iron/Tab., mg.	Sample No.	Iron/Tab., mg.
1	10.18	11	9.74
2	10.43	12	9.87
3	10.10	13	10.37
4	10.17		
5	10.19		
6	10.01		
7	10.19		
8	10.35		
9	10.11		
10	10.07		
Averages	10.18		9.99
Coefficient of variation, %	1.24		

procedures have been performed on numerous additional multivitamin preparations, including capsules, tablets, syrups, and powder mixes.

SUMMARY

An automated chemical method and an X-ray emission procedure have been described for the determination of iron in multivitamin preparations. Following sample preparation, approximately 20 samples can be assayed per hour with the automated procedure as compared to four (in duplicate) by X-ray emission.

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Keyphrases

Automated analysis—iron
Iron–multivitamin preparations—analysis
Colorimetric automated analysis
Diagram—manifold flow system, automated
X-ray emission spectrometry—analysis, nonautomated

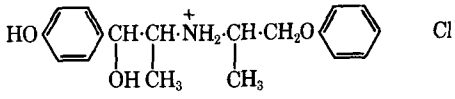
Single-Tablet Assays

Automated Assay of Single Tablets of Isoxsuprine Hydrochloride

By RHYS BRYANT, D. E. MANTLE, D. L. TIMMA, and D. S. YODER

An automatic analyzer system was used to determine the amount of isoxsuprine hydrochloride in single tablets. The intensity of the ultraviolet absorbance of isoxsuprine hydrochloride was a measure of its concentration in the tablet.

ISOXSUPRINE HYDROCHLORIDE[1] (I) is a myovascular relaxant that acts directly and specifically on the smooth muscle of the blood vessels and the myometrium to increase blood flow in vascular disorders, and to reduce uterine hypermotility (1, 2). Isoxsuprine hydrochloride is readily assayed in tablets by means of its characteristic ultraviolet spectrum: $\lambda_{max.}$ 218 mμ (0.1 *M* H_3PO_4) (*a*, 42.2).



The literature on the application of automated analyses to pharmaceutical formulations, including tablets, is extensive, and several comprehensive literature compilations exist (3–5). The application of an automated ultraviolet assay to single tablets of isoxsuprine hydrochloride is now reported. Using this method the probability of meeting the USP test for content uniformity (6) was found to be greater than 99%.

EXPERIMENTAL

Apparatus—The analytical train consisted of the following modules: (*a*) Solidprep Sampler, proportioning pump, and dialyzer, from Technicon Instruments; (*b*) Hitachi Perkin-Elmer spectrophotometer, model 139; (*c*) Microflow cell 9120 No. 5; and (*d*) Sargent model TR-05 recorder.

Reagents—(*a*) Phosphoric acid (H_3PO_4), 0.1 *M*; and (*b*) phosphoric acid (0.1 *M*) containing 0.01% polysorbate 20.[2]

Standards—Aqueous solutions of isoxsuprine hydrochloride were made up at three levels of concentration (0.871, 1.09, and 1.31 Gm./L.) (Table I). Ten-milliliter aliquots of each solution were equivalent to an 8-mg., 10-mg., or 12-mg. tablet, respectively, when they had been diluted with 110 ml. 0.1 *M* H_3PO_4 in the Solidprep sampler. This correction was necessary to compensate for volume differences.

Samples—Production lots of tablets were obtained at both front and back punches of the tableting machine. Theoretical content of each tablet was 10 mg. of isoxsuprine hydrochloride.

Procedure—A schematic of the automatic analyzer[3] system is shown in Fig. 1. Each tablet was individually disintegrated in the presence of 0.1 *M* phosphoric acid. An aliquot of the resulting suspension was dialyzed to remove extraneous solid and the dialyzate passed through the spectrophotometer where its ultraviolet absorbance was measured and recorded (Fig. 2).

Approximately 120 tablets could be analyzed in an 8-hr. day.

The three standard solutions were analyzed several times during the day to check the variation of absorbance. No significant variation was found.

Calculation—The average absorbance for each level of standard was determined. A standard curve [average absorbance *versus* tablet equivalent (mg.)] was prepared. Reference to the standard curve gave for each sample the mg. of isoxsuprine hydrochloride per tablet.

TABLE I—CONCENTRATION AND AVERAGE ABSORBANCE OF STANDARDS

Concn. of Standard, Gm./L.	10 ml. of Standard Equivalent to	Av. Absorbance at 220 mμ
0.871	8 mg. Isoxsuprine HCl	0.253
1.09	10 mg. Isoxsuprine HCl	0.311
1.31	12 mg. Isoxsuprine HCl	0.369

DISCUSSION

Interference at the dialysis stage due to dibasic calcium phosphate (a tablet excipient) was overcome by the use of phosphoric acid. Other acids (*e.g.*, sulfuric acid, hydrochloric acid) were found unsuitable. Sulfuric acid resulted in anomalous dialysis rates; hydrochloric acid attacked the automatic analyzer's stainless steel.

RESULTS

Standard Curve—The average absorbance for each of the three standards was determined. A plot of the values (see Table I) gave a straight line, indicating adherence to Beer's law.

Reproducibility of Standards—The reproducibility of the method was checked by the analysis of 10 standards (Table II).

Reproducibility of Sample Suspension—The precision of the method was checked by the analysis of nine aliquots of a sample suspension (Table II).

Statistical Results[4]—The results of nine lots of 50 tablets have been evaluated. The data are presented in Table III.

Received September 15, 1967, from Mead Johnson Research Center, Mead Johnson & Co., Evansville, IN 47721
Accepted for publication December 20, 1967.

[1] Marketed as Vasodilan by Mead Johnson Laboratories, Division of Mead Johnson & Co.

[2] Tween 20, Atlas Chemical Industries, Inc., Wilmington, Del.

[3] AutoAnalyzer, Technicon, Ardsley, N. Y.

[4] The statistical program was prepared by the Systems Department, Mead Johnson & Co. Results were obtained using an IBM 360 computer.

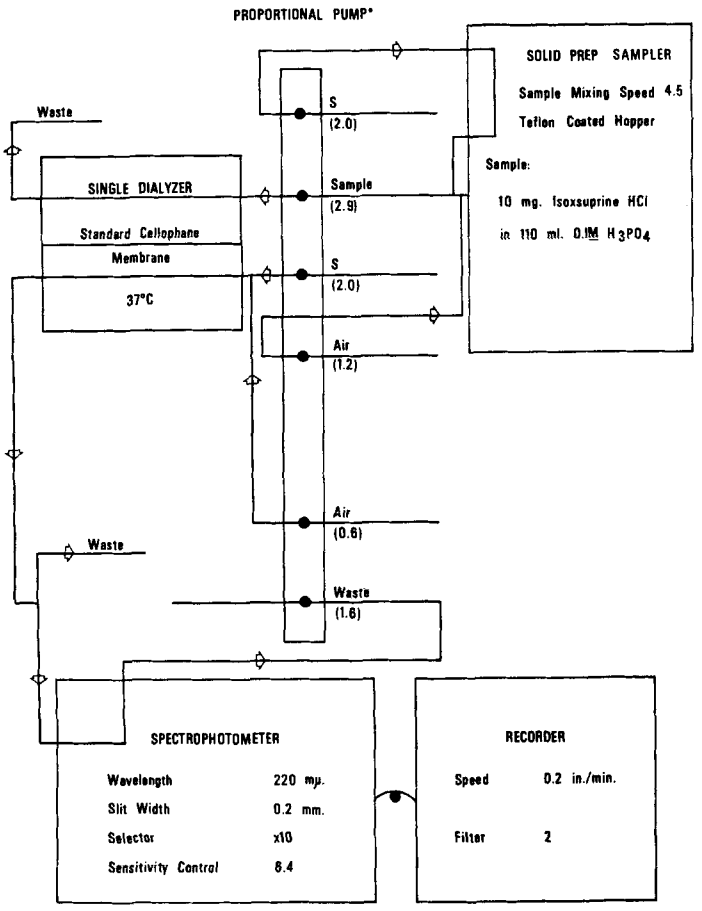


Fig. 1—Schematic for isoxsuprine hydrochloride (values in parentheses are flow rates in ml./min., S = 0.1 M H_3PO_4 with 0.01% polysorbate 20.)

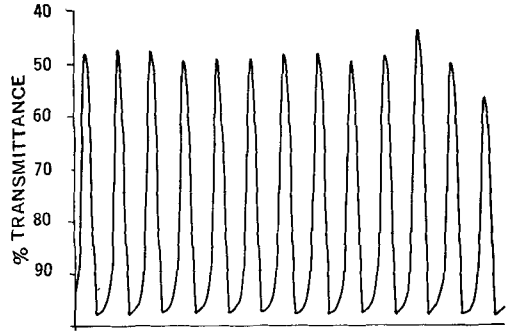


Fig. 2—Typical curve for isoxsuprine hydrochloride.

TABLE II—REPRODUCIBILITY OF STANDARDS AND SAMPLE SUSPENSION

10 ml. of Standard Solution Equivalent to Wt. of Isoxsuprine Hydrochloride			Tablet Suspension Equivalent to 10 mg. Isoxsuprine Hydrochloride
8.0 mg.	10.0 mg.	12.0 mg.	
8.2	10.0	12.0	10.1
8.0	10.0	12.0	9.9
7.9	9.9	12.0	10.0
8.0	10.0	12.0	10.0
7.9	10.0	12.0	10.1
8.0	10.0	12.0	10.2
8.0	10.0	12.0	10.2
8.0	10.0	12.0	10.4
8.0	10.0	11.9	10.4
7.9	10.0	12.0	
Av. 8.0 mg.	10.0 mg.	12.0 mg.	
Coefficient of Variation, %			
1.20	0.33	0.28	1.70

TABLE III—STATISTICAL RESULTS

Lot	Mean mg. Isoxsuprine HCl/Tablet	% Coefficient of Variation
1	9.9	2.4
2	10.1	2.5
3	10.4	3.1
4	10.0	2.5
5	10.3	3.0
6	10.4	1.6
7	10.4	2.3
8	10.6	2.6
9	10.2	1.6

SUMMARY

Single tablets of isoxsuprine hydrochloride have been analyzed using an automated procedure, with greater than 99% probability of meeting the USP content uniformity test.

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(6) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 905.

Keyphrases

Automated analysis—isoxsuprine HCl tablets, single
Diagram—automatic analyzer system
Dialysis—separation
UV spectrophotometry—analysis

Evaluation of Factors Affecting the Encapsulation of Powders in Hard Gelatin Capsules I

Semi-Automatic Capsule Machines

By G. REIER, R. COHN, S. ROCK, and F. WAGENBLAST

A useful model has been developed which relates particulate properties, capsule size, and the operating rate of semi-automatic filling equipment to filled capsule characteristics. The mean capsule fill weight is dependent on machine speed, capsule size, and specific volume. The weight variation as expressed by the standard deviation is a function of machine speed, capsule size, specific volume, flowability, and the presence of a glidant. Particle size considerations of moderately coarse powders (USP designation) are not directly a part of the model because they are reflected in the other evaluated particulate properties. The model is applicable to powder systems other than those from which it was derived. Mean gross capsule weights of such systems may be calculated with an accuracy often exceeding 95 percent. The utility of the model lies in its ability to provide information concerning the relationship of machine speed, capsule size, powder specific volume, and flow to mean capsule fill weight from simple evaluations of small samples of the formulation to be encapsulated.

HARD GELATIN capsules have been known since 1848. They were first prepared in London (1), and quickly attained popularity in the United States where capsule manufacturing and filling operations were carried out manually (2). Hard gelatin capsules are today an important part of solid dosage form drug presentation. A variety of useful, elegant capsule products are available to the physician.

Curiously, the literature is devoid of any reports dealing with the encapsulation of dry powders on an industrial scale. The absence of reported works on this subject is even more marked when one considers the detailed investigations published, for example, by Higuchi *et al.* (3, 4), on the physical factors affecting the tableting process.

Current formulation of powders for encapsulation is carried out on an empirical basis and, while this method has apparently met the needs of the pharmaceutical industry, it would be extremely valuable to have a mathematical model which quantitatively defines the variables encountered in capsule filling. Once these variables are known and quantitated, their application to formulations can be assessed before laboratory work is begun.

The present work was conducted to develop a mathematical model which describes the effect of selected physical powder properties as well as mechanical operating conditions on the capsule filling operation, and to ascertain the relationships of these variables on the encapsulation process.

Received April 28, 1967, from the Squibb Institute for Medical Research, New Brunswick, NJ 08903
Accepted for publication November 15, 1967.
Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
The authors thank S. L. Gitomer, Scientific Computing Center, and A. E. deLorimier and H. B. Humphrey, Pharmaceutical Research, for their assistance.

(2) Lish, P. M., Hillyard, I. W., and Dungan, K. W., *J. Pharmacol. Exptl. Therap.*, **129**, 438(1960).
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The industrial pharmacist encounters drugs of widely differing physical natures, some of which require previous densification before encapsulation can be accomplished. Powders of this sort were not included in this study. Further, there are presently available both semi-automatic and automatic capsule filling machines. This report deals only with the semi-automatic type. The areas of encapsulating densified powders and the effect of variables associated with automatic filling equipment will be the basis of future reports.

EXPERIMENTAL

In order to describe adequately any process, one must have parameters which define that process. Often these parameters are evaluations of the resultant end product. A case in point occurs with capsule production. Mean capsule weight and weight variation are properties which define the completed encapsulation process.

Of the variables affecting encapsulation, those imposed by the filling equipment itself are the speed of operation and the capsule size being filled. Formulation variables include powder particle size and particle size distribution, specific volume, and flowability. The presence or absence of a glidant in the formulation may be expected to have an effect on powder flow. Classically, glidants have been thought to improve flow but recently this concept has been challenged (5).

In order to prepare powder blends varying in mean particle size, particle size distribution, specific volume, and flowability, the following materials were chosen as blend components: microcrystalline cellulose NF,[1] dicalcium phosphate dihydrate unmilled,[2] lactose USP, magnesium stearate USP, and talc USP. Microcrystalline cellulose and dicalcium phosphate dihydrate, unmilled have widely different physical properties, particularly with regard to specific volume and flowability. They were used to vary these properties of the powder blends as desired. Lactose was included as a component because of the need for a filler and its common use as such. A lubricant is necessary for mechanical capsule filling, and magnesium stearate served that purpose. Talc was the glidant.

Three operating speeds, the slowest and the most rapid of which the machine was capable, as well as an intermediate speed, were chosen for study. Three capsule sizes were included in the study.

An experimental design defining the formulation of 42 powder blends from the infinite number that could be formulated was prepared. The lubricant level was maintained throughout at 1%. One-half of the formulations contained no talc, the others 3%. The formulas are given in Tables I and II.

When the two machine operating variables (speed and capsule size) and the three conditions for each are included with the 42 formulations, 378 experimental capsule filling runs are possible. By taking one-third replicates of the operating conditions, the experimental effort was considerably reduced. Each formulation was filled into capsules at one of the nine possible combinations of speed and capsule size, with half of them being studied at two combinations

Table I—Composition of Blends Not Containing Talc

Blend No.	Component %[a] MCC[b]	DPDU[c]	LA[d]
N1	...	...	99.00
N2	...	24.75	74.25
N3	8.25	16.50	74.25
N4	12.37	12.37	74.26
N5	16.50	8.25	74.25
N6	24.75	...	74.25
N7	...	49.50	49.50
N8	16.50	33.00	49.50
N9	24.75	24.75	49.50
N10	33.00	16.50	49.50
N11	49.50	...	49.50
N12	...	74.25	24.75
N13	24.75	49.50	24.75
N14	37.12	37.12	24.76
N15	49.50	24.75	24.75
N16	74.25	...	24.75
N17	...	99.00	...
N18	33.00	66.00	...
N19	49.50	49.50	...
N20	66.00	33.00	...
N21	99.00	...	...

[a] In addition, 1% magnesium stearate was present in each blend. [b] Microcrystalline cellulose. [c] Dicalcium phosphate dihydrate, unmilled. [d] Lactose.

Table II—Composition of Blends Containing Talc

Blend No.	Component %[a] MCC[b]	DPDU[c]	LA[d]
T1	...	...	96.00
T2	...	24.00	72.00
T3	8.00	16.00	72.00
T4	12.00	12.00	72.00
T5	16.00	8.00	72.00
T6	24.00	...	72.00
T7	...	48.00	48.00
T8	16.00	32.00	48.00
T9	24.00	24.00	48.00
T10	32.00	16.00	48.00
T11	48.00	...	48.00
T12	...	72.00	24.00
T13	24.00	48.00	24.00
T14	36.00	36.00	24.00
T15	48.00	24.00	24.00
T16	72.00	...	24.00
T17	...	96.00	...
T18	32.00	64.00	...
T19	48.00	48.00	...
T20	64.00	32.00	...
T21	96.00	...	...

[a] In addition, 1% magnesium stearate and 3% talc were present in each blend. [b] Microcrystalline cellulose. [c] Dicalcium phosphate dihydrate, unmilled. [d] Lactose.

Table III—Operating Conditions for the Encapsulation of Powder Blends

Machine Speed	Capsule Size[a,b] 0	1	2
Fast	N3, 9, 15, 21, T6, 12, 18	N1, 4, 10, 16, T1, 7, 13, 19	N5, 11, 17, T2, 8, 14, 20
Medium	N1, 7, 13, 19, T1, 4, 10, 16	N2, 8, 14, 20, T5, 11, 17	N6, 12, 18, T3, 9, 15, 21
Slow	N2, 8, 14, 20, T5, 11, 17	N6, 12, 18, T3, 9, 15, 21	N1, 4, 10, 16, T1, 7, 13, 19

[a] N, talc absent. [b] T, talc present.

[1] Marketed as Avicel by the FMC Corporation, American Viscose Division, Newark, Delaware.
[2] Marketed by Industrial Division, Stauffer Chemical Company, New York, New York.

TABLE IV—PHYSICAL PROPERTIES OF POWDER BLENDS NOT CONTAINING TALC

Blend No.	Specific Vol., ml./Gm.	Flowability, in.²	Particle Size (Geometric Mean), μ	Particle Size Coefficient of Variation, %
N1	1.27	1.86	69	11.9
N2	1.15	2.57	70	12.7
N3	1.20	3.33	70	11.9
N4	1.41	2.04	72	9.7
N5	1.47	2.87	63	10.2
N6	1.60	2.22	51	15.0
N7	1.07	4.19	110	13.6
N8	1.27	3.66	95	15.2
N9	1.36	3.17	77	16.0
N10	1.59	2.54	49	18.3
N11	1.83	2.49	80	12.2
N12	1.07	3.50	83	13.8
N13	1.30	3.66	82	20.4
N14	1.48	2.78	58	18.3
N15	1.90	2.63	59	18.4
N16	2.06	2.93	42	16.9
N17	1.00	4.15	112	14.8
N18	1.56	3.27	75	14.9
N19	1.76	3.02	58	21.0
N20	2.18	2.63	47	19.4
N21	2.93	2.24	41	12.4

to maintain a balanced design. Table III shows the operating conditions at which each blend was filled.

EXPERIMENTAL

Particulate Properties—*Specific Volume*—Approximately 50 ml. of powder was placed into a tared 100-ml. graduate. The graduate was tapped 15 times mechanically by means of a Numinco Tap-Pak Volumeter.[3] The volume of the powder after tapping was divided by the weight of the powder to give the specific volume in ml./Gm.

Flowability—Powder was allowed to flow gently through a glass funnel by means of a mechanical feeding device[4] onto a calibrated vibrationless flat surface where it formed a cone. The funnel had an orifice of 2.58 mm. and was placed 2.40 cm. above the flat surface. When the apex of the cone reached the funnel orifice powder flow was stopped, and the diameter of the base of the cone determined. The area of the base was calculated (in square inches) rather than the angle of repose. The result was termed "flowability" although the authors realize that such experimental procedures may be measuring interparticle friction rather than flow.

Mean Particle Size and Particle Size Distribution—Particle size determinations were made by sieve analysis using 70, 80, 120, 170, and 325 screens. The Ro-Tap testing sieve shaker[5] was used, and powder samples of 100 Gm. were shaken for 10 min. The best linear plot of percent retained *versus* screen size (in microns) was made on log-probability paper. From the plot the geometric mean diameter and the coefficient of variation were obtained.

Capsule Filling Machine Properties—Throughout the study a Lilly model 8 capsule filling machine[6] was used.

Capsule Size—In order to obtain a meaningful computer analysis it is necessary to have absolute numbers available for data input. This was accomplished in the case of capsule size by measuring the volume in ml. of alcohol USP needed to completely fill the bodies of empty No. 0, No. 1, and No. 2 capsules.

Capsule Machine Speed—A tachometer placed on the turntable spindle yielded r.p.m. data directly.

Filled Capsule Properties—Preliminary work showed that the elimination of ring-to-ring capsule weight differences could be obtained by discarding the first three rings of capsules. There was no statistical difference as measured by a *t* test between the net weights of capsules selected at random from the fourth, fifth, or sixth rings. Also, further study showed that capsule shells do not vary in weight to a statistically significant extent. To simplify analytical weighings, gross capsule weights are reported throughout.

After filling of the powder hopper, the first three rings were filled and discarded, the hopper refilled, and 15 capsules taken at random from the combined fourth, fifth, and sixth rings and individually weighed on an analytical balance. The mean, standard deviation, and coefficient of variation were calculated.

RESULTS

Tables IV and V list the evaluated physical properties of the powder blends.

The machine speed r.p.m. values and the capsule size volume measurements are given in Tables VI and VII.

Filled capsule characteristics are shown in Tables VIII and IX.

A multiple stepwise regression analysis of the dependent variables was made with the aid of an IBM 1620-II computer. A full second-order (quadratic) model with all possible two-factor interactions was employed. The stepwise regression method offers the advantage of including only those variables, quadratic terms, and interactions which demonstrate statistically significant effects on capsule weight and weight variation responses.

During the initial data processing it became evi-

[3] Model JEL-ST, distributed by Numec Instruments and Controls Corporation, Monroeville, Pa.
[4] Eriez Hi-Vi, model V3B style 20A, Eriez Manufacturing Company, Erie, Pa.
[5] Manufactured by W. S. Tyler Company, Cleveland, Ohio.
[6] Eli Lilly and Company, Indianapolis, Ind.

TABLE V—PHYSICAL PROPERTIES OF POWDER BLENDS CONTAINING TALC

Blend No.	Specific Vol., ml./Gm.	Flowability, in.²	Particle Size, (Geometric Mean), μ	Particle Size Coefficient of Variation
T1	1.44	1.56	75	8.4
T2	1.10	3.63	114	9.9
T3	1.27	2.30	76	11.9
T4	1.20	3.14	124	16.8
T5	1.60	1.91	66	13.3
T6	1.64	3.11	88	14.3
T7	1.17	2.43	81	11.3
T8	1.19	3.46	100	12.9
T9	1.38	3.14	74	9.6
T10	1.54	3.24	74	16.4
T11	1.83	2.22	47	15.7
T12	1.08	4.01	96	11.5
T13	1.27	3.02	84	14.3
T14	1.40	3.02	72	16.5
T15	1.83	2.57	51	24.2
T16	2.25	2.81	48	21.4
T17	1.03	2.84	86	13.5
T18	1.43	3.11	74	15.3
T19	1.78	2.96	68	13.9
T20	2.24	2.38	43	22.7
T21	2.50	2.22	43	12.2

TABLE VI—QUANTIFICATION OF CAPSULE FILLING MACHINE SPEEDS

Manufacturer's Designation	Experimental Designation	R.p.m.
A1	Fast	8.7
A2	...	7.3
A3	...	6.0
A4	Medium	4.8
B1	...	4.0
B2	...	3.3
B3	...	2.7
B4	Slow	2.2

TABLE VII—QUANTIFICATION OF CAPSULE SIZES[a]

Size Designation	Vol., ml.
No. 0	0.669
No. 1	0.495
No. 2	0.368

[a] Body only.

dent that a degree of internal correlation existed between the particle size responses and specific volume and/or flowability. Correlation coefficients calculated with and without particle size factors present showed little difference. It was, therefore, possible to eliminate these factors from further analysis since they had no practical effect on capsule characteristics.

The equations below are the result of the computer analysis.

$$Y_1 = 630.71 - 34.37(X_1) + 1.92(X_1)^2 + 1185.99(X_2) + 561.99(X_2)^2 - 421.46(X_3) + 110.34(X_3)^2 - 11.20(X_4) - 24.31(X_1X_2) + 8.27(X_1X_3) - 433.57((X_2X_3). \quad \text{(Eq. 1)}$$

$$Y_2 = 25.73 + 0.27(X_1)^2 + 40.15(X_2) - 10.01(X_3) + 1.83(X_3)^2 - 14.84(X_4) + 3.23(X_4)^2 - 0.60(X_5) - 4.22(X_1X_2) - 0.54(X_1X_4). \quad \text{(Eq. 2)}$$

$$Y_3 = 4.28 + 0.04(X_1)^2 + 0.23(X_3)^2 - 1.86(X_4) + 0.45(X_4)^2 - 0.13(X_5) - 0.15(X_1X_3) - 0.11(X_1X_4). \quad \text{(Eq. 3)}$$

TABLE VIII—FILLED CAPSULE CHARACTERISTICS OF BLENDS WITHOUT TALC

Blend No.	Filling Conditions: r.p.m.	Filling Conditions: Capsule Size	Mean Gross Wt., mg.	S. D., mg.	Coefficient of Variation, %
N1	2.2	2	460.9	8.3	1.8
N1	4.8	0	751.2	15.2	2.0
N1	8.7	1	503.9	13.3	2.6
N2	2.2	0	869.5	19.1	2.2
N2	4.8	1	581.0	10.1	1.7
N3	8.7	0	692.8	7.9	1.1
N4	2.2	2	462.4	6.6	1.5
N4	8.7	1	495.4	11.3	2.3
N5	8.7	2	365.5	7.0	1.9
N6	2.2	1	513.1	11.3	2.2
N6	4.8	2	381.1	7.8	2.1
N7	4.8	0	794.6	24.1	3.0
N8[a]	4.8	1	543.9	8.7	1.6
N9	8.7	0	631.5	8.2	1.3
N10	2.2	2	405.3	14.6	3.6
N10	8.7	1	425.9	8.9	2.1
N11	8.7	2	315.8	3.5	1.1
N12	2.2	1	696.6	21.8	3.1
N12	4.8	2	502.2	8.6	1.7
N13	4.8	0	692.6	12.2	1.8
N14	2.2	0	681.0	22.2	3.3
N14	4.8	1	461.9	6.8	1.5
N15	8.7	0	509.0	8.2	1.6
N16	2.2	2	330.6	12.0	3.6
N16	8.7	1	352.6	5.0	1.4
N17	8.7	2	466.4	12.9	2.8
N18	2.2	1	558.2	11.7	2.1
N18	4.8	2	400.1	4.6	1.1
N19	4.8	0	573.9	11.5	2.0
N20	2.2	0	539.4	18.3	3.4
N20	4.8	1	370.2	4.2	1.1
N21	8.7	0	403.0	7.0	1.7

[a] Excessive binding at 2.2 r.p.m., No. 0 capsule.

where

Y_1 = mean gross capsule weight, mg.,
Y_2 = capsule weight standard deviation, mg.,
Y_3 = capsule weight coefficient of variation, %,
X_1 = machine speed, r.p.m.,
X_2 = capsule size, ml.,
X_3 = specific vol., ml./Gm.,
X_4 = flowability, sq. in.,
X_5 = presence of talc (+1) or absence of talc (−1).

It is obvious from the complexity of the equations that the measured responses are dependent on many factors and interactions between factors. Since no simple relationship exists for each of the evaluated properties, particulate or mechanical, with capsule characteristics, the equations as such are difficult to use for data analysis. Response surface contours, however, may be generated from Eqs. 1–3.

The shape, or contour, of the solid geometrical surface generated by changes in a response produced by continuous variations in values of two interacting factors is known as a response surface contour. Response surface contours may be plotted two dimensionally as contour charts (6). While only two interacting factors can be accommodated on a single contour chart, the preparation of a number of such charts allows for the analysis of the effect of many factors on a response.

DISCUSSION

Mean Capsule Fill Weight—The computer supplied contour charts for all combinations of capsule size and machine speed, including, in the latter case, intermediate machine speeds not a part of the

TABLE IX—FILLED CAPSULE CHARACTERISTICS OF BLENDS WITH TALC

Blend No.	Filling Conditions r.p.m.	Capsule Size	Mean Gross Wt., mg.	S. D., mg.	Coefficient of Variation, %
T1	2.2	2	457.8	6.4	1.4
T1	4.8	0	720.3	17.8	2.5
T1	8.7	1	480.9	14.8	3.1
T2	8.7	2	444.6	12.1	2.7
T3	2.2	1	591.8	16.7	2.8
T3	4.8	2	435.8	6.5	1.5
T4	4.8	0	772.7	10.4	1.4
T5	2.2	0	743.1	19.9	2.7
T5	4.8	1	503.7	9.1	1.8
T6	8.7	0	641.1	7.0	1.1
T7	2.2	2	506.2	6.4	1.3
T7	8.7	1	576.1	9.4	1.6
T8	8.7	2	404.1	9.7	2.4
T9	2.2	1	555.9	8.5	1.5
T9	4.8	2	384.1	9.0	2.3
T10	4.8	0	625.7	7.3	1.2
T11	2.2	0	604.3	9.9	1.6
T11	4.8	1	423.3	5.3	1.2
T12	8.7	0	765.1	13.3	1.7
T13	2.2	2	463.7	3.8	0.8
T13	8.7	1	515.0	9.5	1.9
T14	8.7	2	368.1	6.3	1.7
T15	2.2	1	452.1	6.3	1.4
T15	4.8	2	337.2	4.5	1.3
T16	4.8	0	493.3	8.3	1.7
T17	2.2	0	1006.4	19.0	1.9
T17	4.8	1	647.1	11.4	1.8
T18	8.7	0	641.9	8.1	1.3
T19	2.2	2	377.0	8.4	2.2
T19	8.7	1	406.8	8.9	2.2
T20	8.7	2	277.1	3.2	1.2
T21	2.2	1	373.5	4.9	1.3
T21	4.8	2	271.7	6.1	2.3

original experimental data. Thus, 24 contour charts (8 machine speeds × 3 capsule sizes) were drawn, of which Fig. 1 is representative.

It was seen from these contour charts that, for a given capsule size and machine speed, specific volume almost solely determines the mean capsule weight. A decrease in specific volume (increase in density) increases mean weight. Aside from the obvious fact that dense powders weigh more per unit volume, the weight of the powder head in the hopper above the capsule during filling is increased with dense powders thus increasing the pressure on the material being filled. Of course, the size of the capsule shell and the machine operating speed have a direct effect on capsule weight.

Standard Deviation—Equations 2 and 3 indicate that the presence of talc acts to decrease the filled capsule weight variation. Such a decrease cannot be attributed to improved powder flow as it was measured. The average flowability of powders containing talc was 2.81 sq. in. while that of powders in which talc was not present was 2.94 sq. in. A possible explanation for the decrease in weight variation when talc is present may be that powder flow is more uniform in the presence of a glidant.

In a tableting study, Augsberger and Shangraw (7) reported the opposite result, an increase in weight variability with the addition of a glidant. They also reported a decrease in mean tablet weight in the presence of talc. In the present work, however, there was no such glidant effect on mean capsule weight as evidenced by the absence of a term for talc in Eq. 1.

Two types of contour charts were constructed for studying the interactions of factors contributing to the weight variation of filled capsules. The first type is the result of fixing machine speed, capsule size, and the presence of talc while allowing specific volume and flow to vary. Figure 2 is representative of the charts of this type that were generated showing standard deviation as an estimate of the weight variation. A minimum standard deviation is obtained at intermediate flow values and medium to high specific volumes. Fixing flow at a medium value and increasing specific volume decreases the weight variation. Fixing specific volume and increasing flow results first in a lowering and then an increasing of the standard deviation.

The second type of contour chart results from fixing specific volume, the presence of talc, and capsule size and plotting machine speed *versus* powder flow. Figure 3 is typical. It can be seen that the interaction of machine speed and flow results in a three-dimensional figure, sections of which are ellipse-like in shape. The center area at the top of the figure is the point where the standard deviation is at a minimum. Moving from this area along either the machine speed or flow axis results in an increase in standard deviation. For each capsule size and specific volume combination there are optimum values of machine speed and flow that produce a minimum standard deviation.

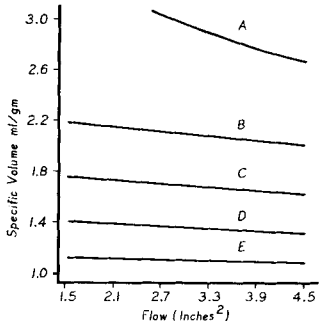

Fig. 1—Mean gross fill weights of No. 0 capsules as a function of specific volume and flow at a machine speed of 4.8 r.p.m. Key: A, 400 mg.; B, 500 mg.; C, 600 mg.; D, 700 mg.; E, 800 mg.

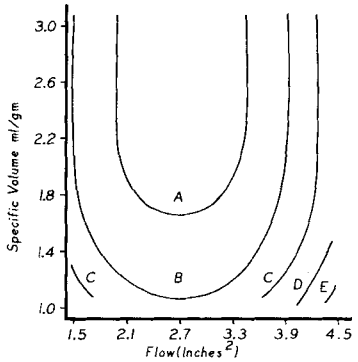

Fig. 2—The standard deviation of filled No. 2 capsules as a function of specific volume and flow at a machine speed of 4.8 r.p.m. Key: A, 3.0 mg.; B, 6.0 mg.; C, 9.0 mg.; D, 12.0 mg.; E, 15.0 mg. (Talc present.)

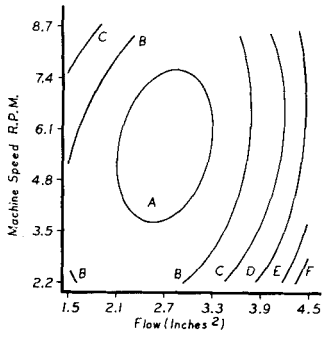

Fig. 3—The standard deviation of filled No. 2 capsules as a function of machine speed and flow at a specific volume of 2.0 ml./Gm. Key: A, 3.0 mg.; B, 6.0 mg.; C, 9.0 mg.; D, 12.0 mg.; E, 15.0 mg.; F, 18.0 mg. (Talc present.)

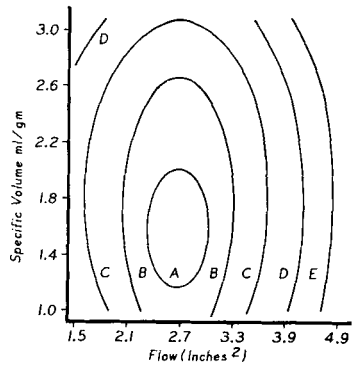

Fig. 4—The coefficient of variation of filled capsules as a function of specific volume and flow at a machine speed of 4.8 r.p.m. Key: A, 1.4%; B, 1.6%; C, 1.8%; D, 2.2%; E, 2.6%. (Talc present.)

Coefficient of Variation—A similar analysis may be made of the influence of the variables on the weight variation as estimated by the coefficient of variation with one exception. Equation 3 states that the coefficient of variation is independent of the capsule size.

Figure 4 is representative of the contour charts generated in which machine speed and the presence of talc are fixed. There is one area in which the coefficient of variation is at a minimum. Any change in specific volume or flow will move the response from the minimum area.

Of the contour charts illustrating the effect of machine speed and flow on the coefficient of variation when the presence of talc and the specific volume are fixed, Fig. 5 is typical. A minimum area occurs at one particular value of machine speed and flow.

Fit of the Model to the Data—The fit of the mathematical model to the data is evidenced by the ability of Eq. 1 to estimate the weights of capsules filled with powders of the same system used in the experimental work but under different filling conditions. The results of this interpolation are shown in Table X where calculated and actual weights of capsules filled with powder blend N7 are compared. Excellent agreement is obtained.

Applicability of the Model—To demonstrate the applicability of the model to other powder systems,

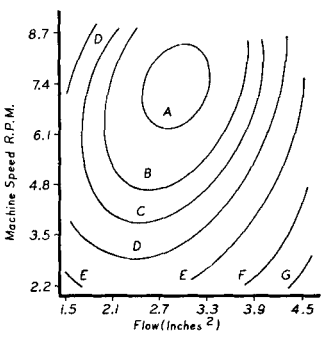

Fig. 5—The coefficient of variation of filled capsules as a function of machine speed and flow at a specific volume of 2.0 ml./Gm. Key: A, 1.2%; B, 1.4%; C, 1.6%; D, 1.8%; E, 2.0%; F, 2.6%; G, 3.2%. (Talc present.)

TABLE X—CALCULATED AND ACTUAL WEIGHTS[a]

Machine Speed	Calculated Weight, mg.	Actual Weight, mg.	Difference, %
A1	440	431	2.0
A2	445	439	1.3
A4	473	460	2.7
B2	501	473	5.6
B4	527	501	4.9

[a] Blend N7, No. 2 capsules.

TABLE XI—CALCULATED AND ACTUAL WEIGHTS[a]

Machine Speed	Calculated Weight, mg.	Actual Weight, mg.	Difference, %
A1	722	760	5.3
A2	737	760	3.1
A4	782	778	0.5
B2	821	798	2.8
B4	855	870	1.8

[a] Formula No. 1, No. 0 capsules.

ascorbic acid and thiamine mononitrate blends were prepared. Small samples of these blends were evaluated for specific volume and flow. The compositions and characteristics of these formulations are given below.

Formula No. 1

Ascorbic acid USP[7] ..... 99 parts
Magnesium stearate USP ..... 1 part
Specific volume = 1.13 ml./Gm.
Flowability = 4.91 sq. in.

Formula No. 2

Thiamine mononitrate USP[8] ..... 98 parts
Magnesium stearate USP ..... 1 part
Pyrogenic silica[9] ..... 1 part
Specific volume = 1.62 ml./Gm.
Flowability = 2.99 sq. in.

Using the specific volume and flow values, capsule weights to be expected at various machine speeds were calculated from Eq. 1. A comparison of calculated and actual weights is given in Tables XI and XII. In three cases the percent difference exceeds 5% but in no case does it exceed 6%.

[7] USP fine crystals, Merck and Company, Inc., Rahway, N. J.
[8] USP extra fine crystals, Merck and Company, Inc., Rahway, N. J.
[9] Marketed as Cab-O-Sil by the Cabot Corporation, Boston, Mass.

TABLE XII—CALCULATED AND ACTUAL WEIGHTS[a]

Machine Speed	Calculated Weight, mg.	Actual Weight, mg.	Difference, %
A1	582	599	2.9
A2	591	618	4.6
A4	626	658	5.1
B2	658	695	5.6
B4	688	[b]	[b]

[a] Formula No. 2, No. 0 capsules. [b] Excessive machine binding, capsules not filled.

The utility of the model as a capsule formulation aid is to be found in the use of Eq. 1. From an evaluation of specific volume and flow properties of a small sample of the powder to be encapsulated, the equation can be used to predict fill weights at various machine speed and capsule size combinations. In a similar manner, the capsule size necessary for the encapsulation of a powder can be determined from the equation without the necessity of a trial. Further, the equation reveals that the alteration of specific volume is necessary if the desired capsule fill weight cannot be obtained from capsule size and machine speed combinations.

SUMMARY

A model has been developed which defines and quantitates the factors affecting the encapsulation of powders in hard gelatin capsules using semiautomatic capsule filling equipment. The model reveals that the mean capsule fill weight is dependent on specific volume, flowability, machine speed, and capsule size. The weight variation as estimated by the standard deviation is a function of machine speed, capsule size, specific volume, flowability, and the presence of a glidant. The coefficient of variation as an estimate of the weight variation is a function of machine speed, specific volume, flowability, and the presence of a glidant but independent of capsule size. Particle size considerations of moderately coarse particles (USP designation) are not directly a part of the model because they are reflected in other measured particulate properties.

The model expresses through equations and contour charts useful information for capsule formulation development. For example, from the contour charts it may be seen that the particulate property influencing fill weight to a major extent at a fixed capsule size and machine speed is specific volume. The interaction of specific volume and powder flowability produces an area where weight variation is at a minimum. The mean capsule fill weights that may be expected for any given powder blend may be calculated with an accuracy often exceeding 95% from simple capsule size, machine speed, and particulate property evaluations on small samples of formulation.

Savings in time and material needed to prepare and evaluate trial formulations are a direct result of the use of this model.

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Keyphrases

Gelatin capsules, hard—powder encapsulation
Powders for encapsulation—physical properties
Capsule fill weight—factors affecting
Weight variation, capsules—factors affecting
Model, mathematical—variables *vs.* capsule characteristics

Powder Flow Studies III

Factors Affecting the Flow of Lactose Granules

By G. GOLD, R. N. DUVALL, B. T. PALERMO, and J. G. SLATER

In an attempt to provide a better understanding of the variables affecting the flow of tablet granulations, the effects of different granulating agents and techniques, particle size distribution, fines, and glidants on the flow of lactose granules were studied. Flow rates were measured with a recording powder flowometer. In binary mixtures the addition of increasing proportion of finer particles to 10/20 mesh granules increased the flow rate to a maximum followed by a decrease. Flow rates of granulations were found to vary considerably, depending upon the particular granulating agent used. For granulations and blended systems of various particle size distributions, a strong negative correlation existed between flow rate of granules and proportion of particles less than 100 mesh in size. The particle size of the granules influenced considerably the effect of glidants.

AN IMPORTANT requirement of tablet formulations is that the powder or granular material possess uniform and free flowing properties. The ability of particulate matter to flow into the dies in a reproducible manner results in uniform tablet weights and uniform doses of active ingredients, as well as increased production rates (1). Using an instrumented rotary tablet machine, Knoechel *et al.* (2) reported that when good flowing formulations were tableted, compression and ejection force levels became more uniform and weight deviations decreased. To achieve better flowability and desirable compression characteristics, it is often necessary to use a granulating technique in the manufacturing procedure. The most common granulating method is wet granulation which involves the moistening of a powder mixture with a granulating solution containing a binder to form a plasticized mass. The mass is then forced through a sieve, dried, and resieved. The finished granulation is a mixture of various size particles and contains some finely powdered material often referred to by industrial pharmacists as "fines." Some finer particles are necessary in limited amounts for producing smoother tablets and for proper functioning of the tablet press. While 10–20% fines has occasionally been set as the maximum amount permissible, it has been pointed out that there is no general limit to the amount of fines that can be present in a granulation and that excessive fines should be determined for each specific formulation (3). Usage of the term fines is rather widespread, yet there is no general agreement as to what mesh size constitutes fines and much confusion exists in the literature due to varying usage of this term.

Hammerness and Thompson (4) observed that the flow rate, ascertained by timed delivery through an orifice, of a tablet granulation increased with added fines to an optimum flow rate, after which additional fines caused the flow rate to decrease. However, fines in this study were particles smaller than 40 mesh. An increase in the amount of lubricant also increased the flow rate, and the combination of fines and lubricant appeared to have a synergistic action. Crosby (5) reported that surface roughness may be reduced by the presence of fines which serve to fill in the depressions or irregularities in the surface of the particles and, thereby, increase the rate of flow. Nelson (6), using angle of repose measurements, found that the addition of fines smaller than 100 mesh to sulfathiazole granules caused an increase in repose angle and concluded that fines increase interparticle friction and are responsible for difficulty in granulation flow. According to Neumann (7) stickiness is produced by the presence of particles smaller than 10 μ. When these "fines" are removed or adsorbed onto larger particles, the powder becomes free flowing.

In many literature reports (8–10) dealing with investigations concerning flow of solid materials, angle of repose measurement was used with assumed correlation to flow. A recent report including a statistical evaluation from this laboratory (11) indicated that angle of repose measurement was not a reliable method to evaluate flow of materials. In view of this and the lack of reports in the literature dealing with factors affecting the flow of tablet granulations, the present work was undertaken in an effort to evaluate the variables

Received April 19, 1967, from the Pharmaceutical Research and Development Laboratory, Consumer Products Division, Miles Laboratories, Inc., Elkart, IN 46514

Accepted for publication December 4, 1967.

Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

The authors thank Mr. R. L. Hurtle for designing and constructing the instrument used in this study.

TABLE I—EFFECT OF GRANULATING AGENTS AND TECHNIQUES ON FLOW RATE

Formulation	Granulating Solution	Method[a]	Flow Rate (Gm./sec.) Complete Formulation	Flow Rate (Gm./sec.) 10/20 Mesh Fraction	Free H_2O %	Mesh Analysis % 10/60	Mesh Analysis % 60/100	Mesh Analysis % Concn. <100
1	10% Starch	A	4.82	6.95	0.30	31	10	59
2	10% Starch	B	5.59	7.69	0.05	36	17	47
3	10% Starch	C	5.72	7.45	0.03	36	15	49
4	Water	A	6.13	7.66	0.30	34	17	49
5	2% Methylcellulose 15 cps.	A	6.83	7.70	0.00	44	9	47
6	10% PVP	A	7.22	7.32	0.10	45	26	29
7	10% Gelatin	A	8.55	7.18	0.07	50	20	30
8	10% Methylcellulose 15 cps.	A	8.90	8.40	0.24	45	16	39
9	Water	D	9.24	9.22	0.23	77	6	17
10	2% Methylcellulose 15 cps.	D	9.71	8.18	0.13	84	4	12
11	Water	E	9.92	8.03	0.30	72	16	12

[a] A, Standardized method as indicated in *Experimental* using 1,000 Gm. granulating solution. B, Starch added to lactose as dry powder and 1,000 Gm. water added. C, Starch added half as dry powder and remainder as 1,000 Gm. of 5% starch paste. D, Additional 2,000 Gm. of water added. E, Additional 1,000 Gm. of water added.

affecting the flow of granules. Lactose granules were used as the model system and the effects of finer particles were ascertained by adding various size particles to 10/20 mesh granules. The effects of different granulating agents and techniques on the flow rate of the resultant granules were also determined. A final objective was to study the effects of commonly used glidants on the flow of lactose granules of varying particle size distribution to ascertain similarities or differences in effects between glidants and granule fines.

EXPERIMENTAL

Materials—Methylcellulose,[1] cornstarch, polyvinylpyrrolidone,[2] gelatin, magnesium stearate, talc, and fumed silicon dioxide[3] were either USP or pharmaceutical grade materials. Glidants were used as supplied by the manufacturers and were finer than 100 mesh. However, cornstarch, when used as a glidant, was dried at 140° F. for 24 hr.

Lactose granulations were prepared in 15 Kg. batches by the following standard procedure: USP lactose powder was passed through a No. 12 screen on an oscillating granulator and placed in a 1.8 cubic foot total capacity Stokes' mixer. The granulating solution, at room temperature, was added in three equal volumes at 2-min. intervals until 1,000 ml. was added. The wet mass was mixed for 20 min., wet sieved through a No. 6 screen on an oscillating granulator, dried at 120° F. for approximately 20 hr., and dry sieved through a No. 12 screen. Moisture content of the granules was determined by the Karl Fischer method and all granules were found to contain moisture corresponding to 1 mole of water of hydration (5.00%) for lactose and 0.3% or less free moisture.

Particle Size Separation and Analysis—Separation of granules into different particle size ranges was accomplished by a Ro-Tap testing sieve shaker with U. S. standard sieves in series 20, 40, 60, 80, 100, and 200 mesh sizes. For particle size analysis of granulations, a 100-Gm. sample was placed in the shaker for 5 min.

Flow Rate Determinations—The Recording Powder Flowometer described in a previous report (12) was modified to contain four strain gauges and a built-in transistorized power supply. The potential across the bridge circuit was adjusted so that each horizontal chart division represented 6.25 Gm. of powder. Flow rates, expressed in Gm./sec., were calculated from the recorder tracing resulting from the flow of 300 Gm. of material passing through a conical stainless steel hopper which measured 20 cm. top diameter, 30 cm. in length, and 10 mm. orifice diameter.

[1] Marketed as Methocel 15 cps. by the Dow Chemical Co., Midland, Mich.
[2] Marketed as Plasdone by the General Aniline and Film Corp., New York, N. Y.
[3] Marketed as Cab-O-Sil by the Cabot Corp., Boston, Mass.

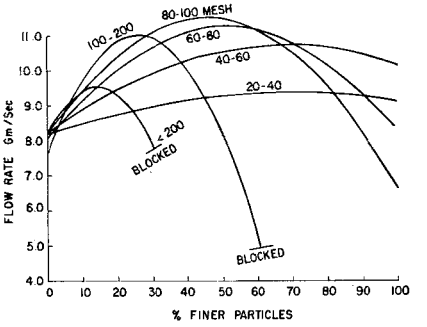

Fig. 1—The effect of finer particles on the flow rate of 10/20 mesh lactose granules.

RESULTS AND DISCUSSION

A 2% methylcellulose granulation (formulation 10—Table I) was sieved into 7 fractions and the effect of finer particles added to 10/20 mesh granules was determined in 5% increments up to 50% and 10% increments thereafter. The flow rates of each system, Fig. 1, are shown in a multiple regression plane (13). From Fig. 1, it will be noted that, as the proportion of the smaller particle sizes increased, the flow rate of 10/20 mesh granules attained a maximum and then decreased. This maximum flow rate was specific for each binary component system and varied with the concentration and size of the added particles. The data indicate that the smaller the particle size, proportionately less was necessary to reach the maximum flow rate. Also, the maximum flow rates did not reflect the flow rates of the individual sieve fractions but did indicate synergistic effect from their combination. The effect of 100/200 and <200 mesh particles was rather surprising, especially in

view of the fact that neither would flow through the funnel orifice used in the study. High concentrations of the 100/200 and <200 sieve fractions did adversely affect flow as concentrations exceeding 30% for <200 and 60% for 100/200 mesh stopped the flow of powder. Jones and Pilpel (14) in evaluating the flow of binary systems of granular magnesia also noted that the percentage of fine component required to produce the maximum flow rate, increases with the size of the fine component. With granular magnesia, however, increased concentrations of 300/350 mesh granules did not block the flow of either 22/36 or 52/72 mesh granules through an 8.98 mm. orifice.

Few guidelines are available for developing tablet granulations having good flow characteristics. Experiments were, therefore, devised to determine the effects of the granulating process on the flow rate. Table I lists the granulating agents and techniques that were evaluated. By using the 1,000 Gm. aqueous granulating solution as the standard for comparing the effectiveness of the various binders and comparing flow rates for significant differences by the method of analysis of variance (15), it may be concluded that the flow rate decreased with the use of 10% starch paste and increased with 2% methylcellulose, 10% PVP, 10% gelatin, and 10% methylcellulose. A significant increase resulted when the concentration of methylcellulose increased from 2–10%. Maximum flow rates were obtained by increasing the quantity of water. However, free moisture content of the resulting granules indicated no relationship between flow rate and free moisture. Flow rates of the 10/20 mesh sieve fractions are also indicated in Table I and suggest that it is risky to characterize the flow properties of a granulation with a separated sieve fraction. It is evident from the mesh analysis data in Table I that increased flow rates are associated with higher concentrations of 10/60 mesh and lower concentrations of <100 mesh particles. The coefficient of correlation (16) has been used to statistically measure the extent of the linear relationship between flow rates and 10/60, 60/100, and <100 sieve sizes. A correlation coefficient close to 0 indicates a weak or nonexistent relationship; whereas, a value close to +1 or −1 indicates a strong relationship between the two variables. The percent dependence (17), a measure of the percentage of the variation in flow rate related to variation in concentration of mesh sizes, has also been calculated. These statistics (Table II) indicate a fairly strong positive correlation between flow rate and proportion of 10/60 mesh particles ($r = +0.88$). A weak or nonexistent correlation exists for the 60/100 size (−0.20) and a strong negative correlation for <100 mesh granules (−0.92). Increasing the degree of wetness was effective in reducing the concentration of <100 mesh particles and, consequently, produced a faster flowing granulation.

To study the effects of particle size distribution, 5 formulations were prepared with varying concentrations of the 7 sieve fractions used in the binary system study. Table III gives the composition of each formulation and its corresponding flow rate. It will be noted that the flow rates ranged from a low of 4.07 Gm./sec. to a high of 10.19 Gm./sec. A high negative correlation coefficient (Table II) was obtained between flow rate and concentration of particles smaller than 100 mesh (−0.99). The 10/60 mesh fraction had a lower positive correlation coefficient of +0.77 while 60/100 particles had a weak correlation (+0.24). The data obtained from this experiment indicate that the proportion of particles smaller than 100 mesh is the main factor influencing the flow rate.

It was noted in binary mixtures that a linear relationship existed on each side of the composition of maximum flow; whereas, the relationship was nonlinear over the entire range. A negative linear relationship between flow rate and proportion of particles less than 100 mesh was obtained in the concentration range of 12–59% for granulations and 13–44% for blended systems. This negative linear relationship is, therefore, apparently representative of mixtures to one side of the composition of maximum flow.

Various materials commonly referred to as glidants are fine particles. As such, the effects of cornstarch, magnesium stearate, fumed silicon dioxide, and

TABLE II—CORRELATION COEFFICIENTS AND PERCENT DEPENDENCE VALUES SHOWING RELATIONSHIP BETWEEN FLOW RATES AND SIEVE SIZES

	Granulations[a]		Blended Granules[b]	
Sieve Size	Correlation Coefficient	% Dependence	Correlation Coefficient	% Dependence
10/60	+0.88	77.33	+0.77	58.65
60/100	−0.20	3.52	+0.24	5.64
<100	−0.92	83.48	−0.99	96.81

[a] Eleven formulations prepared using different granulating solutions and techniques. [b] Five formulations blended from different sieve fractions of 2% methylcellulose granules.

TABLE III—EFFECT OF PARTICLE SIZE DISTRIBUTION ON THE FLOW RATE OF LACTOSE GRANULES

	Formulations				
	A	B	C	D	E
10/20	14.3%	5.0%	23.6%	23.6%	5.0%
20/40	14.3	8.1	20.5	15.0	13.5
40/60	14.3	11.2	17.4	8.9	19.7
60/80	14.3	14.3	14.3	5.0	23.6
80/100	14.3	17.4	11.2	8.9	19.7
100/200	14.3	20.5	8.1	15.0	13.5
<200	14.3	23.6	5.0	23.6	5.0
Flow rate (Gm./sec.)	7.54	4.07	10.19	5.62	8.58

talc were also studied for comparative purposes. These glidants were added to the following lactose granule fractions: 10/60 mesh; 50% 10/60, 50% 60/100; and 50% 10/60, 25% 60/100, and 25% <100. To compare flow rates, a flow factor "f" (18) which is the ratio of the flow rate of discharged granulate and glidant to the flow rate of the discharged granulate without glidant was calculated. A "f" value greater than 1.0 indicates increased flow; whereas, a value below 1.0 indicates decreased flow. It can be seen from Figs. 2–6 that the effectiveness of added particles is dependent upon the particulate size of the granules to which they are added. For example, lactose fines (Fig. 2) and cornstarch (Fig. 3) were effective when added to 10/60 mesh granules, but their effectiveness

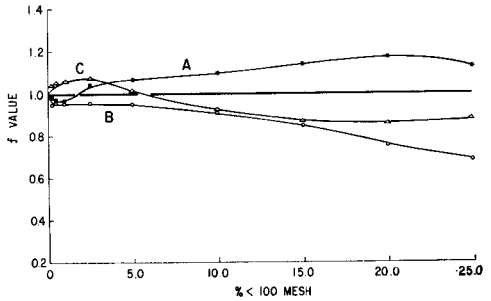

Fig. 2—The effect of <100 mesh particles of the same material on the flow rate of lactose granules. Key: A, 10/60 mesh granules; B, 50% 10/60, 50% 60/100; C, 50% 10/60, 25% 60/100, 25% <100.

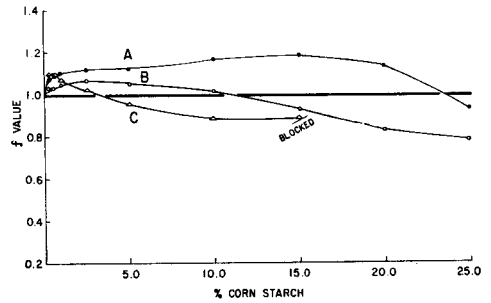

Fig. 3—The effect of cornstarch on the flow rate of lactose granules. Key: A, 10/60 mesh granules; B, 50% 10/60, 50% 60/100; C, 50% 10/60, 25% 60/100, 25% <100.

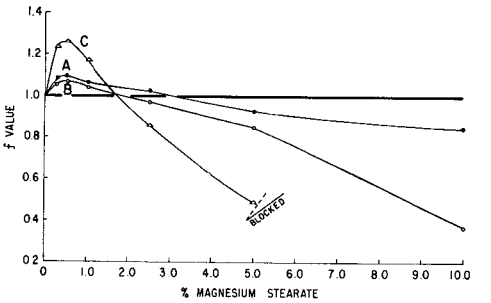

Fig. 4—The effect of magnesium stearate on the flow rate of lactose granules. Key: A, 10/60 mesh granules; B, 50% 10/60, 50% 60/100; C, 50% 10/60, 25% 60/100, 25% <100.

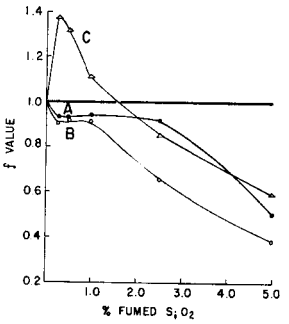

Fig. 5—The effect of fumed silicon dioxide on the flow rate of lactose granules. Key: A, 10/60 mesh granules; B, 50% 10/60, 50% 60/100; C, 50% 10/60, 25% 60/100, 25% <100.

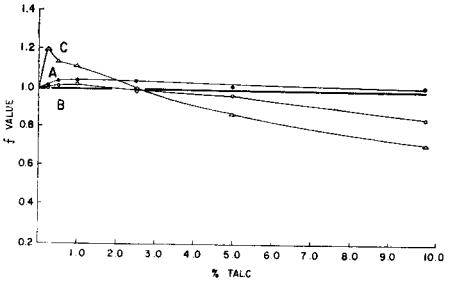

Fig. 6—The effect of talc on the flow rate of lactose granules. Key: A, 10/60 mesh granules; B, 50% 10/60, 50% 60/100; C, 50% 10/60, 25% 60/100, 25% <100.

decreased markedly when added to the other 2 systems which already contained finer particles. Both the fine fraction of the same material and cornstarch appeared to act in a similar manner. In contrast, the optimum concentrations of fumed silicon dioxide, talc, and magnesium stearate were 0.5% or less. All three were more effective in the mixture containing 25% of <100 mesh particles suggesting that these materials act by a different mechanism than fines or cornstarch which were more effective with coarse particles. Both fumed silicon dioxide and magnesium stearate caused sharp decreases in flow rate when used above their optimum concentration.

Jones and Pilpel (14) have classified glidants into two categories: (*a*) those reducing van der Waal's type interparticle cohesive forces and (*b*) those reducing surface rugosity and hence coefficient of interparticle friction. Another possible mechanism is that glidants act by filling void spaces between particles. It appears that fines and cornstarch which were more effective with coarse particles acted either by filling surface irregularities or void spaces; whereas, silicon dioxide, talc, and magnesium stearate possibly acted by reducing interparticle cohesive forces since they were more effective with finer particles and interparticle attraction forces of the van der Waal's type increases as particle size is reduced (19).

SUMMARY

(*a*) In binary mixtures the addition of finer particles increased the flow rate of 10/20 mesh

granules to a maximum, following which a decrease was obtained upon addition of more finer particles. The maximum flow rate was specific for each binary component system and varied with the concentration and size of the added particles.

(*b*) Flow rates of granulations were found to vary considerably, depending upon the degree of wetness and upon the granulating agent used. Granulating agents in order of increasing effectiveness were: 10% starch, 2% methylcellulose, 10% PVP, 10% gelatin, and 10% methylcellulose.

(*c*) Faster flow rates of granulations and blended systems of various particle size distributions were associated with an increase in proportion of 10/60 mesh granules and a decrease in proportion of <100 mesh granules.

(*d*) Effectiveness of glidants was found to be dependent upon particle size of the granules to which they were added. Cornstarch and <100 mesh particles of the same material were found to be more effective for coarse particles; whereas, fumed silicon dioxide, talc, and magnesium stearate were more effective with finer particles.

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Keyphrases

Granule flow—factors affecting
Lactose granules—wet granulation
Flow rate—granulating process effect
Particle size—flow rate effect
Glidant—flow rate, granules

Rheologic Studies on Dermatologic Lotions

By IVO SETNIKAR, GIUSEPPE GÁL, and SERGIO FANTELLI

The rheologic properties of some dermatologic lotions were studied. The changes in the viscosity, thixotropy, and yield value of the lotions during storage and following various degrees of agitation were recorded. The relevance of these phenomena to production processes and to therapeutic use are discussed. The range of optimum viscosity values of dermatologic lotions is indicated.

DERMATOLOGIC LOTIONS are milky, fluid pharmaceutical preparations consisting of a disperse phase of liquid or solid particles in a predominantly aqueous phase.

The rheologic properties of lotions are generally of non-Newtonian type. Indeed, their viscosity often depends upon the shear rate and may be of the dilatant, pseudoplastic, plastic, or Bingham type. They may even exhibit an isothermic reversible decrease in viscosity after an imposed shear rate (thixotropy) or an increase in viscosity (rheopexy).

It is important to know the rheologic properties of a lotion, and the changes they undergo during preparation and during storage in order to adapt the manufacturing operations to these properties and ensure that the lotion possesses the most suitable rheologic properties when it is used therapeutically; few studies on these lines have been published. Woodman and Marsden (1) described the thixotropic properties of a lotion they had prepared. They used "Rheomat" and "Rotovisco" viscosimeters, which involve decanting the lotion into the viscosimeter vessel to measure the viscosity, an operation that alters the rheologic status of a sample with pronounced thixotropic or rheopexic properties.

The ideal instrument for defining the rheologic properties of lotions should (*a*) permit measurement at various shear rates, and (*b*) not require decanting, so that the viscosity of a lotion is not altered before measurement. The latter point is

Received June 13, 1967, from the Research Division, Recordati s.a.s., Milano, Italy.
Accepted for publication October 12, 1967.

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especially important when checking after an interval of time, for the viscosity of a lotion is liable to undergo considerable change during storage. Not even the thixotropic effect can be determined correctly if the lotion has been subjected to shear, *e.g.*, as with decanting. Of the various types of instruments, reviewed by Bottoni (2), neither capillary viscosimeters (Ostwald, Ubbeldholde) nor falling (Hoeppler) or ascending sphere viscosimeters (Cochins) comply with either of the requisites specified above. Brebender, MacMichael, or Ferranti-Shirley viscosimeters permit the study of viscosity in very precisely defined geometric and dynamic conditions, but they require decanting of the liquid and so do not comply with requirement *b*. Brookfield's viscosimeter does not allow the strict definition of geometric and dynamic conditions and so yields conventional viscosity values; on the other hand, it does not require decanting of the liquid, and so it complies with condition *b*.

The authors used Brookfield's viscosimeter for their investigations, as it was felt that it was more important to define thixotropy or rheopexy and changes in viscosity after a long storage than it was to obtain viscosity values that are only formally more accurate using other viscosimeters.

EXPERIMENTAL

Rheologic Determinations—The Brookfield RVF viscosimeter was used, calibrated with Brookfield L-3 standard fluid (10 poise at 25°).

The determinations were made at 24° on 600 ml. of lotion in containers with a diameter of 9 cm. To alter the physical status of the sample as little as possible, the spindle was introduced edgeways and very gently into the sample, thus ensuring the minimal shear and the absence of air bubbles on the undersurface of the spindle. The spindle was then screwed on to the apparatus and readings were taken, starting at the lowest speed (2 r.p.m.). The maximum shear stress value shown on the instrument was recorded and readings were taken in the same way at 4, 10, and 20 r. p. m. ("up" curve). Immediately after the sample was subjected in the same container, for 3 min., to the action of a stirrer with two blades (each of 45 × 25 mm.) spinning at 100 r.p.m. The viscosimeter readings were then taken again at decreasing spindle speeds, from 20 to 2 r.p.m. ("down" curve).

The "initial yield value," that is, the maximum stress value required before shear starts, was determined by extrapolating the rheograms up to the intersection with the abscissa (shear rate = 0).

Composition and Method of Preparing the Lotions—The compositions are specified in Table I. Lotions 1 and 2 were prepared by melting at 70° and mixing stearic acid, esters of saturated fatty acids, sorbitan monopalmitate, and polyoxyethylene sorbitan monolaurate and dissolving the propyl-*p*-oxybenzoate in this mixture. The deionized water was heated in another container to 70° and the methyl-*p*-oxybenzoate was dissolved therein. The oily phase was poured into the aqueous phase and this mixture was passed through an ALM-O homogenizer (Auguste and Moutis, Paris). The lotion was cooled while stirring and, at 30°, the propylene glycol was added.

TABLE I—COMPOSITION OF LOTION BASES

Constituents	Gm./100 ml. Lotion 1	Lotion 2	Lotion 3
Stearic acid	1.00	1.00	...
Esters of saturated fatty acids	6.00	6.00	...
Sorbitan monopalmitate	4.50	6.00	...
Polyoxyethylene sorbitan monolaurate	2.00	2.00	...
Cetostearyl alcohol	...	...	3.00
Cetylpolyglycol ether	...	...	0.60
Propylene glycol	5.00	5.00	5.00
Methyl-*p*-oxybenzoate	0.15	0.15	0.15
Propyl-*p*-oxybenzoate	0.03	0.03	0.03
Deionized water *q.s.* to:	100 ml.	100 ml.	100 ml.

"Farmomac" Metering-Bottling Machine—Some samples of the lotions were put through the metering-bottling machine used in manufacturing, with a view of studying the effects of this machine on the rheologic properties of the lotions. The metering-bottling machine was the "Farmomac F 37," made by Messrs. Farmomac of Bologna. Basically it is a syringe by which a certain volume of lotion is sucked, and through an inlet-outlet valve is injected into the final containers. Suction and filling, advancement, and container closure is automatic and may be performed under sterile conditions. The measured volume can be regulated between 0 and 100 ml. and the advancement speed up to 4,500 bottles/hr. From the hydrodynamic point of view the maximum shear of the liquid is reached in the valve and in the bottling needle, with shear rates depending on the delivered volume and on the advancement speed. Under these working conditions the maximum shear rate was approximately 2000 sec.$^{-1}$ with a duration of 0.5 sec.

RESULTS

Influence of the Number of Revolutions of the Spindle on the Viscosity of the Sample—When the viscosimeter is set in motion the pointer quickly marks the maximum value and then begins to fall back because of the thixotropy of the lotion (Fig. 1). The maximum viscosity value found is thus the closest value to the true viscosity of the sample before it is affected by thixotropy. The viscosity readings were therefore always compared to this maximum value obtained by noting the position of the pointer during the first revolutions of the spindle and stopping the viscosimeter when the pointer reached the highest position on the dial (usually at the third revolution).

Rheologic Properties of the Lotions Being Tested—The consistency curves before and after stirring

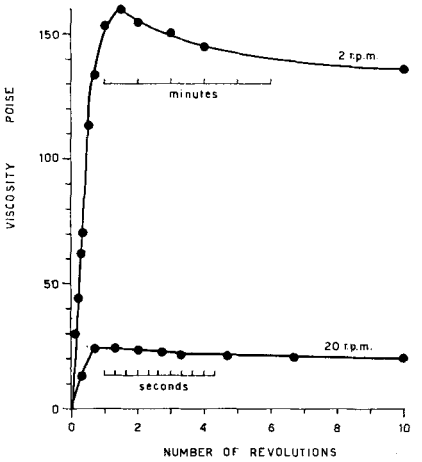

Fig. 1—Changes of viscosity during measurement. Spindle No. 2, 24°, at 2 r.p.m. (approx. 0.6 sec.$^{-1}$ shear rate) and at 20 r.p.m. (approx. 6 sec.$^{-1}$ shear rate). Viscosity appears related to the number of revolutions of the spindle.

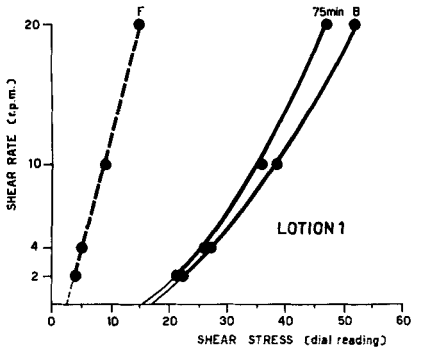

Fig. 2—Lotion 1, spindle No. 2, 24°. Rheogram before (B) and after 75 min. (75) stirring. The dash-line curve (F) is the rheogram after the run through the Farmomac. B is the up curve, the others are down curves.

are given in Figs. 2–4. The viscosity tends to decrease as the shear rate increases and the consistency curve extrapolates to a defined stress value (yield value). The behavior is characteristic of a Bingham-type non-Newtonian liquid.

Stirring diminishes the viscosity and the yield value. The lotions thus show evidence of thixotropy.

Certain types of agitation, such as occur in the Farmomac, may on occasion produce a viscosity which is higher than the lowest viscosity reached by "gentle" stirring.

Dependence of Thixotropy Upon the Type and Duration of Agitation—Figures 2–4 show that the degree of thixotropy depends upon the intensity (*cf.* the F curves) and upon the duration of shear. The two factors must therefore be distinguished and accurately defined when analyzing the thixotropic phenomena. This distinction was drawn by Green and Weltmann (3), who quantified the thixotropic breakdown "by time" and "by velocity."

With traditional methods the thixotropic breakdown measurement is based on the shear imposed by the viscosimeter itself (*cf.* 4) and evaluated by the difference between the "up" and the "down" curve.

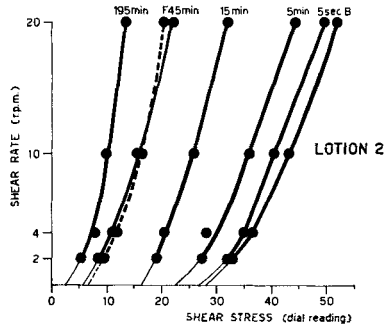

Fig. 3—Lotion 2, spindle No. 2, 24°. Rheogram before (B) and after different times of stirring. The total time of stirring to which the sample was subjected is shown. The dash-line curve (F) is the rheogram of the lotion after the run through the Farmomac. B is the up curve, the others are down curves.

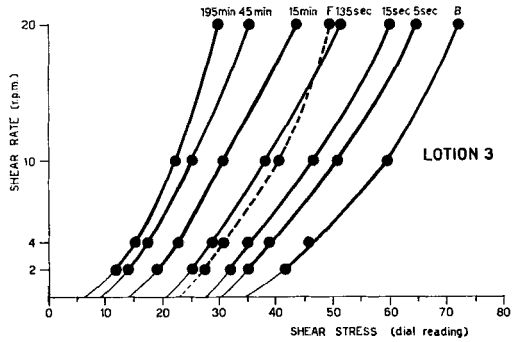

Fig. 4—Lotion 3, spindle No. 2, 24°. Rheogram before (B) and after different times of stirring. The total time of stirring to which the sample was subjected is shown. The dash-line curve (F) is the rheogram of the lotion after the run through the Farmomac. B is the up curve, the others are down curves.

The total shear thus gradually increases from beginning to end of the determination and is not constant in intensity. The result is a loop expressing a superposition of time and intensity of shear that is difficult to analyze.

If a defined quantity of shear is interposed between the consistency measurements, distinct and nearly parallel curves are obtained instead of loops (Figs. 2–4). The distance between the initial (up) curve and the curves after shears of different duration (down curves) expresses thixotropy by time.

The curves obtained differ from those worked out by Green and Weltmann (3) for other types of fluids, because there is also a decrease in yield value [thixotropic coefficient of Goodeve (5)]. Perhaps the difference is due partly to our method, which results in determinations that are less biased by initial thixotropic phenomena, since the operations preceding the determination are less destructive on the structure of the samples.

Effects of the Composition of the Lotion—An alteration of the quantities of the constituents obviously alters the rheologic properties of the lotion, including the thixotropic phenomena. This is illustrated by Figs. 2 and 3, referring to lotions with a different quantitative composition, and by Fig. 4, referring to a lotion with a different qualitative composition.

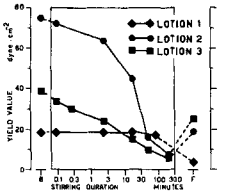

Fig. 5—Yield values at 24° observed for 3 lotions before (B) stirring, after stirring for various periods, and after passage through the Farmomac (F). The values were obtained by extrapolating on the abscissa the consistency curves of Figs. 2–4.

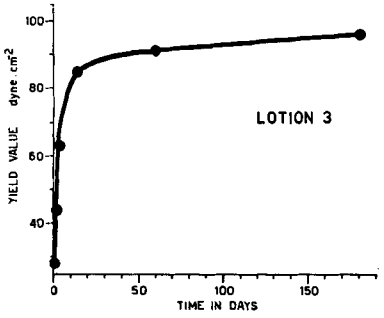

Fig. 6—Lotion 3. Yield values at 24° observed after storage for various periods. This batch of lotion 3 differs from that of Fig. 4.

In Fig. 2 the thixotropy, as a result of 75 min. stirring, is less evident than in the case of Fig. 3. Passage through the bottling machine considerably lowers the viscosity. In Figs. 3 and 4 the thixotropy is marked and the run through the bottling machine determines, in contrast to what happens in Fig. 2, a viscosity intermediate between the initial viscosity and the minimum obtainable with gentle stirring. This effect could be due to a change in particle size or shape or to rheopexic phenomena.

Yield Value—The yield value estimates obtained by means of the Brookfield viscosimeter are represented by a torque and must be referred to the spindle used. To obtain the yield value in dynes·cm.$^{-2}$, the following formula may be used:

$$Y = \frac{m}{2\pi r^2 \cdot r \sqrt[3]{1/2}} = \frac{m}{4{,}986 \cdot r^3} \quad \text{(Eq. 1)}$$

where Y = yield value in dynes·cm.$^{-2}$; m = torque measured in dynes·cm., referred to the intercept of the consistency curves with the shear stress axis; and r = radius of the spindle in centimeters.

Figures 2–4 show that the lotions considered always exhibit a yield value greater than zero.

The yield value changes as a result of stirring. Figure 5 shows the decreases in yield value for the three lotions after different periods of stirring and after passing through the Farmomac.

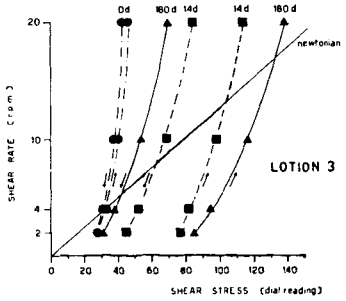

Fig. 7—Lotion 3 (different batch from that of Fig. 4). Consistency curves at 24°. The initial (0 d) up and down curves and the up and down curves after 14 days (14 d) and after 180 days (180 d) are shown. The straight line represents the consistency curve of a Newtonian liquid. As a rule, spindle No. 2 was used. For the 14-day and 180-day samples, spindle No. 3 was used as well. The values were then referred to spindle No. 2 by extending the scale of the abscissas beyond the value 100.

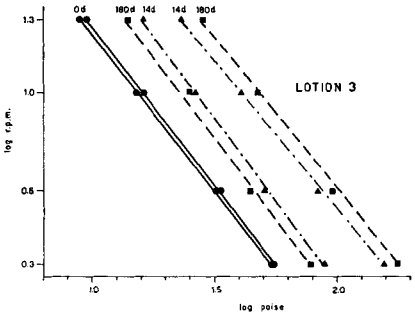

Fig. 8—Results of Fig. 7 plotted on a diagram in which the ordinate is log r.p.m.; the abscissa log poise. By this means the experimental points are on a straight line.

The yield value of a lotion may increase during storage. An example of this is shown in Fig. 6 which refers to lotion 3.

Changes in Viscosity and Thixotropy During Storage—Five samples of lotion 3 were stored in sealed containers at 24° and not moved until viscosimetry. The viscosity and thixotropy values are given in Table II and in Fig. 7.

The viscosity increases considerably with time, *i.e.*, about threefold after 6 months' storage. The thixotropy increases also: evaluated as average percentile decrease of viscosity after standardized stirring it is about 6% in the freshly prepared lotion and rises to 55% after 6 months' storage.

The Fig. 7 viscosity data are drawn in Fig. 8 on a diagram with poise and r.p.m. values in logarithmic

TABLE II—VISCOSITY OF LOTION 3 AFTER MOTIONLESS STORAGE AT 24° FOR VARIOUS INTERVALS OF TIME

r.p.m. of Spindle	Viscosity in Poise: Initial B[a]	Initial A[b]	After 1 day B	After 1 day A	After 3 days B	After 3 days A	After 14 days B	After 14 days A	After 60 days B	After 60 days A	After 180 days B	After 180 days A
2	55	55	83	65	118	76	155	88	165	90	176	78
4	33	31.5	47	37.5	66	44	82.5	50	90	52.5	92.5	44
10	16	15	22.8	18.2	30.8	22	40	26	45	28	47	22
20	9.4	8.8	13.2	11	18	13.6	23	16	27.6	17.5	28	14

[a] Columns B show the values before stirring. [b] Columns A show the values recorded after stirring at 100 r.p.m. for 3 min.

TABLE III—VISCOSITY IN POISE DETERMINED AT 2 r.p.m. AND AT 24° ON FOUR BATCHES OF LOTION 1 OBSERVED AFTER SEVERAL STORAGE PERIODS

No. Days After Preparation	Batch 1		Batch 2		Batch 3		Batch 4	
	N[a]	F[b]	N	F	N	F	N	F
0	23	2.5	19	3.3	15.8	3.8	20.3	2.8
7	39	1.8	32	3				
17	45	1.8	37	3				
35	45	1.8	39	2.5	30.5	5	42	2
56	47	1.7	52	3.5				
75	50	2.8	53	4.3				

[a] Columns N give the values observed on samples not run through the Farmomac. [b] Columns F give the values for the samples run through the Farmomac on the day of preparation.

TABLE IV—VISCOSITY IN POISE RECORDED AT 2 r.p.m. AND AT 24° ON TWO BATCHES OF LOTION 1 IMMEDIATELY AFTER PREPARATION AND AFTER 75 DAYS STORAGE

	Initial			After 75 Days Storage					
				Not Run Through Farmomac			Run Through Farmomac		
	B[a]	A[a]	Δ[b]	B	A	Δ	B	A	Δ
Batch									
1	23	22	1	50	34	16	2.8	3.3	−0.5
2	19	17	2	53	33	20	4.8	4.5	−0.3

[a] Columns B and A show the values before (B) and after (A) agitation at 100 r.p.m. for 3 min. [b] Columns Δ give the difference between A and B, an index of thixotropy.

scales. By this means the experimental points can be aligned in straight lines and described empirically by

$$\ln v = -A \ln s + B \qquad \text{(Eq. 2)}$$

where v = viscosity; s = shear rate; and A and B = two constants.

By imposing a defined quantity of shear a different line is obtained, in which constant A of Eq. 2 does not change and in which constant B decreases. In these conditions the decrease of B quantitizes empirically the thixotropy.

These conclusions do not agree formally with the measurement of thixotropy by coefficient M of Schulte and Kassem (4). It should be noted, however, that in coefficient M thixotropic breakdown by time is superposed on thioxotropic breakdown by shear, whereas our method permits a separate measurement of thixotropic breakdown by shear.

Effects of Initial Agitation on Changes in Viscosity and Thixotropy Occurring Through Time: Role of the Metering-Bottling Machine—A given quantity of lotion was passed through the Farmomac, an operation equivalent to violent agitation. The changes in viscosity during storage of the lotions thus treated were measured and compared with the changes in viscosity of samples of the same lotion not passed through the Farmomac.

Table III shows the results observed on 4 batches of lotion 1. The run through the Farmomac resulted in a considerable decrease of viscosity. Further, during storage, in the samples not passed through the Farmomac viscosity increased greatly (up to 2.5-fold in 75 days) along with thixotropy (up to 8-fold in 75 days, *cf.* Table IV). Both phenomena did not occur in the samples run through the Farmomac. Thus, as a result of the run through this machine an irreversible change in the rheologic properties of the lotion occurred.

Since the implications of these phenomena for lotion manufacture are important, the authors studied one of them. Observations on the performance of the Farmomac showed that the maximum shear rate occurs at the level of the bottling needles, where it may be estimated as high as 2000 sec.$^{-1}$. Viscosity changes due to the bottling process on some lotions using bottling needles with bores of 6 and 3 mm. were therefore studied. The former yielded shear rates of up to 120 sec.$^{-1}$ and the latter up to 2000 sec.$^{-1}$. Table V shows the viscosity values observed for two lotions before and after passage through the Farmomac in these conditions. As suspected, the decrease under viscosity was greater in the samples run through the smaller bore.

TABLE V—VISCOSITY IN POISE MEASURED AT 2 r.p.m. AND AT 24° ON TWO BATCHES OF LOTION 1 BEFORE AND AFTER THE RUN THROUGH THE FARMOMAC FITTED WITH BOTTLING NEEDLES OF DIFFERENT BORE

Batch	Initial	6-mm. Needles	3-mm. Needles
1	23	4	2.5
2	19	4.8	3.3

DISCUSSION

We have already discussed the ways in which thixotropy recorded by the method used in this study differs from that recorded by traditional methods, such as those used by several authors (6–9). Briefly, the thixotropic breakdown obtained by traditional methods depends on the relatively small shear imposed by the measuring instrument itself and is represented by a loop, formed by the up and down curves, in which thixotropy by time and thixotropy by velocity are mingled. The dimensions and shape of the loop depend upon instrumental factors and on the speeds at which the measurements are taken. The very shape of a loop is solely due to the fact that the apical closing point of the loop is the only one to be unaffected, at a given shear rate level by differences in shearing quantity and duration.

Instead of determining immediately the down curve, the authors interposed a set quantity of shear between the initial (up) and the final (down) consistency measurement, and imposed this shear by means that are independent of the measuring instrument. In these conditions the consistency curves before and after shear are almost parallel and may be expressed by Eq. 2.

"Thixotropic hysteresis loop" is an unhappy misnomer, too, as it implies some resemblance to elastic or magnetic hysteresis. The latter have defined physical dimension and meaning and are independent of measuring instruments. The thixotropic loop has neither, and the magnitude of its area supplies no more information than the average decrease in thixotropy between the two consistency curves.

Since thixotropy depends on the type, degree, and duration of shear, no absolute definition of it is possible, and the study of the phenomenon must be approached keeping in mind the actual conditions which may provoke thixotropic phenomena, and the reasons for which thixotropy may be relevant.

Thixotropy also alters the shape of the consistency curve, since any measurement is affected by the shear imposed by the measurement instruments and by the history of the sample (3). This drawback may to some extent be overcome by using Brookfield's "helipath," by which the spindle shifts vertically in the course of measurement so that it keeps changing its position in the sample. For the lotions considered, the helipath did not prove to have advantages over the method adopted, partly because the bars of this instrument are not designed to register the relatively low viscosities of the authors' lotions and partly because, by the authors' method, thixotropy can be prevented from interfering seriously with viscosity measurement.

For the physical stability of a lotion, an important property is that shear does not begin until a stress of some magnitude is applied (Bingham's yield value). Before the stress applied attains this value, the sample behaves as a solid, "imprisoning" the suspended particles, which thus can neither come to the top nor sediment (11). It is even possible to calculate a critical yield value below which the particles separate out into layers. For example, for the lotions studied, it may be assumed that the particles in suspension are spherical, have a diameter of 4 μ, and a density of 0.9, and that the density of the continuous phase is 1.0. In these conditions every particle receives an upthrust of $3.4 \cdot 10^{-9}$ dynes and, as every particle has a surface of $5 \cdot 10^{-7}$ cm.^{2}, the fluid must possess a yield value of at least $6.9 \cdot 10^{-3}$ dynes. cm.$^{-2}$ to prevent the particles from coming to the top. This value is much lower than the actual yield values of our lotions, which are 100–1000 times greater and which thus ensure good stability during storage. It should be noted, however, that the rheologic properties of a lotion are the properties of the fluid as a whole, that is of the continuous phase together with the particles in suspension, and so the foregoing calculations can not be considered very rigidly.

The yield value increases during storage and this improves the physical stability of a lotion. It decreases if the lotion is stirred. If the agitation is very violent the yield value may be abolished altogether and so the stability of the lotion may be affected.

The passage of the lotion through the metering-bottling machine, which is usually the final step in the pharmaceutical process, imposes on the lotion a heavy shear of short duration. This is not comparable to a more gentle stirring of longer duration, since viscosity and yield values may be influenced differently in the two conditions. In fact the violent shear may modify some qualities of the emulsion such as increase of surface area of the internal phase, size and shape of the particles, *etc.*, which may decrease or increase viscosity or yield value, and therefore also the physical stability of the lotion.

Storage increases also the viscosity of the lotions under consideration, thereby slowing down the rate at which the particles come to the top or sediment. An undue increase in viscosity has its snags, however. First, it cancels out the advantages of lotions over creams as regards therapeutic use, second, the lotion becomes more difficult to get out of its container, and third, lotions of different ages exhibit different rheologic properties, a fact that is likely to worry the consumer.

Optimum Viscosity for a Dermatologic Lotion—Although the rheologic definition is an essential technologic datum for dermatologic lotions, the pharmacopeias make no reference to it. The USP XVII, the NF XII, and the BP 1963 simply carry a few examples of lotions without describing their physicochemical properties or specifying methods for determining them.

It is highly desirable for the rheologic properties of lotions to be stated and studied, as knowledge of these properties is indispensable: (*a*) for checking that the formula adopted yields the degree of viscosity suited to the therapeutic use for which the lotion is intended; (*b*) for adjusting the production and bottling equipment to the rheologic properties of the product in the various stages of production, and (*c*) for checking and ensuring the physical stability of the lotion over a period of time.

With regard to point *a*, lotions are usually preferred to creams when the area to be treated is painful or the skin very delicate, in which case the process of rubbing to spread the cream would be painful. Lotions are also preferable when the areas to be treated are large or there are skinfolds or hairy areas or when the lesions are scabbed or chapped, that is when the lotion reaches the target more easily than a cream. For all these reasons viscosity should be kept as low as possible. On the other hand, a liquid with low viscosity tends to run off easily from skin surface, which is never perfectly horizontal. Hence, one has to seek a compromise between these opposite requirements.

This problem does not yet seem to have been approached. We have therefore carried out acceptance tests with lotions of varying viscosity on normal subjects and on patients suffering from skin diseases. The results showed that the extreme range of acceptable viscosity, determined at 20 r.p.m. with a No. 2 spindle at 24°, is between 1 and 20 poise and that the optimum range of viscosity is between 2 and 5 poise. To generalize this indication to other vicosimeters it may be added that the above shear rate corresponds to about 6 sec.$^{-1}$. This shear rate is at the lower levels of those actually applied to lotions when rubbed on skin. In fact the layers of lotion may be estimated between 0.02 and 0.3 cm., the velocity of rubbing between 1 and 5 cm. · sec.$^{-1}$. This is to say that shear rates between 3 and 250 sec.$^{-1}$ may be involved during therapeutic use.

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Keyphrases

Dermatological lotions—rheological properties
Rheology, lotion—changes during aging
Brookfield viscosimeter—shear stress values
Metered bottling machine—rheological effect
Viscosity—shear rate relation
Thixotropy—agitation type, duration

Thermodynamic Effects in the Compression of Solids

By EDWARD J. HANUS* and LOUIS D. KING

The temperature changes of solids undergoing compression were determined. Compounds of known heat capacity were compressed into tablets at varying speeds and pressure and collected into thermally insulated vessels containing a suitable liquid. The resulting increase in temperature of the liquid was measured after equilibrium was established, and it was found that the increases in temperature were directly related to speed of compression and to compression pressure. Extrapolation of the data provided a technique for predicting tablet temperatures at time of compression. Thermal energy was converted to its mechanical equivalent thus providing a means for estimating the energy expended in the tablet compression process. The use of thermochromic indicators qualitatively supported the validity of the conclusions and quantitative confirmation was obtained by direct heating of samples.

MEASUREMENT OF thermal energy developed in a dynamic operation such as the compression of solids with a tablet machine is a difficult and challenging problem. The reasons are numerous: (*a*) the heat content of a compressed compact is very quickly dissipated to its environment, (*b*) the high pressures applied during compression would crush ordinary temperature measuring devices such as thermometers or thermistors, and (*c*) during compression, the granulation is virtually encased within a steel chamber consisting of the die and punches, and thus is not physically accessible at the moment when temperatures are at a maximum.

In the compaction of powders, the following processes contribute to the total energy of compression (1, 2).

(*a*) Packing—Initially within a bed of powder there is essentially only one point of contact between two neighboring particles. As pressure is applied, consolidation takes place by the particles sliding one over the other and heat is generated. The extent to which this occurs is influenced to a large degree by the frictional properties of the two sliding surfaces (3, 4).

(*b*) Elastic and plastic deformation—In this phase of compression, most of the energy is consumed in the deformation of particles and by die-wall friction.

(*c*) Cold working with or without fragmentation—When fragmentation occurs, the specific surface area of tablets increases directly with pressure to a maximum and then decreases (5).

(*d*) Removal of the upper punch from the die after compression.

(*e*) Tablet ejection.

Nelson *et al.* (2), using an instrumented single punch tablet machine, found it possible to estimate the energy expended in the tablet compres-

Received April 18, 1967, from Rutgers, The State University, College of Pharmacy, Newark, NJ 07104
Accepted for publication December 20, 1967.
Portion of a dissertation submitted to the Graduate School, Rutgers, The State University, by Edward J. Hanus in partial fulfillment of the Doctor of Philosophy degree.
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(*d*) Removal of the upper punch from the die after compression.

(*e*) Tablet ejection.

Nelson *et al.* (2), using an instrumented single punch tablet machine, found it possible to estimate the energy expended in the tablet compres-

Received April 18, 1967, from Rutgers, The State University, College of Pharmacy, Newark, NJ 07104
Accepted for publication December 20, 1967.
Portion of a dissertation submitted to the Graduate School, Rutgers, The State University, by Edward J. Hanus in partial fulfillment of the Doctor of Philosophy degree.
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sion process by considering the magnitude and relation between compression variables (upper and lower punch force, ejection force, upper punch displacement), and the linear dimension of the die cavity. Using granulations of sulfathiazole, Nelson calculated a probable temperature rise of about 5° by constructing force–displacement curves and measuring the area under the curve with a planimeter. However, there appears to be a need for a relatively simple and inexpensive method for determining the total heat generated by solids undergoing compression in order to study the tableting process. It was for this reason that the current study was initiated.

The simplest approach to the problem originally appeared to be to include some material in the tablet granulation that would change color in such a way as to give a true indication of the maximum temperature produced during compression. However, results from this portion of the study were only qualitative in nature and a more precise thermometric method was developed.

EXPERIMENTAL

Thermochromic Method—Samples of Thermochrom temperature indicating crayons[1] and Detecto Temp temperature indicating pigments[1] were obtained for evaluation. The exact chemical identity of these highly complex metallic salts could not be provided. When the specified temperature is maintained for 1 or 2 sec. a gas, such as carbon dioxide, ammonia, sulfur dioxide, or water vapor is liberated and the chemical structure and color of the salts undergo changes (6). Eighteen Thermochrom crayons provide temperature measurements from 65°–670°. Thirty-six pigments cover a measurement range from 40 to 1350°; accuracy is estimated to be ±5°. Reportedly changes in air pressures affect all compounds in that the color change temperature thresholds are displaced upward or downward (7). At 5–10 mm. mercury, the materials change color from 5–20% below the temperatures they would indicate under normal atmospheric pressure and when used under vacuum or pressure, suitable calibrations are recommended.

The lowest available temperature indicating crayons of Thermochrom 915–1015 (65°) and 915–1016 (75°) were shaved with a razor blade to small fragments, passed through a No. 40 sieve, added to separate samples of sodium chloride (40/60 mesh) at a concentration of 1%, and mixed for 15 min. by tumbling in a wide-mouth glass bottle. Three-gram tablets were then prepared using a hydraulic press at a pressure of 10,000 lb. Duplicate runs revealed no change in color from gray to blue (65°) or gray to green (75°) in either sample, therefore, it was tentatively concluded that the average temperature of the sodium chloride tablet was less than 65°. However, it was noted that the crayons appeared to contain a waxy base which would undoubtedly act as a lubricant and possibly alter experimental results. For this reason, the Thermochrom crayons were abandoned and attention was directed toward the Detecto Temp pigments which were available as fine powders and could readily be incorporated in tablet granulations.

The first pigment to be examined was Detecto Temp 915–0900 which was reported to change from pink to blue after 2 sec. at 40°. A 5% triturate of sodium chloride (40/60 mesh) was prepared and then compressed into 3-Gm. tablets of $^3/_4$ in. diameter using a flat-faced upper tablet punch and a smooth flat stainless steel plate as a support for the die in lieu of a lower tablet punch. Tablet pressure was brought up rapidly and maintained at 10,000 lb. for 15 sec. and then quickly released. It was noted that tablets made in this manner changed in color from pink to blue indicating that an average tablet temperature of approximately 40° or higher had been attained.

The pigment available at the next higher temperature was Detecto Temp 915–0923 which was reported capable of undergoing two color changes, *i.e.*, pink to lilac at 55° and lilac to blue at 85°. Tablets prepared in the manner described above showed a very faint lilac color which was difficult to distinguish with certainty from the original pink color of the pigment. In order to establish confidence in the observations, tablets were prepared at pressures of 5,000, 10,000, and 30,000 lb. on the basis that increasing pressures might produce higher tablet temperatures. This was borne out in the experimental results which showed no color change at 5,000 lb. pressure, a very faint color change at 10,000 lb., and a definite color change at 30,000 lb.

The experiment was repeated with Detecto Temp 915–0901 which changes from light green to blue at 60°. Pressures of 10,000 and 30,000 lb. were employed; no change in color was observed indicating that a temperature of 60° was not reached regardless of pressure. Tablets containing the 60° pigment (915–0901) were placed in an oven at 80° for $1^1/_2$ hr. A pronounced color change from green to blue was noted indicating that the pigment was operative.

Thus, using a pressure of 30,000 lb., sodium chloride tablets were found to rise in temperature to about 55°, and an increase in thermal energy appeared to be directly related to an increase in pressure.

The temperature developed at points in the compress was probably much higher than the indicated temperature. However, no measure of the maximum temperatures which may have developed was possible because of the time required for the pigments to undergo changes and the possible reversibility of the reactions. Only average temperature changes for the total compress were observed.

Effect of Reduced Pressure—A vacuum pellet die, usually used with infrared procedures, was employed to prepare $^1/_2$-in. flat-faced tablets under reduced pressure. One-gram tablets of sodium chloride were compressed at a pressure of 3,000 lb. while a vacuum of 2 mm. mercury was maintained for 30 min. Two pigments were tested at a concentration of 5%, *i.e.*, 915–0901 (60°) and 915–0924 (65°) and no color change was noted with the 65° pigment while a nonuniform color change from green

[1] Trademarked by the H. V. Hardman Co., Inc.

TABLE I—COLOR CHANGES OBSERVED IN TABLETS OF SODIUM CHLORIDE USING THERMOCHROMIC INDICATORS

Thermochromic Indicator	Color Change and Temp.	Pressure of Hydraulic Press[c] 3	5	10	30	Time Period of Applied Vacuum 2 min.	30 min.
Crayon 915–1015	65°–gray to blue			−			
Crayon 915–1016	75°–gray to green			−			
Pigment 915–0900	40°–pink to blue			+			
Pigment 915–0923[a]	55°–pink to lilac		−	+	+		
	85°–lilac to blue		−	−	−		
Pigment 915–0901	60°–lt. green to blue			−	−		
Pigment 915–0901	60°–lt. green to blue	[b]				−	+
Pigment 915–0924[a]	65°–lt. pink to lt. blue	[b]				−	−
	145°–lt. blue to lt. brown	[b]				−	−

[a] Has two color changes. [b] Vacuum applied. [c] Gauge pressure × 10^3.

to blue was observed with the 60° pigment. The sides of the tablets were mostly blue whereas the upper and lower face of the tablet still showed some evidence of the original green color. Reducing the time period for applying vacuum from 30 min. to 2 min. resulted in no color change in tablets prepared with either one of the pigments. This indicates that the thermochromic materials change color significantly below the temperatures that they would under normal atmospheric pressure. A summary of the above findings is presented in Table I.

Dynamic Compression—The behavior of the thermochromic materials under dynamic conditions of compression using a Stokes model E single punch tablet machine equipped with 1/2-in. flat-faced punches was studied. Two pigments, 915–0900 (40°) and 915–0293 (55°) were added at a level of 5% to a number of common pharmaceutical compounds; no tablet lubricant was used. Approximately 10 to 50 tablets were compressed depending on the severity of binding or capping experienced because of the absence of lubrication. In all cases, the tablets were made as hard as practical. However, hardness, tablet weight, and thickness measurements were not attempted because the resulting tablets were not satisfactory *per se.*

As shown in Table II, sodium bicarbonate, alumina, citric acid, lactose, and sodium chloride attained average temperatures of about 40°, representing a temperature rise of about 16° above room temperature (24° ± 2°); polyethylene glycol did not exhibit a comparable rise in temperature which is not unexpected considering its lubricating properties (low coefficient of friction) and its relatively low yield value.

Thermometric Method—A single punch Stokes Tablet Machine model E as shown in Fig. 1 was converted to a variable speed tablet press by the use of appropriate pulley arrangements. Other modifications included the use of 1/8 in.-Teflon laminated to the bottom of the feed shoe in order to reduce friction and heat transfer between the feed shoe and die table. In addition, the hopper and die table were ringed with a combination of copper and rubber tubing, covered with aluminum foil and then connected to a constant temperature bath. The granulation, die table, punches, and die were maintained at constant temperature by circulating water through the system. The hopper was covered and a thermometer was inserted into the granulation with the bulb centered 1 in. from the bottom of the hopper. An additional thermometer was attached to the die table in close proximity to the punches and die in order to monitor possible temperature changes in that area of the tablet machine which was critical to the operation. A closed chute was prepared of cardboard and polyethylene and attached to the tablet machine in order to permit transfer of tablets to a Thermos bottle with a minimum loss of heat. Flat-faced punches of 1/2 in. diameter were used throughout the study.

TABLE II—COLOR CHANGES IN TABLETS OF PHARMACEUTICAL COMPOUNDS USING THERMOCHROMIC INDICATORS

Compound	Pigment 915–0900 Color Change[a]	Pigment 915–0923 Color Change[b]
Sodium bicarbonate	+	−
Alumina	+	−
Citric acid	+	−
Lactose	+	−
Polyethylene glycol 4000	−	−
Sodium chloride	+	−

[a] Changes at 40° from pink to blue. [b] Changes at 55° from pink to lilac.

Granulations—Calcium carbonate and sodium chloride, two common materials of known heat capacity, were selected for study. The first was formulated into granules by a wet granulating technique whereas the latter, which can be compressed directly into tablets, was used in its pure form. The calcium carbonate granulation was prepared as follows:

Calcium carbonate USP	10.000 Kg.
Magnesium stearate	0.105
Cellulose acetate phthalate	0.465
Acetone USP	2.068
Methanol	6.204

The cellulose acetate phthalate (CAP) was slowly added with stirring to the acetone. After solution was effected, the methanol was added with stirring until the solution was homogeneous and the solution was then filtered through gauze.

The calcium carbonate was weighed, transferred to a pony mixer, and the granulating solution was added slowly with blending until the mixture was sufficiently wet. The wet mass was forced through a No. 8 mesh screen, air-dried for 18 hr., and then dried at 40° for a period of 40 hr. The dry granules were forced through a No. 14 screen and transferred to a steel drum. Magnesium stearate was added to an equal weight of 60 mesh fines, blended for 15

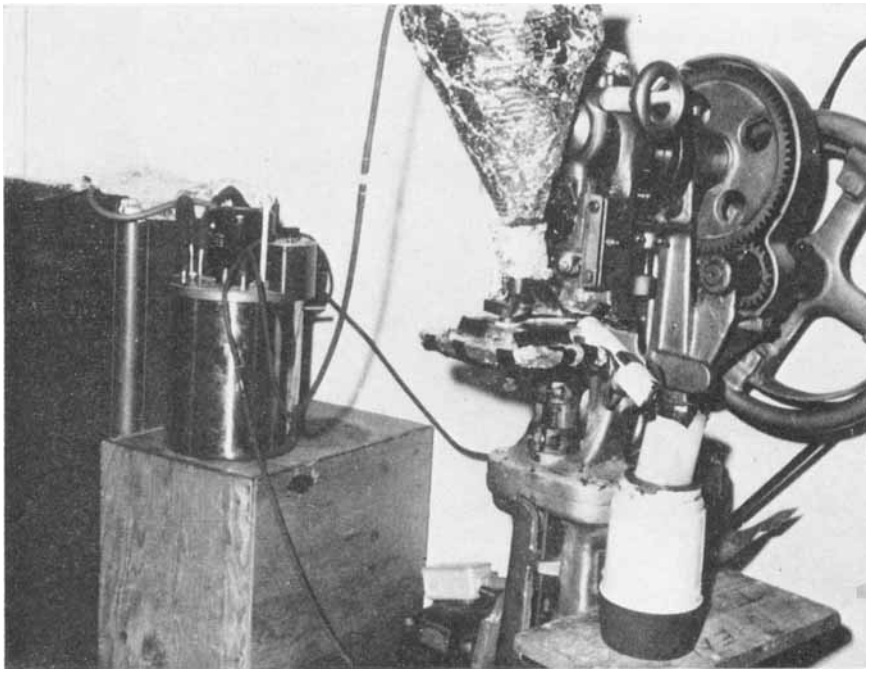

Fig. 1—Modified Stokes model E tablet machine.

min., and then added to the calcium carbonate granulation and mixed for 30 min. to form a homogeneous mixture.

One 25-lb. lot of sodium chloride was used throughout this study. Results of particle size analyses are recorded in Table III.

All operations were conducted in a sealed, air-conditioned room in which a constant temperature of 24° ± 1° was maintained at all times. Wide-mouth Thermos bottles were tared and 160 Gm. of distilled water was added. The bottles were capped and allowed to come to equilibrium for a period of time not less than 15 min. The speed of the tablet machine was then checked with a stopwatch, and the tablets collected were checked for initial weight, thickness, and hardness. Ten tablets were weighed to obtain an average weight; thickness was measured with a pair of vernier calipers and hardness was determined with the Monsanto tablet hardness tester. The plastic cap was removed from one of the Thermos bottles and quickly replaced with a square of Lucite (5 × 5 × ½ in.) to prevent any changes of temperature. A Beckman differential thermometer was inserted through a hole in the Lucite cover into the water to a point ½ in. from the bottom of the container. The temperature of the water was measured after allowing a period of 5 min. for equilibration. The Thermos bottle was then transferred to the tablet machine and inserted snugly under the tablet chute equipped with a round plastic adapter. The tablet machine was activated and approximately 350 tablets each weighing 500 mg. were collected directly into the Thermos. The bottle was immediately capped and allowed to come to equilibrium for 30 min. Exact tablet weight was determined by weighing the Thermos again at this point. At the end of a 30-min. period, the container was gently tumbled for 1 min. and the temperature of the mixture measured. All determinations were made in triplicate at machine, speeds of approximately 27, 40, 60, 80, 100, 120, and 140 tablets/min. Temperatures of the granulation, the room, and the die table were recorded at the beginning and end of each run.

As a control, tablets prepared as previously described were allowed to cool, and then stored at 24° for 15 hr. in preweighed quantities of exactly 175 Gm. The stored tablets were quickly added to the Thermos bottle containing 160 Gm. of distilled water. Temperature changes were measured using the exact procedure described above.

To check the accuracy of tablet temperatures predicted, 175 Gm. of preweighed 500-mg. tablets were stored in sealed glass bottles and heated in a constant temperature oven at a mean temperature of 45.60°. The heated tablets were quickly transferred to the Thermos bottles following precisely the procedure described above and appropriate temperature measurements made.

The procedure employed with sodium chloride was identical to that reported above for calcium carbonate with the following exceptions: (*a*) Two hundred 700-mg. sodium chloride tablets weighing 140 Gm. were compressed and collected in each

Table III—Particle Size Analysis of Calcium Carbonate and Sodium Chloride Granulations

Particle Size, μ	Calcium Carbonate, %	Sodium Chloride, %
840	24.0	5.1
420–840	33.3	65.2
177–420	21.4	29.6
177	21.3	0.1

Thermos bottle containing 150 Gm. of carbon tetrachloride. (*b*) In checking the accuracy of the method, the sodium chloride tablets were heated at a mean temperature of 32.06°.

Effect of Pressure—Although an instrumented tablet press, equipped with a strain gauge for measuring pressure was not available, it seemed desirable to determine the effect of pressure on thermal effects developed during tablet compression. In this connection, the pressure adjustment screw was measured; the circumference was calculated and found to be 8.3254 cm. The screw was then suitably calibrated so that each quarter turn of the screw would move through a distance of 2.0814 cm. Using sodium chloride as the test material, tablets were compressed at 8 different pressure settings ranging from 0 to 2.5 turns of the pressure adjustment set screw. All determinations were conducted in triplicate using the process detailed heretofore.

CALCULATIONS

The temperature of a tablet at a given machine speed was calculated from the thermal capacities of the tablet material and the liquid to which the tablets were added. Heat capacity values utilized are listed below (8):

Calcium carbonate granulation	0.206
Sodium chloride	0.220
Distilled water	1.000
Carbon tetrachloride	0.202

The following equation was used for calculating the increase in thermal energy of the compressed solid.

$$\Delta T = \frac{\Delta T_L W_L C_L}{W_t C_t}$$

where ΔT is the measured increase in temperature (°C.) of the tablets as a result of compression, ΔT_L is the increase in the temperature of the liquid (°C.), W_L the weight of the liquid, C_L the heat capacity of the liquid, W_t the weight of tablets, and C_t the heat capacity of the tablet granulation.

The calculated temperature (T) of the tablet just before striking the surface of the liquid was determined by summing ΔT and T_f and making a correction for the heat of wetting, mixing, dissolution, *etc.* (T_w) as follows:

$$T = (\Delta T + T_f) - T_w$$

where T_f represents the final temperature of the tablet-liquid mixture after equilibrium is established.

It was not feasible to collect a predetermined exact weight of tablets because of normal tablet weight variations, and practical counting difficulties. Thus, temperature readings were adjusted for a common weight, *i.e.*, 175 Gm. for the calcium carbonate granulation and 140 Gm. in the case of the sodium chloride. Temperature increases obtained with the unheated control tablets (T_w) were subtracted from the calculated temperature (T) in the case of the calcium carbonate granulation only since the T_w for the sodium chloride was found to be insignificant. This correction factor was 4.90° for the $CaCO_3$.

RESULTS AND DISCUSSION

Tables IV and V present a summary of the results obtained with the calcium carbonate and sodium chloride granulation, respectively. These data show that as the speed of the tablet machine increases, the temperature of the tablets collected also increases. In all cases, temperature ranges were not more than 1.61° indicating excellent reproducibility of results considering the nature of the experiments.

As can be observed, tablet hardness was reasonably well maintained throughout the study since variation in pressure imparts significant effects on tablet temperature. The relationship of tablet temperature to machine speed, Fig. 2, is almost

Table IV—Temperatures of Compressed 500-mg. Calcium Carbonate Tablets as a Function of Speed of Compression in °C.

Speed (Tablets/Min.)	Temperature Increase (T)	Tablet Temperatures[a] (T)	Range (°C.)	Tablet Hardness,[b] Kg.	Tablet Thickness,[b] cm.
26.74	16.27	36.42	36.26–36.61	10.6	0.248
40.32	17.69	38.32	37.48–39.08	10.5	0.247
61.43	19.70	40.84	40.48–41.53	10.6	0.245
77.72	20.47	41.79	41.43–42.14	10.5	0.247
100.00	21.28	42.74	42.11–43.34	10.2	0.245
122.95	22.13	43.67	43.00–44.17	10.1	0.248
140.85	22.20	43.74	43.66–43.82	10.7	0.242

[a] Average of 3 experiments—corrected for T_w. [b] Average of 30 measurements.

Table V—Temperatures of Compressed 700-mg. Sodium Chloride Tablets as a Function of Speed of Compression in °C.

Speed (Tablets/Min.)	Temperature Increase (T)	Tablet Temperatures[a] (T)	Range (°C.)	Tablet Hardness,[b] Kg.	Tablet Thickness,[b] cm.
25.64	2.69	28.69	28.67–28.88	7.5	0.269
39.47	4.18	31.89	31.16–32.36	7.8	0.261
60.00	5.41	34.21	33.49–34.58	7.5	0.261
81.97	6.32	35.13	34.85–35.32	7.4	0.261
102.74	7.04	36.30	36.11–36.51	7.4	0.261
140.87	7.11	36.51	36.35–36.82	7.4	0.257

[a] Average of 3 experiments. [b] Average of 30 measurements.

linear at low speeds, whereas at high speeds the temperature values tend to become constant. This indicates that a limiting value was approached and that relatively little thermal energy is lost to either the tablet press or atmosphere at the highest attainable machine speed.

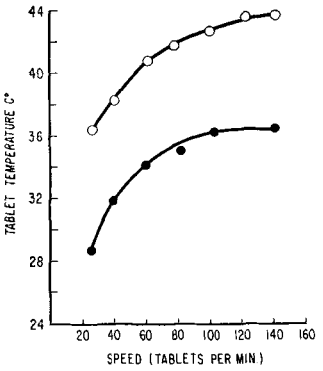

Fig. 2—Tablet temperature as a function of machine speed. Key: ●, 700-mg. sodium chloride tablets; ○, 500-mg. calcium carbonate tablets.

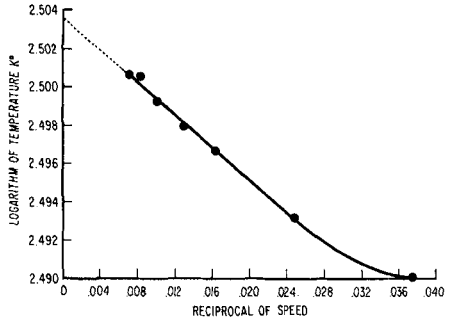

Fig. 3—Relationship of the logarithm of tablet temperature in degrees Kelvin (K.°) and the reciprocal of machine speed for tablets of calcium carbonate.

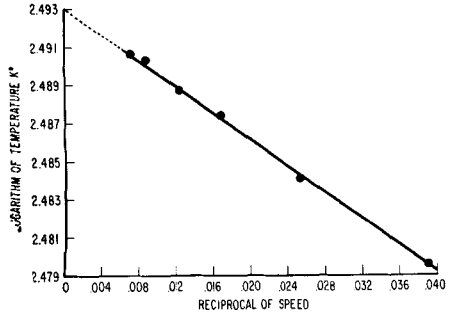

Fig. 4—Relationship of the logarithm of tablet temperature in degrees Kelvin (K.°) and the reciprocal of machine speed for tablets of sodium chloride.

Plotting the logarithm of the tablet temperature in degrees Kelvin (°K.) against the reciprocal of machine speed produced a linear relationship as shown in Figs. 3 and 4. Extrapolation of the plot to the y axis gave the predicted temperature of the solid at the instant of maximum compression. At this theoretically infinite speed, losses in thermal energy by conduction to the tablet machine or radiation to the atmosphere approached zero. Thus, were it possible to run a tablet press at infinite speed, the enthalpy of the compressed compact would be completely transposed to the liquid in the thermally insulated collecting vessel. Some slight departure from linearity was noted only in the case of the calcium carbonate granulation at a slow speed of 27 tablets/min. The temperature of the compressed sodium chloride and calcium carbonate tablets was then determined at time of compression from their respective plots and is reported in Table VI.

The average increase in temperature as a result of the tablet compression process was found to be 20.9° for calcium carbonate and 14.2° for sodium chloride. The local temperatures in the granulation at the points of contact during compaction may momentarily be several hundred degrees, thus the temperatures reported above actually represent the mean tablet temperature immediately after compression.

In order to establish further confidence in the procedure, tablets were first heated to an exact temperature and subjected to the experimental method detailed above. Predicted and observed temperatures were compared and are reported in Table VII. The observed and predicted temperatures for calcium carbonate are in good agreement. However, there is a difference of approximately 9% between the 2 values for sodium chloride. This difference appears to be due to difficulty in obtaining exact values for the specific heat in the indicated temperature range and slight inherent errors in the experimental methods. Variability within individual experiments was not more than 0.16° in all cases.

Pressure has a profound thermodynamic effect on materials undergoing compression as can be deduced from Table VIII. At a low pressure mea-

Table VII—A Comparison of Calculated and Observed Temperatures Using Preheated Tablets of Calcium Carbonate and Sodium Chloride

Tablet Identification	Predicted Temperature, °C.	Observed Temperature, °C.	Temperature Difference, °C.
Calcium carbonate tablets, 500 mg.	44.9	45.6	0.7
Sodium chloride tablets, 700 mg.	38.2	35.0	3.2

Table VI—Predicted Tablet Temperatures as the Result of Compression

Tablet Identification	Predicted Tablet Temperature °K.	Predicted Tablet Temperature °C.	Temperature of Hopper Granulation, °C.	Increase in Temperature as a Result of Compression, °C.
Calcium carbonate tablets, 500 mg.	317.9	44.9	24.0	20.9
Sodium chloride tablets, 700 mg.	311.2	38.2	24.0	14.2

TABLE VIII—EFFECT OF PRESSURE ON 700-mg. TABLETS OF SODIUM CHLORIDE AT A CONSTANT TABLET MACHINE SPEED[a]

Pressure, cm.[b]	Tablet Temperature[c] (T), °C.	Range, °C.	Increase in Temperature, °C.	Tablet Thickness,[d] cm.	Tablet Hardness,[e] Kg.
0.0000	25.51	25.32–25.48	1.51	0.335	*e*
4.163	27.40	27.30–27.42	3.40	0.315	*e*
6.244	27.48	27.41–27.58	3.48	0.310	*e*
8.325	28.29	28.23–28.40	4.29	0.300	*e*
10.407	29.37	29.29–29.43	5.37	0.290	*e*
12.488	30.77	30.56–31.04	6.77	0.280	4.0
16.651	32.38	31.81–32.72	8.38	0.270	5.9
20.814	35.12	34.67–35.42	11.12	0.260	7.1

[a] 81.97 tablets/min. [b] Each half turn (180°) of pressure set screw equals 4.163 cm. [c] Average of 3 experiments. [d] Average of 15 measurements. [e] Too soft to measure accurately.

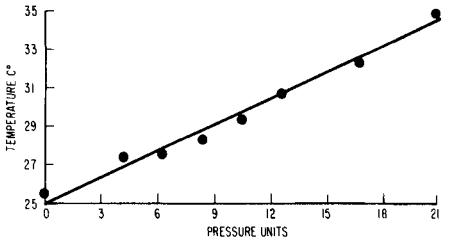

Fig. 5—The relationship of tablet temperature and applied pressure during the compression of sodium chloride tablets.

sured as 0 "cm." the tablet formed was observed to be extremely fragile however its shape was retained; the temperature increase noted was only 1.51°. On the other hand, when the pressure was grossly increased to a near maximum at 20.814 "cm." pressure, the increase in thermal energy was more than seven fold. Plotting pressure as represented by calibrated pressure adjusting screw *versus* temperature, produced a linear relationship as shown in Fig. 5. The reproducibility of the individual experiments at various pressure was excellent with the range of results being not more than 0.91° in all cases. As expected, tablet thickness was found to be inversely related to pressure and tablet hardness directly related.

Having determined tablet temperature as a result of compression, the heat in calories input, was then obtained from the following relationship:

$$Q = \Delta TCM$$

where Q is calories of heat, ΔT the increase in temperature, C the heat capacity, calorie/degree/Gm. of the granulation, and M the weight of one tablet in Gm. As shown in Table IX, it was found that the increase in heat per tablet for the calcium carbonate and sodium chloride tablet was 3.0 and 2.0 cal., respectively. Observing the law of conservation of energy and assuming that all the useful energy expended in the compression process appeared as heat, the mechanical equivalents of heat appearing in Table IX were derived from the following relationships: (9)

$$1 \text{ cal.} = 4.1858 \times 10^7 \text{ ergs} = 4.1858 \text{ abs. joules}$$

Thus by determining the increase in thermal energy in powdered solids as a result of compression, it has been possible to estimate the total useful energy expended in the tablet compression process.

SUMMARY AND CONCLUSIONS

The thermal changes occurring in powdered solids undergoing compression have been studied. Granulations of calcium carbonate and sodium chloride were compressed into tablets at varying machine speeds and pressures and collected into a thermally insulated vessel containing a suitable liquid. The resulting increase in temperature was measured after equilibrium was established. It was found that the increases in temperature were directly related to speed of compression and to the applied pressure. Plotting the reciprocal of time *versus* the logarithm of the temperature provided a linear relationship. An extrapolation provided techniques for predicting tablet temperature at the time of compression. Good agreement was observed between predicted tablet temperatures and results obtained by heating tablets by direct means. Further qualitative confirmation was obtained by the use of thermochromic materials which changed color at predetermined temperatures.

The measured thermal energy was converted to its mechanical equivalent thus providing a means for determining the useful work required for the tablet compression process. It is felt that the techniques reported here should find future utility as a tool in more quantitatively evaluating various parameters in tabletting technology related to the study of lubricants, excipients, glidants, particle size, moisture, and hardness.

TABLE IX—ENERGY REQUIREMENTS PER TABLET FOR THE COMPRESSION OF CALCIUM CARBONATE AND SODIUM CHLORIDE GRANULATIONS

Tablet Identification	Temperature Increase,[a] °C.	Thermal Energy Increase, Cal.	Mechanical Energy Expended ergs × 10^{-7}	Mechanical Energy Expended abs. joules
Calcium carbonate tablets, 500 mg.	20.9	3.0	12.6	12.6
Sodium chloride tablets, 700 mg.	14.2	2.0	8.5	8.5

[a] From Table VI.

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Keyphrases

Compression—solids
Thermodynamic effects—compression
Tablets—heat of compression
Temperature indicating pigments—heat of compression
Heat of compression, measured—water temperature change
Pressure relation—thermal effects
Speed of compression—thermal effects

Notes

Synthesis of Several Dibasic Acid Esters of 3-Quinuclidinol as Potential Pharmacological Agents

By HILLEL LIEBERMAN and SAMUEL ELKIN

Eight new dibasic acid esters of 3-quinuclidinol have been synthesized and isolated as their free bases using an alcoholysis reaction. A ninth compound, 3-quinuclidinyl hydrogen homophthalate, could not be isolated with sufficient purity to obtain an elemental analysis. However, this compound showed characteristic ester bands and gave a positive hydroxamic acid test. In a preliminary pharmacological evaluation in dogs, 3-quinuclidinyl hydrogen phthalate and 3-quinuclidinyl hydrogen 2,3-pyridinedicarboxylate gave a substantial increase in blood pressure and produced central stimulation as evidenced by a marked increase in respiration. Five compounds showed varying degrees of local anesthetic activity.

SERIES OF ESTERS of 3-quinuclidinol have been prepared by Sternbach and Kaiser (1) and by Mikhlina and Rubtsov (2). All of these esters produced marked spasmolytic activity. Of great interest was the observation that many of these esters showed a much higher antiacetylcholine activity than the analogous esters derived from diethylaminoethanol and other generally used basic alcohols. One of the most active of the series was 3-diphenylacetoxyquinuclidine.

Many other therapeutically active quinuclidine derivatives have been synthesized and pharmacological activity has been found in both mono- and disubstituted products. Several 3-substituted derivatives of quinuclidine have been reported by Grob (3) to stimulate the central nervous system. A series of 2-aminomethyl quinuclidines has been prepared and tested by Rubtsov *et al.* (4). Some members of the series were found to have strong ganglionic blocking activity while one member, diethylaminoethylquinuclidine-2-carboxylate (5), was found to be a good hypotensive. Ganglionic blocking was also noticed with 2,3-disubstituted derivatives (6). Perrine (7) has synthesized a series of 4-phenyl substituted compounds for investigation as possible analgesics.

The research involved in this work devotes itself to the synthesis of a series of dibasic acid esters of 3-quinuclidinol and the preliminary pharmacological testing of these products. These compounds evoked one or more of the following pharmacological responses: local anesthetic activity, respiratory and vasomotor stimulation.

Eight new compounds were synthesized and are represented by the general formula, I. All products

Received July 19, 1967, from Temple University School of Pharmacy, Philadelphia, PA 19140

Accepted for publication October 23, 1967.

Abstracted, in part, from a thesis presented by Hillel Lieberman in partial fulfillment of the requirements for the Master of Science degree.

The authors thank Dr. David Mann, Temple University School of Pharmacy, for his guidance in the pharmacological studies.

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Keyphrases

Compression—solids
Thermodynamic effects—compression
Tablets—heat of compression
Temperature indicating pigments—heat of compression
Heat of compression, measured—water temperature change
Pressure relation—thermal effects
Speed of compression—thermal effects

Notes

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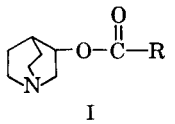

I

Compound	R
1	—CH_2CH_2COOH
2	HOOC—
3	—CH=CHCOOH
4	—$(CH_2)_3COOH$
5	HOOC—
6	HOOC—
7	—$CH_2CH(C_{10}H_{21})COOH$
8	—$CH_2CH(C_{16}H_{33})COOH$

All compounds were prepared by alcoholysis, an acylation reaction between an anhydride and an alcohol (8). All types of alcohols are acylated by anhydrides. Cyclic anhydrides of dibasic acids are cleaved by alcohols to the monoester of the corresponding dibasic acid (9). The method employed for the preparation of the aforementioned compounds was a modification of that used by Eliel and Burgstahler (10).

The dibasic cyclic anhydrides were dissolved in boiling dry benzene containing varied amounts of pyridine. 3-Quinuclidinol, dissolved in hot dry benzene, was added to a refluxing solution of the anhydride and the mixture was allowed to reflux for 12 hr. In the case of the phthalic acid ester and the 2,3-pyridinedicarboxylic acid ester, white precipitates appeared during the reflux. These were filtered and subsequently purified. In all other cases, a clear solution was obtained at the end of the reflux period from which the product precipitated upon cooling. All yields were greater than 70%.

One of the compounds, 3-quinuclidinyl hydrogen, homophthalate was very hygroscopic. Attempts were also made to prepare the hydrochloride and methiodide salts. The methiodide salt was prepared by adding excess methyl iodide dropwise to an isopropanol solution of the ester and precipitating the methiodide salt with anhydrous ether. The gummy residue which formed was extremely hygroscopic and could not be crystallized. The hydrochloride salt was prepared by bubbling dry HCl into an alcoholic solution of the ester and precipitating the hydrochloride salt with anhydrous ether. This gummy precipitate could not be crystallized; however, it gave a positive hydroxamic acid test and the IR spectrum showed a characteristic ester band at 5.8 mμ.

EXPERIMENTAL

All melting points are uncorrected. Analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y., and Clarke Microanalytical Laboratory, Urbana, Ill.

Preparation of 3-Quinuclidinyl Hydrogen Succinate—Into a 3-necked flask equipped with a stirrer, dropping funnel, and condenser were added 7.5 Gm. (0.075 mole) succinic anhydride and 100 ml. of dry benzene. This mixture was heated and 15 ml. of pyridine was added. Then 9.6 Gm. (0.075 mole) of 3-quinuclidinol[1] dissolved in 50 ml. of hot dry benzene was added all at once. Upon addition, the solution immediately turned greenish yellow. The mixture was refluxed for 12 hr. whereupon a clear, colorless solution resulted. The solution was distilled at atmospheric pressure to remove 100 ml. of benzene. The remaining solution was then allowed to stand at room temperature for 36 hr. at the end of which time a white precipitate had separated. The precipitate was filtered and recrystallized three times from isopropanol and ether to yield 14.1 Gm. (82.8%) of a white, crystalline product; m.p. 141–143°.

Anal.—Calcd. for $C_{11}H_{17}NO_4$: C, 58.13; H, 7.54; N, 6.16. Found: C, 58.24; H, 7.92; N, 5.90.

Preparation of 3-Quinuclidinyl Hydrogen Phthalate—Into a 3-necked flask equipped with a stirrer, dropping funnel, and condenser were added 11.1 Gm. (0.075 mole) of phthalic anhydride and 75 ml. of dry benzene. This mixture was heated and 5 ml. of pyridine was added. Then 9.6 Gm. (0.075 mole) of 3-quinuclidinol dissolved in 50 ml. of hot dry benzene was added all at once. The mixture was refluxed for 5 hr. whereupon a white precipitate formed. The mixture was cooled to room temperature and filtered with suction. This precipitate was recrystallized three times from hot isopropanol to yield 18.0 Gm. (85.9%) of a white, crystalline product; m.p. 253–255°.

Anal.—Calcd. for $C_{15}H_{17}NO_4$: C, 65.44; H, 6.22; N, 5.09. Found: C, 65.70; H, 6.41; N, 4.99.

Preparation of 3-Quinuclidinyl Hydrogen Maleate—Into a 3-necked flask equipped with a stirrer, dropping funnel, and condenser were added 7.5 Gm. (0.075 mole) of maleic anhydride and 75 ml. of dry benzene. This mixture was kept at room temperature and 9.6 Gm. (0.075 mole) of 3-quinuclidinol dissolved in 50 ml. of hot dry benzene was added all at once. Upon addition, the solution immediately assumed a yellow color which turned brown within 10 min. After the solution turned brown, 50 ml. of pyridine was added all at once and a brown gelatinous mass was deposited on the bottom of the flask. The mixture was refluxed for 4 hr. at the end of which time, a brown gelatinous mass remained at the bottom of the flask. The solution was distilled at atmospheric pressure to remove 75 ml. of benzene. The remaining solution was then allowed to stand at room temperature for 36 hr. and filtered with suction. The precipitate was washed with ether and then dissolved in hot isopropanol The solution was cooled and the product reprecipitated with anhydrous ether. This pre-

[1] Supplied by U. S. Army Chemical Center, Edgewood, Maryland. Can be prepared by the method of Aaron, H. S., Elkin, S., Owens, O. O., Rosenstock, P. D., Leonard, S., and Miller, J. I., *J. Org. Chem.*, 30, 1331(1965).

cipitate was recrystallized three times from hot isopropanol and ether to yield 12.5 Gm. (73.9%) of product; m.p. 160–164°.

Anal.—Calcd. for $C_{11}H_{15}NO_4$: C, 58.65; H, 6.71; N, 6.22. Found: C, 58.65; H, 6.50; N, 5.95.

Preparation of 3-Quinuclidinyl Hydrogen Glutarate—Into a 3-necked flask equipped with a stirrer, dropping funnel, and condenser were added 8.6 Gm. (0.075 mole) of glutaric anhydride and 100 ml. of dry benzene. This mixture was heated and 25 ml. of pyridine was added. Then 9.6 Gm. (0.075 mole) of 3-quinuclidinol dissolved in 50 ml. of hot dry benzene was added all at once. The mixture was refluxed for 15 hr. whereupon a clear, colorless solution resulted. The solution was distilled at atmospheric pressure to remove 100 ml. of benzene. After allowing the solution to cool to room temperature, anhydrous ether was added until cloudiness developed. The mixture was then permitted to stand at room temperature for 30 hr. at the end of which time a white precipitate separated. The mixture was filtered and the residue washed with ether and dried at 50° for 30 min. The white crystalline powder was then dissolved in hot isopropanol and the solution allowed to come to room temperature. Anhydrous ether was added until a white precipitate formed. This precipitate was recrystallized three times from hot isopropanol and ether to yield 14.6 Gm. (80.7%) of a white crystalline product; m.p. 91–94°.

Anal.—Calcd. for $C_{12}H_{19}NO_4$: C, 59.73; H, 7.94; N, 5.81. Found: C, 59.29; H, 7.95; N, 5.63.

Preparation of 3-Quinuclidinyl Hydrogen 1,2-Cyclohexane Dicarboxylate—Into a 3-necked flask equipped with a stirrer, dropping funnel, and condenser were added 11.6 Gm. (0.075 mole) of 1,2-cyclohexanedicarboxylic anhydride and 125 ml. of dry benzene. The mixture was heated and 20 ml. of pyridine was added. Then 9.6 Gm. (0.075 mole) of 3-quinuclidinol dissolved in 50 ml. of hot dry benzene was added all at once. The mixture was refluxed for 15 hr. whereupon a clear, colorless solution resulted. The solution was distilled at atmospheric pressure to remove 125 ml. of benzene. The remaining solution was then allowed to stand at room temperature for 36 hr. at the end of which time a white precipitate had separated. The mixture was filtered and the gummy white residue was washed with anhydrous ether. The residue was dissolved in hot isopropanol and, after cooling, anhydrous ether was added until a white precipitate formed. This precipitate was recrystallized three times from isopropanol and ether to yield 18.1 Gm. (87.7%) of a white, crystalline product; m.p. 205–206°.

Anal.—Calcd. for $C_{15}H_{23}NO_4$: C, 64.03; H, 8.24; N, 4.98. Found: C, 63.88; H, 8.30; N, 4.87.

Preparation of 3-Quinuclidinyl Hydrogen 2,3-Pyridinedicarboxylate—Into a 3-necked flask equipped with a stirrer, dropping funnel, and condenser were added 11.18 Gm. (0.075 mole) of 2,3-pyridinedicarboxylic anhydride and 150 ml. of dry benzene. The mixture was heated and 10 ml. of pyridine was added. Then, 9.6 Gm. (0.075 mole) of 3-quinuclidinol dissolved in 50 ml. of hot dry benzene was added all at once. The mixture was refluxed for 6 hr. whereupon a white precipitate formed. The mixture was cooled to room temperature and filtered with suction. This precipitate was recrystallized three times from isopropanol and ether to yield 19.6 Gm. (94.7%) of a white crystalline product; m.p. 218°.

Anal.—Calcd for $C_{14}H_{16}N_2O_4$: C, 60.86; H, 5.84; N, 10.14. Found: C, 59.86; H, 6.09; N, 10.10.

Preparation of 3-Quinuclidinyl Hydrogen n-Decylsuccinate—Into a 3-necked flask equipped with a stirrer, dropping funnel, and condenser were added 18.0 Gm. (0.075 mole) of *n*-decylsuccinic anhydride and 100 ml. of dry benzene. The mixture was heated and 25 ml. of pyridine was added. Then, 9.6 Gm. (0.075 mole) of 3-quinuclidinol, dissolved in 50 ml. of hot dry benzene, was added all at once. The mixture immediately developed an amber color. The mixture was refluxed for 18 hr. whereupon a clear, colorless solution resulted. The solution was distilled at atmospheric pressure until only a viscous oil remained. Upon cooling, the oil solidified to a white, waxy material. This was dissolved in a minimum amount of hot isopropanol and after cooling, anhydrous ether was added until a white precipitate formed. This precipitate was recrystallized three times from isopropanol and ether to yield 24.8 Gm. (89.8%) of a white, crystalline product; m.p. 100–103°.

Anal.—Calcd. for $C_{21}H_{37}NO_4$: C, 68.63; H, 10.15; N, 3.81. Found: C, 67.94; H, 10.18; N, 3.77.

Preparation of 3-Quinuclidinyl Hydrogen n-Hexadecylsuccinate—Into a 3-necked flask equipped with a stirrer, dropping funnel, and condenser were added 24.3 Gm. (0.075 mole) of *n*-hexadecylsuccinic anhydride and 125 ml. of dry benzene. The mixture was heated and 25 ml. of pyridine was added. Then 9.6 Gm. (0.075 mole) 3-quinuclidinol, dissolved in 50 ml. of hot dry benzene, was added all at once. The mixture immediately developed an amber color. The mixture was refluxed for 18 hr. whereupon a clear, colorless solution resulted. The solution was distilled at atmospheric pressure leaving a viscous oil. Upon cooling, the oil solidified to a white, waxy material. This was dissolved in the minimum amount of hot isopropanol and, after cooling, anhydrous ether was added until a white precipitate formed. This precipitate was recrystallized three times from isopropanol and ether to yield 30.7 Gm. (89.5%) of a white, crystalline product; m.p. 82–85°.

Anal.—Calcd. for $C_{27}H_{49}NO_4$: C, 71.79; H, 10.94; N, 3.10. Found: C, 71.05; H, 11.20; N, 3.27.

Attempted Preparation of 3-Quinuclidinyl Hydrogen Homophthalate—Into a 3-necked flask equipped with a stirrer, dropping funnel, and condenser were added 12.2 Gm. (0.075 mole) of homophthalic anhydride and 12.5 ml. of dry benzene. The mixture was heated and 15 ml. of pyridine was added. Then 9.6 Gm. (0.075 mole) of 3-quinuclidinol, dissolved in 50 ml. of hot, dry benzene, was added all at once. A dark brown gelatinous mass formed immediately. The mixture was refluxed for 8 hr. at the end of which time a clear amber solution resulted.

All attempts to extract the product from this solution yielded a brown, gelatinous, hygroscopic mass. Repeated trials to produce a crystalline compound (by purification and salt formation) failed.

The IR spectrum of this compound showed the characteristic ester bands and the compound gave a positive hydroxamic acid test.

PHARMACOLOGY

All compounds were tested as aqueous solutions of the ester free base. Although each of the compounds synthesized has not, as yet, been investigated, information is available for a preliminary report. The conclusions drawn from the results should be accepted as tentative and preliminary to more thorough investigation.[2]

Local Anesthetic Activity: Procedure—Four rabbits were placed in rabbit boxes and the hair around the eyes was carefully clipped. The winking reflex was tested by gently touching the cornea with a glass rod. The lower lid of the rabbit's conjuctival sac was held to form a cup, and then four drops of a 1% solution of either procaine or one of the drugs tested were instilled. After instillation of the drug, the solution was allowed to remain in contact with the surface of the eyeball for 2 min. by gently pressing the lids together. Anesthesia was tested for, after 2 min., by touching the glass rod to the cornea. This testing was done at regular intervals in order to note the time required for the winking reflex to return to normal. After recovery from the effects of the drugs, a second trial was made (following the above procedure) in order to compare the duration of anesthesia with that attained in the first trial.

Results—Procaine was utilized for comparative anesthetic effects. Of the seven esters tested, five produced anesthesia in both trials for varying lengths of time. The two esters that gave negative results failed to produce anesthesia in either trial. All results are tabulated in Table I.

Vasomotor and Respiratory Activity—Compounds tested were 3-quinuclidinyl hydrogen glutarate and 3-quinuclidinyl hydrogen 2,3-pyridinedicarboxylate. A male dog was anesthetized with an i.p. injection of sodium pentobarbital. The dose given was 30 mg./Kg. of body weight. The animal was strapped to a bench and the hair was shaved from the neck region. A thin, longitudinal strip of skin in the middle of the neck was cut with a scissors to expose the longitudinal muscles and these were separated along the midline with a blunt forceps. The left carotid artery located in the carotid sheath beside and slightly below the trachea was exposed. A ligature was tied around the artery peripherally and a loose ligature was placed around the artery centrally. A bulldog clamp was placed on the most central portion (beyond the loose ligature) of the artery. During this procedure the entire exposed area was flooded with 10% sodium citrate solution. Then, a fluid-filled system for recording blood pressure was prepared. The system included a Bourdon blood pressure transducer attached to a sodium citrate filled glass cannula. This cannula, which was inserted into the artery, lead to the transducer which detected pressure changes and sent impulses to the recorder. A fluid filled system which included a U-shaped glass tube filled with mercury was used to standardize the blood pressure at intervals to be used as reference points. This was done by squeezing a rubber bulb to change the height of the mercury. After standardization, this manometer-type apparatus was disconnected.

[2] Procedures employed in the pharmacological evaluation were taken from: Mann, D. E., "A Laboratory Manual of Pharmacology," 3rd ed., Temple University, School of Pharmacy, Philadelphia, Pa., 1962–1964.

TABLE I—LOCAL ANESTHETIC ACTIVITY[a]

Compound Tested	Duration of Anesthesia, min.
Trial I	
Procaine	42
1	...
2	20
3	12
4	10
5	...
7	6
8	14
Trial II	
Procaine	28
1	...
2	15
3	10
4	10
5	...
7	12
8	15

[a] The duration of anesthesia does not include the 2 min. when the lids were compressed. All drugs were administered in a dose of 4 drops of 1% aqueous solution.

A V-shaped opening was cut halfway through the artery close to the peripheral ligature to allow for adequate insertion space. The artery was placed over the tip of the cannula and the loose ligature was tied securely around the arterial flap on the tip of the cannula. The bulldog clamp was removed and the area was checked for leakage of blood. The stopcock was opened and the pressure was recorded on the physiograph. Respiration was recorded by means of an impedance pneumograph which was attached to the skin on the chest of the animal.

The thigh area of the dog was shaved and the femoral vein exposed. It was cleared of extraneous matter and two ligatures were slipped around the vein. The vein was cut open and a needle was inserted. Each compound to be tested was injected through the exposed femoral vein. All exposed areas on the dog were kept saturated with the citrate solution.

There was a substantial increase in blood pressure produced upon the administration of 10 mg. of each of the compounds tested. Both compounds produced a marked increase in respiration.

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(12) *Ibid.*, pp. 161–163.
(13) *Ibid.*, p. 153.

Keyphrases

3-Quinuclidinol dibasic acid esters—synthesis
Alcohol-anhydride acylation reaction
Anesthetic activity, local—3-quinuclidinol esters
Vasomotor activity—3-quinuclidinol esters
Respiratory activity—3-quinuclidinol esters

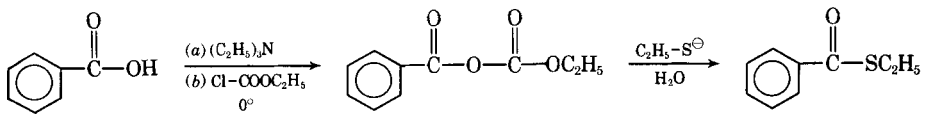
α-Alkyloximino Acid Thiol Esters: New Class of Compounds Showing Antimalarial Activity

By DOMINICK A. COVIELLO and NORMAN L. HINES

New derivatives of α-alkyloximino acids, the thiol esters, were prepared by modification of an application of mixed carboxylic-carbonic anhydrides. In general, the compounds show modest antimalarial activity, though one prolongs the lives of infected mice for more than 3 days longer than controls.

IT IS THE PURPOSE of this communication to report the antimalarial activity of α-alkyloximinocarboxylic acid thiol esters prepared by a modification of a reported method (1). The method utilizes mixed carboxylic-carbonic anhydrides (acyl alkyl carbonates) which are decomposed by a mercaptan in the presence of triethylamine. The authors have found that aqueous solutions of mercaptides are equally efficient in the anhydride decomposition. The modification was inspired by a report that carboxamides are produced by reacting the acyl alkyl carbonates with aqueous ammonia (2) indicating that anhydrous conditions are not essential for success in the anhydride reaction.

In a model reaction, ethyl thiolbenzoate was prepared as follows:



It was found that a better elemental analysis for the product could be obtained in the case of the title compounds through the modified procedure.

The compounds prepared are shown in Table I.

Biological Testing—Groups of five mice were infected with *Plasmodium berghii*. Three days after challenge test compounds were administered subcutaneously The data are reported in Table II.

EXPERIMENTAL

α-Ethyloximinopropionic Acid—This was obtained in 50% yield according to the procedure described by Ferris (3). It melted at 68–69°. Reported: 68–70°.

α-Benzyloximinopropionic Acid—The product was obtained by hypochlorite oxidation of the previously reported α-benzyloximinobutanone (3). The product melting at 84–85° was obtained in 52% yield. Reported melting point 84–85° (3).

α-Methyloximinobutyric Acid—Oxidation of α-methyloximino-2-pentanone in the manner described above gave a 13% yield of acid melting at 75–77°. Its confirmation was by elemental analysis of the thiol ester derivative (see Table I).

α-Ethyloximinobutyric Acid—The acid was prepared according to the procedure of Waters and Hartung (4). The yield was 48% and the melting point 58–59° was in agreement with the reported value.

α-Benzyloximinobutyric Acid—The product was obtained in 55% yield and had a melting point of 89–94° which was in agreement with the reported value (5).

α-Ethyloximinocaproic Acid—This was prepared in 82% yield according to the method of Waters and Hartung (5) and its boiling point of 76–80°/0.05

Received August 8, 1966, from the College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612

Accepted for publication October 13, 1967.

This investigation was supported by the University of Illinois Research Board and grant RH-00293-01 (MCHB) and RH-00293-02 (MCHB), National Center for Radiological Health, U. S. Public Health Service, Bethesda, Md.

Antimalarial testing and test data have been made available through the courtesy of Dr. David Jacobus, Walter Reed Army Institute of Research, Washington, D. C.

The technical assistance of J. C. Ziebell, W. L. Jacobson, and L. Sternson is also acknowledged.

Keyphrases

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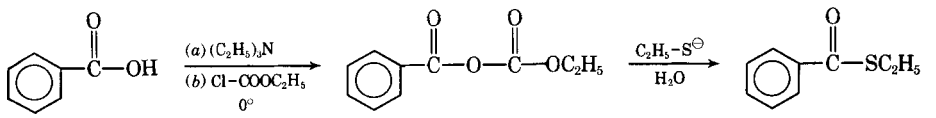
By DOMINICK A. COVIELLO and NORMAN L. HINES

New derivatives of α-alkyloximino acids, the thiol esters, were prepared by modification of an application of mixed carboxylic-carbonic anhydrides. In general, the compounds show modest antimalarial activity, though one prolongs the lives of infected mice for more than 3 days longer than controls.

IT IS THE PURPOSE of this communication to report the antimalarial activity of α-alkyloximinocarboxylic acid thiol esters prepared by a modification of a reported method (1). The method utilizes mixed carboxylic-carbonic anhydrides (acyl alkyl carbonates) which are decomposed by a mercaptan in the presence of triethylamine. The authors have found that aqueous solutions of mercaptides are equally efficient in the anhydride decomposition. The modification was inspired by a report that carboxamides are produced by reacting the acyl alkyl carbonates with aqueous ammonia (2) indicating that anhydrous conditions are not essential for success in the anhydride reaction.

In a model reaction, ethyl thiolbenzoate was prepared as follows:



It was found that a better elemental analysis for the product could be obtained in the case of the title compounds through the modified procedure.

The compounds prepared are shown in Table I.

Biological Testing—Groups of five mice were infected with *Plasmodium berghii*. Three days after challenge test compounds were administered subcutaneously The data are reported in Table II.

EXPERIMENTAL

α-Ethyloximinopropionic Acid—This was obtained in 50% yield according to the procedure described by Ferris (3). It melted at 68–69°. Reported: 68–70°.

α-Benzyloximinopropionic Acid—The product was obtained by hypochlorite oxidation of the previously reported α-benzyloximinobutanone (3). The product melting at 84–85° was obtained in 52% yield. Reported melting point 84–85° (3).

α-Methyloximinobutyric Acid—Oxidation of α-methyloximino-2-pentanone in the manner described above gave a 13% yield of acid melting at 75–77°. Its confirmation was by elemental analysis of the thiol ester derivative (see Table I).

α-Ethyloximinobutyric Acid—The acid was prepared according to the procedure of Waters and Hartung (4). The yield was 48% and the melting point 58–59° was in agreement with the reported value.

α-Benzyloximinobutyric Acid—The product was obtained in 55% yield and had a melting point of 89–94° which was in agreement with the reported value (5).

α-Ethyloximinocaproic Acid—This was prepared in 82% yield according to the method of Waters and Hartung (5) and its boiling point of 76–80°/0.05

Received August 8, 1966, from the College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612

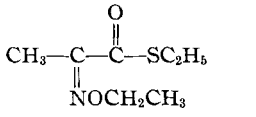
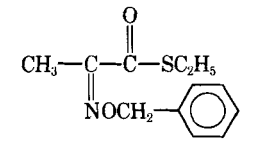
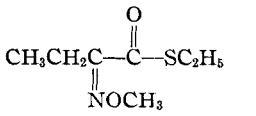
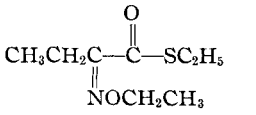
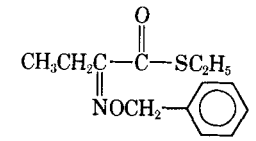
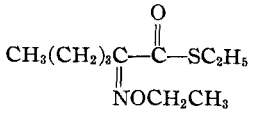
Accepted for publication October 13, 1967.

This investigation was supported by the University of Illinois Research Board and grant RH-00293-01 (MCHB) and RH-00293-02 (MCHB), National Center for Radiological Health, U. S. Public Health Service, Bethesda, Md.

Antimalarial testing and test data have been made available through the courtesy of Dr. David Jacobus, Walter Reed Army Institute of Research, Washington, D. C.

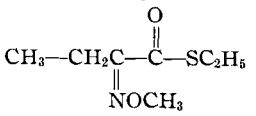
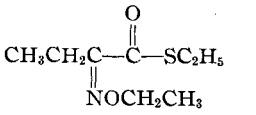
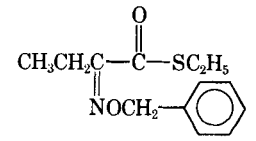
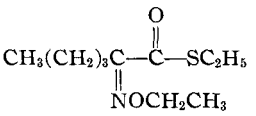
The technical assistance of J. C. Ziebell, W. L. Jacobson, and L. Sternson is also acknowledged.

TABLE I—α-ALKYLOXIMINO ACID THIOL ESTERS

Compd.	B.p., °C.	Yield, %	$\eta_{D/t}$	Anal.—Calcd.	Anal.—Found
	49–50/0.03 mm.	67	1.4864/24°	C, 48.00 H, 7.43 N, 8.00 S, 18.30	47.85[a] 7.61 8.09 18.21
	110–112/0.01 mm.	47	1.4861/28°	C, 60.72 H, 6.39 N, 5.91 S, 13.49	60.95[a] 6.29 5.95 13.38
	48–49/0.01 mm.	66	1.4851/30°	C, 48.00 H, 7.43 N, 8.00 S, 18.30	48.24[b] 7.60 8.00 18.41
	50–52/0.01 mm.	63	1.462/25°	C, 50.79 H, 7.94 N, 7.41 S, 16.93	51.23[b] 8.14 7.56 16.79
	114–16/0.07 mm.	71	1.541/21°	C, 62.15 H, 6.77 N, 5.58 S, 12.75	62.18[b] 6.86 5.51 12.73
	67–68/0.02 mm.	37	1.4756/25°	C, 55.23 H, 8.84 N, 6.45 S, 14.76	55.35[a] 8.87 6.38 14.71

[a] Analyses performed by Kurt Eder, Geneva, Switzerland. [b] Analyses performed by Micro-Tech Laboratories, Skokie, Ill.

TABLE II—EFFECT OF α-ALKYLOXIMINO THIOL ESTERS ON MALARIA-INFECTED MICE

Compd.	Dose, mg./Kg.	Mean Survival Time, Days		Change in Survival Time, Days	Toxic Deaths
	40	7.2	6.4	+0.8	
	160	7.4	6.4	+1.0	
	640	7.8	6.4	+1.4	
	40	6.8	6.4	+0.4	
	160	6.8	6.4	+0.4	
	640	6.8	6.4	+0.4	
	40	7.8	6.7	+1.1	
	160	10.0	6.7	+3.3	
	640				5[a]
	40	7.0	6.4	+0.6	
	160	7.0	6.4	+0.6	
	640	8.2	6.4	+1.8	

[a] Deaths occurring on days 2, 3, 4, and 5, after infection, are attributed to drug action and counted as toxic deaths. Control animals do not die before day 6.

mm. was in agreement with the reported value.

Ethyl Thiolbenzoate—In a 1-L., round-bottom flask was placed 200 ml. of anhydrous ether and 12.2 Gm. (0.1 mole) of benzoic acid. The solution was then cooled to 0° with an ice bath and 10.12 Gm. (0.1 mole) of triethylamine was added followed by 10.85 Gm. (0.1 mole) of ethyl chlorocarbonate, at such a rate as to maintain the temperature at 0–5°. The solution was then stirred for 30 min. in the ice bath. The triethylamine hydrochloride was

filtered off and 50 ml. of a 4 *M* aqueous solution of sodium ethylmercaptide was added. The solution was allowed to stir for 1 hr. at room temperature. The ether layer was separated and the aqueous layer was extracted with two 50-ml. portions of ether. The ethereal layers were combined and dried overnight with anhydrous magnesium sulfate. The magnesium sulfate was removed by filtration and the ether removed by distillation. The residual oil was then distilled under reduced pressure and the fraction distilling between 59–60°/.075 mm. was collected. The product weighed 11.95 Gm. (71.98%) and its refractive index was 1.5690 at 20.5°. The reported boiling point is 83–84° at 2.5 mm. and refractive index is 1.5678 at 25° (5).

α-Alkyloximinocarboxylic Acid Thiol Esters—The title compounds were prepared in the manner described for the preparation of ethyl thiolbenzoate. Pertinent data on the compounds are summarized in Table I.

REFERENCES

(1) Ciba Ltd., Brit. pat. 780,943 (August 14, 1957); through *Chem. Abstr.*, **52**, 11904c(1958); Schwyzer, R., Ciba Pharmaceutical Products, Inc., U. S. pat. 2,824,863 (Feb. 25, 1958); through *Chem. Abstr.*, **52**, 14689c(1958).

(2) Woolley, D. W., Hershey, J. W. B., and Jodlowski, H. A., *J. Org. Chem.*, **28**, 2012(1963).

(3) Ferris, A. F., *ibid.*, **24**, 1726(1959).

(4) Waters, K. C., and Hartung, W. H., *ibid.*, **12**, 469 (1947).

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(6) "Handbook of Chemistry and Physics," Chemical Rubber Publishing Co., Cleveland, Ohio, 1959.

Keyphrases

α-Alkyloximino acid thiol esters—synthesis
Mercaptan decomposition of acyl alkyl carbonates
Refractive index
Antimalarial activity

Molecular Association of the Antibiotic Prasinomycins

By JOEL KIRSCHBAUM

The molecular weight of the antibiotic prasinomycins in ethanol is 1600–2300. In aqueous solvents the prasinomycins associate, forming two aggregates with molecular weights of approximately 31,000 and 61,000. The aggregates may be disrupted by organic solvents, dilution below 0.012 percent, or the addition of salts.

THE prasinomycins are a family of closely related antibiotics isolated from *Streptomyces prasinus* (1). Although the molecular weight of one major component of the mixture, as calculated from phosphate group analysis (1), was 1550 ± 10%, the inability to dialyze these antibiotics through a cellulose membrane indicated the possibility of molecular association. The molecular weight in ethanol, determined by means of an analytical ultracentrifuge,[1] ranged between 1600 and 2300. In aqueous solvents the apparent molecular weights were 31,000 and 61,000. These solvent-induced differences in molecular weight indicate association or aggregation. Some of the properties of these aggregates will be described in this paper.

EXPERIMENTAL

Sedimentation coefficients of prasinomycin solutions of concentrations from 10 mg./ml. to 1.25 mg./ml. were measured in 4°, 12-mm. cells centrifuged in an An-D rotor at 42,040 r.p.m. and 20.7°. The phaseplate angle of the schlieren optical system was 75°. For solutions with concentrations of 1.25 mg./ml. to 0.25 mg./ml., the sedimentation coefficients (s) were determined in a 12-mm. synthetic boundary centerpiece in an interference cell.

Molecular weights were determined using equilibrium and Archibald approach-to-sedimentation equilibrium methods.

For the Archibald (2) method, 5–10 mg./ml. solutions in 12-mm. interference cells were centrifuged in an An-D rotor at 20.7°. Concentration gradients were formed at speeds of 6995, 8766, 12,590, 20,410, 29,500, and 42,040 r.p.m. to test for any dependence of molecular weight on speed, which, in turn, affects concentration of the sedimenting material. The schlieren optical system was used with a phaseplate angle of either 75° or 80°. The concentration gradients at the meniscus and cell bottom were measured from data recorded on Kodak metallographic plates magnified 10-fold on a Nikon magnifier. Engelberg's technique (3) for evaluating the concentration gradient integral was used. Total concentrations were determined in separate experiments using a synthetic boundary centerpiece. An IBM 1620 computer was used for some of the repetitive calculations.

Equilibrium centrifugation (4) aided in obtaining the molecular weights of prasinomycin solutions of 0.15 mg./ml. and lower in concentration. Using a calibrated syringe, 0.03 ml. of prasinomycin solution was injected into one compartment of an interference cell fitted with sapphire windows and layered with fluorochemical FC 43 (Beckman Instruments, Inc.). Solvent was injected into the reference compartment. The final percentage

Received September 5, 1967, from the Squibb Institute for medical research, New Brunswick, NJ 08903

Accepted for publication October 27, 1967.

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[1] Model E Analytical Ultracentrifuge, Spinco Division, Beckman Instruments, Inc.

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of prasinomycin in the solution compartment was determined by two methods: (*a*) photographically measuring the height of liquid in each compartment of the rotating cell, before and after flow of solvent into the solution compartment and measuring the cell centerpiece cross-section, and (*b*) combining the weight of solvent and solution added to a cell with the densities of liquids.

Diffusion constants (5) were evaluated in the analytical ultracentrifuge at 20.7°, using a synthetic boundary centerpiece and the schlieren optical system. Initial solute concentrations were from 10 to 2 mg./ml. The first image was recorded at two-thirds of the final speed of 29,500 r.p.m. This first picture was considered to be the zero-time image for diffusion constant calculations

The partial specific volume was determined at 20° in a 1-ml. pycnometer. Viscosities were determined with the aid of capillary and Zimm (6) viscometers.

RESULTS AND DISCUSSION

The minimum molecular weights of the prasinomycins, as determined by approach-to-equilibrium sedimentation in either 100% ethanol or in 90% ethanol–10% 0.2 *M* NaCl–0.02 *M* sodium phosphate buffer (pH 6.85), are identical within experimental error and are between 1,600 and 2,300 daltons (Table I). The small quantity of inorganic buffer in the ethanol diminishes charge-charge interactions that may lead to 5 to 20% erroneously lower molecular weight. A minor component, molecular weight approximately 7,200, is evident which may be a tetramer of the prasinomycins. In NaCl–sodium phosphate buffer the molecular weight of the prasinomycins was calculated to be 31,000 daltons, within a 3% experimental error. This is an aggregate since almost all of the solute prasinomycin could be accounted for as the 31,000 dalton component. Occasionally, traces of a component of higher molecular weight were evident; apparent mol. wt. 60,000 daltons. In 0.072 *M* borate buffer, pH 8.6, only the higher molecular weight component, mol. wt. = 61,500, was present. No dependence of molecular weight on speed was found.

The 31,000 dalton aggregate of prasinomycin is stable in various aqueous buffers ranging from pH 4.05 to pH 10.4 (Table I). In pH 12 glycine–NaOH buffer the aggregate is disrupted since the molecular weight is approximately 2,000, with a trace of a 3,600 molecular weight component present.

Verification of the 31,000 dalton molecular weight determined in aqueous solvents and the 1,600–2,300 molecular weight determined in ethanolic solutions was achieved by combining the sedimentation and diffusion constant measurements (7). The aggregate sedimentation constant (*s*) of 4.0 *S* ($S = 10^{-13}$ cm. sec.$^{-1}$) at zero concentration and 20°, together with the diffusion constant, $D^0_{20,w} = 9.0 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ of the aggregate, yields a mol. wt. of 30,000 daltons. The diffusion constant of prasinomycin in ethanol is 2.2×10^{-6} cm.^{2} sec.$^{-1}$ (which itself indicates that prasinomycin in ethanol has a lower molecular weight than prasinomycin in aqueous solvents). Combination with the $D^0_{20,w}$ value of $0.82–1.02 \times 10^{-13}$ cm.^{2} sec.$^{-1}$ gives an approximate mol. wt. of 2,100 daltons for unaggregated prasinomycin, in good agreement with the 1,600–2,300 mol. wt. determined by approach-to-equilibrium sedimentation.

Since the molecular weight of the prasinomycins in ethanolic solutions is 1,600–2,300 and the apparent molecular weight of the prasinomycins in aqueous solvents is 31,000, an obvious question is: what is the apparent molecular weight of the prasinomycins in mixtures of ethanol and aqueous solvents. In Fig. 1 are the curves depicting the changes in molecular weight of the prasinomycins as (*a*) the free acid of prasinomycin dissolved in ethanol is diluted with NaCl–sodium phosphate buffer, and (*b*) sodium salt, of prasinomycin, initially dissolved in NaCl–sodium phosphate buffer is diluted with ethanol. Molecular weights were determined after a 3–18 hr. equilibration. Per-

TABLE I—MOLECULAR WEIGHTS OF THE THREE MAJOR PRASINOMYCINS A, B, AND C DISSOLVED IN VARIOUS SOLVENTS

Form of Prasinomycin	Prasinomycin	Solvent	Mol. Wt. Major Component	Mol. Wt. Minor Component
Free acid	A	Ethanol–phosphate buffer	2300	7300
Methyl ester	A	Ethanol	2100	6400–7600
Free acid	A	Ethanol	2000	7400
NH_4^+ Salt	A	Ethanol–phosphate buffer	2150	6800
Free acid	B	Ethanol	1600	7400
Free acid	B	Ethanol–phosphate buffer	1850	7500
Na^+ Salt	B	Ethanol–phosphate buffer	1700	7400
Free acid	C	Ethanol–phosphate buffer	2200	7100
Free acid	C	Ethanol	1850	7200
Na^+ Salt	C	Ethanol	1650	...
Methyl ester	C	Ethanol	2100	7200
K^+ Salt	A,B	Ethanol–phosphate buffer	1700	6000
Free acid	A,B,C	Phosphate buffer	31000 ± 3%	61000 ± 5%
Na^+ Salt	A,B,C	Phosphate buffer	31000 ± 3%	61000 ± 5%
Na^+ Salt	B	pH 4.05, 1 *M* acetate buffer	32000	
Na^+ Salt	B	pH 10.4, 0.05 *M* glycine–NaOH buffer	32000	
Na^+ Salt	B	pH 12, 0.17 *M* glycine–NaOH buffer	2000	3600
Na^+ Salt	B	Isotonic buffer, containing K^+, Na^+, Ca^{2+}, Mg^{2+}, Cl^-, $SO_4^=$ and $PO_4^=$	31000	

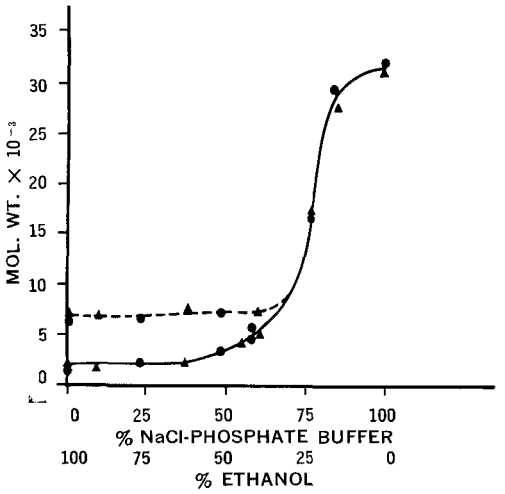

Fig. 1—Changes in mol. wt. of prasinomycin caused by changing the solvent: ●, free acid of prasinomycin initially dissolved in ethanol diluted with NaCl-sodium phosphate buffer; ▲, sodium salt of prasinomycin dissolved in NaCl-sodium phosphate buffer diluted with ethanol. Note existence of tetramer of mol. wt. 7,200–7,600 daltons (broken line) at ethanol concentrations from 30 to 100%.

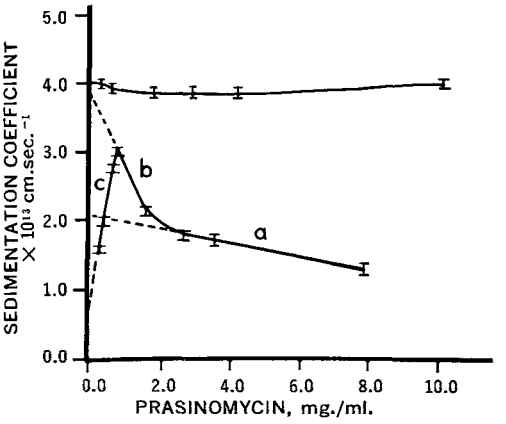

Fig. 2—Dependence of the sedimentation coefficient of prasinomycin on its concentration in 0.2 M NaCl–0.02 M sodium phosphate buffer, pH 6.9, upper curve and in 5.0 M NaCl–0.02 M sodium phosphate buffer, pH = 6.9, lower curve.

sistently found below 65% NaCl–sodium phosphate buffer–35% ethanol is the 7,200 dalton compound identified tentatively as tetramer. The two molecular weight curves, including tetramer branching, are superimposable, as expected, since this procedure of adding the free acid form to buffered solvent is analogous to adding acid to aqueous sodium phosphate.

As seen in Fig. 2, upper curve, variation of concentration from 10 mg./ml. to 0.25 mg./ml. for prasinomycin dissolved in 0.2 *M* NaCl–0.02 *M* sodium phosphate buffer does not appear to result in the dissociation of the aggregate, since $s_{20,w}$ = 4.0 *S* at both the initial and final concentrations. However, the curvature of the line is indicative of some dissociation or rearrangement. When the NaCl concentration of the buffer was increased to 5 *M*, a complex figure was obtained (Fig. 2 lower curve). If the three portions of the curve, labeled a, b, and c are extrapolated to zero concentration (broken lines) and the *s* values measured, a is equal to 2.05 *S*, b is equal to 3.9 *S*, and c is equal to 1.1 *S*; all values corrected for viscosity and density. The sedimentation coefficient of b, *s* = 3.9 *S*, may be considered to be equivalent to a molecular weight of 31,000 daltons. Substituting these values into the formula, molecular weight = $f_s^{2.2}$, *f* is equal to 1,550. The calculated molecular weight of *a* is approximately 7,500 daltons and that of *c* is approximately 1,700 daltons. These molecular weight values approximate closely monomer and tetramer molecular weights.

Since sedimentation coefficient values of prasinomycin solutions below 0.25 mg./ml. cannot be measured by the schlieren optical system, the interference optical system was used, and molecular weights were determined by sedimentation equilibrium methods. At a concentration of 0.012%, two components, with approximate molecular weights of 3,600 and 2,000 were calculated. At 0.005% only a 1,800–2,000 molecular weight component was discernible.

The various molecular weights of prasinomycin found in this study are 1,800–2,300, 3,600, 7,200–6,600, 15,000, 31,000 and, under certain conditions, 60,000–62,000 daltons. It appears that aggregation proceeds by repeated dimerization of the monomer to the 31,000 dalton aggregate.

The aggregate may be stabilized by electrostatic (8) interactions since increasing the ionic strength can dissociate the complex. Conversely, by using water alone as solvent (0 ionic strength but for added prasinomycin), the observed sedimentation coefficient is 5.4 *S*. This *s* value is equivalent to the 61,000 dalton aggregate and is the highest molecular weight aggregate observed.

Cleavage (9) of one prasinomycin species gives a 1,400 dalton and a 350 dalton fragment. The 350 dalton moiety was found to be a hydrocarbon (9). The 1,400 dalton fragment did not aggregate, indicating that the association of the prasinomycins may also require hydrophobic bonding (10).

Hydrogen bonding may not have a role in prasinomycin aggregation because 8 *M* urea did not affect the sedimentation coefficient of the aggregate (*s* = 3.9 *S*), after best correction (11) for density and viscosity.

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Keyphrases

Prasinomycins—molecular association

Molecular weight—approach-to-equilibrium method

Sedimentation coefficients—schlieren, interference optical systems

Diffusion constants—centrifuge, schlieren optical system

Gas-Liquid Chromatographic Determination of Lincomycin

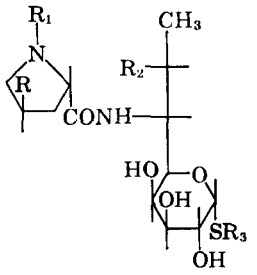
By R. L. HOUTMAN, D. G. KAISER, and A. J. TARASZKA

The gas-liquid chromatography of lincomycin and some of its analogs, determined as trimethylsilyl ethers, is reported. The application of this procedure to quantification of lincomycin in bulk material and pharmaceutical preparations is described.

THE APPLICATION of gas-liquid chromatography to polyhydroxy compounds has been greatly enhanced by the advent of methods for the quantitative conversion of these compounds to their trimethylsilyl ethers (1, 2). The silylation procedure consists of dissolving the material in pyridine and adding hexamethyldisilazane and trimethylchlorosilane.

Lincomycin (I) (3), a medium spectrum antibiotic containing an octopyranose moiety, was silanized by this procedure and chromatographed as the intact tetra trimethylsilyl ether. U-21699 (II), an antibiotic produced at about the 3% level in the microbiological synthesis of lincomycin (4) was chromatographed under the same conditions and gave a retention time of 0.85 relative to lincomycin. This separation enabled the quantification of small amounts of U-21699 in lincomycin as well as the determination of lincomycin in bulk material and pharmaceutical formulations.

Since the preparation of the trimethylsilyl ether derivatives of these compounds is rapid, quantitative, and can be applied on a micro scale, GLC analysis of lincomycin and some of its analogs has become a practical laboratory procedure.



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III. (U-21251F)
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R_3 = —CH_2CH_3

V. (U-11973E)
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R_2 = —OH
R_3 = —CH_3

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EXPERIMENTAL

Reagents and Materials—The lincomycin and analogs used in this study were prepared by the Research Division of The Upjohn Company. Hexamethyldisilizane and trimethylchlorosilane were obtained from the Dow Corning Co., Midland, Mich. A solution of tetraphenylcyclopentadienone[1] in pyridine (5 mg./ml.) was employed as the internal standard. The column packing utilized for gas-liquid chromatography was 3% (w/w) SE-30 on 100–120 mesh Gas Chrom Q.[2] All solvents were reagent grade and used as supplied.

Instrumentation—An F & M model 402 high efficiency gas chromatograph,[3] equipped with a flame ionization detector and U-shaped glass columns (2 m. long × 3 mm. i.d.) was utilized throughout the study. Helium was used as the carrier gas at a flow rate of 60 ml./min. Air and hydrogen flow rates were adjusted to give maximum response. The column oven was operated isothermally at 240°, the flash heater at 270°, and the detector at 290° Peak areas were measured with an Infotronics 11HSB/42 electronic integrator.[4]

Procedures—In general, the silanization technique of Makita and Wells (5) was used. For quantification of lincomycin, 50 mg. of the bulk drug was accurately weighed and dissolved in 10.0 ml. of the internal standard solution. One milliliter of hexamethyldisilizane and 0.5 ml. of trimethylchlorosilane were added. The solutions were allowed

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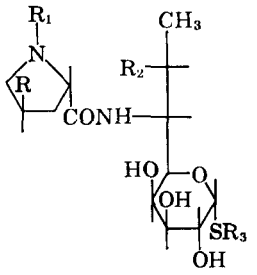

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TABLE I—ANALYTICAL DATA OF TMS DERIVATIVES

Lincomycin TMS			U-21251F TMS		
Assay	Theory	Found	Assay	Theory	Found
C	51.82	51.65	C	50.55	50.20
H	9.57	9.58	H	8.94	8.72
N	4.03	4.03	N	4.37	4.14
S	4.61	4.72	S	5.00	4.96
Mol. Wt.[a]	695	695	Cl	5.53	5.66
			Mol. Wt.[a]	640	640

[a] Determined by mass spectrometry.

to stand for 10 min. and then centrifuged prior to chromatography.

Silanizable excipients, such as sucrose and lactose, in formulations required some adjustment in the aforementioned reagent ratios but did not interfere with the quantification of lincomycin. One extreme example was an aqueous solution containing 650 mg. of sucrose and 50 mg. of lincomycin per ml. of formulation. Two milliliters of the solution was lyophilized, dissolved in 10 ml. of internal standard solution, and silanized with 5 ml. of hexamethyldisilazane and 2.5 ml. of trimethylcholorosilane. It was necessary to allow 30 min. for complete reaction. Similarly, centrifuged fermentation beers may be analyzed following lyophilization.

For qualitative examination or determination of analog ratios from fermentation samples, 10 mg. of material was dissolved in 1 ml. pyridine. Hexamethyldisilizane (0.2 ml.) and trimethylchlorosilane (0.1 ml.) were added. Reaction conditions were the same as described for the determination of lincomycin in the bulk drug.

For each series of samples, a reference solution was prepared by accurately weighing an amount of lincomycin reference standard approximately equivalent to that in the sample and treating it identically.

Calculations—The lincomycin concentration in the sample solutions was determined by a direct comparison of peak area ratios.

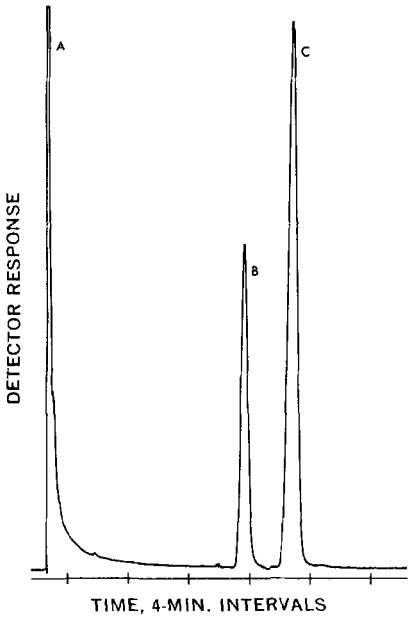

Fig. 1—Chromatogram of U-21699 and lincomycin, A = solvent, B = U-21699, C = lincomycin.

Characterization of Derivatives—Samples of the trimethylsilyl derivatives were collected at the exit port of the chromatograph for characterization. The collected samples were compared either by infrared spectroscopy or mass spectrometry to materials isolated from the reaction solutions. In the case of lincomycin and U-21251F (III) (6) sufficient sample was isolated to enable complete elemental and spectral analysis. These data are summarized in Table I.

RESULTS AND DISCUSSION

Quantification of U-21699 in Lincomycin—In the search for a suitable liquid phase for the separation and quantification of U-21699 in lincomycin, it was observed that any of the nonpolar liquid phases were acceptable. The SE-30 column described in the procedure was selected for its stability and general availability. After establishing conditions

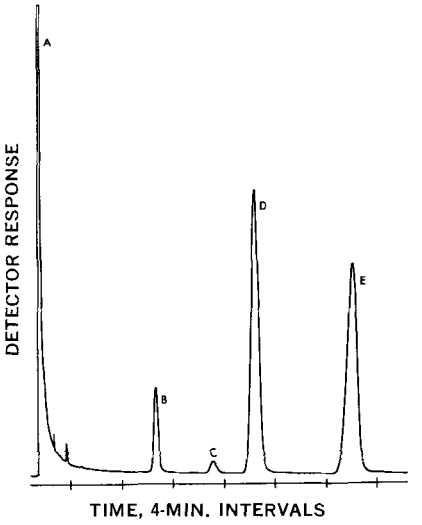

Fig. 2—Chromatogram of an assay preparation from a hard-filled capsule: A = solvent, B = lactose, C = U- 21699 and lactose, D = lincomycin, E = internal standard.

TABLE II—EXCIPIENT RETENTION TIMES

Compound	Relative Retention Time
Sorbitol	0.08
Sucrose	0.56
Lactose	0.81 and 0.54
Lincomycin	1.0
Internal standard (tetraphenylcyclopentadienone)	1.46

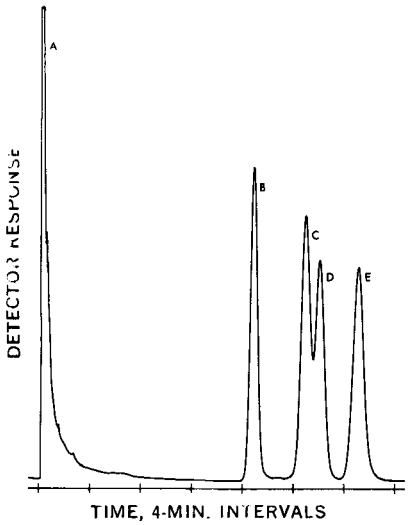

Fig. 3—Chromatogram of lincomycin analogs. A = solvent, B = U-21699, C = lincomycin, D = U-11921, E = U-11973.

for chromatography, the response factors for lincomycin and U-21699 were determined by silylating and chromatographing a 50–50 mixture of the two compounds. The difference in response per mg. for the two materials was less than 1%.

This small difference was insignificant in the determination of small amounts of U-21699 in lincomycin. The comparable response for lincomycin and U-21699 might have been predicted from the similarity of the compounds (I and II). One milligram each of lincomycin and U-21699, when weighed as the monohydrate hydrochloride, yield 0.781 and 0.778 mg. of carbon, respectively, as the trimethylsilylethers. This response relationship was further established by a recovery study. Eight prepared mixtures ranging from 0–10% U-21699 in lincomycin by weight were assayed. Statistical analysis of the data indicated a slope of 0.99.

A chromatogram of a production lot of lincomycin containing 2.7% of U-21699 is presented in Fig. 1. Note the change in attenuation allowing a measurable area for the minor component.

Twenty replicate assays, each on different days, of a sample of lincomycin containing 2.33% U-21699 gave a standard deviation of 0.108% for U-21699. This represents a coefficient of variation of 4.8% for the minor constituent.

Quantification of Lincomycin in Bulk Drug and Formulations—Tetraphenylcyclopentadienone, the internal standard used in the assay, had a retention time relative to lincomycin of 1.46 which minimized the interference of possible decomposition products. The relative retention times of common excipients used in various formulations were established and are shown in Table II.

Table III—Retention Times of Lincomycin Analogs

Compd.	Relative Retention Time	Derivative
II (U-21699)	0.81	Tetra TMS ether
III (U-21251F)	0.86	Tri TMS ether
I (Lincomycin)	1.00	Tetra TMS ether
IV (U-11921A) (7)	1.05	Tetra TMS ether
V (U-11973E) (8)	1.20	Tetra TMS ether
VI (U-24166A) (9)	1.22	Tetra TMS ether
Tetraphenylcyclopentadienone	1.46	...

Precision studies conducted on several formulations indicated that the coefficients of variation were all less than 1%. A chromatogram of an assay preparation from a hard-filled capsule is shown in Fig. 2.

Chromatography of Other Analogs—Figure 3 is a chromatogram of a synthetic mixture of lincomycin and three analogs to illustrate the degree of separation. It should be noted, however, that this is a hypothetical mixture and that only U-21699 is normally produced in the microbiological synthesis of lincomycin. Two other analogs, U-21251F and U-24166A, not shown in Fig. 2, were chromatographed using this technique and a summary of the retention times relative to lincomycin are shown in Table III.

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Keyphrases

Lincomycin—analysis
Trimethylsilyl ether—lincomycin
Analogs, lincomycin—characterized
GLC—analysis

Antitumor Agents from *Bursera microphylla* (*Burseraceae*) I. Isolation and Characterization of Deoxypodophyllotoxin

By E. BIANCHI, M. E. CALDWELL, and J. R. COLE

Deoxypodophyllotoxin has been isolated and characterized as one of several antitumor agents from *Bursera microphylla* (*Burseraceae*). β-Sitosterol has also been shown to be present in this plant.

As a result of a routine screen of Southwestern plants for potential antitumor activity, the chloroform extract of *Bursera microphylla* A. Gray (*Burseraceae*)[1] demonstrated activity toward the human epidermoid carcinoma of the nasopharynx test system (cell culture) of the CCNSC (9KB).

Bursera microphylla is a tree usually less than 30 ft. tall and can be found in Southwestern Arizona, Baja California, Sonora, and southward into Central Mexico. The material used in this research was collected in March and June from rocky dry slopes in the Gila mountains near Ligurta in Yuma County, Arizona. *Bursera microphylla*, as well as other *Bursera* species, have been the subject of previous investigations: various substances such as phellandrene and phellandral (1), α-hydroxylupene and α-amyrin (2), and others (3) have been reported.

The above materials did not show any tumor inhibition activity (4). The plant was then subjected to a systematic fractionation in order to characterize the active principle. The fractionation procedure involved the utilization of solvent extraction followed by adsorption chromatography on alumina, thick-layer chromatography on silica gel, and paper chromatography.

Four substances (I, II, III, and IV) were separated. Substance IV was identified as β-sitosterol on the basis of mixed melting point, superimposable infrared spectra of an authentic specimen, and a comparison of R_f values with a known specimen utilizing thin-layer chromatography. The solvent systems employed were dichloromethane–benzene–ethyl acetate (12:24:3) and ethyl ether–dichloromethane (1:5). Substances I, II, and III showed a strong tumor inhibition against the 9KB system. Substance III was identified as deoxypodophyllotoxin. This is somewhat unique as there are apparently no reports in the literature of this compound being isolated from this plant or any closely related genus and species. In addition, III was treated with a mild alkali and was converted into deoxypicropodophyllin (the "picro" or "B" series). Substance II was subjected to paper and thin-layer chromatography and showed a single spot but all attempts at crystallization failed. The isolation, purification, and characterization of this compound will be reported in a forthcoming publication. Substance I was not present in sufficient quantities to warrant further chemical investigation at this time but will also be investigated at a later date.

The preliminary chloroform extract exhibited activity of less than 1.0×10^{-2} mcg./ml. Deoxypodophyllotoxin showed activity in this system of 2.6×10^{-3} mcg./ml. Activity is defined as $ED_{50} \leqq 10$ mcg./ml. for plant extracts. Results are expressed as the dose that inhibits growth to 50% of control growth 3 days after drug addition (5).

EXPERIMENTAL

Extraction—The stems (12 Kg.) of *Bursera microphylla* were dried in the air, ground, and extracted exhaustively in a Soxhlet with chloroform. After removal of the solvent, the residue (470 Gm.) was treated several times with petroleum ether (b.p. 40–60°). The material obtained from the solution was inactive and, therefore, discarded. The insoluble part separated from the liquid by centrifugation was extracted with ethyl ether. The insoluble portion was inactive and discarded. The organic layer was stirred (not shaken in order to prevent emulsion) three times with a 10% aqueous solution of sodium carbonate. The ethereal extract, after removal of the alkali aqueous solution yielded the active material (Scheme I).

Isolation and Identification of Deoxypodophyllotoxin—The active material was chromatographed through a column of alumina (grade III). The second fraction obtained by elution with benzene yielded material showing improved activity. The fractions obtained by elution with chloroform, ethyl ether, acetone, and methanol were inactive and were discarded. The active fraction was then subjected to thin-layer chromatography. The matrix used was Silica Gel G, Merck. The solvent system employed was dichloromethane–benzene–ethyl acetate (12:24:3). A double run was employed. There appeared to be four predominant materials. In order to obtain each of these in a pure state, the mixture was applied on preparative chromatoplates which were run under the same general conditions. This procedure afforded more precise separation of the four substances designated as I, II, III, and IV. Substance I was neglected because the amount of material obtained was insufficient for further study at this time. Substance IV was identified as β-sitosterol by the procedures indicated above. The yield of β-sitosterol from the dry plant was approximately 0.021%. Since the resolution of II and III was not satisfactory because of the closeness of their R_f values when the above indicated solvent system was used, they were eluted together, applied on preparative chromatoplates (1 mm. thick), and

Received September 18, 1967, from the Division of Pharmaceutical Chemistry, College of Pharmacy, University of Arizona, Tucson, AZ 85721

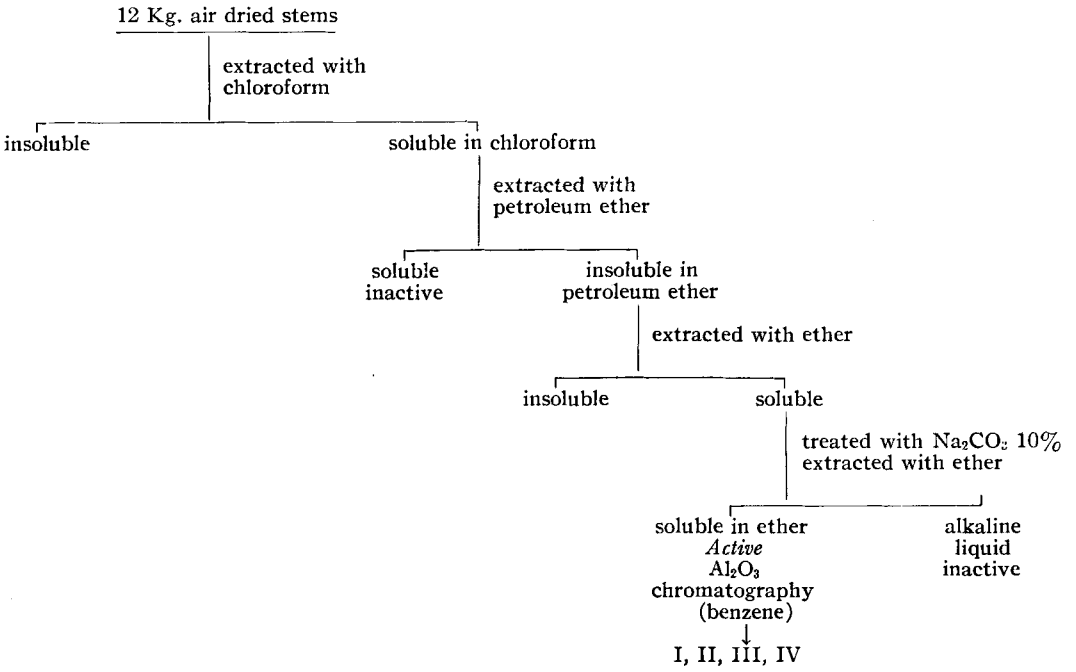
Accepted for publication November 1, 1967.

This investigation was supported in part by contract PH 43-63-1136, Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, and research grant CA 05076-MCHB, National Cancer Institute, U. S. Public Health Service, Bethesda, Md.

Presented at the IUPAC National Product Symposium, June 1966, Stockholm, Sweden.

[1] Identification confirmed by Robert Barr, College of Pharmacy, and Dr. Charles Mason, Botany Department University of Arizona, Tucson. A reference specimen was also deposited.

Schematic Presentation of Extraction
B. microphylla



Scheme I

run with a dichloromethane–ethyl acetate (5:1) system. This procedure gave good resolution for the separation of III. It was eluted with acetone and recrystallized from methanol, m.p. 162–163°; $[\alpha]_D$ −112° (chloroform). These data are in agreement with that reported by the literature for deoxypodophyllotoxin. Mass spectrometry indicated a parent ion of *m/e* 398 compatible with this compound. In addition, no depression in a mixed melting point and a superimposable infrared spectrum with an authentic sample further proved the identification of III as deoxypodophyllotoxin.[2] The yield of deoxypodophyllotoxin from the dry plant was approximately 0.09%.

Conversion of Deoxypodophyllotoxin into Deoxypicropodophyllin—To a solution of deoxypodophyllotoxin in methanol, a few drops of an aqueous methanolic solution of potassium bicarbonate was added and the mixture was allowed to stand overnight. The mixture was treated as indicated above and yielded deoxypicropodophyllin. The same result was obtained using sodium acetate as the basic catalyst for this conversion (6). The compound showed an inversion of the sign of the optical rotation, $[\alpha]_D$ +30° in chloroform. Its mass spectrum pattern again indicated a parent ion *m/e* 398 indicating that only a geometrical rearrangement took place during the alkali treatment.

CONCLUSIONS

Bursera microphylla has been the subject of a phytochemical investigation in order to determine the chemical agents responsible for the apparent antitumor activity in the 9KB system of the CCNSC. Deoxypodophyllotoxin has been isolated and characterized as being one of these agents. Two other agents have been isolated and will be the subject of future reports. β-Sitosterol has also been shown to be present in this plant.

[2] The authors are indebted to Dr. J. L. Hartwell of the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md., who provided a sample of deoxypodophyllotoxin.

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Keyphrases

Antitumor agents—*Bursera microphylla*
Deoxypodophyllotoxin—isolation, characterization
Chromatography, adsorption, thick layer, paper—separation
IR spectrophotometry—identity
TLC—identity
Mass spectroscopy—identity
Tumor inhibition, ED_{50}—deoxypodophyllotoxin

Effect of Some Nonionic Surfactants on the Rate of Absorption of Aminophylline from Suppositories in Rabbits

By J. M. PLAXCO, JR., and F. FOREMAN*

The rate of absorption of aminophylline from theobroma oil base suppositories containing 5 percent of various nonionic surfactants was determined. The results were erratic and unpredictable, with no correlation with *in vitro* results reported in a previous communication.

SUPPOSITORIES HAVE received much attention in recent years as a means of administering medication. In the U. S. more attention has been directed toward *in vitro* investigations of drug release. European literature (1–9) contains many reports of *in vivo* work using different drugs and various animals. A previous paper from this laboratory (10) reported the *in vitro* effects of some nonionic surfactants on the rate of release and dialysis of aminophylline and of ephedrine and ephedrine hydrochloride from theobroma oil suppositories.

The purpose of the current investigation was to determine the effects, *in vivo*, of these same surfactant-containing aminophylline suppositories. Because of the reports of Waxler and Schack (11) and Glass and his coworkers (12), it was felt that the concentration of aminophylline (theophylline) in the plasma was a reliable indicator of the rate of absorption of the drug. Waxler and Schack (11) showed that aminophylline (theophylline) is restricted to the plasma, does not penetrate the red cell membrane, and is bound only slightly to the proteins of the blood and to tissue proteins.

EXPERIMENTAL

Materials—The suppositories were those prepared and investigated for *in vitro* release (10). Each suppository contained 5% surfactant and 10 mg. of aminophylline in theobroma oil.

Procedure—Young male white rabbits weighing initially approximately 2 Kg. were used. At the conclusion of the investigation they weighed approximately 3–4 Kg. each. At least 1 week lapsed between tests on each rabbit to allow the rabbits to recuperate and to eliminate completely all traces of drug from the system. As many tests were run on each suppository as time permitted, usually 5 or 6 different rabbits per suppository, though in a few instances only 3 were used. Twenty rabbits were used, mainly in succession, which resulted in different animals testing one suppository. In a few instances in which repeat tests were run, the same animal was used. The variability was less using the same animal than when using different animals but unfortunately insufficient data were available for analysis.

The rabbits were fasted overnight to rid the rectum of fecal matter, and each experiment was started at approximately the same time each morning (9:00 a.m.) for uniformity and to allow ample time to use another rabbit if the suppository was expelled. After weighing the rabbit, a suppository was inserted into the rectum and pushed about 2 cm. with a glass rod lubricated with white petrolatum. No satisfactory method of closing the anus to prevent expulsion of the suppository was found, and occasionally it was expelled, necessitating the use of another rabbit. After insertion of the suppository the animal was returned to its cage for about one-half hr.

The rabbit was then placed in a rabbit holder with its head exposed. At the stated times, approximately 4 ml. of blood was collected from the marginal or medial ear veins in a heparinized tube and 3 ml. of this was pipeted into a separator containing 30 ml. of chloroform-isopropyl alcohol (20:1) mixture. The aminophylline (theophylline) concentration was determined by the method of Schack and Waxler (13) with modifications as noted.

Chloroform NF and isopropyl alcohol NF were freshly distilled weekly and stored in glass-stoppered bottles of 500 ml. capacity. This precaution of fresh solvents had previously been noted by Glass *et al.* (12) and others.

The 3 ml. of blood was extracted for 3 min. with two 30-ml. portions of chloroform–isopropyl alcohol mixture and the portions mixed. Following filtration, 40 ml. of the solution was extracted with 6.0 ml. of 0.1 *N* NaOH and centrifuged. The absorbance at 277 mμ was determined on a Beckman model DB spectrophotometer and compared with a standard curve prepared by adding a known amount of aminophylline to bovine blood and extracting, using the same technique as described. This method gave more uniform and reproducible results than using a standard curve prepared from the absorbance of varying concentrations of aminophylline in 0.1 *N* NaOH and correcting for the absorbance of the blood. Schack and Waxler (13) found "the mean blank value of human blood (300 analyses) to be an optical density of 0.07 ± 0.02 per cc. 0.1 *N* NaOH. Blood and tissues of other species follow this closely." Our experiments confirmed these values for rabbit, swine, and cattle blood. It was also found that following the insertion of blank surfactant-containing suppositories (no aminophylline) the same values were obtained.

A concentration of 10 mg. of aminophylline per suppository was satisfactory for the *in vitro* work but the plasma levels for this concentration were at the lower end of the curve for the *in vivo* work. It is believed that more uniform results would have been obtained with more than 10 mg. of aminophylline per suppository. However, the object of the investigation was to compare *in vitro* and *in vivo* release from the same batches of suppositories.

Received June 15, 1967, from the School of Pharmacy, University of South Carolina, Columbia, SC 29208
Accepted for publication October 27, 1967.
This investigation was supported in part by Grant GM-11801-02 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
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TABLE I—AMINOPHYLLINE BLOOD LEVELS FROM THEOBROMA OIL SUPPOSITORIES CONTAINING 5% NONIONIC SURFACTANTS

Surfactant Chemical Type or Composition	HLB	Mg. Dialyzed, 1 hr.	Blood Levels, mcg./100 ml. Blood 1 hr.	3 hr.	4 hr.	5 hr.
Theobroma oil only	0	1.0	400	250		200
Sorbitan fatty acid esters[a]						
Sorbitan monostearate	4.7	1.2	250	300		200
Sorbitan monopalmitate	6.7	1.7	240	160		80
Sorbitan monolaurate	8.6	2.1	220	230		100
Polyoxyethylene sorbitan fatty acid ester-ethers[b]						
POES monostearate	9.6	2.9	230	160		80
POES tristearate	10.5	2.7	260	150	190	...
POES monolaurate	13.3	4.1	200	220		80
Polyoxyethylene fatty acid esters[c]						
POE (8) stearate	11.1	6.0	200	150		80
POE (40) stearate	16.9	1.5	400	90		50
POE (50) stearate	17.9	1.7	200	120		negligible
Polyoxyethylene fatty ethers[d]						
POE (4) lauryl ether	9.7	9.8	300	300		80
POE (23) lauryl ether	16.9	6.6	340	240	250	...
POE (2) cetyl ether	5.3	1.3	200	240		200
POE (10) cetyl ether	12.9	7.8	400	100		50
POE (20) cetyl ether	15.7	2.6	150	300		300
POE (10) stearyl ether	12.4	7.4	350	...		200
POE (20) stearyl ether	15.3	2.1	400	450		50
POE (20) oleyl ether	15.3	5.6	50	50		negligible

[a] Trademarked as Spans 60, 40, and 20, respectively. [b] Trademarked as Tweens 61, 65, and 21, respectively. [c] Trademarked as Myrjs 45, 52, and 53, respectively. [d] Trademarked as Brijs 30, 35, 52, 56, 58, 76, 78, and 98, respectively, all trademarked by the Atlas Chemical Industries, Inc., Wilmington, Del.

RESULTS AND DISCUSSION

Table I shows the concentration of aminophylline in the blood plasma. A study of this table reveals no pattern of release. The addition of the surfactants did not result in an increased rate of absorption as compared to theobroma oil. In most instances there was even a decrease in plasma concentration. HLB values, which had a pronounced effect in our and other *in vitro* work, had no discernible effect in the *in vivo* work. No relationship was found between chemical type or structure and effect. There was also no correlation between *in vitro* and *in vivo* results.

Figures 1 and 2 show the scatter pattern of the individual aminophylline plasma concentrations from selected suppositories. Individual differences were great but were more uniform than data obtained using human subjects (11, 12). This was to be expected as conditions were more closely controlled with rabbits than would be possible with humans.

Generally, it has been found that the addition of surfactants to oleaginous bases (suppository or ointment) increased the *in vitro* rate of release (10–15).

Reports of *in vivo* work have been contradictory. Riegelman and Crowell (16) found that the rate of absorption of sodium iodide was accelerated by the presence of surfactants but the rate of absorption of the sodium salt of 2,4,6-triiodophenol was retarded. Kakemi *et al.* (17) showed that the rectal absorption of sulfonamides in the rat was reduced by the presence of nonionic surfactants due to the entrapment of part of the drug within the micelles of the surfactants.

Various attempts have been made to correlate *in vitro* and *in vivo* data regarding drug release from

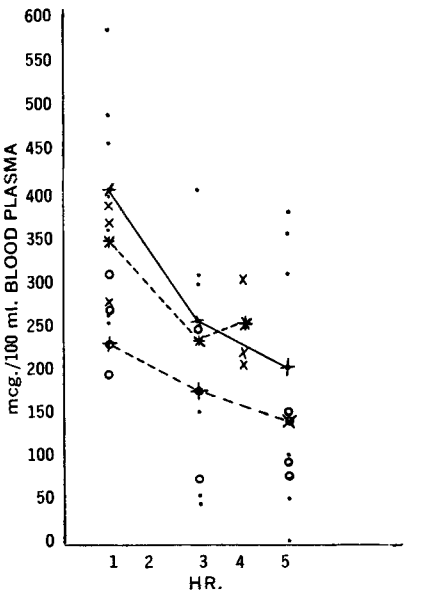

*Fig. 1—Aminophylline plasma concentrations. Key: •, theobroma oil; ⊠—, theobroma oil-av.; O, POES monostearate; ⊠- - -, POES monostearate-av.; ×, POE (23) lauryl ether; *- - -, POE (23) lauryl ether-av.*

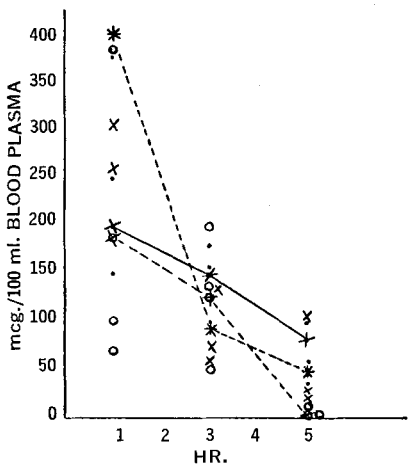

*Fig. 2—Aminophylline plasma concentrations. Key: •, POE (8) stearate; ✕—, POE (8) stearate-av.; O, POE (50) stearate; ✕- - -, POE (50) stearate-av.; ×, POE (40) stearate.; *- - -, POE (40) stearate.*

suppositories. Since *in vitro* conditions cannot take into account the conditions prevailing in the rectum, especially regarding fluid content, the viscosity and chemical composition of the mucus, and the pressure of the walls on the suppository, they have been unsuccessful. Recently Setnikar and Fantelli (18) described an apparatus which more closely reproduces the rectal environment. Using this apparatus, the *in vitro* disintegration time compared favorably with the disintegration time of radiopaque suppositories in man. This was an improvement over other techniques but still omits the effect of many factors.

Kuhne (4) reported results of studies relating *in vitro* to *in vivo* experiments. He prepared suppositories of two pairs of drugs, phenobarbital and its sodium salt, and atropine and atropine sulfate, in several bases. The suppositories were administered to rabbits for the *in vivo* experiments, and dialyzed through a membrane for the *in vitro* experiments. The water-insoluble drugs, phenobarbital and atropine, did not pass from the suppositories through the membrane in the *in vitro* work, but all drugs passed readily through the rectal mucosa into the blood in the *in vivo* experiments. He concluded that *in vitro* results cannot serve as a basis for the prediction of *in vivo* results.

Neuwald and Kunze (5) compared *in vitro* results with blood levels in man of three salicylates: acetylsalicylic acid, calcium acetylsalicylate, and sodium salicylate. The plasma concentrations of all three salicylates were about the same after 2 hr., but there was very wide variation in the amounts of drug diffused *in vitro*. Their *in vivo* results do not agree with animal experiments of others and contradict their own *in vitro* results. They drew the conclusion that the evaluation of suppository medication for man cannot be based on animal experiments, much less on *in vitro* diffusion experiments.

Our data support the unpredictability of absorption of drugs from suppositories. Several explanations may be advanced as to why the addition of the nonionic surfactants retarded the absorption of aminophylline by the rabbits.

It has been shown (19) that, at least for salicylates, drugs are initially absorbed as rapidly as they are released from the suppository—*i.e.*, the rate of release and diffusion to the rectal mucosa is the rate-limiting step. Our *in vitro* study (10) revealed that the addition of nonionic surfactants increased the rate of release and diffusion of aminophylline. Hence, it is evident that the interference with absorption is not due to lessened release.

The cellophene membrane used in the *in vitro* study cannot be compared with the living rectal mucosa. While most drugs are absorbed from the rectum by a simple diffusion process, this diffusion through a lipoidal membrane is different from a cellophene membrane. As reported by Riegelman and Crowell (16) and Kakemi *et al.* (17) it is probable that part of the drug is trapped within the micelles of the surfactant. The nature of the membranes and the size openings were probably such that the surfactants were able to pass through the cellophane membrane much more readily than through the rectal membrane.

Other possible explanations are that the surfactants irritated or in some other manner changed the permeability of the rectal membrane. This is unlikely as Nissim (20) has shown that some nonionic polyoxy surfactants (polyoxyethylene sorbitan esters) exhibited no untoward action on the mucosa.

Another possible explanation is that the surfactants emulsified or in some other manner modified the mucus lining the rectum, causing it to trap or interfere with the absorption of the aminophylline.

SUMMARY AND CONCLUSIONS

(*a*) The rate of absorption of aminophylline from theobroma oil and from theobroma oil containing 5% nonionic surfactants was determined.

(*b*) The addition of the surfactants generally resulted in decreased absorption of the drug.

(*c*) There was little, if any, difference noted in results related to HLB, chemical structure, or chemical type of the surfactant.

(*d*) There was no correlation between *in vitro* and *in vivo* data of aminophylline release and absorption. The evaluation of suppository medication, in rabbits and probably for man, cannot be based on *in vitro* dialysis experiments.

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Keyphrases

Aminophylline suppositories—*in vivo* absorption rate
Nonionic surfactant effect—*in vivo* absorption rate
Blood levels—aminophylline from suppositories
UV spectrophotometry—analysis

Datura Tissue Cultures: Production of Minor Alkaloids from Chlorophyllous and Nonchlorophyllous Strains

By E. JOHN STABA and ANTONIN JINDRA*

The highest medium concentration of manganese used (14 p.p m.) appeared to stimulate the production of cuscohygrine, choline, and pseudotropine from *Datura stramonium* L., strain 5450, suspension cultures. Amino acid precursors, or chlorophyllous tissue had no significant effect upon the production of these alkaloids. No hyoscyamine or scopolamine was detected. Significant amounts of *Datura* suspension tissues were grown in multiliter fermentors.

RECENTLY, ALKALOIDS have been reported produced by *Catharanthus* (1), *Conium* (2), *Datura* (3), *Ipomoea* (4), *Nicotiana* (5), and *Rauwolfia* (6) tissue cultures. In the past a number of investigators have studied alkaloid production by plant tissue or organ cultures, and their results are comprehensively discussed in review articles (7–9). Plant tissue cultures have been used to biochemically alter alkaloids (10) and cardenolides (11).

The tropane alkaloids are reportedly produced by *Datura* (3, 12, 13) and *Hyoscyamus* (14) callus tissue cultures, and to be potentiated by the addition of certain amino acids to the medium (13). The tropane alkaloids may also be degraded and metabolized by microorganisms (15). Manganese ion supplements stimulated alkaloid production and arginase activity in both sand and field cultures of *Datura stramonium* plants (16).

The principal objective of this study was to determine the nature of the alkaloids present in *Datura* suspension cultures and if they might be increased or modified by altering the manganese ion concentration in the medium, by adding tropane alkaloid precursors to the medium, or by inducing chloroplast formation.

EXPERIMENTAL

Tissue Cultures—The *Datura* tissue culture principally studied was 36 month-old seed callus of *Datura stramonium* L., strain 5450. This culture was established in June 1963 (13) from plants believed to be high in alkaloid content and subcultured to the liquid medium in June 1966. The long-term experiment shown in Table I represents seven continuous subcultures of this strain which were grown as previously described (13, 17).

Forty-one month-old seed callus of *D. stramonium* (13); 36 month-old seed callus of *D. quercifolia* L., strain 52146 and *D. innoxia* Mill. (13); and 4 month-old seed callus of *D. stramonium* (seed origin: Drug Plant Greenhouse, Univ. of Nebraska) were grown as suspension cultures for approximately 3 months, and subsequently the suspension cultures were analyzed for their alkaloid content. A continuous light period was provided (150 ftc. from 40-w. cool-white, fluorescent tubes; Amplex Corp.) to establish and maintain chlorophyllous suspension cultures (*D. stramonium* L., strain 5450).

Three 7.5-L. fermentors (model FS-300, New Brunswick Sci. Co., New Brunswick, N. J.) containing 3.4 L. of medium with 14 p.p.m. of manganese were each inoculated with 12 day-old suspension

Received April 28, 1967, from the College of Pharmacy, University of Nebraska, Lincoln NB 68508
Accepted for publication December 21, 1967.
Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This investigation was supported by a National Science Foundation senior foreign scientist fellowship (FY-1965) to Dr. A. Jindra, and by grant GM 13440-01 from the National Institute of General Medical Sciences, U. S. Public Health Service, Bethesda, Md.
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cells (approx. 5 Gm. dry wt.). The tissues were grown in the fermentors for 21 days at 28°, and each fermentor received an aeration rate of 1 L. of sterile air per min. and an agitation rate of 80 r.p.m.

Alkaloid Analysis—Approximately 2 Gm. of dry tissue was finely ground and mixed in a porcelain, dish with 1.5 ml. of ammonium hydroxide (28%), 2.0 ml. of ethanol (95%), and 4.0 ml. of ethyl ether. After 12 hr. the dried material was extracted with chloroform in a Soxhlet apparatus for 24 hr. The chloroform extract was evaporated *in vacuo* and the residue redissolved in chloroform. The resultant solution was placed in a glass tube and the solvent removed using infrared heat. The residue was then mixed with 0.50 ml. of 0.1 *N* HCl (in 90% ethanol) and the mixture centrifuged.

Extracts (100–200 μl.) were applied to Whatman paper No. 3 and developed by descending chromatography using the organic phase of the mixture, *n*-butanol–glacial acetic acid–water (50:3:25) (18). The alkaloids were detected by dipping the chromatograms in modified Dragendorff's reagent.[1] The R_f values obtained for the standards were: cuscohygrine[2] 0.08, choline 1.17, pseudotropine[2] 0.29, tropine 0.36, scopolamine 0.50, hyoscyamine 0.69. The alkaloids in the sample were identified by co-chromatography with the standard alkaloids.

Some extracts were applied to Whatman paper No. 3 impregnated with 0.5 *M* KCl and developed in *n*-butanol–concentrated hydrochloric acid (98:2) saturated with water (19). Alkaloid detection was achieved as described above.

Concentrations of alkaloids were estimated either by eluting the Dragendorff-positive zone from the chromatogram with acetic anhydride and photometrically comparing the resultant color with standard hyoscyamine concentrations (20), or by comparing the Dragendorff-positive zone with known concentrations of alkaloids on the chromatogram.

Amino Acid Analysis—The tissue (10 Gm. wet wt.) was ground with washed sand and extracted with 70% ethanol in a conical flask for several hours. This extraction process was repeated five times. The combined extracts (approximately 150 ml.) were filtered and evaporated to dryness *in vacuo*. The residue was dissolved in 5.0 ml. of 70% ethanol.

The amino acid extracts (200 μl.) were analyzed on Whatman paper No. 1 by two-dimensional chromatography[3] (21). The first solvent system was a mixture of phenol–ethanol–chloroform (2:1:1) with 0.1% of oxime. To each liter of this mixture was added 100 ml. of 25% ammonium hydroxide solution. Tropeolin 000 was added to the amino acid mixture to indicate the extent of amino acid resolution. The paper was allowed to develop for approximately 48 hr. After drying at normal room temperature, the chromatograms were developed twice in the organic phase of the second solvent system, *n*-butanol–glacial acetic–water (10:1:3).

The analysis of basic amino acids, especially arginine and ornithine, was achieved by purifying and concentrating a sample through a system of ion exchangers. An Amberlite IR 120 (H^+) column (23.8 Gm. wet wt.) was prepared, and after applying the extract, it was eluted with 25–30 ml. of 1 *N* NH_4OH. The eluate was evaporated to dryness at 50–55°, and the resultant residue dissolved in 5.0 ml. of water. This solution was applied to an Amberlite IRA 400 (CO_3^{-2}) column (11.8 Gm. wet wt.) and the effluent collected and evaporated to dryness at 50–55°. The residue was dissolved in 2.0 ml. of water and either 100 or 200 μl. applied to Whatman paper No. 1 for one-dimensional chromatography using a mixture of amyl alcohol–pyridine–water (35:30:30) (22). The amino acids were detected by dipping the chromatograms in a 0.5% ninhydrin solution (acetone–pyridine; 9:1). After drying at normal room temperature for 10 min. the chromatograms were heated at 60° for 2–3 min. The zones were fixed by dipping in a solution of cupric nitrate.[4] The chromatograms were allowed to dry in the dark for 30 min.

RESULTS AND DISCUSSION

The principal alkaloids detected in the tissue cultures were cuscohygrine (CC), choline (CH), and pseudotropine (PT). Their identification was based upon reported R_f values (23) in two chromatographic procedures previously described (18, 20), and by co-chromatography with reference compounds. At no time was hyoscyamine or scopolamine detected in the tissue cultures. These minor alkaloids are present in *Datura* (24), *Withania* (25, 26), and *Scopolia* (23). No relationship could be established between the minor alkaloid content and callus, suspension, or chlorophyllous tissue cultures. These minor alkaloids were sometimes not found, and also could not be related to tissue age, or tissue morphology (creamy-white suspension cells or vesicular brown tissue). The *D. stramonium*, strain 5450, tissue culture used for this study was previously reported to contain scopolamine and a total alkaloid content of 15 mg.% (13). West and Mika (12) reported *Datura* root callus to contain 47–53 mg. % total alkaloids, and Netien and Combet (3) reported *D. metel* L. stem callus to contain approximately 15.5 mg.% of hyoscyamine and scopolamine.

The results obtained with suspension cultures are shown in Table I. The first two experiments of *Datura* suspension cultures grown in medium with 7 p.p.m. of manganese contained no Dragendorff-positive spots. Tissue grown in Experiment III with amino acid precursors grew very poorly and did not contain Dragendorff-positive spots. However, CC, CH, PT were present in some of the cultures without precursors. The tissue from Experiments IV *a*, *b*, *c* did not contain Dragendorff-positive spots, and was grown in media without precursors and with lower manganese concentrations (1 and 7 p.p.m.). Only 21 day-old tissue grown on medium with 14 p.p.m. of manganese contained the three alkaloids: CC, CH, and PT. Twenty-eight day-old tissue

[1] Modified Dragendorff's reagent: Mix together basic bismuth carbonate 2.6 Gm. and sodium iodide 7.0 Gm. Add 25 ml. glacial acetic acid and boil for 3–4 min., set overnight and filter. The filtrate (20 ml.) was added to ethyl acetate (80 ml.) to make the stock solution. Final detecting solution was formed by mixing and storing the following for at least 24 hr.: 20 ml. of stock solution, 4 ml. of glacial acetic acid, 120 ml. of ethyl acetate, and 10 ml. of water.

[2] Supplied through the courtesy of Dr. W. C. Evans, University of Nottingham, England.

[3] The two-dimensional chromatographic analysis was performed by Dr. V. Jiracek, Department of Biochemistry, Charles University, Praha, Czechoslovakia.

[4] Aqueous saturated solution of cupric nitrate (2 ml.) is mixed with concentrated nitric acid (0.04 ml.), and acetone added to 200 ml.

TABLE I—*D. stramonium* SUSPENSION CULTURE: INFLUENCE OF AGE, MANGANESE CONCENTRATION, AND AMINO ACIDS ON ALKALOID PRODUCTION

Experiment (Age of Culture: Months)	Age of Tissue Analyzed (Days)	Tissue Dry Wt./Flask[a] (Gm.)	Mean Concn. (p.p.m.)	Amino Acids Present[b] (Days)	Alkaloids Detected[c]		
					Cusco-hygrine	Choline	Pseudo-tropine
I (36)	23	...	7	0			
II (37)	27	...	7	0			
III (38)							
a	31	0.485	1	0	+	+	
b	31	0.270	7	0			
c	31	0.340	14	0	+		+
d	31	0.077	1	31			
e	31	0.120	7	31			
f	31	0.050	14	31			
IV (39)							
a	21	0.366	1	0			
b	21	0.474	7	0			
c	21	0.425	14	0	+	+	+
d	28	0.533	1	0		+	
e	28	0.200	7	0			
f	28	0.425	14	0		+	+
g	28	0.675	1	7		+	
h	28	0.725	7	7		+	+
i	28	0.40	14	7			+
V[d] (40)							
a	8	0.167	14	0			
b	20	0.267	14	0	+	+	+
c	34	0.792	14	0			
d	48	0.725	14	0			
e	20	0.244	7	0	+	+	
f	20	0.096	14	20	+	+	+
g	27	0.833	14	7	+	+	+
VI (42)							
a	16	0.765	7	0			
b	16	0.744	14	0			
c	16	0.808	7	8			
d	23	1.336	7	0			
e	23	1.340	14	0			
f	23	1.524	7	15			

[a] Average dry weight tissue obtained from 4–10 250-ml. conical flasks containing 75 ml. of medium. Tissue was dried at 90° for 24 hr. [b] Final concentration in medium: 0.02% of L-ornithine HCl and of L-arginine HCl; 0.01% of L-phenylalanine; 0.05% of L-asparagine (anhydrous). The amino acid stock solution used was sterilized by filtration and aseptically added to the medium. [c] Paper chromatographic procedure according to Trabert (18) and detection by modified Dragendorffs reagent. *Rf*: Cuscohygrine, 0.08; Choline, 0.17; Pseudotropine, 0.29. [d] The tissue from Experiment V*a–d* was combined, dried, and assayed as one sample.

grown on similar medium contained a strong PT spot (estimated concentration 0.035%). Pseudotropine was also present (estimated concentration 0.015%) in 28 day-old tissue on similar medium with precursors (Experiment IV). The tissue appearance varied from a creamy-white suspension to a brown vesicular tissue. Supplemental analysis of these two forms indicated that they were similar with regard to their alkaloid content, and the creamy-white suspension tissue was selected for Experiments V and VI, and the multiliter experiment. The three alkaloids, CC, CH, and PT, were present in Experiment V tissues grown in media with high manganese levels (14 p.p.m.), and with or without amino acid precursors. The highest concentration of CC (estimated 0.01%) was in Experiment V*e* tissue, and the highest concentration of PT (estimated 0.017%) in Experiment V *a–d*. A growth curve for Experiment V *a–d* is shown in Fig. 1. At the time of maximum growth (34 days) the growth index value was 8.12; the growth efficiency index (G.E.I.)[5] value 3.18; and the Gm. of tissue (wet wt.) produced per liter per day was 6.2. The moisture content, determined by drying the tissue at 90° for approximately 24 hr. was 95.5%. The tissue grown for Experiment VI in medium with either 7 or 14 p.p.m. of manganese, with and without precursors, contained no Dragendorff-positive spots.

Chlorophyllous cultures grew very rapidly as a green, finely dispersed cellular culture. The cultures analyzed contained CH, but no other Dragendorff-positive spots.

The tissues obtained from the three fermentors contained no Dragendorff-positive spots. As much as 10 Gm. dry wt. of tissue was taken for an analysis, and yet the result was negative for alkaloids. The growth index values for the three fermentors are given in Fig. 1. The fermentor with the maximum growth had a growth index value of 9.34; a G.E.I. value of 0.13; and the Gm. of tissue (wet wt.) produced per liter per day was 14.1. These values were calculated for the growth yield obtained and do not necessarily represent the maximum growth yield possible. There is continued interest in the production of large amounts of plant cells (27, 28). The amount of fresh tissue obtained from the best multiliter fermentation was 14.1 Gm./L./day, and compares favorably with that reported for *Ammi visnaga* (19.0 Gm./L./day) (9).

The amino acids found in the two-dimensional chromatograms of tissue from Experiments III *a, b, c*

[5] G.E.I. = Growth index at maximum growth/medium (L.) × days required for maximum growth.

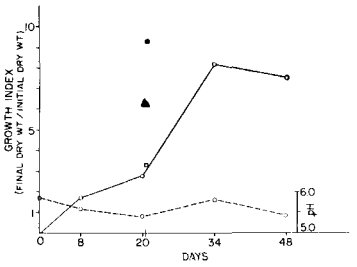

Fig. 1—Datura stramonium *L., strain 5450, growth in flasks (Experiment V), and from multiliter fermentors. Key:* O, *250-ml. conical flasks (75-ml. medium). Growth (average dry wt.) from 4 flasks. Inoculum dry weight: 0.098 Gm.;* □, *fermentor I;* Δ, *fermentor II;* ■, *fermentor III. Fermentors contained 3.4-L. medium and a tissue inoculum of approximately 5 Gm. dry weight. Final medium pH was 5.5.*

and IV *a*, *b*, *c* (Table I) in high concentrations were arginine, glutamic acid, and glutamine; in intermediate concentrations were alanine, γ-aminobutyric acid, and serine; and in low concentrations were aspartic acid, isoleucine, leucine, phenylalanine, proline, threonine, tyrosine, and valine. One-dimensional chromatograms of all samples had a high concentration of arginine and only traces of ornithine. The amino acids ornithine and phenylalanine, which are known to be the principal precursors of the tropane alkaloids (24), did not show any significant stimulating effects on the production of either minor or major alkaloids in the *Datura* tissue cultures studied. An analysis of the amino acids in *Datura* suspension cultures was qualitatively similar, except for the absence of lysine and tryptophan, to that reported for *D. stramonium* plants (16). Large amounts of arginine, and trace amounts of ornithine, are present in *Datura* tissue cultures.

Higher concentrations of manganese, a known activator of arginase (16), appeared to stimulate the production of the minor alkaloids present in *Datura* suspension cultures.

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Keyphrases

Datura tissue cultures
Alkaloid production—seed callus cultures
Chlorophyllous cultures—*Datura* tissue
Manganese—alkaloid growth in cultures
Ion exchange, column chromatography—separation, amino acids
Paper chromatography—analysis, identity

Quantitative Analysis of Flavonoids

By HARUO OGURA, YŌKO SHIKIBA, and YUKIO YAMAZAKI

Some flavonoids, quercitrin, quercetin, and rutin are analyzed quantitatively using zirconium chloride or diphenylboric acid β-aminoethyl ester as a color reagent. These methods are suitable for the quantitative analysis of flavonoids contained in vegetable crude drugs and pharmaceutical preparations.

THE QUANTITATIVE analysis of rutin and its preparations by the determination of the ultraviolet absorption (UV) has been well known (1–4). In the present investigation on the quantitative analysis of rutin (I), quercitrin (II), and quercetin (III), their preparations were investigated. Studies on the quantitative analysis were restricted to colorimetry, and zirconium chloride (*A*) and β-aminoethyl diphenylborate (*B*) (5) were used as the color reagents.

The relative concentration of the colored complex seemed to depend on the pH of the reaction medium. The pH profile for the appearance of the colored complex was determined in acetic acid-sodium acetate buffer (Figs. 1 and 2). The buffer solutions

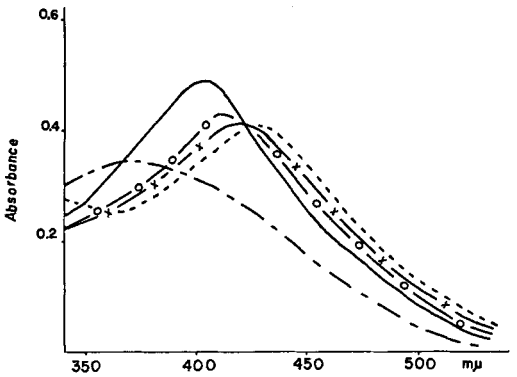

Fig. 1—UV spectra of flavones colored by method A *(rutin) in various buffer solutions. Key:* — — —, *2* M *AcOH;* ———, *2* M *AcOH–2* M *AcONa (3:1);* O, *2* M *AcOH–2* M *AcONa (1:1);* X, *2* M *AcOH–2* M *AcONa (1:3);* – – – –, *2* M *AcONa.*

suitable for method *A* were acetic acid–sodium acetate (3:1) and for method *B*, sodium acetate. The characteristic reactions of these flavones produced a yellow color with method *A* ($\lambda_{max.}$: I, 400 mμ; II, 402 mμ; III, 468 mμ) and method *B* ($\lambda_{max.}$: I, 404 mμ; II, 394 mμ; III, 406 mμ). Calibration curves (Fig. 3) for complexes of I, II, and III by both methods *A* and *B* suggested the possibility of quantitative analysis.

The quantitative analytical data for pharmaceutical preparations are summarized in Table I. Some of the crude drugs analyzed as quercitrin (No. 1) or quercetin (No. 2), and simple preparations of flavones (I, II) and containing ascorbic acid (Nos. 3~6) also gave good quantitative analytical data. The data also revealed that the presence of hesperidin, vitamin K, and carbazochrome showed no interference. On the other hand, preparations containing methylhesperidin, vitamin K_4 diacetate, and/or extracts of coptis, rheum, and scutellaria showed rather a large observational error.

EXPERIMENTAL

Sample solutions were prepared by dissolving 5 mg. each of the materials in 50 ml. of methanol in a

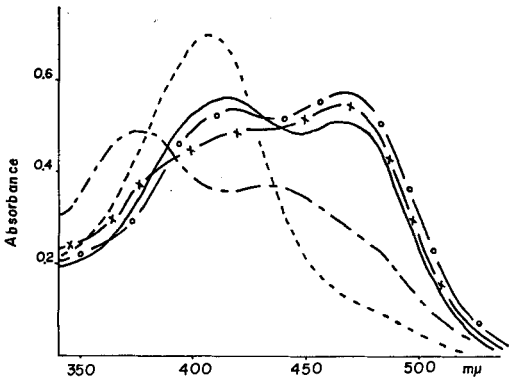

Fig. 2—UV spectra of flavones colored by method B *(quercetin) in various buffer solutions. Key:* — — —, *2* M *AcOH;* ———, *2* M *AcOH–2* M *AcONa (3:1);* O *2* M *AcOH–2* M *AcONa (1:1);* X, *2* M *AcOH–2* M *AcONa (1:3);* – – – –, *2* M *AcONa.*

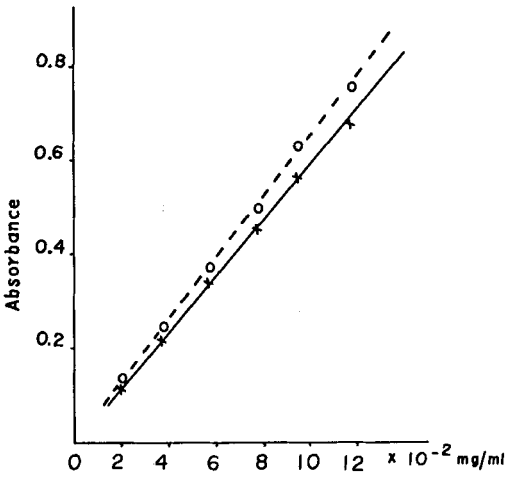

Fig. 3—Calibration curves of rutin by methods A *and* B. *Key: X, method* A; O, *method* B.

Received July 13, 1967, from the College of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo, Japan.
Accepted for publication November 15, 1967.

TABLE I—QUANTITATIVE ANALYSES OF RUTIN IN PHARMACEUTICAL PREPARATIONS

No.	Preparation (Components)	% of Theory—Method *A*	% of Theory—Method *B*
1	Extract *Aesculus* leaf (quercitrin)(6)	42.6[a]	40.6[a]
2	Extract *Aesculus* seed (quercetin) (6)	0[a]	1.3[a]
3	Quercitrin (0.8 mg.) Ascorbic acid (0.25 mg.)/ml.	103.1[a]	99.4[a]
4	Rutin (×10)	100.4	100.4
5	Rutin (50 mg.) Ascorbic acid (25 mg.)/ml.	98.0	100.0
6	Rutin (70 mg.) Ascorbic acid (70 mg.)/tab.	98.9	100.1
7	Rutin (20 mg.) Ascorbic acid (20 mg.) Hesperidin (50 mg.)/tab.	101.8	103.7
8	Carbazochrome 3-sodium sulfonate (25 mg.) Hesperidin (50 mg.) Rutin (40 mg.) Ascorbic acid (100 mg.) Vitamin K (5 mg.) Dibasic calcium phosphate (300 mg.)/Gm.	100.3	103.7
9	Methylhesperidin (40 mg.) Rutin (40 mg.) Ascorbic acid (100 mg.) Adrenochrome monosemicarbazone (2 mg.) Vitamin K_4 diacetate (2 mg.)/Gm.	124.9	116.0
10	Rutin (100 mg.) Scutellaria root (50 mg.) Coptis (25 mg.) Rheum (25 mg.) Extract rheum, coptis, scutellaria root (85 mg.)/Gm.	120.3	120.3
11	Rutin (20 mg.) Scutellaria root (10 mg.) Coptis (5 mg.) Rheum (5 mg.) Extract rheum, coptis, scutellaria root (17 mg.)/tab.	114.4	118.1

[a] Each value represents a % of quercitrin (Nos. 1 and 3) and quercetin (No. 2) in the preparations.

volumetric flask and diluting with water to make 100 ml.

(A) Zirconium Chloride—To 5 ml. of the sample solution in a 25-ml. volumetric flask, 1 ml. of a buffer solution [*a*, 2 *M* acetic acid; *b*, 2 *M* acetic acid–2 *M* sodium acetate (3:1); *c*, 2 *M* acetic acid-2 *M* sodium acetate (1:1); *d*, 2 *M* acetic acid–2 *M* sodium acetate (1:3); *e*, 2 *M* sodium acetate] and 1 ml. of 3% zirconium chloride were added, and diluted with water to the mark.

(B) β-Aminoethyl Diphenylborate—To 5 ml. of the sample solution in a 25-ml. volumetric flask, 1 ml. of a buffer solution (same as above) and 1 ml. of methanolic 1% β-aminoethyl diphenylborate were added, and diluted with methanol to the mark.

CONCLUSION

As shown in Fig. 1, 2 *M* acetic acid–2 *M* sodium acetate (3:1) solution (*b*) was suitable for method *A*. On the other hand, 2 *M* sodium acetate solution (*e*) was suitable for method *B* as shown in Fig. 2. Calibration curves for I are shown in Fig. 3.

It is considered that further investigation should be carried out to introduce a new quantitative analytical method for rutin preparations and/or vegetable crude drugs. In addition to the two methods mentioned above, there is another method for the quantitative analysis of flavonoids under investigation in our laboratories.

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Keyphrases

Flavonoids—analysis
Colorimetric analysis
Zirconium chloride—color reagent
β-Aminoethyl diphenylborate—color reagent
pH profile—colored complex

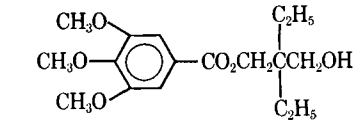
Preparation of 2,2-Diethyl-3-(3,4,5-trimethoxybenzoyloxy)propyl Carbamate

By JOSEPH SAM*

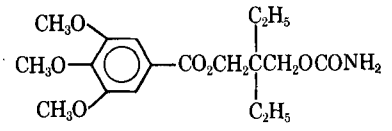
The preparation of the 3,4,5-trimethoxybenzoate of 2,2-diethyl-1,3-propanediol, and the conversion of the ester to the carbamate are described.

THE USEFUL depressant properties of propanediols (1) and the presence of the 3,4,5-trimethoxybenzoyl group in the rauwolfia alkaloids (2) prompted the preparation of a hybrid involving the two groups.

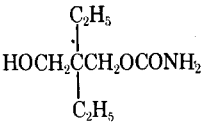
The esterification of an excess of 2,2-diethyl-1,3-propanediol with 3,4,5-trimethoxybenzoyl chloride provided the mono ester, I. The treatment of I



I



II



III

with phosgene, followed by ammonolysis yielded the mixed ester-carbamate, II. Compound II also was prepared by the esterification of 2,2-diethyl-3-hydroxypropyl carbamate (III).

Preliminary pharmacological studies in mice demonstrated that compounds I and II exhibit mild central nervous system depressant properties.

EXPERIMENTAL[1]

2,2-Diethyl-3-hydroxypropyl-3,4,5-trimethoxybenzoate—A mixture of 46 Gm. (0.2 mole) of 3,4,5-trimethoxybenzoyl chloride, 53 Gm. (0.4 mole) of 2,2-diethyl-1,3-propanediol, and 300 ml. of toluene was refluxed for 7 hr. The mixture was cooled and washed with a 5% sodium carbonate solution. The toluene solution was dried over anhydrous sodium sulfate and then distilled to yield 25 Gm. (39%) of product, b.p. 193–196° (0.2 mm.).

Anal.—Calcd. for $C_{17}H_{26}O_6$: C, 62.6; H, 8.03. Found: C, 62.0; H, 7.88.

2,2-Diethyl-3-(3,4,5-trimethoxybenzoyloxy)propyl Carbamate—*Method A*—A solution of 17.5 Gm. (0.1 mole) of 2,2-diethyl-3-hydroxypropyl carbamate (3, 4) and 23 Gm. (0.1 mole) of 3,4,5-trimethoxybenzoyl chloride in 300 ml. of benzene was refluxed for 3 hr. and then cooled. The benzene solution was washed with water and 5% sodium bicarbonate, respectively, and then dried over anhydrous sodium sulfate. Distillation of the solvent and crystallization of the residual material from a mixture of benzene and *n*-hexane gave 15 Gm. (41%) of light brown solid, m.p. 110–118°. Recrystallization from benzene gave product, m.p. 124.5–126°.

Anal.—Calcd. for $C_{18}H_{27}NO_7$: C, 58.5; H, 7.37. Found: C, 58.3; H, 7.41.

Method B—The procedure (5) described for the preparation of carbamates of aryl glycerol ethers was followed using the same molar ratios of reactants. From 3.3 Gm. (0.01 mole) of 2,2-diethyl-3-hydroxypropyl-3,4,5-trimethoxybenzoate there was obtained 0.4 Gm. (10%) of product which was washed with petroleum ether (b.p. 60°) and recrystallized from benzene; m.p. 124.5–126°. A mixed melting point with the product obtained by method *A* showed no depression.

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Received March 30, 1967, from Bristol Laboratories, Syracuse, NY 13201
Accepted for publication October 2, 1967.
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[1] All melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected.

Keyphrases

2,2-Diethyl-3-(3,4,5-trimethoxybenzoyloxy) propyl carbamate—synthesis
Esterification and ammonolysis—reactions
Pharmacological screening—CNS depressant activity

Correlation of Pharmacologic Activity and Dissolution Rates of Reserpine-Desoxycholic Acid Dispersions

Sir:

The usefulness of solid-state dispersions for enhancing the dissolution rate of drugs has been demonstrated in a number of recent reports (1–5). The principal reason for the observed effects appears to be particle size reduction. An interesting example of the enhanced pharmacologic activity of such dispersions has been provided by Malone *et al.* (6). These workers found a significant increase in the blepharoptotic activity of reserpine in mice after oral administration of the drug as a solid dispersion in desoxycholic acid (DCA). A correlation between the composition of the reserpine-DCA dispersion and apparent biologic activity was also noted in that an increase in the DCA:reserpine ratio resulted in an increase in activity (see Table I). Results of studies to be reported in this communication suggest that particle size reduction of reserpine in the dispersion, leading to increased dissolution and absorption rate, and possibly increased availability of reserpine, is likely to be a major factor in the enhancement of pharmacologic activity.

Reserpine[1] and DCA[2] dispersions were prepared according to the methods of Malone *et al.* (6) which involve co-precipitation of the materials from chloroform–absolute alcohol solutions upon evaporation of the solvent system. Attempts to determine the dissolution rate of reserpine in aqueous systems were unsuccessful because of assay difficulties and adsorption problems introduced by the extremely low aqueous solubility of the drug. Hence, dissolution rates of reserpine from 12 to 20-mesh particles of the various preparations were determined in the following manner. Each sample, containing an equivalent of 150 mg. reserpine, was added to a round-bottom flask containing 300 ml. of ethyl acetate (certified A.C.S. grade) at 37° ± 0.1°. Stirring was provided by means of an overhead motor, connected to a Teflon blade, operating at 50 r.p.m. Samples were withdrawn at periodic intervals, filtered, diluted 1:10 with methanol, and assayed for reserpine using a Beckman DB-G spectrophotometer at 268 mμ. Desoxycholic acid appeared to be essentially "insoluble" in ethyl acetate. Solutions of DCS equilibrated in ethyl acetate at room temperature and 37°, upon 1:10 dilution, showed no spectral activity from 350 mμ to 220 mμ. In addition, the spectrum of reserpine at various concentrations was found to be identical in ethyl acetate and ethyl acetate saturated with DCA.

The dissolution data plotted in Fig. 1 indicate an increased dissolution rate of reserpine from the DCA dispersions. The greater the percentage of DCA in the dispersion, the faster the dissolution of reserpine. The raw dissolution data were replotted assuming that dissolution followed an apparent first-order process under nonsink conditions, as previously discussed by Gibaldi and Feldman (7). These plots are shown in Fig. 2.

TABLE I—CORRELATION OF DISSOLUTION DATA AND BIOLOGIC ACTIVITY OF VARIOUS RESERPINE-DCA DISPERSIONS

Molar Composition of Dispersions (Reserpine: DCA)	$k \times 10^4$ (mg.$^{-1}$min.$^{-1}$)	Ratio of Rate Constants	Oral Potency[a]
1:0	2.55	—	—
1:4	3.68	1.4	2.6
1:8	5.38	2.1	2.8
1:16	8.10	3.2	3.8
1:32	16.3	6.4	1.9

[a] Peak blepharoptotic activity after oral administration, as compared to the peak activity of reserpine base after oral administration (from *Reference 6*).

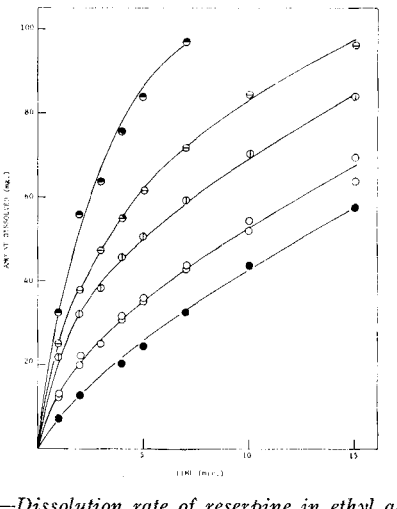

Fig. 1—Dissolution rate of reserpine in ethyl acetate from various preparations. Key: ●, reserpine-DCA, 1:0; ○, 1:4; ◑, 1:8; ⊖, 1:16; ◓, 1:32.

[1] Generously supplied by Ciba Pharmaceutical Company.
[2] Special enzyme grade, Mann Research Laboratories, Inc.

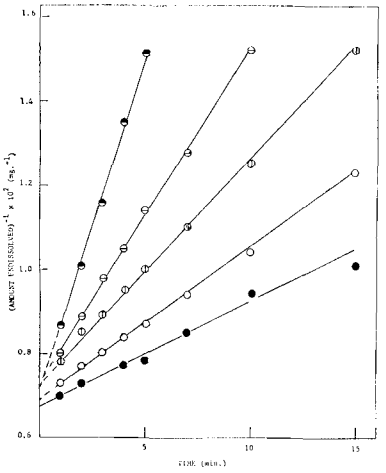

Fig. 2—Second-order plot of reserpine dissolution data assuming dissolution was apparent first order under nonsink conditions. Key: ●, reserpine–DCA, 1:0; ○, 1:4; ◑ 1:8; ⊖, 1:16; ◓, 1:32.

The apparent second-order rate constants were calculated and these are reported in Table I together with the corresponding ratios. A rank correlation is noted between the apparent second-order dissolution rate constants and the biologic activities of the various reserpine preparations. The sole exception is the 1:32 reserpine–DCA dispersion which is significantly less biologically active than would be predicted by the dissolution data.

The use of organic solvents in correlation studies between *in vitro* and *in vivo* data is rarely desirable or logical. However, as noted by Levy (8) dissolution in organic solvents does reflect adequately the particle size of the drug. Furthermore, dissolution in an organic solvent may reflect changes in crystal structure. To explore the latter possibility, diffraction spectra were obtained for reserpine and DCA, separately precipitated from the solvent system, and for various dispersions, using a General Electric XRD-6 diffractometer.[3] All samples appeared to be noncrystalline. In view of the absence of solution interaction between DCA and reserpine in ethyl acetate, and the lack of change in crystal form, it is concluded that the physical state of reserpine (*i.e.*, an extremely fine particle size attained by co-precipitation with DCA) is responsible for the increased dissolution rates observed with the reserpine-DCA dispersions.

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[3] The authors thank Dr. Eli Shefter for providing the diffraction data.

Keyphrases

Reserpine–desoxycholic acid dispersions—solid state
Solid state dispersions—pharmacologic activity effect
Dissolution rate–reserpine–desoxycholic acid dispersion

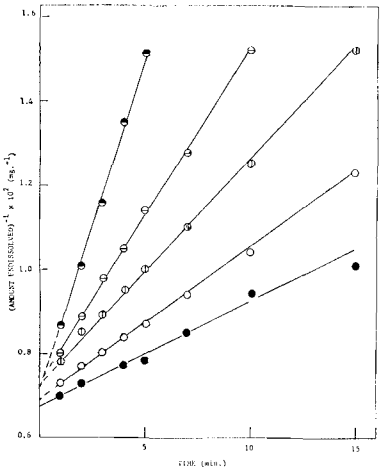
Antidepressive Effect of N-3,4,5-Trimethoxybenzoyl Heptamethylenimine

Sir:

It was Luts *et al.* (1) who reported on the synthesis and preliminary pharmacological investigation of *N*-3,4,5-trimethoxybenzoyl heptamethylenimine. As stated by these authors, unlike other amines containing trimethoxybenzoyl group the compound is void of depressant and analgesic action; moreover, it exerts a mild stimulating effect.

In a series of studies concerned with a large number of derivatives of heptamethylenimine substituted by benzoic acid the above-mentioned compound has also been synthesized and subjected to close pharmacological investigation.

Synthesis was carried out principally by the method reported by Luts *et al.* (1).



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Synthesis was carried out principally by the method reported by Luts *et al.* (1).

In mice the LD_{50} was 170 ± 5.15 i.p. 200 ± 21.52 s.c.

Investigated after Zetler's (2) cataleptic method, in i.p. doses of 20 mg./Kg., the compound was found to be a strong antagonist of catalepsy caused by reserpine or chlorpromazine. It has furthermore strongly antagonized the toxicity of reserpine under acute and subacute conditions. Alone it gave rise to slight hyperthermia. In doses of 40 mg./Kg. it has reversed hyperthermia due to reserpine. In rotarod test (3) the compound alone failed to exert any effect in doses of 10–80 mg./Kg. i.p., but doses from 10 mg./Kg. have antagonized incoordination caused by 2.5 mg./Kg. reserpine. In doses of 10 mg./Kg. the compound has intensified the hypertensive effect of epinephrine in cats. After preliminary treatment for 5 days, the effect of epinephrine and norepinephrine on blood pressure has been greatly enhanced. As shown by Knoll's motimeter (4), the compound induced minimal hypermotility when administered in i.p. doses of 20 mg./Kg.

Hypermotility caused by amphetamine has been inhibited in the first 60 min., but in the period from 60–210 min. it has been increased in an extraordinary measure. When administered simultaneously with hexobarbital, the compound has slightly prolonged the narcosis time. However, after premedication over 24 hr., anaesthesia was considerably shortened. The compound has exerted practically no influence on pentylenetetrazol,[1] strychnine, and electroconvulsions.

No analgesic effect could be demonstrated. Monoamino oxidase has not been inhibited either *in vitro* or *in vivo* after premedication for 1 week The compound has shown no noteworthy harmful effect in subacute toxicity tests.

Upon due consideration of the obtained findings, the compound would seem to display an extraordinary likeness in pharmacological properties to tricyclic antidepressants, and as such it may be denoted as a potential antidepressive drug of new chemical type.

A report on detailed pharmacological results is to be published later.

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Received July 31, 1967.
Accepted for publication January 19, 1968.

Keyphrases

N–3,4,5–Trimethoxybenzoyl heptamethylenimine—antidepressive activity
Reserpine activity—antagonism
Epinephrine hypertensive effect—increased
Amphetamine hypermotility effect—antagonism

Validity of the Doty Reaction as a Stability-Indicating Assay for Isoproterenol in an Aerosol Preparation

Sir:

The sensitivity, accuracy, precision, and manipulative simplicity of the ferro-citrate color reaction described by Doty (1) have led to its wide use for assay of preparations containing isoproterenol, epinephrine, and other catecholamine drugs. The reaction is selective for the catechol function, and it will therefore be stability indicating where the degradation reaction involves the catechol group. Higuchi, Sokoloski, and Schroeter (2) assayed known mixtures of epinephrine and its oxidative degradation products by four procedures; they reported close correspondence between the result obtained by the Doty reaction and the true value, indicating that the reaction is stability indicating where degradation occurs by oxidation. Schroeter, Higuchi, and Schuler (3) described a nonoxidative degradation reaction of epinephrine with bisulfite, used as an antioxidant, where a physiologically inactive substituted benzylsulfonic acid is formed. A similar reaction occurs between bisulfite and isoproterenol (4). The epinephrine-bisulfite reaction product was prepared by the literature method (5). As expected, this product was found to give a color with the Doty reagent, vitiating this assay method for catecholamines in solutions preserved by bisulfite. The Doty method would have obvious limitations in following the stability of those catecholamines subject to racemization in solution (6); however this limitation does not exist with the racemic isoproterenol salts official in the compendia.

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Our concern related to the validity of the ferrocitrate assay as a stability method for isoproterenol in a nonaqueous aerosol preparation[1] with thonzonium bromide, ascorbic acid, alcohol, and propellants. In order to ascertain whether or not degradation of isoproterenol in this preparation furnished a product with an intact catechol function, we subjected aged samples to thin-layer chromatography and sprayed the developed chromatograms with Doty reagent prepared according to the NF (7). (The use of this reagent as a selective spray for detection of catechol derivatives on chromatograms has not been described previously. A fuller description of its application will be published elsewhere.)

The aerosol containers were chilled by immersion in a freezing bath of solid carbon dioxide–acetone, opened with pliers, then allowed to warm to room temperature to evolve propellants. The remaining solution was diluted with an equal volume of absolute methanol, and 25-μl. volumes of it and a 600 mcg./ml. solution of reference isoproterenol HCl were spotted 3 cm. from the bottom edge of a commercial precoated 20 × 20 cm. thin-layer plate consisting of a 0.25-mm. thickness of microcrystalline cellulose without binder or phosphor on glass.[2] Before use, the plates were developed in the solvent to about 15 cm. from the starting line and air dried to remove contaminants from the plate. Pretreatment of the plate in this manner eliminated an apparent second front across the chromatogram which resulted in artifactual spots such as those described by Choulis (8). The chromatogram was developed to about 15 cm. from the starting line with a mixture of 2-propanol and 0.1 *N* hydrochloric acid (5:1). After air drying and spraying with the Doty reagent, only one purple spot was obtained at R_f 0.4, corresponding to intact isoproterenol. The detection limit for isoproterenol HCl or epinephrine HCl is below 0.3 mcg. (2% of the amount spotted from the formulation). A volume of 0.5 μl. of 0.6 mg./ml. epinephrine HCl (0.3 mcg.) was added to a sample preparation spot. It was readily detected at R_f 0.2 after chromatographic separation and treatment with Doty reagent. Thus, the method was shown to be capable of separating isoproterenol from a closely related catecholamine and detecting at least 2% of the amount spotted.

The procedure was applied to isoproterenol and thonzonium bromide aerosol samples stored at room temperature for more than 5 years. Examination of stability data obtained for five lots of the formulation during this time revealed no consistent pattern of degradation. Instead, it appears that each sample reacts to the extent of the oxygen present and remains substantially unchanged thereafter. Although some degradation was evident in all of the stability samples, isoproterenol content remained satisfactory throughout the testing period.

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Received December 26, 1967.
Accepted for publication February 16, 1968.

Keyphrases

Isoproterenol, aerosol—stability assay
Doty reaction—validity confirmed
Catechol oxidation detection—Doty reagent
TLC—analysis, separation

Absolute Stereochemistry and Analgesic Potency of Prodine Enantiomers

Sir:

Stereochemical factors are known to be of importance in analgesic action (1). Among the structures in this well-known class of biologically active compounds, α and β-prodine (Ia and Ib, respectively) (2) distinguish themselves by being highly potent synthetic analgesics possessing two asymmetric centers. While the relative stereochemistries of Ia and Ib are known (3), the absolute configurations and potencies of the optically pure enantiomers have not been reported and are of great interest because they possess an asymmetric center in common with isomethadone (4). In this communication evidence is presented which permits assignment of the complete absolute stereochemistry of these enantiomers.

Treatment of racemic IIa (2) with an equivalent of (+)-tartaric acid in methanol–acetone

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Received December 26, 1967.
Accepted for publication February 16, 1968.

Keyphrases

Isoproterenol, aerosol—stability assay
Doty reaction—validity confirmed
Catechol oxidation detection—Doty reagent
TLC—analysis, separation

Absolute Stereochemistry and Analgesic Potency of Prodine Enantiomers

Sir:

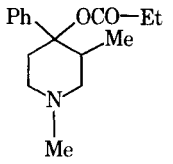
Stereochemical factors are known to be of importance in analgesic action (1). Among the structures in this well-known class of biologically active compounds, α and β-prodine (Ia and Ib, respectively) (2) distinguish themselves by being highly potent synthetic analgesics possessing two asymmetric centers. While the relative stereochemistries of Ia and Ib are known (3), the absolute configurations and potencies of the optically pure enantiomers have not been reported and are of great interest because they possess an asymmetric center in common with isomethadone (4). In this communication evidence is presented which permits assignment of the complete absolute stereochemistry of these enantiomers.

Treatment of racemic IIa (2) with an equivalent of (+)-tartaric acid in methanol–acetone

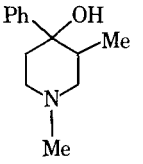
afforded the acid tartrate salt, m.p. 161–162°, $[\alpha]_D^{22}$ + 13.5° (c 1, H_2O), from which the amine, m.p. 89–90°, $[\alpha]^{22}$ + 10.2° (c 1, acetone), was regenerated. This was transformed to the methiodide, m.p. 169–170°, $[\alpha]_D^{22}$ + 1.6° (c 1, EtOH), which was converted to the quaternary hydroxide and then heated at 170° for 1 hr. The structure of the elimination product, III, $[\alpha]_D^{26}$ + 59.8° (c 2, Et_2O), (75% yield) was confirmed by the NMR spectrum which showed a doublet methyl resonance at 0.82 p.p.m. (J = 6.5 c.p.s.) and three olefinic proton resonances; H_a = 5.13 p.p.m., H_b = 5.44 p.p.m. (J_{ab} = 2 c.p.s.), H_c = 6.40 p.p.m. (J_{bc} = 17 c.p.s., J_{ac} = 10 c.p.s.). Hydroxylation of III with osmium tetroxide followed by *in situ* cleavage of the triol with sodium metaperiodate afforded (−)-IV which was isolated as the maleate salt, m.p. 127–129°, $[\alpha]_D^{20}$ − 52.6° (c 1, EtOH). The physical properties were found to be in accord with those of the maleate salt, m.p. 127–128°, $[\alpha]_D^{20}$ − 53.2° (c 1, EtOH), derived from authentic IV (5) which has been determined to be in the (*R*)-series. Esterification of (+)-IIa with propionyl chloride afforded (+)-α-prodine hydrochloride, m.p. 191–193°, $[\alpha]_D^{21}$ + 26.5° (c 1, EtOH). Employing the same procedure, (−)-α-prodine hydrochloride, m.p. 192–193°, $[\alpha]_D^{21}$ − 27.9° (c 1, EtOH), was prepared from (−)-IIa, m.p. 88–89°, $[\alpha]_D^{21}$ − 10.7° (c 1, acetone), obtained by using (−)-tartaric acid in the resolution of the racemic alcohol. The IR spectra of both antipodes were superimposable and virtually identical to that of the racemate.

The above chemical correlation establishes (+)-Ia·HCl as having the (3*R*) configuration. Since it is known (3) that the phenyl and methyl groups in Ia have a *trans* relationship, it follows therefore that the complete stereochemistry of (+)-α-prodine hydrochloride is (3*R*:4*S*) and is depicted by structure VIa. Due to its enantiomeric relationship with (+)-Ia·HCl, the stereochemistry of (−)-α-prodine hydrochloride is designated as (3*S*:4*R*).

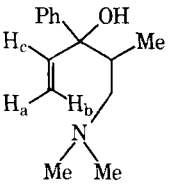
Treatment of (±)-IIb (2) with one equivalent of (−)-dibenzoyltartaric acid in H_2O–MeOH afforded the acid dibenzoyltartrate salt, m.p. 163–164°, $[\alpha]_D^{22}$ − 42.6° (c 1, MeOH), from which (+)-IIb, m.p. 136–137°, $[\alpha]_D^{20}$ + 74.5° (c 1, acetone) was obtained. Employing (+)-dibenzoyltartaric acid in an identical procedure gave (−)-IIb, m.p. 136–137°, $[\alpha]_D^{20}$ − 75.0° (c 1, acetone). Dehydration of (+)-IIb with aqueous HCl yielded (−)-V, $[\alpha]_D^{22}$ − 115° (c 1, Et_2O), whose IR and NMR spectra were identical to that of the racemic olefin (6). Pyrolysis of (+)-Ia according to the procedure of Diamond (7)



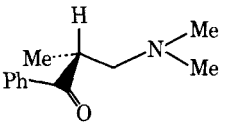
Ia *trans*-Ph:Me
Ib *cis*-Ph:Me



IIa *trans*-Ph:Me
IIb *cis*-Ph:Me



III



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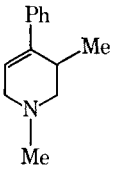
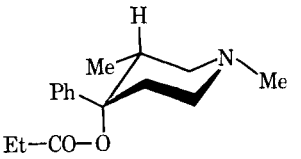
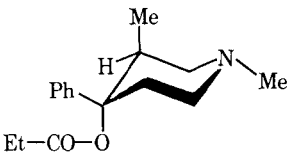


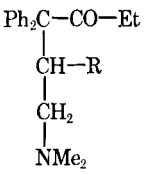
V



VIa



VIb



VII, R = Me
VIII, R = H

afforded (+)-V, $[\alpha]_D^{22}$ + 93.2° (c 1, Et_2O), which possessed IR and NMR spectra identical to that of enantiomeric and racemic material. Esterification of (+)-IIb with propionyl chloride gave (+)-Ib·HCl, m.p. 186–188°, $[\alpha]_D^{20}$ + 74.5° (c 1, EtOH). Employing (−)-IIb in an identical procedure afforded (−)-Ib·HCl, m.p. 186–188°, $[\alpha]_D^{20}$ − 73.7° (c 1, EtOH). The IR spectra of both enantiomers were virtually identical to that of the racemate.

The preceding stereochemical correlation and the known *cis* relationship (3) between phenyl and methyl groups in β-prodine permits the configurational assignment of (+)- and (−)-Ib·HCl as (3*S*:4*S*) (VIb) and (3*R*:4*R*), respectively.

It was found that most of the analgesic activity of both α- and β-prodine resides in the (+)-antipodes (Table I). The analgesic potency of the diastereomeric tartrate salts of Ib has been reported (8). However, in view of the reported low enantiomeric potency ratio and our inability to effect complete optical resolution by this

TABLE I—ANALGESIC POTENCY OF PRODINE ENANTIOMERS

Compound	ED_{50}[a], mg./Kg.
dl-α-Prodine HCl	1.7
(+)-α-Prodine HCl	0.90
(−)-α-Prodine HCl	22.0
dl-β-Prodine HCl	0.35
(+)-β-Prodine HCl	0.25
(−)-β-Prodine HCl	2.6
3-Desmethylprodine HCl	1.3
Meperidine HCl	12.0

[a] Analgesic activity was determined by a modified hot plate method (13).

method, it is very probable that Ib was only partially resolved.

It is significant that the C-3 asymmetric centers in the more potent isomers have opposite configurations. This, coupled with the observation that desmethylprodine (9) possesses greater potency than either of the less active prodine isomers (Table I) suggests that the 3-methyl group does not contribute to the drug-receptor interaction in a totally positive fashion. The difference in potency between (+)-Ia and (+)-Ib most probably is related to different brain levels[1] and it is conceivable that the lower potency of desmethylprodine, when compared to the more active antipodes, may largely be due to lower brain levels rather than to the absence of involvement of the 3-methyl group at the receptor level.

It is noteworthy that the more active enantiomers of isomethadone (VII) (10) and Ia are configurationally related (11). Since the desmethyl analog (VIII) (12) possesses potency which is comparable to VII, there exists the possibility that in this case the methyl group of (*S*)-isomethadone is also playing primarily a passive role in the interaction with analgesic receptors.

[1] Preliminary experiments indicate that the brain levels of β-prodine in rats are greater than those of the α-isomer and that this is the primary factor determining the potency difference between these diastereomers.

(1) Portoghese, P. S., *J. Pharm. Sci.*, **55**, 865(1966).
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Received November 15, 1967
Accepted for publication February 9, 1968.

This investigation was supported by research grant NB 05192 from the National Institutes of Health.

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Keyphrases

Prodine enantiomers—absolute sterochemistry
Optical rotation—structure
IR spectrophotometry—structure
NMR spectroscopy—structure
ED_{50}—prodine enantiomers
Absolute configuration—analgesic activity

4,4′-Dihydroxybibenzyl, a Reduction Metabolite of *trans*-Stilbene

Sir:

Our investigations of the metabolism of certain stilbenes has revealed a unique conversion of *trans*-stilbene in rabbits (2.1%)[1] to 4,4′-dihydroxybibenzyl.

The above metabolite (originally designated X) was found in a phenolic fraction obtained from an ether extract of urine of rabbits, which had been administered *trans*-stilbene (25 mg./Kg., i.m.). Paper, thin-layer, and vapor phase chromatography (VPC) (1) of this phenolic fraction disclosed the presence of the *trans*-stilbenes, 4-hydroxy-, 4,4′-dihydroxy-, 3-hydroxy-4-methoxy-, and 4-hydroxy-3-methoxystilbene, and X, which was not comparable to these or other simple phenolic stilbenes. A 2-mg. sample of the trimethylsilyl (TMS) either of X, obtained by preparative VPC, was subjected to IR, UV, and mass spectral analysis.

The IR spectrum of the TMS derivative of X,

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The IR spectrum of the TMS derivative of X,

[1] Determined by VPC (TMS ether). Chromatographic analysis of urine obtained from guinea pigs administered *trans*-stilbene has indicated an even greater (10.6%) conversion to 4,4′-dihydroxybibenzyl.

except for differences in the fingerprint region, was similar to that of reference 4-trimethylsiloxy- and 4,4′-di(trimethylsiloxy)stilbene. The UV spectrum, however, with a single $\lambda_{max.}$ at 280 mμ, was atypical for the *trans*-stilbene chromophore (2). Mass spectral analysis[2] of the isolated derivative showed a weak parent peak at m/e = 358, a base peak at m/e = 179, and other prominent peaks at m/e = 286 and m/e = 107.

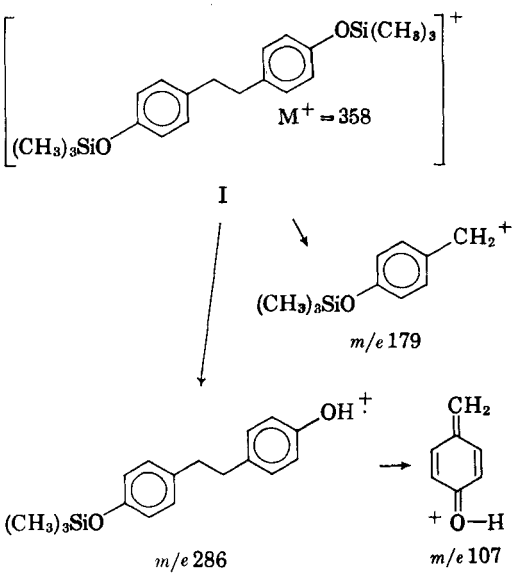
With these data, structure I was proposed for the TMS derivative of X and its mass spectral breakdown was rationalized as in Scheme I.

Based upon this determined molecular weight of 358, a molar absorptivity of 3460 (at 280 mμ) was calculated for the TMS derivative of X and this provided additional evidence for the proposed structure (3).

The TMS derivative of X was found to co-chromatograph (VPC) with the di-TMS ether of authentic 4,4′-dihydroxybibenzyl. Hydrolysis of the isolated TMS derivative of X and comparison of the product (X) with 4,4′-dihydroxybibenzyl showed complete agreement in mobility and reaction to diazotized sulfanilic acid in three thin-layer systems and further substantiated the identity of X as 4,4′-dihydroxybibenzyl.

The formation of a bibenzyl derivative, a reduction product from *trans*-stilbene, involves a novel metabolic transformation for this class of compounds. In contrast, metabolic schemes incorporating reductions are well established for nitro- and azo-compounds, including the reduction of the stilbene isostere, azobenzene to hydrazobenzene (4), a postulated intermediate in the reductive cleavage of azobenzene (5). Reports of metabolic reductions of carbon-carbon double bonds are primarily limited to Δ^4-3-keto-steroids (6, 7) and certain cinnamic acid derivatives (8). These reductions are analogous to the well-known hydrogenations of α,β-unsaturated carbonyl compounds which occur in intermediary metabolism. Reductions resembling the one found in the present investigation, however, are rare and in combination with the implied reductive step in the formation of 1,2-dihydronaphthols (9), advance the significance of hydrogenation in the metabolism of similar conjugated ethylenic compounds.

[2] The authors are indebted to Luther H. Smithson, Jr., and Rick Faubert of Varian Associates, Palo Alto, Calif., for the mass spectral data.



Scheme I

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Received December 5, 1967.
Accepted for publication January 10, 1968.
This investigation was supported in part by Public Health Service training grant GM 1367.
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Keyphrases

trans-Stilbene—conversion in rabbits
4,4′-Dihydroxybibenzyl—urinary metabolite *trans*-stilbene
Vapor phase chromatography—identity
TLC—identity
IR spectrophotometry—identity
UV spectrophotometry—identity
Mass spectroscopy—structure

JOURNAL OF Pharmaceutical Sciences

May 1968 volume 57, number 5

Review Article

The Mannich Reaction

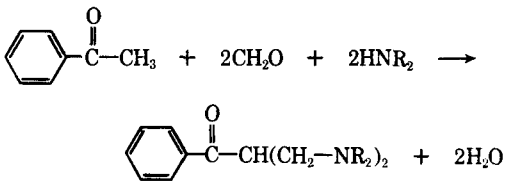
Mechanistic and Technological Considerations

By B. BLACKBURN THOMPSON

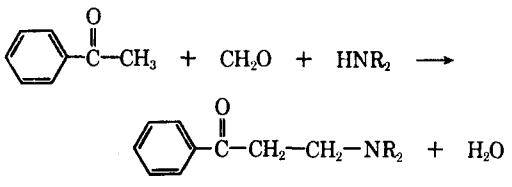
THE EARLIEST EXAMPLES of the Mannich reaction were published in succession by Tollens and co-workers (1, 2), Petrenko-Kritschenko and co-workers (3–6), and by Mannich and Krösche (7). Mannich was the first to recognize the reaction as a general one, and a detailed investigation was begun in 1917. The Mannich reaction was reviewed by Blicke (8), Karbe (9), Nobles (10, 11), Reichert (12), and others (13, 14) and it was the subject of recent books by Reichert (15) and by Hellmann and Optiz (16).

The Mannich reaction consists of the condensation of ammonia or a primary or secondary amine usually as the hydrochloride salt, an aldehyde, and a compound capable of supplying one or more active hydrogen atoms. As a result of the reaction an aminomethyl group generally replaces the active hydrogen atom. Products of the condensation are known as "Mannich bases." A typical condensation, with acetophenone as the active hydrogen compound, may be illustrated as in *Scheme I.*

As cited in the excellent review by Blicke (8), multiple product formation is possible. For example, multiple aminomethylations may occur at the α-position of compounds such as acetophenone of the previous example, *viz.*, *Scheme II.* Similarly, mono- and disubslituted products including position isomers, result from multiple aminomethylation of phenols.[1] In this case the replaceable hydrogens are not all equivalent as indicated in *Scheme III.* These examples illustrate the complications which may result from the presence of multiple replaceable hydrogen atoms in the molecule.



Scheme I



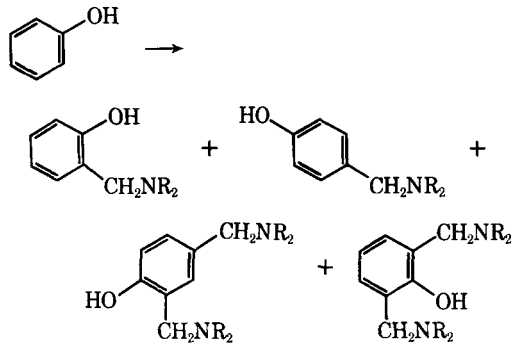
Scheme II

The amine used also promotes formation of mixed products. With secondary amines, only a single product is possible, however, with primary amines and ammonia, reacting ratios of active hydrogen compounds, formaldehyde, and amine may be 2:2:1 and 3:3:1, respectively. In the reaction of antipyrine salicylate, formalde-

Received from the School of Pharmacy, University of Georgia, Athens, GA 30601

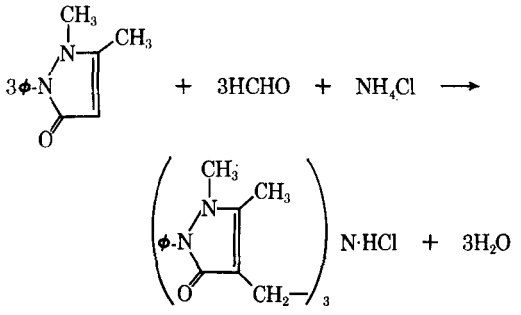
The author acknowledges financial support from The Office of General Research, The University of Georgia, Athens, GA 30601.

[1] The observation that *ortho*-mono-substitution predominates has been the subject of the mechanistic studies (17, 36).



Scheme III

hyde, and ammonium chloride (7), the reaction which reportedly stimulated Mannich's pioneering interest in the condensation which bears his name, the reaction ratio is 3:3:1, respectively, and may be illustrated as in Scheme IV.



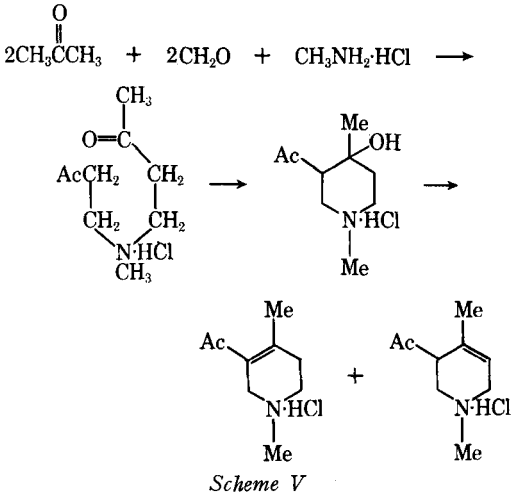
Scheme IV

Gautier *et al.* (18) have reported on the problem of isomer formation associated with utilization of compounds with mixed functionality such as ethynylalkyl aryl ketones as the active hydrogen compound. In this case both the hydrogen of the acetylenic substituent and the ketone's α-hydrogens are reactive under Mannich conditions.

A somewhat different isomer problem is presented in the case of methyl isopropyl ketone in which seemingly sound evidence for aminomethylation of the methyl group (19, 20) and of the isopropyl group (21) have been reported.

In many cases, by-products may be formed by side-reactions. In not a few cases, such processes may occur to the exclusion of normal Mannich reaction or, if sequential to the Mannich reaction, completely destroy the normal products. Such processes include (*a*) formation of α, β-unsaturated ketones (8, 22), a process which could result from deamination (elimination of amine) of the Mannich base, or alternatively, through hydroxymethylation followed by dehydration of the active hydrogen compound; (*b*) formation of methylene-bis derivatives of the active hydrogen compound; and (*c*) formation of methylene-bis-amines or similar compounds. These processes will be discussed within the framework of the Mannich reaction mechanism.

Mannich and Ball (23) and Mannich and Ritsert (24) reported formation of an unstable Mannich base derived from acetone, formaldehyde, and methylamine (2:2:1 reacting ratio). This Mannich base readily cyclizes as Scheme V shows:

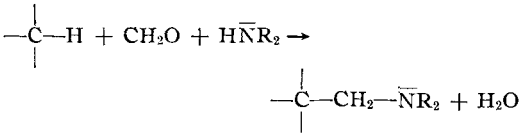


Scheme V

The stability of the products of the Mannich reaction will be covered more thoroughly in the succeeding sections.

MECHANISM OF THE CONDENSATION

The classical examples of the Mannich reaction involved reaction of an active hydrogen compound (reactive $\diagup$C—H $\diagdown$ linkage present) with formaldehyde and ammonia or a primary or secondary amine, *i.e.*, Scheme VI.



Scheme VI

The product, known as a "Mannich base," is an unsymmetrical derivative of methylene. Modern theoretical considerations permit inclusion of acidic $\diagup$NH, —OH, or —SH $\diagdown$ containing compounds as qualified "active hydrogen" compounds susceptible to the Mannich aminomethylation process.

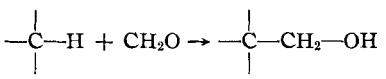
A great deal of work has been done relevant to the progress of the Mannich reaction (17–18, 22, 25–47), and several workers have proposed mechanisms (17, 22, 25, 34, 35, 40–47). A his-

torical development of the mechanism studies will be presented in the following paragraphs.

An evaluation of Scheme VI reveals that formaldehyde, an electrophilic agent, reacts with two nucleophilic components, the amine and a carbanic center derivable from the replaceable hydrogen compound. The product of the Mannich reaction is the result of an unsymmetrical condensation, and any satisfactory mechanism must take this factor into consideration.

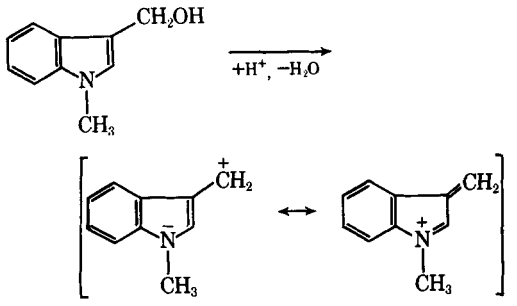
According to Hellmann and Opitz (25), the course of the condensation might be expected to follow one of the following sequences: (*a*) reaction of the labile hydrogen compound with formaldehyde to yield the hydroxymethyl derivative which then condenses with the amine to produce a Mannich base; or (*b*) addition of the amine to formaldehyde to form an *N*-hydroxymethyl or related derivative which then reacts with the labile hydrogen compound to produce a Mannich base.

In consideration of proposition *a*, if the nucleophilicity of the carbanion derived from the labile hydrogen compound be greater than that of the amine, formation of a hydroxymethyl derivative of the labile hydrogen compound would be favored (proposition *a*) over formation of an aminomethylol (proposition *b*). Thus the initial reaction, Scheme VII, would be expected to occur.



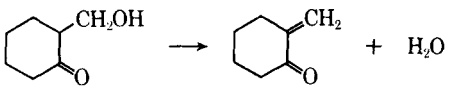
Scheme VII

This hydroxymethyl derivative will now react further if it can form either a resonance-stabilized carbonium ion, *e.g.*, Scheme VIII,



Scheme VIII

or if the loss of water can occur according to Scheme IX.



Scheme IX

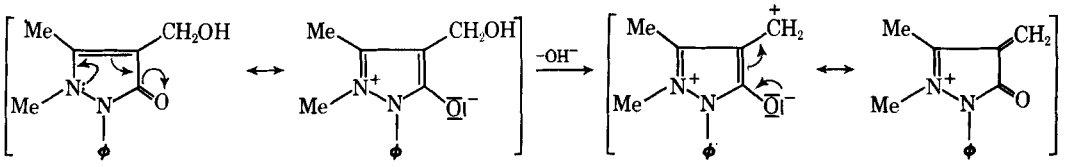
Either of these intermediates, *i.e.*, the resonance-stabilized carbonium ion in Scheme VIII or the unsaturated dehydration product shown in Scheme IX, would then be expected to condense with another molecule of the compound or ion with the highest nucleophilic potential, in this case, the carbanion derived from the labile hydrogen compound. Such a pathway could only lead to a symmetrical methylene derivative of the labile hydrogen compound and not to a Mannich base. If such reasoning is supported by experimental evidence, proposition *a* would seem untenable. Such evidence is available and will be presented below.

4-Hydroxymethylantipyrine, which forms a resonance-stabilized carbonium ion according to Scheme X, was studied by Bodendorf and Koralewski (31) as a possible intermediate in the Mannich reaction. 4-Hydroxymethylantipyrine, according to these workers, does not react with either free amine or with amine hydrochloride. Furthermore, free amine and variable amounts of acid likewise fail to yield the Mannich base of antipyrine. These observations were offered as proof against a hydroxymethyl derivative of the labile hydrogen compound as the intermediate in the Mannich reaction.

In their investigation of the Mannich reaction, Bodendorf and Koralewski (31) reported that the formation of methylene-bis(antipyrine) took place when 4-hydroxymethylantipyrine was warmed with dimethylamine hydrochloride but not with the free base. Hellmann and Opitz (25, 26) reinvestigated, based on the assumption that 4-hydroxymethylantipyrine is not the true intermediate in the Mannich, but that it is an intermediate in the synthesis of methylene-bis(antipyrine). If these be reasonable assumptions, then as the acidity of the media increases the yields of Mannich base and of methylene-bis(antipyrine) should not be coordinated closely. In fact, these workers (26) obtained 11.51% of Mannich base with piperidine, formaldehyde, and antipyrine with no methylene-bis(antipyrine), while piperidine hydrochloride under the same conditions gave 61.54% of Mannich base. When the quantity of acid present reached a 10% excess over the molar requirements, the yield of Mannich base fell to 16.8% and a yield of 9.7% of methylene-bis(antipyrine) also resulted.

While the above results disprove the participation of 4-hydroxymethylantipyrine as a resonance-stabilized derivative (formed *via* Scheme VIII) in the Mannich reaction is it equally unlikely that 2-methenylketones[2] (formed *via*

[2] These α-methenylketones may be viewed as vinylogs of formaldehyde. Free amines, but not their salts, will add to such compounds.



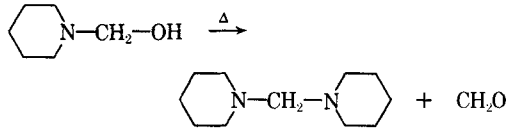
Scheme X

Scheme IX) could represent true intermediates? In answer to this question, such intermediates were discounted since dehydration of hydroxymethyl derivatives of the active hydrogen component often requires temperatures far in excess of those required by the Mannich reaction (25). Furthermore the observation that piperidine, formaldehyde, and cyclohexanone gave a 37% yield of Mannich base (31) while use of piperidine hydrochloride provided a 62% yield (48) appears to rule out addition to an α,β-unsaturated derivative as a possible course.

These data seem conclusive in proving that hydroxymethylation of the active hydrogen component does not represent a true course for the Mannich reaction. The conclusion that may, therefore, be drawn is that proposition *b* above probably is valid. This second postulate, that formaldehyde interacts with the amine as the initial step in the condensation, will now be discussed.

In order to present an effective discussion of the mechanistic aspects of *N*-hydroxymethylamine and related derivatives of aldehyde and amine, a brief discussion of the chemistry of these intermediates based on articles by Henry (49), Stewart and Bradley (50), Hellmann and Opitz (25–30), and by Wagner (51) will be covered below. For a more detailed discussion the reader should consult the original articles.

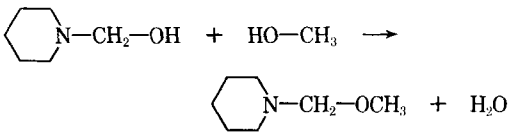
The *N*-hydroxymethylamines, as reported by Henry (49) in 1895, may be obtained from aqueous formaldehyde and the amine with cooling. These oily, nondistillable *N*-hydroxymethylamines are difficult to purify, and exist only in the cold. Warming causes decomposition to formaldehyde and free amine along with some recondensation to form methylenediamine, *e.g.*, Scheme XI.



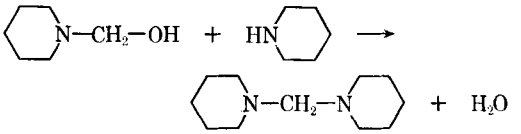
Scheme XI

Methylenediamines are formed exclusively when formaldehyde and amine are allowed to react without cooling.

In contrast to oxygen hemi-acetals, nitrogen hemi-acetals are converted to *O,N*-acetals with alcohols or to *N,N*-acetals (methylenediamine) with amines without added catalysts. For example, Schemes XII and XIII.



Scheme XII



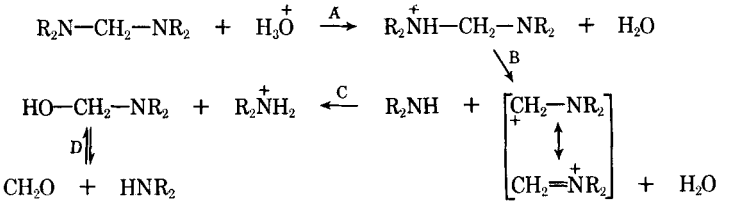
Scheme XIII

The members of both compound classes are very acid-sensitive and follow a well-defined reaction course in acid hydrolysis (50) from the symmetrical methylenediamine as illustrated in Scheme XIV.

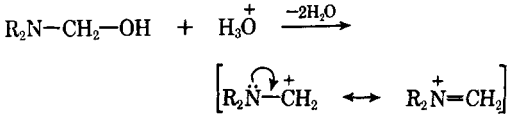
The ammonium salt of methylenediamine which formed in step A decomposes immediately in water to form an aminomethyl carbonium ion (reaction B). The acid in this system may react further to neutralize the free amine formed in equilibrium (D), or it may act directly on the intermediate, *N*-hydroxymethylamine. The latter action was discussed by Stewart and Bradley (50), who reported that addition of an *N*-hydroxymethylamine to a solution of aqueous acid followed the course illustrated in Scheme XV. The reverse order of mixing appeared to favor formation of the salt of the free amine.

The above cleavage at first glance appears to be unlikely since nitrogen, the most basic center of the molecule, ought to bind the proton. Experimentally, however, it has been demonstrated that the ammonium salt is unstable in aqueous solution and can be formed only under anhydrous conditions (31, 52).

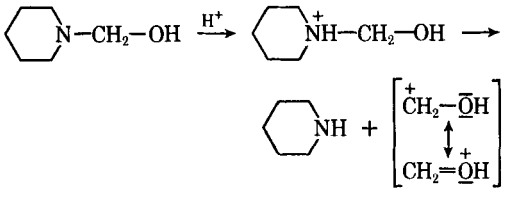
If the stability of the ionic species formed in the cleavage is considered, the formation of the aminomethyl carbonium ion according to Scheme XIV is understandable. The two possible courses for the cleavage are shown in Schemes XVI and XVII. The hydroxymethyl carbonium ion of Scheme XVI is considerably less stable than the aminomethyl carbonium ion of Scheme XVII, hence the greater resonance stabilization of the



Scheme XIV

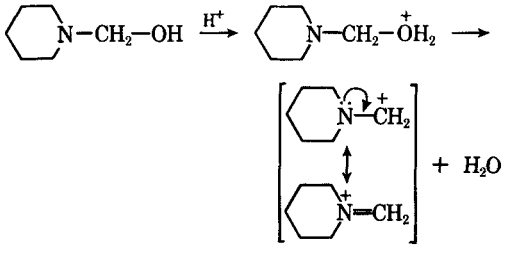


Scheme XV



Scheme XVI

or,



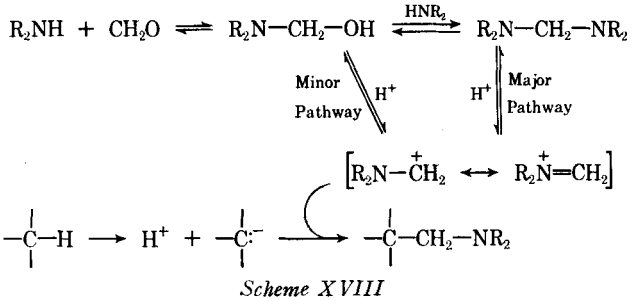
Scheme XVII

latter ion makes Scheme XVII the favored pathway.

In studying the possible role of *N*-hydroxymethylamines in the Mannich condensation, Bodendorf and Koralewski (31) condensed antipyrine with formaldehyde and piperidine hydrochloride to obtain the Mannich base, 4-piperidinomethylantipyrine, in 62% yield. With free piperidine and formaldehyde a 37% yield of the Mannich base was obtained. With preformed *N*-hydroxymethylpiperidine, the compound suggested by Mannich as the reaction intermediate, a yield of only 20% was obtained. These workers concluded that *N*-hydroxymethylamine is not the intermediate in the Mannich reaction.

Hellmann and Opitz later proved that the Mannich base in the latter two combinations is formed during the isolation procedure when acid conditions prevail (26). If the active aminomethylating agent is the electrophilic aminomethyl carbonium ion, which arises only under acidic conditions, then the conclusion of Bodendorf and Koralewski (31) against *N*-hydroxymethylamine as the intermediate was based on inaccurate information. Certainly knowledge of the formation of this ion, contributed by Stewart and Bradley (50), supports the work of Hellmann and Opitz (25–30).

The next significant studies of the mechanism of the Mannich reaction were published in 1949 by Liebermann and Wagner (32) and by Alexander and Underhill (33). Liebermann and Wagner concluded that each combination of reactants requires a different pH for the best results and that an initially high acidity is required. These workers concluded that the aminomethyl carbonium ion previously mentioned is the final electrophilic intermediate and that it reacts with a carbanion which arises from the acidic or labile hydrogen compound. According to the mechanism proposed by these authors, methylenediamine is the intermediate formed in the Mannich reaction prior to formation of the aminomethyl carbonium ion. However, the possibility that *N*-hydroxymethylamine might be required in a few reactions demands its retention in the overall



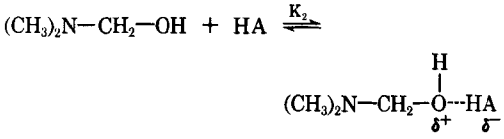
Scheme XVIII

mechanism. The complete sequence is shown in Scheme XVIII.

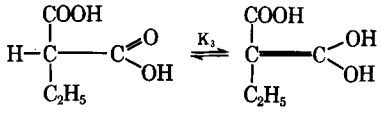
Alexander and Underhill (33) offered a mechanism which involved *N*-hydroxymethylamine as the intermediate and in which the acidic hydrogen compound was suggested to react in the enol form rather than to ionize as Liebermann and Wagner proposed. The reason for suggesting an enolic intermediate rather than an ionic intermediate was based on ionic strength effect experiments which failed to alter the rate of the reaction. Had an ionic intermediate been involved prior to the rate-controlling step, the reaction rate should have changed in proportion to the change in the ionic strength of the media. The reaction is observed to proceed immediately if preformed *N*-hydroxymethyl-*N*,*N*-dimethylamine is employed, but an induction period is required if formaldehyde and free amine are used instead. This observation indicates that a slow reaction between formaldehyde and dimethylamine is required before typical third-order kinetics are observed. It also indicates that the rate of this condensation between formaldehyde and dimethylamine, although faster than the rate-controlled step, is of the same general magnitude.

The mechanism proposed for the condensation of ethylmalonic acid with formaldehyde and dimethylamine shown in Schemes XIX–XXIII.

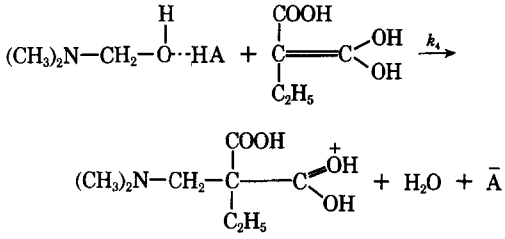
Scheme XIX



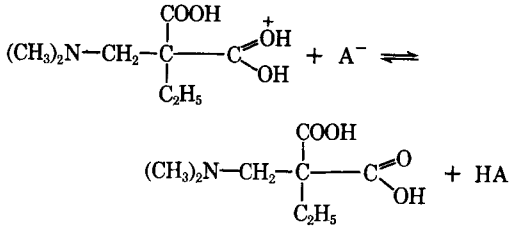
Scheme XX



Scheme XXI



Scheme XXII



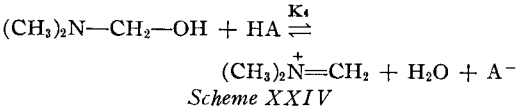
Scheme XXIII

This sequence calls for general acid catalysis by HA with Scheme XXII as the rate-controlling step. The rate expression reduces to:

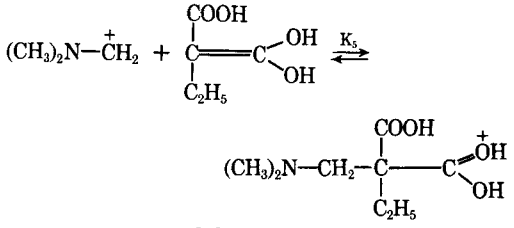
$$\frac{dx}{dt} = k[CH_2O][(CH_3)_2NH][C_2H_5 \cdot CH(COOH)_2]$$

where k includes the concentration of HA which remains constant.

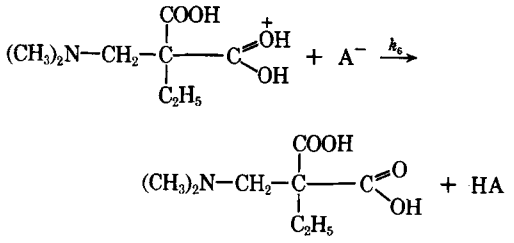
The possibility was considered that Schemes XX, XXII, and XXIII might be replaced by the sequence in Schemes XXIV–XXVII.



Scheme XXIV



Scheme XXV



Scheme XXVI

This was ruled out since Scheme XXVI is very unlikely to be the rate-determining step. Consideration of Scheme XXV as rate determining requires that the reaction velocity be dependent upon the oxonium ion concentration. Alexander and Underhill decided against this course because of the somewhat complex pH dependency of the reaction. As Hellmann and Opitz later indicated (25), the complicated nature of the pH dependency may be solved when one considers that acid retards formation of *N*-hydroxymethyldimethyl amine to such an extent that Scheme XIX, which

appears to be only slightly faster than the rate-controlling step, may finally become rate determining. Since dimethylaminomethyl carbonium ion is used up faster than it can be formed, the reaction may decrease with decreasing pH. This problem was also dismissed by Cummings and Shelton (34), who pointed out that the reaction in Scheme XXVII properly belongs in the kinetic

$$R_2\overset{+}{N}H_2 + \bar{A} \rightleftharpoons R_2NH + HA$$

Scheme XXVII

equation since, in acid solution, the amine exists largely in the salt form. This fact accounts for the conclusion (above) by Hellmann and Opitz (25) and simultaneously rids the kinetic expression of its "specific oxonium requirement." Therefore, Schemes XXIV, XXV, and XXVI cannot be eliminated as possibilities.

When, as in the example by Alexander and Underhill (33), an acid is employed as the active hydrogen compound and reacts in the acid form, a term to account for the acid to anion dissociation reaction must be included. It is probably in part for this reason that these workers observed the complex interaction between pH and the rate constant. Contrary to the opinion of Alexander and Underhill (33) that specific oxonium ion catalysis cannot explain this complex variation of rate constant with pH, it seems likely that the above-discussed factors could easily account for this complexity (34).

Hellmann and Opitz (25–30), in a series of comprehensive studies, proved that *N*-hydroxymethylamine, *N*-methoxymethylamine, and methylenediamine could all be formed in the course of the Mannich reaction, depending upon the conditions employed. Since highest yields were obtained when one of these materials was added to a mixture of excess aqueous acid and the acidic or labile hydrogen compound, a method which gives the highest concentration of the aminomethyl carbonium ion (50), it was concluded that this ion was the active aminoalkylating agent. The reverse order of addition, *i.e.*, adding acid to a mixture of the reactants, does not offer favorable pH conditions until the addition is essentially complete. It was also found that a pH as low as 1.0 is successful in some cases if the proper order of addition is used.

Based on the extensive experimentation carried out, Hellmann and Opitz (25) concluded that rather strong inductive effects must be operating in order to activate the labile hydrogen compound sufficiently to react as a carbanion, or there must be enol formation in the absence of strong inductive effects (28). The proposed mechanism offered is quite similar to the mechanism in acid media offered by Cummings and Shelton (34) and represents an S_E2 mechanism. The S_N2 mechanism under basic conditions was covered under the title of transmethylation owing to the opinion by Hellmann and Opitz that the Mannich reaction is that aminomethylation occurring under acidic conditions (25, 30).

Hellmann and Opitz (25, 26) reinvestigated the condensation between antipyrine, formaldehyde, and piperidine in the belief that the data of Bodendorf and Koralewski (31) simply did not seem to fit available postulates. Among other things they determined that the purification procedure of Bodendorf and Koralewski failed to remove methylene-bis(4-antipyrine), a major contaminant of the Mannich base. Following the published procedure (31), in which a 37% yield had been reported but purifying by *in vacuo* distillation, Hellmann and Opitz (26) were able to remove methylene-bis(4-antipyrine) (b.p. 238° at 760 mm.; 100° at 2 mm.) and thus effect an improved purification of the Mannich base. The yield of Mannich base amounted to only 11.51% (26). The use of *N*-hydroxymethylpiperidine and antipyrine gave 13.89% (26) instead of 20% as reported by Bodendorf and Koralewski (31). Using antipyrine, formaldehyde, piperidine, and an equimolar portion of hydrogen chloride provided 62% yields according to both groups (26, 31). These data point to an acid-catalyzed reaction for production of the Mannich base of antipyrine. Of itself, antipyrine is too weakly acidic to catalyze the reaction.

The negligible yields with basic conditions in the antipyrine example suggested that perhaps the Mannich reaction on antipyrine does not occur at all under these conditions. A careful study of the procedures of Bodendorf and Koralewski (31) led Hellmann and Opitz (26) to the conclusion that the small quantity of Mannich base formed in the procedure arises during product isolation, when acidic conditions prevail. By redesigning the workup procedure as little as 3–5% of product was obtained, an observation which certainly supports the "no-reaction" hypothesis.

According to Stewart and Bradley (50) the maximum concentration of the aminomethyl carbonium ion occurs on addition of *N*-hydroxymethylamine *N*-alkoxymethylamine or methylene-bis(amine) to aqueous acid. Reversing the order of addition leads to little or at best very slow production of the necessary ion. It may be inferred from these data that proper order of addi-

tion is important for the successful testing of these intermediates.

In order to evaluate the relative effectiveness of these intermediates, Hellmann and Opitz (25, 26) compared yields using the two different orders of addition with each of the three possible intermediates. *N*-Hydroxymethylpiperidine, when added to a solution of antipyrine and excess acid (10% excess, pH 2.5), gave a 71% yield of Mannich base, while the same ingredients gave only 56% of Mannich base along with some methylene-bis(4-antipyrine) when the acid was added to the antipyrine and *N*-hydroxymethylpiperidine mixture. Use of *N*-methoxymethylpiperidine gave, on addition to a mixture of antipyrine and excess acid (6% excess, pH 3.0), gave an 87% yield of Mannich base. When the acid was present to the extent of a 40% excess (pH 1.0), the reaction still gave a 77% yield of Mannich base. They found that with similar conditions but reversing the order of addition completely inhibited the Mannich reaction. Finally, Hellmann and Opitz report an 81% yield of Mannich base when methylene-bis(*N*-piperidine) is added to antipyrine and excess acid (12% excess, pH 2.0).

The above data certainly support the concept that *N*-hydroxmethylpiperidine, and methylene-bis(*N*-piperidine) can serve as a source of the aminomethyl carbonium ion. The apparent success of this procedure supports the contention that the Mannich reaction does require one of these substances as an intermediate. The possibility certainly exists that no one of these intermediates can serve for all examples of the Mannich reaction (17, 25–47). Additional results which should be cited for this study include the observation that addition of aldehyde decreases the pH of the solution (25), *i.e.*, Scheme XXVIII.

$$R_2\overset{+}{N}H_2 + \bar{A} + CH_2O \rightleftharpoons R_2N{-}CH_2{-}OH + HA$$

Scheme XXVIII

Hellmann and Opitz (25) also expressed doubt regarding the possible formation of methylene-bis(*N*-amines) under acidic conditions, particularly if water is present. These derivatives of formaldehyde and amine simply are not stable under these conditions (50).

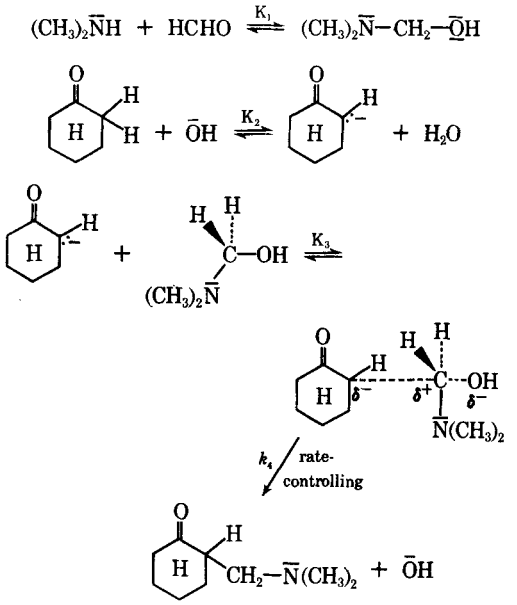
In the two major contributions to the mechanism of the Mannich reaction which had appeared in 1949, a divergence of views was obvious. Liebermann and Wagner (32) had proposed a process in which two ions, a carbanion derived from the active hydrogen compound and the aminomethyl carbonium ion, derived from an aminomethylol or a methylene-bis-amine, neutralized each other. Alexander and Underhill (33) found that no significant change in the rate occurred with changes in ionic strength of the media. If a carbanion-carbonium ion neutralization is present in the transition state of the rate-controlling process, a large change in rate with change in ionic strength ought to have been observed (53). Secondly, based on mechanistic pathways visualized by Alexander and Underhill, the outright liberation of a free aminomethyl carbonium ion for reaction with a carbanion would require kinetic independence of the reaction from pH influence. Alexander and Underhill had, however, observed a pH maxima at 3.8. They reasoned that below this pH, the rate began to be severely limited by extensive conversion of amine into its salt form.

Hellmann and Opitz (25–30) pointed out that the conversion of amine and formaldehyde into aminomethylol was reported by Alexander and Underhill to be only slightly faster than the rate-controlling step. They pointed out that the complex pH effects could be explained when one recalls that the effect of mounting acidity is to decrease the concentration of free amine. This deficiency of free amine can make the already slow conversion to aminomethylol finally become rate controlling. The mounting acidity, which does not favor carbanion formation, coupled with the absence of ionic strength effects, convinced Hellmann and Opitz that the reaction proceeded without involving the carbanion of the active hydrogen compound. The aminomethyl carbonium ion, derived from an *N*-hydroxymethylamine, was concluded to be the aminomethylating agent.

A recent kinetic study by Cummings and Shelton (34) reached conclusions quite similar to those of Hellmann and Opitz (25–30) concerning the mechanism of the Mannich reaction. The mechanisms proposed by these workers are given for basic media in Scheme XXIX and for acidic media in Scheme XXX. Several comments by Cummings and Shelton (34) were made relevant to the previous kinetic and qualitative studies. They pointed out, as did Hellmann and Opitz (25–30), that the amine is largely present as the salt under acidic conditions. Incorporating this factor into the reaction sequence they obtained a rate equation in which the rate was independent of the pH. At sufficiently low pH, this is precisely what was observed with cyclohexanone as the active hydrogen compound (34). The example chosen by Alexander and Underhill (33) involved an acid as the active hydrogen compound. If the reactive form is the acid, as shown in their mechanism, then another term to account for the acid form and ionic form equilibria should be present

in the equation. This would make the rate equation dependent upon the hydronium ion concentration.

It is of interest to note that the mechanistic pathway and rate equations of Cummings and Shelton (34) in acid media involve production of a dimethylaminomethyl carbonium ion from *N*-hydroxymethyldimethylamine and that this ion then reacts with an α-carbanic center developed



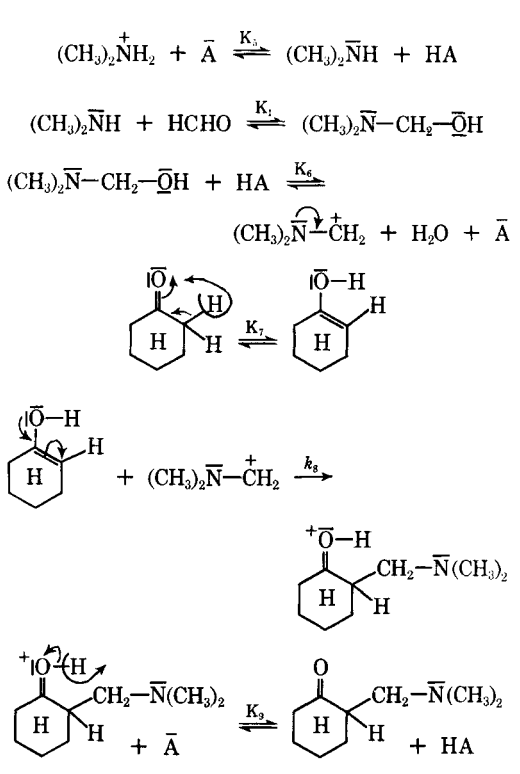
$$\frac{dx}{dt} = k[(CH_3)_2\bar{N}H]\cdot[CH_2O]\cdot[C_6H_{10}O]\cdot[\bar{O}H]$$

Scheme XXIX

on the enol form of cylcohexanone. The rate expression does not contain an HA or an H_3O^+ term. The view is, therefore, a composite of previous suggestions (33, 25–30).

The mechanism for basic media does not suggest formation of the dimethylaminomethyl carbonium ion, which indeed should only be present in acidic media. Instead dimethylaminomethanol is the intermediate on which an S_N2 displacement of OH^- by the cyclohexanone carbanion takes place. The appropriate carbanion presumably arises by base attack on the reactive α-protons of cyclohexanone.

The above-mentioned kinetic study does much to clarify the general views on the course of the Mannich reaction but have been unable to resolve problem of deciding among methylene-bis-amines, *N*-alkoxymethylamines, or *N*-hydroxymethylamines as the true intermediate in the Mannich reaction. Several recent kinetic studies provide further information on the mechanism and technology of the Mannich reaction (17, 35, 36, 43–47, 54–58).



$$\frac{dx}{dt} = k[(CH_3)_2\overset{+}{N}H_2]\cdot[CH_2O]\cdot[C_6H_{10}O]$$

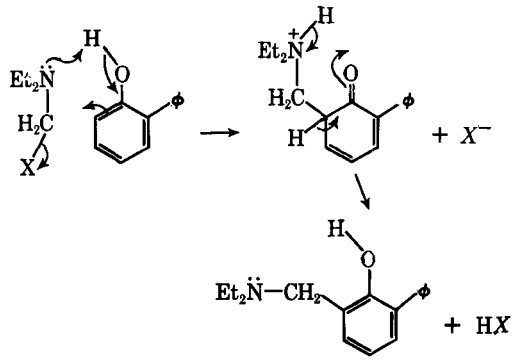
Scheme XXX

Burckhalter and co-workers (17, 36) have studied the mechanism of the Mannich reaction on phenols with emphasis on the observed preponderance of *ortho* substitution. For example, Burckhalter (59) obtained a preponderance of the *ortho*-substituted product (42%) of 2-phenylphenol and less than a third (13%) of the *para*-substituted isomer. Several additional references are cited by Burckhalter and Leib (36) in reviewing the subject.

In the mechanistic and kinetic studies carried out in basic media by Burckhalter and co-workers (17, 36), the rate of condensation of formaldehyde and morpholine with 2,4-dimethylphenol is controlled by formaldehyde if the concentration of formaldehyde is *less* than half the morpholine concentration. Conversely, morpholine concentration is critical if it is *less* than *twice* the formaldehyde concentration. These data suggest the participation of methylene-bis(*N*-morpholine) as an intermediate. A pure sample of this compound gave data kinetically indistinguishable from those of the formaldehyde and morpholine mixture. Calorimetric data by Fernandez and Butler (44) also supported a methylene-bis-(amine) intermediate.

Burckhalter and co-workers (17, 36) suggested that *ortho*-substitution predominates in amino-

methylation of phenols because of hydrogen bond participation. The process was visualized as in Scheme XXXI.



$X = -OH, -OR, -NR_2$.

Scheme XXXI

In view of preceding comments the most likely intermediate would be the methylene-bis(amine) ($X = -NR_2$). Since phenols are acidic and capable of existing as free phenol or phenolate ion and, since the reactive intermediate amine conceivably might react as a neutral molecule or as a positively charged molecule, a maximum rate ought to be observed at some definite pH if two neutral (phenol plus amine) or two charged ions (phenolate ion plus positively charged amine derivative[3]) interact. Conversely the reaction rate should increase with (*a*) decreasing pH if free phenol and protonated amine interact, and (*b*) with increasing pH if phenolate and free amine interact. The observed rate *versus* pH profile provided a maximum between pH 9.6 and 10.2 (maxima at approximately pH 10.0) indicating that the reaction occurs between either a pair of neutral reactants (phenol plus amine) or between a pair of ions (phenolate plus amine salt). Several factors weaken the case for the latter possibility. First, the pH at which the maximum occurs is basic. The methylenebis-(morpholine) salt should not be stable under strongly acidic conditions (25, 51) unless present in an anhydrous medium (52), and is not likely to form in the basic pH studied. Secondly, the preponderance of *ortho*-substitution isomers previously observed (36, 59) ought to involve a concerted mechanism (36) which is most favorably accommodated by a hydrogen bonded complex between the neutral phenolic molecule and the methylene-bis(amine) structure (Scheme XXXI).

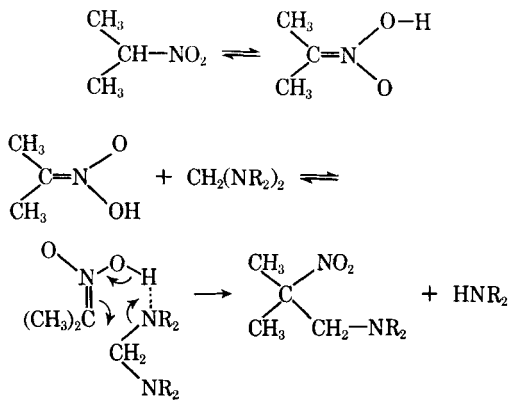
Fernandez and associates (44–47, 55–58) have contributed calorimetric, cryoscopic, and kinetic studies recently. Based on the colorimetric data obtained in the conversion of formaldehyde and amine to *N*-hydroxymethylamine and then, with a second mole of amine, to methylene-bis(amine), Fernandez and Butler (44) concluded that the reaction proceeds in most cases to methylene-bis-(amine). There seems to be little *N*-hydroxymethylamine at equilibria in most of the examples studied. This is not surprising in view of the technique used, *viz.*, amine (two parts), was treated slowly with aqueous formaldehyde (one part). It has been reported that the intermediate, *N*-hydroxymethylamine, is unstable in the presence of excess amine, reacting to yield the bis(amine) (49, 51). The previous study by Burckhalter *et al.* (17, 36) certainly supports the concept of a methylene bis(amine) as the most likely intermediate under basic conditions as did this calorimetric study (44).

Fernandez and Fowler (45) in 1964 reported the kinetics associated with the reaction of 2-nitropropane and methylene-bis(piperidine) or methylene-bis(morpholine). Choice of the methylene bis(amines) simplified the process and circumvented the necessity of speculation concerning identity of the intermediate form of the formaldehyde and amine. To further ensure the integrity of this intermediate, water was excluded by use of anhydrous solvents. The choice of methylene bis(piperidine) and methylene-bis(morpholine) provided widely different basicities (pKb 2.78 and 5.64 for the starting amines, respectively). Solvents included dioxane, DMF, nitrobenzene, and triethylamine.

Briefly, results of the experiments confirmed that the more basic methylene-bis(piperidine) exhibited much faster rates than did the morpholine derivative. Excessive basicity, as with excess methylene-bis(piperidine) or triethylamine as solvent, caused a slowing of the condensation. Fernandez and Fowler (45) reasoned that under these conditions the nitropropane reacts in the anionic form and produces an extremely reactive amide ion (R_2N^-) as the conjugate base. The latter is not a favored state and thus slows the overall rate. The rate also increased with an increase in dielectric constant. In media of low dielectric constant the mechanism is suggested to occur *via* a hydrogen-bonded complex of the *aci*-form of 2-nitropropane interacting with methylene-bis(amine). (See Scheme XXXII.)

At higher dielectric constant the activation parameters suggested to Fernandez and Fowler (45) that the mechanism changes to one involving a higher enthalpy of activation and lower entropy of activation. The mechanism visualized involves incorporation of a molecule of methylene-

[3] The charged amine was referred to as a protonated methylene-bis(amine) (17). The aminomethyl carbonium ion might also be a possibility.



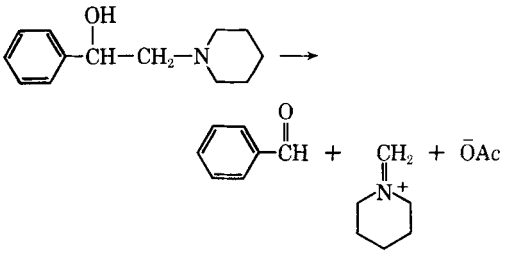
Scheme XXXII

bis(amine) into the solvent shell of 2-nitropropane. The transition state visualized thus involves coordination of the hydroxyl proton of *aci*-2-nitropropane with solvent, thus differing from the above cyclic or quasi-6-membered transition in which this hydroxyl proton is coordinated with the amino group which will be eliminated. The transition state for high dielectric media is, therefore, less rigid and restrained but yields the reactive amide ion, R_2N^-, on conversion to product. The value for ΔS^* is thus less and ΔE^* greater than for the low dielectric media transition state.

Methylene-bis(piperidine) gave accelerated rates when compared to the morpholine derivative. This was attributed to the enhancement of electron density at the α-methylene by the more basic piperidine moieties (45).

The mechanism studies on 2-nitropropane (45) were extended by Fernandez, Fowler, and Glaros (46) to nitromethane and nitroethane. Methylene-bis(piperidine) and methylene-bis(morpholine) again were used as the aminoalkylating agents. Previously all attempts to prepare mono-aminomethylated nitromethane resulted in multiple substitution (40, 60–63). In the study by Fernandez *et al.* (46) mono-substitution of nitromethane resulted when it was treated with methylene-bis(amines) under anhydrous conditions. When a small amount of water is present, or if *N*-ethoxymethylpiperidine is used, disubstitution results. These data suggest that the second alkylation step probably requires *N*-hydroxymethylamine or *N*-alkoxymethylamine in order to be successful. By inference, the initial aminoalkyl substitution probably may occur with any one of the three possible aminoalkylating agents. The evidence presented by Fernandez *et al.* (45, 46, 55) and by Burckhalter and coworkers (17, 36) certainly supports the hypothesis that basic conditions favor *formation* of methylene-bis(amines) (49, 51) and that these intermediates may function as aminoalkylating agents (25, 26). This does not lead to the conclusion that such an intermediate is necessarily the *best* agent for aminoalkylation based on rate or position of equilibria.

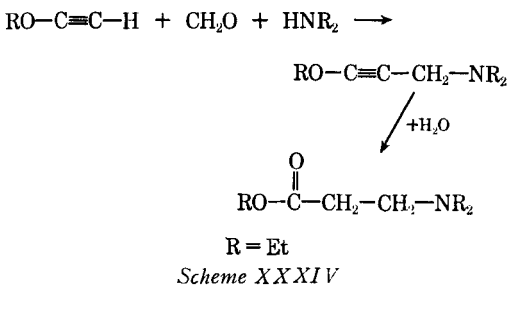
Roth, in 1961, suggested that the lead tetraacetate cleavage of 1, 2-amino-alcohols in which the amine is tertiary leads to formation of aminomethyl carbonium ions according to Scheme XXXIII (64).



Scheme XXXIII

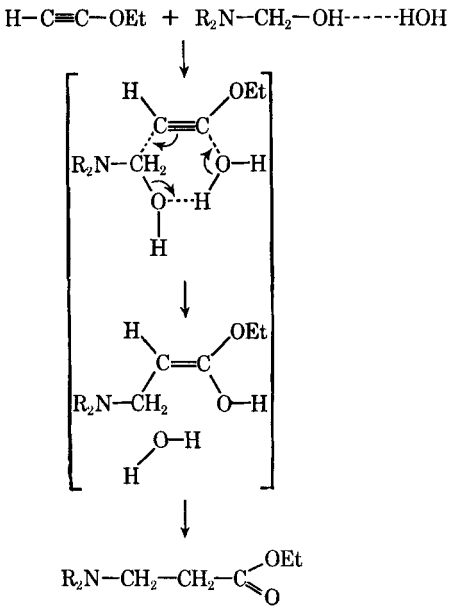
In a later study Roth was able to demonstrate that the aminomethyl carbonium intermediate did indeed occur in this cleavage (35). By adding typical Mannich reaction components (acidic C—H and N—H containing compounds) during the above type cleavage reaction, Roth was able to isolate the corresponding Mannich bases in high yields. Although this did not afford absolute proof that the aminomethyl carbonium ion is the true aminomethylating agent, it provided additional support for this hypothesis. In the absence of a suitable trapping agent, the aminomethyl carbonium ion formed in the study cited above (64) was hydrolyzed yielding formaldehyde and secondary amine hydrochloride or acetate. Among the compounds selected by Roth as suitable acidic hydrogen-containing compounds (either CH or NH) are phthalimide, theophylline, succinimide, carbazol, benzoxazolone, benzamide, isatin, saccharin, barbital, acetophenone, antipyrine, cyclohexanone, and indole (35).

Studies involving use of acetylenic compounds in the Mannich reaction have afforded successful condensations in the case of phenylacetylene and its substituted analogs (65). When ethoxyacetylene was employed in the Mannich reaction, Arens and co-workers (66) obtained what appeared to be the hydrolysis product of the corresponding Mannich base, *i.e.*, Scheme XXXIV. Under anhydrous conditions (dioxane as solvent) no trace of Mannich base was isolable. The mechanism proposed suggests that a hydrated *N*-hydroxymethylamine interacted as shown in Scheme XXXV. The alkoxy group in such acetylene derivatives tends to create electron-rich



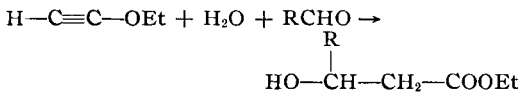
R = Et

Scheme XXXIV



Scheme XXXV

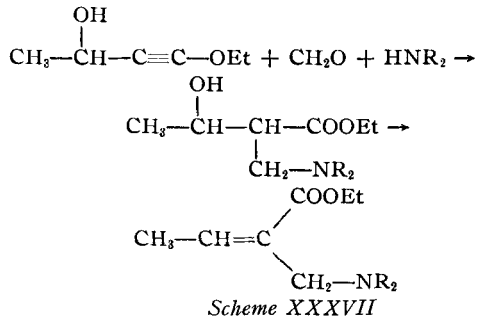
character about the carbon bearing the "replaceable" hydrogen. The above process obviously is not a Mannich reaction and the "replaceable" hydrogen never leaves the acetylenic group. Further proof was offered when, in 1957, Vieregge and Arens (67) demonstrated that ethoxyacetylene, water and aldehyde would react as in Scheme XXXVI.



Scheme XXXVI

The obviously similar product was formed in the absence of added amine. In the same paper, these workers cited experiments by van der Werf and Arens (unpublished data) in which the aminomethylation and hydrolysis had occurred in a molecule that did not contain a replaceable hydrogen (Scheme XXXVII).

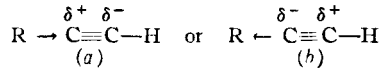
Fernandez *et al.* (55) reported that propargyl alcohol was aminomethylated at the hydroxyl group in preference to the acetylenic group in the absence of added catalyst. Salvador and Simon



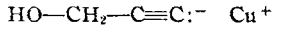
Scheme XXXVII

(43) undertook a more detailed study of the behavior of propargyl alcohol under Mannich reaction conditions. Under conditions chosen for the study, Salvador and Simon were not able to isolate the aminomethyl propargyl ethers said to be the preferred product under previously used conditions (55). Instead methylene-bis(amines) were the sole isolable products (43).

According to Salvador and Simon (43) the electronic nature of acetylenes can be represented as follows:



The presence of an α-hydroxyl group as in propargyl alcohol precludes formation of form *b* and, since this is thought to be the type of electron distribution required for a successful Mannich condensation with acetylenic substances, propargyl alcohol should not undergo the Mannich condensation. By conversion of propargyl alcohol to a copper salt,



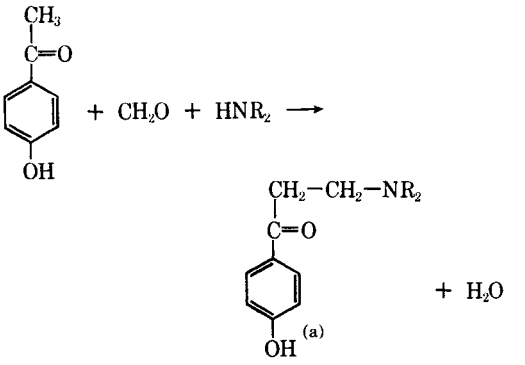
the problem of providing a suitable carbanic species for reaction with a suitable derivative of formaldehyde and amine was easily solved. In practice, the addition of a quantity of cupric sulfate to the buffered, aqueous solution was sufficient to catalyze the reaction (43).

Since the reaction was carried out in aqueous media, insoluble reaction products separated and were accessible for identification. During condensation of propargyl alcohol, piperidine, and formaldehyde, an oily layer separated (43). On distillation the oil produced a strong evolution of formaldehyde and gave methylene-bis(piperidine) as the distillate. Since formaldehyde and piperidine originally were introduced in 1:1 ratio infrared spectral data were obtained on the original oil prior to distillation. The original oil was thus identified as *N*-hydroxymethylpiperidine by comparison with an authentic sample (49). Salvador and Simon (43) then combined propargyl alcohol and *N*-hydroxymethylpiperidine in aqueous cupric sulfate and obtained poorer yields than was

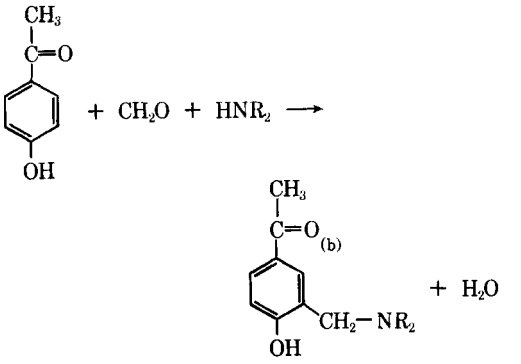
obtained with simple starting materials. Substitution of methylene-bis(piperidine) for the *N*-hydroxymethylpiperidine gave yields similar to those of the regular ingredients. These data indicate that methylene-bis(piperidine) is the true reaction intermediate. It should be recalled that the above-mentioned intermediates were isolated and identified from the reaction itself.

Based on isolation of *N*-hydroxymethylpiperidine from the reaction mixture, its ready conversion to methylene-bis(piperidine) on heating, and the significantly more favorable results on using the latter as a starting material, Salvador and Simon proposed the reaction pathway shown in Scheme XXXVIII. In this pathway the formation and existence of *N*-hydroxymethylpiperidine in mildly acidic conditions is not a very favorable pathway hence its further conversion to the aminomethyl carbonium ion is slow. This effect plus the decreasing availability of the acetylide ion in more acidic media results in slow condensation under mildly acidic conditions (about 80% yield in 5.5 hr. at pH 6.0) and no condensation below pH 3.0. In basic media the cupric acetylide formed more readily as did methylene-bis(piperidine). Thus an 80% yield was obtained in only 1.0 hr. at pH 8.4. The formation of a protonated methylene-bis(piperidine) in mildly basic media, and its subsequent decomposition to an aminomethyl carbonium ion is not very plausible. In this respect the proposed pathway of Salvador and Simon (43) probably is in error since much evidence to support methylene-bis(piperidine) *per se* as the intermediate under basic conditions has been presented (17, 36, 45, 46, 55).

Examples are available in which the Mannich reaction may occur at two different sites within a molecule, each of which represents a different class of active hydrogen. For example, with *p*-hydroxyacetophenone the following reaction courses are conceivable (68): Schemes XXXIX and XL.

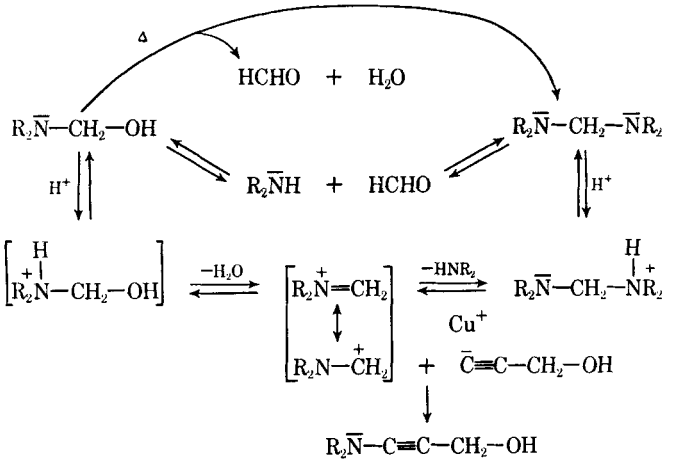


Scheme XXXIX



Scheme XL

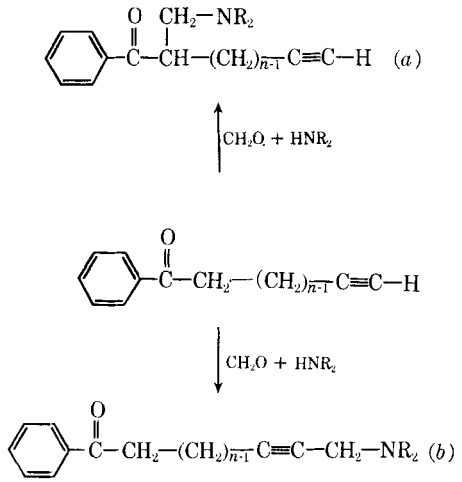
However, only examples of the first class of reaction were available (69–74) prior to 1964. Recently, the selective occurrence of reactions, illustrated as Scheme XL, has been recorded by Gautier *et al.* (68). Products were obtained in 50–60% yields. In general these workers found



Scheme XXXVIII

that acidic conditions favored condensation at the ketonic site, the suggested mechanism being similar to the Cummings and Shelton (34) proposal (Scheme XXX). Under basic conditions interaction with the neutral form of the aminomethylating agent [*N*-hydroxymethylamine or methylene-bis(amine)] occurs preferentially at the ring positions situated *ortho* to the phenolic group. Unfortunately, no examples were given for testing these "selective" conditions in which the phenolic group and ketonic carbonyl groups were not conjugated. This ought to affect the relative reactivities of the two sites to a noteworthy extent.

Gautier *et al.* (18) also have reported success in selective aminomethylation of ketonic and acetylenic moieties within the same molecule. The possible pathways, Scheme XLI, *a* and *b*, proved



Scheme XLI

to be accessible under acidic and basic conditions, respectively. Yields of type *a* compounds were in the 10–55% range while type *b* products were obtained in 48–63% yields. Gautier *et al.* (18) postulated an acetylide carbanion attack (SN2 mechanism) on *N*-hydroxymethylamine as the course of the reaction. In view of earlier discussions the methylene-bis(amine) is more likely to be present as the reactive intermediate. In acidic media, the reaction was considered to occur by means of an aminomethyl carbonium ion attack on the enol form of the ketone (18).

Faberov and Mironov (54) recently conducted a kinetic study of the Mannich reaction applied to ketones and aldehydes with subsequent amine elimination. Much of the data presented parallel data offered by Alexander and Underhill (33), Cummings and Shelton (34), and Fernandez *et al.* (45, 46). Both the product identity in one case (75) and the high yield reported (54) have been challenged (76). Such confusion concerning product identity in the case of unsymmetrical aliphatic ketones has been the subject of many publications (19–21, 75–82). Faberov and Mironov also calculated activation energies for some of the active hydrogen compounds used in the study.

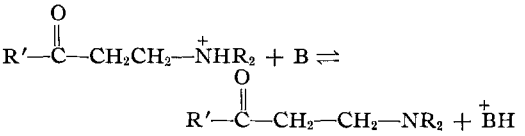
DECOMPOSITION REACTIONS OF MANNICH BASES

Mannich bases frequently are unstable. One type of instability, *i.e.*, propensity for amine elimination, has long been recognized (8, 11, 83–87). This type of decomposition can occur only if a proton is situated vicinal to the amine group and appears to be more prevalent when β-aminoketone salts or the free base form of β-aminocarboxylic acids are heated with water, *e.g.*, as in steam distillation. Faberov and Mironov (54) have reported obtainment of very high yields of α,β-unsaturated ketones and aldehydes by conducting the Mannich condensation in acidic solution and steam distilling without prior isolation of the intermediate Mannich base. Success of the method was reported to be dependent upon successful formation of Mannich base salts in high yields. These workers studied the relationship of pH to the rate of the Mannich reaction in order to assure rapid attainment of maximum yields (54).

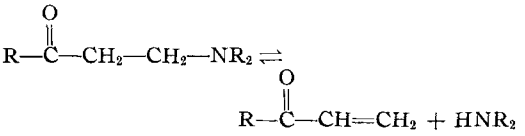
Angeloni and Tramontini (89) studied the rate of decomposition of β-aminoketone hydrochlorides in boiling water. They observed pseudo first-order kinetics for the decomposition. Their data are summarized as follows. The stronger the base (amine) present in the structure, the greater the stability of the Mannich base hydrochloride; addition of sulfuric acid slows the deamination reaction thus suggesting that the free Mannich base and not its salt is the reacting species. The unsaturated ketone product can add free amine but not amine salts. Because of this fact, elimination should be more complete with an equivalent of acid present even though the rate is greater in basic solution.

Horák and co-workers (88), using a polarographic method, were able to follow the deamination of 2-piperidinomethylcyclohexanone and of ω-piperidinopropiophenone and its methiodide. As they expected, different mechanisms for Mannich base salts and their quaternized derivatives were suggested by the data. As the pH increased, the value for the measured rate increased steadily for the methiodide but followed a typical "dissociation" curve in the case of the Mannich

bases. These data indicate that decomposition of the methiodide salt is a base-catalyzed reaction although other data suggest it to be more complex than an E2 mechanism. The data, including that relating occurrence of a dissociation reaction as given above, suggest that the Mannich base salts first undergo a dissociation followed by a unimolecular deamination of the free Mannich base. The process may be illustrated as given in Schemes XLII and XLIII.



Scheme XLII



Scheme XLIII

It is of interest to note that type and concentration of the buffer had no effect on the rate constant for the above process while the methiodide salt decomposition was very decidedly dependent upon these factors.

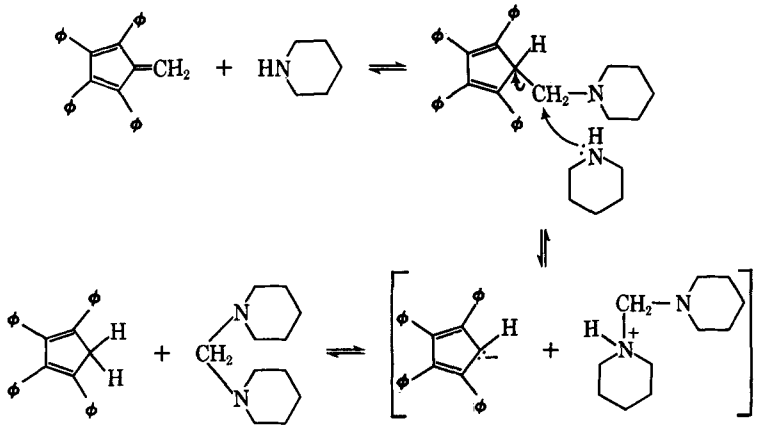
Rivière (22), in a study on the reversibility of the Mannich reaction, also discussed amine elimination to a limited extent. Principal among the stated conclusions is that elimination is especially important as a pathway if further, irreversible changes involving the unsaturated ketone can take place. Dimerization is a frequently seen irreversible process which forces the elimination reaction toward completion. Michael-type addition reactions also can force an elimination reaction toward completion (90–92). The presence of acid which can tie up the eliminated amine preventing reversal or the removal of the unsaturated ketone by steam distillation are adequate procedures.

Several studies on the application of the Mannich reaction to sulfones have been conducted (28, 92–98). With simple aryl alkyl sulfones the reaction failed (28, 92) but gave amine elimination products when arylsulfonylalkanoic acids and esters were used (92–98). Some Mannich base could be isolated in addition to the α,β-unsaturated sulfones when primary amines or ammonia were used along with aromatic aldehydes (92, 94–98). Either use of a secondary amine or of formaldehyde sufficed in order to result in complete elimination. The most likely mode of formation of the unsaturated sulfone is amine elimination (97, 98) rather than the concerted decarboxylation-amine elimination process previously suggested (93).

REVERSIBILITY OF THE MANNICH REACTION

Spoerri and co-workers (37, 38), in studying the addition of bases to 2,3,4,5-tetraphenylfulvene, obtained 1,2,3,4-tetraphenyl-1,3-cyclopentadiene when the starting material was heated with excess cold piperidine. They suggested that the phenomenon represents a reverse Mannich reaction and proposed the course shown in Scheme XLIV for the reaction. With brief contact time at 0° it was possible to isolate the intermediate Mannich base. Heating resulted in amine elimination while refluxing with excess piperidine yielded a reverse Mannich reaction. In literature references cited by Spoerri *et al.* (37) the most successful reversals took place in the presence of excess amine.

Larramona and Tchoubar (99) presented an informative review of the reversibility of the Mannich reaction. It is significant that they



Scheme XLIV

reported reversal with β-aminoalkyl aryl ketones but not with those Mannich bases containing only *alkyl* groups. Both water and amine were effective in catalyzing this reverse reaction.

Riviére (22) contributed much to an understanding of factors which can influence the reversibility of the Mannich reaction. His research further proves the reversibility of the Mannich condensation. In this report the reaction of free amines and amine hydrochlorides on β-aminoketones was studied and found to produce three competitive reactions: (*a*) desaminomethylation (reverse Mannich), (*b*) deamination (elimination), and (*c*) amine exchange between the β-aminoketone and reactant amine. The reversal of the Mannich reaction was found to be dependent on three factors. These are: (*a*) the carbon structure of the β-aminoketone must contain a C_6H_5—C=O— group although this, by itself, was not sufficient for desaminomethylation; (*b*) the presence of anilino as the amino group was unfavorable for reversal of the Mannich reaction; (*c*) aminoketones which were unable to undergo desaminomethylation could, after undergoing amine exchange with amines contained in the media, then undergo a reverse Mannich reaction.

Riviére concluded that the Mannich reaction is reversible in basic media and that its success therefore depends on the position of the equilibria. The position of the equilibria can be altered by irreversible side reactions or by amine exchange. The solvent also has a profound effect: in toluene the equilibria position could be approached from either side; in ethanol, the solvent seems to interfere with simple equilibria and provides different positions depending upon direction of approach.

FACTORS AFFECTING SELECTION OF CONDITIONS

Formaldehyde for use in the Mannich condensation may be provided by aqueous formalin, trioxymethylene (trioxane), or paraformaldehyde (100, 101). Unfortunately selection of one of these forms is not as simple as it may appear.

Aqueous *formalin* (37–40% usually) is a generally satisfactory source of formaldehyde for Mannich reactions which form stable products and have very favorable equilibria positions (8, 22). It must be remembered that the water present can both serve as a solvent and/or lead to reversal of the Mannich reaction in basic media (22). Aqueous formaldehyde may also be used for acidic media condensations, again providing some reversibility difficulties in certain cases.

Trioxymethylene (trioxane) is a cyclic trimer of formaldehyde (100, 101) and is prepared by allowing formaldehyde, obtained by distillation of 60% aqueous formaldehyde solution containing 2% of sulfuric acid, to polymerize and extracting the trioxane and linear polymer mixture with methylene dichloride. Trioxymethylene is a true acetal and can yield formaldehyde only in the presence of acid even in nonaqueous solvent systems. Studies have shown that trioxymethylene can provide formaldehyde in aqueous acid solution at a uniform rate (pseudo first order) (101). Unfortunately, the weakly acidic conditions of the Mannich reaction frequently result in such a slow depolymerization that trioxymethylene is impractical as a source of formaldehyde. A 75% depolymerization requires 2.3 hr. at 70° with 4 N H_2SO_4 and 2.3 days at 70° with 0.5 N H_2SO_4.

The third form of formaldehyde is a linear polymer called *paraformaldehyde* (100), more properly called polyoxymethylene, and may exist in three different forms. The first is *α-polyoxymethylene*, a linear polymer of moderate size (n of 30) and belonging to the "hemiacetal" class. The internal bonds are of the acetal type, hence depolymerization in base can occur, albeit from the terminal ends of the molecules. Cleavage may occur in aqueous or nonaqueous acid solutions all along the polymer chain and at both ends. The second type of polymer is *β-polyoxymethylene* which is similar chemically to the α form. The polymer size is significantly larger, hence this is a less soluble form. This β form of paraformaldehyde is formed by adding sulfuric acid during dehydration of formaldehyde. The third *polyoxymethylene* is the γ isomer which is formed from formaldehyde in methanol by sulfuric acid dehydration. This isomer is stable to bases since it is a true acetal formed from α- and β-polyoxymethylene and methanol. The rate of depolymerization probably is slow as it is with trioxymethylene.

Some chemical supply houses have persisted in using the name "trioxymethylene" as a substitute for "paraformaldehyde." This, of course, creates confusion regarding identity of the material. Furthermore, use of the name "polyoxymethylene" by other suppliers without specifying α-, β-, or γ-isomers may be an occasional source of difficulty.

In many cases it will be advantageous to use derivatives of the amine component and formaldehyde in order to overcome disadvantageous acidity, basicity, or stability of the acidic hydrogen compound or of the product (25).

In the acid-catalyzed Mannich reaction the aminomethylating agent seems to be an amino-

methyl carbonium ion (25, 34) which may be formed most easily and in highest yields by adding *N*-hydroxymethylamine, *N*-alkoxymethylamine, or methylene bis(amine) to a solution of the replaceable hydrogen compound in excess acid. The disadvantages of this procedure include the retarding influence of excess acid on formation of a carbanic center on the replaceable hydrogen compound. Addition of aliquot or aliquant quantities of the total acid followed by addition of a similar quantity of the aldehyde and amine derivative being used often overcomes this difficulty. The acid must always be in excess but, for most reactants, this excess should never be very great. Methylene-bis(amines) will require double the stoichiometric quantity of acid in order to neutralize the mole of amine given off. When amine hydrochloride and active hydrogen compound are dissolved in absolute ethanol and paraformaldehyde is added portionwise (8, 102), excellent results are likewise obtained. The addition of amine hydrochloride assures only moderate acidity and, as formaldehyde is gradually released and reacts with the amine salt, the observed release of free acid will likewise be gradual. The Mannich base, as it forms, will take up the acid and the acidity never builds up in this procedure. A similar procedure for free amine is used when the amine salt is not available (102).

In basic media the same three aminomethylating agents may be utilized with good results (25, 26, 45, 46). Although the actual aminomethylating agent under basic conditions probably is the methylene bis(amine) the experimental evidence offered by Fernandez presents a strong case for *N*-hydroxymethylamine as being a more forceful reagent (45, 46). In basic media in particular, methylene bis(amine) yields an equivalent of free amine which can, as previously shown, accelerate Mannich reaction reversal. Since the amine occurs as part of the equilibria, the position of equilibria will also be affected. When such unfavorable equilibrium positions occur with methylene bis(amine) the use of *N*-alkoxymethylamine or *N*-hydroxymethylamine will give differently constituted equilibria involving alcohols or water, respectively, and hence a totally different equilibrium constant. It is entirely possible that the new equilibria will now provide the desired product in high yield. It is sometimes possible to force the condensation if a solvent such as toluene is used in conjunction with a Dean-Stark trap to remove water from the equilibria. For small quantities of water a Soxhlet thimble containing a nonreacting drying agent is even more effective than the Dean-Stark trap.

With regard to the active hydrogen component of the condensation, two major methods of developing a carbanic center are applicable (25). These are ionization and electronic localization in enols and heterorings. For strongly acidic materials ionization may even take place in moderately acidic media, although it is rare that very low pH values will be conducive to formation of the necessary carbanion or to formation of the required aminomethyl carbonium ion if amine hydrochloride and formaldehyde are used *per se*. Excessive acidity also binds the free electron pairs in enols and heterocyclic systems sufficiently to prevent condensation with the aminomethyl carbonium ion.

The use of basic conditions with strongly acidic components often yields carbanions with greater nucleophilicity than that of the amine. Under such conditions a symmetrical methylene derivative of the active hydrogen compound results (25, 31, 32, 97, 98, 103–105). Basic conditions are favored over acidic conditions in the case of nitroalkanes, acetylenes, phenols, and related materials. The acetylenes in particular undergo the Mannich reaction more readily in the presence of cupric sulfate (43) or under more strongly basic conditions than most active hydrogen sources (18, 43, 68).

Choice of solvent frequently is of great importance (22). The common solvents for the Mannich reaction include water, acetic acid, ethanol, isoamyl alcohol, and toluene (8). The first four are possessed of varying degrees of polarity and thus foster the formation of ionic species to different degrees. Water, acetic acid, and isoamyl alcohol have more elevated boiling points than does ethanol, and their use can therefore speed up an otherwise slow approach to equilibria. The higher temperature also can hasten the elimination of amine from the Mannich base product (37, 38) and thus complicate the process. In base this elimination is reversible while in acid it is slower but tends to go to completion if heated long enough. Acetic acid as a medium can suppress ionization of the acidic compound and thus alter its reactivity. This phenomenon may sometimes be used to advantage. Toluene on the other hand tends to act uniformly to suppress all ion formation. For condensations which seem to involve nonionic reactants, *e.g.*, simple enols or heterosystems reacting with *N*-hydroxy- or *N*-alkoxymethylamines or methylene bis(amines), such media may afford excellent results.

The preceding comments have been quite general in order to afford a broad view of Mannich reaction technology. Other comments of value are to be found in Blicke's review (8) and in the discussions of Hellmann and Opitz (25), and others (11, 22, 99). When difficulties are encountered one must decide if these be in the nature of poor equilibrium position, formation of elimination products, incorrect solvent, or inappropriate conditions or ingredients for proper formation of the intermediates in the reaction. Often steps, such as those discussed above, may be taken which will circumvent these problems.

The value of varying the pH, changing solvents, and, in general, attempting to ascertain why difficulties seem to be occurring in an attempted Mannich condensation cannot be valued too highly. Such knowledge may lead to easily successful variations in the procedure. The observed simplicity of the Mannich condensation procedure in the successful cases should not lead anyone to believe that the process is not indeed complicated and one frequently beset with pitfalls. The author sincerely hopes that this review will serve a useful purpose and that it will aquaint those in the field with more of the work which has been published in other languages.

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Keyphrases

Mannich reaction—review
Mechanism, technology—Mannich reaction
Condensation, Mannich reaction—mechanism
Decomposition reactions—Mannich bases
Reversibility—Mannich reaction
Formaldehyde source—Mannich reaction

Research Articles

Electrolyte Alterations in Vascular Smooth Muscle and Hypotensive Activity of a New Chalcone Derivative

By GERALD P. SHERMAN, ELIAS W. PACKMAN, and G. VICTOR ROSSI

Chalcone R-2803 [2-(2-dimethylaminoethoxy)-3′,4′,5′-trimethoxy chalcone hydrochloride] is an effective and long-acting depressor agent when administered intravenously and orally to dogs and rats. In the intact animal, R-2803 is essentially devoid of adrenolytic or ganglioplegic activity. Cross-circulation studies indicate that R-2803 does not possess central hypotensive activity. A direct action at the vascular level is demonstrated by inhibition of norepinephrine- and angiotensin-induced contractions of isolated aortic muscle. Single intravenous doses increase the sodium and potassium content of rabbit aorta, but decrease serum sodium and potassium values, reflecting an apparent shift in the equilibrium of electrolytes between blood and vascular tissue. Although a decrease in hypotensive activity is not observed after administration of R-2803 for 3 days, the electrolyte changes are less than those observed after single intravenous doses. An equidepressor dose of hesperidin methyl chalcone produces similar elevations of aortic sodium and potassium levels. The electrolyte alterations are not a consequence of blood pressure reduction as evidenced by failure of other hypotensive agents to alter aortic electrolyte balance. Sodium and potassium changes in vascular muscle may play a role in the initial phase of the hypotensive effect of the chalcones.

PHARMACOLOGIC EVALUATION by Rossi and Packman (1) of 14 chalcone derivatives synthesized by Packman and Rubin (2) indicated 2-(2-dimethylaminoethoxy) chalcone to be the most active hypotensive compound in the series. Further modification of this molecule by Packman (3) resulted in a derivative, 2-(2-dimethylaminoethoxy)-3′,4′,5′-trimethoxy chalcone hydrochloride (compound R-2803), characterized by greater depressor potency and extended duration of action.

Based primarily on studies on isolated intestinal smooth muscle, Riedesel and Combs (4) postulated that hesperidin methyl chalcone, which

Received November 13, 1967, from the Department of Biological Sciences, Philadelphia College of Pharmacy and Science, Philadelphia, PA 19104
Accepted for publication December 27, 1967.
This study was supported in part by a research grant from Wm. H. Rorer, Inc., Fort Washington, Pa.
The authors wish to thank Dr. Joseph P. Buckley, Chairman of the Department of Pharmacology, University of Pittsburgh School of Pharmacy, for permission to report the results of cross-circulation studies performed in his laboratories. The authors also wish to thank Mr. Richard Kostrzewa for performance of the spasmolytic assays with aortic tissue.

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Keyphrases

Mannich reaction—review
Mechanism, technology—Mannich reaction
Condensation, Mannich reaction—mechanism
Decomposition reactions—Mannich bases
Reversibility—Mannich reaction
Formaldehyde source—Mannich reaction

Research Articles

Electrolyte Alterations in Vascular Smooth Muscle and Hypotensive Activity of a New Chalcone Derivative

By GERALD P. SHERMAN, ELIAS W. PACKMAN, and G. VICTOR ROSSI

Chalcone R-2803 [2-(2-dimethylaminoethoxy)-3′,4′,5′-trimethoxy chalcone hydrochloride] is an effective and long-acting depressor agent when administered intravenously and orally to dogs and rats. In the intact animal, R-2803 is essentially devoid of adrenolytic or ganglioplegic activity. Cross-circulation studies indicate that R-2803 does not possess central hypotensive activity. A direct action at the vascular level is demonstrated by inhibition of norepinephrine- and angiotensin-induced contractions of isolated aortic muscle. Single intravenous doses increase the sodium and potassium content of rabbit aorta, but decrease serum sodium and potassium values, reflecting an apparent shift in the equilibrium of electrolytes between blood and vascular tissue. Although a decrease in hypotensive activity is not observed after administration of R-2803 for 3 days, the electrolyte changes are less than those observed after single intravenous doses. An equidepressor dose of hesperidin methyl chalcone produces similar elevations of aortic sodium and potassium levels. The electrolyte alterations are not a consequence of blood pressure reduction as evidenced by failure of other hypotensive agents to alter aortic electrolyte balance. Sodium and potassium changes in vascular muscle may play a role in the initial phase of the hypotensive effect of the chalcones.

PHARMACOLOGIC EVALUATION by Rossi and Packman (1) of 14 chalcone derivatives synthesized by Packman and Rubin (2) indicated 2-(2-dimethylaminoethoxy) chalcone to be the most active hypotensive compound in the series. Further modification of this molecule by Packman (3) resulted in a derivative, 2-(2-dimethylaminoethoxy)-3′,4′,5′-trimethoxy chalcone hydrochloride (compound R-2803), characterized by greater depressor potency and extended duration of action.

Based primarily on studies on isolated intestinal smooth muscle, Riedesel and Combs (4) postulated that hesperidin methyl chalcone, which

Received November 13, 1967, from the Department of Biological Sciences, Philadelphia College of Pharmacy and Science, Philadelphia, PA 19104
Accepted for publication December 27, 1967.
This study was supported in part by a research grant from Wm. H. Rorer, Inc., Fort Washington, Pa.
The authors wish to thank Dr. Joseph P. Buckley, Chairman of the Department of Pharmacology, University of Pittsburgh School of Pharmacy, for permission to report the results of cross-circulation studies performed in his laboratories. The authors also wish to thank Mr. Richard Kostrzewa for performance of the spasmolytic assays with aortic tissue.

possesses blood pressure reducing activity, inhibits potassium efflux thus preventing effective depolarization of the muscle membrane. Preliminary investigations conducted with chalcone R-2803, including spasmolytic activity *in vitro* and effect on regional perfusion rate *in situ*, suggested an action directly at the level of vascular smooth muscle. This report characterizes the depressor activity of chalcone R-2803 in experimental animals and examines the possible relationship between the blood pressure reducing effect of chalcones and alterations in the electrolyte composition of vascular tissue.

EXPERIMENTAL

Hypotensive Activity in Anesthetized Dogs—Blood pressure was monitored in 20 male and 17 female mongrel dogs (9–18 Kg.), anesthetized with allobarbital[1]–urethan (0.6 ml./Kg., intravenously), by coupling a femoral arterial cannula *via* a photoelectric transducer to a physiograph (E & M Instrument Co., Houston, Texas). In this series of experiments all drugs were administered intravenously. The intensity and duration of the hypotensive response to 5, 10, and 20 mg./Kg. of chalcone R-2803 were determined, and the effect of prior administration of 10 mg./Kg. of R-2803 was evaluated on the pressor or depressor response to: bilateral occlusion (30 sec.) of the common carotid arteries, electrical stimulation (30 v., 120 p.p.s. for 15 sec.) of the central and peripheral stumps of the severed vagus nerve, epinephrine (25–50 mcg.), norepinephrine (5–10 mcg.), acetylcholine (5–10 mcg.), dimethylphenylpiperazinium (12.5 mcg.), 5-hydroxytryptamine (250 mcg.), and angiotensin amide (10 mcg.).

Three dog cross-circulation pairs were prepared according to the procedure detailed by Bickerton and Buckley (5). Compound R-2803 was administered into the arterial inflow to the recipient's head, and subsequently into the recipient's femoral vein in doses of 1 and 5 mg./Kg.

Hypotensive Activity in Unanesthetized Dogs—The hypotensive effect of 10 and 20 mg./Kg. of R-2803, administered orally in gelatin capsules, was evaluated in 2 male and 2 female unanesthetized mongrel dogs (7–11 Kg.). Femoral arterial systolic and diastolic pressures were monitored externally by means of an automatic cuff inflation pump functioning in conjunction with an electrosphygmograph which provides a system for continuous recording of occluding cuff pressure and superimposed Korotkoff sounds.

Hypotensive Activity in Unanesthetized Rats—The depressor response to oral administration of 20 mg./Kg. of R-2803 was determined in unanesthetized normotensive and experimental hypertensive male Sprague-Dawley rats (150–190 Gm.). Sustained elevated blood pressure (*i.e.*, systolic pressure in excess of 170 mm. Hg) was induced by extirpation of the left kidney and replacement of drinking water by 2% saline solution. Caudal arterial pressure was estimated indirectly by means of an electrosphygmographic system.

Effect on Contraction of Rabbit Aortic Spirals In Vitro—Spiral strips prepared, according to the procedure described by Furchgott (6), from the descending thoracic aorta of male New Zealand white rabbits (1–2 Kg.) were suspended in Krebs-bicarbonate solution at 37.5°, aerated with 95% O_2 + 5% CO_2, and arranged for kymographic recording of contractions. The concentration of *l*-norepinephrine bitartrate required to elicit a submaximal response was determined individually for each muscle preparation, after which the extent of inhibition of norepinephrine-induced contraction was evaluated with concentrations of R-2803 ranging from 1.67 to 20 mcg./ml. This assay procedure was repeated with other aortic strips using angiotensin amide as the spasmogenic stimulus.

Sodium and Potassium Levels in Rabbit Aorta—Analyses of the sodium and potassium content were performed with aortic tissue samples obtained from male New Zealand white rabbits (1–2 Kg.) 2 hr. after intravenous administration of: R-2803 (10 mg./Kg.), hesperidin methyl chalcone (40 mg./Kg.), mecamylamine (5 mg./Kg.), guanethidine (15 mg./Kg.), chlorothiazide (100 mg./Kg.), and normal saline (volumes equal to injected solution of the experimental drugs). The descending thoracic and abdominal aorta was excised from animals sacrificed by air embolism; the tissue was rinsed in deionized water, trimmed of fat and connective tissue, weighed and ashed (600°, 12–14 hr.). Sodium and potassium concentrations were determined by means of a Perkin-Elmer 303 atomic absorption spectrophotometer, and results expressed in terms of meq./Kg. wet weight of tissue.

RESULTS

Hypotensive Activity in Anesthetized Dogs—Reduction of systolic and diastolic pressure was apparent within 60 sec. after intravenous injection of 5 mg./Kg. of chalcone R-2803 in anesthetized dogs. Blood pressure decreased progressively and attained a hypotensive plateau within 60–90 min. (Fig. 1). A dose-response relationship was evident with the 5, 10, and 20 mg./Kg. dosage range examined.

The chalcone had a more pronounced effect on diastolic than on systolic blood pressure. There was

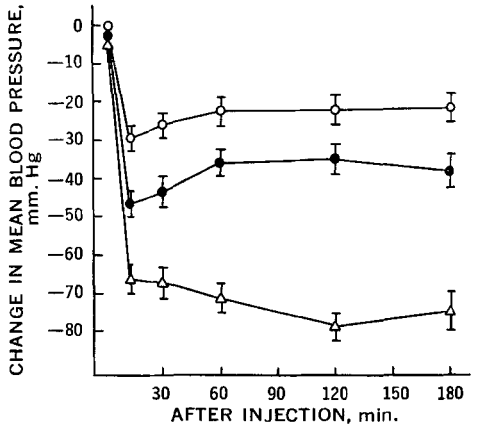

Fig. 1—Reduction of mean blood pressure in anesthetized dogs after intravenous injection of chalcone R-2803. Key: ○, 5 mg./Kg. (N = 3); ●, 10 mg./Kg. (N = 4); △, 20 mg./Kg. (N = 3). Vertical bars indicate standard errors of the mean.

[1] Dial, Ciba Pharmaceutical Co., Morris Plains, N. J.

little change in heart rate and no apparent alteration in the electrocardiogram. A short period of hyperpnea occurred following intravenous administration of the chalcone.

In no case was there a substantial return to pretreatment pressure levels after 3 hr. (arbitrary termination of most assays in this series), and the blood pressure remained depressed 6 hr. after drug administration in those experiments ($N = 5$) conducted for an extended time period. There were no detectable differences in the depressor response to intravenous administration of 10 mg./Kg. of R-2803 in nonpretreated dogs ($N = 4$) and in dogs ($N = 3$) pretreated with 3 daily intraperitoneal injections of 10 mg./Kg. of the chalcone.

A moderate additional reduction in blood pressure (Δ 20 mm. Hg) occurred when the position of anesthetized dogs administered 10 mg./Kg. of R-2803 intravenously, and secured to a tilt table, was abruptly changed from the horizontal plane to an angle of 60°. In contrast, this postural change elicited a further marked reduction in pressure (Δ 50 mm. Hg) in dogs injected intravenously with 15 mg./Kg. of phenoxybenzamine.

Intravenous injection of 10 mg./Kg. of R-2803 partially blocked the carotid occlusion pressor reflex and the responses to electrical stimulation of the central and peripheral ends of the severed vagus nerve, but did not significantly alter the characteristic pressor or depressor effects of epinephrine, norepinephrine, acetylcholine, dimethylphenylpiperazinium, 5-hydroxytryptamine, and angiotensin.

Administration of the antihistaminic compound chlorpheniramine (6 mg./Kg., intravenously) 30 min. prior to intravenous injection of 5, 10, and 20 mg./Kg. of R-2803 in anesthetized dogs ($N = 3$, 4, 3, respectively) decreased by 26–42% the extent, but did not significantly affect the duration of the hypotensive activity of the chalcone. Pretreatment, intravenously, of anesthetized dogs with 150 mcg. of the histamine-releasing agent 48/80 ($N = 3$), or 150 mg. of the antiserotonin agent cyproheptadine ($N = 3$) did not alter the intensity or duration of the depressor response to intravenous injection of 10 mg./Kg. of R-2803.

Administration of R-2803 [1 mg./Kg. ($N = 1$) and 5 mg./Kg. ($N = 2$)] into the arterial inflow to the recipient's head in cross-circulation preparations reduced blood pressure in the donor dog but did not elicit a hypotensive response in the recipient animal. However, administration of the chalcone into the arterial inflow resulted in a marked reduction in perfusion pressure to the recipient's head, indicative of cerebral vascular dilation.

Hypotensive Activity in Unanesthetized Dogs—Blood pressure reduction was detectable within 30 min. after oral administration of 10 and 20 mg./Kg. of R-2803 to unanesthetized dogs; the maximal response was evident within 2 hr. (Fig. 2). The extent of blood pressure reduction following oral administration of 20 mg./Kg. was approximately equivalent to that produced by intravenous injection of 10 mg./Kg. of the chalcone. In those animals monitored for an 8-hr. period after oral administration, the blood pressure had not returned to the predrug level at the termination of the assay.

That the anesthetic employed in a previous phase of this study had no appreciable effect on the depressor response was evidenced by the lack of a significant difference between the hypotensive activity of the chalcone (10 and 20 mg./Kg., orally) in unanesthetized dogs and in dogs anesthetized (allobarbital–urethan) immediately following administration of R-2803.

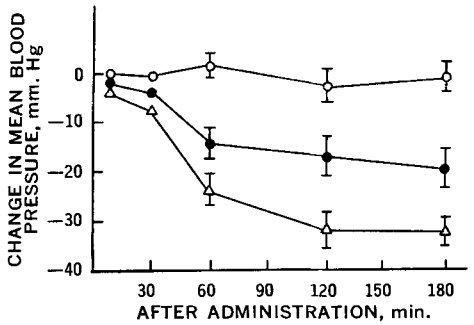

Fig. 2—Reduction of mean blood pressure in unanesthetized dogs after oral administration of chalcone R-2803. Key: ○, placebo capsule (N = 4); ●, 10 mg./Kg. (N = 5); △, 20 mg./Kg. (N = 5). Vertical bars indicate standard errors of the mean.

Hypotensive Activity in Unanesthetized Rats—Oral administration of 20 mg./Kg. of R-2803 produced essentially the same degree and duration of blood pressure reduction in normotensive rats and in renal hypertensive rats (Fig. 3). Although the extent of depressor activity following oral administration of R-2803 in unanesthetized rats was comparable to that observed in unanesthetized dogs, the duration of blood pressure reduction was considerably shorter in the rat (approximately 2.5 hr.) than in the dog (exceeding 6 hr.).

Inhibition of Contraction of Rabbit Aortic Spiral In Vitro—With reference to blocking the contractile response of isolated spiral strips of rabbit aorta to norepinephrine, the ED_{50} concentration of R-2803 was found to be 3.2 mcg./ml. ($N = 22$). A concentration of 20 mcg./ml. of R-2803 reduced angiotensin-induced contractions of aortic smooth muscle by approximately 30% ($N = 8$). It was not possible, however, to determine an ED_{50} value for angiotensin-antagonism inasmuch as concentrations of chalcone in excess of 20 mcg./ml. elicited contracture in aortic strips previously exposed to angiotensin.

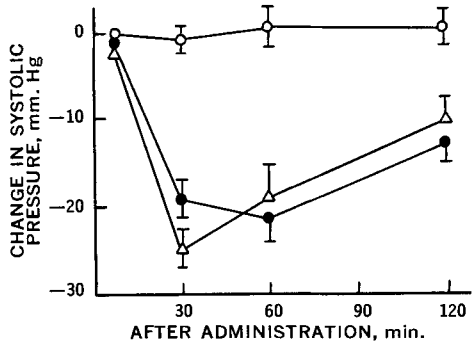

Fig. 3—Reduction of systolic blood pressure in unanesthetized rats after oral administration of chalcone R-2803. Key: ○, saline administration in normotensive rats (N = 2); ●, 20 mg./Kg. in normotensive rats (N = 12); △, 20 mg./Kg. in hypertensive rats (N = 11). Vertical bars indicate standard errors of the mean.

TABLE I—MEAN SODIUM AND POTASSIUM LEVELS IN RABBIT AORTA AND SERUM 2 HR. AFTER INTRAVENOUS ADMINISTRATION OF VARIOUS HYPOTENSIVE AGENTS

Treatment	Dose mg./Kg.	No. Animals	Sodium meq./Kg. $\bar{X}^a \pm$ S.E.	*P* Value	Potassium meq./Kg. $\bar{X}^a \pm$ S.E.	*P* Value
Aortic Electrolyte Levels						
Control (saline)		32	1.195 ± 0.019		1.178 ± 0.016	
Chalcone R-2803	10	20	1.295 ± 0.024	<0.05	0.642 ± 0.022	<0.001
Hesperidin methyl chalcone	40	20	1.304 ± 0.019	<0.01	0.449 ± 0.026	<0.001
Control (saline)		14	1.176 ± 0.027		0.228 ± 0.025	
Chalcone R-2803[b]	10	17	1.138 ± 0.020	...	0.328 ± 0.018	<0.05
Control (saline)		27	1.229 ± 0.020		0.323 ± 0.015	
Mecamylamine	5	18	1.196 ± 0.022	...	0.245 ± 0.001	<0.01
Guanethidine	15	17	1.177 ± 0.024	...	0.353 ± 0.017	...
Control (saline)		5	1.246 ± 0.042		0.204 ± 0.025	
Chlorothiazide	100	6	1.188 ± 0.033	...	0.287 ± 0.021	...
Serum Electrolyte Levels						
Control (saline)		14	2.125 ± 0.713		0.816 ± 0.052	
Chalcone R-2803	10	14	2.078 ± 0.580	<0.05	0.705 ± 0.036	<0.05

[a] Mean logarithmic values ± standard error. [b] Pretreatment with R-2803, 10 mg./Kg., intraperitoneally in single doses for 3 days and sacrified 2 hr. after administration of R-2803, 10 mg./Kg., intravenously.

Sodium and Potassium Levels in Rabbit Aorta—Chalcone R-2803 (10 mg./Kg., intravenously) increased aortic sodium and potassium levels, and decreased the serum concentration of these electrolytes (Table I). The aortic potassium elevation following intravenous administration of R-2803 was less in rabbits pretreated with the chalcone (10 mg./Kg./day, intraperitoneally, for 3 days) than in those animals which received only the single intravenous injection.

An approximate equidepressor dose (40 mg./Kg., intravenously) of hesperidin methyl chalcone produced alterations in aortic sodium and potassium values similar to those obtained with R-2803.

With the exception of a decreased potassium level following treatment with mecamylamine, the intravenous administration of mecamylamine (5 mg./Kg.), guanethidine (15 mg./Kg.), and chlorothiazide (100 mg./Kg.) did not elicit significant changes in the sodium or potassium content of aortic tissue.

DISCUSSION

Chalcone R-2803 [2-(2-dimethylaminoethoxy)-3′,4′,5′-trimethoxy chalcone HCl] was found to be an effective and long-acting depressor agent when administered intravenously to anesthetized normotensive dogs, and orally to unanesthetized normotensive dogs, normotensive and hypertensive rats. Prolonged moderate reductions in arterial pressure were consistently obtained following intravenous injection of 5–10 mg./Kg., and oral administration of 10–20 mg./Kg. of the chalcone in dogs.

In the intact animal, R-2803 does not possess significant ganglionic blocking or alpha-adrenergic blocking activity. Cross-circulation studies with R-2803 did not reveal any basis for a central mechanism of hypotensive action. A direct action at the vascular level was indicated by an increase in the regional perfusion rate *in situ*, and inhibition of the contractile effect of norepinephrine and angiotensin on isolated aortic smooth muscle.

The possibility of histamine release as a component of the hypotensive action was explored by comparing the blood pressure patterns following intravenous administration of R-2803 in anesthetized saline-pretreated (control) dogs, in dogs pretreated with an effective histamine-blocking dose of chlorpheniramine, and in dogs administered repeated doses of compound 48/80 until the onset of refractoriness. Chlorpheniramine appreciably diminished the degree of blood pressure reduction at all dosage levels of R-2803 examined, but did not consistently affect the duration of the depressor response. No comparable inhibition of the hypotensive action of R-2803 was observed in dogs pretreated with compound 48/80. These latter results may be considered inconclusive inasmuch as there is no definitive method for determining the completeness of histamine depletion in the intact animal, although Slomka and Goth (7) have proposed that refractoriness to subsequent doses of 48/80 is indicative of depletion of available histamine stores. It has frequently been noted that the histamine release which occurs with the peak blood levels resulting from the intravenous injection of certain drugs may not occur or may be less apparent following the relatively slow absorption of the same drugs from the gastrointestinal tract. However, the hypotensive response to intraduodenal administration of R-2803 was also diminished in dogs pretreated with chlorpheniramine. Partial reduction rather than complete abolition by chlorpheniramine of the hypotensive response suggests that histamine release may contribute to, but cannot completely account for, the blood pressure reducing effect of R-2803.

Correlations among the ionic and bioelectric properties of smooth muscle provide a foundation for the proposed roles of altered electrolyte composition of the vascular tree in the increased peripheral resistance characteristic of essential hypertension, and in the mechanism of action of certain blood pressure reducing agents. Su and Bevan (8) have suggested that intracellular antagonism of potassium may be involved in the reduction of arteriolar tone produced by the alpha-adrenergic blocking agents, phenoxybenzamine and SY-14 [*N*-(2-chloroethyl)-*N*-ethyl-1-naphthalenethylamine HCl]. It has been theorized that the gradient of sodium and/or potassium ions existing between the intracellular and extracellular spaces is related to inherent vascular responsiveness and that the thiazides may elicit their hypotensive effect by altering these electrolyte gradients (9).

This basic hypothesis remains plausible although the thiazides have not been found to alter the concentration of sodium in the plasma of hypertensive patients (10), or the sodium content of the arterial walls of experimental animals (11).

The studies with intestinal smooth muscle conducted by Riedesel and Combs (4) on the basis of which they postulated that hesperidin methyl chalcone inhibits potassium efflux, and our observations that chalcone R-2803 increased regional perfusion rate and decreased the reactivity of isolated aortic smooth muscle to norepinephrine and angiotensin, prompted an investigation of the effect of R-2803 on the electrolyte composition of vascular tissue. Single intravenous hypotensive doses of R-2803 increased the sodium and potassium content of rabbit aorta and decreased the serum sodium and potassium levels. These data may reflect a shift in the equilibrium of electrolytes between blood and vascular muscle, *i.e.*, alterations in the intracellular/extracellular gradients of sodium and potassium ions. An approximate equidepressor dose of hesperidin methyl chalcone, which was about one-fourth as potent as R-2803 on a milligram basis, produced similar changes in aortic sodium and potassium levels.

The electrolyte alterations in vascular muscle following administration of R-2803 and hesperidin methyl chalcone are apparently not a consequence of the blood pressure reduction as evidenced by the failure of other potent hypotensive agents (*i.e.*, mecamylamine, guanethidine, and chlorothiazide) to similarly alter the aortic electrolyte concentrations.

Sodium and potassium imbalances in vascular smooth muscle may play a role in the initial phases of the hypotensive action of the chalcones, with the inhibition of potassium efflux preventing effective depolarization of the muscle membrane. However, due possibly to compensatory mechanisms, the electrolyte concentrations in vascular tissue revert toward normal levels within a time period during which refractoriness to the blood pressure reducing effect does not develop.

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Keyphrases

Chalcone derivatives—pharmacological activity
Depressor activity—chalcone R-2803
Hypotensive activity—chalcone R-2803
Norepinephrine activity—antagonized
Angiotensin activity—antagonized
Electrolyte changes—smooth muscle, blood

Cyclic α,β-Unsaturated Ketones Related to Ethacrynic Acid

By JOHN G. TOPLISS and LEROY M. KONZELMAN

2-Carboxymethoxy-8,9-dihydro-6-methyl-5*H*-benzocyclohepten-5-one and 5-carboxymethoxy-2-methylindone, which are structurally related to ethacrynic acid, have been synthesized and the acute renal excretory response to intravenous injections of the compounds in the anesthetized dog has been evaluated. Only marginal activity, compared to ethacrynic acid, was observed.

THE DISCOVERY of a new class of diuretic agents, α,β-unsaturated ketone derivatives of aryloxyacetic acids of the general structure I, was reported by Schultz *et al.* in 1962 (1). Subsequently, pharmacological and clinical reports (2) have appeared on a compound in this series, ethacrynic acid (II).

The authors were interested in determining the effect on diuretic activity of the incorporation of the α,β-unsaturated ketonic function in a ring system in compounds of this general structural type. Consequently, it was decided to synthe-

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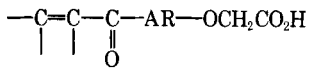

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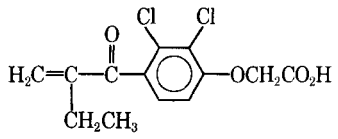

II

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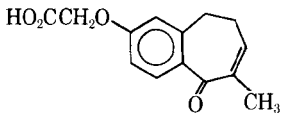
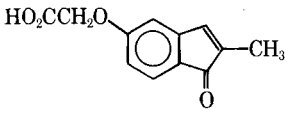


III

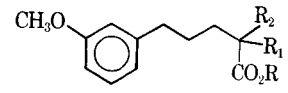


IV

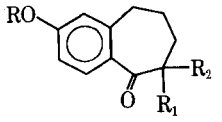
DISCUSSION

The route for the synthesis of the benzocycloheptenone (III) started from *m*-methoxycinnamylidenemalonic acid (Va) which was esterified with methanol in the presence of hydrogen chloride and the resulting dimethyl ester (Vb) was reduced in methanol solution with a palladium catalyst to give dimethyl 3-(*m*-methoxyphenyl)-propylmalonate (VIa). Methylation with methyl iodide and sodium hydride in dimethylformamide gave the malonic ester (VIb) which was then hydrolyzed with alkali and the resulting malonic acid (VIc) was decarboxylated by heating to give 5-(*m*-methoxyphenyl)-2-methylvaleric acid (VId). Cyclization of VId was effected using polyphosphoric acid in high dilution at 70° furnishing 2-methoxy-6-methylbenzocycloheptan-5-one (VIIa). The four-step sequence VIa through VIIa could be performed without purification of the individual intermediates in an overall yield of 80%. Demethylation of VIIa with hydrogen iodide in acetic acid afforded 2-hydroxy-6-methylbenzocycloheptan-5-one (VIIb) which was then transformed into its acetic acid derivative, VIIc, with chloroacetic acid in aqueous alkali. Bromination of VIIc using bromine in methylene chloride yielded the α-bromoketone VIId and this compound on dehydrobromination with refluxing 2,4-lutidine afforded the desired 2-carboxymethoxy-8,9-dihydro-6-methyl-5*H*-benzocyclohepten-5-one (III).

Va, R = H
Vb, R = CH_3

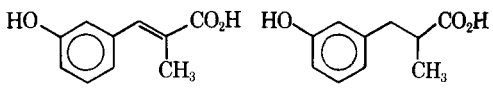


VIa, R = CH_3; R_1 = CO_2CH_3; R_2 = H
VIb, R = CH_3; R_1 = CO_2CH_3; R_2 = CH_3
VIc, R = H; R_1 = CO_2H; R_2 = CH_3
VId, R = H; R_1 = H; R_2 = CH_3

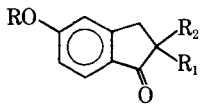


VIIa, R = CH_3; R_1 = H; R_2 = CH_3
VIIb, R = H; R_1 = H; R_2 = CH_3
VIIc, R = CH_2CO_2H; R_1 = H; R_2 = CH_3
VIId, R = CH_2CO_2H; R_1 = Br; R_2 = CH_3

For the synthesis of the indone (IV), *m*-hydroxy-α-methyl cinnnamic acid (VIII) was hydrogenated in dilute sodium hydroxide solution in the presence of a palladium catalyst to give *m*-hydroxy-α-methylhydrocinnamic acid (IX). Cyclization of



VIII IX



a, R = H; R_1 = H; R_2 = CH_3
b, R = CH_2CO_2H; R_1 = H; R_2 = CH_3
c, R = CH_2CO_2H; R_1 = Br; R_2 = CH_3

IX was effected with a boron trifluoride catalyst in chloroform solution to give 5-hydroxy-2-methyl-1-indanone (Xa)[1] which was converted into its acetic acid derivative Xb. The α-bromoketone, Xc, was obtained by bromination of Xb with bromine in methylene chloride solution and Xc was converted into the desired 5-(carboxymethoxy)-2-methylindone (IV) by treatment with refluxing 2,4-lutidine.

Renal Pharmacology[2]—The acute renal excretory response to intravenous injections of the compounds was examined in dogs anesthetized with 50 mg./Kg. of vinbarbital. Physiological saline was infused intravenously at a rate of 1.0 to 3.0 ml./min. continuously except for the experiment on IV. In this instance the infusion fluid (3.0 ml./min.) contained 5% mannitol and 0.25% sodium chloride. Ureters were cannulated with polyethylene tubing to facilitate urine collections. Compounds were dissolved in alkali and, after adjustment of pH, injected into the infusion tube. A standard diuretic, ethacrynic acid, dissolved in 5% sodium bicarbonate was given at a dose of 5 mg./Kg. after at

[1] Cyclization of *m*-hydroxy-α-methylcinnamic acid (VIII) under various conditions to give 5-hydroxy-2-methylindone was unsuccessful.

[2] Determined by the late Dr. R. M. Taylor, Biological Research Division, Schering Corporation.

least 1 hr. had elapsed from the time of injecting the test compound. Sodium and chloride ions were determined in 10-min. urine collections by flame photometery and electrometric titration with silver ion, respectively.

The activity of each compound, included in Table I, represents the percent increase in sodium

TABLE I—NATRIURETIC ACTIVITY OF SOME CYCLIC KETONES RELATED TO ETHACRYNIC ACID

Compd.	Dose, mg./Kg.	Natriuretic Activity[a]
IV	20	30
Xb	10	26
III	10	16
VIIc	10	11
Ethacrynic acid	5	411

[a] Percent increase in sodium excretion based on mean values of three 10-min. control and treated periods. Changes in chloride and urine flow were roughly similar to sodium excretion in magnitude and duration.

excretion over control values using averages for three 10-min. periods before (control) and after (treated) injection. The activity of ethacrynic acid given in Table I is an average value for these experiments.

Biological Results—The compounds showed only marginal diuretic activity compared to ethacrynic acid. The latter appears to exert its diuretic effect through interaction of the α,β-unsaturated acyl group with functionally important sulfhydryl groups (3). Structure-activity studies in the series indicated that for maximum biological activity one position in the aromatic nucleus *ortho* to the unsaturated ketone function must be substituted (1) and that monosubstitution of the methylene of the unsaturated acyl group causes some loss of activity (4). Saturation of the double bond results in almost complete loss of activity (1, 4). With regard to the compounds synthesized in the present study the very low order of activity exhibited by the cyclic saturated ketones VIIc and Xb, was in accord with expectations. In the case of the cyclic unsaturated ketones III and IV, although the known structural requirements for activity appear to be satisfied, the observed diuretic activity was of a marginal character. It seems unlikely that the double bond in these compounds would be chemically unreactive to sulfhydryl groups. However, the relative orientations in space of the double bond, carbonyl group, and phenyl ring are fixed which may interfere with the ability of the compounds to achieve an adequate fit at the receptor site.

EXPERIMENTAL[3]

Dimethyl m - Methoxycinnamylidenemalonate (Vb)—A solution of Va (5) (150 Gm.) in methanol (1,000 ml.) and concentrated HCl (25 ml.) was refluxed for 16 hr. The solution was evaporated *in vacuo* leaving a brown viscous oil which was used in the next step without further purification. Crystallization of the oil from $CHCl_3$-hexane gave the analytical sample, m.p. 61–63°.

[3] Melting points (uncorrected) were determined on a Thomas-Hoover capillary melting-point apparatus and ultraviolet absorption spectra were determined in methanol solution.

Anal.—Calcd. for $C_{15}H_{16}O_5$: C, 65.21; H, 5.84. Found: C, 65.06; H, 5.89.

Dimethyl 3 - (m - Methoxyphenyl)propylmalonate (VIa)—A solution of crude Vb (161 Gm.) in methanol (600 ml.) was hydrogenated for 16 hr. at 3.4 Kg./cm.^{2} pressure and room temperature in the presence of 5% Pd/C catalyst (6 Gm.). Removal of the catalyst by filtration followed by evaporation of the methanol *in vacuo* gave the product (148 Gm.) as a brown oil which was used without further purification in the next step.

Dimethyl 3 - (m - Methoxyphenyl)propylmethylmalonate (VIb)—Sodium hydride (3.54 Gm., 0.079 mole, of a 53.5% suspension in mineral oil) was added in small portions to a stirred solution of VIa (20.0 Gm., 0.071 mole) in dry DMF (100 ml.) at room temperature. The solution was heated at 90° for 0.5 hr., cooled to room temperature, and methyl iodide (30.4 Gm., 0.214 mole) was then added dropwise. After heating the stirred reaction mixture at 90° for 5 hr., the solvent was removed *in vacuo* to give the crude product which was used without further purification in the next step.

3-(m-Methoxyphenyl)propylmethylmalonic Acid (VIc)—NaOH (20 Gm.) in water (110 ml.) was added to the crude VIb obtained in the previous step and the reaction mixture stirred under reflux for 5 hr. The resulting solution was acidified with concentrated HCl, extracted with $CHCl_3$, and the $CHCl_3$ extract washed with water, dried (Na_2SO_4), and the solvent evaporated. A small portion of the residue solidified on trituration with hexane. This was recrystallized twice from ether-hexane to give the analytical sample, m.p. 93–95°.

Anal.—Calcd. for $C_{14}H_{18}O_5$: C, 63.14; H, 6.81. Found: C, 63.20; H, 6.78.

5-(m - Methoxyphenyl) - 2 - methylvaleric Acid (VId)—The crude malonic acid (VIc) was decarboxylated by heating at 160–170° for 1 hr. to give crude VId (17.2 Gm.) characterized as the *S*-benzylisothiuronium salt, m.p. 125–126°.

Anal.—Calcd. for $C_{21}H_{18}N_2O_3S$: C, 64.92; H, 7.26; N, 7.21; S, 8.25. Found: C, 64.89; H, 7.51; N, 7.26; S, 7.83.

2- Methoxy - 6 - methylbenzocycloheptan - 5 - one (VIIa)—Crude VId (16.7 Gm.) was added to polyphosphoric acid at 70° with rapid stirring. The reaction mixture was stirred at 70° for 1.5 hr. and then poured onto ice and extracted with $CHCl_3$. The $CHCl_3$ extracts were washed with 10% NaOH followed by water, dried (Na_2SO_4), and the solvent was evaporated. The residual crude ketone was chromatographed on an alumina column and elution with 1:1 benzene-hexane gave VIIa (10.5 Gm.), $\lambda_{max.}$ 222 mμ (ϵ 11,700); 270 mμ (ϵ 12,400). The semicarbazone crystallized from methanol–water, m.p. 191–192°.

Anal.—Calcd. for $C_{14}H_{19}N_3O_2$: C, 64.34; H, 7.33; N, 16.08. Found: C, 64.01; H, 7.46; N, 16.14.

2- Hydroxy - 6 - methylbenzocycloheptan - 5 - one (VIIb)—A solution of VIIa (6.8 Gm.) in glacial acetic acid (35 ml.) and 47% hydriodic acid (35 ml.) was refluxed for 1 hr. under nitrogen. The reaction mixture was diluted with water (100 ml.) and extracted with ether. The ether extracts were washed with saturated $NaHCO_3$ and then extracted with 10% NaOH. Evaporation of the ether gave recovered VIIa (2.7 Gm.). Acidification of the NaOH extracts gave crude VIIb (3.2 Gm.), m.p. 120–125°.

Recrystallization from ether–hexane gave 2.8 Gm., m.p. 135–137°. The analytical sample had m.p. 136–137°.

Anal.—Calcd. for $C_{12}H_{14}O_2$: C, 75.76; H, 7.42. Found: C, 75.90; H, 7.36.

2-Carboxymethoxy-6-methylbenzocycloheptan-5-one (VIIc)—Chloroacetic acid (2.2 Gm., 0.023 mole) was added to a solution of VIIb (2.9 Gm., 0.015 mole) in NaOH (from 1.7 Gm. NaOH and 25 ml. water) and the resulting solution was refluxed for 5 hr., cooled and acidified with concentrated HCl. The precipitated solid was collected and stirred with saturated $NaHCO_3$ solution (80 ml.) for 1 hr. Filtration gave starting material (0.65 Gm.) and acidification of the filtrate afforded the crude product (2.6 Gm.), m.p. 148–150°. The analytical sample was obtained on recrystallization from ether, m.p. 152–153°.

Anal.—Calcd. for $C_{14}H_{16}O_4$: C, 67.72; H, 6.50. Found: 67.59; H, 6.74.

6-Bromo-2-carboxymethoxy-6-methylbenzocycloheptan-5-one (VIId)—A solution of bromine (1.7 Gm., 0.011 mole) in CH_2Cl_2 (20 ml.) was added dropwise to a stirred refluxing suspension of VIIc (3.0 Gm.) in CH_2Cl_2 (100 ml.). Stirring was continued for 20 min. at room temperature on completion of the bromine addition, and the solvent was then evaporated in an air current giving crude α-bromoketone m.p. 206–209°. Recrystallization from $CHCl_3$ gave 3.1 Gm., m.p. 212–214°. The analytical sample, obtained by recrystallizing once more from the same solvent, had a m.p. 213–214°.

Anal.—Calcd. for $C_{14}H_{15}BrO_4$: C, 51.39; H, 4.62. Found: C, 51.32; H, 4.76.

2-Carboxymethoxy-8,9-dihydro-6-methyl-5H-benzocyclohepten-5-one (III)—A solution of VIId (3.1 Gm.) in 2,4-lutidine (12 ml.) was heated at 160° (oil bath) for 1.5 hr., cooled, diluted with water (40 ml.), acidified with concentrated HCl, and filtered. The crude product, 1.8 Gm., m.p. 158–160°, was recrystallized from $CHCl_3$-hexane to give 1.7 Gm., m.p. 161–162°, unchanged on further recrystallization, $\lambda_{max.}$ 237 mμ (ϵ 10,700); 291 mμ (ϵ 7,060).

Anal.—Calcd. for $C_{14}H_{14}O_4$: C, 68.28; H, 5.73. Found: C, 68.28; H, 5.63.

m-Hydroxy-α-methylhydrocinnamic acid (IX)—A solution of VIII (6) (90.0 Gm.) in 10% NaOH (300 ml.) was hydrogenated in a Parr shaker over 5% Pd/C (2.0 Gm.) at 3.4 Kg./cm.^{2} pressure and room temperature. After filtration to remove the catalyst, the solution was acidified with concentrated HCl, extracted with ether, and the extracts washed with water and dried (Na_2SO_4). Evaporation of the ether gave the product as a viscous brown oil (89.5 Gm.) which was characterized as the piperidine salt, m.p. 142–144° after crystallization from acetone.

Anal.—Calcd. for $C_{15}H_{22}NO_3$: C, 67.89; H, 8.75. Found: C, 67.70; H, 9.00.

5-Hydroxy-2-methyl-1-indanone (Xa)—Boron trifluoride was bubbled into a cold stirred solution of IX (20.0 Gm.) in $CHCl_3$ (500 ml.) for 4 hr. The reaction mixture was stirred at room temperature for 16 hr. and then 10% Na_2CO_3 (400 ml.) was slowly added, after which stirring was continued at room temperature for 1 hr. more. The chloroform layer was separated, washed with water, dried (Na_2SO_4), and the solvent was evaporated to give the crude indanone (16.0 Gm.) m.p. 136–142°. Recrystallization from ether-hexane gave 11.6 Gm., m.p. 153–155° [reported (7) m.p. 155.5–156.5°, prepared by different method].

5-Carboxymethoxy-2-methyl-1-indanone (Xb)—The procedure for the preparation of VIIc was employed using Xa (7.0 Gm., 0.043 mole), chloroacetic acid (6.2 Gm., 0.065 mole), and NaOH (4.6 Gm.) in water (200 ml.) yielding crude Xb (6.1 Gm.), m.p. 125–135° and recovered Xa (2.7 Gm.). The product was recrystallized from chloroform-hexane giving 4.9 Gm., m.p. 147–149°. The analytical sample had m.p. 148–150°.

Anal.—Calcd. for $C_{12}H_{12}O_4$: C, 65.64; H, 5.49. Found: C, 65.19; H, 5.39.

2-Bromo-5-carboxymethoxy-2-methyl-1-indanone (Xc)—The technique employed for the preparation of VIId was used. Bromination of Xb (31.9 Gm.) gave crude bromoketone (42.0 Gm.) m.p. 110–115°. Recrystallization from ether–hexane gave 37.0 Gm., m.p. 138–140°. The analytical sample had m.p. 138–139°.

Anal.—Calcd. for $C_{12}H_{13}BrO_4$: C, 48.14; H, 3.71; Br, 26.72. Found: C, 48.17; H, 3.90; Br, 27.04.

5-Carboxymethoxy-2-methylindone (IV)—A solution of Xc (18.29 Gm.) in 2,4-lutidine (73 ml.) was heated at 160° for 1.5 hr., poured into dilute HCl and filtered giving crude IV (10.1 Gm.), m.p. 165–170°. Recrystallization from chloroform–hexane furnished 6.1 Gm. of yellow crystals, m.p. 183–185°. The analytical sample had m.p. 187–188°. $\lambda_{max.}$ 218 mμ (ϵ 20,300); 257 mμ (ϵ 52,500); 330 mμ (ϵ 6,900).

Anal.—Calcd. for $C_{12}H_{10}O_4$: C, 66.05; H, 4.62. Found: C, 66.39; H, 4.33.

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Keyphrases

α,β–Aryloxyacetic acid ketones, unsaturated—synthesis
Ethacrynic acid related compounds
Structure–activity relationship
UV spectrophotometry—structure
Diuretic activity—α,β-aryloxyacetic acid ketones
Flame photometry—sodium ion analysis
Electrometric titration—chloride ion analysis

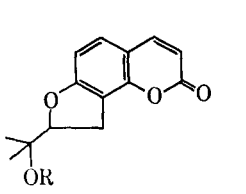
Coumarins V

Mass Spectra of Columbianetin and Its Esters

By M. SHIPCHANDLER and T. O. SOINE

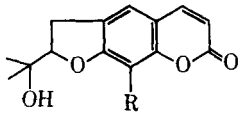
The mass spectra of columbianetin and its acetate, angelate, tiglate, and 3-bromoangelate have been determined. Columbianetin shows M →188 → 187 (base peak 187) as the principal fragmentation pathway whereas the esters show M→ 228 → 213 (base peak 213) as the major fragmentation route. Probable mechanisms for fragmentation are suggested.

In connection with our interest in physiologically active naturally occurring coumarins (1) a number of new representatives of this class have been isolated and structurally characterized. Among these were two dihydrofurocoumarins, columbianin and columbianadin, which were reported by Willette and Soine (2) as the D-glucoside (I) and angelate (II), respectively, of the parent alcohol, columbianetin (III). Since then, the structure of columbianin has been revised (3) to that of a β-D-gentiobioside of columbianetin (IV). During the research leading to the revised structure, employment of mass spectrometry for a molecular weight determination also led to an examination of the mass spectral characteristics of III and certain of its esters. The present report concerns itself with the spectra of III, its acetate (V), angelate (II), tiglate (VI), and 3-bromoangelate (VII).



R

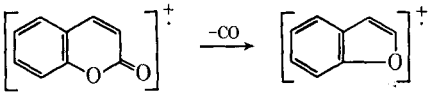
I—D-Glucosyl
II—Angeloyl
III—H
IV—β-D-Gentiobiosyl
V—Acetyl
VI—Tigloyl
VII—3-Bromoangeloyl



VIII—H
IX—OH

Mass spectrometry has been employed by others on similar coumarins both for structural elucidation and for determination of fragmentation patterns. Abdel-Hay *et al.* (4), for example, carried out mass spectral studies on linear dihydrofurocoumarins including marmesin (VIII) which is the linear isomer of III and, similarly, Schneider *et al.* (5) examined rutaretin (IX). The mass spectra of VIII, IX, and III have common features except that, in IX, the peaks are displaced to 16 higher *m/e* units because it possesses an extra phenolic group. Virtually all of the important peaks found in the case of III are to be found in the spectra of VIII and IX. It may be pointed out that, in the case of IX, the fragmentation mechanisms have been supported by accurate mass measurements. Seshadri *et al.* (6) reported the mass spectral characteristics of selinidin, the angelate of lomatin (7), which is a closely related coumarin of the dihydroseselin type. Here, again, the spectrum bore similarities to the isomeric II.

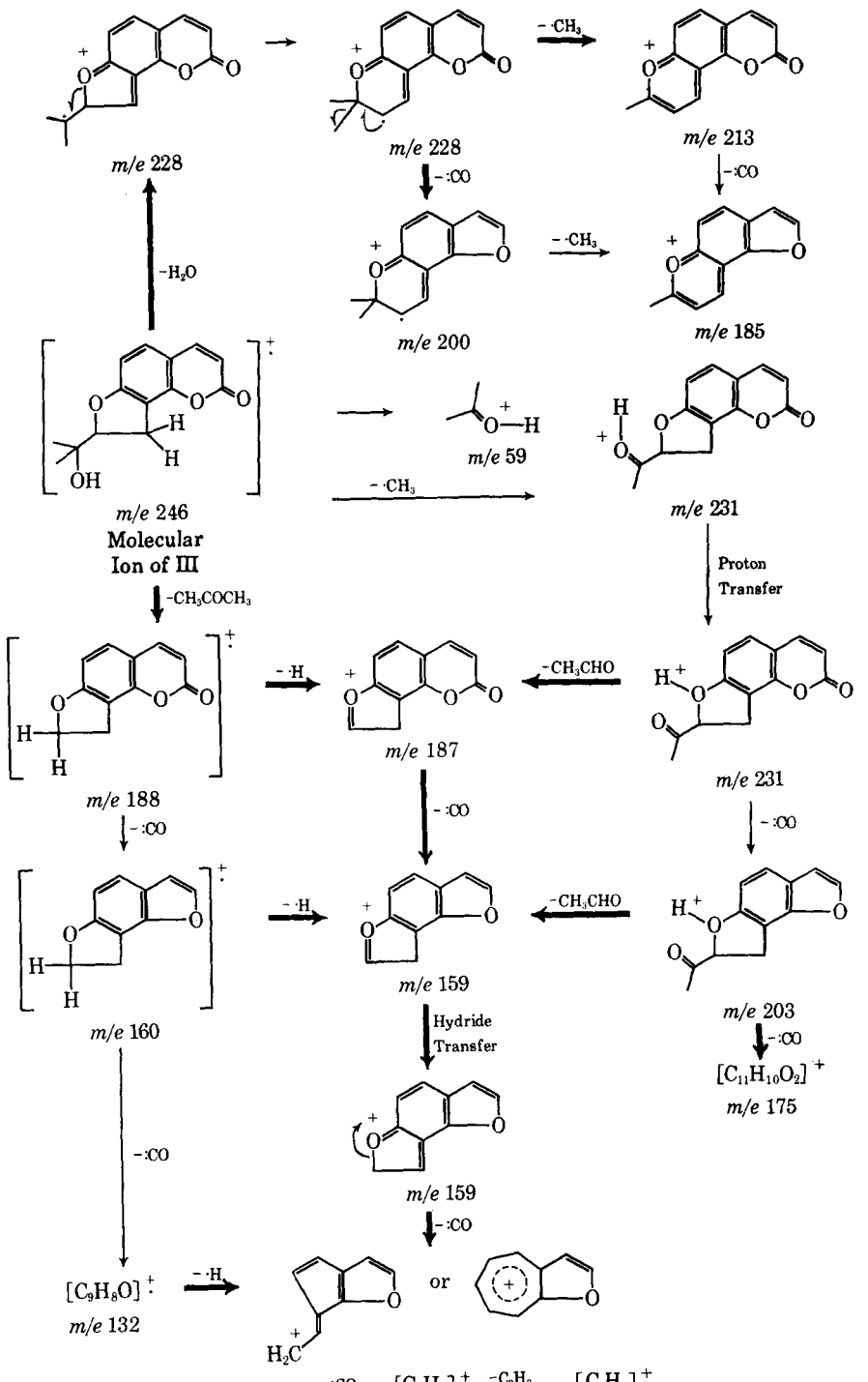
Barnes and Occolowitz (8) studied the mass spectra of a number of oxygen-containing heterocyclic compounds occurring in nature and Budzikiewicz *et al.* (9) reviewed the work done on this aspect up to 1964. According to the latter authors, and agreeing with the present study, CO is a highly stable neutral particle and, therefore, fragments arising due to its loss are characteristic features of all coumarins. For example, coumarin itself shows a stepwise loss of two molecules of CO and umbelliferone (7-hydroxycoumarin) loses 3 molecules of CO in a similar manner. Barnes and Occolowitz (8) suggest that the molecular ion of coumarin (X) loses the first molecule of CO by way of the lactone carbonyl and that the resulting ion has the structure of the molecular ion of benzofuran (XI) as indicated by a close similarity



m/e 146 X — *m/e* 118 XI

of the spectra of these two compounds. The validity of the benzofuran structure, however, has been challenged by Pirkle (10). Nevertheless, structures written for certain ions are often simply a convenient means of representing fragmentation pathways and are not intended to imply an actual ion structure. In this sense, the present paper employs the benzofuran system as resulting from loss of CO from the coumarinic

Received September 14, 1967, from the Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455
Accepted for publication December 1, 1967.
This investigation was supported in whole by grant HE-07101 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.


m/e 228
m/e 228
m/e 213
m/e 200
m/e 185
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m/e 246
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Ion of III
m/e 231
Proton
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m/e 188
m/e 187
m/e 231
m/e 160
m/e 159
m/e 203
[C11H10O2]+
m/e 175
Hydride
Transfer
m/e 159
[C9H8O]+
m/e 132
or
m/e 131
[C8H7]+
m/e 103
[C6H5]+
m/e 77

Scheme I

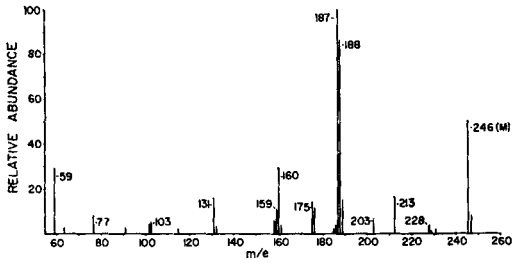
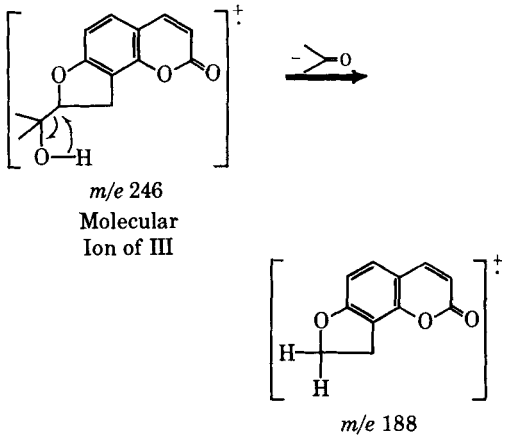


Fig. 1—Mass spectrum of III. Peaks of abundance ratio less than 3% have been omitted in all spectra unless a reference has been made to them in the text. Numbers in all figures correspond to those cited in the text.

lactone carbonyl. In many cases, the proposed fragmentation pathways are supported by the appearance of metastable ions, these being indicated in the several schemes by heavily lined arrows.

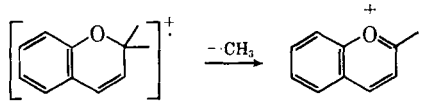
The mass spectrum of III (see Fig. 1) shows a molecular ion which fragments by different routes as shown in Scheme I. The principal pathway of fragmentation involves loss of acetone from the side chain of the molecular ion giving rise to a rearranged ion of *m/e* 188 which has the probable structure shown in Scheme I. This ion then loses an H atom to give rise to a highly stabilized ion of *m/e* 187 which forms the base peak in the spectrum. The *m/e* 188 and 187 ions each lose two molecules of CO in a stepwise manner to generate the ions of *m/e* 160 and 132 in the first case and ions of *m/e* 159 and 131 in the latter case. The ion of *m/e* 159 can also arise from the ion of *m/e* 160 by loss of H atom as indicated by the corresponding metastable ion peak. In a similar fashion the ion of *m/e* 131 can also arise from the ion of *m/e* 132. The ion of *m/e* 131 could be a highly stabilized allylic carbonium ion or could be a rearranged furotropylium ion as proposed in the case of osthol and dihydroosthol (8). This ion then shows loss of another molecule of CO to generate an ion of *m/e* 103 which fragments by losing a molecule of C_2H_2 to give an ion of *m/e* 77. Aromatic systems are known to lose C_2H_2 as a neutral fragment (11). The mechanism of fragmentation M → 188 can be rationalized as shown in Scheme II.

The peak at *m/e* 228 is due to an ion arising through loss of H_2O from the molecular ion. A mechanism similar to that suggested in the case of VIII (4) can be proposed for its formation. This ion then loses a methyl radical, giving rise to an ion of *m/e* 213 which probably has a stabilized benzopyrilium structure. The appearance of this type of ion is the most prominent feature in the mass spectra of the esters of III, selinidin (6),



Scheme II

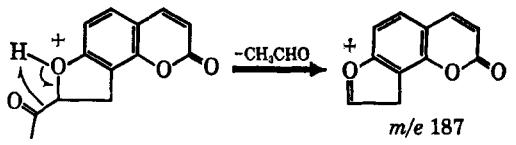
seselin (8), braylin (8), 2,2-dimethylchromene (8), and substituted 2,2-dimethylchromenes (8). The molecular ion of 2,2-dimethylchromene (XII), for example, shows a loss of methyl radical with the resulting ion (XIII) being assigned the structure of a stabilized benzopyrilium ion (8). This ion forms the base peak and there are no other peaks of comparable intensity in its entire spectrum. The ion of *m/e* 213 shows a loss of CO giving rise to an ion of *m/e* 185. The pathway M → 228 → 213 → 185 is very prominent in



the spectra of the esters of III. The alternate pathway for the formation of the ion of *m/e* 185 is probably 228 → 200 → 185 as shown in Scheme I.

The M-15 peak at *m/e* 231 is apparently due to the loss of methyl radical from the molecular ion. This ion, in turn, can lose a molecule of CO to generate an ion of *m/e* 203 which can further lose another molecule of CO to give rise to an ion of *m/e* 175. The ion of *m/e* 231 can also lose CH_3CHO by rearrangement and thus give rise to the ion of *m/e* 187 which represents an alternate pathway for the formation of the latter ion. Similarly, the ion of *m/e* 203 can give rise to an ion of *m/e* 159. Both of these pathways are substantiated by the appearance of metastable ions in the present study as well as in the case of IX (5). However, the metastable peak at *m/e* 151.1 can represent either the transformation 231 → 187 or 203 → 175 or both. A possible rearrangement mechanism leading to formation

of the ion of *m/e* 187 from the ion of *m/e* 231 is shown in Scheme III.



Scheme III

A peak at *m/e* 59 is due to the protonated form of acetone arising from the side chain of III. The appearance of this ion is a characteristic feature of coumarins containing the 2-hydroxyisopropyl grouping [*e.g.*, marmin (12)]. This ion forms the base peak in the spectrum of *tert*-butyl alcohol (13).

The probable fragmentation pathway for V (see Fig. 2) is shown in Scheme IV. The molecular ion loses acetic acid giving rise to a peak at *m/e* 228 which further loses a methyl radical providing an ion of *m/e* 213 which forms the base peak. This fragmentation is quite similar to that of the parent compound and, as mentioned before, is the principal pathway. The ion of *m/e* 213 loses a molecule of CO giving an ion of *m/e* 185. An alternate pathway, 228 → 200 → 185, could also operate in this case as shown in Scheme I. The molecular ion also shows loss of ketene which generates the molecular ion of III by rearrangement. Hence, it is expected that all the ions found in the spectrum of III should also be found in this case. An additional pathway is available for formation of the ion of *m/e* 187, *i.e.*, M → 229 → 187 as shown in Scheme IV. The transformation 229 → 187 is substantiated by a metastable peak at *m/e* 152.8 in the case of the D-glucoside of III (14). The ion of *m/e* 229 is produced from the molecular ion by loss of an acetate radical. This ion can lose an H atom to give the ion of *m/e* 228. Finally, the peak at *m/e* 231 is quite intense in the case of V as compared to III. The reasons for this difference are not entirely clear.

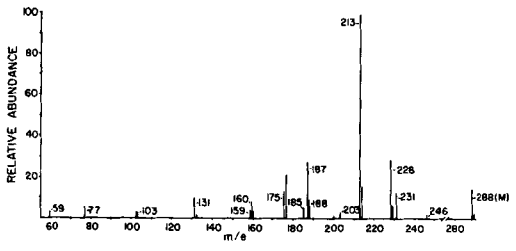
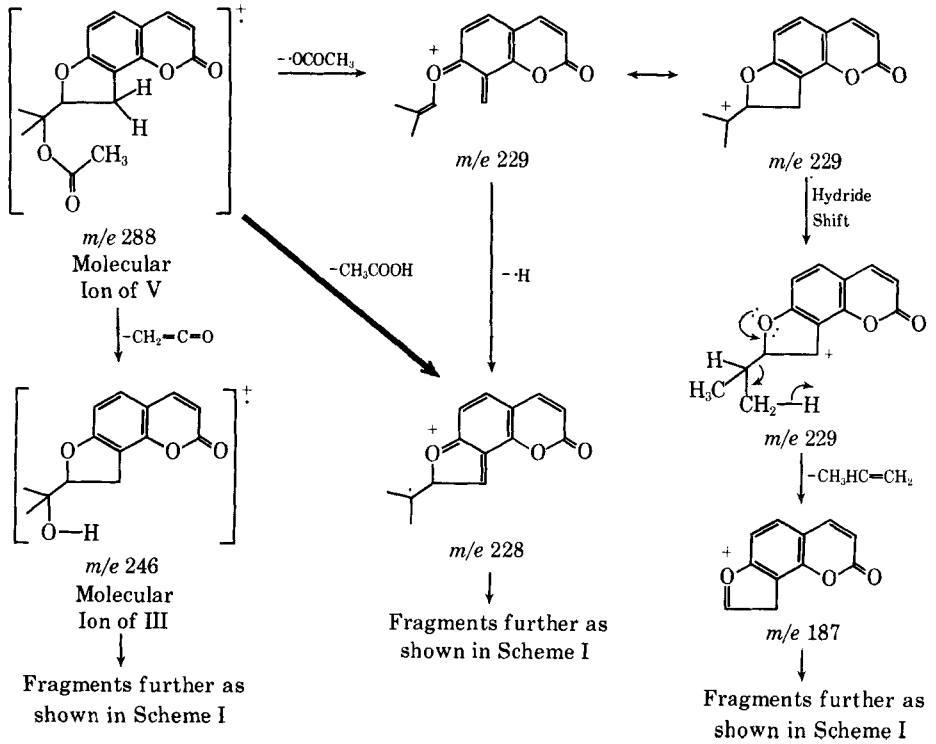


Fig. 2—Mass spectrum of V.

The major fragmentation pathways for II (see Fig. 3) are represented in Scheme V and are similar to those of selinidin (5), the dihydroseselin-type coumarin isomeric with II. As in the case of the acetate ester, the principal pathway for fragmen-



VI Scheme

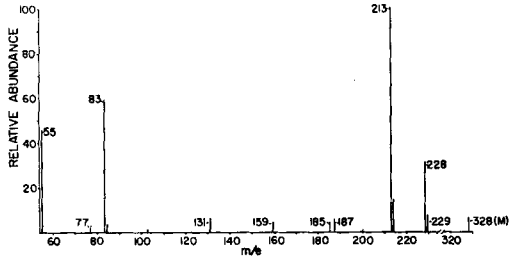


Fig. 3—Mass spectrum of II.

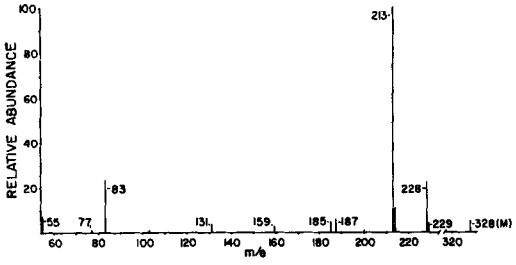
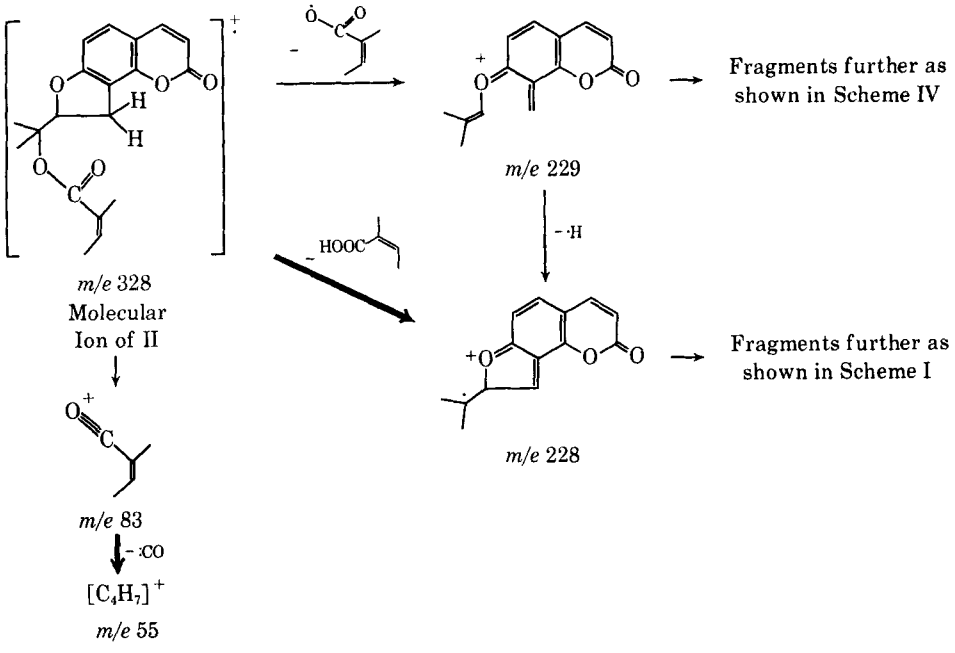


Fig. 4—Mass spectrum of VI.



Scheme V

tation is M → 228 → 213 → 185. Another important pathway is the generation of an ion of *m/e* 83 arising by α-cleavage from the acid part of the ester which further loses a molecule of CO to give an ion of *m/e* 55. This latter transformation is substantiated by the corresponding metastable ion peak. The peak at *m/e* 188 is quite inconspicuous and, therefore, the ion of *m/e* 187 can only arise from the ion of *m/e* 229.[1] Furthermore, a rational rearrangement mechanism cannot be written for the generation of the molecular ion of III which then fragments further to generate the ion of *m/e* 188 as in the case of V. Peaks at *m/e* 160 and 132, due to the ions arising from the ion of *m/e* 188, are also inconspicuous.

The spectrum of VI (see Fig. 4), the geometric isomer of II, shows all the peaks present in the spectrum of II. However, there are substantial differences in the abundance ratios in certain cases, namely the peaks that can be ascribed to the acid portion of the ester. Presumably, the fragmentation pathways as represented for II in Scheme V are valid for VI.

The principal fragmentation pathways for VII are shown in Scheme VI (see Fig. 5). They are quite similar to those for II and show twin peaks of molecular ions at *m/e* 406 and 408. These ions fragment in the same manner as does the corresponding ion for II. The ions which do not involve the acid part of the ester appear at the

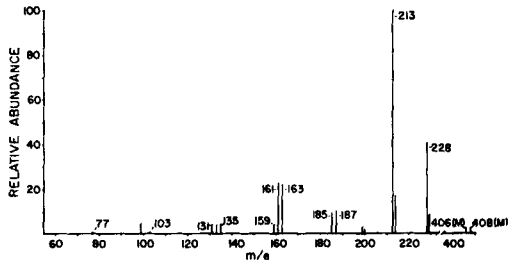
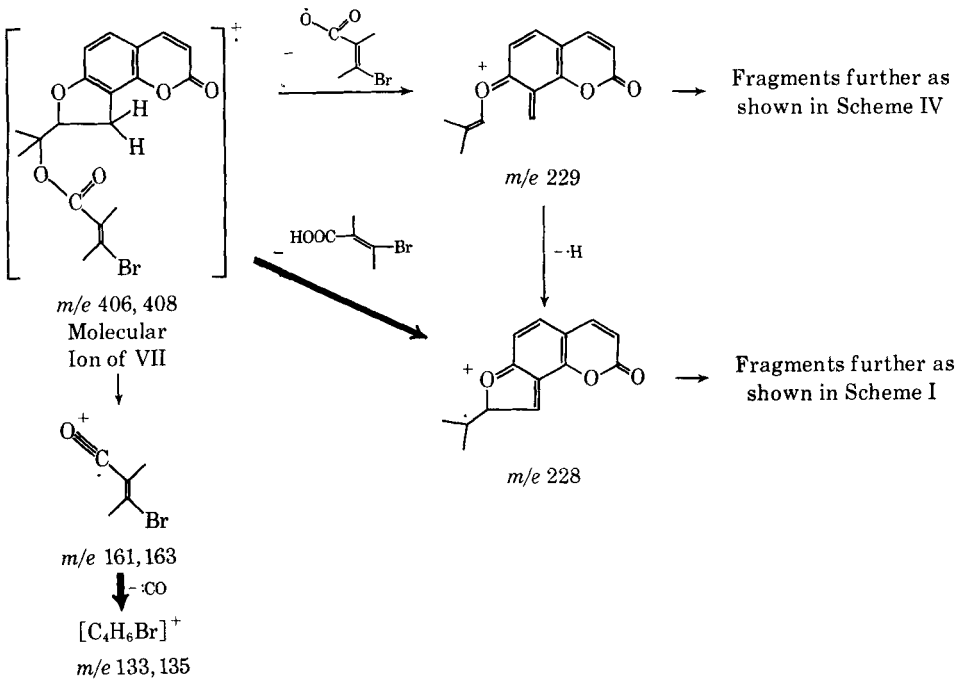


Fig. 5—Mass spectrum of VII.

[1] The peak at *m/e* 229 is extremely small in the case of III. However, this peak (corrected for the *P* + 1 satellite peak of 228) is observable in the case of the esters of III. This is probably due to the expulsion of ·OOC—R radical as against ·OH radical, the former being much more stable.



Scheme VI

same *m/e* units as in the case of II. Once again, the ion of *m/e* 213 forms the base peak. However, the peak at *m/e* 83 in the case of II which arises from the acid part is displaced to *m/e* 161 and 163. Similarly, the peak at *m/e* 55 is displaced to *m/e* 133 and 135.

EXPERIMENTAL

Materials—Columbianetin (III) and its acetate (V), angelate (II), tiglate (VI), and 3-bromoangelate (VII) were available in these laboratories as products isolated or synthesized during the studies of Willette and Soine (2) on *Lomatium columbianum* Mathias and Const.

Mass Spectra—These were carried out by Mr. A. R. Swanson and Mr. R. D. Berg, School of Chemistry, University of Minnesota, employing a Hitachi Perkin-Elmer RMU-6D mass spectrometer. The instrument was operated with a source temperature of 250° and an ionizing voltage of 50 ev. The sample inlet temperature was 70° in the case of columbianetin, 90° for the acetate, 120° in the case of the angelate, and 125° for the tiglate and 3-bromoangelate. Assistance in the interpretation of the spectra was given by Dr. Thomas Watson, Pharmacy Department, University of Sydney, Sydney, Australia.

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Keyphrases

Coumarins
Columbianetin—structure
Esters, columbianetin—synthesis
Mass spectrometry—structure

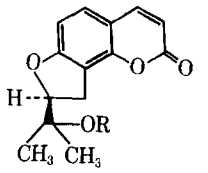
Coumarins VI

A Revised Structure of Columbianin and Pertinent Mass Spectral Studies

By M. SHIPCHANDLER and T. O. SOINE

The previous erroneous assignment of the structure of columbianin as a D-glucoside of columbianetin has been revised to indicate that it is a β-D-gentiobioside. The mass spectral characteristics of columbianin, its acetate, the true β-D-glucopyranoside of columbianetin, and its acetate have been examined and probable fragmentation pathways proposed.

THE ISOLATION of a new coumarin from the umbelliferous plant, *Lomatium columbianum* Mathias and Const., was reported in 1964 (1). The name assigned to this coumarin, a glycoside, was *columbianin* and it subsequently has been shown to be present in *L. dissectum* var. *multifidum* (Nutt.) Mathias and Const. (2) as well as in *L. nuttallii* (A. Gray) Macbr. (3). Upon acid hydrolysis, columbianin yielded D-glucose and a tertiary alcohol, columbianetin (I). Various chemical and spectral studies (1) indicated the validity of the structure assigned to I which has since been confirmed by Nielsen and Lemmich (4) who extended their studies (5) to show that an 8(S) configuration could be assigned as well. The erroneous assignment of a D-glucoside structure (II) for columbianin was based on the fact that only D-glucose and I were obtained from acid hydrolysis and that the elemental analyses obtained for both the glycoside and its acetate could reasonably have been interpreted as dihydrates, the originally proposed formulase. More recently, an examination of the nuclear magnetic



R

I —H
II —D-Glucosyl
III —β-D-Gentiobiosyl
IV —β-D-Glucopyranosyl

resonance (NMR) spectrum of the acetate of columbianin showed that it was inconsistent with the proposed structure and, therefore, rendered untenable the structure of the parent glycoside.[1] Specifically, the spectrum of the glycosidic acetate showed the *O*-acetyl methyls appearing as four peaks at 7.92, 8.00, 8.04, and 8.07 τ and the *gem*-dimethyls of the columbianetin moiety at 8.69 τ. The denouement, however, was the surprising peak ratio of 3.52:1 between the *O*-acetyl methyls and the *gem*-dimethyls since a ratio of 2:1 was expected. Additional analytical and degradative studies were indicated by this finding.

An ebullioscopic determination on the acetate, while not necessarily definitive, suggested the molecular weight to be about 800 rather than the lower value required by the original structure. Supporting this value was the observance of a rather weak molecular ion peak at *m/e* 864 (±4) in the mass spectrum. More definitive was an *O*-acetyl value that was about 7% higher than the expected value for the dihydrate of a tetraacetate. Finally, elemental analysis of a carefully purified sample of the acetate showed close correspondence to the required $C_{40}H_{48}O_{21}$ from the mass spectral study.

These data, of course, suggested that a disaccharide moiety was present in columbianin since the acetate was obviously a heptaacetate rather than the earlier suggested tetraacetate. To identify this disaccharide moiety, columbianin was subjected to a partial hydrolysis under mild conditions using a cation-exchange resin in the acid cycle (6). The hydrolysate was examined by paper chromatography using the silver nitrate technique for carbohydrate spot detection (7). Two spots appeared, one of which was identical in mobility with D-glucose and the other having a mobility consistent with a 1 → 6 linked disaccharide. The two possibilities, isomaltose (1 → 6,α) and gentiobiose (1 → 6,β) have nearly the same R_f values. Preparative paper chromatography made possible the isolation of this disaccharide ($[\alpha]_D^{20}$ −6° [c 2.0, H_2O]). The specific rotations (8) of the two possibilities are rather far apart—*isomaltose* $[\alpha]_D^{24}$ +120° (H_2O); *gentiobiose* $[\alpha]_D^{20}$ +8.7° (H_2O)—and, therefore, it ap-

Received October 12, 1967, from the Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455

Accepted for publication December 15, 1967.

This investigation was supported in whole by Grant HE-07101 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

The authors acknowledge with gratitude the assistance of Dr. B. A. Lewis, Biochemistry Department, St. Paul Campus, University of Minnesota, St. Paul, in providing valuable guidance and authentic samples for comparison. The authors are also indebted to Dr. Thomas Watson, Department of Pharmacy, University of Sydney, Sydney, Australia, for assistance in the interpretation of the mass spectra contained herein.

[1] A preliminary communication covering the revised structure appeared in *J. Pharm. Sci.*, **56**, 661(1967).

pears that gentiobiose must be the disaccharide under consideration with the small differences being attributed to impurities extracted from the paper during the workup. All doubt on this score was eliminated by showing that acetylation of the disaccharide provided β-D-gentiobiose octaacetate which was identical in all respects to an authentic sample. These data prove that columbianin is a gentiobioside of I but do not clarify the configurational α- or β-linkage of the disaccharide to the aglycone.

Gentiobiose is known to occur in nature in the glycosidic form (8). A well-known example is the cyanogenetic glycoside, amygdalin, which was studied by Fischer (9). He showed that amygdalin liberates a molecule of glucose and a new glucoside when treated with an enzyme isolated from yeast extract. Very recently, the hydrolysis of amygdalin, catalyzed by β-glucosidase from almonds, has been studied by Haisman and Knight (10). The enzyme has been shown to cleave β-linked glucose units in a stepwise manner from the nonreducing end of the sugar chain (10, 11). Amygdalin is hydrolyzed in three steps, the last being the hydrolysis of the cyanohydrin to benzaldehyde and HCN. In a similar manner, if columbianin is a β-gentiobioside, it should be hydrolyzed in two steps and no gentiobiose should be observed in the hydrolysate as opposed to acid hydrolysis where the cleavage is random and gentiobiose is observed (10). In considering whether the disaccharide unit is bonded to I by an α- or β-linkage it would be expected that enzymatic hydrolysis with β-glucosidase would stop after the first step if columbianin is an α-gentiobioside. On the other hand, if columbianin is a β-gentiobioside, it would proceed to I, albeit slowly, since tertiary alkyl β-glucosides are known to hydrolyze slowly (12). Experiment showed that columbianin liberated the aglycone in good yield when

$$\text{columbianin} \xrightarrow{\text{fast}} \text{glucose} + \text{monosaccharidic coumarin} \xrightarrow{\text{slow}} \text{glucose} + \text{aglycone (I)}$$

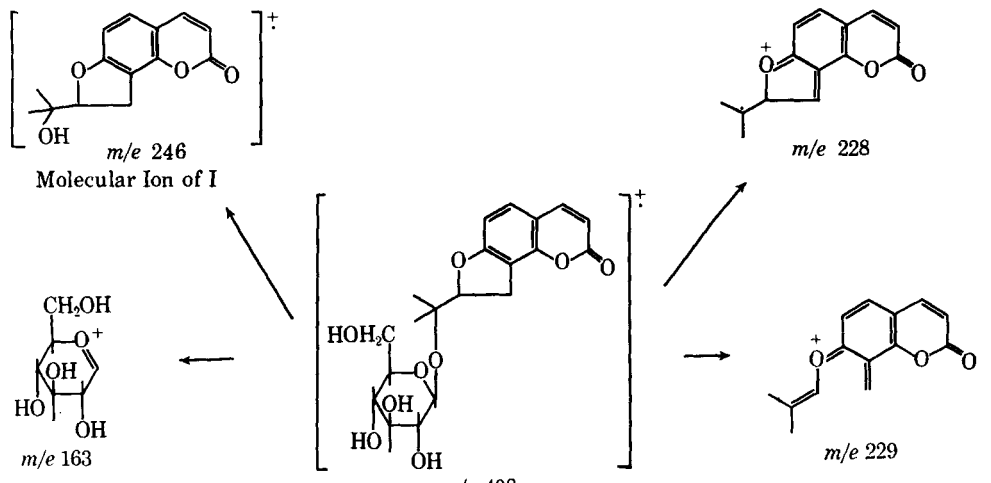
hydrolyzed with β-glucosidase in a prolonged experiment, paper chromatograms showing no gentiobiose but an abundance of glucose. However, paper and thin-layer silica gel chromatograms showed a fluorescent spot having a lesser polarity than the original glycoside but having a greater polarity than I. This material has been isolated in fairly good yield by the use of silica gel and preparative paper and thin-layer chromatography on an enzymatic hydrolysate that had been allowed to run for only a limited amount of time. The isolated compound did not exhibit a definite melting point but did give a small molecular ion peak in the mass spectrum corresponding to *m/e* 408 (*i.e.*, $C_{20}H_{24}O_9$). It provided a tetraacetate, m.p. 163.5–164.5°, in which the ratio of *O*-acetyl methyl peaks and *gem*-dimethyl peaks in the NMR spectrum was 2:1 as expected. The acetyl value as well as the elemental analysis was consistent with the $C_{28}H_{32}O_{13}$ formula of a tetraacetate. The acetate showed a fairly strong molecular ion peak at *m/e* 570 which was in accord with the assigned formula. The enzymatic study of columbianin proves conclusively that columbianin is the β-D-gentiobioside (III) of I. The name, columbianetin β-D-glucopyranoside, is assigned to the monosaccharidic coumarin since all of the analytical and spectral data are consistent with the structure (IV) assigned to it.

Mass Spectral Considerations—The mass spectrum of IV retains almost all of the features characterizing the fragmentation of I (13). The principal fragmentations of the molecular ion have been described in Scheme I[2] whereas the abundance ratios of the ions have been shown in Fig. 1. A peak at *m/e* 246 is probably the molecular ion of I arising from rearrangement of the molecular ion of IV, the probable mechanism being shown in Scheme II. Hence, it is expected that all of the peaks found in the mass spectrum of I will also be found in the present case, assuming that the ion arising by direct electron impact on I is similar to the one arising by the fragmentation shown in Scheme II. The fragments shown in Scheme I would follow the same fragmentation pathways as described previously (13). Many of these pathways have been supported in our earlier paper (13) by the appearance of the corresponding metastable ion peaks.

The peak at *m/e* 229 is very conspicuous as compared to the corresponding peak in the mass spectrum of I. This is probably due to the increased stability of the glucosyl radical as against the hydroxyl radical. Furthermore, the peak at *m/e* 229 is quite conspicuous in the spectra of the esters of I (13) and may, probably, be ascribed to being due to a similar reason.

The ion of *m/e* 188 could either be generated from the ion of *m/e* 246 (*i.e.*, the molecular ion of I) or could arise directly from the molecular ion of IV or both. This ion could then fragment by two routes as in the case of I, *i.e.*, 188 → 187 → 159 → 131 → 103 → 77 and 188 → 160 → 132 with the latter route being of relatively minor consequence.

[2] The ion structures given are not necessarily accurate representations, being used primarily as a convenient means for showing the fragmentation pathways. Further fragmentation pathways for the coumarinic ions have been discussed in the previous paper.



Scheme I

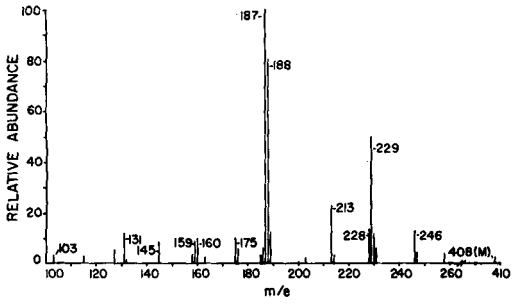


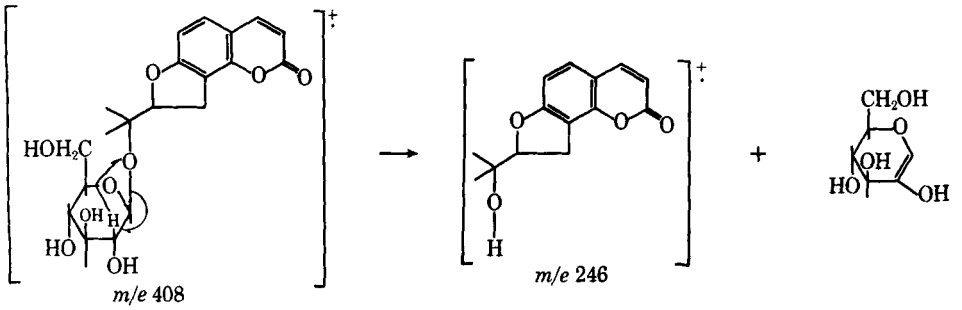
Fig. 1—Mass spectrum of IV. Peaks of abundance ratio less than 3% have been omitted in all spectra unless a reference has been made to them in text. Numbers in all figures correspond to those cited in the text.

The ion of *m/e* 187 forms the base peak which, in addition, could arise by two more routes, *i.e.*, M → 246 → 231 → 187 and M → 229 → 187. The transformation 229 → 187 is supported by the appearance of a metastable peak at *m/e* 152.8. A pathway of minor consequence is 231 → 203 → 175.

The ion of *m/e* 228 arises from the molecular ion by loss of a molecule of glucose. A mechanism similar to that proposed in the case of I for its formation (13) can be proposed in this case also. The ion fragments by the route 228 → 213 → 185.

In addition to the features that can be ascribed to the coumarinic part, there are peaks which arise from the carbohydrate portion although these are of lesser consequence. Reed *et al.* (14) have studied the mass spectrum of methyl β-D-glucopyranoside and suggest that the peak at *m/e* 163 can be ascribed to an ion arising from the cleavage of the bond between the anomeric carbon and its substituent. This ion, in turn, loses H_2O to generate an ion of *m/e* 145. The latter ion has been shown to fragment into ions of *m/e* 85, 74, 71, and 60 (14).

The tetraacetate of IV shows quite a predictable mode of fragmentation (see Fig. 2) characteristic of the parent compound and also of the polyacetate derivatives of glucose. The peaks which can be said to have originated from the sugar part of the molecule are quite prominent with the peak at *m/e* 169 forming the base peak. Pearl and Darling (15) have recently studied a number of natural glucoside acetates and have found that all of these, in general, possessed a similar pattern as far as the sugar portion is con-



Scheme II

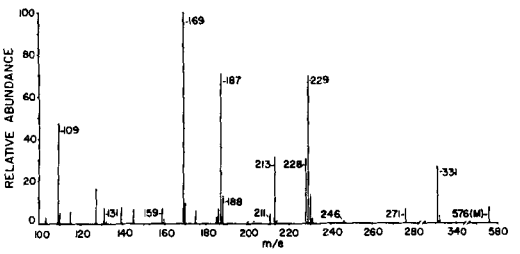


Fig. 2—Mass spectrum of the acetate of IV.

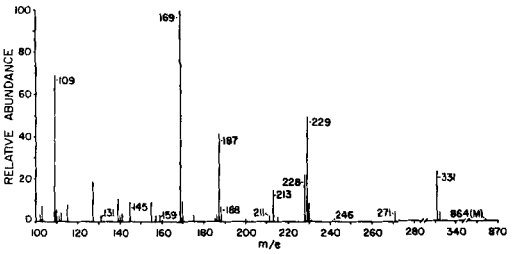
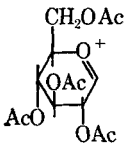


Fig. 4—Mass spectrum of the acetate of III.

cerned. Biemann *et al.* (16) have shown that the peak at *m/e* 331 in the spectra of glucose pentaacetate and the tetraacetates of methyl and phenyl glucosides is due to V, *i.e.*, the ion arising from cleavage between the anomeric carbon and its substituent. Pearl and Darling (15), likewise, found this type of ion in a number of glucoside acetates. This ion fragments by two routes, *i.e.*, 331 → 271 → 229 → 169 → 109 and 331 → 211 → 169 (16,17). Triacetyl and diacetyl oxonium ions at *m/e* 145 and 103 are also observed. One would expect to find almost all of the ion peaks in



m/e 331

V

the spectrum of the tetraacetate as are found in the spectrum of IV as far as fragments arising from the coumarinic portion of the molecule are concerned.

Columbianin shows almost all the principal features of IV in the mass spectrum (see Fig. 3), the difference being in the peak ratios. The base peak appears at *m/e* 187 just as in the mass spectrum of IV. We failed, however, to observe a molecular ion peak.

The mass spectrum of the heptaacetate of III (see Fig. 4) resembles that of the tetraacetate of IV, once again the differences being mainly in the abundance ratios. The ion of *m/e* 169 forms the base peak. The molecular ion peak, however, is rather weak.

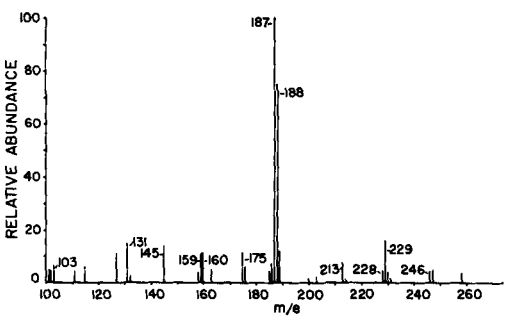


Fig. 3—Mass spectrum of III.

EXPERIMENTAL

Melting points were determined in capillary tubes in a Thomas-Hoover melting point apparatus and are uncorrected.

Infrared spectra were determined on a Perkin-Elmer 237B grating infrared spectrophotometer.

Values of $[\alpha]$ were determined on a Perkin-Elmer 141 polarimeter.

Nuclear magnetic resonance (NMR) spectra were determined on a Varian Associates A-60 instrument in $CDCl_3$ using tetramethylsilane (TMS) as the internal standard.

Mass spectra were obtained by Mr. A. R. Swanson and Mr. R. D. Berg, School of Chemistry, University of Minnesota, employing a Hitachi Perkin-Elmer RMU-6D mass spectrometer. The instrument was operated with a source temperature of 250° and an ionizing voltage of 50 ev. The sample inlet temperature was 300° in the case of IV, 250° for the tetraacetate of IV, 350° in the case of III, and 380° for the heptaacetate of III.

Silica gel for column chromatography refers to Baker Analyst No. 3405 and silica gel for thin-layer chromatography refers to "Chromagram" sheets supplied by Distillation Products Industries, Rochester, N. Y.

Columbianin—The product isolated during the studies of Willette and Soine (1) in these laboratories was employed.

Columbian Acetate—The acetate of columbianin was prepared in the manner previously described (1) and was crystallized from chloroform–cyclohexane, m.p. 218–219°, undepressed on admixture with the authentic acetate.

Anal.[3]—Calcd. for $C_{40}H_{48}O_{21}$: C, 55.55; H, 5.59; mol. wt., 864; *O*-acetyl, 34.84. Found: C, 55.63; H, 5.53; mol. wt. (ebullioscopic), 800; *O*-acetyl, 34.92.

Partial Hydrolysis of Columbianin—Columbianin (20 mg.) was dissolved in 0.5 ml. of water in a small test tube and to this was added 40 mg. of cation-exchange resin in the acid cycle[4] and the mixture placed into a boiling water bath for 1 hr. The

[3] Microanalyses were determined by the Microanalytical Laboratory, School of Chemistry, University of Minnesota, Minneapolis, or by the Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

[4] Marketed as Amberlite IR-120 by Rohm and Haas Co., Philadelphia, Pa., and converted to the acid cycle with 10% sulfuric acid.

hydrolysate was spotted on standard size Whatman paper No. 1 together with authentic samples of glucose, isomaltose, maltose, and gentiobiose. The paper was developed by the descending chromatographic technique using a pyridine–ethyl acetate–water (2:5:7, upper layer) mobile phase for about 16 hr. The paper was dried and then developed with silver nitrate in acetone in the usual manner (7). Two spots appeared, one of which had moved the same distance as glucose and another which had moved the same distance as isomaltose and gentiobiose, the latter two having traveled almost identically.

Isolation of Gentiobiose—Columbianin (400 mg.) was dissolved in 10 ml. of water and 800 mg. cation-exchange resin in the acid cycle was then added and the reaction mixture heated in a boiling water bath for 5.5 hr. The water lost by evaporation was replaced from time to time. After the end of the heating period the hydrolysate was filtered and the filtrate streaked on standard length Whatman paper No. 3 and developed in the manner described above. After drying, narrow strips were cut from each side of the paper and developed with silver nitrate to locate the zone containing the disaccharide. This zone was cut out, divided into small pieces, and eluted with water. Removal of the water under vacuum followed by drying gave several mg. of the crude disaccharide $\{[\alpha]_D^{20} -6°$ (c 2.0, $H_2O)\}$. The crude product was acetylated with acetic anhydride and sodium acetate in the usual manner to give 30 mg. of β-D-gentiobiose octaacetate, m.p. 191–192°; $[\alpha]_D^{20} -1.86°$ (c 0.64, $CHCl_3$) {lit., m.p. 193°; $[\alpha]_D^{19.5} -5°$ (c 1.8, $CHCl_3$) (8)}. The melting point was not depressed on admixture with an authentic sample of β-D-gentiobiose octaacetate and the infrared spectra were identical.

Isolation of the Aglycone from the Enzymatic Hydrolysis—Columbianin (200 mg.) was dissolved in 20 ml. of water and the aid of heat and to the cooled solution was added 100 mg. of β-glucosidase.[5] The mixture was slowly stirred at room temperature for 56 hr. at which time it was boiled for a few minutes and, after cooling, treated with another 100 mg. of enzyme followed by an additional 36 hr. of stirring. Silica gel thin-layer chromatographic examination in the beginning showed blue fluorescent coumarinic spots when developed with chloroform–ethanol–ethyl acetate (2:3:10) and examined under ultraviolet light. The R_f values of the three principal spots were: 0.07, 0.37, and 0.66 with the first (R_f = 0.07) corresponding to columbianin and the last to columbianetin. Presumably, the spot of intermediate R_f value was an intermediate product of hydrolysis. Paper-chromatographic examination using the pyridine–ethyl acetate–water system previously employed also showed three blue fluorescent spots under ultraviolet light. However, development of the paper chromatogram of the hydrolysate using the silver nitrate technique failed to show the presence of gentiobiose although a spot corresponding to glucose was observed. As the hydrolysis proceeded, the spot corresponding to columbianin slowly lost its fluorescence, whereas the spot corresponding to columbianetin gained in brilliance. When the hydrolysis was terminated the hydrolysate showed only a spot corresponding to columbianetin (R_f = 0.66) and the spot of intermediate polarity (R_f = 0.37). The hydrolysate was extracted with ether until a portion of the ether failed to fluoresce under ultraviolet light when spotted on filter paper. The ether extract was dried with anhydrous sodium sulfate, stripped of solvent, and crystallized to provide 35 mg. (40% of theory) of columbianetin, m.p. 160–161°, which failed to depress the melting point of an authentic sample with which it showed an identical infrared spectrum.

Isolation of Columbianetin β-D-Glucopyranoside—Columbianin (500 mg.) was dissolved in 25 ml. of water with the aid of heat and, after cooling, 100 mg. of β-glucosidase was added. The mixture was stirred for 12 hr. at room temperature at which time thin-layer chromatographic examination showed only traces of unhydrolyzed columbianin, only a small amount of columbianetin, and the major part of the fluorescence in the spot of intermediate polarity. The hydrolysis was then stopped by heating the reaction mixture on a steam bath for several minutes. The isolation, in several runs, was carried out by three different chromatographic techniques. Preparative paper or thin-layer chromatographic procedures employing the previously described solvent systems were employed effectively followed by final elution of the product with methanol. Column chromatography employing silica gel activated at 110° and impregnated with 5% of water appeared to be quite satisfactory also. Elution with ethyl acetate–chloroform (1:1) yields the aglycone and, subsequently, elution with ethyl acetate provides the compound of intermediate polarity (R_f = 0.37) which can be crystallized with difficulty from ethyl acetate–cyclohexane. This compound does not have a definite melting point, softening at *ca* 117–120°, honeycombing at about 130°, and finally becoming transparent at about 180°; $[\alpha]_D^{18.5}$ +206 (c 1.0, CH_3OH). Because of the indefinite melting point and difficulty in crystallization, this compound was analyzed as the acetate derivative.

Columbianetin β-D-Glucopyranoside Acetate—Preparation of the acetate in the usual manner employing acetic anhydride and sodium acetate yielded white crystals from a mixture of ethyl acetate and cyclohexane. Since solvation of the product with cyclohexane was evident from the NMR it was dried at 85° for several hours in vacuum to give a product with m.p. 163.5–164.5°; $[\alpha]_D^{19.5}$ +146° (c 1.0, $CHCl_3$).

Anal.—Calcd. for $C_{28}H_{32}O_{13}$: C, 58.33; H, 5.59; *O*-acetyl, 29.86. Found: C, 58.33; H, 5.73; *O*-acetyl, 29.60.

[5] Product of General Biochemicals, Chagrin Falls, Ohio.

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Keyphrases

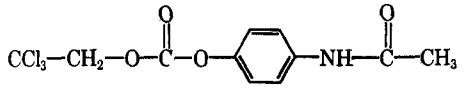
Coumarins
Columbianin structure—revised
Column chromatography—separation
IR spectrophotometry—structure
Mass spectrometry—structure
Paper chromatography—identity
TLC—identity
NMR spectrometry—identity
Polarimetry—identity

4-Acetamidophenyl 2,2,2-Trichloroethyl Carbonate Synthesis, Physical Properties, and *In Vitro* Hydrolysis

By J. V. SWINTOSKY*, H. C. CALDWELL, C. W. CHONG, G. M. IRWIN, and L. W. DITTERT*

A trichloroethyl carbonate prodrug ester of acetaminophen has been made by reacting acetaminophen with trichloroethyl chloroformate. It is a virtually tasteless crystalline compound retaining the pharmacologic effects of acetaminophen and trichloroethanol. It is lipophilic; its solubility in water is less than 0.05%. It undergoes base-catalyzed hydrolysis, and at pH 7.4 has a half-life in water solution of about 7 hr. Its stability in the solid state makes this compound amenable to use in dosage forms such as tablets and capsules. Hydrolysis of this compound is not catalyzed by human gastric fluid, but it is catalyzed by human intestinal fluid, rat intestinal mucosa, rat plasma, rat liver, and human plasma. It was surmised, therefore, that following oral administration to animals, a portion of the prodrug may be absorbed intact, but some cleavage may occur before and during absorption from the intestines. Furthermore, since enzymes that hydrolyze it are prevalent in plasma and liver, the prodrug on entering the blood will be hydrolyzed very rapidly.

THIS PAPER deals primarily with the preparation and *in vitro* hydrolysis characteristics of a new prodrug (1, 2) form of acetaminophen and trichloroethanol possessing the following structure:



4-Acetamidophenyl 2,2,2-Trichloroethyl Carbonate (ATC)

Albert (3) has used the term "prodrug" to describe compounds which undergo biotransformation prior to exhibiting their pharmacologic effects. Harper (4) has used the term "drug latentiation" to describe chemical modification of a biologically active compound to form a new compound which upon *in vitro* enzymatic attack will liberate the parent compound.

During the past few decades there have been numerous *in vitro* studies describing hydrolysis of esters using serum and other body tissues as enzyme sources. Glick (5) measured the enzymatic hydrolysis of a variety of esters including those of choline. Related studies were undertaken by Adams and Whittaker (6) and Alles and Hawes (7). Levine and Clark (8) studied the relationship between structure and *in vitro* hydrolysis of various esters and amides in human serum. Frazer (9) performed hydrolysis studies on succinylcholine esters using cholinesterases. Other studies of these types have been reported

Received July 12, 1967, from Smith Kline & French Laboratories, Philadelphia, PA 19101
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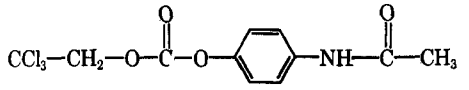

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in the literature as, for example, those of Kalow (10) and Casida and Augustinsson (11).

The carbonate ester, ATC, was designed to exhibit analgesic and sedative properties only after biotransformation following ingestion. Combining the sedative and analgesic activities of the parent drugs, acetaminophen and trichloroethanol, into a single molecule seemed logical since their pharmacologic activities are complementary, and their molar doses are somewhat equivalent. But the primary objectives were to impart new physical-chemical properties to the prodrug so that its absorption rate might be slowed, its dose-time-action profile modified in a useful way, and its profile of physical-chemical properties would be more desirable.

Both acetaminophen and trichloroethanol have an unpleasant taste, and trichloroethanol, a liquid, is especially unsuited for convenient pharmaceutical formulation. It was surmised that blocking the hydroxy groups through a carbonate bridge and linking the molecules together would suppress hydrophilic properties, improve taste, and yield a chemically neutral solid with more lipophilic quality than its parent drugs. Implicitly in the work underlying the preparation of ATC was the assumption that the compound would undergo cleavage in the body releasing the parent drugs at a rate that would result in a useful therapeutic action.

When administered to mice, ATC was shown to possess low acute oral toxicity, to be analgesic, and to reduce spontaneous motor activity, suggesting that it acted in a manner that would be expected of a prodrug of acetaminophen and trichloroethanol (1). Since the pharmaceutic and pharmacologic profiles of this compound seemed to have possible practical value in therapeutics, the authors set out to determine: (*a*) its hydrolysis rate in buffered water, (*b*) its cleavage rates in gastric and intestinal fluids, and (*c*) its cleavage rates in blood and tissue homogenates. It was hoped that these data would yield information about the hydrolytic stability of ATC in dosage forms, the importance and distribution of enzymes in the body that would cause it to be cleaved hydrolytically, the influence of enzymes on the cleavage rates of this apparent prodrug, and the cleavage, if any, of the compound in the gastrointestinal tract before absorption into the blood stream.

EXPERIMENTAL

Hydrolysis Studies

Chromatographic Procedure—The hydrolyses were carried out in a beaker with stirring at 37° as follows: 110 ml. of the biologic material (human gastric fluid or rat liver homogenate, *etc.*) was placed in the beaker and stirred until its temperature reached 37°. A 10-ml. blank sample of the biologic material was withdrawn. Three milligrams of ATC dissolved in 1 ml. of 95% ethanol was added to the biologic material and samples were withdrawn for analysis at appropriate time intervals.

Analyses were carried out as follows: samples (usually 10 ml.) removed from the reaction mixture were placed immediately in glass mortars containing three drops of concentrated hydrochloric acid (sufficient to bring the pH below 1). One gram of diatomaceous earth[1] for each ml. of reaction mixture plus 1 Gm. excess was added to each mortar, and this was triturated. This material was then packed on chromatography columns containing 5 Gm. of diatomaceous earth wet with 4 ml. of water. The mortars were "rinsed" with 2 Gm. of diatomaceous earth wet with 1 ml. of water, and this material was packed onto the columns. The columns were capped with a pledget of glass wool, and 50 ml. of reagent chloroform was poured in. The first 25 ml. of eluate was evaporated to dryness in a jet of air on a steam bath, and the residue was dissolved in 95% ethanol with gentle heating. The ethanol solution was transferred to a volumetric flask, brought to 25 ml. with ethanol, and analyzed spectrophotometrically using the absorption maximum of ATC at 240 mμ. The blank sample of the enzyme preparation was treated in exactly the same way, and the resulting alcohol solution was used in the reference cell of the spectrophotometer.

Initial experiments with mixtures of ATC and acetaminophen showed that the chromatographic procedure separated these two compounds completely.

Spectrophotometric Procedure—Hydrolysis reactions utilizing biologic materials as enzyme sources which were sufficiently dilute to allow direct UV analysis were carried out in the thermostated cell compartment of a Cary model 15 spectrophotometer. The biologic material (50 ml.) was warmed to 37° in a 125-ml. conical flask and a portion placed in the spectrophotometer reference cell. One-half milliliter of 95% ethanol containing 1.5 mg. of ATC was injected by means of a hypodermic syringe, and the flask was swirled gently until mixing was complete. A portion of this mixture was transferred to the sample cell of the spectrophotometer and the absorbances at 240 mμ (for ATC) or 300 mμ (for acetaminophen) were followed with time. The reaction was allowed to proceed until no further change in absorbance was observable, and the data were plotted in terms of Δ absorbance *versus* time (12).

At pH 7.4 the spectra of ATC and acetaminophen are similar; but there are sufficient differences in their molar absorptivities at 240 and 300 mμ to readily follow the hydrolysis reactions at either of these wavelengths. Since the hydrolysis reactions all followed first-order kinetics, plots of absorbance *versus* time, as the absorbance of the reaction mixture approached an equilibrium value, yielded the first-order rate constant. The values of many of the rate constants were verified by following the reactions at both wavelengths and, in several cases, by following the reactions using the chromatographic procedure. The same half-lives were obtained by all three techniques.

[1] Celite, Johns-Manville Corp., New York, N. Y.

Syntheses

2,2,2-Trichloroethyl Chloroformate (13)—Conversion of trichloroethanol to the chloroformate was done in a hood. Vapors of phosgene and the chloroformate[2] were trapped in a sodium hydroxide solution.

A mixture of 150 ml. of toluene and 41 ml. (0.5 mole) of pyridine was cooled wih a dry ice–isopropanol bath. Phosgene (0.75 mole) was added with stirring to this cold mixture. Freshly distilled trichloroethanol (0.5 mole) was then dripped into the mixture, and the dry ice–isopropanol bath was removed after 10 min. The mixture was then stirred for 1 hr. with ice water cooling and for 4 hr. at room temperature. The excess phosgene was removed *in vacuo* at room temperature and the pyridine hydrochloride was removed by filtration.

4-Acetamidophenyl 2,2,2-Trichloroethyl Carbonate—The reaction between acetaminophen and 2,2,2-trichloroethyl chloroformate was carried out in a hood.

Seventy-five and one-half grams (0.5 mole) of 4-hydroxyacetanilide was dissolved in a solution of 20 Gm. (0.5 mole) of sodium hydroxide in 300 ml. of water. This solution was dripped into a chilled toluene solution of the above chloroformate with stirring over a period of 15 min., and stirring was continued for 90 min. The precipitate was recrystallized from dilute alcohol (charcoal) to give 107 Gm. (66%) of product, a white crystalline solid, m.p. 151–153.5°.

Anal.—Calcd. for $C_{11}H_{10}Cl_3NO_4$: C, 40.46; H, 3.09; Cl, 32.57. Found: C, 40.67; H, 3.14; Cl, 32.47.

ATC has a solubility in water of about 35 mcg./ml., and in cyclohexane of 56 mcg./ml., at 37°. It is much more soluble (> 1%) in alcohol and chloroform. As a fine powder, ATC is poorly wetted by water and tends to float.

Materials

Phosphate Buffer—A 0.02 *M* phosphate buffer solution was used to study hydrolysis rates of ATC in water at various pH's. A 0.1 *M*, pH 7.4 phosphate buffer was used in hydrolysis studies involving biological materials, with the exception of human gastric fluid. All hydrolysis studies were done at about body temperature, 37°.

Human Gastric and Intestinal Fluids—These materials were obtained from patients at the Presbyterian University of Pennsylvania Medical Center, Philadelphia. The gastric fluid had a pH of 1.1 and was used undiluted in the hydrolysis studies. The intestinal fluid was used for hydrolysis studies in dilutions of 1, 2, 3, and 4% in phosphate buffer, pH 7.4. The hydrolysis studies in gastric fluid were carried out using the chromatographic procedure, whereas the studies in intestinal fluid employed the spectrophotometric procedure.

Human Plasma—Samples of human plasma were obtained from the Philadelphia Serum Exchange, Children's Hospital. Studies utilizing fresh plasma, 1 part in 50 of phosphate buffer, were carried out using the spectrophotometric procedure to follow hydrolysis rates.

Rat Plasma—The plasma was separated from fresh blood collected in a heparinized beaker by centrifuging the blood at about 20,000 r.p.m. for 5 min.; then the plasma was frozen immediately and stored frozen until used. Hydrolysis studies were carried out utilizing rat plasma at a concentration of 1% in phosphate buffer. The spectrophotometric method was used to follow concentrations of ATC or acetaminophen.

Rat Liver—A Dierolf rat (about 200 Gm.) was killed, and its liver was removed, weighed, and homogenized in a small volume of ice cold normal saline solution using a Waring blender. The homogenate was strained through cotton gauze and brought to 100 ml. with cold normal saline solution. This suspension was then diluted with sufficient phosphate buffer to make a final concentration of 0.1% liver tissue based on the wet weight of the original liver. Hydrolysis studies in this suspension were carried out using the chromatographic procedure.

Rat Intestinal Mucosa—A Dierolf rat (about 200 Gm.) was killed, and its entire small intestine [about 61 cm. (24 in.)] was removed. The intestine was opened, and the solid contents were removed by gentle rinsing with cold water. Mucosal scrapings from the entire segment were obtained by means of a microspatula with a curved blade. The scrapings were mixed with cold normal saline solution (10 ml.) in a tissue grinder, and the resulting suspension was filtered through filter paper. The filtrate was diluted 1:20 (volumetrically) with phosphate buffer, and hydrolysis rate studies were carried out in this solution using the spectrophotometric procedure.

RESULTS AND DISCUSSION

Nonenzymatic Cleavage of ATC—The influence of pH on the hydrolysis rates of this compound was studied: (*a*) to determine to what extent this might influence shelf life of dosage forms prepared from it, and (*b*) to estimate if these rates alone would be sufficiently fast to produce drug from prodrug at a rate which would elicit a pharmacologic response.

In Fig. 1 there are plotted the half-lives ($t_{1/2}$) of ATC as a function of pH in the pH range 2 to 10.5. Hydrolysis reactions all followed first-order kinetics. Studies on the effect of phosphate ion, though not reported here, were done to determine how this might influence the hydrolysis rate. Some catalysis due to phosphate was observed at pH's above 8. However, 0.02 *M* phosphate buffer exerted almost no effect on the hydrolysis rate in this pH range. Below pH 8, phosphate buffer exerted virtually no catalytic effect on hydrolysis.

Between pH 7.6 and 10.5 the plot of log $t_{1/2}$ *versus* pH (Fig. 1) is a straight line with a slope of approximately −1, indicating that the hydrolytic process is first-order with respect to hydroxyl ion in this pH range. Below pH 7, the hydrolytic rate approaches a constant value perhaps due to simple water hydrolysis. At 7.4, the pH of blood and other tissue fluids, the $t_{1/2}$ is about 7 hr. At pH's of 1 to 3, corresponding to stomach pH, the $t_{1/2}$ is about 15 hr. Therefore, at pH's normally encountered in the body, *i.e.*, about 1 to 7.4, it is apparent that the $t_{1/2}$ in the absence of enzymes is always more than about 7 hr. From these data we surmise that at the pH's of body fluids the cleavage of this compound would

[2] Vapors of the chloroformate are very irritating, and they are reported to be as toxic as chlorine (14).

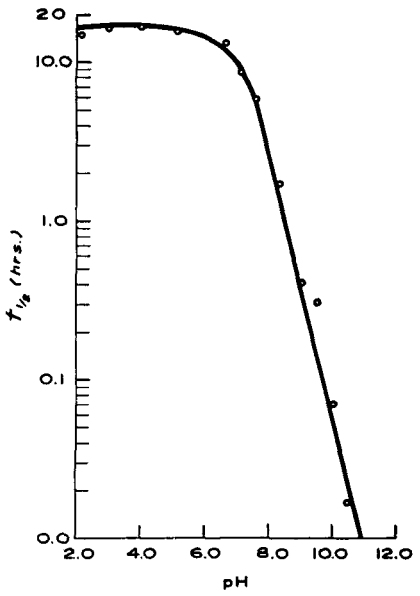

Fig. 1—Plot showing half-lives at various pH's for hydrolysis of 4-acetamidophenyl 2,2,2-trichloroethyl carbonate (ATC) in 0.02 M phosphate buffer at 37°.

not occur at a sufficient rate in the body to elicit a useful response from the acetaminophen and trichloroethanol that were formed unless the hydrolysis reaction was catalyzed by enzymes.

Hydrolysis of ATC Catalyzed by Enzymes from Human and Animal Materials—Since preliminary pharmacologic and biochemical studies, to be reported in another paper, indicated that ATC was an active compound with the predicted pharmacologic properties, knowledge pertaining to sites in the body where the presence of enzymes might induce cleavage of the compound took on added significance.

Thus, it became of interest to assess the hydrolytic effects of various body fluids and tissues, for example, gastrointestinal fluids, blood, and liver. These studies were concerned primarily with the enzymic cleavage rate of the compound relative to its cleavage rate in buffer. No attempts were made to elucidate the mechanism of the enzyme-induced cleavage of ATC, nor to identify the specific enzymes responsible for this cleavage.

All the reactions studied followed, or were assumed to follow, first-order kinetics; and all the data were plotted as log Δ absorbance *versus* time from which the half-lives were determined. Occasionally, it was either impossible or impractical to allow a reaction to go to completion in order to determine the terminal absorbance of the reaction mixture, and, occasionally, apparent terminal absorbance values observed experimentally produced curved semilog plots. In these cases, the terminal absorbances were adjusted to straighten the semilog plots. This approach was found to be as reliable and somewhat more convenient than the Guggenheim method of plotting first-order data (12).

Although enzyme reactions often are not simple first-order reactions, the half-lives determined in these experiments are indicative that the enzyme systems we studied have a definite catalytic effect on the hydrolysis of ATC and that enzyme systems obtained from human and animal sources vary widely in their ability to accelerate the hydrolysis reaction.

In cases of enzyme catalyzed reactions, it is often observed that the rate of decrease of substrate concentration, $-dS/dt$ varies with the substrate concentration [S] according to the Michaelis-Menten equation,

$$\frac{-dS}{dt} = \frac{V[S]}{K_S + [S]}$$

where V is the maximum velocity reached at infinite concentrations of substrate, and K_S is the dissociation constant of the enzyme-substrate complex. When $[S] \ll K_S$, the velocity of the reaction is proportional to the substrate concentration, *i.e.*, the reaction follows first-order kinetics. Thus, in enzyme-catalyzed hydrolysis reactions, pseudo first-order kinetics are often observed through a significant portion of the reaction.

In our studies of ATC hydrolysis using body fluids and tissues as enzyme sources, first-order kinetics were observed usually beyond 50% hydrolysis of the substrate. Thus, in these screening studies it was convenient to determine approximate first-order hydrolysis rate constants. Enzyme-catalyzed hydrolyses, therefore, are expressed as half-lives or in terms of the quotient

$$\frac{t_{1/2}\text{ (water)}}{t_{1/2}\text{ (biologic material)}}$$

at pH 7.4 and 37°.

Hydrolysis of ATC in Human Gastric Fluid—Half-life values in human gastric fluid were in excess of 5 hr. indicating that if there were enzymes here that could catalyze the hydrolysis reaction their effects were insignificant.

Hydrolysis of ATC in Human Intestinal Fluid (HIF)—Typical rates of hydrolysis of ATC in human intestinal fluid, diluted with 0.1 *M* phosphate buffer, pH 7.4, are summarized in Table I.

These results support the view that the fluids of the lumen of the intestine possess substances which have rather pronounced catalytic effects on the hydrolysis of ATC. For example, measured in 4% human intestinal fluid, the hydrolysis rate proceeds 18 times faster than in buffer alone. Though ATC might dissolve and be absorbed intact to some extent from the stomach, it appears that some hydrolysis must occur in the fluids of the intestinal lumen prior to absorption. Therefore, both ATC and its cleavage products could be absorbed from the small intestine.

Effect of Mucosal Scrapings from Rat Small Intestine on ATC Cleavage—Mucosal scrapings appear to be high in esterases that cleave ATC. When ATC

TABLE I—EFFECT OF HUMAN INTESTINAL FLUID ON CLEAVAGE RATES OF ATC AT 37°

Concentration of Human Intestinal Fluid (HIF) in 0.1 *M* Phosphate Buffer, pH 7.4, %	$t_{1/2}$, Min.	$t_{1/2}$ (buffer)/ $t_{1/2}$ (HIF in buffer)
0	420	1
1	98	4
2	46	9
3	33	13
4	23	18

was dissolved to a concentration of 30 mcg./ml. in 200 ml. of phosphate buffer containing the mucosal scrapings of the entire small intestine of a rat, it underwent hydrolysis with a half-life of 5.5 min. This is about 65 times the hydrolysis rate observed in buffer alone. These data support the hypothesis that a drug such as ATC might undergo considerable hydrolytic cleavage in the process of passing through the mucosal lining as it is being absorbed in the intestine.

Hydrolysis by Rat Liver—When ATC was dissolved to a concentration of 30 mcg./ml. in a 0.1% suspension of rat liver in phosphate buffer, it hydrolyzed with a half-life of 5 min. This is about 70 times as fast as the hydrolysis in buffer alone.

Hydrolysis in Plasma—A dilution of 1:50 human plasma in phosphate buffer caused a 30 mcg./ml. solution of ATC to cleave with a half-life of 20 min. This is about 18 times faster than the hydrolysis rate in buffer; on the other hand, it is a much slower rate than was observed in rat plasma of the same dilution. Figure 2, depicting hydrolysis in diluted human plasma, represents typical data observed in the catalyzed hydrolysis of ATC in the presence of body fluids and tissues.

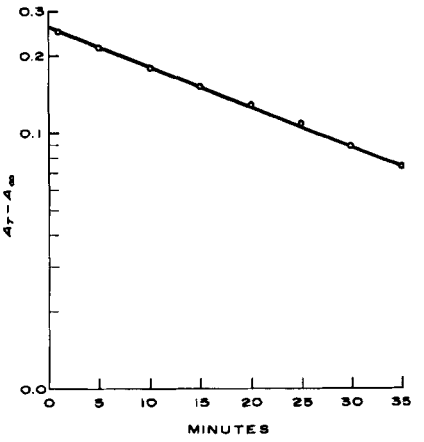

Fig. 2—First-order plot of the hydrolysis of 4-acetamidophenyl 2,2,2-trichloroethyl carbonate (ATC) in pH 7.4 phosphate buffer (0.1 M) containing 2% (v/v) human plasma at 37°. Absorbances were determined at 240 mμ. A_t, absorbance at time t; A_∞, absorbance when reaction is apparently complete; half-life, about 20 min.

CONCLUSIONS

These studies have shown the utility of the carbonate linkage in creating a prodrug from two well-known drugs. They show how the physical-chemical properties of both acetaminophen and trichloroethanol were modified substantially to produce a new compound with good lipid solubility but with low water solubility and a relatively slow dissolution rate. The compound is virtually tasteless apparently as a result of "locking" the hydrophilic hydroxy groups of the parent drugs and markedly decreasing the aqueous solubility of the parent drugs. The profile of new physical properties makes it possible to use ATC in certain dosage forms, such as tablets, which would have been impossible with a simple physical combination of the parent drugs.

A hydrolysis rate corresponding to a half-life of 15 hr. in water at pH 4–7 suggests that suspensions of ATC in water would not be stable for prolonged periods despite its very low aqueous solubility. On the other hand, the low water solubility, coupled with a dose that would be relatively large, suggest that suspensions might be prepared that would retain appreciable potency, at least for several weeks.

A hydrolysis rate at pH 2 corresponding to a $t_{1/2}$ of 15 hr. coupled with an intrinsic low water solubility and slow cleavage rate in human gastric fluids suggest that this compound, when administered orally, is absorbed from the stomach primarily in the form of ATC. The presence in human intestinal fluid and rat intestinal mucosa of enzymes which catalyze the cleavage of ATC suggest that absorption from the intestine of both ATC and its cleavage products could occur simultaneously. The fact that esterases are abundant in the plasma and liver would suggest that in animals and humans the ATC that was absorbed intact would be very short lived and might not even be detectable in the blood but rather, would be quickly converted to acetaminophen and trichloroethanol.

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Keyphrases

4-Acetamidophenyl 2,2,2-trichloroethyl carbonate (ATC)—synthesis
Prodrug—acetaminophen, trichloroethyl carbonate ester
Hydrolysis, ATC—pH, enzyme effect
Column chromatography—separation
UV spectrophotometry—analysis

Synthesis of D- and L-Thalidomide and Related Studies

By Y. FULMER SHEALY, CLYDE E. OPLIGER*, and JOHN A. MONTGOMERY

The optical isomers of thalidomide were synthesized by a route based on D- and L-isoglutamine. The thalidomide isomers were obtained in high yields by treating the optical isomers of N^2-phthaloylisoglutamine at 0° with thionyl chloride in dimethylformamide. L-Thalidomide was also obtained by using N,N'-carbonyldiimidazole to close the glutarimide ring. Application of these two methods, together with the active (cyanomethyl) ester method, to both N^2-phthaloyl-L-isoglutamine and N^2-phthaloyl-L-glutamine showed that L-thalidomide is formed more readily and with less risk of racemization from the isoglutamine derivative. DL-Thalidomide was also prepared under much milder conditions than those previously used.

THE DESIRABLE SEDATIVE, the undesirable neurotoxic, and the tragic teratogenic properties of thalidomide (IX) are well known. Summaries of these properties have been published (*e.g.*, 1–3), and numerous other publications of biological and biochemical studies have appeared. The synthesis of thalidomide consists, in essence, of the formation of two imide rings. In most of the recorded methods of synthesis, the final step was the formation of the glutarimide ring at temperatures of 150–250° (4–15). The glutarimide ring has also been formed before the introduction of the phthalimido group (16, 17). Prior to the development of *N*-ethoxycarbonylphthalimide for the phthaloylation of amino acids (18), the preparation of phthaloylglutamic acid derivatives was usually accompanied by some racemization (19, 20), although procedures have been devised to obviate or minimize racemization (*e.g.*, 19, 21). However, even when optically active glutamic acid or glutamine was employed for thalidomide synthesis, racemization apparently occurred, either during the phthaloylation with phthalic anhydride, or at the high temperatures used for glutarimide-ring formation. Syntheses of L-thalidomide (22) and of both D- and L-thalidomide (23) have been reported in preliminary communications. Several biological studies (24–29) that have included D- and L-thalidomide have been published without revealing the method of preparation of the optical isomers. In this report a full account of the studies resulting in a synthesis of D- and L-thalidomide is presented (23).

These investigations began with attempts to form the glutarimide ring from N^2-phthaloyl-L-glutamine (L-I) by using peptide-forming reagents. The preliminary investigations produced evidence of the formation of some thalidomide under relatively mild conditions and, also, of partial conversion of the amide group to a nitrile group by dehydrating agents. Dehydration of glutamine and asparagine derivatives to nitriles by certain peptide-forming reagents has been reported (30–35). The active-ester method was investigated by utilizing the cyanomethyl ester of L-I, since it is conveniently prepared. In the strongly basic medium of one equivalent of sodium methoxide in dimethyl sulfoxide (DMSO), the L-I cyanomethyl ester (L-II, $[\alpha]_D^{25}$ −46.6° in dimethylformamide [DMF]) was rapidly cyclized at room temperature to thalidomide in a yield of 70%. Cyclization in good yield also occurred, at a slower rate, with less than one equivalent of methoxide. The conditions employed were much milder than those previously reported; even so, DL-thalidomide (DL-IX) was the product of both experiments.

The preliminary evidence of difficulties in basing a synthesis of L-thalidomide on L-I was suggestive, rather than conclusive, because the application of peptide-forming reactions was not thoroughly investigated. During the course of these studies, Liberek presented evidence that phthaloyl derivatives (36) and cyanomethyl esters (37) of amino acids are especially susceptible to base-catalyzed racemization. Since our evidence and evidence from the literature suggested that dehydrating-type reagents might produce considerable nitrile from glutamine derivatives (30–35) and that base-catalyzed cyclizations might cause racemization, these studies were subsequently based on isoglutamine for the following reasons. Activation of the carboxyl group of glutamine derivatives increases the acidity of the C—H bond of the adjacent asymmetric carbon atom and, thereby, enhances the tendency to racemize. In isoglutamine

Received October 9, 1967, from Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, AL 35205
Accepted for publication December 21, 1967.
This work was supported by the C. F. Kettering Foundation and by Contract PH43-64-51 from the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
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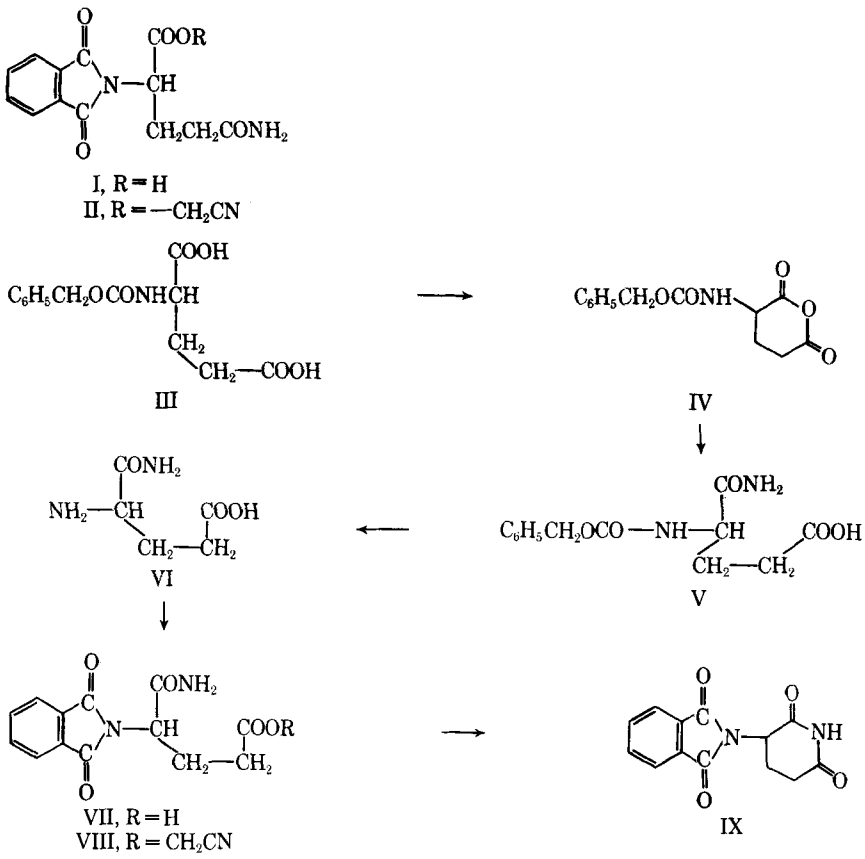

Scheme I

(VI) derivatives, however, the carboxyl group to be activated for ring-closure is separated from the asymmetric carbon atom by two methylene groups. In addition, there is evidence from peptide chemistry (*e.g.*, 38–40) of rather facile imide formation from α-glutamyl peptides.[1]

The synthetic route to D-and L-thalidomide is outlined in Scheme I, and details, including some refinements of procedures reported in the literature for intermediates (III–VI), are described in *Experimental.* The method of Nefkens *et al.* (18), which introduces the phthaloyl group without racemization, was used to prepare the phthaloyl derivatives (I, VII) of L-glutamine (already mentioned), L-isoglutamine, and D-isoglutamine. After work on the synthesis of these compounds had been completed, Schröder and Klieger (41) reported the synthesis of L-I and L-VII by this method, although the optical rotation of L-VII was not recorded. The formation of the glutarimide ring was effected readily by thionyl chloride in DMF at 0° or, over a longer period of time, by *N,N'*-carbonyldiimidazole (42). The thionyl chloride-DMF method afforded L- and D-thalidomide (L-IX and D-IX), with optical rotations comparable to those of subsequently purified specimens, in yields of 75–85%. The optical rotations[2] of L- and D-thalidomide recrystallized from DMF–water were −64.6° and +64°, respectively, in dry DMF at 25°. The optical isomers and commercial DL-thalidomide moved identically on thin-layer chromatograms and gave identical nuclear magnetic resonance spectra in DMSO-d_6. The infrared spectra, determined by the potassium bromide disk method, of D- and L-thalidomide were identical, as expected, and were also identical with a published spectrum (43) of DL-thalidomide, except for minor differences ascribable to the fact that the spectra were determined in the solid state.

Tipson (20) had found that the phthalimide ring of phthaloylglutamic acid is readily opened by dilute aqueous base, and Williams (45) reported that DL-thalidomide undergoes hydrolysis in the neutral pH range to at least 12 compounds.[3] These findings led us to design our

[1] Battersby and Reynolds (40) reported the failure of a γ-glutamyl (glutamine-type) peptide to cyclize under conditions that gave a low yield of a glutarimide from an α-glutamyl (isoglutamine-type) peptide.

[2] Values reported in the literature for L-thalidomide are $[\alpha]_D^{20}$ −62° in dioxane (22) and $[\alpha]_D^{20}$ −45.7° in DMF (26) and for D-thalidomide, $[\alpha]_D^{20}$ +53.2° in DMF (26). A very recent publication (44) on some biological properties of L- and D-thalidomide gave $[\alpha]_D^{20}$ −58° and +60°, respectively, in DMF.

[3] A complete description of the studies of Williams and coworkers on hydrolysis has subsequently been published (26). Other reports of facile hydrolysis have also been published (*e.g.*, 46, 47).

preparative and isolation procedures with the aim of preventing, or minimizing insofar as possible, hydrolysis of the imide rings, as well as racemization. In addition, after the thalidomide isomers had been obtained, we attempted to determine the stability of L-thalidomide and its immediate precursor (L-VII) in DMF and aqueous DMF. Both compounds were in DMF solution (containing trace amounts of water) during the preparation of L-thalidomide and during the determination of their optical rotations, and L-thalidomide was exposed to aqueous DMF during isolation and recrystallization. Optical rotation data indicated that L-thalidomide suffered little or no change in dry DMF or in 80% DMF during observation periods of 18 and 43 hr., respectively, and that L-VII was stable in both of these solutions for at least 24 hr. However, a specimen of L-thalidomide that had not been recrystallized (after isolation from a thionyl chloride–DMF reaction) exhibited a decrease in specific rotation in both dry DMF and in 80% DMF; the decrease was more rapid in the latter solution. Subsequently, at least one recrystallization from DMF–water was performed on the day of the preparation of L- or D-IX. As a further precaution, the preparation of L-VII at a pH lower than that of the customary Nefkens phthaloylation was performed, but there appeared to be no advantage in this modification.

In the course of our studies, comparison of L-glutamine and L-isoglutamine derivatives as precursors of thalidomide was made. L-I and L-VII were treated with thionyl chloride in DMF in experiments identical in procedure and isolation methods. One pair of reactions was allowed to proceed for 0.5 hr.; another pair, for 2 hr. L-VII gave L-thalidomide in yields of 75–80%; L-I gave a low yield (9–10% calculated as thalidomide) of crude material that contained sulfur, had an optical rotation greater in magnitude than that of L-thalidomide, and produced 2–3 spots (one of which was thalidomide) on thin-layer chromatograms. From the filtrate of the 2-hr. reaction of L-I, a yield of 11% of crude thalidomide was obtained. Although L-I gave a small amount of thalidomide of unknown optical purity, L-VII obviously gave vastly superior results in this procedure.

In another pair of identical experiments, reactions of L-I and of L-VII with *N,N'*-carbonyldiimidazole gave a 41% yield of L-thalidomide ($[\alpha]_D^{25}$ −58°) from L-VII and a 30% yield of DL-thalidomide from L-I.

Cyclization of the cyanomethyl ester (L-VIII) of phthaloyl-L-isoglutamine with sodium methoxide in DMSO in experiments identical with those employed for the L-glutamine derivative (L-II) likewise gave DL-thalidomide. However, during the preparation of L-VIII in the presence of triethylamine, optically active thalidomide, as well as L-VIII, was obtained. This observation alone did not necessarily demonstrate a greater propensity of L-VIII to cyclize, since the reaction time required to prepare L-VIII was greater than that used to prepare L-II. However, it did show that L-VIII would cyclize under less basic conditions than those of the sodium methoxide–DMSO reagent. The product isolated after treating L-VIII with triethylamine in DMSO was partially racemized L-thalidomide ($[\alpha]_D^{25}$ −45°), whereas L-II in an identical experiment was racemized but failed to cyclize. Thus, isoglutamine derivatives were cyclized much more readily by the three methods investigated and were much less susceptible to racemization than the corresponding glutamine derivatives.

EXPERIMENTAL[4]

Infrared spectra were recorded with a Perkin-Elmer model 521 spectrophotometer using samples in potassium bromide disks. Melting points were determined on a Kofler Heizbank melting point apparatus unless otherwise noted. TLC was performed by the ascending method on layers of Silica Gel H[5] of standard 0.25 mm. thickness supported on glass plates. Crude and purified specimens of the thalidomides were applied to the adsorbent in DMF, although D- and L-thalidomide could also be applied in ethyl acetate. Generally, 20- and 80-mcg. portions of each sample were spotted, and commercial DL-thalidomide and the starting material were used as reference compounds. At 80 mcg. the thalidomide spots were usually convex. Spots were detected with an ultraviolet lamp, which emits principally at 254 mμ, after the developed chromatograms had been sprayed with Ultraphor WT.[6] A spray of basic potassium permanganate was sometimes also used to detect nonabsorbing impurities. Unless otherwise indicated, either 95:5 (v/v) chloroform–methanol or 85:15 (v/v) benzene–methanol was used to develop the chromatograms. In these solvent systems L- and D-VII remain at the origin, but they migrate in 1:1 chloroform–methanol.

N^2-Benzyloxycarbonyl-L-isoglutamine (L-V)—*N*-Benzyloxycarbonyl-L-glutamic acid anhydride (L-IV) was prepared by the method of Bergmann and Zervas (48) and recrystallized from chloroform–ether (49): yields, 80–83%; m.p. 94–95°; $[\alpha]_D^{25}$ −43 to −45.0° (c = 10.0 in glacial acetic acid).

[4] The authors are indebted to Drs. W. J. Barrett, W. C Coburn, Jr., and P. D. Sternglanz of the Analytical and Physical Chemistry Division of this Institute for spectral determinations and elemental analyses; to Mrs. Frances Dean for optical rotations; and to Misses Kathleen Hewson and Imogene Baswell for thin-layer chromatographic determinations.

[5] Brinkmann Instruments Inc., Westbury, N. Y.

[6] An optical whitening agent by BASF Colors and Chemicals, Inc., Charlotte, N. C.

Lit. values $[\alpha]_D$, m.p.: −44.1°, 94° (48); −43°, 91–92° (49); −45.1° (50). Although the melting point and optical rotation of a batch of commercial *N*-benzyloxycarbonyl-L-glutamic acid (L-III) were in agreement with values reported in the literature, partially racemized anhydride was obtained unless the commercial material was purified as follows. A solution of 98 Gm. of commercial *N*-benzyloxycarbonyl-L-glutamic acid in 600 ml. of ethyl acetate was washed twice with 100-ml. portions and 4 times with 50-ml. portions of water, dried with magnesium sulfate, and diluted with 2.5 L. of petroleum ether. The white precipitate was washed twice on a filter with 500-ml. portions of petroleum ether: yield, 83.5 Gm.; m.p. 120–122°; $[\alpha]_D^{25}$ −7.8° (c = 3.00 in glacial acetic acid).

The procedure of Bergmann and Zervas (48) for the preparation of L-V was modified. Anhydrous ammonia was introduced during approximately 30 min. into a stirred solution (protected from atmospheric moisture with a tube of calcium sulfate) of 84 Gm. of the anhydride (L-IV) in 1 L. of freshly distilled chloroform, 500 ml. of anhydrous ether, and 150 ml. of dry, purified dioxane at 0°. The mixture was stirred at 0° for an additional 75 min., the solvents were removed *in vacuo* at 30–40°, the residue was dissolved in 700 ml. of water, and the aqueous solution was acidified to pH 3.4 at 0°. The precipitated product was washed four times with 300-ml. portions of water and recrystallized from 1.8 L. of water: yield, 54.6 Gm. (61%); m.p. 174–175°; $[\alpha]_D^{25}$ −7.1° (c = 2.00 in methanol). Lit. m.p. 175° (48), 174.5–175° (51); $[\alpha]_D^{19}$ −6.0° (methanol) (51).

N^2-Benzyloxycarbonyl-D-isoglutamine (D-V)—*N*-Benzyloxycarbonyl-D-glutamic acid (D-III: m.p. 122°; $[\alpha]_D^{25}$ +7.7°, c = 10.0 in glacial acetic acid) was prepared by the method of Klieger and Gibian (52) and purified by the procedure used for the L-isomer. It was converted *via* *N*-benzyloxycarbonyl-D-glutamic anhydride (52) (D-IV: 84% yield; m.p. 92–93°; $[\alpha]_D^{25}$ +42.6°, c = 10.0 in glacial acetic acid) to D-V (56% yield; m.p. 174°; $[\alpha]_D^{25}$ +6.1°, c = 1.96 in methanol) by the procedures used in the preparation of the L-isomer. Klieger and Gibian report specific rotations of +7.6° and +43.1° for D-III and D-IV, respectively.

D-Isoglutamine (D-VI)—A solution of 8.0 Gm. of D-V, 680 ml. of 50% aqueous methanol, and 2 ml. of acetic acid was prepared and was treated with hydrogen in the presence of 5% palladium-charcoal catalyst (2 Gm.) at atmospheric pressure with stirring for 2 hr. (Slight warming may be required to prepare the 50% methanol solution of D-V, and rewarming to 35–40° during the early part of the hydrogenation may be necessary if some precipitation occurs.) The system was flushed with fresh hydrogen after 15- and 75-min. intervals. The filtrate from the catalyst was evaporated to dryness *in vacuo* at 25–30°, and the residue was dissolved in 25 ml. of water and reprecipitated with 250 ml. of acetone. The white precipitate was collected by filtration from the chilled mixture, washed with acetone, and dried *in vacuo* over phosphorus pentoxide at room temperature: yield, 3.78 Gm. (90%); m.p. 188°; $[\alpha]_D^{22}$ −20.6° (c = 5.39 in water). Recrystallization of 2.1 Gm. of crude product from water (20 ml.) and acetone (120 ml.) gave 1.95 Gm. (93% recovery); m.p. 188°; $[\alpha]_D^{22}$ −21.3° (c = 5.41 in water).

Anal.—Calcd. for $C_5H_{10}N_2O_3$: C, 41.09; H, 6.90; N, 19.17. Found: C, 41.17; H, 6.94; N, 19.03.

L-Isoglutamine (L-VI) was prepared from L-V by the procedure used for D-VI: yield, 89%; m.p. 187° (Heizbank), 178–179° (capillary); $[\alpha]_D^{22}$ +20.6°(c = 5.4 in water). Lit. m.p. 186–186.5° (51); $[\alpha]_D^{22}$ + 21.1° (48), + 21.2° (51).

N^2-Phthaloyl-L-isoglutamine (L-VII)—To a cold (0–5°) solution consisting of 6.9 Gm. (47 mmoles) of L-isoglutamine (L-VI), 7.0 Gm. (56.5 mmoles) of sodium carbonate monohydrate, and 100 ml. of water was added 14.6 Gm. (66.6 mmoles) of *N*-ethoxycarbonylphthalimide (18), and the resulting suspension was stirred vigorously for 10 min. at 0–5°. The initial pH was approximately 9. The ice bath was removed, and the mixture was stirred for 50 min. as it warmed to room temperature. The unreacted *N*-ethoxycarbonylphthalimide was removed by filtration, the filtrate was acidified at 0° to pH 4 with concentrated hydrochloric acid, and the solution was allowed to stand (for about 10 min.) until a white precipitate began to form. The mixture was then acidified further to pH 2, stirred at 0–5° for 1 hr., and filtered. The precipitate was washed with three 20-ml. portions of water at 0–5° and dried *in vacuo* over phosphorus pentoxide at room temperature. A solution of the product (m.p. 150–151°) in 125 ml. of hot ethanol was diluted with 100 ml. of petroleum ether (b.p. 30–60°), cooled slowly, and stored at 0–5° for 2 hr. The recrystallized product was collected by filtration, washed with petroleum ether, and dried as before: yield, 8.3 Gm. (63%); m.p. 151–152° and 158–160°; $[\alpha]_D^{25}$ −27±0.5°(c = 2 in DMF).

Anal.—Calcd. for $C_{13}H_{12}N_2O_5$: C, 56.52; H, 4.38; N, 10.14. Found: C, 56.46; H, 4.42; N, 10.11.

In view of the facile formation of thalidomide from L-VII by methods described below, the product of this reaction was examined by TLC (95:5 chloroform–methanol). At the high spotting level of 500 mcg. a trace amount of thalidomide was detected in the crude product, but not in the recrystallized sample. Schröder and Klieger (41) reported a melting point of 122–124° and did not give $[\alpha]_D$. We have sometimes observed melting of apparently pure samples in this temperature range or lower. For example, one specimen melted at 110–120°, resolidified, and remelted at 150°; the analytical data (C, 56.41; H, 4.40; N, 10.24) and rotation (−27.2°) were satisfactory. Most specimens would melt at 150–152° (Heizbank or capillary) and, when induced to resolidify, would remelt at 158–161°.

The procedure described above was similar to that used by Nefkens, Tesser, and Nivard (18) to prepare phthaloyl-L-glutamic acid. The studies of Williams (45) on the stability of DL-thalidomide and those of Tipson (20) on phthaloyl-L-glutamic acid suggested that reopening of the phthalimide ring might occur in some degree in the basic solution. Therefore, a similar experiment was performed in a less basic solution in which the initial, millimolar proportions of L-isoglutamine, *N*-ethoxycarbonylphthalimide, and sodium carbonate monohydrate were 10:13.8:1.58 in 25 ml. of water. The pH of the mixture at the beginning was 7.4, and solid sodium carbonate monohydrate (6.22 mmoles) was added in portions

so as to maintain the pH at 7.3–7.7. The reaction time after the removal of the mixture from the ice bath was 70 min. This modified procedure did not give improved results; the product, isolated as before, was obtained in 56% yield; m.p 150°; $[\alpha]_D^{25}$ −27.2°(c = 2 in DMF).

N^2-Phthaloyl-D-isoglutamine (D-VII) was prepared by the first procedure described above: yield, 64%; m.p. 151°; $[\alpha]_D^{25}$ + 26.5 ± 0.5° (c = 2.0 in DMF). Found: C, 56.79; H, 4.09; N, 10.06. The infrared spectrum was identical with that of the L-isomer.

L-Thalidomide (L-N-(2,6-Dioxo-3-piperidyl)phthalimide) (L-IX)—A solution of 2.00 Gm. (7.25 mmoles) of phthaloyl-L-isoglutamine (L-VII) in 50 ml. of dry DMF was cooled to −5 to 0° in an ice-salt bath. To the cold solution, which was under a current of nitrogen and was protected from moisture with a tube of calcium sulfate, 0.65 ml. (9 mmoles) of thionyl chloride was added dropwise with vigorous stirring during 4 min.[7] The solution was then stirred at −5 to 0° for 1 hr. and poured slowly (4 min.) into a mechanically stirred mixture of 1.78 Gm. (18.1 mmoles) of potassium acetate, 150 ml. of ice-cold water, and 75 Gm. of crushed ice. The white precipitate was collected after 30 min. of stirring, washed with three 10-ml. portions of cold water, and dried *in vacuo* over phosphorus pentoxide at room temperature: yield, 1.63 Gm. (87%); $[\alpha]_D^{25}$ −65.5°(c = 2.00 in dry DMF). Values of $[\alpha]_D^{25}$ (c = 2 in dry DMF) of the products of several experiments in which the reaction times were 0.5–2 hr. were 64 ± 1.5°; melting temperatures were similar to those of purified specimens.

Specimens of L-thalidomide were purified by reprecipitation from 1:4 (v/v) DMF–water[8] as follows. A solution of a specimen of L-thalidomide (55–65 mg./ml.) in dry DMF was filtered and chilled to −5 to 0°. Cold water (4 volumes) was introduced dropwise with vigorous mechanical stirring at a rate (20–40 min.) that kept the temperature at 0 ± 4°. The mixture was stirred at 0° for an additional 0.5 hr.; and the precipitate was collected on a filter, washed with cold water, and dried *in vacuo* over phosphorus pentoxide at room temperature. The recovery after each reprecipitation was 90–94%. The product obtained from the reaction described above was recrystallized three times from DMF–water, the first recrystallization being performed on the same day that the reaction was performed. The optical rotation was essentially unchanged after the first reprecipitation; $[\alpha]_D^{25}$ −64.6 ± 0.5° (c = 2.00 in DMF).

Anal.—Calcd. for $C_{13}H_{10}N_2O_4$: C, 60.46; H, 3.91; N, 10.85. Found: C, 60.63; H, 3.73; N, 10.94.

D-, L-, and DL-Thalidomide (applied in DMF) moved identically and near the front on thin-layer plates of silica gel developed in 95:5 chloroform–methanol or in 85:15 benzene–methanol. DMSO-d_6 solutions (prepared without heating) of D-, L-, and DL-thalidomide gave identical NMR spectra. The melting point observed for L-thalidomide depended on the techniques used in the determination, the variability being due evidently to thermal racemization. Pure specimens melted at 244–245° when sprinkled along the gradiently heated bar of a Kofler Heizbank apparatus. Specimens that melted in this range would resolidify and remelt at a higher temperature, usually near the melting point of DL-thalidomide. Melting in the region of 240–250° could be observed in a capillary tube, provided that the specimen was inserted near its melting point.

Reference infrared spectra recorded for specimens obtained by recrystallizing commercial DL-thalidomide from dioxane, from 1:4 DMF–water by the procedure described for the optical isomers, or from glacial acetic acid displayed minor differences. The spectra of an untreated commercial sample, the specimen from dioxane, and several specimens prepared by the cyanomethyl ester method (below) were all identical with a subsequently published spectrum (43). The spectra of L- and D-thalidomide were identical with the published spectrum of DL-thalidomide except that a broad, weak band near 860–850 cm.$^{-1}$ in the spectrum of DL-IX was replaced by a weak, poorly resolved doublet near 850 and 830 cm.$^{-1}$ in the spectra of the optical isomers. The spectra of specimens of DL-IX (either commercial or prepared from L-II or L-VIII) that had been recrystallized from DMF–water (by the procedure described for the optical isomers) differed somewhat more from the spectra of the optical isomers, although the spectra were very similar. The principal differences have already been listed (23); the difference in the 860–800 cm.$^{-1}$ region was more pronounced since the band near 850 cm.$^{-1}$ was considerably weaker in the spectrum of DL-IX recrystallized in this way. Differences between the spectra of D- or L-IX and DL-IX are undoubtedly due to the fact that all spectra were determined in the solid state.

D-Thalidomide (D-IX) was prepared by the thionyl chloride–DMF procedure described for the L-isomer: yield of crude product, 85%; m.p. (Kofler Heizbank), 243°; $[\alpha]_D^{25}$ + 63.2° (c = 2.00 in DMF). The D-isomer was reprecipitated from DMF–water by the procedure described for the L-isomer: m.p. 244–245° (variable as for the L-isomer); $[\alpha]_D^{25}$ + 64 ± 0.5° (c = 2.00 in dry DMF). The infrared spectrum was identical with that of L-IX.

Anal.—Found: C, 60.52; H, 3.68; N, 10.76.

Optical Rotatory Stability of L-Thalidomide and L-VII—Since it was considered possible that trace amounts of water in DMF solutions of thalidomide and L-VII might effect some hydrolysis, observations of the specific rotation over a period of time were first made with solutions prepared from DMF containing small amounts of water. The amount of water was determined by vapor-phase chromatography. A solution of a recrystallized specimen of L-thalidomide in DMF, containing less than 0.1% water, was allowed to stand for 18 hr. The initial (−63.4°, determined within 20 min. of the addition of DMF) and the final (−63.2°) rotations (c = 2.0) were in agreement within experimental error. Addition of sufficient water to form an 80% (v/v) DMF solution caused the rotation to drop to −61.4°. This change was presumably due to the change of solvents (DMF to 80% DMF) since values of $[\alpha]_D^{25}$ were in the range of 60.9 ± 0.5° during the next 43 hr., at which time the experiment was dis-

[7] Another experiment was identical except that the thionyl chloride was first dissolved in a small portion of DMF, and this solution was then added similarly. Both the yield (20%) and the optical rotation (−60°) were lower.

[8] A decrease in optical rotation was observed after a crude specimen of L-thalidomide had been recrystallized from acetic acid. Dioxane gave a low recovery when used to recrystallize DL-thalidomide.

continued. These data indicate little, if any, degradation. In contrast, a specimen of L-thalidomide obtained directly from a thionyl chloride–DMF procedure showed a decrease in $[\alpha]_D^{25}$ (c = 2) in dry DMF (less than 0.01% water, distilled from calcium hydride and dried further over molecular sieves) from an initial value of −63.5° (determined 5–10 min. after the addition of DMF) to the following values (hr. after initial determination in parentheses): −60.1°(7), −52.8°(24), −47.6°(96), −46.7°(103), −46.4°(119). Values of $[\alpha]_D^{25}$ (c = 1.6) for a solution of the same specimen of L-thalidomide in 80% DMF decreased from an initial value of −61.7° as follows (hr. in parentheses): −58.8°(1), −56.8°(2), −54.7°(3), −52.6(4), −51.3°(6), −43.3°(24), −42.4°(29.5), −39.5°(94), −39.4(118). The infrared spectra of the residues recovered (by evaporation of the solvents *in vacuo*) from the last two solutions after approximately 5 days were intermediate between that of L-thalidomide and that of DL-thalidomide recrystallized from DMF–water.

The specific rotations of a specimen of N^2-phthaloyl-L-isoglutamine (L-VII) in dry DMF (less than 0.01% water) and in 80% (v/v) DMF were within the range of −26.5 ± 0.2° (c = 2.0) and 26.8 ± 0.2° (c = 1.6), respectively, during a 24-hr. observation period.

N^2-Phthaloyl-L-glutamine Cyanomethyl Ester (L-II)—Chloroacetonitrile (1.63 ml.) was added to a mixture (protected from atmospheric moisture with a tube of calcium sulfate) consisting of 3.0 Gm. of phthaloyl-L-glutamine (L-I) (18, 39), 1.6 ml. of anhydrous triethylamine, and 10 ml. of purified, anhydrous dioxane. The reaction mixture was kept at 60°, with stirring, for 1 hr. The solid phase dissolved, and a new solid later precipitated. The reaction mixture was chilled in an ice bath; and the precipitate was separated by filtration, washed with several portions of water, and dried *in vacuo* over phosphorus pentoxide at room temperature: yield, 1.5 Gm. (44%); m.p. 189–190°; $[\alpha]_D^{25}$ −46.4° (c = 2.01 in DMF). A specimen was recrystallized twice from dioxane: m.p. 189–190°; $[\alpha]_D^{25}$ −46.6° (c = 1.98 in DMF).

Anal.—Calcd. for $C_{15}H_{13}N_3O_5$: C, 57.14; H, 4.16; N, 13.33. Found: C, 57.38; H, 4.00; N, 13.12.

N^2-Phthaloyl-L-isoglutamine Cyanomethyl Ester (L-VIII) and Thalidomide from Phthaloyl-L-isoglutamine (L-VII)—A solution of 4.0 Gm. of L-VII 3.2 ml. of triethylamine, 3.2 ml. of chloroacetonitrile, and 20 ml. of purified, anhydrous dioxane was stirred at 60–65° for 4 hr. Examination of aliquots from an earlier experiment by TLC had shown that a considerable quantity of L-VII remained after 1.25 hr. The mixture, containing a precipitate, was poured into 150 ml. of ether; the new mixture was stirred at 0° for 30 min.; the ether was decanted; the gummy residue was treated again with ether (50 ml.), which was also decanted; and the flocculent residue was stirred at 0° with 150 ml. of water. After the solid had been separated by filtration, washed with water, and dried *in vacuo* at room temperature over phosphorus pentoxide, it weighed 3.2 Gm.; m.p. 163° to a cloudy liquid; $[\alpha]_D^{25}$ −35° (c = 2.0 in DMF). TLC (85:15 benzene–methanol) indicated that this fraction was a mixture of L-VIII and thalidomide; the latter presumably was formed from L-VIII.

Evaporating the solvent from the ether decantates and stirring the residue with cold water gave 0.4 Gm. of solid (m.p. 238–244°, $[\alpha]_D^{25}$ −56° in DMF) that was shown by TLC (1 spot) and infrared spectroscopy to be thalidomide.

Recrystallization of the first fraction (3.2 Gm.) from dioxane–petroleum ether gave a first crop of 1.9 Gm. (m.p. 170°, $[\alpha]_D^{25}$ −26.8°) and a second crop, by further dilution with petroleum ether, of 701 mg. of impure thalidomide (m.p. 230–250°, $[\alpha]_D^{25}$ −42.6°), which was identified by TLC. Recrystallization of the first crop (1.9 Gm.) from dioxane–petroleum ether gave 1.5 Gm. of L-VIII: m.p. 173–174°; $[\alpha]_D^{25}$ −25.3° (c = 2.00 in dry DMF); TLC (85:15 benzene–methanol), 1 spot.

Anal.—Calcd. for $C_{15}H_{13}N_3O_5$: C, 57.14; H, 4.16; N, 13.33. Found: C, 57.22; H, 4.31; N, 13.25.

DL-Thalidomide from the Cyanomethyl Esters (L-II and L-VIII)—A solution of 368 mg. (1.17 mmoles) of phthaloyl-L-glutamine cyanomethyl ester (L-II) in 5 ml. of dry dimethyl sulfoxide was added dropwise during 10 min. to a stirred mixture of 63 mg. (1.17 mmoles) of sodium methoxide and 10 ml. of dry dimethyl sulfoxide at 20°. The dimethyl sulfoxide had been distilled from calcium hydride, and the reaction mixture was protected from atmospheric moisture by a stream of nitrogen and a calcium sulfate drying tube. After the addition had been completed, 0.1 ml. of glacial acetic acid was added to neutralize the base and the solution was concentrated *in vacuo* at room temperature to a paste. Addition of 20 ml. of water produced a solid that was collected by filtration, washed with water, and dried *in vacuo* at room temperature: yield, 212 mg. (70%); m.p. 269–273° (cap.); $[\alpha]_D^{25}$ 0.0°(c = 2.06 in dioxane).

In another experiment the amount of sodium methoxide was reduced to 13% on a molar basis, and the appearance of thalidomide and the disappearance of the cyanomethyl ester (L-II) were monitored by TLC (9:1 chloroform–methanol). The disappearance of L-II was essentially complete within 3 hr.; and thalidomide, isolated by the procedure described above, was obtained in 80% yield. It also was racemic.

Treatment of phthaloyl-L-isoglutamine cyanomethyl ester (L-VIII) with sodium methoxide in dimethyl sulfoxide likewise caused racemization. DL-Thalidomide was isolated in 78% yield from an experiment, analogous to the first experiment described above, in which L-VIII was added to an equimolar amount of sodium methoxide during 10 min. From an experiment in which L-VIII was treated with 16 mole percent of sodium methoxide for 2.5 hr. by the second procedure described for L-II, DL-thalidomide was also obtained in 78% yield.[9] Isolation of thalidomide from both of these experiments was effected by pouring the reaction solutions into a mixture of crushed ice and water. All samples of DL-thalidomide obtained from L-II and L-VIII were identified by their melting points and infrared spectra (compared with commercial material), by TLC, and by the absence of optical rotation in DMF or dioxane.

[9] The reaction time employed in this experiment was similar to that used for L-II. Since L-VIII cyclizes more readily, a much shorter reaction time might be adequate and might afford optically active thalidomide.

Comparison of L-I and L-VII as Precursors of L-Thalidomide—*Thionyl Chloride–DMF Method*—In identical experiments carried out simultaneously, phthaloyl-L-glutamine (L-I) and phthaloyl-L-isoglutamine (L-VII) were treated with thionyl chloride in DMF. The procedures were identical with that described above for the preparation of L-thalidomide except that the reactions were allowed to proceed for 2 hr. From L-VII, L-thalidomide was obtained in 76% yield; $[\alpha]_D^{25}$ −64.5° (c = 2.00 in DMF). From L-I, only a small quantity of a pink solid was obtained (by exactly the same isolation procedure) $[\alpha]_D^{25}$ −83° (c = 1.0 in DMF). This material contained sulfur (5.9%), and TLC revealed the presence of 2–3 components, one of which was thalidomide. The amount (150 mg. from 1.600 Gm. of L-I) corresponded to a yield of 10%, calculated as thalidomide. A second crop amounted to 12 mg., and finally a third portion of 173 mg. (11.5%) of material shown by its infrared spectrum and by TLC (2 spots) to be impure thalidomide was isolated from the filtrate residue.

Another pair of identical experiments in which the reaction time was 0.5 hr. gave similar results in that L-VII gave L-thalidomide (80% yield, $[\alpha]_D^{25}$ −64.2°, c = 2.00 in DMF), whereas L-I afforded only a small amount (9% calculated as thalidomide) of material that was similar [judged by its infrared spectrum, optical rotation (−88°), and thin-layer chromatogram] to the crude product of the 2-hr. reaction.

N,N′-Carbonyldiimidazole Method—To a solution of 1.265 Gm. of L-VII in 40 ml. of dry DMF (distilled from calcium hydride) was added 0.810 Gm. (91% pure by gas chromatographic determination of liberated carbon dioxide) of *N,N′*-carbonyldiimidazole (40). The reaction solution (protected from moisture by calcium sulfate) was stirred in an atmosphere of nitrogen for 96 hr. at room temperature. Glacial acetic acid (0.34 ml.) was added to the solution, the mixture was concentrated *in vacuo* at room temperature to a viscous oil, and the mixture resulting from the addition of 20 ml. of water was stirred at 0° for 30 min. A white precipitate was removed by filtration, washed with water, and dried *in vacuo* at room temperature: yield, 491 mg. (41%); $[\alpha]_D^{25}$ −57.9° (c = 1.99, DMF). Two reprecipitations from DMF–water (1:4) effected a modest increase in the specific rotation; $[\alpha]_D^{25}$ −59° (c = 2 in DMF).

An identical experiment with phthaloyl-L-glutamine (L-I) afforded DL-thalidomide in 30% yield. The infrared spectrum was identical with that of a commercial specimen. Recrystallization of the DL-thalidomide obtained from L-I from DMF–water (1:4) gave a specimen, m.p. 274–276° (cap.) with an infrared spectrum identical with that of the commercial specimen after it had been recrystallized in the same way; m.p. 275–276° (cap.).

Cyanomethyl Ester Method—As shown above, the cyanomethyl esters of both phthaloyl-L-glutamine and phthaloyl-L-isoglutamine were converted to DL-thalidomide by sodium methoxide in DMSO. In view of the fact that optically active thalidomide was isolated during the preparation of L-VIII, a weaker base was tried. A solution (under a nitrogen atmosphere and protected from moisture with a tube of calcium sulfate) of 315 mg. (1 mmole) of L-VIII, 16.5 ml. of dry DMSO, and 3.5 ml. of a solution containing 0.04 ml. of triethylamine per ml. of DMSO (1 mmole of triethylamine) was stirred at 34–37° for 72 hr. The formation of thalidomide was monitored by TLC (85:15 benzene–methanol). The solution was neutralized with glacial acetic acid and poured into a mixture of 40 Gm. of water and 40 Gm. of crushed ice. After 30 min. of stirring, the product was collected on a filter, washed with water, and dried *in vacuo* at room temperature: yield, 189 mg. (73%, not corrected for the removal of aliquots for TLC); $[\alpha]_D^{25}$ −45.3° (c = 2.02, DMF); TLC showed only thalidomide. The infrared spectrum was identical with that of L-thalidomide except that the doublet at 850 and 830 cm.$^{-1}$ in the L-thalidomide spectrum was replaced by a band at 850 cm.$^{-1}$ with a shoulder at 830 cm.$^{-1}$.

An identical experiment with the glutamine derivative (L-II) caused racemization without appreciable cyclization. Phthaloyl-DL-glutamine cyanomethyl ester was isolated by pouring the reaction solution into 40 ml. of a mixture of water and crushed ice: yield, 263 mg. (84%); m.p. 176–177°; $[\alpha]_D^{25}$ 0.0° (c = 2.02, DMF). This material and the starting material (L-isomer) traveled together on thin-layer plates (9:1 chloroform–methanol) and gave very similar infrared spectra.

Anal.—Calcd. for $C_{15}H_{13}N_3O_5$: C, 57.14; H, 4.16; N, 13.33. Found: C, 56.93; H, 3.95; N, 13.22.

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Keyphrases

Thalidomide, D and L—synthesis
TLC separation
Optical rotation—identity
IR spectrophotometry—structure

Study of Absorption, Translocation, and Residue Properties of 2,3,5-Triiodobenzoic Acid in Field-Grown Soybeans

By L. A. SPITZNAGLE, J. E. CHRISTIAN, A. J. OHLROGGE, and C. E. BRECKINRIDGE, JR.*

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INCREASED KNOWLEDGE of plant metabolism has led to a search for chemicals which stimulate or inhibit the growth of plants. One such chemical is 2,3,5-triiodobenzoic acid, commonly referred to as TIBA. TIBA has been shown by Galston (1) to decrease apical dominance and to affect the flowering of soybean plants, causing an increased number of flowers. Anderson (2) reported that the result of several combined effects is an increased yield of soybeans per acre, making TIBA a useful agricultural chemical. However, since soybean products are used in animal feeds and for human consumption, the residue and metabolism properties of TIBA must be determined prior to general usage.

Wheeler and Johns (3) reported the first synthesis of TIBA by reacting anthranilic acid with iodine monochloride. The product was diazotized, and allowed to react with potassium iodide yielding TIBA. Olivier and Combe (4) were able to obtain 95% of the theoretical yield of TIBA by making minor changes in the method of Wheeler and Johns (3). Munakata and Nakai (5), and Ice, Breckinridge, and Christian (6) described the synthesis of 2(^{131}I),3,5-triiodobenzoic acid. They followed closely the procedure of Olivier and Combe (4) substituting sodium iodide-131 for potassium iodide. Jarboe (7) synthesized 2,3(^{131}I),5(^{131}I)-triiodobenzoic acid by substituting iodine-131 monochloride for iodine monochloride.

EXPERIMENTAL

Synthesis—To facilitate tracing the TIBA molecule through the entire growth period of the soybean plant from the flowering to the bean stage, radioactive TIBA was synthesized containing carbon-14 in the carboxyl position. One hundred millicuries (2.584 Gm.) of anthranilic acid[1] ($C_6H_4{}^{14}CO_2H$-2-NH_2), was suspended in 60 ml. of

Received November 24, 1967, from the Bionucleonics and Agronomy Departments, Purdue University, Lafayette, IN 47907
Accepted for publication January 30, 1968.
This research was conducted under the auspices of The Institute for Environmental Health, and was supported in part by a grant from the International Minerals and Chemical Corporation, Skokie, Ill.
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[1] Volk Radiochemical Co., Inc., New York, N. Y.

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0.7 *N* hydrochloric acid and cooled to 0°. Eleven grams (3.5 ml.) of iodine monochloride[2] and 30 ml. of 0.7 *N* hydrochloric acid were added to the cold suspension with stirring. To allow for completion of the reaction, stirring was continued for 48 hr. at room temperature after which excess iodine was reacted with sodium bisulfite. The solution was cooled to 0°, the precipitated reaction product (3,5-diiodoanthranilic* acid)[3] was collected by filtration. The reaction product was partially purified by acid-base reaction, ammonium hydroxide solution was added to give a pH of 9.5. The alkaline solution was filtered through a sintered-glass funnel. Glacial acetic acid was added to give a pH of 3. The solution was cooled to 0° and the resulting crystals of 3,5-diiodoanthranilic* acid collected on a sintered-glass funnel.

The 3,5-diiodoanthranilic* acid was diazotized by dissolving it in 40 ml. of concentrated sulfuric acid and cooling to 0°, followed by slowly adding 4 Gm. of sodium nitrite with stirring. After 2 hr. of cooling and stirring, the diazotized product was poured on ice. Nitrogen dioxide was removed from the solution by bubbling air through the solution for 30 min.

To the cold solution, 12 Gm. of potassium iodide in 12 ml. of water were added slowly with stirring. Nitrogen was removed from the solution by heating, and excess iodine was reacted with a sodium bisulfite solution.

After the solution was cooled to 0°, crude TIBA* was collected on a sintered glass-funnel. Fifteen milliliters of ether and enough hot ethanol to effect solution was added to the crude TIBA* and the solution was filtered. Ten milliliters of water was added, and the solution cooled to 0°. The crystals of TIBA* which formed were dried and weighed, the compound melted at 223–225.5°. The reported melting point for TIBA is 223–226° (3, 8). Approximately 55% of the theoretical yield (5.15 Gm.) of TIBA* (specific activity 16.240 dpm/mcg.[4]) was obtained.

The radiochemical purity of the compound was studied using thin-layer chromatography and autoradiography. Adsorbosil-1 (Applied Science Laboratories, Inc.), 250-μ thick, and a solvent system of petroleum ether (30–60° fraction)–propionic acid (10:1) were used. Nonradioactive compounds were chromatographed simultaneously and located by spraying with a 0.1% methanol solution of bromocresol green. Radioactive spots on the thin-layer plate were scraped from the plate and analyzed by internal liquid scintillation counting techniques to obtain a quantitative evaluation of the components.

The following compounds in addition to the TIBA* were separated: 3,5-diiodobenzoic* acid (1.36% of the radioactivity spotted), 2,5-diiodobenzoic* acid (1.16% of the radioactivity spotted), and an unidentified compound remaining at the origin (3.88% of the radioactivity spotted). Repeated recrystallization failed to improve the radiochemical purity of the compound. Work by another investigator (9) indicates that photochemical degradation occurs during chemical manipulation, resulting in the presence of impurities.

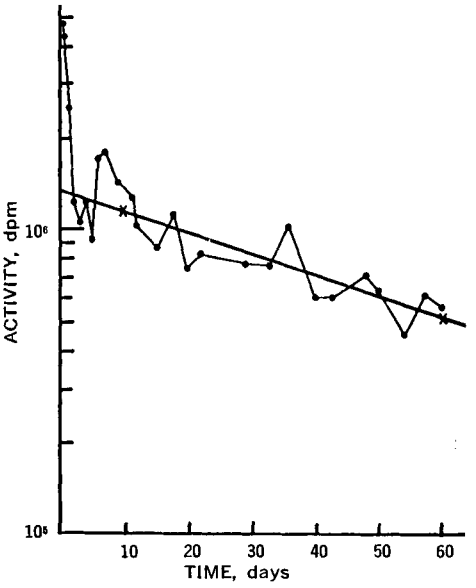

Fig. 1—Carbon-14 activity in soybean plant.

Plant Studies—A soybean plot, which at the beginning of the flowering stage contained approximately 600 plants averaging in the ninth trifoliate stage and about 35.5 cm. (14 in.) in height, was sprayed with a solution of TIBA* in water (650 mg. of TIBA* dissolved in ethanol 95% and brought up to a volume of 3 L. with distilled water). The spray was applied to the soybean plants with a hand sprayer to provide even application at the level of about 1 mg. of TIBA* per plant.

Three plants were taken from the plot at various time intervals (see Fig. 1) up to 60 days after application of the TIBA*, and the remaining plants were harvested at 110 days. Each sample was separated into the following parts: roots, stems, old leaves and old petioles, new leaves and new petioles (those which formed after the ninth trifoliate stage), pods, and seeds. After weighing, the samples were frozen in liquid nitrogen and ground in a blender to reduce the particle size and to provide a uniform mixture.

Five- to ten-gram samples of the plant parts were quantitatively converted to carbon dioxide using a modification of the Van Slyke-Folch wet oxidation procedure described by Jeffay and Alvarez (10). Each sample plus 7 Gm. of potassium iodate and 25 Gm. of chromium trioxide was placed in a suction flask connected to the fritted-glass dispersion tube of a gas-washing tower. Two hundred fifty milliliters of acid mixture[5] was added slowly to the suction flask from a separator. The mixture was boiled for 10 min. and the emitted carbon dioxide collected in 200 ml. of a mixture of ethanolamine and 2-ethoxyethanol (1:2). Heating was then discontinued and the system flushed with dry, carbon dioxide-free air. Controls were run using a known

[2] Eastman Organic Chemicals, Rochester, N. Y.
[3]* Denotes carboxyl-^{14}C.
[4] Determined by dissolving 10 mg. of TIBA* in 100.0 ml. of ethanol and counting 0.100-ml. samples and correcting for quenching by the addition of an internal standard.
[5] Prepared by mixing one part 85% phosphoric acid with two parts fuming sulfuric acid (20% SO_3) and allowing the mixture to cool before use.

TABLE I—CARBON-14 ACTIVITY FOUND IN THE WHOLE PLANT AND IN THE OLD LEAVES OF SOYBEAN PLANTS AFTER TREATMENT WITH CARBOXYL-^{14}C-2,3,5-TRIIODOBENZOIC ACID

Days After Application	Whole Plant		Old Leaves	
	dpm/Gm.[a]	dpm Total[b]	dpm/Gm.	dpm Total[b]
0	53,240	4,767,000	115,600	4,215,000
0.5	44,800	4,307,000	87,500	3,713,000
1	36,630	2,516,000	74,600	2,105,000
2	14,190	1,200,000	26,630	852,000
3	14,470	1,062,000	24,330	570,000
4	15,600	1,230,000	24,500	512,000
5	9,800	930,000	16,900	506,000
6	12,100	1,718,000	19,400	1,009,000
7	18,800	1,782,000	39,400	2,280,000
9	12,360	1,423,000	19,200	714,000
11	11,350	1,284,000	18,900	594,000
12	9,910	1,029,000	16,000	448,000
15	7,600	867,000	12,300	400,000
18	8,300	1,119,000	13,900	525,000
20	6,500	749,000	7,700	222,000
22	5,430	834,000	10,500	358,000
29	4,820	772,000	8,000	226,000
33	4,690	754,000	10,200	305,000
36	5,340	1,021,000	16,600	502,000
40	3,570	593,000	11,600	264,000
43	3,760	600,000	3,800	70,000
48	2,970	704,000	8,200	200,000
50	4,100	633,000	10,100	206,000
54	1,780	454,000	4,600	119,000
57	2,900	601,000	10,000	217,000
60	2,340	551,000	8,500	151,000

[a] Disintegrations/min./Gm. of plant material. [b] Calculated by multiplying the weight in grams of plant material by the dpm/Gm.

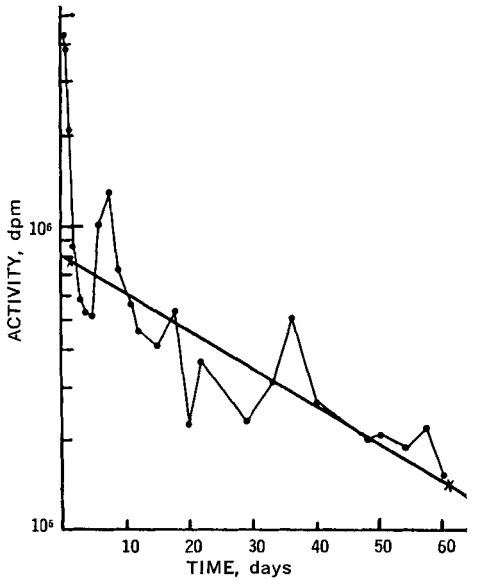

Fig. 2—Carbon-14 activity in old soybean leaves.

amount of TIBA* and 10 Gm. of plant material.

The liquid scintillation solution consisted of toluene and 2-ethoxyethanol (1:1), PPO[6] (4 Gm./L.) and dimethyl-POPOP[7] (100 mg./L.). All samples (3 ml. of the ethanolamine solution) were counted in a Tricarb liquid scintillation counter[8] and the results corrected for background and quenching following the method suggested by Schrodt, Gibbs, and Cavanaugh (11).

Results and Discussion—The radioactivity data for the whole soybean plant and the old leaves, Table I, indicated a rapid initial loss of radioactivity. Figures 1 and 2 are semi-log plots of the radioactivity against time for the soybean plant and the old leaves, respectively. At application the total

TABLE II—% CARBON-14 FOUND IN THE VARIOUS PLANT PARTS OF SOYBEAN PLANTS AFTER TREATMENT WITH CARBOXYL-^{14}C-2,3,5-TRIIODOBENZOIC ACID

Days After Application	%[a] in New Leaves	% in New Petioles	%[b] in Pods	%[b] in Seeds
6	5.0	2.2	...	...
7	6.4	1.3	...	...
9	10.4	3.6	...	...
11	21.3	4.7	...	...
12	21.1	5.4	...	...
15	21.1	5.5	...	...
18	21.0	2.7	...	...
20	27.6	5.5	2.4	...
22	21.0	7.7	2.2	...
29	25.8	6.4	8.7	0.2
33	14.5	2.5	5.6	3.5
36	11.9	3.1	8.0	2.2
40	11.0	1.2	6.5	4.9
43	21.1	3.4	6.8	21.5
48	14.3	3.3	12.0	12.3
50	15.0	3.0	11.4	14.4
54	13.4	3.0	9.8	13.2
60	8.3	3.7	5.4	31.1

[a] Calculated by dividing the total carbon-14 radioactivity in the plant by the amount in each of the plant parts and multiplying by 100. [b] TIBA* was applied at the flowering stage; consequently pods and seeds were not sufficiently formed for sampling and analysis until 20 and 29 days after application, respectively.

[6] 2,5-Diphenyloxazole.
[7] Dimethyl-1,4-bis-2-(5-phenyloxazolyl)-benzene.
[8] Model 3003, Packard Instrument Co., La Grange, Ill.

activity of a plant was 4,767,000 dpm; by 2 days after application the activity found on a plant was 1,200,000 dpm. Since absorption was being studied, the first 2 days were not included in the calculations of the linear regression for the soybean plant and the old leaves. From the linear regression lines shown on the graphs, the biological half-lives were calculated to be 42.5 and 24 days from the soybean plant and the old leaves, respectively. The difference between the half-lives indicated a translocation to other parts of the plant.

To better indicate this translocation, the percent of the carbon-14 in the various plant parts at the various sampling times was determined (Table II) and plotted against time. Figures 3–6 show the

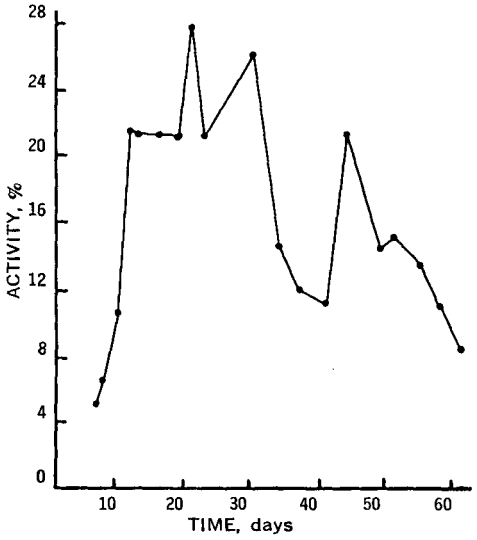

Fig. 3—Percent carbon-14 activity of the soybean plant in new leaves.

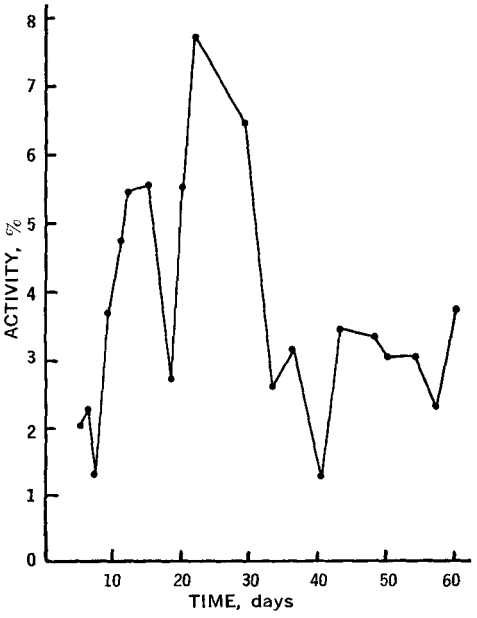

Fig. 4—Percent carbon-14 activity of the soybean plant in new petioles.

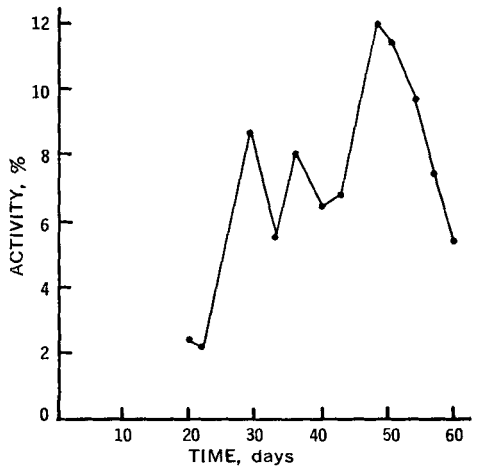

Fig. 5—Percent carbon-14 activity of the soybean plant in pods.

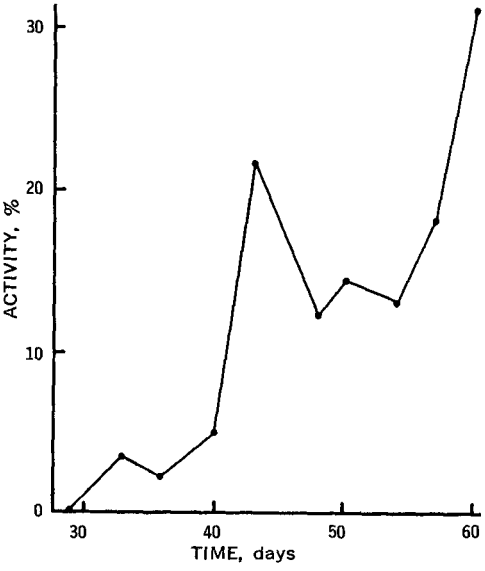

Fig. 6—Percent carbon-14 activity of the soybean plant in seeds.

percent of the carbon-14 activity in the soybean plant found in the new leaves, new petiole, pods, and immature seeds, respectively. From these figures it can be seen that carbon-14 was translocated to sites of new growth within the plant, and that as these parts aged, there was a translocation to newer parts of the plant. Although the seeds accounted for an increasing percentage of the radioactivity in the plant, the actual dpm/Gm. of soybeans did not increase after day 50.

Eleven samples of harvested soybeans were analyzed and the 95 percentile confidence level for the average radioactivity per Gm. of seeds was calculated. The 95 percentile confidence limits equaled 2709 dpm ± 950 dpm/Gm. of seeds, equivalent to 167 ± 59 ng. of TIBA* and/or metabolites per Gm. of seeds.[9]

[9] The 95% confidence interval = mean ± (1.96) (standard error of the mean).

SUMMARY

The plant growth regulator 2,3,5-triiodobenzoic acid containing carbon-14 in the carboxyl position was synthesized and applied to field-grown soybeans. A biological half-life of 42.5 days was found for the radioactivity in the soybean plant. Radioactivity was translocated to sites of new growth within the plant. At harvest the seeds contained a residue equivalent to 167 ng. of TIBA* and/or its metabolites per Gm. Before an assessment of the health hazard associated with the use of this chemical on soybeans can be made, there must be further study as to the nature of the residue.

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Absorption, translocation, residue—soybean plants
TLC—analysis
Autoradiography—analysis
Liquid scintillation counting—^{14}C, soybean plants

Mechanism of Action of Phenolic Disinfectants VIII

Association of Phenolic Disinfectants With Proteins

By JON E. STARR and JOSEPH JUDIS

Association of phenol-^{14}C (P-C-14), *p-tert*-amylphenol-^{14}C (PTAP-C-14), and 2, 4-dichlorophenol-^{14}C (DCP-C-14) with human serum and bacterial (*Micrococcus lysodeikticus*) proteins was investigated by means of density gradient ultracentrifugation and Sephadex gel filtration. Definite association between the phenols, PTAP-C-14 and DCP-C-14, and human serum proteins could be demonstrated by sucrose density gradient ultracentrifugation and with Sephadex gel filtration. The major protein in human serum involved in this association appeared to be albumin. Sucrose density gradient ultracentrifugation provided data indicating association of bacterial proteins with the three phenolic compounds, but most clear-cut in the case of PTAP-C-14. Protein binding could explain interference of serum with germicidal effects of phenolic disinfectants and enzyme inhibition and structural damage may account for bactericidal action.

TWO MAJOR CONSIDERATIONS motivated the investigation of the possible association of proteins and phenolic disinfectants. The interference of organic matter with the action of many disinfectants including phenol derivatives is a well recognized, troublesome phenomenon (1) and the authors desired to gain direct evidence for the basis of this interference, namely, whether binding of disinfectant by protein occurs. Secondly, an understanding of the mechanism by which phenolic disinfectants are lethal to bacteria would be much more complete if it could be shown that certain microbial cell components are specifically attacked by these disinfectants. One could presume that if a chemical component of the microbial cell bound phenolic disinfectants, this component could be, hypothetically, the one damaged by these germicides. Proteins, of course, play a major structural and functional role in all living cells and the general toxicity of phenolic disinfectants could be best explained on the basis of affinity for a generally distributed cell component, such as proteins.

The proteins selected for the preliminary studies were human serum, human serum albumin,

Received April 28, 1967 from the College of Pharmacy, University of Toledo, Toledo, OH 43606
Accepted for publication January 10, 1968.
Abstracted in part from a thesis submitted by Jon E. Starr to the University of Toledo, Toledo, Ohio, in partial fulfillment of Master of Science degree requirements.

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Association of Phenolic Disinfectants With Proteins

By JON E. STARR and JOSEPH JUDIS

Association of phenol-^{14}C (P-C-14), *p-tert*-amylphenol-^{14}C (PTAP-C-14), and 2, 4-dichlorophenol-^{14}C (DCP-C-14) with human serum and bacterial (*Micrococcus lysodeikticus*) proteins was investigated by means of density gradient ultracentrifugation and Sephadex gel filtration. Definite association between the phenols, PTAP-C-14 and DCP-C-14, and human serum proteins could be demonstrated by sucrose density gradient ultracentrifugation and with Sephadex gel filtration. The major protein in human serum involved in this association appeared to be albumin. Sucrose density gradient ultracentrifugation provided data indicating association of bacterial proteins with the three phenolic compounds, but most clear-cut in the case of PTAP-C-14. Protein binding could explain interference of serum with germicidal effects of phenolic disinfectants and enzyme inhibition and structural damage may account for bactericidal action.

TWO MAJOR CONSIDERATIONS motivated the investigation of the possible association of proteins and phenolic disinfectants. The interference of organic matter with the action of many disinfectants including phenol derivatives is a well recognized, troublesome phenomenon (1) and the authors desired to gain direct evidence for the basis of this interference, namely, whether binding of disinfectant by protein occurs. Secondly, an understanding of the mechanism by which phenolic disinfectants are lethal to bacteria would be much more complete if it could be shown that certain microbial cell components are specifically attacked by these disinfectants. One could presume that if a chemical component of the microbial cell bound phenolic disinfectants, this component could be, hypothetically, the one damaged by these germicides. Proteins, of course, play a major structural and functional role in all living cells and the general toxicity of phenolic disinfectants could be best explained on the basis of affinity for a generally distributed cell component, such as proteins.

The proteins selected for the preliminary studies were human serum, human serum albumin,

Received April 28, 1967 from the College of Pharmacy, University of Toledo, Toledo, OH 43606
Accepted for publication January 10, 1968.
Abstracted in part from a thesis submitted by Jon E. Starr to the University of Toledo, Toledo, Ohio, in partial fulfillment of Master of Science degree requirements.

and an extract of *Micrococcus lysodeikticus* cells. The methods chosen for demonstrating possible binding of phenolic disinfectants by these proteins or more specifically, a protein-disinfectant complex, were density gradient centrifugation and Sephadex gel filtration. In previous work (2) three carbon-14 labeled phenol derivatives were used, phenol, 2, 4-dichlorophenol, and *p-tert*-amylphenol. The same compounds were utilized in the studies described here.

MATERIALS AND METHODS

Materials—The radioactive phenol derivatives used in the experiments were synthesized by New England Nuclear Corporation, Boston, Mass., and had the following specific activities: phenol-^{14}C (P-C-14), 50 μc. per 3.04 mg.; 2, 4-dichlorophenol-^{14}C (DCP-C-14), 50 μc. per 12.0 mg.; and *p-tert*-amylphenol-^{14}C (PTAP-C-14), 50 μc. per 30.4 mg. The label in all three compounds was uniformly distributed in the ring. Phenol-C-14 was used in aqueous stock solution while the other derivatives were dissolved in 0.1% sodium hydroxide and all stock solutions contained 5 μc./ml.

Human serum (tissue culture grade) was obtained from Difco Laboratories, Inc., Detroit, Mich. Albumin, human crystalline 4x, and crystalline D-sucrose, C.P., were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. The albumin was reconstituted in distilled water to a concentration of 42 mg./ml. Sephadex G-200 and G-25 obtained from AB Pharmacia, Uppsala, Sweden, were used in the gel filtration experiments. All other materials used were reagent grade.

For the preparation of the bacterial extracts, *M. lysodeikticus* ATCC 4698 was grown on the medium previously used (2) and prepared in the following manner. Cells were washed twice with distilled water, and resuspended in distilled water to give a 1:3 w/v ratio. Glass beads measuring 25 μ in diameter were added in a 1:3 v/v ratio and the suspension subjected to sonic disintegration for 25 min. at 5° using a Branson sonifier, Branson Instruments Inc., Danbury, Conn., at the maximum power output. The material was then centrifuged at 12,000 × g for 10 min., and the supernatant, recentrifuged once. The supernatant contained 12.5 mg. of protein per ml., using the Lowry-Folin method (3) with crystalline human serum albumin as a standard.

Methods—Radio-assay was carried on in a Nuclear-Chicago Liquid Scintillation System and an aliquot of a sample was diluted when necessary with enough distilled water to produce a total volume of 2.0 ml., which was added to 13 ml. of a phosphor solution (2). Enough counts were accumulated in all experiments to give a nine-tenths error of less than 5%.

Density gradient separations were carried out as follows. In all experiments, 0.3 ml. of protein solution was added to 0.2 ml. of diluted radioactive phenol derivative. The stock solutions of the latter were diluted 8:50 (0.8 ml. of phenol derivative plus 4.2 ml. of 0.1% sodium hydroxide). In the experiments in which bacterial extract was used the ratio of the latter to the phenol derivative solutions was 9:1, using the stock solution of the phenol derivative undiluted. All mixtures were incubated for 10 min. at 25°. Gradients of 2–20% sucrose in 0.067 *M* tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl), with a pH of 7.4, were used in the density gradient analysis involving human serum, serum albumin, and the radioactive phenols. Gradients of 1–20% sucrose in 0.067 *M* TRIS-HCl pH 7.4, were used in the studies with the bacterial extract. The gradients were produced in 5 ml. lusteroid centrifuge tubes using a gradient former constructed after the design of Britten and Roberts (4). In all of the analyses except those involving the bacterial extracts, 0.1 ml. of the sample was layered on the top of the sucrose gradient. In the experiments in which bacterial extract was used, 0.15 ml. of sample was taken for density gradient analysis. The tubes were centrifuged for 2–10 hr. at 165,000 × g at 3.5° in a Spinco model L-2 preparative ultracentrifuge using a SW50-L swinging bucket rotor. The contents of the tube were fractionated by piercing the bottom of the tube with a needle and eight drops were collected per fraction. Each fraction was diluted with 2.0 ml. of distilled water in all cases except those experiments involving the bacterial extract, in which case the fractions were diluted with 1.5 ml. of distilled water. One milliliter of each fraction was analyzed for protein using the Lowry-Folin method (3) and another aliquot of each diluted fraction was taken for measurement of radioactivity.

The procedure for gel filtration studies involving PTAP-C-14 was as follows. Sephadex G-200 was allowed to come to equilibrium in 1.0 *M* sodium chloride solution and a bed 30 cm. in height was prepared in a 2.5 × 45 cm. column obtained from AB Pharmacia, Uppsala, Sweden. The column was washed with 1.0 *M* sodium chloride for 3 days to stabilize the bed and a nylon gauze was placed on the surface of the bed to minimize its disruption when layering the samples (5). Reaction mixtures containing 2.0 ml. of human serum and 0.5 ml. of PTAP-C-14 (0.2 ml. of stock solution and 9.8 ml. of 0.1% sodium hydroxide), were incubated for 10 min. at 25°. Two milliliters of the mixture was layered on the bed and eluted with 1.0 *M* sodium chloride. Three-milliliter fractions of the eluant were collected on a fraction collector and 1 ml. of each fraction was diluted with 2.0 ml. of distilled water and the protein content determined by measuring light absorption at 279 mμ in a Bausch and Lomb Spectronic 600 spectrophotometer. The remaining 2.0 ml. of each fraction was used for measurement of radioactivity. In the experiments in which DCP-C-14 was used, Sephadex G-25 replaced G-200; 0.3 *M* sodium chloride was used as the eluant; and the reaction mixture consisted of 1.0 ml. of human serum, 1.0 ml. of 0.067 *M* phosphate buffer at pH 7.4, and 0.5 ml. DCP-C-14 (0.3 ml. stock solution and 2.7 ml. of 0.1% sodium hydroxide). Sephadex G-25 was used in place of Sephadex G-200 in the experiments with DCP-C-14 because Sephadex G-25 interacts less than G-200 with phenolic compounds (5) thus offering less competition to the proteins in the formation of complexes of the latter.

RESULTS AND DISCUSSION

Figures 1 through 3 represent the results of the density gradient studies of mixtures of human

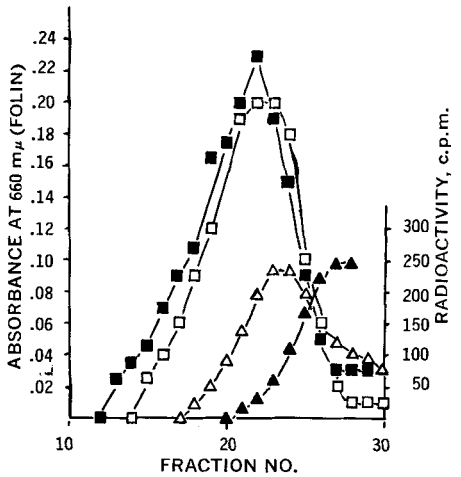

Fig. 1—Density gradient ultracentrifugation of human serum, PTAP-C-14, and a mixture of human serum and PTAP-C-14. The mixture was incubated for 10 min. at 25°. One-tenth ml. of the mixture was layered on a 2–20% sucrose gradient, centrifuged at 165,000 × g at 3.5° for 300 min., fractionated, and analyzed for protein and radioactivity. The initial mixture contained 0.3 ml. serum and 0.2 ml. of isotope (see Materials and Methods). *Key:* ■, *human serum alone;* □, *human serum mixed with PTAP-C-14;* ▲, *PTAP-C-14 alone;* △, *PTAP-C-14 mixed with human serum. In all figures,* □ ■ *refers to data obtained by analysis for protein and* △ ▲ *refers to radioactivity.*

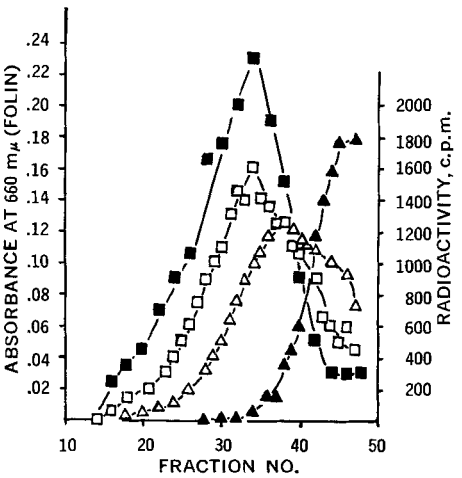

Fig. 2—Density gradient ultracentrifugation of human serum, DCP-C-14, and a mixture of human serum and DCP-C-14. Experimental conditions were the same as in Fig. 1 except the samples were centrifuged for 600 min. Key: ■, *human serum alone;* □, *human serum mixed with DCP-C-14;* ▲, *DCP-C-14 alone;* △, *DCP-C-14 mixed with human serum.*

serum and the radioactive derivatives of phenol. As one would expect, the phenol derivatives moved very little in the centrifugal field employed while the human serum proteins moved a considerable distance. The radioactive peaks obtained upon analysis of the mixtures of phenol derivatives with human serum are taken to represent bound phenol derivative. In most cases, the radioactive peak

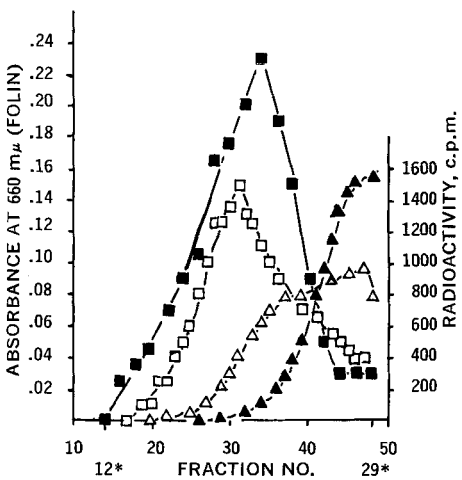

Fig. 3—Density gradient ultracentrifugation of human serum, P-C-14, and a mixture of human serum and P-C-14. Experimental conditions were the same as in Fig. 1. Key: ■, *human serum alone;* □, *human serum mixed with P-C-14;* ▲, *P-C-14 alone;* △, *P-C-14 mixed with human serum.*

from the mixtures did not correspond exactly with that of free protein and this slight difference in mobility may be due to the change of properties of the proteins when they are in combination with phenol derivatives. Chen (6) points out that serum albumin may have different physical properties when combined with fatty acids. The data in Fig. 3 would suggest that phenol does not form as tight a complex with serum proteins since the radioactive peak obtained from the phenol-human serum mixture is not as distinct nor was it located as close to the protein peak as it was in the cases of the other two phenol derivatives.

The data obtained from the experiments in which human serum albumin was substituted for human serum are given in Figs. 4 through 6. The results are similar to those obtained with human serum including the suggestion of relatively poor binding of phenol. While the radioactive peak obtained on analysis of phenol-albumin mixtures was in a significantly different position from that of free phenol (Fig. 6), the former certainly did not correspond well to the curve representing albumin.

Previous work from this laboratory (2) indicated that bacterial extracts did bind phenol derivatives in that the extracts interfered with binding of the phenol derivatives to whole cells. Density gradient analysis of mixtures of bacterial extract and radioactive phenols did show evidence for protein-phenol derivative complexes but most conclusively in the case of PTAP-C-14 (Fig. 7). The radioactive peak obtained in mixtures of DCP-C-14 and P-C-14 (Figs. 8 and 9) were significantly different in location from free phenol derivatives but not as distinctly as in the case of PTAP-C-14.

Evidence for protein-phenol derivative complexes was more unequivocal in the experiments utilizing gel filtration as the analytical method (Figs. 10 through 13). For example, PTAP-C-14 had a distinctly different rate of elution from the human serum proteins (Fig. 10) when subjected to gel filtration individually, but in a mixture, a radioactive peak appeared at a position (5) identical

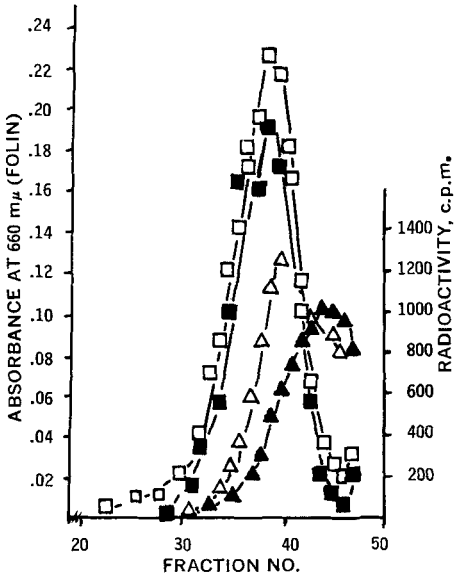

Fig. 4—Density gradient ultracentrifugation of human serum albumin, PTAP-C-14, and a mixture of serum albumin and PTAP-C-14. Experimental conditions were the same as in Fig. 1. Key: ■, albumin alone; □, albumin mixed with PTAP-C-14; ▲, PTAP-C-14 alone; △, PTAP-C-14 mixed with albumin.

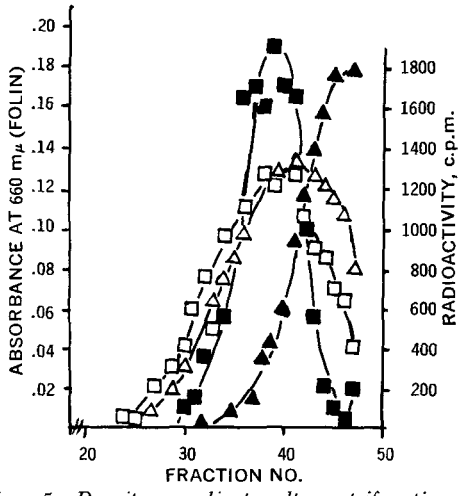

Fig. 5—Density gradient ultracentrifugation of human serum albumin, DCP-C-14, and a mixture of serum albumin and DCP-C-14. Experimental conditions as for Fig. 1. Key: ■, albumin alone; □, albumin mixed with DCP-C-14; ▲, DCP-C-14 alone; △, DCP-C-14 mixed with albumin.

with that of the human serum albumin peak (Fig. 11). Similar results were obtained with human serum and DCP-C-14 (Figs. 12 and 13) although in these experiments, the human serum proteins did not separate since Sephadex G-25 was used instead of G-200. Phenol-C-14 was not included in this series because preliminary experiments demonstrating definite association of the other two phenol derivatives with serum proteins indicated no binding of phenol-C-14.

In the opinion of the authors, the experimental

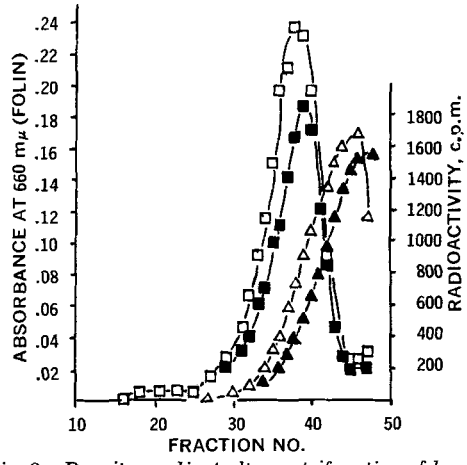

Fig. 6—Density gradient ultracentrifugation of human serum albumin, P-C-14, and a mixture of serum albumin and P-C-14. Experimental conditions as in Fig. 1. Key: ■, albumin alone; □, albumin mixed with P-C-14; ▲, P-C-14 alone; △, P-C-14 mixed with albumin.

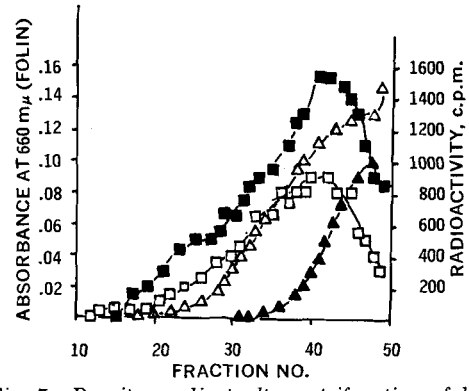

Fig. 7—Density gradient ultracentrifugation of bacterial extract, PTAP-C-14, and a mixture of bacterial extract and PTAP-C-14. Changes in experimental conditions from those in Fig. 1 were that 0.15 ml. of sample was layered on a 1–20% sucrose gradient, and centrifuged for 180 min. The initial mixture contained 0.9 ml. of bacterial extract and 0.1 ml. of phenol derivative (see Methods and Materials*). Key: ■, bacterial sonicate alone; □, bacterial sonicate mixed with PTAP-C-14; ▲, PTAP-C-14 alone; △, PTAP-C-14 mixed with bacterial sonicate.*

results present evidence for a protein-phenol disinfectant complex and would thus indicate that phenol derivatives are bound to serum proteins and presumably bacterial proteins, since the latter should constitute the major component of the bacterial extracts and the analytical method used would detect no other high molecular weight compounds other than proteins. These results are in accord with work previously reported from this laboratory (2) and that of Weinbach and Garbus (7–9) who reported the binding of several halophenols including DCP, to albumin. In all of the methods used in the studies reported here, the evidence for a protein-PTAP or DCP complex was much more clear-cut than for a protein-P complex in that the former were characterized by radioactive and protein curves corresponding rather

closely but in the case of the latter, correspondence was relatively poor. It is conceivable that the phenol derivatives did not associate with albumin as such but perhaps some other protein in the albumin fraction. Another explanation for the noncorrespondence of protein and phenol derivative curves could be that the phenol-protein complex had different mobility or rate of elution from those of the protein only. The same data might be considered in terms of relative affinities of the three phenol derivatives for proteins and one might expect that phenol is a poorer disinfectant because it is capable of less or looser binding to proteins. Although the specific activities of the three phenol derivatives were not identical, tightness of binding to proteins was considered to be related to identity of location of radioactivity and protein.

It was hoped that the experiments concerning bacterial extracts would result in clear-cut evidence for the existence of a protein-phenol derivative complex since previous work (2) described experiments indicating binding of phenol derivatives by whole cells. In mixtures of bacterial extracts and disinfectants PTAP-C-14 seemed to be located in a

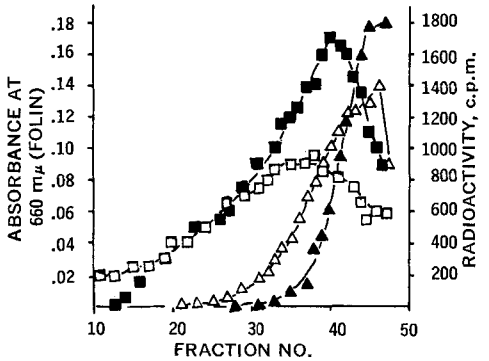

Fig. 8—Density gradient ultracentrifugation of bacterial extract, DCP-C-14, and a mixture of bacterial extract and DCP-C-14. Experimental conditions were the same as those in Fig. 7. Key: ■, bacterial sonicate alone; □, bacterial sonicate mixed with DCP-C-14; ▲, DCP-C-14 alone; △, DCP-C-14 mixed with bacterial sonicate.

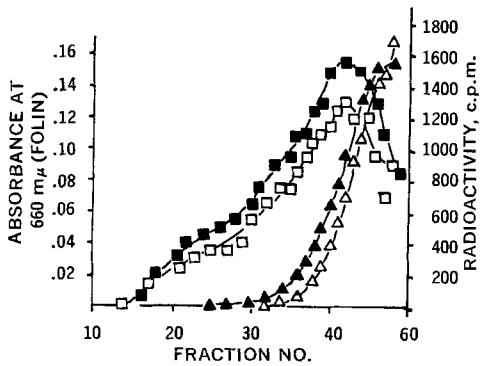

Fig. 9—Density gradient ultracentrifugation of bacterial extract, P-C-14, and a mixture of bacterial extract and P-C-14. The experimental conditions were the same as those used in Fig. 7. Key: ■, bacterial sonicate alone; □, bacterial sonicate mixed with P-C-14; ▲, P-C-14 alone; △, P-C-14 mixed with bacterial sonicate.

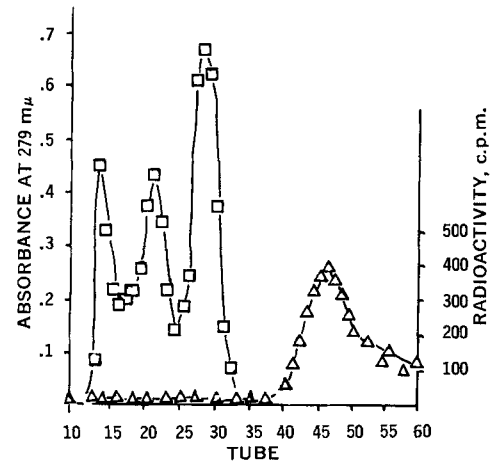

Fig. 10—Gel filtration of human serum and PTAP-C-14 separately. A 2.0-ml. sample was eluted from a Sephadex G-200 column with 1.0 M sodium chloride and 3.0 ml. fractions were collected and analyzed for protein and radioactivity. Key: □, human serum; △, PTAP-C-14.

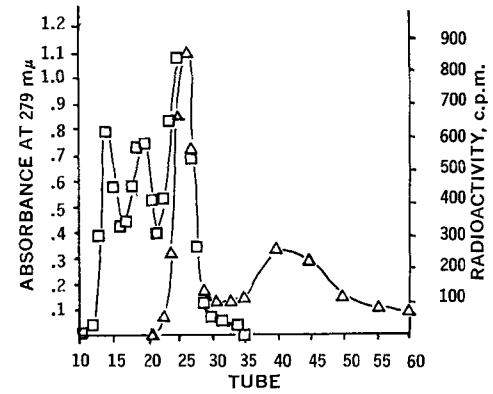

Fig. 11—Gel filtration of a mixture of human serum and PTAP-C-14. Experimental conditions were the same as in the case of Fig. 10. Key: □, human serum; △, PTAP-C-14.

centrifugal field similarly to protein in the bacterial extract (see Fig. 7), but the same was not true of DCP-C-14 or P-C-14 (Figs. 8 and 9). It is possible that in the process of preparing the bacterial extracts, major components responsible for binding of the phenol derivatives to whole cells were not obtained or were lost in the process of centrifugation of the sonic disintegrates. It is equally conceivable that the major component responsible for binding of phenol derivatives is present in such low concentrations in the extracts that it would not be detected by the method for protein analysis used. In any case, further work is presently in progress to obtain more conclusive evidence for binding of phenol derivatives by a component of these bacterial cells and characterization of this component. In addition, additional studies are being carried on to characterize the nature of binding of serum albumin to phenol derivatives, especially the number and nature of the binding sites involved. In conclusion, it is felt that the data herein presented suggest that phenols do bind to proteins including bacterial proteins and that this phenomenon could well

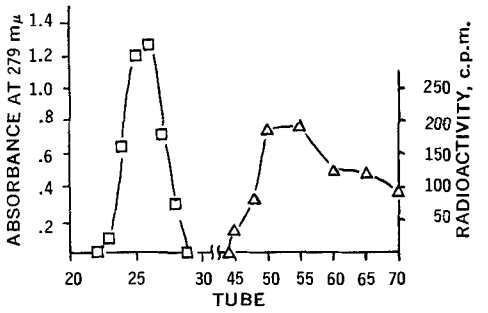

Fig. 12—Gel filtration of human serum and DCP-C-14 separately. Experimental conditions were the same as those used in the experiment represented by Fig. 10 except Sephadex G-25 was used and elution was done with 0.3 M sodium chloride. Key: □, human serum; △, DCP-C-14.

account for the antibacterial action of phenols. If binding is to the proteins in bacterial membranes, this association could well lead to disruption of the integrity of bacterial membranes, a phenomenon which has been demonstrated (10). Inactivation of enzymes by phenol is well documented (7, 11–15) and the latter could be a result of this binding process. It is interesting to note that there appears to be a correlation between the phenol coefficient of a given derivative and the extent of binding to whole bacterial cells. It is hoped that it may be possible to demonstrate a similar correlation in regard to binding to proteins in general.

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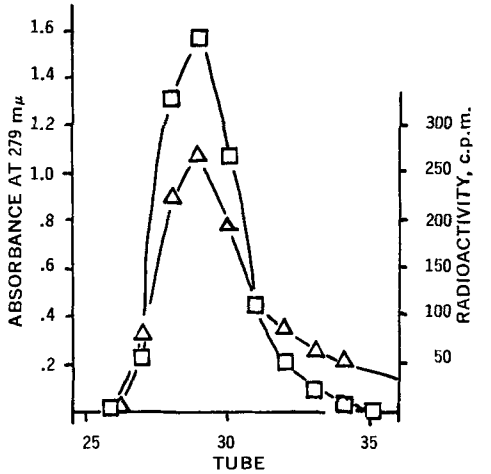

Fig. 13—Gel filtration of a mixture of human serum and DCP-C-14. The experimental conditions were the same as those of Fig. 12. Key: □, human serum mixed with DCP-C-14; △, DCP-C-14 mixed with human serum.

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Keyphrases

Phenolic disinfectants—activity mechanism
Radioactive phenol derivates—test compounds
Protein–phenol disinfectant—association
Density gradient, sucrose—association confirmed
Gel filtration—association confirmed
Liquid scintillation counting—analysis
Spectrophotometry—analysis

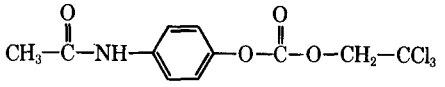
Acetaminophen Prodrugs I

Synthesis, Physicochemical Properties, and Analgesic Activity

By L. W. DITTERT*, H. C. CALDWELL, H. J. ADAMS†, G. M. IRWIN, and J. V. SWINTOSKY*

Carbonate and carboxylic acid ester prodrugs of acetaminophen were prepared. As expected, they had lower water solubilities and higher oil/water partition coefficients than acetaminophen itself. At pH 7.4 the prodrugs were slowly hydrolyzed in phosphate buffer, but human plasma enzymes markedly accelerated the hydrolyses. The LD_{50}'s of some of the compounds in mice suggest that, following oral administration, their solubility properties play a greater part in controlling the availability of acetaminophen than do their hydrolysis rates. Several of the compounds were found to have weak analgesic activity orally in rats supporting the theory that their analgesic activities and toxicities are due to release of acetaminophen. The results show how the carbonate linkage may be employed to create prodrugs and how it is possible to obtain a series of pharmacologically similar compounds possessing vastly different physicochemical properties.

THE SYNTHESIS, physical properties, and *in vitro* hydrolysis behavior of 4-acetamidophenyl 2,2,2-trichloroethyl carbonate (ATC)



ATC

have been reported previously (1). This compound was found to have the same spectrum of pharmacologic activity as a physical mixture of trichloroethanol and acetaminophen, but its analgesic and sedative properties were less intense and persisted for a longer period of time than those of the physical mixture (2). These results suggested that the pharmaceutical properties and dose-time-action profiles of trichloroethanol and acetaminophen can be modified, without modifying their qualitative pharmacologic activity, by combining them in a readily cleavable prodrug carbonate ester. The prodrug ester has physicochemical properties different from those of the parent compounds and may be absorbed at a different rate following oral administration.

It was the purpose of the present study to pursue further the principles of chemical synthesis and physicochemical and biologic evaluation of carbonate prodrugs. The authors wished to determine: (*a*) whether carbonate esters of acetaminophen, other than the trichloroethanol carbonate, would behave as prodrugs; (*b*) whether carboxylic acid esters of acetaminophen would behave as prodrugs; (*c*) which physicochemical properties are important in determining the *in vivo* activities of these compounds; (*d*) to what extent the physical properties of the prodrugs vary with structure; and (*e*) to what degree their *in vitro* cleavage rates vary with structure.

Received July 19, 1967, from Smith Kline & French Laboratories, Philadelphia, PA 19101

Accepted for publication January 22, 1968.

The authors wish to acknowledge the assistance of the following: Philip Goldman and Andrew Sivak for assistance in the syntheses; Dr. R. Walter Thompson and Margaret Carrol for analytical support; William Mann for assistance in the animal work, and Elisabeth Rattie for assistance in the solubility and hydrolysis studies.

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EXPERIMENTAL

Chemical Synthesis

Melting points were determined in capillary tubes on a Thomas-Hoover apparatus and are uncorrected. Most of the reactions were run only once. Reported yields are of pure compound.

***p*-Acetamidophenyl Butyrate–Procedure A**—A mixture of 30.2 Gm. (0.2 mole) of 4′-hydroxyacetanilide, 32.4 ml. (0.2 mole) of butyric anhydride, 1 ml. of pyridine, and 50 ml. of tetrahydrofuran was heated on a steam bath for 2.5 hr. The solvent was removed *in vacuo*, and the residue was recrystallized from benzene and washed with ether to give the pure compound in 87% yield.

***p*-Acetamidophenyl Chloroacetate–Procedure B**—To a solution containing 30.2 Gm. (0.2 mole) of 4′-hydroxyacetanilide and 16.2 ml. (0.2 mole) of pyridine in 100 ml. of tetrahydrofuran was added with stirring 15.2 ml. (0.2 mole) of chloroacetyl chloride in 30 min. The mixture was stirred at ambient temperature for 2 hr. The solid was collected and recrystallized twice from ethanol to yield 21% of pure compound.

***p*-Acetamidophenyl 4-Morpholinoacetate Hydrochloride–Procedure C**—A mixture of *p*-acetamidophenyl chloroacetate, morpholine, and triethylamine (0.05 mole of each) in 800 ml. of benzene was heated at reflux for 1 hr. to give an oily precipitate. This precipitate was decolorized with activated carbon in acetone. The hydrochloride salt was then prepared and recrystallized from dilute alcohol.

Bis (*p*-Acetamidophenyl) Succinate–Procedure D—To a chilled solution containing 7.8 Gm. (0.05 mole) of succinoyl chloride and 50 ml. of toluene was added slowly with stirring, a mixture of 4 Gm.

(0.1 mole) of sodium hydroxide and 15.1 Gm. (0.1 mole) of 4′-hydroxyacetanilide in 100 ml. of water. The addition required 15 min., and the reaction was stirred for an additional 1.5 hr. at room temperature. The product was filtered off and washed with water. Two recrystallizations from ethanol gave 33% of a white solid.

p-Acetamidophenyl Hydrogen Succinate–Procedure E—To a mixture of 20 Gm. (0.2 mole) of succinic anhydride in 75 ml. of toluene was added with stirring and cooling under nitrogen a solution of 30.2 Gm. (0.2 mole) of 4′-hydroxyacetanilide and 8 Gm. (0.2 mole) of sodium hydroxide in 100 ml. of water. The addition required 15 min. and the solution was stirred for 1.5 hr. with cooling. The mixture was filtered and the aqueous layer was acidified to give the desired product. The product was dissolved in sodium bicarbonate solution and precipitated with HCl. Finally it was dissolved in tetrahydrofuran, dried over anhydrous magnesium sulfate, diluted with 3 volumes of warm benzene, and cooled to give 28% of pure compound.

Bis (p-Acetamidophenyl) Carbonate–Procedure F—Phosgene was added with stirring under nitrogen to a solution containing 1 mole of 4′-hydroxyacetanilide and 1 mole of sodium hydroxide in 2 L. of water until the reaction mixture was neutral. The product was collected and recrystallized from dilute alcohol containing activated carbon and sodium sulfite to give 50% of the hydrate, m.p. 207–208°.

Anal.—Calcd. for $C_{17}H_{16}N_2O_5 \cdot H_2O$: C, 58.96; H, 5.24; N, 8.09. Found: C, 59.37; H, 5.25; N, 8.16.

An additional recrystallization from absolute alcohol gave the desired product.

p-Acetamidophenyl Hexylcarbonate–Procedure G—To 4′-hydroxyacetanilide (0.2 mole) and pyridine (0.22 mole) in 150 ml. of methylene chloride was added with cooling and stirring hexyl chloroformate (0.2 mole) in 75 ml. of methylene chloride. The resulting mixture was stirred for 2 hr., washed with water, and dried over anhydrous magnesium sulfate. The solvent was removed and the product was recrystallized from dilute alcohol.

Solubilities

The saturation solubilities of the compounds at 37° were determined in water and spectral grade cyclohexane (Fisher) in rotating bottles utilizing an assembly that has been described previously (3). To minimize hydrolysis, the aqueous samples were rotated for only 6 hr. The cyclohexane samples were rotated for 16 hr. (overnight). Samples of the clear supernatant saturated solutions were withdrawn into hypodermic syringes through Millipore filters held in Swinney filter adapters (Millipore Filter Corporation). The samples were analyzed spectrophotometrically.

Partition Coefficients

The partition coefficients of the compounds between cyclohexane and water at 25° were determined using a method and apparatus previously described (4).

In Vitro Hydrolysis Rates

*M*Half-lives for hydrolysis of the compounds in 0.1 pH 7.4 phosphate buffer with and without 2% human plasma at 37° were determined by a spectrophotometric method previously described (1). Frozen human blood plasma (Type O^+) was obtained in approximately 100-ml. quantities from single donors through the Philadelphia Serum Exchange.

Particle Size Reduction

Before *in vivo* testing, all compounds were micronized by one or more passes through a fluid energy mill (Trost Jet Mill, Helme Products, Inc., Helmetta, N. J.). This procedure produced particles in the 2–20 μ size range.

Oral Toxicity in Mice

Male Carworth Farms mice, weighing between 12–18 Gm. were randomly divided into groups of 10 and dosed orally with drug suspended in 0.5% tragacanth. Concentrations were calculated so that each animal received a dose volume of 20 ml./Kg. Control animals received 20 ml./Kg. of 0.5% tragacanth. After dosing, the animals were housed 10 per cage and observed daily for 5 days; if no deaths occurred during the subsequent 48 hr. (a total of 7 days), the study was terminated. If any deaths occurred during this 48-hr. period, the study was continued until no further deaths occurred for 2 consecutive days.

Analgesic Activity in Rats

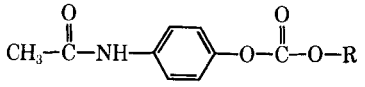
The method of Randall and Selitto was employed (5). Male Dierolf rats (100–148 Gm.) were used after fasting 16–18 hr. Inflammation was produced by the injection of 0.1 ml. of a 20% suspension of brewer's yeast into the plantar surface of the rat's left hind foot. Pressure was applied to the foot at a constant rate of 20 mm. Hg/sec. by a glass cone attached to the plunger of a 10-ml. syringe connected to an air pressure line and pressure gauge. The pain threshold was determined as the amount of pressure in mm. Hg required to induce struggle. Control threshold measurements (referred to as the Yeast Control) were obtained for both the inflamed foot and normal foot 2 hr. after injection of the yeast suspension, immediately before administration of the drug. All changes in pain threshold within each control and each treated group are reported as: mm. Hg pressure at time "t" after treatment minus; mm. Hg pressure at time "t_0" immediately preceding administration of the drug (Yeast Control). Subsequent threshold determinations were made 30, 60, 120, 180, and 240 min. after administration of the test compounds.

On the basis of preliminary trials, a dose of 400 mg./Kg. of acetaminophen was selected, and the prodrugs were administered in molar equivalent doses. Because of its low oral toxicity, the 4-acetaminophenyl ester was administered in twice the molar equivalent dose, *i.e.*, equivalent to 800 mg./Kg. of acetaminophen. The drugs were administered orally as suspensions in 0.5% tragacanth, and concentrations were adjusted so that each animal received 20 ml./Kg. The control animals received 20 ml./Kg. of 0.5% tragacanth. There were 5 animals per treated group and 5 per control group for the acetaminophen carbonates; there were 11 treated animals and 11 controls for acetaminophen. All testing was done on a "blind" basis.

RESULTS AND DISCUSSION

Synthesis and Physical Properties—Synthesis of the compounds, some of which are new composi-

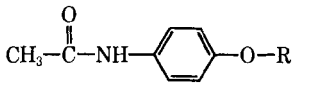
TABLE I—SYNTHESIS PROCEDURES AND PHYSICAL PROPERTIES FOR CARBONATE ESTERS OF ACETAMINOPHEN



R	Formula	Mol. Wt.	Yield, %	Synthesis Procedure	M.p.°C.[a]	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
Methyl[b]	$C_{10}H_{11}NO_4$	209.2	54	D	115.5–116.5	57.41	57.51	5.30	5.36	6.70	6.76
Ethyl	$C_{11}H_{13}NO_4$	223.2	71	D	121–122[c]	...	...	...	...	...	...
Isopropyl[b]	$C_{12}H_{15}NO_4$	237.3	45	D	131.5–132	60.75	60.62	6.37	6.06	5.90	5.87
Butyl	$C_{13}H_{17}NO_4$	251.3	46	D	119.5–120[d]	62.14	61.95	6.82	6.69	5.57	5.59
Isobutyl[b]	$C_{13}H_{17}NO_4$	251.3	24	D	119–121	62.14	62.12	6.82	6.77	5.57	5.54
Hexyl[b]	$C_{15}H_{21}NO_4$	279.3	68	G	112.5–113.5	64.50	64.26	7.58	7.34	5.01	4.99
Octyl[b]	$C_{17}H_{25}NO_4$	307.4	68	G	82.5–83	66.42	66.15	8.20	8.30	4.56	4.53
Chloroethyl[b]	$C_{11}H_{12}ClNO_4$	257.7	89	D	122.5–123	51.27	51.45	4.69	4.79	5.44	5.48
Phenyl[b]	$C_{15}H_{13}NO_4$	271.3	59	D	139–140.5	66.41	66.16	4.83	4.64	5.16	5.19
p-4-Acetaminophenyl	$C_{17}H_{16}N_2O_5$	328.3	50	F	219.5–220[e]	62.19	62.16	4.91	4.75	8.53	8.56

[a] Recrystallization solvent: ethanol–water. [b] These represent new compositions of matter. [c] Literature m.p. 120° (6). [d] Literature m.p. 117–120°(6). [e] Literature m.p. 200° (6).

TABLE II—SYNTHESIS PROCEDURES AND PHYSICAL PROPERTIES FOR CARBOXYLATE ESTERS OF ACETAMINOPHEN



R	Formula	Mol. Wt.	Yield, %	Synthesis Procedure	M.p.°C.	Recryst. Solvent[a]	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
Acetyl	$C_{10}H_{11}NO_3$	193.2	88	A	154–155[b]	A	62.17	61.98	5.74	5.78	7.25	7.40
Butyryl[c]	$C_{12}H_{15}NO_3$	221.3	87	A	139.5–141.5	D	65.14	64.93	6.83	6.73	6.33	6.61
Hexanoyl[c]	$C_{14}H_{19}NO_3$	249.3	36	B	108–109	A	67.45	67.75	7.68	7.68	5.62	5.79
Stearoyl[c]	$C_{26}H_{43}NO_3$	417.6	89	B	117–118	A	74.77	74.55	10.38	10.32	3.35	3.34
Pivalyl[c]	$C_{13}H_{17}NO_3$	235.3	34	D	162.5–163	C	66.36	65.92	7.28	7.09	5.95	6.11
Crotonyl[c]	$C_{12}H_{13}NO_3$	219.2	7	B	146–147	C	65.74	65.82	5.98	6.01	6.39	6.44
Fumaryl[c]	$C_{20}H_{18}N_2O_6$	382.4	5	D	278–279	G	62.82	63.09	4.74	4.78	7.33	7.36
Acid succinyl	$C_{12}H_{13}NO_5$	251.2	28	E	145.5–146.5[d]	F	57.37	57.39	5.22	5.10	5.58	5.50
Succinyl[c]	$C_{20}H_{20}N_2O_5$	384.4	33	D	229–230	B	62.49	62.41	5.24	5.22	7.29	7.28
Benzoyl	$C_{15}H_{13}NO_3$	255.3	61	D	170.5–171.5[e]	E	70.58	70.38	5.13	5.08	5.49	5.50
Cinnamyl	$C_{17}H_{15}NO_3$	281.3	62	B	200–201[f]	B	72.58	72.69	5.37	5.45	4.98	4.96
Chloroacetyl[c]	$C_{10}H_{10}ClNO_3$	227.7	21	B	184.5–185	B	52.76	52.92	4.43	4.41	6.15	6.18
Morpholinoacetyl·HCl	$C_{14}H_{18}N_2O_4\cdot HCl$	314.8	29	C	258–259	C	53.42	53.61	6.08	6.14	8.90	8.80

[a] Recrystallization solvents are: A, toluene; B, ethanol; C, ethanol–water; D, benzene; E, ethanol–hexane; F, tetrahydrofuran–benzene; G, acetic acid–water. [b] Literature m.p. 150° (7). [c] These represent new compositions of matter. [d] Literature m.p. 220° (8). [e] Literature m.p. 136–137° (9). [f] Literature m.p. is 171° (7).

tions of matter, did not present unusual problems. The molecular weights, yields, melting points, recrystallization solvents, and elemental analyses of the compounds are shown in Tables I and II.

The solubilities of acetaminophen and the various esters in water and cyclohexane at 37° and the cyclohexane/water partition coefficients for the compounds at 25° are shown in Table III. As expected, the prodrug esters of acetaminophen, in most cases, were much less soluble in water and much more soluble in cyclohexane than acetaminophen. The aqueous solubilities of the carbonate esters decreased rapidly as the chain length of the alcohol moiety increased.

Branching in the alcohol chain tended to increase both aqueous and cyclohexane solubilities as illustrated by the solubilities of the butylcarbonate compared with those of the isobutylcarbonate. This effect could be attributed to loss of symmetry in the molecules which reduces crystal lattice energy. As the alcohol chain length increased, the cyclohexane solubilities and cyclohexane/water partition coefficients of the carbonate esters also increased. Although chain branching tended to increase the cyclohexane solubility, it did not increase the partition coefficient because of the concomitant increase in aqueous solubility. It would seem, however, that branching would tend to increase the oral availability of these compounds, since both the aqueous and lipid solubilities were increased. Chlorine substitution in the alcohol moiety decreased aqueous solubility and increased cyclohexane solubility. Thus, the chloroethylcarbonate had the highest partition coefficient of all the carbonate esters. The acetaminophencarbonate esters of phenol and of 4-acetaminophenol (bisacetaminophenyl carbonate) were relatively high melting compounds and, consequently, were not very soluble in either water or cyclohexane. The discrepancies between the measured partition coefficients shown in Table III, and the partition coefficients that could be calculated for these two compounds from the solubility data, are due to ex-

TABLE III—SOLUBILITIES, PARTITION COEFFICIENTS, AND AVAILABILITY FACTORS FOR ACETAMINOPHEN PRODRUGS

Derivative	Solubilities (37°) Water mg./ml.	Solubilities (37°) Cyclohexane mg./ml.	Cyclohexane/Water Partition Coefficients 25°	f.d.[a]	Availability Factor[b]
Acetaminophen	20	0.0015	0.000075	0.000075	0.10
Carbonate Esters					
Methyl	6.0	0.048	0.008	0.0079	2.3
Ethyl	1.1	0.036	0.03	0.032	1.6
Isopropyl	1.1	0.05	0.09	0.043	2.1
Butyl	0.16	0.081	0.48	0.34	2.2
Isobutyl	0.38	0.13	0.50	0.25	3.8
Hexyl	0.037	0.12	0.84	0.76	1.0
Octyl	0.0044	0.14	0.94	0.97	0.14
Chloroethyl	0.39	0.55	1.2	0.59	8.9
Phenyl	0.063	0.02	0.32	0.24	0.56
4-Acetaminophenyl	0.060	0.001	0.015	0.016	0.03
Carboxylate Esters					
Acetyl	3.4	0.019	0.0043	0.0056	1.0
Butyryl	0.54	0.019	0.053	0.034	0.83
Hexanoyl	0.057	0.083	0.85	0.59	1.3
Stearoyl	0.015	0.018	1.0	0.55	0.2
Pivalyl	0.11	0.042	0.3	0.28	1.3
Crotonyl	0.43	0.015	...	0.034	0.67
Fumaryl	...	...	...	...	...
Acid succinyl	6.5	0.0007	0.0009	0.0001	0.03
Succinyl	0.0068	<0.0001	<0.0001	...	...
Benzoyl	0.017	0.012	0.55	0.41	0.3
Cinnamoyl	0.0014	0.0013	1.3	0.48	0.023
Chloroacetyl	0.28	0.11	0.18	0.28	3.4
Morpholinoacetyl·HCl	102	0.0087	0.019	0.00008	0.26

[a] *f.d.* = fractional distribution of the compound in a cyclohexane/water system calculated by dividing the cyclohexane solubility by the sum of the cyclohexane solubility and the water solubility. [b] Availability factor = *f.d.* × molar aqueous solubility × 10^4.

perimental analytical error resulting from the low concentrations measured in the solubility studies. Nonetheless, it would seem that addition of the polar acetamido group significantly reduces lipid solubility and consequently the cyclohexane/water partition coefficient is reduced.

The solubility behavior of the carboxylate esters was essentially the same as that of the carbonate esters. Increasing chain length in the acid moiety decreased aqueous solubility and increased lipid solubility, although the cyclohexane solubilities of the carboxylate esters were not as high as their analogous carbonate esters. Apparently, carboxylates are inherently more polar than carbonates. Branching in the acid moiety seemed to have less effect on the solubilities of the carboxylates than it did on the solubilities of the carbonates (compare butyryl with pivalyl and butylcarbonate with isobutylcarbonate) and unsaturation in the acid moiety also seemed to have little effect on the solubility (compare butyryl with crotonyl). The high melting fumaryl ester was extremely insoluble in both water and cyclohexane so that no measurement of its solubilities could be made by our methods. The succinyl ester, also a high melting compound, was also very insoluble in both water and cyclohexane. The acid succinyl ester was quite soluble in water due to its free carboxyl group, but its lipid solubility was very low. As with the carbonate esters, aromatic groups in the carboxylate esters tended to decrease both aqueous and lipid solubilities, and chlorine tended to decrease aqueous, but increase lipid, solubilities. Because it is a salt, the morpholinoacetyl·HCl derivative was very soluble in water and very insoluble in cyclohexane.

Availability Factors—In evaluating the availability of a relatively insoluble compound for oral absorption, one must take into consideration both the aqueous solubility, which is directly related to the rate at which the compound dissolves in the gut lumen, and the lipid solubility, which is directly related to the rate at which the compound partitions into the lipid gut barrier. In order to compare compounds, it would be convenient to express both these factors in one number. The "availability factors" listed in Table III are an attempt to do this. They are products of the molar aqueous solubilities and a factor related to the lipid/water distribution characteristics of the compounds. Because partition coefficients can be extremely low or extremely high and thus offset significant changes in aqueous solubilities, the lipid/water distribution characteristics of the compounds were expressed in terms of "fractional distributions" (*f.d.*). These values were calculated using the following empirical equation:

$$f.d. = \frac{\text{cyclohexane solubility}}{\text{cyclohexane solubility} + \text{aqueous solubility}}$$

Whereas partition coefficients may vary from 0 to infinity, fractional distributions, as defined above, vary from 0 to 1.

The availability factors for nearly all the acetaminophen carbonate esters are of the same order of magnitude and are larger than that for acetaminophen itself. The isobutylcarbonate derivative is more soluble in both water and cyclohexane than the other carbonates; and, consequently, its availability factor is somewhat higher than that of the other aliphatic carbonates. How-

ever, the chloroethylcarbonate has an even higher factor which is primarily due to its high cyclohexane solubility. The factor for the octylcarbonate is low due to its low aqueous solubility, while the factors for the phenyl- and 4-acetaminophenylcarbonates are low because both their aqueous and cyclohexane solubilities are low.

The factors for the carboxylate esters show a similar degree of variability and are generally lower than those of the carbonates. Factors for some of the carboxylates could not be calculated because their solubilities in water and cyclohexane are too low. The factor for the acid succinyl derivative is notably low because of its low cyclohexane solubility and in spite of its relatively high aqueous solubility. This is also true of the morpholinoacetyl·HCl derivative, although this compound would probably have a higher factor in its free base form than in its salt form. The benzoyl and cinnamoyl derivatives have low factors due to low aqueous and cyclohexane solubilities.

It should be mentioned that conjecture based on availability factors, such as those shown in Table III, must be restricted to compounds of closely similar structure. Cyclohexane and most other organic solvents probably have solvent properties which are markedly different from those of the G.I. barrier lipids. However, cyclohexane was chosen for these solubility and partitioning studies because it is one of the most nonpolar pure solvents available commercially. Moderate solubilities in cyclohexane should reflect a high degree of lipophilic character in the drugs. However, the G.I. barrier is not pure hydrocarbon, and drugs which have a relatively low solubility in cyclohexane may have a much greater solubility in G.I. barrier lipids due to polar interactions. As long as one is dealing with drugs of similar enough structure that the polar interactions are relatively equal and the structural differences result in changes in the nonpolar portions of the molecule, it is relatively safe to make comparative predictions of oral absorbability. However, when the structures differ as much as the difference between a carbonate ester and a carboxylate ester of equal chain length, or even as little as the difference between an aliphatic carbonate and a chlorine-substituted aliphatic carbonate ester, such predictions must be made with great caution. Therefore, it is dangerous to say that acetaminophen itself is less "available" than the prodrugs; but it is reltively safe to say that the aliphatic carbonates are all about equally available, with the isobutyl derivative being perhaps slightly more rapidly available for absorption than the rest.

In Vitro Hydrolysis Behavior—It was expected that the prodrug carbonate and carboxylate esters of acetaminophen would be hydrolyzed *in vivo* before, during, or after absorption to release free acetaminophen which would then exert its characteristic pharmacologic actions. It was, therefore, desirable to determine the relative susceptibility of these compounds to both nonenzymatic and enzymatic hydrolysis *in vitro*. It was of particular interest to look for relationships between the hydrolysis rates and the pharmacologic activities of the compounds.

Table IV shows half-lives for the hydrolyses of the prodrugs at 37° in pH 7.4 phosphate buffer with and without 2% human plasma. In general, all the compounds hydrolyzed slowly in pH 7.4 buffer; and most exhibited half-lives in excess of 24 hr. (1440 min.). The carbonate and carboxylate esters behaved very similarly in this regard, but the cinnamoyl and chloroacetyl (carboxylate ester) derivatives were unusually labile to hydrolysis exhibiting half-lives of 1.2 min. and 12 min., respectively, in buffer.

Table IV—Half-Lives for the Hydrolysis of Acetaminophen Prodrugs in pH 7.4 Phosphate Buffer (0.1 *M*) With and Without 2% v/v Human Plasma (37°)

Compd.	2% Human Plasma (min.)	Buffer (min.)	$t_{1/2}$ Buffer/ $t_{1/2}$ Plasma
Carbonate Esters			
Methyl	180	9,000	50
Ethyl	45	12,000	267
Isopropyl	55	18,000	327
Butyl	15	18,000	1200
Isobutyl	19	21,000	1105
Hexyl	11	22,800	2073
Octyl	14	19,800	1414
Chloroethyl	25	1,320	53
Phenyl	25	240	9.6
4-Acetaminophenyl	130	420	3.2
Carboxylate Esters			
Acetyl	38	>1,440	...
Butyryl	34	>1,440	...
Hexanoyl	30	>1,440	...
Stearoyl	...	...	...
Pivalyl	462	...	...
Crotonyl	258	>1,440	...
Fumaryl	58	138	2.4
Acid succinyl	1.1	660	60
Succinyl	38	390	10
Benzoyl	98	>1,440	...
Cinnamoyl	2.8	1.2	0.43
Chloroacetyl	4.6	12	2.6
Morpholinoacetyl · HCl	138	438	3.2

In pH 7.4 buffer containing 28% human plasma, the hydrolysis rates were much more rapid. The degree of acceleration is illustrated in the column headed "$t_{1/2}$ buffer/$t_{1/2}$ plasma." This column shows that some of the compounds hydrolyzed as much as 2,000 times faster in the presence of 2% human plasma than in plain pH 7.4 buffer. This accelerated hydrolysis rate is attributed to the esterases present in the plasma. In other experiments, it was observed that as plasma concentrations increased, hydrolysis rates likewise increased.

Toxicity and Analgesic Activity—On the basis of the hydrolysis data, it might be concluded that all of the carbonate and carboxylate compounds should behave as prodrugs of acetaminophen, hydrolyzing to release free acetaminophen in the tissues of humans and animals. On the basis of the availability factors shown in Table III for the carbonate esters, for example, it might be concluded that these compounds should not differ appreciably in their rates of dissolution and absorption from the gastrointestinal tract. To test the validity of these conclusions *in vivo*, the oral LD_{50}'s were determined of some of the compounds in mice and the analgesic

TABLE V—HALF-LIVES FOR HYDROLYSIS IN HUMAN PLASMA, AVAILABILITY FACTORS, AND ORAL LD_{50}'s IN MICE FOR PRODRUG CARBONATE ESTERS OF ACETAMINOPHEN

Derivative	Availability Factor[a]	LD_{50} (Limits) moles/Kg.	LD_{50} (Limits)[b] mg./Kg.
Acetaminophen	0.10	7.1 (5.4–9.4)	1080 (824–1415)
Carbonate Esters			
Methyl	2.3	8.4 (6.7–10.5)	1750 (1400–2188)
Ethyl	1.6	6.5 (5.3–7.9)	1450 (1190–1770)
Isopropyl	2.1	14.8 (10.5–16.9)	3500 (2500–4000)
Butyl	2.2	6.4 (4.4–9.8)	1614 (1093–2449)
Isobutyl	3.8	8.6 (6.7–10.9)	2150 (1693–2731)
Phenyl	0.56	12.5 (8.9–17.7)	3400 (2411–4794)

[a] See Table III for definition. [b] LD_{50}'s and 95% confidence limits were calculated by the method of Litchfield and Wilcoxon (12).

activities of some of the compounds in rats using the Randall-Selitto technique.

Table V shows half-lives for enzymatic hydrolysis, availability factors, and oral LD_{50}'s in mice for some of the carbonate esters. The LD_{50}'s are given in moles/Kg. since 1 mole of each prodrug would release 1 mole of acetaminophen regardless of the molecular weight of the prodrug. Thus, comparison of toxicities on a molar basis gives a more precise evaluation of *in vivo* availability than comparison on a mg./Kg. basis. The molar LD_{50}'s of the carbonate prodrugs are virtually identical, and their confidence limits overlap each other and those of acetaminophen in almost all cases. In those cases in which the confidence limits do not overlap, the lack of agreement does not appear to be the result of either availability or slow enzyme hydrolysis. For example, the isobutyl derivative has the highest availability factor; but its toxicity overlaps that of the phenyl compound which has the lowest factor. The methyl compound is the slowest hydrolyzing compound, but it is equitoxic to the isobutyl compound, which is relatively rapidly hydrolyzed. It would seem that normal biological variation or some physical factor, such as poor wetability, may have caused the variations in the toxicities of these compounds. All of the compounds were micronized to reduce the particles to the 2–20 μ size range before biological testing.

The oral LD_{50}'s of the acetyl, butyryl, hexanoyl, and stearoyl carboxylate esters in mice were found to be in excess of 12 moles/Kg. The stearoyl derivative was so insoluble that no deaths could be produced at any dose. Thus, it would appear that the generally lower availability factors given for these compounds in Tabe III indicate that they are less available than the carbonates. However, the LD_{50}'s were so high that meaningful comparisons could not be made.

It is well known that it is difficult to quantitate the activity of mild nonnarcotic analgesics in laboratory animals (10); therefore, the authors attempted only to establish whether or not the acetaminophen carbonates possess analgesic activity in the range of acetaminophen. The method of Randall and Selitto was employed, and a control group of animals was included to show that the yeast-injected rat paw retains its increased sensitivity to pain for the duration of the study (4 hr.). The results of a statistical analysis of the postdrug pain threshold data are shown in Table VI. The isopropyl and phenyl carbonates had significant analgesic activity at doses equimolar to a 400 mg./Kg. dose of acetaminophen. These compounds have availability factors of 2.1 and 0.56, respectively (Table V); but the difference between these values is apparently not great enough to cause a significant difference in analgesic activity by this test. If prodrug derivatives had been prepared of a drug with a more potent and more easily quantitated pharmacologic activity, perhaps a difference in availability factor such as that found for these prodrugs would have made a significant difference in the pharmacologic responses. However, with drug activity as weak and difficult to measure as mild analgesia, it is impossible to tell whether or not the carbonate prodrugs are absorbed at any different rates or with greater or lesser efficiencies than acetaminophen itself.

TABLE VI—ANALGESIC ACTIVITY (RANDALL-SELITTO) OF ACETAMINOPHEN AND CARBONATE PRODRUGS OF ACETAMINOPHEN ORALLY IN RATS

Compd.	Time Postdrug (Min.)[a] 30	60	120	180	240
Acetaminophen	+	+	+	+	+
Carbonate Esters					
Isopropyl	+	0	+	+	0
Phenyl	0	0	+	+	+

[a] + = Significant ($p \leq 0.05$) analgesic activity; 0 = no significant analgesic activity. Significance was determined by means of Student's t test (13).

Because this study was concerned primarily with establishment of principles rather than a determination of the pharmacologic activity profiles of every compound made, a number of the acetaminophen esters were not studied *in vivo* for their activity profiles. Nevertheless, since each of the compounds that were thus studied exhibited the gross pharmacologic activities of acetaminophen, and each underwent relatively rapid hydrolysis in the presence of plasma, it seems fair to assume that most of the compounds reported here would behave as acetaminophen prodrugs.

CONCLUSIONS

Carbonate and carboxylate esters prepared as potential prodrugs of acetaminophen were shown to have a variety of physicochemical properties, particularly lipid and water solubilities. *In vitro* enzyme-catalyzed hydrolysis studies suggested that most of the compounds would be hydrolyzed to release acetaminophen following oral administration to animals and humans. Toxicity and analgesic

activity studies in mice and rats suggested that the lipid and water solubilities of the compounds, rather than their enzyme-catalyzed hydrolysis rates, probably control the availability of acetaminophen following oral administration. These experiments provide additional evidence that the carbonate linkage may be an importantly useful one for the creation of variant physical and chemical properties in an entity with a singular pharmacologic action (11). This might then offer the opportunity to the formulator to choose among a variety of compounds with a singular pharmacologic action for the one which is: (*a*) amenable to physical formulation in a given dosage form; (*b*) stable in a given dosage form where all forms of the drug may not be equally stable; (*c*) satisfactory from the standpoint of taste or consumer acceptability; and (*d*) appropriate therapeutically from the standpoint of its time-action profile.

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Keyphrases

Acetaminophen prodrugs
Carbonate esters, acetaminophen–synthesis
Carboxylic acid esters, acetaminophen–synthesis
Hydrolysis rates, *in vitro*–acetaminophen prodrugs
Toxicity, oral–acetaminophen prodrugs
Analgesic activity–acetaminophen prodrugs

Acetaminophen Prodrugs II

Effect of Structure and Enzyme Source on Enzymatic and Nonenzymatic Hydrolysis of Carbonate Esters

By L. W. DITTERT*, G. M. IRWIN, C. W. CHONG, and J. V. SWINTOSKY*

Hydrolysis rates are reported for acetaminophen prodrugs with the structure CH_3CONH-ϕ-OCOOR at pH 7.4 in phosphate buffer alone or containing 1% human plasma or serum from several animal species. The hydrolysis rates in buffer decreased as the electrophilic character of the R group decreased. Dilute plasma or serum accelerated the hydrolysis; and the number of carbon atoms, the degree of chain branching, aromaticity, and chlorine substitution in the R group variously affected the degree of acceleration. In general, the sera of small rodents (mouse, guinea pig, and rat) were more potent catalysts of the hydrolyses of all types of acetaminophen carbonates than that of other animals (cat, dog, sheep, and rabbit) or human plasma.

THE METHODS of preparation and the physical properties of a series of carbonate esters of acetaminophen have been previously reported (1). These compounds were found to hydrolyze at various rates in dilute (2% v/v) human plasma solutions, and some of them had analgesic activity on the order of acetaminophen in rats. It was postulated that the analgesic activity was due to free acetaminophen released in the blood streams of the rats following oral administration of the prodrugs.

This report discusses the influence of the structure of acetaminophen carbonate prodrugs on the nonenzymatic hydrolysis of the compounds at pH 7.4 and on the enzymatic hydrolysis of the compounds catalyzed by blood plasma from

Received July 19, 1967, from Smith Kline & French Laboratories, Philadelphia, PA 19101
Accepted for publication January 22, 1968.
The authors wish to thank Elisabeth Rattie for technical assistance.
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Hydrolysis rates are reported for acetaminophen prodrugs with the structure CH_3CONH-ϕ-OCOOR at pH 7.4 in phosphate buffer alone or containing 1% human plasma or serum from several animal species. The hydrolysis rates in buffer decreased as the electrophilic character of the R group decreased. Dilute plasma or serum accelerated the hydrolysis; and the number of carbon atoms, the degree of chain branching, aromaticity, and chlorine substitution in the R group variously affected the degree of acceleration. In general, the sera of small rodents (mouse, guinea pig, and rat) were more potent catalysts of the hydrolyses of all types of acetaminophen carbonates than that of other animals (cat, dog, sheep, and rabbit) or human plasma.

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humans and blood sera from various animal species.

EXPERIMENTAL

Frozen citrated human blood plasma (Type O^+) was obtained in approximately 100-ml. quantities from single donors through the Philadelphia Serum Exchange. Frozen pooled animal sera were obtained from Colorado Serum Company, Denver, Colorado.

Half-lives for the hydrolysis of the acetaminophen carbonates at 37° in pH 7.4 phosphate buffer (0.1 *M*), with and without human plasma or animal serum, were determined by direct UV analysis in the thermostatted cell compartment of a Cary model 15 spectrophotometer. Fifty milliliters of the plasma or serum solution was warmed to 37° in a 125-ml. glass-stoppered conical flask and a portion placed in the spectrophotometer reference cell. One-half milliliter of 95% ethanol containing an appropriate amount of the acetaminophen carbonate was injected by means of a hypodermic syringe, and the flask was swirled gently until mixing was complete. A portion of this mixture was transferred to the sample cell of the spectrophotometer and the absorbance followed until no further change was observable. In cases in which the reactions were very slow, the final absorbance values were calculated. The half-lives were determined from plots of log Δ absorbance *versus* time (see Fig. 1). In most cases, the spectrophotometer was set at 240 mμ (the absorbance maximum of the esters), where a decrease in absorbance reflected the disappearance of the ester. In several experiments, the spectrophotometer was set at 300 mμ (the absorbance maximum of acetaminophen), where an increase in absorbance reflected the appearance of acetaminophen. For a given ester in a given enzyme system, the same half-lives were obtained at the two wavelengths.

RESULTS AND DISCUSSION

Half-lives for the hydrolysis of 11 carbonate esters of acetaminophen in pH 7.4 phosphate buffer (0.1 *M*), with and without 1% (v/v) human plasma, are shown in Table I. The rates of nonenzymatic cleavage of acetaminophen carbonate esters of straight-chain alcohols decreased with increasing chain length to four carbons; thereafter, they remained essentially constant at a minimum value.

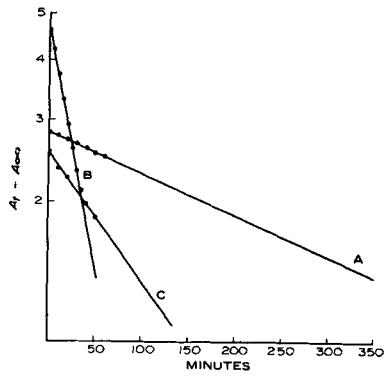
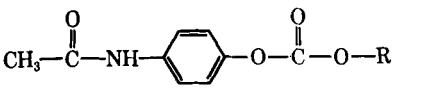


Fig. 1—Representative plots showing the first-order nature of the hydrolysis of carbonate esters of acetaminophen in pH 7.4 phosphate buffer (0.1 M*) containing 1% (v/v) human plasma at 37°. Absorbances were determined at 240 mμ, the absorbance maximum of the esters.* (A_t = *absorbance at time* t; A_∞ = *absorbance when the reaction is apparently complete.*) The *half-lives are as follows*—A, *methylcarbonate*, $t_{1/2}$ = *345 min.;* B, *butylcarbonate*, $t_{1/2}$ = *30 min.;* C, *isopropylcarbonate*, $t_{1/2}$ = *111 min.*

TABLE I—HALF-LIVES FOR HYDROLYSIS OF CARBONATE ESTERS OF ACETAMINOPHEN IN pH 7.4 PHOSPHATE BUFFER (0.1 *M*)[a]



R	Half-lives, min. 1% Human Plasma	Half-lives, min. Buffer	$t_{1/2}$ buffer/ $t_{1/2}$ plasma
$-CH_3$	345	9,000	26
$-C_2H_5$	90	12,000	133
$-C_4H_9$	30	18,000	600
$-C_6H_{13}$	22	22,800	1036
$-C_8H_{17}$	28	19,800	707
$-CH(CH_3)_2$	111	18,000	162
$-CH_2-CH(CH_3)_2$	37	21,000	568
$-C_6H_5$	52	240	4.6
$-C_6H_4-NH-C(=O)-CH_3$	230	420	1.8
$-CH_2-CH_2-Cl$	50	1,320	26
$-CH_2-C-Cl_3$	52	240	4.6

[a] With and without 1% v/v human plasma (37°).

These results are in agreement with the findings of Gordon *et al.* (2), who studied the effect of the carbon chain length of aliphatic alcohols on the ammonolysis of their acetate esters. They found that the rate of ammonolysis decreased to a constant minimum value at alcohol carbon chain lengths of about five. These results suggest that the hydrolysis of the acetaminophen carbonates is subject to essentially the same electronic effects as the hydrolysis of carboxylic acid esters and of other carbonate esters (3); that is, the alkyl groups tend to act as "electron pumps" depolarizing the carbonyl group and making it less susceptible to nucleophilic attack. As with carboxylic acid esters, this inductive effect levels off at alkyl chain lengths of about four or five carbons.

The rates of enzymatic cleavage of the acetaminophen carbonates of straight-chain alcohols in 1% human plasma increased with increasing chain length up to about six carbons. The figures shown in Table I in the column headed $t_{1/2}$ buffer/$t_{1/2}$ enzyme, which indicate the degree of enzyme catalysis relative to the nonenzymatic rate, follow the same pattern. Adams and Whittaker (4), in studies on human plasma cholinesterase, reported that the optimum chain length for either the acyl group or the alkyl group in a homologous series of carboxylic acid esters is four carbons. Hofstee (5) found that esters of fatty acids with seven carbons in the acyl chain are more susceptible to chymotrypsin catalyzed hy-

TABLE II—HALF-LIVES FOR HYDROLYSIS OF CARBONATE ESTERS OF ACETAMINOPHEN IN pH 7.4 PHOSPHATE BUFFER (0.1 *M*)[a]

R	Buffer	Half-lives, min. ($t_{1/2}$ buffer/$t_{1/2}$ enzyme)[b] Mouse	Guinea Pig	Rat	Cat	Human Plasma	Rabbit	Dog	Sheep
$-CH_3$	9000	30 (700)	51 (176)	95 (95)	294 (31)	345 (26)	79 (114)	40 (225)	335 (27)
$-C_2H_5$	12000	15 (800)	35 (343)	101 (119)	86 (140)	90 (133)	115 (104)	156 (77)	557 (22)
$-C_4H_9$	18000	6.9 (2610)	3.7 (4865)	6.7 (2687)	18 (1000)	30 (600)	50 (360)	27 (667)	520 (35)
$-CH(CH_3)_2$	18000	40 (450)	20 (900)	108 (167)	118 (153)	111 (162)	238 (76)	...	...
$-CH_2-CH(CH_3)_2$	21000	7.5 (2800)	5.6 (3750)	7.9 (2658)	12 (1750)	37 (568)	143 (147)	55 (382)	318 (66)
$-C_6H_5$	240	0.17 (1412)	0.25 (960)	1.3 (185)	10 (24)	52 (4.6)	...	100 (2.4)	165 (1.5)
$-C_6H_4-NHCOCH_3$	420	1.5 (280)	1.5 (280)	3.6 (117)	11 (38)	130 (3.2)	71 (5.9)	111 (3.8)	63 (6.7)
$-CH_2-CCl_3$	240	0.21 (1143)	0.93 (258)	1.1 (218)	10 (24)	52 (4.6)	18 (13)	100 (2.4)	50 (4.8)

[a] Containing 1% serum from animals or 1% human plasma (37°). [b] The numbers in parentheses are $t_{1/2}$ buffer/$t_{1/2}$ enzyme ratios which represent the degree of enzymatic catalysis.

drolysis than esters of other fatty acids. The enzymes in human plasma responsible for the hydrolysis of acetaminophen carbonate esters appear to behave in a similar way. However, since human plasma contains a mixture of esterolytic enzymes, at least part of the dependence of the enzymatic rates on structure may be attributed to variations in the contributions made by the several plasma enzymes to the overall hydrolysis rates of the esters.

Half-lives for the hydrolyses of two acetaminophen carbonate esters of branched chain aliphatic alcohols are also shown in Table I. Branching slows both the nonenzymatic and the enzymatic hydrolysis reactions slightly, but the effect is less pronounced in the isobutyl than in the isopropyl case. The slowing effect of branching is probably due to inductive electronic effects similar to those observed by Gordon *et al.* (2) in their studies of the ammonolysis of acetate esters of ethanol, butanol, isopropanol, and isobutanol. Their results show a direct parallel with the nonenzymatic cleavage data for the corresponding acetaminophen carbonate esters shown in Table I.

Chain branching apparently has comparatively little effect on the degree of enzymatic catalysis of the acetaminophen carbonates by human plasma as shown by the closeness of the $t_{1/2}$ buffer/$t_{1/2}$ enzyme values for the ethyl and isopropyl derivatives and for the butyl and isobutyl derivatives. Thus, it would appear that branching in the aliphatic alcohol chain has much less of an effect on the human plasma catalyzed hydrolysis reaction than does the total number of carbons in the chain. This is an important finding with regard to the selection of derivatives for the preparation of carbonate ester prodrugs. Derivatives of branched alcohols generally have lower melting points and higher aqueous and nonaqueous solubilities than those of straight-chain alcohols (1). Since branching seems to have little effect on the hydrolysis of the carbonates, branched derivatives with their greater aqueous solubilities might be more rapidly available for oral absorption but equally susceptible to enzymatic hydrolysis as the straight-chain derivatives.

Table I shows hydrolysis data for the phenyl- and the *p*-acetaminophenylcarbonates of acetaminophen. The results suggest that aromatic groups apparently affected both the electronic state and the affinity of human plasma enzymes for these molecules. The phenolic carbonates were much more rapidly hydrolyzed in pH 7.4 buffer than the hexylcarbonate, probably because of the electrophilic character of the aromatic rings which polarize the carbonyl group and make it more susceptible to nucleophilic attack. The phenolic carbonates, however, were much less susceptible to enzymatic cleavage than is the hexylcarbonate, possibly because the hexyl group has a greater affinity for the catalytic sites on the esterolytic enzymes of human plasma.

The influence of chlorine substitution on the enzymatic and nonenzymatic hydrolysis of the ethylcarbonate of acetaminophen is illustrated in Table I. These data show that increasing chlorine substitution beta to the carbonate linkage progressively increased the nonenzymatic hydrolysis rate. The effect was quite pronounced in going from the ethyl to the monochloroethyl derivative and was apparently due to the capacity of chlorine to act as an "electron sink" causing increased polarization of the carbonyl

group. On the other hand, progressive chlorine substitution appears to decrease the susceptibility of the ethylcarbonate ester to enzymatic hydrolysis. Again this effect might be due either to deteriorating enzyme substrate "fit," or to changes in the contributions made by the various enzymes to the overall hydrolysis rate as chlorine substitution is increased.

The results of the studies on the phenolic carbonates and the chlorine substituted ethylcarbonates of acetaminophen suggest that prodrug carbonates of phenols and of alcohols containing electrophilic substituents are relatively more susceptible to nonenzymatic cleavage but relatively less susceptible to enzymatic cleavage than prodrug carbonates of aliphatic alcohols. Thus, phenols and electrophilic alcohols may form carbonate ester prodrugs which are less stable pharmaceutically and less labile to enzymatic cleavage *in vivo* than those formed by aliphatic alcohols.

Table II shows half-lives for the hydrolysis of eight carbonate esters of acetaminophen in pH 7.4 phosphate buffer (0.1 *M*) containing 1% blood serum from seven animals or 1% human plasma. Values for $t_{1/2}$ buffer/$t_{1/2}$ enzyme for each of the enzyme systems are shown in parentheses. The results show that serum from all the animals contains esterases capable of catalyzing the hydrolysis of the acetaminophen carbonate esters, although in several instances the catalysis is very weak.

For almost every species, the aliphatic carbonate esters can be ranked in the following order of susceptibility to enzymatic attack: butyl $\cong$ isobutyl > isopropyl $\cong$ ethyl > methyl. In serum from small rodents (mouse, guinea pig, and rat) the phenyl- and 2,2,2-trichloroethylcarbonates were about equally susceptible to enzymatic attack and the *p*-acetaminophenylcarbonate was somewhat less susceptible. In serum from the other animals and in human plasma, the *p*-acetaminophenylcarbonate was somewhat more susceptible to enzymatic attack than the phenyl- and 2,2,2-trichloroethylcarbonates.

For almost every compound, the sera of the animals can be ranked in the following approximate order of catalytic potency: mouse > guinea pig > rat > cat > human > rabbit > dog > sheep, although in several cases guinea pig and mouse could be interchanged in this ranking.

Augustinsson (6) reported wide species differences in both the concentrations and the specificities of the esterases normally found in the sera of animals and man. Since serum contains several esterolytic enzymes, each with its own specificities for the substrates, at least part of the dependence of the enzymatic rates on structure in a given sample of serum may be attributed to variations in the contributions made by the plasma enzymes to the overall hydrolysis rates. The specificities and the concentrations of the enzymes in the serum vary from species to species, and are probably largely responsible for the various hydrolysis rates observed for a given substrate in the sera of various animals.

Thus, it is not surprising that it is difficult to make broad generalizations based on the data of Table II. However, the following conclusions can be drawn: (*a*) if an acetaminophen carbonate ester has a comparatively large $t_{1/2}$ buffer/$t_{1/2}$ enzyme ratio in the serum of one species, it will probably also have comparatively large $t_{1/2}$ buffer/$t_{1/2}$ enzyme ratios in the sera of all species, and (*b*) the sera of small rodents seem to possess either higher concentrations of esterases, or esterases which have a greater affinity for acetaminophen carbonate esters than the sera of the other animals or human plasma.

All the compounds hydrolyzed in 1% human plasma. It would be expected that all would release free acetaminophen in the blood of humans fairly rapidly if they indeed reached the blood intact following oral administration. In almost every case, the compounds were hydrolyzed more rapidly in the serum of common laboratory test animals (except rabbit and dog) than in human plasma, and it would be expected that free acetaminophen would be rapidly released following oral absorption in these animals. Thus, human plasma is a convenient test system for studying the hydrolysis of carbonate ester prodrugs because hydrolysis in this system suggests that hydrolysis would also occur in the blood of humans and laboratory test animals *in vivo*. Lack of hydrolysis in dilute human plasma, however, would not necessarily mean that hydrolysis in the tissues of laboratory test animals and humans is not possible.

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Keyphrases

Acetaminophen prodrugs
Carbonate ester, acetaminophen—hydrolysis
Hydrolysis—enzymatic, nonenzymatic
UV spectrophotometry—analysis

Basically Substituted Dioxolanes and Dioxanes

By OLE GISVOLD and H. V. MAULDING, JR.*

The synthesis of a group of basically substituted 1,3-dioxolanes and 1,3-dioxanes is reported. Cyclic acetals and ketals were prepared by the acid catalyzed, azeotropic distillation method, utilizing either a 1,2- or a 1,3-diol and either *p*-nitrobenzaldehyde or *p*-nitroacetophenone as the carbonyl compound, followed by catalytic reduction of the aromatic nitro group to the corresponding primary amine. All members of this series have a *p*-amino-phenyl substituent at position 2 of the dioxolane or dioxane ring in order to conform structurally as closely as possible to the sulfonamide and procaine prototypes. These compounds exhibited no antibacterial action *in vitro*. One compound had local anesthetic activity.

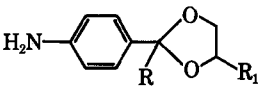
REPLACEMENT OF ESTERS by either substituted or unsubstituted 1,3-dioxolanes and/or 1,3-dioxanes has upon occasion led to effective medicinal agents. Bovet's acetal (1) is one of the most active muscarinic agents ever found. Blicke and co-workers (2–4) produced potent antispasmodics by replacement of the —CHCOO— of adiphenine with basically substituted 1,3-dioxolane and 1,3-dioxane rings. Recently, Hardie and co-workers (5) have reported on the local anesthetic and spasmolytic properties of a series of 4-(2-piperidyl)-1, 3-dioxolanes.

The ester linkage usually found in the procaine prototype is not a necessity as aminoalkyl ethers of the general formula $ArO(CH_2)_3NR_2$ (6) are efficient local anesthetics. Thus it was hoped that dioxolanes and dioxanes of the general formulas (I) and (II) might possess some degree of local anesthetic activity, as they closely resemble procaine and the alkylamino benzoates, with substitution of the ester group by the oxygenated ring representing the major structural modification.

Although there are certain general requirements for anti-*p*-aminobenzoic acid activity these stipulations are not nearly so specific as they may appear at first glance. The structural features requisite for antagonism of PAB are basically: (*a*) a primary aromatic amino group, and (*b*) an electronegatively charged group *para* to the amine (7). Sulfur is not necessary as the sodium *p*-amino-phenyl-arsonate (8) and diaminobenzil (9) are antimetabolites of the acid. As the critical distances between (*a*) the two ring oxygens and (*b*) the ring oxygens and the hydrogens of the primary aromatic amine in the dioxolanes (I) and the dioxanes (II) are quite similar to those stated for sulfanilamide and the *p*-aminobenzoate anion (7) (about 2.4 and 6.8 Å., respectively), it was thought that they might possess some degree of anti-*p*-aminobenzoic acid activity.

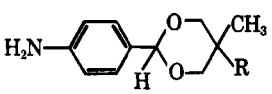
The intermediate halodioxolanes and halodioxanes (Tables I and II) required for the synthesis of the corresponding amino compounds were prepared by condensation of the aldehyde or ketone with glycerol-α-monobromohydrin or 2-methyl-2-bromomethyl-1,3-propanediol (10) in the presence of *p*-toluenesulfonic acid according to the procedure of Salmi (11). The nonhalogenated dioxolanes were prepared in the same manner using the appropriate 1,2-glycol. As it had been found previously that 5-methyl-5-bromomethyl-1,3-dioxanes reacted sluggishly with secondary amines, the bromo dioxane was converted to the iodo derivative (12) so as to obtain the wanted amines.

The 4-aminomethyl-1,3-dioxolanes (Table I) and the 5-methyl-5-aminomethyl-1,3-dioxanes (Table II) were produced by heating the intermediate halo compound, the amine and benzene in a pressure bottle on either a steam bath or in an oven for 6 to 7 days (2–4). In the case of the intermediate amino dioxolanes, these compounds were oils which decomposed on attempted distil-



(I)

R = H, CH_3
R_1 = H, CH_2Ar, CH_2NR_2, CH_2N cyclic alkyl

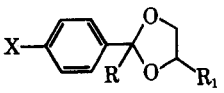


(II)

R = CH_2NR_2, CH_2N cyclic alkyl

Received May 19, 1966, from College of Pharmacy, University of Minnesota, Minneapolis, MN 55455
Accepted for publication January 30, 1968.
Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.
The authors wish to thank Dr. W. T. Sumerford of Mead Johnson Research Center, Evansville, Indiana, for microbiological screening, and Dr. Harvey Kupferberg of the University of Minnesota for the preliminary tests for anesthetic activity.
* Present address: Sandoz Pharmaceutical, Hanover, N. J.

TABLE I—PHYSICAL CONSTANTS OF 1,3-DIOXOLANES



Compd.	X	R	R_1	M.p.[a] °C.	Yield, %	Molecular Formula	Anal., %[b] Calcd.	Found
1	NO_2	H	H	89–91[c]	73	$C_9H_9NO_4$[d]	C ...	...
							H ...	...
2	NH_2	H	H	75–79[e]	86	$C_9H_{11}NO_2$[e]	C, 65.43	65.52
							H, 6.71	6.79
3	NO_2	CH_3	H	73–75[c]	77	$C_{10}H_{11}NO_2$	C, 57.41	57.38
							H, 5.30	5.28
4	NH_2	CH_3	H	105–109[c]	44	$C_{10}H_{13}NO_2$	C, 67.02	67.01
							H, 7.31	7.37
5	NO_2	H	CH_2Br	70–106[c, f]	60	$C_{10}H_{10}BrNO_2$	C, 41.70	42.08
							H, 3.47	3.51
6	NO_2	H	$CH_2N(C_2H_5)_2$	165[c, g]	70[h]	$C_{20}H_{23}N_5O_{11}$[g]	C, 47.10	47.03
							H, 4.55	4.61
7	p-$NO_2C_6H_4CH{=}NH$[i]	H	$CH_2N(C_2H_5)_2$	68–78[c]	59	$C_{21}H_{25}N_3O_4$	C, 65.78	65.37
							H, 6.58	6.73
8	NO_2	H	$CH_2NC_5H_{10}$[j]	175–178[k]	73[h]	$C_{16}H_{23}N_2O_4I$[k]	C, 44.24	44.21
							H, 5.33	5.38
9	p-$NO_2C_6H_4CH{=}NH$[i]	H	$CH_2NC_5H_{10}$[j]	90–105[c]	55	$C_{22}H_{25}N_3O_4$	C, 66.81	67.02
							H, 6.37	6.54
10	NO_2	H	$CH_2NC_4H_8O$[l]	173–176[k]	84[h]	$C_{15}H_{21}N_2O_5I$[k]	C, 41.26	41.60
							H, 4.85	4.74
11	NH_2	H	$CH_2NC_4H_8O$[l]	104–114[c]	82	$C_{14}H_{20}N_2O_3$	C, 63.61	63.65
							H, 7.62	7.46
12	NO_2	H	$CH_2N(C_4H_9)_2$	135–137[c, g]	74[h]	$C_{24}H_{31}N_5O_{11}$[g]	C, 50.96	51.06
							H, 5.52	5.36
13	p-$NO_2C_6H_4CH{=}NH$[i]	H	$CH_2N(C_4H_9)_2$	60–64[c]	69	$C_{25}H_{33}N_3O_4$	C, 68.31	68.69
							H, 7.56	7.65
14	p-$NO_2C_6H_4CH{=}NH$[i]	H	$CH_2NC_6H_{12}$[m]	95–104[c]	49	$C_{23}H_{27}N_3O_4$	C, 67.46	67.39
							H, 6.64	6.71
15	NO_2	H	$CH_2C_6H_5$	45–59[c]	61	$C_{16}H_{15}NO_4$	C, 67.35	67.27
							H, 5.30	5.21
16	p-$NO_2C_6H_4CH{=}NH$[i]	H	$CH_2C_6H_5$	108–118[c]	22	$C_{23}H_{20}N_2O_4$	C, 71.12	71.53
							H, 5.19	5.41
17	NO_2	CH_3	$CH_2C_6H_5$	123–135[c]	66	$C_{17}H_{17}NO_4$	C, 68.21	68.27
							H, 5.73	5.82
18	p-$NO_2C_6H_4CH{=}NH$	CH_3	$CH_2C_6H_5$	104.5–109[c]	42	$C_{24}H_{22}N_2O_4$	C, 71.37	71.49
							H, 5.51	5.62

[a] Uncorrected melting points were taken on a Thomas Hoover capillary melting point apparatus. [b] Microanalytical data were provided by the University of Minnesota Microanalytical Laboratory, Minneapolis. [c] Recrystallized from ethanol. [d] *Reference 24.* [e] Previously reported as an oil, (b.p. 150°, 1 mm.), *Reference 25.* [f] Mixture of diastereoisomers. [g] Analyzed as picrate. [h] Yield of base. [i] All primary aromatic amines in this series with the exceptions of compounds 2, 4, and 11 were isolated as p-nitrobenzylidene derivatives due to their instability in the free form. [j] Piperidino. [k] Analyzed as methiodide. [l] Morpholino. [m] 1-Methylpiperidino.

lation and were isolated as picrates or methiodides for analytical purposes.

The 2-p-aminophenyl dioxolanes and dioxanes (Tables I and II) were obtained by reduction of the corresponding 2-p-nitro-compounds using platinum oxide as the catalyst (13–15). In all cases where the 2-p-aminophenyl group was present, the compounds showed some degree of instability with the dioxolanes apparently being less stable than the dioxanes. All attempts at salt formation with the reduced substances failed resulting in a yellow polymeric material which was possibly due to the hydrolysis of the acetal or ketal to p-aminobenzyaldehyde and its subsequent Schiff base self-condensation (16, 17).

The Schiff bases were quite easily formed by treatment of the 2-p-aminophenyl-dioxanes and dioxolanes with p-nitrobenzaldehyde (18, 19). They were stable and the only method by which some of the primary aromatic amino compounds could be isolated without decomposition.

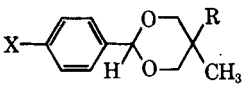
It should be noted that in all cases where the possibility of diastereoisomerism existed in preparation of these compounds it appeared to occur. However, upon preparation of methiodides or picrates from the mixture of isomers a sharp melting point was shown indicative of molecular compound formation (20, 21).

EXPERIMENTAL

The compounds prepared are listed in Tables I and II. The general preparative procedures are illustrated with specific examples.

2-p-Nitrophenyl-5-methyl-5-bromomethyl-1,3-dioxane(II,1)—A mixture of 16.5 Gm. (0.1 mole) of p-nitrobenzaldehyde, 20 Gm. (0.11 mole) of 2-methyl-2-bromomethyl-1,3-propanediol (10),

TABLE II—PHYSICAL CONSTANTS OF 1,3-DIOXANES



Compd.	X	R	M.p.[a] °C.	Yield, %	Molecular Formula	Anal., %[b] Calcd.	Found
1	NO_2	CH_2Br	111–112[c]	46.5[d]	$C_{12}H_{14}NO_4Br$	C, 45.58	45.36
						H, 4.46	4.59
2	NO_2	CH_2I	121.5–124[c]	94	$C_{12}H_{14}NO_4Br$	C, 39.68	40.08
						H, 3.88	4.09
3	NO_2	$CH_2N(C_2H_5)_2$	58–60[c]	64	$C_{16}H_{24}N_2O_4$	C, 62.31	62.29
						H, 7.79	7.89
4	p-$NO_2C_6H_4CH{=}NH$[e]	$CH_2N(C_2H_5)_2$	146–149[c]	48[f]	$C_{23}H_{29}N_3O_4$	C, 67.13	67.20
						H, 7.10	7.25
5	NO_2	$CH_2NC_5H_{10}$[g]	88–89[h]	75	$C_{17}H_{24}N_2O_4$	C, 63.73	63.93
						H, 7.55	7.33
6	NH_2	$CH_2NC_5H_{10}$[g]	122–123[h]	23	$C_{17}H_{26}N_2O_2$	C, 70.34	70.61
						H, 9.05	9.11
7	p-$NO_2C_6H_4CH{=}NH$[e]	$CH_2NC_5H_{10}$[g]	150–153[c]	46	$C_{24}H_{29}N_3O_4$	C, 67.83	67.57
						H, 6.90	7.07
8	NO_2	$CH_2NC_4H_8O$[i]	146–149[c]	39	$C_{16}H_{22}N_2O_5$	C, 59.59	59.51
						H, 6.88	7.05
9	NH_2	$CH_2NC_4H_8O$[i]	129–132[c]	91	$C_{16}H_{24}N_2O_3$	C, 67.73	66.05
						H, 8.27	8.43
10	p-$NO_2C_6H_5CH{=}NH$[e]	$CH_2NC_4H_8O$[i]	184–186[c]	51	$C_{23}H_{27}N_3O_5$	C, 64.93	64.91
						H, 6.39	6.54

[a] Uncorrected melting points were taken on a Thomas-Hoover capillary melting point apparatus. [b] Microanalytical data were provided by the University of Minnesota Microanalytical Laboratory, Minneapolis. [c] Recrystallized from ethanol. [d] A second fraction, m.p. 52–76°, was isolated which gave correct analysis and was presumably the second diastereoisomer. [e] Primary aromatic amines isolated as stable p-nitrobenzylideneanilino-derivatives. [f] Yield based on weight of compd. 3 used in reduction. [g] Piperidino. [h] Recrystallized from methanol. [i] Morpholino.

one crystal of p-toluenesulfonic acid, and 100 ml. of benzene was refluxed (11, 22) for 20 hr. in a flask to which a Dean Stark water trap (23) was attached. The warm mixture was filtered and shaken out three times with 10% sodium carbonate followed by distilled water. The benzene was removed *in vacuo* and the resultant solid dissolved in 350 ml. of ethanol. The precipitate was collected and recrystallized from ethanol yielding 17.0 Gm. (46.5%), m.p. 111–112°.

2-p-Nitrophenyl-4-diethylaminomethyl-1,3-dioxolane (I, 6)—A solution of 14.4 Gm. (0.05 mole) of 2-p-nitrophenyl-4-bromomethyl-1,3-dioxolane and 14.6 Gm. (0.20 mole) of diethylamine in 50 ml. of benzene was heated in a pressure bottle on a steam bath for 6 days (2–4). The diethylamine hydrobromide was removed by filtration and the solvent and excess diethylamine evaporated. The resultant oil was taken up into 100 ml. of benzene followed by extraction with 10% citric acid. The acidic extracts were neutralized with solid sodium carbonate. The oily amine was again dissolved in benzene and the preceding acid-base extraction repeated twice more. The amine was taken up in ether and dried with anhydrous sodium sulfate. Upon evaporation of the ether 9.9 Gm. (70%) of a yellow oil, n_D^{21} 1.5262, was obtained which was found to decompose on attempted distillation (0.5 mm.). A picrate (m.p. 165°) was prepared for analysis by adding dropwise an ethanolic solution of picric acid to a solution of the amine in ethanol.

2-p-Aminophenyl-4-(1-piperidylmethyl)-1,3-dioxolane (Not Isolated)—A solution of 2.1 Gm. of 2-p-nitrophenyl-4-(1-piperidylmethyl)-1,3-dioxolane in 50 ml. of absolute ethanol with 100 mg. of platinum oxide (13) was reduced on the Parr hydrogenator (15) at 30 lb. pressure and the shaking stopped when the calculated amount of hydrogen had been taken up. The catalyst was removed by filtration, the solvent removed *in vacuo*, and the pale yellow oil used immediately for preparation of the Schiff base. All attempts at salt preparation failed.

2-[4-(N-4'-Nitrobenzylideneanilino)]-4-(1-piperidylmethyl)-1,3-dioxolane (I, 9)—An equimolar quantity of p-nitrobenzaldehyde (995 mg.) was added to the 2-p-aminophenyl-4-(1-piperidylmethyl)-1,3-dioxolane obtained from the preceding step and the mixture heated under reduced pressure on a water bath (18, 19). After 15 min. the oil had turned bright orange. It was removed from the water bath and refluxed for 20 min. with 10 ml. of ethanol. The Schiff base began to crystallize as bright gold needles on cooling to room temperature and on recrystallization from absolute ethanol yielded 1.5 Gm. (55%), m.p. 90–105°.

2-p-Aminophenyl-4-(4-morpholinomethyl)-1,3-dioxolane(I, 11)—A solution of 6.35 Gm. of 2-p-nitrophenyl-4-(4-morpholinomethyl)-1,3-dioxolane in 30 ml. of absolute ethanol with 100 mg. of platinum oxide (13) was reduced on the Parr hydrogenator (15) at 30 lb. pressure until hydrogen uptake ceased (about 10 min.). The resulting mixture was filtered through a sintered-glass filter to remove catalyst and the filtrate placed in the deep freeze. After 2 weeks a white crystalline precipitate was noted which was removed and recrystallized three times from absolute ethanol, yielding 4.8 Gm. (82.5%) of small white plates, m.p. 104–114°. The infrared spectrum showed the presence of a primary aromatic amino group.

MICROBIOLOGY AND PHARMACOLOGY

These series of compounds were subjected to microbiological screening on *E. coli*, *S. aureus*, two

species of Mycobacteria, *S. cerevisiae*, *C. albicans*, and *G. candidum*. None showed appreciable inhibition of any test organisms at concentrations of 100 mcg./ml.

None of the compounds had surface anesthetic activity when tested in the rabbit's eye.

Intracutaneous injection of a 0.25% solution of compound 13 (Table I) in guinea pigs caused anesthesia of 30 min. duration.

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Keyphrases

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Azeotropic distillation reaction—acid catalyzed
Microbiological screening
Anesthesia, local—screening

Dissolution Rates in Surfactant Solutions Under Stirred and Static Conditions

By M. GIBALDI, S. FELDMAN, R. WYNN, and N. D. WEINER*

The dissolution rate of benzoic acid was determined in water and solutions of polyoxyethylene (23) lauryl ether by the rotating disk and static disk methods. The results under stirred and static conditions substantially deviated from the Noyes-Whitney equation. Dissolution rates in surfactant solutions were much less than anticipated on the basis of solubilization data. The ratios of dissolution rate in surfactant solution to that in pure solvent were found to be significantly greater under static conditions as compared to the ratios determined under stirred conditions, suggesting a possible change in dissolution mechanism. Certain aspects of dissolution rate theory are explored to explain this unusual phenomenon.

THE INFLUENCE of interacting colloids such as polymers or micellar aggregates on drug solubility has been investigated extensively and the literature contains numerous reports concerning drug solubilization in colloidal systems. However, only a limited amount of information is available on the influence of solubilization on dissolution rate.

Taylor and Wurster (1) found significant increases in both the solubility and dissolution rate of various forms of prednisolone in 0.1% solutions of sodium lauryl sulfate. Since this concentration is considerably below the critical micelle concentration (CMC) and since the extent of solubiliza-

Received September 1, 1967, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication January 25, 1968.
This investigation was supported in part by grant 50-1077A from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service, Bethesda, Md.
* Present address: College of Pharmaceutical Sciences, Columbia University, New York, NY 10023

species of Mycobacteria, *S. cerevisiae*, *C. albicans*, and *G. candidum*. None showed appreciable inhibition of any test organisms at concentrations of 100 mcg./ml.

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Dissolution Rates in Surfactant Solutions Under Stirred and Static Conditions

By M. GIBALDI, S. FELDMAN, R. WYNN, and N. D. WEINER*

The dissolution rate of benzoic acid was determined in water and solutions of polyoxyethylene (23) lauryl ether by the rotating disk and static disk methods. The results under stirred and static conditions substantially deviated from the Noyes-Whitney equation. Dissolution rates in surfactant solutions were much less than anticipated on the basis of solubilization data. The ratios of dissolution rate in surfactant solution to that in pure solvent were found to be significantly greater under static conditions as compared to the ratios determined under stirred conditions, suggesting a possible change in dissolution mechanism. Certain aspects of dissolution rate theory are explored to explain this unusual phenomenon.

THE INFLUENCE of interacting colloids such as polymers or micellar aggregates on drug solubility has been investigated extensively and the literature contains numerous reports concerning drug solubilization in colloidal systems. However, only a limited amount of information is available on the influence of solubilization on dissolution rate.

Taylor and Wurster (1) found significant increases in both the solubility and dissolution rate of various forms of prednisolone in 0.1% solutions of sodium lauryl sulfate. Since this concentration is considerably below the critical micelle concentration (CMC) and since the extent of solubiliza-

Received September 1, 1967, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication January 25, 1968.
This investigation was supported in part by grant 50-1077A from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service, Bethesda, Md.
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tion appeared to be related to the activity of the crystal form, the authors suggested that a cooperative phenomenon was responsible for the increased solubility. Furthermore, a major factor in the increased dissolution rate appeared to be an apparent increase in the interfacial rate constant by virtue of the surfactant assisting in the release of surface molecules through interfacial tension lowering. Hence, the influence of micellar solubilization, if any, on the dissolution rate of prednisolone is this study cannot be assessed.

Bates, Gibaldi, and Kanig (2) reported substantial increases in the dissolution rates of griseofulvin and hexestrol in micellar solutions of bile salts. In each case, because of the extremely low solubility of both drugs, dissolution rate was followed in a concentration region which was above the saturation solubility in water.

Until recently the influence of interacting colloids on dissolution rate in a drug concentration region below saturation solubility had received only theoretical consideration. Higuchi (3) presented a theoretical analysis pertinent to the dissolution of solids in colloidal solutions. The equations resulting from this analysis predict that the effect on dissolution rate as a function of colloidal solubilizer concentration will be far less than the effect predicted by the Noyes-Whitney relationship (4). The latter suggests a direct proportionality between dissolution rate and total solubility. The Higuchi equations (3) further predict that substantial effects on dissolution rate will be observed only when the drug concentration in solution approaches or exceeds saturation solubility. This prediction is in agreement with the findings of Bates *et al.* (2) and the earlier findings of Wurster and Polli (5).

Experimental evidence confirming the theoretical predictions were presented in a recent review (6). The dissolution rate of benzocaine in various concentrations of polysorbate 80[1] was found to be in agreement with the diffusional model proposed by Higuchi rather than the Noyes-Whitney equation. Higuchi (6) has further suggested that the magnitude of effects of interacting colloids on dissolution rates could be used for the differntiation of dissolution mechanism.

The present report concerns a study of the solubilization and dissolution rate of benzoic acid in solutions of a nonionic ether-type surfactant. Dissolution rate was followed under stirred and static conditions. It is the purpose to demonstrate that the effect of micellar solubilization on dissolution rate cannot be predicted by the Noyes-Whitney equation and to propose that different mechanisms of dissolution may be operative under stirred and static conditions.

[1] Tween 80, Atlas Chemical Industries, Wilmington, Del.

EXPERIMENTAL

Materials—Baker analyzed reagent benzoic acid was used as the dissolving solid and polyoxyethylene (23) lauryl ether,[2] served as the solubilizing agent.

Solubility Determinations—Benzoic acid solubility was determined in a series of aqueous solutions containing various concentrations of polyoxyethylene (23) lauryl ether. In each case, an amount of benzoic acid in excess of the amount required for saturation solubility was added to the surfactant solutions contained in 25-ml. culture tubes. The tubes were sealed, placed in a Metabolyte incubator shaker (New Brunswick Scientific Co., N.J.), and equilibrated at 37° for 3–5 days. Equilibrium was determined by repetitive sampling.

Determination of Dissolution Rate—Dissolution rates were determined at 37° by the rotating disk (7, 8) and static disk (9) methods, using 300 ml. of water or aqueous solutions of polyoxyethylene (23) lauryl ether. Speed of rotation for the rotating disk experiments was 100 r.p.m.

Assay—Aliquot samples were passed through a Millipore filter (0.45 μ pore size), when necessary, and appropriately diluted with 0.1 *N* HCl. Benzoic acid concentration was determined spectrophotometrically at 230 mμ using a Beckman DB-G recording spectrophotometer. In each case, an appropriate concentration of surfactant was contained in the blank to avoid interference.

RESULTS AND DISCUSSION

Figure 1 shows the saturation solubility of benzoic acid as a function of surfactant concentration. The results describe a typical solubilization curve with a linear relation between solubility and polyoxyethylene (23) lauryl ether concentration above the CMC. Solubilization studies at low surfactant concentrations indicate a CMC of less than 0.1 Gm./100 ml.

According to the Noyes-Whitney relationship (4), under sink conditions and with constant surface area, dissolution rate (*d.r.*) may be described by the following equation:

$$d.r. = KC_s \qquad \text{(Eq. 1)}$$

where K is a constant and C_s is the solubility. In nonmicellar systems, $K = D/h$ where D is the solute molecule diffusion coefficient, and h is the effective diffusion layer thickness. If one assumes that both D and h remain invariant if the drug is dissolved in a medium containing colloidal solubilizer, then it may be deduced from Eq. 1 that

$$R = \frac{Cs^*}{Cs} \qquad \text{(Eq. 2)}$$

where R is the ratio of dissolution rate in surfactant solution to that in pure solvent and Cs^* is the drug solubility in surfactant solution.

Using the solubility data obtained in the present

[2] Brij 35 SP, Batch No. 1340B, generously supplied by Atlas Chemical Industries, Wilmington, Del.

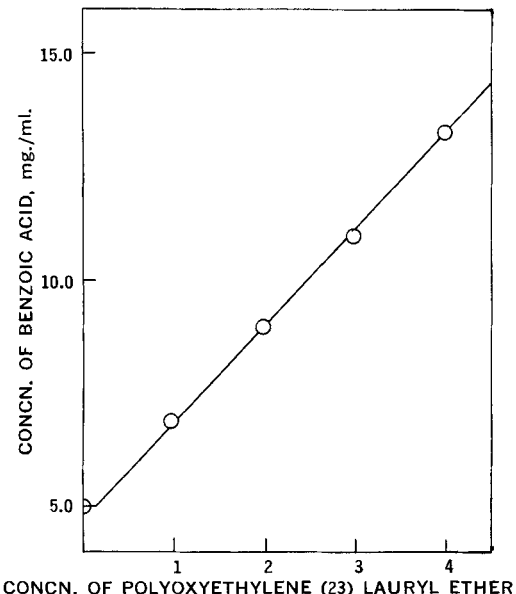

Fig. 1—Saturation solubility of benzoic acid as a function of surfactant concentration.

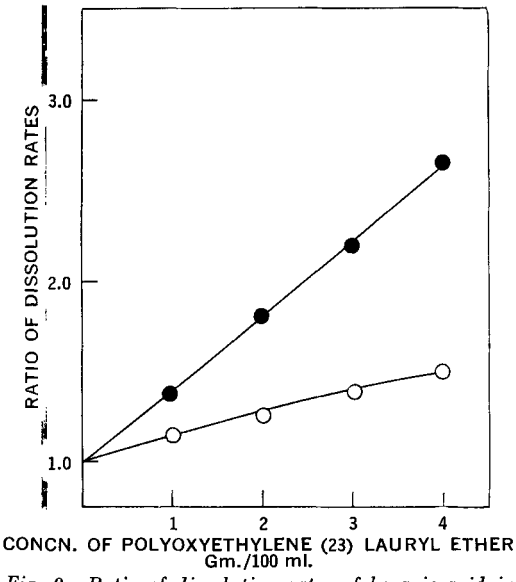

Fig. 2—Ratio of dissolution rates of benzoic acid in surfactant solution to that in water. Key: ●, upper curve was constructed by means of the Noyes-Whitney equation and solubility data: ○, lower curve represents experimental dissolution rate data.

study the theoretic dissolution rate ratios employing Eq. 2 were determined. The results are shown in Fig. 2 in contrast to the experimental dissolution rate ratios determined at 100 r.p.m. The curves in Fig. 2 clearly demonstrate that the Noyes-Whitney relation fails to predict dissolution behavior in systems containing colloidal solubilizers. Dissolution rates of benzoic acid in solutions containing polyoxyethylene (23) lauryl ether are substantially less than anticipated on the basis of solubilization data and the application of the Noyes-Whitney equation.

The dissolution of a drug in the presence of an interacting colloid such as a micelle involves consideration of two diffusing species, *viz.*, the free drug and the drug–micelle interaction product (3). In view of the large difference in molecular weight between the drug molecule and the micellar species, one anticipates a significant difference with respect to the diffusion coefficient of each species. For this situation, the diffusion layer theory gives the following equation (3):

$$d.r. = \frac{DCs}{h} + \frac{D'Cs'}{h} \qquad \text{(Eq. 3)}$$

where D' is the diffusion coefficient of the micelle-solubilized drug and Cs' is the solubility increase due to solubilization. If we assume that h, D, and D' are independent of the concentration of colloidal solubilizer then the following ratio (R) of dissolution rate in surfactant solution to that in pure solvent is predicted by diffusion layer theory.

$$R = \frac{DCs + D'Cs'}{DCs} \qquad \text{(Eq. 4)}$$

As indicated in Eq. 4 the greater the difference between D and D' the greater the negative deviation of R from the ratio value predicted by the Noyes-Whitney equation.

If one assumes that the data under stirred conditions adheres to the diffusion layer theory, a theoretic diffusion coefficient for the micelle-solubilized drug may be determined. Using the experimental R values, the appropriate solubilization data, and a value of 1.2×10^{-5} cm.²/sec. as the diffusion coefficient for benzoic acid (10), an apparent mean diffusion coefficient of 3.8×10^{-6} cm.²/sec. for the micelle-solubilized benzoic acid was calculated.

The ratios predicted by the diffusion layer theory are shown as the dashed line in Fig. 3. The predicted curve fits the experimental data reasonably well. The fit suggests, in agreement with Higuchi (3, 6), that a diffusion layer model can be used to explain the effect of colloidal solubilizers on dissolution rate.

The dissolution studies under static conditions were prompted by the possibility that different mechanisms of dissolution exist under extremely low agitation conditions and under stirred conditions. The results of these studies were used to calculate dissolution rate ratios which are plotted in Fig. 3. At each polyoxyethylene (23) lauryl ether concentration studied the ratio under static conditions was significantly higher than the ratio under stirred conditions.

An advantage of studying dissolution in systems involving interacting materials which result in diffusing species with significantly different diffusion coefficients is the possibility of mechanism differentiation (6). For example, if hydrodynamic conditions are maintained constant, dissolution rate ratios as a function of solubilizer concentration will be the same regardless of the intensity of agitation. A change in the magnitude of a dissolution rate ratio suggests a change in the hydrodynamics of the system and a possible change in the dissolution mechanism. Thus, the difference in dissolution rate ratios observed under static conditions provides evidence for the existence of different dissolution mechanisms.

An alternative to the diffusion layer theory to consider in describing dissolution rate mechanism

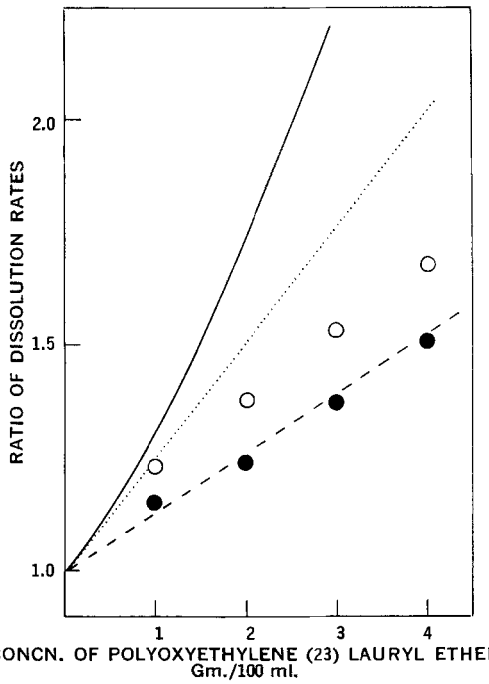

Fig. 3—Ratio of dissolution rates of benzoic acid in surfactant solution to that in water under static (○) *and stirred conditions* (●). *Key: theoretical ratios predicted by Noyes-Whitney theory* (——); *Danckwerts' theory* (· · ·); *and diffusion layer theory* (– – –).

under static conditions is one originally proposed by Danckwerts (11) to describe dissolution phenomenon under turbulent conditions. This model envisions macroscopic packets of solvent impacting the solid-liquid interface by eddy diffusion. Uptake of solute into the packet occurs upon impaction and these saturated surface elements are continually replaced by new packets of solvent. Although the Danckwerts' model was proposed originally to explain dissolution under turbulent conditions, a type of eddy diffusion may exist under static conditions and a transference of concept may be possible.

The appropriate rate expression for dissolution rate per unit area under sink conditions in accord with the Danckwerts' model is

$$d.r. = S^{1/2}D^{1/2}Cs \qquad \text{(Eq. 5)}$$

where S is the mean rate of production of fresh surface. When considering dissolution of a material in a medium of colloidal solubilizer, Higuchi (6) has shown that Danckwerts' theory leads to the following rate equation:

$$d.r. = S^{1/2}\sqrt{(Cs + Cs')(DCs + D'Cs')} \qquad \text{(Eq. 6)}$$

where the terms have the same meaning as previously described. If we assume that S, D, and D' are independent of concentration of solubilizer, then the following ratio of dissolution rate in surfactant solution to that in pure solvent is predicted by Danckwerts' theory.

$$R = \frac{\sqrt{(Cs + Cs')(DCs + D'Cs')}}{D^{1/2}Cs} \qquad \text{(Eq. 7)}$$

Theoretical values of R were calculated from Eq. 7 using an apparent mean diffusion coefficient of 3.8×10^{-6} cm.²/sec. for the micelle-solubilized benzoic acid. The theoretical relation of R and concentration of surfactant is shown as the dotted line in Fig. 3. It is apparent that the Danckwerts' model fits the static dissolution data only at the 1% level. At higher concentrations of polyoxyethylene (23) lauryl ether, progressive deviation is observed.

Several possibilities may be explored to rationalize the lack of agreement of the experimental data with the model. One possibility is that surface renewal rate is dependent on the surfactant concentration, *i.e.*, $S^{1/2} \alpha 1/(\text{surfactant})$. The dependence could involve viscosity or some other physical-chemical property of the system which is a function of polyoxyethylene (23) lauryl ether, concentration.

A second possibility is that the mechanism of dissolution under static conditions involves a combination of the diffusion layer and Danckwerts' models. Such a combination has been termed the film-penetration model (12). This model is similar to the Danckwerts' model except that solvent packets impact a diffusion layer rather than the solid-liquid interface. Interestingly, the film-penetration model has been shown to predict dissolution under turbulent conditions, more accurately than the Danckwerts' model (12).

A further possibility which cannot be ruled out is that dissolution under static conditions actually adheres to Dankwerts' theory, while dissolution under stirred conditions (under the present experimental conditions) manifests negative deviation from the results predicted by this model. The evidence, to date, that dissolution under stirred conditions follows diffusion layer theory is strictly empirical. There has been no independent verification of the model, as for example, by the independent determination of the diffusion coefficient of the drug-micellar species. Hence, the various possibilities cannot be resolved without further investigation.

BIOLOGIC IMPLICATIONS

The present report describes certain dissolution phenomena which may have an important bearing on dissolution in the gastrointestinal tract as well as on the general area of *in vivo-in vitro* correlation. The most successful methodology employed to date to relate *in vitro* dissolution rate with *in vivo* absorption has been described by Levy (13–15) and appears to involve a dissolution mechanism describable by diffusion layer theory. By means of this dissolution method Levy *et al.* (16) have reported quantitative correlation of gastrointestinal absorption in man of aspirin from three markedly different types of dosage forms. The correlation may be expressed as

$$\%\text{ – absorbed at time } T = \%\text{ – dissolved in } \frac{T - \text{lag time}}{2}$$

There is no doubt as to the precision of the correlation. However, in terms of absolute amounts, about twice the amount of drug was dissolved *in vitro* than was absorbed *in vivo* over the same time period. Correlation was observed at 50–60 r.p.m. and failed to exist at higher or lower r.p.m. Thus, it is not possible to correct the correlation in absolute

terms by simply decreasing the agitation rate.

It is proposed that the problem we face in developing *in vivo-in vitro* correlations could involve the mechanism of dissolution as well as the intensity of agitation. The nature of the dissolution mechanism in the gastrointestinal tract is not at all clear. In view of the type of agitation a dissolving solid encounters in the tract as a result of gastrointestinal motility, it is quite conceivable that dissolution will not follow pure diffusion layer theory. The process may be obscured by periodic and irregular diffusion currents which could result in a highly complex dissolution mechanism. Perhaps a combination of diffusion layer and Danckwerts' mechanisms may be operative. A great deal of further experimentation on dissolution is required to resolve these questions.

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Static conditions—dissolution rates

Kinetics of Reaction of Dehydroacetic Acid II

Reaction With Primary Amines

By S. GOTO, A. KONO, and S. IGUCHI

It was concluded that the conversion of 2,6-bis-(phenethylamino)-2,5-heptadiene-4-one (BPH) into *N*-phenethyl-lutidone (PL) in 80 percent ethanol is accounted for in terms of aminolysis reaction. In general, the aminolysis reaction proceeds *via* a two-step reaction path involving a *gem*-diamine as an intermediate. The first step in the above reaction of BPH becomes reversible by the addition of excess β-phenethylamine (PE-NH). The mechanistic pathway was discussed. Moreover, it was supported with kinetic investigation using an analog computer that BPH is the most important intermediate in the conversion of 3-(1-phenethylamino)ethylidene-6-methyl-3*H*-pyran-2,4-dione (Schiff's base) to PL in the presence of large excess PE-NH.

IN A PREVIOUS paper (1), the kinetic study of Schiff's base formation between dehydroacetic acid (DHA) and β-phenethylamine (PE-NH) has been shown. A further kinetic investigation on the transformation of Schiff's base to *N*-phenethyllutidone (PL)[1] is described, and also an attempt was made to confirm kinetically the reaction mechanism that was proposed by Iguchi *et al.* (2).

EXPERIMENTAL

Materials—Ethanol of superior special grade for precision analysis was used. PE-NH (Wako pure reagent) was redistilled twice (b.p. 178–179°). DHA (Taito Pfizer Co.) was recrystallized from ethanol–water, m.p. 109–110°. Commercial acetic acid was redistilled, and sodium acetate and sodium chloride of pure reagent grade were used for the kinetic studies.

Preparation of 3-(1-Phenethylamino)ethylidene-6-methyl-3H-pyran-2,4-dione (Schiff's Base)—DHA (10 Gm.) was dissolved in 15 ml. ethanol solution containing an equivalent mole of PE-NH and the solution was warmed on a steam bath. After 30 min. the solvent of reaction mixture was distilled *in vacuo*, the residue was washed once with ethyl ether, and recrystallized from ethyl ether–benzene, m.p. 89–91°.

Preparation of 2,6-Bis-(phenethylamino)-2,5-heptadiene-4-one (BPH)—To 5 ml. of ethanol solution of DHA (3.4 Gm.) was added an excess of

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PE-NH and kept at room temperature for 24 hr. After the solvent was evaporated, the residue was washed once with ethyl ether and then recrystallized, m.p. 115–116°.

Preparation of *N*-Phenethyl-lutidone (PL)—One gram of BPH was dissolved in 20 ml. of water, and a few drops of hydrochloric acid or acetic acid were added. After 10 hr. heating, the reaction solution was dried *in vacuo*. The residue was recrystallized from aqueous methanol, m.p. 167–168°.

Kinetic Studies—BPH was dissolved in 80% v/v ethanol which had previously been adjusted to the desired pH with the addition of acetate buffer solution. The pH values were determined with a Hitachi-Horiba pH meter using glass-saturated calomel electrodes. The ionic strength of the solution was adjusted to 0.1. The solution was transferred to a glass 100-ml. volumetric flask and placed in a constant temperature bath maintained at 60°. Aliquots (1 ml.) were withdrawn at regular time intervals, and the reaction was quenched by cooling in ice water and diluted with distilled water. The progress of the reaction was determined by measuring the samples for residual BPH spectrophotometrically. The products, intermediate (X) and PL, were also determined spectrophotometrically. In the case of conversion reaction of BPH or Schiff's base in the presence of excess PE-NH, the sample solutions were prepared by dissolving substrates in various concentrations of PE-NH (0.0–1.0 *M*).

Calculations of Amounts of Products, BPH, and Schiff's Base—The absorbances of the sample solution were measured against a blank solution at 266 mμ, 322 mμ, and 370 mμ. The calculations are based on the relations;

$$A_{266} = \epsilon^{PL}_{266}(PL)$$

$$A_{322} = \epsilon^{X}_{322}(X) + \epsilon^{BPH}_{322}(BPH)$$

$$A_{370} = \epsilon^{BPH}_{370}(BPH) + \epsilon^{X}_{370}(X)$$

Molar absorptivity values are $\epsilon^{PL}_{266} = 19{,}900$, $\epsilon^{X}_{322} = 20{,}000$, $\epsilon^{BPH}_{322} = 3{,}600$, $\epsilon^{BPH}_{370} = 41{,}600$, $\epsilon^{X}_{370} = 2{,}500$,

where $A\lambda$ is the absorbance at a wavelength λ in mμ for the sample solution, and (PL), (X), and (BPH) are molar concentrations of PL, intermediate, and BPH, respectively. The molar absorptivity value of intermediate could be estimated from the initial concentration of BPH, the molar absorptivity value of BPH, and the absorbances of the sample solution at 322 mμ and 370 mμ in the initial reaction time when the formation of PL was not observed.

In the case of conversion reaction of Schiff's base in the presence of excess PE-NH, the absorbances of the sample solution were measured at 266 mμ, 310 mμ, and 370 mμ. The calculations for each compound follow:

$$A_{266} = \epsilon^{PL}_{266}(PL) + \epsilon^{S}_{266}(S)$$

$$A_{310} = \epsilon^{S}_{310}(S) + \epsilon^{BPH}_{310}(BPH)$$

$$A_{370} = \epsilon^{BPH}_{370}(BPH)$$

Molar absorptivity values are: $\epsilon^{S}_{266} = 1{,}800$, $\epsilon^{S}_{310} = 16{,}100$, $\epsilon^{BPH}_{310} = 2{,}600$. Other values were already described, where (S) is the concentration of Schiff's base in the aliquot. The percentage deviation for above analytical procedure was ±4%. The existence of PE-NH did not interfere with their determinations.

Thin-Layer Chromatographic Analysis—The thin-layer plates (5 × 20 cm.) were coated with a 0.5-mm. layer of Silica Gel B-5 (Wako pure reagent). After the sample solution was spotted on the plates, the plates were developed with ethyl ether. The solvent front was allowed to advance 13 cm. When the developed plates were exposed to iodine vapor in a sealed glass box, the visible spots with R_f values corresponding to BPH (0.47) and PL (0.0) were recognized. When acetone was used, the R_f value of PL ascended 0.15. In the case of the reaction between Schiff's base and PE-NH, the R_f value for Schiff's base was 0.22. Unfortunately, it was impossible to get an R_f value for the intermediate on the developed plates.

Computer Analysis—A Beckman Instrument Inc., Ease analog computer was utilized for this investigation.

RESULTS AND DISCUSSION

Conversion of 2,6-Bis-(phenethylamino)-2,5-heptadiene-4-one (BPH) in Acetate Buffer Region—An attempt was made at first to investigate the conversion of BPH to PL in acetate buffer region. The apparent first-order rate constants for the loss of BPH decreased rapidly with the increase of experimental pH as shown in Table I.

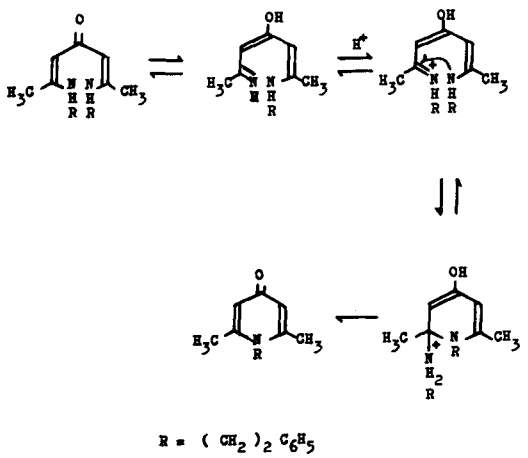
The conversion of BPH was followed by the loss of spectrophotometric absorbance. Because absorbance at 370 mμ decreased with time concurrent with increase in absorbance over the region 322 to 266 mμ, the following route was postulated:

$$\text{BPH} \xrightarrow{k_1} \text{intermediate (X)} \xrightarrow{k_2} \text{PL}$$

TABLE I—APPARENT FIRST-ORDER RATE CONSTANT FOR THE CONVERSION OF BPH IN ACETATE BUFFER REGION AT 60°[a]

Observed pH	k_1	k_2
4.82	...	7820
6.41	...	226
7.11	630, 620[b]	46.0, 46.0[b]
7.53	219	22.0
8.18	51.3, 54.5[c]	8.80, 8.80[c]
8.50	25.1, 27.0[d]	2.00, 2.00[d]
9.85[e]	6.97	1.13
10.1[f]	7.00	3.37

[a] $10^4\ k_1$ and $10^6\ k_2$ in sec.$^{-1}$. Initial concentration: BPH, 0.8 to 3.0 × $10^{-3}M$. The ionic strength was adjusted to 0.1 by the addition of sodium chloride. [b] When the concentration of acetate buffer solution was at twofold ranges, no significant differences in the apparent rate constants were observed. [c] When the concentration of acetate buffer solution was at a half range, no significant differences in the apparent rate constants were observed. [d] When the concentration of substrates was twofold ranges, no significant differences in the apparent rate constants were observed. [e] No acetate buffer solution was used. [f] Sodium acetate was used.



R = $(CH_2)_2C_6H_5$

Scheme I—Proposed route for the conversion of BPH into PL.

The first-order rate constants for the conversion of BPH at pH values below 6.4 were quite large. The reason for this behavior is considered as follows. Under more acidic conditions, all BPH is predominantly protonated and the intramolecular amine attached to the protonated carbon atom becomes an important reaction pathway, and it is expected that the conversion rate of BPH is related to the existence of protonated BPH. The pH-independent region for k_1 is observed under a weakly basic condition and the rate becomes small in this region. It is probably the consequence of the minimum protonated BPH existence. These results may be accounted for in terms of intramolecular aminolysis reaction that is the replacement of the amine component by another amine in the same molecule. Numerous papers (3–5) have mentioned that the imines, particularly when existing in cationic form $\left(\diagup C{=}\overset{+}{N}\diagdown\right)$, readily react with various nucleophilic reagents involving water and amines, and that the reaction is accelerated with an addition of hydrogen ions or an increase in negative charge of nucleophilic reagents. The conversion mechanism of BPH to PL was proposed as shown in Scheme I.

The reaction proceeds *via* a two-step reaction path involving a *gem*-diamine as an intermediate. Such an intermediate is analogous to the carbinolamine for hydrolysis and formation of Schiff's base. Moreover, the formation and decomposition of the *gem*-diamine are expected to be a function of pH. Koehler *et al.* (3) have stated "although the existence of an intermediate in the aminolysis reaction of Schiff's base has yet to be demonstrated, the nature of the reaction almost certainly required that they proceed via a two-step reaction path involving a gem-diamine as intermediate." In our experiment, the existence of the intermediate was not shown by the thin-layer chromatogram. But it is expected that the absorbance at 322 mμ may show the existence of the intermediate.

Conversion of 2,6-Bis-(phenethylamino)-2,5-heptadiene-4-one (BPH) in the Presence of Excess β-Phenethylamine (PE-NH)—The first step in BPH conversion reaction became reversible in the presence of excess PE-NH. When a great excess of PE-NH was added, the equilibrium constant (K) governing the formation of the intermediate (X) from BPH was smaller than in the presence of small excess PE-NH. It may be explained by the acceleration of reverse reaction (X → BPH) in more basic conditions because it was observed that the reverse reaction rate became quite large in a 0.04 N NaOH solution. The conversion rate of the intermediate (X) into PL was also accelerated proportionally to the increasing PE-NH. This fact may be rationalized in terms of the general acid catalytic reaction by the protonated PE-NH in this step. Therefore, the process of the conversion of BPH to PL can be described as follows:

$$\text{BPH} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{intermediate (X)} \overset{k_2}{\longrightarrow} \text{PL}$$

Based on the assumption, $k_1 + k_{-1} \gg k_2$, the molar concentrations of BPH, intermediate (X), and PL over the whole reaction period can be described by approximate equations (6) as shown in the following:

$$(\text{BPH}) = \frac{k_{-1}}{(k_1 + k_{-1})} \cdot e^{-\frac{k_1}{(k_1 + k_{-1})} \cdot k_2 t} \quad \text{(Eq. 1)}$$

$$(\text{X}) = \frac{k_1}{(k_1 + k_{-1})} \cdot e^{-\frac{k_1}{(k_1 + k_{-1})} \cdot k_2 t} \quad \text{(Eq. 2)}$$

$$(\text{PL}) = 1 - e^{-\frac{k_1}{(k_1 + k_{-1})} \cdot k_2 t} \quad \text{(Eq. 3)}$$

Where (BPH), (X), and (PL) are the molar concentrations of BPH, intermediate, and PL, respectively. The k_1 and k_{-1} are the rate constants for forward and reverse reactions, respectively, and k_2 is the rate constant for formation of PL from the intermediate. The equilibrium constant ($K = k_1/k_{-1}$) shown in Table II, can be estimated from experimental results.

Under the experimental condition (BPH: $1 \times 10^{-2}M$, PE-NH: 1.0 M) in which PE-NH is

TABLE II—SUMMARY OF RATE CONSTANT[a] AND EQUILIBRIUM CONSTANT ON THE REACTION OF BPH IN THE PRESENCE OF EXCESS β-PHENETHYLAMINE AT 60°

REACTION: $\text{BPH} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{X} \overset{k_2}{\longrightarrow} \text{PL}$

(BPH) M	(PE-NH) M	$\frac{(\text{PE-NH})}{(\text{BPH})}$	$K\left(=\frac{k_1}{k_{-1}}\right)$	k_1	k_{-1}	k_2
0.00146	0.0	0.0	...	6.97	0.0	0.0113
0.00139	0.017	12.2	9.7	6.53	0.673	0.0219
0.00155	0.024	15.5	6.5	6.22	0.957	0.0246
0.00105	0.100	95.4	1.8	6.40	3.56	0.140

[a] 10^4k in sec.$^{-1}$.

TABLE III—SUMMARY OF RATE CONSTANT[a] AND EQUILIBRIUM CONSTANT ON THE REACTION OF BPH IN THE PRESENCE OF EXCESS β-PHENETHYLAMINE AT 60°

REACTION: $BPH \underset{}{\overset{K}{\rightleftharpoons}} X \xrightarrow{k_2} PL$, with $BPH \xrightarrow{k_3} C$

(BPH) M	(PE-NH) M	(PE-NH)/(BPH)	K	k_2	k_3
0.010	0.20	20.0	0.33	0.120	0.008
0.010	0.50	50.0	0.18	0.190	0.008
0.010	1.00	100.0	...	0.019	0.007
				$\widetilde{k_{app.}}$[b]	

[a] k in hr.$^{-1}$. [b] $k_{app.}$ represents the apparent first-order rate constant for the conversion of BPH into PL.

greatly in excess of substrate, the reaction appearance has been changed, that is, the concentration of the intermediate (X) was maintained quite small or almost zero over the entire experimental period of kinetic run. On the other hand, it was determined experimentally that the amount of PL formed was not equal to the amount of BPH decreased. Based on the above result, another route must be established. Three pathways are considered:

$BPH \rightleftharpoons X \rightarrow PL$, with $BPH \rightarrow C$ (Route 1)

$BPH \rightleftharpoons X \rightarrow PL$, with $X \rightarrow C$ (Route 2)

$BPH \rightleftharpoons X \rightarrow PL \rightarrow C$ (Route 3)

But Route 3 should be neglected because no degradation was observed for PL over the entire experimental time. The selection of a suitable route was conducted by using an electronic analog computer. Since the equilibrium between BPH and the intermediate (X) is quickly formed at the initial stage of conversion reaction, the rate constant in each reaction step can be easily estimated. When their validity was checked by the analog computer, a good agreement was found between the computer calculated concentration-time curves and the experimental results in the case of Route 1. And the Route 2 may be omitted. The sign C shows the overall products except PL and they are assumed to be mainly various condensation products. The obtained values for rate constants and equilibrium constants are shown in Table III.

Conversion of Schiff's Base in the Presence of Excess β-Phenethylamine (PE-NH)—The experimental condition of treating a great excess of PE-NH (1.0 M) over the Schiff's base (1×10^{-2} M) was selected for the purpose of deciding on a major reaction route. The route for the conversion of Schiff's base to BPH would be attacked by an amine molecule on 6 position of carbon atom, opening the pyronl ring and continuous decarboxylation. But, unfortunately, no intermediates in the above reaction route could be isolated synthetically and spectrophotometrically. Based on the assumption that the concentrations of intermediates are almost zero, the apparent first-order rate constant on the conversion of Schiff's base into BPH was calculated. Typical changes of the spectral curves as a function of time are given in Fig. 1.

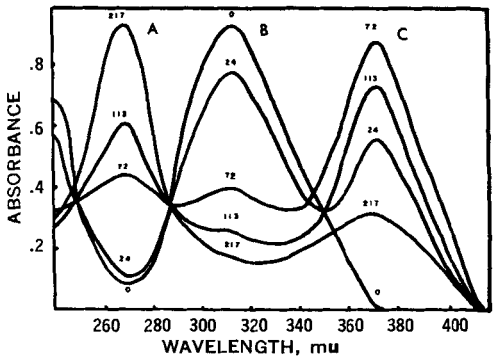


Fig. 1—The spectral changes of the reaction between Schiff's base and excessive β-phenethylamine in 80% ethanol at 60°. Initial concentration: Schiff's base, 1×10^{-2}M; β-phenethylamine 1.0 M. Key: A, PL; B, Schiff's base; C, BPH. The time of the readings after the start of the reaction is given in hours.

The changes of maximum absorbance at 310 mμ are characteristic of the first-order conversion of Schiff's base, because the intermediate (X) ($\lambda_{max.}$ = 322 mμ) formed remains almost zero during the solution conversion. The obtained first-order rate constant for loss of Schiff's base is adapted to Eqs. 4–7 together with the rate constants which were calculated in the previous section for BPH conversion in the presence of a great excess of PE-NH.

Schiff's base (S) $\xrightarrow{k}$ (0.0145 hr.$^{-1}$) BPH $\xrightarrow{k'}$ (0.019 hr.$^{-1}$) PL; BPH $\xrightarrow{k''}$ (0.007 hr.$^{-1}$) other products (C)

Therefore, the differential equations for the above route are:

$$d(S)/dt = -k(S) \quad \text{(Eq. 4)}$$

$$d(BPH)/dt = k(S) - k'(BPH) - k''(BPH) \quad \text{(Eq. 5)}$$

$$d(PL)/dt = k'(BPH) \quad \text{(Eq. 6)}$$

$$d(C)/dt = k''(BPH) \quad \text{(Eq. 7)}$$

The circuit diagram used in an electronic analog computation for the above equations is shown in Fig. 2.

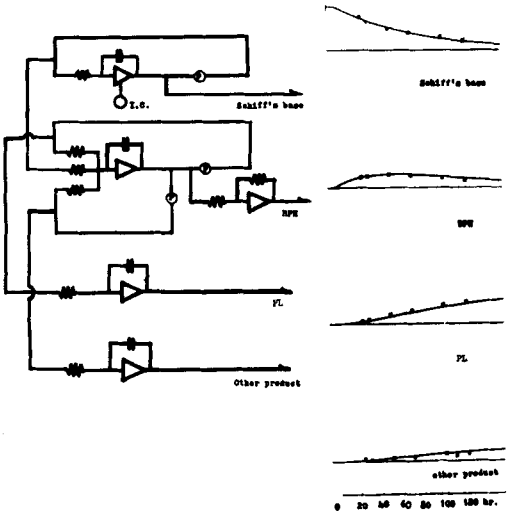


Fig. 2—Block diagram and computer curves obtained by computer analysis. Key: ——, obtained curve; •, experimental value

The agreement between the obtained and experimental values supports the idea that the reaction proceeds on the strength of the above mechanism represented by the simplified scheme and that BPH is the most important intermediate in the conversion route of Schiff's base into PL in the presence of a great excess of PE-NH.

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Keyphrases

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Aminolysis reaction
UV spectrophotometry—kinetic analysis
TLC—analysis

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Received December 8, 1967, from the Department of Biological Sciences, Philadelphia College of Pharmacy and Science, Philadelphia, PA 19104
Accepted for publication January 9, 1967.
This research was supported in part by a grant from Smith Kline & French Laboratories, Philadelphia, PA 19101
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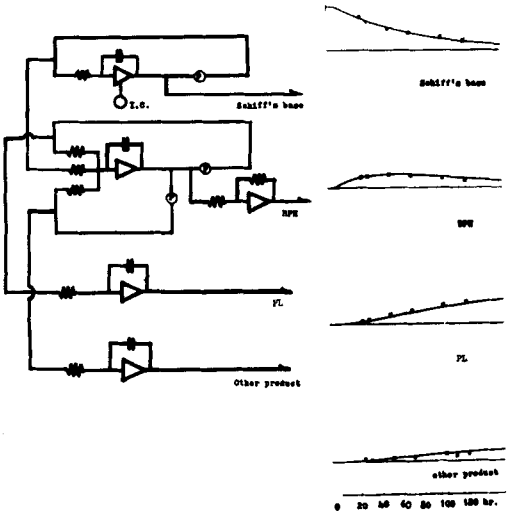

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The Thiry-Vella dog (1) provides a chronic *in situ* loop preparation consisting of a segment of small intestine, isolated with its blood, nerve, and lymph supplies intact, in which both the oral and aboral ends of the segment are exteriorized through the abdominal wall and sutured in place. Thus there is formed a readily accessible U-shaped loop of intestine having considerable physiologic integrity. With proper postsurgical care the fistula remains patent, enabling each animal to be used repeatedly and to serve as its own experimental control, thus providing a high degree of stability and reproducibility. Previous investigations utilizing the Thiry-Vella loop have been concerned primarily with the study of intestinal secretions (2, 3), the absorption of nutrient factors (4–8), and the effect of drugs on the motility of intestinal smooth muscle (9). The basic objective of the research described in this report was to explore the feasibility of the Thiry-Vella preparation as a biologic model for the evaluation of the absorption of drugs from the intestinal lumen.

EXPERIMENTAL

Preparation of Thiry-Vella Fistulae—Five male and 7 female mongrel dogs (9–13.6 Kg.) were used in the various phases of this investigation. Eight of these animals were surgically prepared with a Thiry-Vella fistula according to the procedure described by Markowitz (10). A postsurgical period of not less than 3 weeks elapsed prior to investigational use of each animal. All animals were maintained on a standard laboratory diet, and housed in temperature-, humidity-, and, light-controlled quarters.

Intestinal Instillation—Solutions for intestinal instillation contained either 75, 150, 300, or 450 mg. of *N*-acetyl-*p*-aminophenol (SKF ISO No. 141819) in 25 ml. of phosphate buffer at pH 5.8. Following anesthetization (sodium pentobarbital, 35 mg./Kg., intravenously) of the test animal, previously fasted for 18–24 hr., the fistula was washed with normal saline solution, warmed to 37° to remove any secretion or particulate matter. Washing was continued until the effluate was clear. Thirty minutes after termination of washing, clamped polyethylene tubing was inserted 2 cm. into each end of the fistula. One clamp was opened and a 25-ml. volume of *N*-acetyl-*p*-aminophenol buffered solution, warmed to 37°, was introduced into the fistula. The tubing was again clamped. Care was taken to insure that none of the instilled fluid was lost through leakage. Venous blood samples were obtained prior to and 6, 12, 18, 24, 30, 60, and 120 min. after instillation, and analyzed for total *N*-acetyl-*p*-aminophenol in the plasma.

Oral Administration—Gelatin capsules, containing either 75, 150, 300, or 450 mg. of *N*-acetyl-*p*-aminophenol, were administered orally. Immediately after swallowing the capsule, the test animal was anesthetized. Blood samples were obtained prior to and 15, 30, 60, 120, 180, 240, 300, and 360 min. after oral administration and analyzed for total *N*-acetyl-*p*-aminophenol in the plasma.

Intravenous Administration—Solutions for intravenous administration contained either 75, 150, 300, or 450 mg. of *N*-acetyl-*p*-aminophenol in 25 ml. of 0.9% saline. After anesthetization of the test animal, 25 ml. of the solution, previously warmed to 37°, was injected over a period of 30 sec. Blood samples for analysis were obtained prior to and 15, 30, 60, 120, 180, and 240 min. after injection.

Analytical Procedures—The blood plasma volume of each animal was determined by means of an isotope dilution technique using radio-iodinated (^{131}I) serum albumin (11). A modification (12) of the method of Brodie and Axelrod (13) was employed in assaying the total *N*-acetyl-*p*-aminophenol content of plasma samples.

RESULTS AND DISCUSSION

A critical consideration in the laboratory evaluation of new drugs and new dosage formulations intended for oral administration is the availability of feasible and relevant methodology with which to assess gastrointestinal absorption. Although various *in vitro* and *in vivo* techniques have contributed fundamentally to an understanding of the physicochemical principles involved in the absorption process, the applicability, and predictive value of current methodology in this field leaves much to be desired. Theoretical considerations led us to explore the Thiry-Vella dog as a potential pharmacometric system and, in our opinion, this research has provided support for the utility of the fistula preparation as a biologic model for the evaluation of drug absorption from the intestinal mucosa.

One of the technical problems requiring resolution was the method of administration of drug solution into the *in situ* intestinal loop. Constant rate perfusion of drug solution through the Thiry-Vella loop was found to produce swelling and distention of tissue which did not occur following introduction of relatively small fixed volumes into the isolated intestinal segment. Furthermore, pressure exerted on the intestinal wall, as occurs during perfusion, may artificially facilitate drug absorption. Comparative studies indicated that maintenance of the physiological integrity of the intestinal mucosa could best be achieved by the instillation of fixed volumes of solution.

Selection of *N*-acetyl-*p*-aminophenol as a tool for studying the feasibility of the Thiry-Vella dog as a biologic model for evaluation of drug absorption was based primarily on two considerations: availability of a quantitative procedure for the determination of *N*-acetyl-*p*-aminophenol in biological fluids and the predicted physical state of the compound at the pH of the mucosal segment. The pH of the jejunum in the dog has been reported (2) to range from pH 4 to 7.6. Under the conditions of this study, the pH of the Thiry-Vella jejunal loop was found to be pH 5.3 to 6.3. *N*-Acetyl-*p*-aminophenol has a pKa in excess of 12 and therefore would not be appreciably ionized at pH 5.8, the pH selected for the buffered solution of drug to be instilled in the Thiry-Vella loop. In view of the well-established concepts of the relative lipid solubility of the unionized moiety, it could be anticipated that the compound would be effectively absorbed from the jejunal loop at pH 5.8.

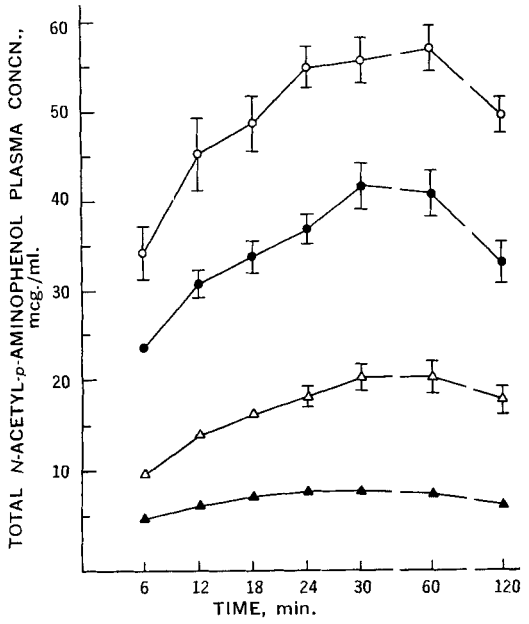

Fig. 1—Total N-*acetyl*-p-*aminophenol plasma concentration (mcg./ml.) in Thiry-Vella dogs following intestinal instillation of designated doses. Key:* ▲, *75 mg.;* △, *150 mg.;* ●, *300 mg.;* ○, *450 mg. in 25 ml. of phosphate buffer (pH 5.8). Each point represents the mean (± S.E.) response of 2 trials in each of 4 dogs. Where not indicated, the S.E. values are equivalent to less than 2 mcg./ml.*

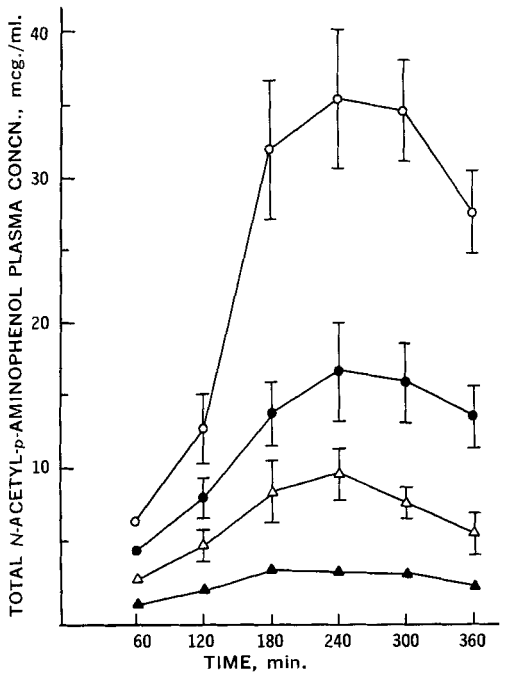

Fig. 2—Total N-*acetyl*-p-*aminophenol plasma concentration (mcg./ml.) in dogs without a Thiry-Vella fistula following oral administration of designated doses. Key:* ▲, *75 mg.;* △, *150 mg.;* ●, *300 mg.;* ○, *450 mg. Each point represents the mean (± S.E.) response of 2 trials in each of 4 dogs. Where not indicated, the S.E. values are equivalent to less than 2 mcg./ml.*

Since absorption was estimated as a function of the concentration of drug in the plasma, and since the blood plasma volume percent was essentially identical (4.35 ± 0.25%) in those animals prepared for this investigation, the experimental drug was administered on a mg./animal basis rather than on a mg./Kg. basis.

Following intestinal instillation of buffered solution of *N*-acetyl-*p*-aminophenol into the chronic Thiry-Vella fistula dog, absorption was initiated within the first 6 min., and the plasma concentration reached a peak within 30–60 min. Graphical representation of the data (Fig. 1) shows that within the dosage range examined, an increase in dose was reflected by an increase in the concentrations present in the plasma commensurate with the dose increments, and that the curves representing the plasma concentrations at the various posttreatment time periods for doses of 75, 150, 300, and 450 mg. were parallel. Therefore, as the dose of the experimental drug was increased the amount absorbed into the blood was increased indicating that the model quantitatively reflects drug absorption from the intestinal mucosa.

The apparent differences in the resulting plasma concentrations of *N*-acetyl-*p*-aminophenol among the various dosage levels were established by analysis of variance as being statistically significant (P = 0.01–0.001). Statistical analysis also indicated no lack of parallelism among the time-response curves.

Analysis of the plasma samples following intravenous injection revealed that the rate of disappearance of *N*-acetyl-*p*-aminophenol from the blood was relatively slow. A comparison of the plasma concentrations following intestinal instillation and those occurring subsequent to intravenous injection of identical doses indicated that, for the dosage range examined, from 80 to 98% of the drug introduced into the intestinal fistula was absorbed.

The characteristics of the Thiry-Vella fistula suggested that drug absorption from this relatively standardized mucosal segment would be subject to fewer variables than encountered upon exposure of the drug to the entire gastrointestinal tract. Therefore, it was of interest to compare absorption following instillation of the experimental drug into the *in situ* loop with that occurring after oral administration. Plasma levels subsequent to the oral administration of *N*-acetyl-*p*-aminophenol to dogs without a Thiry-Vella fistula evidenced (Fig. 2) substantially greater variability than those observed after instillation of identical doses of drug into the intestinal loop.

The applicability of the Thiry-Vella dog to the study of the kinetics of drug absorption is apparent. Following instillation, simultaneous sampling of the blood and the contents of the intestinal loop may be performed to determine the extent of retention, if any, of test agent in the intestinal compartment. Further, the drug may be injected intravenously and periodic samplings taken subsequently from the *in situ* loop. Thus, by determining the concentration of drug in the blood, that which remains in the loop, and that which may be transferred from the blood into the intestinal loop, it may be possible to

establish the kinetics of the absorption phenomena in this biologic model.

Although major abdominal surgery is required, the chronicity of the fistula dog permits each animal to serve as its own control. The same animal can be used repeatedly to compare the absorption characteristics of the same drug at different dosage levels, as well as different drugs at the same and alternate dosage levels. It may also be possible to evaluate absorption from various dosage formulations such as solutions, suspensions, single dose, and sustained-release solid dosage forms. Investigations of the interactions of drug combinations with regard to intestinal absorption are also feasible because the animal is serving as its own control. This is in contrast to other techniques, such as the ligated rat intestine, where the viability of the isolated segment is in question after a relatively short period of time.

Furthermore, the animal and *in situ* loop may be considered essentially identical for each experiment. The uniformity of the absorbing surface (*i.e.*, the loop) does not vary in a given animal as contrasted with the use of different animals. The mucosal epithelium, the blood, lymph, and nerve supplies remain essentially constant from one experiment to another.

The absorbing surface of the intestinal mucosa of the dog is closely related to that of man. Thus, preliminary investigations using Thiry-Vella dogs can provide data from which at least semi-quantitative predictions may be made of the absorption of a compound in man.

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Keyphrases

Drug absorption—intestinal mucosa
Thiry-Vella fistula dog—biologic model
Jejunal loop—Thiry-Vella fistula
N-Acetyl-*p*-aminophenol—absorption
Plasma-*N*-acetyl-*p*-aminophenol—analysis

Factors Affecting the Dissolution Rate of Medicaments from Tablets I

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By JAMES T. JACOB* and ELMER M. PLEIN

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THE IMPORTANCE of determining physiological availability of medicaments from tablets was recognized only recently. Even though the USP tablet disintegration test is not designed to show a measure of physiological availability of medicament, this test is used by manufacturers, as well as drug control agencies, as one of the criteria in determining the quality of tablets. There is evidence in the literature that this test leaves much to be desired as a control test in the production of tablets (1–3).

Nelson (4) reported that dissolution rate was the rate-determining factor in absorption of

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establish the kinetics of the absorption phenomena in this biologic model.

Although major abdominal surgery is required, the chronicity of the fistula dog permits each animal to serve as its own control. The same animal can be used repeatedly to compare the absorption characteristics of the same drug at different dosage levels, as well as different drugs at the same and alternate dosage levels. It may also be possible to evaluate absorption from various dosage formulations such as solutions, suspensions, single dose, and sustained-release solid dosage forms. Investigations of the interactions of drug combinations with regard to intestinal absorption are also feasible because the animal is serving as its own control. This is in contrast to other techniques, such as the ligated rat intestine, where the viability of the isolated segment is in question after a relatively short period of time.

Furthermore, the animal and *in situ* loop may be considered essentially identical for each experiment. The uniformity of the absorbing surface (*i.e.*, the loop) does not vary in a given animal as contrasted with the use of different animals. The mucosal epithelium, the blood, lymph, and nerve supplies remain essentially constant from one experiment to another.

The absorbing surface of the intestinal mucosa of the dog is closely related to that of man. Thus, preliminary investigations using Thiry-Vella dogs can provide data from which at least semi-quantitative predictions may be made of the absorption of a compound in man.

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tetracycline, provided that the dosage form restricted the initial surface area. He reported also that absorption of benzylpenicillin and of acetylsalicylic acid was proportional to the dissolution rate of the drugs in the gastrointestinal tract (5). Levy (6) has pointed out that the dissolution rate of a drug in particulate form is determined by a number of factors including intrinsic dissolution rate, particle size distribution, speed and type of agitation, anisotropism, and presence of other solids.

A variety of techniques is used to determine *in vitro* dissolution rates of medicaments from tablets. Levy and Hayes (7) used a beaker method to determine the dissolution rates of nationally advertised brands of aspirin. Later Levy devised the oscillating tube method and the rotating disk method (8). A revolving plastic dialysis cell containing a paper membrane was used by Marlowe and Shangraw (9) to determine dissolution rate of sodium salicylate from tablets. Schroeter and Wagner (10) used an automatic dissolution apparatus consisting of a USP tablet disintegration apparatus connected through a flow cell to a spectrophotometer, with attachments for filtration and dilution of solution. Several solid dosage forms were studied by this procedure.

A survey of recent literature shows several instances of clinical ineffectiveness of commercial tablets which met USP requirements including disintegration tests. These reports include prednisone tablets evaluated by Campagna *et al.* (3), studies by Carter (11), by Levy (12), and Brudney *et al.* (13) of tolbutamide tablets, and a study by Lozinski (14) of bishydroxycoumarin tablets. In each case the *in vitro* dissolution rate of the drug was found to be poor.

An *in vivo* dissolution and absorption test would likely give a more complete evaluation of a tablet formulation than could an *in vitro* test; however since an *in vivo* test would involve such factors as individual and species variation in absorption as well as tablet dissolution, interpretation of the results of such tests is very complex. Whereas *in vitro* dissolution tests do not register physiological availability of a drug *per se*, yet carefully designed *in vitro* dissolution tests can be used as an index of the physiological availability of active constituents from tablets. Different formulation and manufacturing procedures are known to influence the disintegration rate and the dissolution rate of tablets. The objectives of this study were (*a*) to determine the USP disintegration rate and the *in vitro* dissolution rate of a generic name product, namely phenobarbital tablets manufactured by several companies and (*b*) to study how some variable factors in manufacture and storage affect the availability of phenobarbital from the uncoated tablet. This portion of the report is concerned with commercially available tablets.

Phenobarbital is supplied in tablets of various strengths by a number of different pharmaceutical companies. Phenobarbital is a weak acid with a pKa of 7.5. It is readily absorbed from stomach, small intestine, rectum, and intramuscular sites. The mechanism of absorption from gastrointestinal sites is by nonionic diffusion (15).

EXPERIMENTAL

Commercial Phenobarbital Tablets—Forty-two lots of uncoated phenobarbital tablets of various strengths (1/4 gr., 1/2 gr., and 1 1/2 gr.) manufactured by 24 different pharmaceutical concerns within the United States were purchased either directly from the manufacturer or from wholesale drug suppliers. The dissolution rate of each lot of tablets was determined. The USP disintegration test was carried out on those lots of tablets which showed poor dissolution rate in the acid medium.

In Vitro Dissolution Test—A modified oscillating tube method originally designed by Levy (8) was used. The modified apparatus consisted of an oscillating tube which was a Plexiglas cylinder (19 cm. in length and 1.9 cm. in diameter) with a 40-mesh stainless steel screen fused to one end and a wire hook attached to the other end. The cylinder was attached to the basic unit of USP tablet disintegration apparatus by means of the wire hook (oscillating tube replacing basket-rack assembly). The cylinder was inserted through the opening of a plastic lid into a 400-ml. beaker containing 300 ml. of 0.1 *N* HCl of pH 1.2 (acid medium) or a buffer solution[1] (alkaline medium) of pH 9.5 maintained at 37° ± 0.5°. A 2-blade stirring shaft was introduced into the beaker through another opening in the plastic lid. The shaft was attached to a variable speed external stirrer.[2] The apparatus was set in motion, and a weighed tablet was dropped into the cylinder. At the end of 5 min. the apparatus was stopped, the cylinder was detached and gently raised above the level of the medium. The solution was then stirred at 600 r.p.m. for 30 sec. and 5 ml. was pipeted for analysis. The oscillating tube was then slowly immersed into the medium and the apparatus was set in motion once again. Five milliliters of medium was added to the solution in the beaker to replace that amount withdrawn. Samples were taken similarly at 10-, 20-, 30-, and 60-min. intervals and analyzed. Three runs were made on each lot of tablets and their average was recorded. If readings were not within 2% of average, then the experiment was repeated three more times and the average of the six readings was recorded.

Analytical Method—Phenobarbital was determined spectrophotometrically with a Beckman

[1] The buffer solution (boric acid–potassium chloride–sodium hydroxide) was prepared according to the method described by Mattson (16).

[2] Manufactured by Gerald K. Heller Co., Las Vegas, Nev.

TABLE I—DISSOLUTION RATES AND DISINTEGRATION TIMES[a] OF SOME COMMERCIAL PHENOBARBITAL TABLETS

Manufacturer	Strength, gr.[b]	% Phenobarbital Dissolved in Acid, 30 Min.	% Phenobarbital Dissolved in Acid, 60 Min.	% Phenobarbital Dissolved in Alkali, 30 Min.	Disintegration Time, Min., with Disks	Disintegration Time, Min., without Disks	Hardness[c]
A	1/2	69.9	100	74.2	12 (11–13.5)	48 (40–60)	4.5
B	1/4	25.0	32.0	100	12 (11–13)	134 (100–168)	2.5
C	1/2	43.0	100	100	8 (7–12)	23 (16–28)	3.5
D	1/4	80.0	100	86.6	6 (6–7)	34 (28–40)	5.5
E	1/2	18.4	29.8	22.2	20 (18–21)	65 (55–90)	9.0
F	1/2	42.0	82.5	50.0	16 (13–18)	48 (46–49)	5.5
G	1 1/2	11.2	18.3	20.4	45 (38–51)	...[d]	11.0
H	1 1/2	80.6	100	88.1	20 (19–20)	50 (39–64)	10.5
I	1 1/2	43.6	67.2	84.8	7 (6–8)	...[d]	7.0
J	1/2	89.3	100	46.9	19 (15–20)	...[e]	7.0
K	1 1/2	60.9	74.0	53.7	4 (3–5)	202 (150–270)	5.5
L	1/2	84.7	100	35.6	7 (7–8)	33 (30–35)	2.5
M	1 1/2	51.7	78.6	49.6	24 (20–25)	...[d]	6.5

[a] Determined by USP XVII procedure. [b] The approximate metric equivalents of 1/4 gr., 1/2 gr., and 1 1/2 gr. are 15 mg., 30 mg., and 100 mg., respectively. [c] As Strong-Cobb units. [d] All 6 tablets remained on the screen at the end of 120 min. [e] Three tablets disintegrated in 100–115 min.; 3 remained on the screen at the end of 120 min.

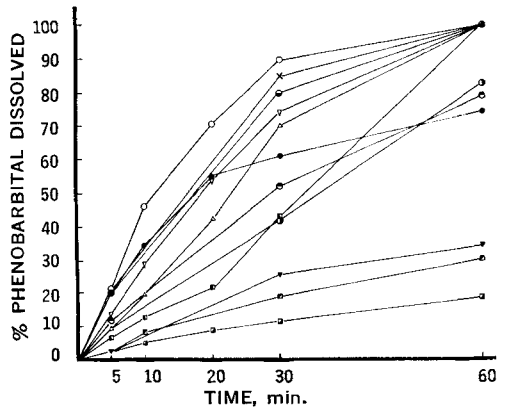

Fig. 1—Dissolution rates in 0.1 N HCl, of some commercial phenobarbital tablets. Key: ○, J 1/2 gr.; ×, L 1/2 gr.; ◒, D 1/4 gr.; ▽, H 1 1/2 gr.; △, A 1/2 gr.; ●, K 1 1/2 gr.; ◓, M 1 1/2 gr.; ◧, C 1/2 gr.; ◑, F 1/2 gr.; ▼, B 1/4 gr.; ◩, E 1/2 gr.; ◪, G 1 1/2 gr.

model DU spectrophotometer. Solutions were filtered through Whatman No. 41 filter paper and were diluted appropriately with buffer solution of pH 9.5 and the absorbance was measured at 240 mμ.

Disintegration Test—The USP XVII tablet disintegration test with and without disks was carried out on 13 lots of tablets which showed poor dissolution rates in acid medium. Actual disintegration times were recorded.

RESULTS AND DISCUSSION

Dissolution—Twenty lots of tablets out of 42 lots studied released 100% phenobarbital within 5 min., 4 lots within 5–10 min., 3 lots within 10–20 min., and 2 lots within 20–30 min. when a 0.1 *N* HCl bath was used. Thirteen lots of tablets failed to release 100% phenobarbital into the acid medium within 30 min. (Table I). At the end of 60 min. of test, 3 out of the 13 lots of tablets did not release at least 50% of the drug. A comparison of dissolution pattern of 12 out of these 13 lots of tablets is illustrated in Fig. 1. One lot (manufacturer I, 1 1/2 gr.) showed considerable tablet-to-tablet variation in dissolution rate. In order to get an approximation of the dissolution rate of the tablets in the intestinal tract, the 13 lots of tablets were subjected to dissolution test in an alkaline medium consisting of a buffer solution of pH 9.5. Despite the fact that phenobarbital is highly soluble in buffer solution of pH 9.5, 11 out of the 13 lots of tablets did not release 100% phenobarbital in 30 min. In some cases the dissolution rate in alkaline medium was slower than in acid medium (manufacturers J, K, L, and M). In most cases the tablets broke apart into fairly large pieces, but failed to disintegrate any further, thereby failing to release phenobarbital into the medium. The failure of medium to penetrate the granules may be attributed to formulation factors such as types and concentrations of binder, disintegrant, filler, and lubricant, and hardness of compression.

Another interesting finding from the dissolution study in the acid medium was the great difference in dissolution rate of phenobarbital from tablets of various strengths manufactured by the same company (Table II). The difference in dissolution rates of these tablets was thought possibly to be due to several factors such as size, hardness, shape, whether scored or unscored, quantity of excipients, *etc.*, but Table II seems to indicate that these properties were not responsible for the variable rates. Since the two lots of tablets were formulated separately, it has to be presumed that some of the formulation factors have influenced the dissolution rate of these tablets.

Disintegration—The USP XVII tablet disintegration test with and without disks showed remarkable difference in results. From Table I it can be seen that all lots of tablets except one passed the test with disks, whereas 12 lots of tablets failed the test without disks. There was absolutely no correlation between disintegration times (with disks) and dissolution rates. The tablet that disintegrated within 4 min. (manufacturer K, 1 1/2 gr.) had released only 61% of the drug into acid medium in 30 min., whereas the same lot of tablets took 202 min. to disintegrate completely when disks were not used in the test. The disks exerted a slight pounding action on tablets and resulted in fast and fairly uniform disintegration times. Although it can be generalized that tablets which took a long time to disintegrate (without disks) had relatively poor dissolution rates, no quantitative correlation can be made between the two tests. Tablets which dis-

TABLE II—VARIATIONS IN THE DISSOLUTION RATES OF PHENOBARBITAL TABLETS OF DIFFERENT STRENGTHS PRODUCED BY THE SAME MANUFACTURERS

Manufacturer	Strength, gr.[a]	% Phenobarbital Dissolved in 30 min.	% Excipient in the Tablet	Hardness[c]	Shape
A	1 1/2	100 (3)[b]	42.3	7.0	S.C.;[d] scored
A	1/2	70	73.4	4.5	S.C.; unscored
I	1/2	100 (4)[b]	69.8	10.0	Scored pellet[e]
I	1 1/2	44	50.0	6.5	S.C.; scored
J	1/4	100 (10)[b]	92.9	6.5	Large scored pellet
J	1/2	89	70.1	7.0	S.C.; unscored
K	1/4	100 (30)[b]	84.1	3.5	Unscored pellet
K	1 1/2	57	47.4	5.5	S.C.; scored

[a] The approximate metric equivalents of 1/4 gr., 1/2 gr., and 1 1/2 gr. are 15 mg., 30 mg., and 100 mg., respectively [b] Actual time in minutes for complete dissolution. [c] As Strong-Cobb units. [d] S.C. = Standard concave. [e] Flat-faced tablet.

integrate into particles small enough to pass through 10-mesh screen may still contain drug within the cores which may not be released into the medium.

Experimental data indicated usefulness of test apparatus for determining *in vitro* availability of active ingredients from tablet bases. It was found that stirring the solution using an external stirrer (not by movement of oscillating tube alone) was essential to get accurate and reproducible results. Since the solution was maintained at 37°, covering the beaker during the experiment also helped to obtain more reproducible results.

SUMMARY AND CONCLUSIONS

A modification was made in the dissolution, apparatus described by Levy, and this apparatus was used to determine the *in vitro* dissolution rate of phenobarbital from tablets. Forty-two lots of commercial phenobarbital tablets from 24 companies were tested. Thirteen out of the 42 lots of tablets failed to release 100% phenobarbital in 30 min. when 0.1 *N* HCl was used as the medium. Out of these 13 lots 11 lots failed to release 100% of the drug in 30 min. when the acid medium was replaced by an alkaline medium. The dissolution rate in the two media differed markedly and this fact has to be taken into consideration in evaluating the physiological availability of the drug. Since the dissolution rates of tablets of different strengths made by the same manufacturers showed considerable variation, factors such as types and concentrations of diluent, binder, disintegrant, and lubricant, as well as hardness of compression, should be considered in the formulation of tablets.

There was absolutely no correlation between the dissolution rate and the disintegration time (with and without disks). The USP disintegration test should be replaced by a dissolution test and the limits of dissolution rate should be based on *in vitro* determination or *in vitro-in vivo* correlations (wherever possible to determine) specific for the drug in question and the type of the tablet.

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Keyphrases

Dissolution rates, phenobarbital tablets—factors affecting
In vitro dissolution test—method
Disintegration—dissolution rates compared
UV spectrophotometry—analysis

Factors Affecting Dissolution Rate of Medicaments from Tablets II

Effect of Binder Concentration, Tablet Hardness, and Storage Conditions on the Dissolution Rate of Phenobarbital from Tablets

By JAMES T. JACOB* and ELMER M. PLEIN

A series of formulations is described in which several binders are used in varying proportions and the tablets are compressed at different pressures. Results show the effects of these variables on dissolution rates of phenobarbital tablets in an acid medium of pH 1.2. Some data on the effects of storage of tablets under controlled conditions are presented.

IN ORDER that a clinical response be elicited by administration of a tablet, not only should the tablet contain the labeled amount of medicament, but also the medicament should be fully available to the body. When drugs are administered in dosage forms other than solutions, the rate at which the dosage form releases the medicament into gastrointestinal fluid may be more important than the effective surface area of the absorbing agent. For this reason with solid dosage forms (tablets, capsules, powder, *etc.*) the absorption of medicament may depend upon the dissolution rate of the formulation and/or the medicament (1).

Several workers have studied the effects of various formulation factors on dissolution rates of medicaments from compressed tablets. Sunkes (2) found an inverse relationship between the amount of salicylamide dissolved from granules and the percent of granulating agent used in preparing these granules. He found also that tablets released salicylamide at a faster rate than did granules from which tablets were made. An opposite observation was reported by Harris (3) who found that granules released medicament faster than did tablets made from the same granules. Henderson's (4) studies indicated that the effect of particle size upon either tablet hardness or dissolution time is characteristic of a particular compound and that no general prediction could be made as to quantitative correlation among these factors for all drugs. Levy *et al.* (5) studied effects of granule size, starch concentration, and compression pressures on the dissolution rate of salicylic acid from granules prepared by double compression. They found that the dissolution rate increased with decreasing granule size, with increasing starch content, and with increasing precompression pressures. Levy and Gumtow (6) in a study of the effect of lubricants on the dissolution rate of salicylic acid from tablets reported that a hydrophobic lubricant (magnesium stearate) retards dissolution rate, whereas a water-soluble surface-active agent (sodium lauryl sulfate) increases dissolution rate. Yen (7) conducted an extensive study on various factors affecting the dissolution rate of triamterene tablets and concluded that besides studying individual factors affecting the dissolution rate the combination of various factors should be considered for a true evaluation. Parrot *et al.* (8) reported that when neutral, ionic, and nonionic organic compounds were employed as additives, the dissolution rate of benzoic acid was linearly dependent upon its solubility in the particular solvent system.

In a study of commercial phenobarbital tablets it was found that several lots of tablets showed poor dissolution rates in an acid medium and also in an alkaline medium (9). Since one or more various formulation factors could contribute to the slow dissolution rate of these commercial tablets, this investigation was undertaken to study two factors, in particular—namely, binder concentration and compression hardness. Effects of certain storage conditions on dissolution rate also were studied. Fumed silicon dioxide[1] which is used chiefly as a free flowing agent and arabinogalactan[2] which is a natural gum exudate were evaluated for their tablet-binding properties.

Received November 17, 1967, from the University of Washington, College of Pharmacy, Seattle, WA 98105

Accepted for publication January 2, 1968.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

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This investigation was supported in part through grants provided by the Washington Initiative 171 funds for research in biology and medicine.

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[1] Marketed as Cab-O-Sil by Cabot Corp., Boston, Mass.

[2] Marketed as Stractan by Stein, Hall and Co., Inc., New York, N. Y.

TABLE I—COMPOSITION, HARDNESS, AND DISSOLUTION RATES OF PHENOBARBITAL TABLETS[a]

Batch No.	Lactose %	Fumed SiO_2 %	Arabinogalactan %	Hardness[b]	Time for 100% Dissolution, sec.
1	36	1	2	6.0	30
2	36	0	3	6.5	55
3	33	1	5	5.0	600
4	36	1	2[c]	5.0	40
5	33	1	5[c]	6.5	3600[d]
6	34	5	0	7.0	40
7	37	2[c]	0	6.0	60
8	34	5[c]	0	6.5	20

[a] Each tablet contained 50% phenobarbital, 10% starch, and 1% magnesium stearate in addition to the ingredients listed in table. [b] As Strong-Cobb units. [c] As solution or gel in water. [d] 94% released in 30 min.

TABLE II—EFFECT OF HARDNESS ON DISSOLUTION RATES OF PHENOBARBITAL TABLETS PREPARED WITH 5% ARABINOGALACTAN

Batch No.	Hardness[a]	% Phenobarbital Dissolved per Time Interval, min. 5	10	20	30	60
3A	5.0	92.6	100.0	...	...	...
3B	6.0	44.2	95.9	100.0	...	...
5A[b]	5.0	100.0	...	...	...	...
5B[b]	6.5	20.0	34.3	84.4	94.3	100.0
5C[b]	9.5	13.4	[c]	37.2	76.3	98.4

[a] As Strong-Cobb units. [b] Binder added as solution. [c] Did not determine.

EXPERIMENTAL

Formulation of Tablets—A number of batches of phenobarbital tablets was manufactured by wet granulation (approximately 250 tablets to a batch). Each tablet was made to contain 100 mg. of phenobarbital and 100 mg. of excipients composed of varying quantities of binders. Besides fumed silicon dioxide and arabinogalactan, a few commonly used binders also were used (acacia, gelatin, ethyl cellulose,[3] and hydroxyethyl cellulose[4]). Various concentrations of fumed silicon dioxide and arabinogalactan were used either as powders or as solutions or gels in water to make the tablets. Tablets were compressed from 20/40-mesh granules on a Stokes single-punch machine model 511-5 using $^1/_{16}$-in. (~0.8 cm.) die and standard concave punches.

Hardness—Tablets were compressed at various pressures maintaining a minimum hardness of 5 Strong-Cobb units.

Dissolution Test—Rate of release into 0.1 *N* HCl (pH 1.2) was determined by the use of the test method previously described (9).

USP XVII tablet disintegration test with and without disks was carried out only on those batches of tablets which did not release 100% phenobarbital in 30 min.

Aging—Six tablets from each batch were kept in an open 2-dram (~10 ml.) vial in an oven set at 55° for 16 days and then were tested for hardness and dissolution rate at the end of the storage period.

Humidity Studies—Six tablets from each batch containing fumed silicon dioxide or arabinogalactan were stored at 98% relative humidity in a desiccator containing a saturated solution of potassium dichromate kept at 25° (10), for 30 days, and the following tests were carried out: amount of absorbed moisture (tablets were weighed before and after storage in the desiccator), hardness, and dissolution rate. The dissolution rates were determined immediately after removing the tablets from the desiccator and also after exposing them to room temperature for 24 hr. during which time they lost moisture and attained constant weight.

[3] Marketed as Ethocel by Dow Chemical Co., Midland, Mich.

[4] Marketed as Natrosol by Hercules Powder Co., Glens Falls, N. Y.

RESULTS AND DISCUSSION

Table I gives the dissolution rates of phenobarbital from tablets manufactured using fumed silicon dioxide or arabinogalactan as a binder. Granules made with fumed silicon dioxide in concentrations of 2% and 5% as a gel in water and 5% as powder possessed good binding qualities and produced smooth, shiny tablets of satisfactory hardness and fast dissolution rates (maximum dissolution time 60 sec.). Arabinogalactan was found to be a good binder when used in concentrations of 2–5%. An increase in concentration of arabinogalactan, however, resulted in a decrease in dissolution rate, especially when the binder was used in the form of a gel in water (dissolution time 3600 sec.).

Table II shows effect of hardness on dissolution rates of phenobarbital tablets prepared with 5% arabinogalactan added as a powder and granulated with water or used as a solution in water to granulate the powder mixture.

Tests on tablets formulated with acacia, gelatin, ethyl cellulose, and hydroxyethyl cellulose used as binders in various concentrations clearly indicate how dissolution rates can be affected by altering the concentration of binder as well as hardness of the tablet (Table III). Satisfactory tablets were obtained with acacia or gelatin as a binding agent, although differences in hardness of the tablets affected their dissolution rates. An increase in hardness by 2 Strong-Cobb units was responsible for about 50% reduction in dissolution rate of tablets prepared with acacia (Batch No. 9A and 9B). Hardness of tablets is also a factor in dissolution rates of tablets prepared with 2% gelatin (Batch No. 10A and 10B), with 4% gelatin (Batch No. 11A and 11B), and perhaps with 5% ethyl cellulose. The effects of concentration of gelatin and hardness of compression

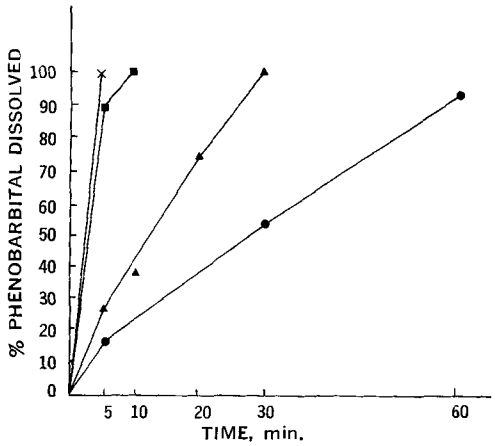

Fig. 1—Dissolution rates in 0.1 N HCl, of phenobarbital tablets made with gelatin as a binder. Key: ×, 2% gelatin (No. 10A) compressed at a hardness of 6.5 Strong-Cobb units; ■, 2% gelatin (No. 10B) compressed at a hardness of 10.0 Strong-Cobb units; ▲, 4% gelatin (No. 11A) compressed at a hardness of 6.5 Strong-Cobb units; ●, 4% gelatin (No. 11B) compressed at a hardness of 13.0 Strong-Cobb units.

on dissolution rates of tablets are illustrated in Fig. 1. Ethyl cellulose and hydroxyethyl cellulose possess good binding properties and produced tablets with good physical appearance; however, the poor dissolution rates shown in Table III indicate they are unsatisfactory binding agents in these formulations.

The USP tablet disintegration test with and without disks was carried out on tablets with slow dissolution rates in 0.1 *N* HCl and the results are shown in Table III. A comparison of dissolution rates and disintegration times (with and without disks) showed no correlation between the two tests. Tablets formulated with 1% ethyl cellulose took 60 min. to release 98% of the drug into acid medium, but the disintegration time in water was only 30 sec. (No. 12, Table III). This great disparity can be attributed to the fact that tablets swelled on contact with water and broke apart into small particles, whereas in the acid medium no such swelling was observed.

The results of aging tablets at 55° for 16 days, which storage time is approximately equivalent to a storage time at room temperature for 2 yr. (11), showed slight increase in tablet hardness (Table IV). This observation substantiates earlier reports found in the literature (12, 13). There was no decrease in dissolution rates as a result of increased hardness due to aging.

Considerable quantities of moisture were absorbed by tablets when they were exposed to 98% relative humidity for 30 days as a result of which the dissolution rates of these tablets were markedly decreased. A comparison of data in Tables I and V shows the differences in the dissolution rates. The quantity of moisture absorbed and retained depended upon the type and concentration of binder used. Tablets made with arabinogalactan absorbed more moisture than did those made with fumed silicon dioxide (Table V). The quantity of absorbed moisture was directly proportional to the concentration of binder used. There were remarkable differences in dissolution rates of tablets determined immediately after removal from the humidity chamber and after they had lost most of the absorbed moisture. Tablets made with fumed silicon dioxide clearly indicated this variation (Batch No. 6, 7, and 8, Table V). The absorbed moisture retarded the dissolution rates of drug by preventing the acid solution from penetrating into the tablets. After losing most of the absorbed moisture (by evaporation at

TABLE IV—EFFECT OF ACCELERATED AGING OF PHENOBARBITAL TABLETS AT ELEVATED TEMPERATURE ON HARDNESS AND DISSOLUTION TIME

Batch No.	Hardness[a] A[b]	Hardness[a] B[c]	Time for 100% Dissolution in 0.1 *N* HCl (sec.) A[b]	Time for 100% Dissolution in 0.1 *N* HCl (sec.) B[b]
1	6.0	7.0	30	40
2	6.5	7.0	55	60
3	5.0	5.0	600	300
4	5.0	6.0	40	50
5	6.5	7.0	3600[d]	600
6	7.0	9.0	40	35
7	6.0	6.0	60	60
8	6.5	7.3	20	30

[a] As Strong-Cobb units. [b] Immediately after manufacture. [c] After storing for 16 days at 55°. [d] 94% release in 30 min.

TABLE III—EFFECT OF HARDNESS ON DISSOLUTION RATES AND DISINTEGRATION TIMES OF PHENOBARBITAL TABLETS PREPARED WITH SOME COMMON BINDERS

Batch No.	Binder	Concentration of Binder %	Hardness[a]	% Phenobarbital Dissolved per Time — Interval, min. 5	10	20	30	60	Disintegration Time, min. with Disks	Disintegration Time, min. without Disks
9A	Acacia	4	6.5	71.8	100	...	...	...	...	...
9B	Acacia	4	8.5	36.8	52.2	89.3	100	...	...	...
10A	Gelatin	2	6.5	100	...	...	...	...	...	...
10B	Gelatin	2	10.0	89.1	100	...	...	...	...	...
11A	Gelatin	4	6.5	26.1	38.9	73.8	100	...	...	...
11B	Gelatin	4	13.0	16.4	[b]	[b]	52.3	92.1	15.0	35.0
12	Ethyl cellulose	1	6.5	70.1	[b]	[b]	93.3	98.3	0.5	10.0
13A	Ethyl cellulose	5	6.5	5.0	[b]	[b]	11.6	15.6	57.0	[c]
13B	Ethyl cellulose	5	9.0	4.1	[b]	[b]	8.0	14.0	[d]	[e]
14	Hydroxyethyl cellulose	2	5.0	19.2	30.9	43.7	59.5	83.2	16.0	20.5
15	Hydroxyethyl cellulose	4	6.5	4.3	11.0	18.8	22.3	36.8	[f]	82.0

[a] As Strong Cobb units. [b] Did not determine. [c] Over 120 min. [d] Approximately 75% of the tablet remained on the screen after 120 min. [e] Approximately 90% of the tablet remained on the screen after 120 min. [f] Could not determine accurately since the tablets remained glued to the disks from the beginning of the test.

TABLE V—EFFECT OF MOISTURE ON THE DISSOLUTION RATES OF PHENOBARBITAL TABLETS EXPOSED TO 98% RELATIVE HUMIDITY FOR 30 DAYS

Batch No.	% Moisture Absorbed	% Moisture Retained	% Phenobarbital Dissolved in 30 min. A[a]	% Phenobarbital Dissolved in 30 min. B[b]	% Phenobarbital Dissolved in 60 min. A[a]	% Phenobarbital Dissolved in 60 min. B[b]
1	4.69	0.36	17.0	21.9	30.6	34.7
2	5.64	0.29	17.9	19.4	31.1	42.5
3	7.81	0.37	21.2	24.6	33.1	37.6
4	4.82	0.33	18.0	22.0	31.5	33.9
5	7.73	0.35	16.0	17.1	30.0	31.8
6	6.54	0.23	24.9	77.1	30.5	90.7
7	4.57	0.07	15.1	33.5	21.4	61.4
8	6.78	0.36	20.0	72.2	37.1	93.7

[a] Dissolution rate determined immediately after removal from the humidity chamber. [b] Dissolution rate determined following storage in the humidity chamber and after the tablet had been exposed to room temperature to a constant weight.

room temperature) the tablets were more vulnerable to penetration of 0.1 *N* HCl resulting in an increased dissolution rate. However, the increased dissolution rates were considerably lower than the dissolution rates of the same tablets prior to their storage in the humidity chamber.

SUMMARY AND CONCLUSIONS

1. Fumed silicon dioxide and arabinogalactan in concentrations of 2–5% possess good tablet-binding qualities.

2. Increase in binder concentration and hardness of compression resulted in a decrease in the dissolution rates of phenobarbital tablets. Selection of binder, its concentration, and hardness at which tablets are compressed are important factors which should be properly controlled so that the medicament will be completely released for quick physiological availability.

3. No apparent change in dissolution rate was observed when phenobarbital tablets were aged at 55° for 16 days.

4. Depending upon type and concentration of binder, phenobarbital tablets absorbed varying quantities of moisture when exposed to 98% relative humidity for 30 days. As a result of moisture absorption, dissolution rates of phenobarbital from those tablets were considerably decreased. An increased dissolution rate was observed when the moist tablets were tested after exposure to room temperature for 24 hr. during which time they lost most of the absorbed moisture. However, this increased dissolution rate was much lower than the original dissolution rate of the tablet.

5. No correlation was found between dissolution rate and USP tablet disintegration test with and without disks.

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Keyphrases

Tablets—compressed
Dissolution rates, phenobarbital tablets—factors affecting
Binders—tablet dissolution rate
Hardness—tablet dissolution rate
Aging—tablet dissolution rate
Moisture—tablet dissolution rate

Identification of Antibiotics in Sensitivity Disks

By LOLA G. WAYLAND and PETER J. WEISS

Identity tests for antibiotic sensitivity disks have been developed. Suitable tests previously reported in the literature have been modified and adapted to the microquantities involved in these disks. Once a system of testing had been established, it was screened for interference from all other disk antibiotics. Penicillins are identified generally by an iodometric method and specifically by thin-layer chromatography (TLC). The polypeptide antibiotics are differentiated by TLC in combination with nitration, reduction, and diazotization to further distinguish colistin from polymyxin. Lincomycin, oleandomycin, and kanamycin are identified by TLC systems, the tetracyclines by paper chromatography. Under varying conditions of solvents and wavelengths, streptomycin, erythromycin, nystatin, vancomycin, novobiocin, cephalothin, and viomycin are identified spectrophotometrically. Color tests have been devised for chloramphenicol, dihydrostreptomycin, and neomycin.

THE FIRST antibiotic disks, made by Bondi *et al.* in 1947, were widely recognized as "diagnostic aids for the rapid determination of the susceptibility of microorganisms to antibiotics" (1). Since then, they have proved as valuable as anticipated, and have widespread use in hospitals and clinical laboratories throughout the world. Because of the reliance placed upon information elicited from their use, confirmatory evidence of the labeled identity of the antibiotics in the disks is of great importance. This paper describes laboratory procedures developed for this purpose.

Regulations describing the tests and methods of assay and requirements for certification of antibiotic disks are published in the Code of Federal Regulations, title 21, chapter 1, part 147. Table I lists the antibiotic disks now included in these regulations with references to the applicable identity tests described in this paper.

Although antibiotic disks are tested by a "performance" method for inhibitory response against specific microorganisms and are compared with control disks of known potencies, the zone of inhibition obtained does not always specifically identify the antibiotic. For example, from microbiological assay alone, positive differentiation cannot be made between colistin and polymyxin, and between chloramphenicol and the tetracyclines; among the various tetracyclines, and among the various penicillins; between kanamycin and neomycin, and between dihydrostreptomycin and streptomycin, particularly if the potencies deviate greatly from the label claims. While it may not be of the utmost medical importance to differentiate one tetracycline from another, it is important to distinguish a tetracycline from chloramphenicol, or a semisynthetic penicillin from cephalothin or penicillin G. Some antimicrobial agent other than an antibiotic may conceivably be present and under certain conditions may be undetected by microbial assay. Therefore, a system of chemical identity tests for antibiotics in disks was established to supplement the quantitative information obtained by microbiological assay techniques.

Received August 22, 1967, from National Center of Antibiotics and Insulin Analysis, Bureau of Science, Food and Drug Administration, Washington, DC 20204

Accepted for publication January 22, 1968.

The standard disks used in developing these tests were kindly supplied by Mary Ann Garth and the Special Investigations Branch of the Division of Antibiotics and Insulin Certification.

IDENTIFICATION PROCEDURES

I. Penicillins—(*a*) *Iodometric Test*—This general screening procedure for penicillin and cephalothin involves a modification of the iodometric assay method (2). Place enough disks in each of two tests tubes to provide 30 mcg. of sodium cephalothin, 20 units of penicillin G, or 4 mcg. of other penicillins. To both tubes add 1 ml. of 1% phosphate buffer (pH 6) and shake well. To one tube, add 1 ml. of 1 *N* sodium hydroxide, shake, and let stand 15 min. Then add 1 ml. of 1.2 *N* hydrochloric acid and 0.2 ml. of 0.001 *N* iodine solution; after 15 min. add 1 drop of starch indicator solution. To the second tube add 2 ml. of distilled water, 0.2 ml. of iodine solution, and 1 drop of starch indicator solution. If penicillin or cephalothin is present, the first tube remains colorless, while the second develops the characteristic blue of the starch–iodine complex.

(*b*) *Chromatography*—Blend 30 Gm. of cellulose MN 300 without binder (Macherey, Nagel, Westbury, N.Y.) with 200 ml. of distilled water at high speed for 15 min. Apply this slurry to 20 × 20 cm. glass plates with a Brinkmann-Desaga applicator set for a film depth of 0.250 mm. Air dry the plates overnight and use without preactivation. Place enough disks to provide not less than 10 mcg. of penicillin in a sintered-glass microfi ter. Pipet 1.0 ml. of methanol onto the disks and allow to steep, with occasional stirring, for 5 min. Then apply suction and collect the filtrate in a small, flat-bottomed vial (15 × 75 mm.). Evaporate the extract to dryness under a stream of air, and redissolve in 50 μl. of methanol. Spot this solution on a TLC plate in 1-μl. increments until 1 mcg. has been applied.

Prepare a standard for each antibiotic from standard disks and spot each on the plate in the same manner. Standard and sample extracts may be spotted both individually and as a mixture to

insure homogeneity. Use only freshly extracted solutions. When the plate has been spotted, place it in a rectangular tank lined with heavy filter paper saturated with the solvent system, a 0.1 *M* sodium chloride solution (3). Place a glass trough in the bottom of the tank with solvent and allow the plate to rest directly in it. Allow the solvent front to travel 10 cm. from the starting line, then remove the plate from the development tank and allow to air dry. Lightly spray the plate with a freshly prepared solution of 2 parts 10% aqueous ferric chloride, 1 part 5% aqueous potassium ferricyanide, and 7 parts 20% sulfuric acid. After 5 min. for color development, the penicillins appear as blue spots on a green background. Directly compare the positions of the sample spots with the standards. The average R_f values of 10 runs are: sodium nafcillin, 0.45; cloxacillin, 0.62; sodium oxacillin, 0.68; potassium phenethicillin, 0.76; penicillin G, 0.85; and methicillin 0.89.

The tetracyclines have R_f values from 0.50 to 0.65 but are yellow before the color reagent is applied and in addition, yield negative iodometric reactions. Vancomycin hydrochloride appears at an R_f of about 0.90, but also yields a negative iodometric test.

(*c*) *Ninhydrin Test*—Ampicillin is the only penicillin that reacts with ninhydrin. After identifying it as a penicillin by the iodometric test, staple one disk to a sheet of Whatman No. 1 chromatographic paper, and apply one drop of ninhydrin reagent (1% ninhydrin in 100 ml. of water-washed *n*-butanol containing about 1 ml. of pyridine). Dry and heat the disk with a hot-air gun. Ampicillin yields a characteristic purple color.

II. Bacitracin, Colistin Sulfate, Kanamycin Sulfate, Neomycin Sulfate, and Polymyxin B Sulfate—(*a*) *Chromatography*—Apply Silica Gel G on glass plates (20 × 20 cm.) manually with a Brinkmann-Desaga applicator to a depth of 0.250 mm. Air dry overnight. Commercial TLC sheets may also be used.

Extract the antibiotics from the disks in the manner described for the penicillins, using water instead of methanol. Extract a minimum of 20 mcg. from the disks so that, when the residue is dissolved in 50 µl. of water, a total of 4 mcg. will be spotted on a TLC plate. For each antibiotic, extract standard disks and spot the extracts on the plate. Prepare the tank in the manner described for the penicillins, and use the same type of tank. The two-phase resolving system consists of chloroform–methanol–17% ammonia (40:25:20) (4), and the portion in the glass trough must be freshly prepared for each run. Both phases are used together. After the plate has been placed in the tank to develop, seal the lid with masking tape. When the solvent front has moved 10 cm. (in about 1 hr.), remove the plate from the tank and allow it to air dry. Spray the plate with ninhydrin reagent (see Ic) or use a commercial aerosol containing 0.3% ninhydrin in ethanol. Heat the plate with a hot-air gun to make the spots visible. Bacitracin yields a very characteristic spot at the front. The R_f values are: colistin sulfate, 0.80; polymyxin B sulfate, 0.70; kanamycin sulfate, 0.50; and neomycin sulfate, 0.30.

(*b*) *Diazotization*—Since phenylalanine is one of the decomposition products of polymyxin B sulfate but not of colistin sulfate, one may be differentiated from another by nitration, reduction, and diazotization of this aromatic moiety (5). Extract 10–15 polymyxin B sulfate 50-unit or colistin sulfate 10-mcg. disks with 1 ml. of water into a 50-ml. conical flask and evaporate the extract to dryness under a stream of air. Simultaneously evaporate 1.0 ml. of solution containing 10 mcg. of phenylalanine. Add 1.0 ml. of fuming nitric acid to each flask, cover with a watch glass, and heat on a vigorously boiling steam bath for 30 min. Remove from heat and add 10 ml. of water, 2 ml. of concentrated ammonium hydroxide, and about 50 mg. of sodium hydrosulfite. Mix and allow to stand 20 min. Then cool the flasks in a large evaporating dish containing crushed ice. Add 5.0 ml. of a 5% sodium nitrite solution and 1.5 ml. concentrated hydrochloric acid. Let stand 30 min., maintaining the temperature below 5°. Add 10 ml. of 5% sulfamic acid, shake, and dispel the nitrous oxide fumes with a current of air. Add 4.0 ml. of freshly prepared 0.8% solution of *N*-(1-naphthyl) ethylenediamine dihydrochloride. If polymyxin B sulfate is present, a red-purple color similar to that of the phenylalanine will develop. Colistin sulfate will remain colorless or only faintly pink tinged.

Bacitracin gives a positive result in this test, but may be identified by the TLC system described for group II*a*. Ampicillin yields a positive reaction with ninhydrin and diazotization; it also moves with the solvent front in the TLC system for group II*a*, but may be distinguished from them by the iodometric test for penicillin.

III. Tetracyclines—*Chromatography*—Extract the disks in the same manner described for the penicillins, using methanol as solvent. Extract enough disks to yield 50 mcg. in 50 µl. Subject the solutions, along with appropriate standards, to ascending paper chromatography, using the method of Selzer and Wright (6). Use a stationary phase of MacElvaine's buffer (pH 3.5) and a solvent system of chloroform–nitromethane–pyridine (10:20:3). The resulting spots are visible under ultraviolet light in the presence of ammonia fumes. No interference arises from any of the other 23 antibiotics listed in Table I.

IV. Lincomycin Hydrochloride and Oleandomycin Phosphate—*Chromatography*—Extract ten 2-mcg. disks in the manner described for penicillins, but with 0.05% phosphate buffer (pH 6) instead of methanol. After evaporation, dissolve the residue in 50 µl. of the buffer and apply ten 1-µl. spots to a TLC plate prepared as for procedure II*a*. Treat standard disks in the same manner. Use the resolving system, methylethyl ketone–acetone–water (9.3:2.6:1), developed by Brodasky and Lummis (7). Develop the chromatogram and air dry the plate. Place the plate in an iodine atmosphere (a closed rectangular glass tank containing a small beaker with several iodine crystals warmed on a steam bath) until yellow spots appear. Remove the plate from the tank, allow it to stand several minutes, and then spray it lightly with starch indicator solution until the lincomycin hydrochloride or oleandomycin phosphate shows up as a dark purple spot. Make direct comparisons of the positions of the spots with the standards.

All the penicillins give two spots in this system. Cloxacillin, methicillin, penicillin G, potassium phenethicillin, sodium nafcillin, and sodium oxacillin

TABLE I—IDENTIFICATION PROCEDURES FOR ANTIBIOTIC SENSITIVITY DISKS

Antibiotic	Usual Potency Level[a] Low	High	Identification Procedure
Ampicillin	2	10	I*a,c*
Bacitracin	2 units	10 units	I*a*,II*a*
Sodium cephalothin	30	...	XI
Chloramphenicol	5	30	VI
Chlortetracycline hydrochloride	5	30	III
Sodium cloxacillin	1	...	I*a,b*
Colistin sulfate	2	10	II*a,b*
Demethylchlortetracycline hydrochloride	5	30	III
Dihydrostreptomycin sulfate	2	10	VIII*a,b*
Erythromycin	2	15	XIII
Kanamycin sulfate	5	30	II*a*
Lincomycin hydrochloride	2	...	IV
Methacycline hydrochloride	5	30	III
Methicillin	5	...	I*a,b*
Sodium nafcillin	1	...	I*a,b*
Neomycin sulfate	5	30	II*a,V*
Sodium novobiocin	5	30	VII
Nystatin	100 units	...	IX
Oleandomycin phosphate	2	15	IV
Sodium oxacillin	1	...	I*a,b*
Oxytetracycline	5	30	III
Penicillin G	2 units	10 units	I*a,b*
Potassium phenethicillin	3 units	...	I*a,b*
Polymyxin B sulfate	50 units	300 units	II*a,b*
Streptomycin sulfate	2	10	VIII*a,b*
Tetracycline hydrochloride	5	30	III
Vancomycin hydrochloride	5	30	XII
Viomycin sulfate	2	10	X

[a] Potency is given as mcg./disk unless otherwise stated.

have R_f values ranging from about 0.35–0.44 for the major spot, while ampicillin has its major spot at 0.16. Sodium cephalothin has one spot at R_f 0.35; the tetracyclines and nystatin all have one or two spots which are yellow *before* being exposed to iodine; and oleandomycin phosphate has one spot at R_f 0.24. Lincomycin hydrochloride is readily distinguished from all these, since it yields a single spot at an R_f of 0.56.

V. Neomycin Sulfate—Extract enough disks with 1 ml. of water to give at least 75 mcg. of antibiotic. Collect the filtrate in a 50-ml. round-bottom distillation flask. Add 5 ml. of 40% sulfuric acid; reflux for 100 min. and cool. Transfer the solution to a glass-stoppered test tube and extract with 2.5 ml. of xylene. Transfer the upper xylene layer with a syringe with a cannula tip to a second tube. Add 2.5 ml. of *p*-bromoaniline reagent (8), and shake vigorously. If the disks contain neomycin sulfate, a pink color develops on standing.

No other disk antibiotic has been found to interfere in this test but the pink color will not develop in the presence of water.

VI. Chloramphenicol—Steep at least four 30-mcg. disks in 1 ml. of methanol with occasional stirring for 2 min. Filter with suction into a test tube and wash into a 50-ml. glass-stoppered centrifuge tube with 10 ml. of water. Add 10 ml. of 12 *N* sodium hydroxide and 10 ml. of α-naphthol reagent (a 2 mg./ml. solution in 6 *N* sodium hydroxide, freshly prepared) (9). Heat the tube for 30 min. in a boiling water bath, cool in tap water for about 5 min., and dilute to mark with methanol. Immediately upon the addition of the methanol a blue color appears which deepens slightly upon standing.

None of the other 27 antibiotics in Table I gives a blue color.

VII. Sodium Novobiocin—Extract enough disks with 2 ml. of methanol to give 40 mcg. of novobiocin. Evaporate to dryness under a stream of air and dissolve the residue in 2 ml. of 0.1 *N* sodium hydroxide. Record the UV spectrum from 200 to 400 mμ in 1-ml. cells. A characteristic absorption curve is obtained with a maximum at 305 mμ, shoulders at 288 and 248 mμ, and a minimum at 260 mμ. Standard disks are similarly treated for comparison.

VIII. Streptomycin Sulfate and Dihydrostreptomycin—(*a*) Place single disks of sample and standard side by side on a spot plate. Add one drop of oxidized nitroprusside reagent to each disk (10). With either streptomycin sulfate or dihydrostreptomycin a red-orange color develops rapidly. Viomycin sulfate gives a faint response. None of the other disk antibiotics interferes. If a ninhydrin spot test on a single disk (method I*c*) is negative, viomycin sulfate is eliminated.

(*b*) Differentiate streptomycin sulfate from dihydrostreptomycin by extracting 60 mcg. from disks into a test tube with 2 ml. of water. To the extract add 0.4 ml. of 1 *N* sodium hydroxide. Place the same solution in both 1-ml. cells and, with a double-beam spectrophotometer, record the spectrum from 300 to 350 mμ. Return the contents of the cell to the tube and heat it in a boiling water bath for 5 min. Cool the tube to room temperature and record the spectrum from 300 to 350 mμ with the unheated solution in the reference cell. Streptomycin sulfate produces a broad maltol peak at 320 mμ (11). The ferric chloride color of this method (11) is too faint to be visible at this concentration.

No other antibiotic in Table I gives this maltol reaction; however, the lower potency streptomycin sulfate disks give inconclusive results, possibly

because of methylcellulose interference from the higher proportion of paper.

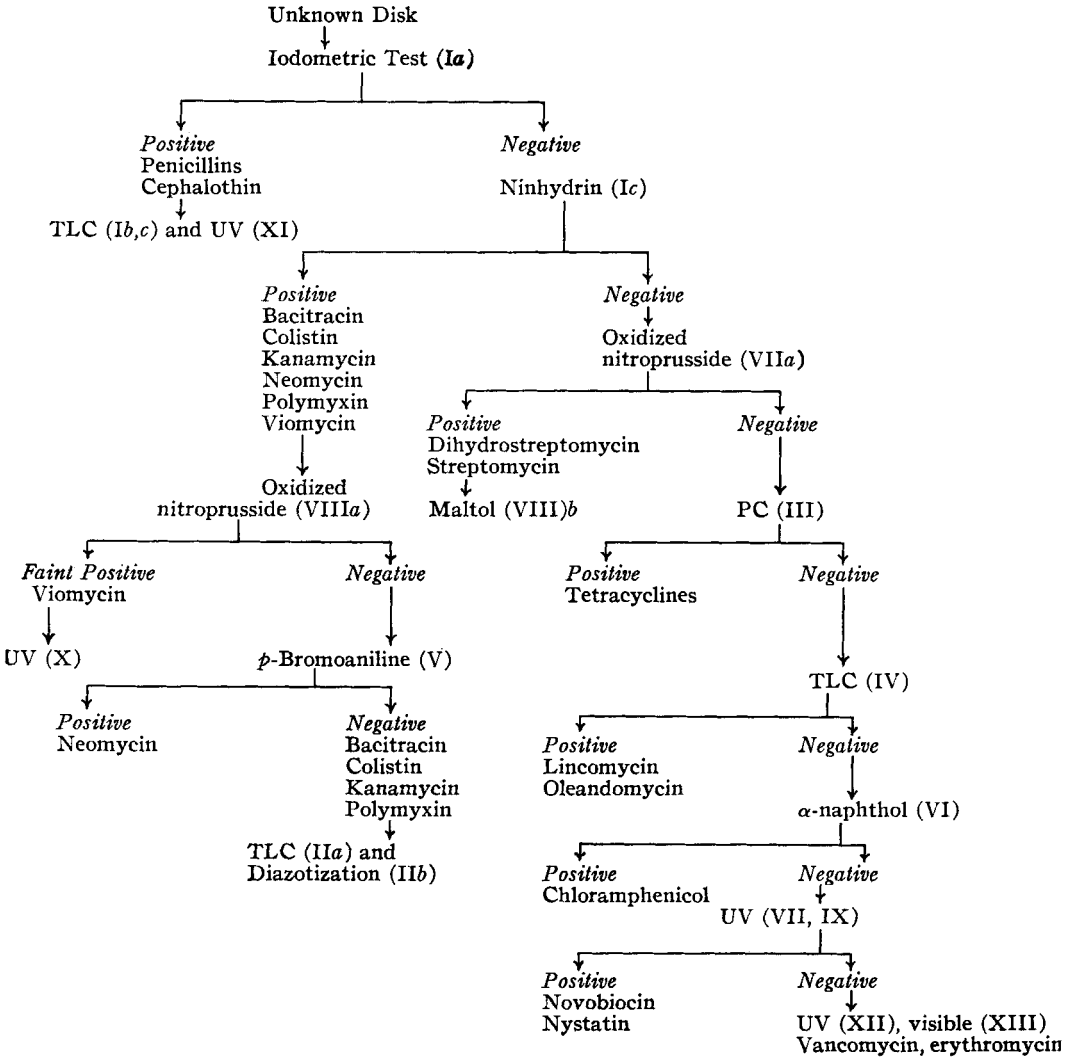
IX. Nystatin—Extract one 100-unit disk with three 1-ml. portions of methanol; collect and dilute to volume with methanol in a 5-ml. volumetric flask. Record the UV spectrum from 210 to 400 mμ. Characteristic peaks occur at 318, 304, and 291 mμ. Treat standard disks in a similar manner and compare spectra. No other antibiotic has a UV spectrum closely resembling that of nystatin.

X. Viomycin Sulfate—Extract five 10-mcg. or ten 2-mcg. disks with 1 ml. of 0.05% phosphate buffer, pH 6.0 Record the UV spectrum from 250–350 mμ against a buffer blank, using 1-ml. cells. Viomycin sulfate has a strong absorbance at 268 mμ. Although potassium phenethicillin absorbs at this wavelength, it is 10 times weaker in magnitude, and the shape of the potassium phenethicillin curve is more complex.

XI. Sodium Cephalothin—Extract four 30-mcg. disks with 1 ml. of water. Wash the disks with two additional 1-ml. portions of water and collect all filtrates in a 5-ml. volumetric flask. Dilute to 5 ml. with water and record the UV spectrum from 220 to 310 mμ. Treat standard disks similarly. Sodium cephalothin yields a peak at 237 mμ and a shoulder at 265 mμ. No other antibiotic in Table I gives this spectrum.

XII. Vancomycin Hydrochloride—Extract enough disks to yield 50 mcg. in 1 ml. of water. Evaporate the extract to dryness under a stream of air and redissolve in 1 ml. of 0.02 *N* sulfuric acid. Record the UV spectrum from 250–350 mμ, using water in the 1-ml. reference cell. With vancomycin hydrochloride a peak occurs at about 278 mμ.

Extract the same number of disks, evaporate the extract to dryness, and redissolve in 1 ml. of 0.02 *N* sodium hydroxide. Record the UV spectrum from 250–350 mμ, using 1-ml. cells. The vancomycin hydrochloride absorption peak shifts to about 302 mμ. In each case standard disks are similarly treated. This acid-base shift in absorbance wavelength is characteristic only of vancomycin hydrochloride (12) among the antibiotics listed in Table I.



XIII. Erythromycin—Extract two to four 15-mcg. disks with 1 ml. of chloroform, evaporate the extract to dryness under a stream of air, and redissolve in 1 ml. of 40% sulfuric acid. Heat in a water bath at 50° for 30 min. and cool to room temperature. Treat standard disks in the same manner. Record the visible spectrum from 450–500 mμ, using water in the 1-ml. reference cell. Erythromycin has a strong absorbance at about 482 mμ (13). Under these conditions none of the other 27 antibiotics listed in Table I absorbs strongly at 482 mμ.

DISCUSSION

The system of chemical testing outlined in the flow chart (Scheme I) and described here in detail can completely identify an unknown disk sample, if enough disks (80–100) are available. Four unknown disk samples were carried through this identification system and successfully identified as colistin, kanamycin, nafcillin, and oxytetracycline.

In practice, testing begins from the labeled identity and proceeds to confirm or deny the label. In the identification system described, as in many others, positive reactions to any single test may not be sufficient to characterize definitively the compound in question. The composite picture from supplemental chemical tests, in combination with microbiological response and potency data, results in satisfactory identification. For example, ampicillin and bacitracin react with ninhydrin, move with the solvent front in the TLC method for group II*a* antibiotics (although bacitracin has a very distinctive spot), and undergo the nitration, reduction, and diazotization procedure for the differentiation of colistin sulfate and polymyxin B sulfate. The iodometric test would identify a properly labeled ampicillin disk as a penicillin, but if an ampicillin disk were labeled as bacitracin and tested as such, the error might not be discovered. Because of this possibility, the iodometric test is carried out on bacitracin disks routinely. However, the discrepancy would be detected by microbial assay, because in that test the responses of ampicillin and bacitracin are very different.

The identity tests described have been applied satisfactorily to 250 lots of disks received as part of the antibiotic certification program. Each antibiotic was represented at least twice. Often these disks are color coded with dyes. Thus far, none of the dyes has been found to interfere in the tests.

This system of identity tests applies to those antibiotics presently in use in sensitivity disks but the use of new antibiotics may bring new problems. If such new antibiotics are closely related to the ones now in use, some of these identity tests may not be as specific as at present. The special problems of antibiotic sensitivity disk identity testing, dealing with microgram quantities of highly complex, related materials on a paper matrix, require frequent reevaluation for the possible improvement of the existing tests. Nevertheless, the system of methods just described should serve as a useful starting point for developing new test methods as the need arises.

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Keyphrases

Antibiotics in sensitivity disks—identity
Iodometric assay
TLC—identity
Ninhydrin test—identity
Diazotization-reaction—identity
UV spectrophotometry—identity

Kinetics of Degradation of Glutethimide

By J. W. WESOLOWSKI*, SEYMOUR M. BLAUG, TING-FONG CHIN, and JOHN L. LACH

The hydrolysis of glutethimide (2-ethyl-2-phenyl-glutarimide) in buffered aqueous solutions in the pH range 1.5–8.0 has been studied. This region exhibited specific base catalysis. The overall rate may be expressed by the equation, rate = k' (glutethimide) at constant pH. The energy of activation, corrected for heat of ionization of water, was calculated. A mechanism of hydrolysis is proposed.

GLUTETHIMIDE is a widely used nonbarbiturate sedative hypnotic (1). It belongs to a relatively new medicinal group of compounds, the glutarimides, which exhibit CNS depressant as well as stimulant properties (2–4). A review of the literature showed that very little kinetic work has been done on glutarimides (5–8). In view of the increasing importance of this group of compounds it was felt that a quantitative investigation of the hydrolysis rate of glutethimide was warranted.

Investigations on the hydrolysis of succinimide (9) and phthalimide (10, 11) have shown that the ionization of the imide has a strong effect on the course of hydrolysis. In the acid region where the imide is undissociated, specific base catalysis has been observed. The presence of the imide anion causes a decrease in the hydrolysis rate. A complex rate expression has been derived to explain the observed effect (10).

Glutethimide hydrolyzes in weakly basic solution to 4-ethyl-4-phenyl glutaramic acid (EPG) (8, 12, 13) and to the corresponding glutaric acid in 40% potassium hydroxide (12). In the pH range studied, 1.5–8.0, it was expected that glutethimide ionization would be very small and that the predominant product of hydrolysis would be EPG.

An ion-exchange procedure was used to follow the course of glutethimide degradation. A strongly basic quaternary amine anionic-exchange resin specially purified (washed with 10% phosphoric acid and with methanol), was used to separate the glutethimide from EPG. The glutethimide was then eluted from the column by methanol and assayed spectrophotometrically at 257.5 mμ.

EXPERIMENTAL

Reagents and Apparatus—Glutethimide, recrystallized m.p. 85–86°; glutethimide NF reference standard; EPG m.p. 156–157° (prepared by refluxing glutethimide in a 10% sodium hydroxide solution for 1 hr.); sodium hydroxide, sodium chloride, C.P. Merck; monobasic, dibasic, and tribasic sodium phosphate; anhydrous methanol, reagent grade, Mallinckrodt; orthophosphoric acid, reagent grade, Allied Chemical; Anion-exchange (Cl), 50 mesh, analytical reagent grade resin[1]; Beckman DU spectrophotometer and 5-cm. cells; glass columns, 1 × 20 cm. with a glass stopcock at one end and a 100-ml. reservoir at the other end; glass columns, 2 × 45 cm. with Teflon stopcocks (Fisher); constant temperature bath with Sargent heater, circulator, and regulator, Model No. S-PN-2770; thermometer calibrated to 0.1°.

The buffers used in this study were: pH 1.5—0.4 *M* phosphoric acid; pH 2.35, 3, 4, and 5—0.4 *M* Na_2HPO_4–0.4 *M* H_3PO_4; pH 6, and 6.5—0.4 *M* Na_2HPO_4–0.4 *M* NaH_2PO_4; pH 7, 7.5 and 8—0.4 *M* Na_3PO_4–0.4 *M* NaH_2PO_4. All buffer solutions were adjusted with the aid of a Beckman model H2 pH meter. The same phosphate buffer system was used throughout the study in that the pH of this buffer is relatively independent of temperature.

Procedures—The following general procedure was used for studying the effect of pH and temperature on the rate of hydrolysis of glutethimide.

An accurately weighed glutethimide stock solution was prepared in anhydrous methanol, such that 5 ml. contained 100 mg. of glutethimide. The buffer solutions were preheated to the temperature at which the particular run was made. A 100-ml. volumetric flask was filled with 50 ml. of the preheated buffer and 5 ml. of the methanolic glutethimide stock solution was pipeted in with constant swirling. The solution was diluted with buffer to volume, mixed, and placed in the bath. After a lapse of 10 min. to allow the solution to thermally equilibrate, 5 ml. representing the zero hour sample was withdrawn, transferred onto a column of the anion-exchange resin, and 20 ml. of cold deionized water was added to the column. The sample was passed through the column at the rate of 1.5 ml./min. When the solution level reached the top of the resin, anhydrous methanol was added as the eluent and the flow rate was increased to 5–7 ml./min. One-hundred milliliters was collected in a volumetric flask and assayed spectrophotometrically at 257.5 mμ, using the eluent collected from a blank column as a blank.

Samples were removed from the volumetric flask at definite time intervals and analyzed by this procedure.

pH and Temperature Effect on the Rate of Hydrolysis—In order to study the effect of pH and

Received April 14, 1967, from College of Pharmacy, University of Iowa, Iowa City, IA 52240
Accepted for publication January 12, 1968.
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[1] Dowex 2-4x. Obtained from J. T. Baker Chemical Co.

temperature on the rate of hydrolysis of glutethimide, the reaction was carried out at the different temperatures, in buffer solution, as follows: 50°—pH 6, 6.5, 7, 7.5, and 8.0; 60°—pH 5, 6.5, 7, 7.5, and 8.0; 65°—pH 6, 6.5, 7, 7.5, and 8.0. Since glutethimide solutions at pH's 1.5 to 5.0 show insignificant degradation after 90 hr. at 65°, the data are not included.

Effect of Ionic Strength—The effect of ionic strength on the rate of hydrolysis was determined by studying the hydrolytic rate at 65° of a solution of glutethimide in 0.4 *M* phosphate buffer at pH 7.5 and comparing the rate of hydrolysis with a solution of glutethimide of the same concentration containing 1% potassium chloride. The effect of buffer concentration on hydrolytic rate was also studied by following the hydrolysis rate at 65°–pH 7.5 in 0.2 *M* phosphate buffer.

Effect of Glutethimide Concentration—Solutions of glutethimide containing 2.217×10^{-4}, 1.915×10^{-c}, and 1.495×10^{-4} moles/L. in 0.4 *M* phosphate buffer of pH 7.5 were prepared and the rate of hydrolysis followed at 65° in the manner described.

DISCUSSION

In the pH range 5–8 studied (Figs. 1–3), the hydrolysis of glutethimide appears to be base

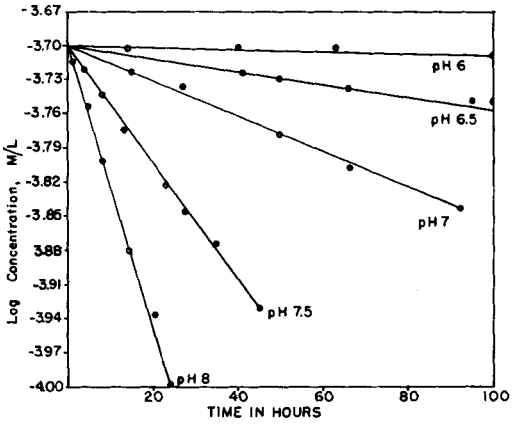


Fig. 1—Log of concentration of glutethimide against time at 50° at various pH's.

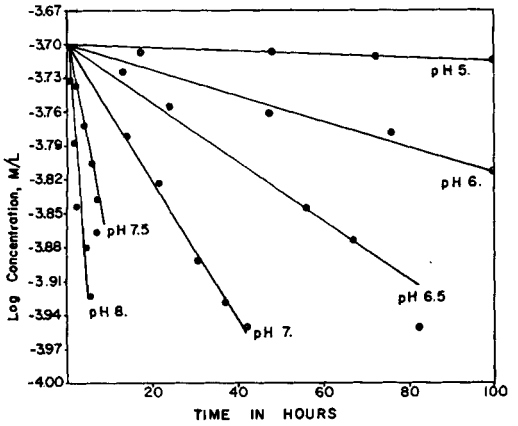


Fig. 2—Log concentration of glutethimide against time at 60° at various pH's.

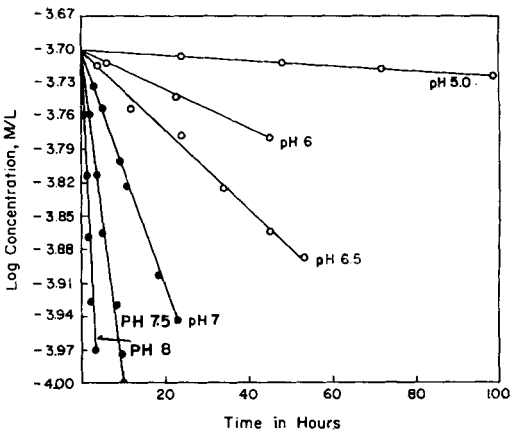


Fig. 3—Log concentration of glutethimide against time at 65° at various pH's.

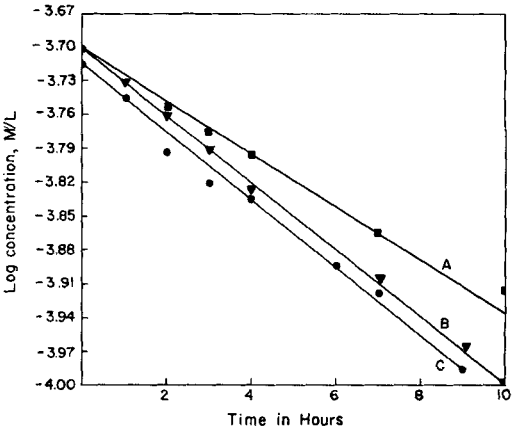


Fig. 4—Effect of buffer concentration and ionic strength on the hydrolysis rate of glutethimide at 65° in phosphate buffer at pH 7.5. Key: A, 0.4 M buffer + 1% KCl; B, 0.4 M buffer; C, 0.2 M buffer.

catalyzed. Figure 4 shows that although the phosphate buffer system was the only one used in this study, no discernible change in the hydrolytic rate was observed when the reaction was carried out in 0.2 and 0.4 *M* buffer solutions, suggesting a specific base catalysis.

It has been reported that the rate of hydrolysis of succinimide (9) and phthalimide (10, 11) is dependent upon the concentration of ionized as well as unionized form of the cyclic imide. Therefore, a decrease in the rate should be observed as the proportion of dissociated to undissociated imide increases. The imide anion is not readily attacked by the hydroxyl ion, in that greater energy would be required to overcome the electrostatic repulsive forces present in such a system.

In a previous work (10) a general second-order rate expression was reported which took into consideration the contribution of the ionized as well as the unionized species on the rate observed. Since the present work dealt with the lower pH region, k_a was considered negligible in comparison to the hydrogen ion concentration since the anionic species is insignificant in this pH range.

The hydrolysis rate is first order with respect to glutethimide since a straight line is obtained when

the logarithm of concentration is plotted against time (Figs. 1–3). This can be further verified (Fig. 5) by plotting the effect of initial glutethimide concentration on the hydrolysis rate. The plot shows the first-order dependence of the reaction with respect to glutethimide, the rate of the reaction being directly proportional to the concentration of the substrate.

The order of the reaction with respect to hydroxyl ion concentration can be determined from the relationship

$$\log k = \log C + n\text{pH}$$

A plot of log *k' versus* pH should give a straight line with a slope of *n*. Figure 6 shows that the slope, 0.897, is not in agreement with the expected value of 1.0. The deviation may be due to the analytical

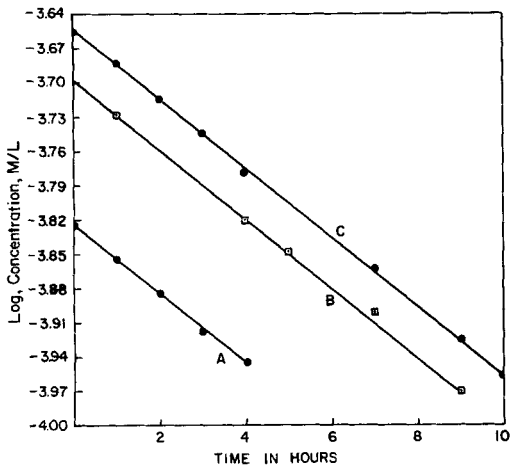


Fig. 5—Effect of initial concentration on the hydrolysis rate of glutethimide at 65°, pH 7.5. Key: A, *1.495 × 10⁻⁴ moles/L.;* B, *1.915 × 10⁻⁴ moles/L.;* C, *2.217 × 10⁻⁴ moles/L.*

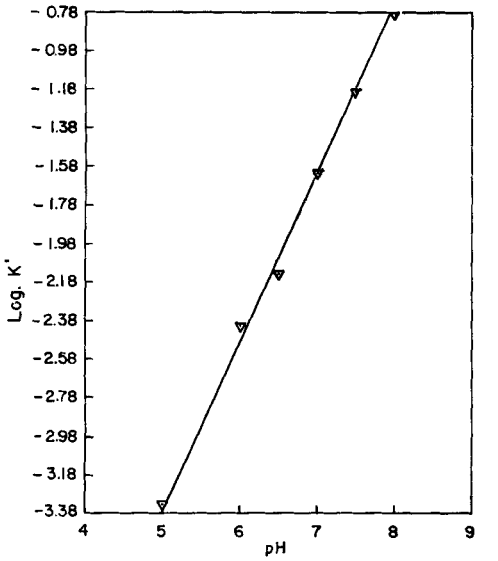


Fig. 6—Log K', the specific reaction constant against pH at 65°.

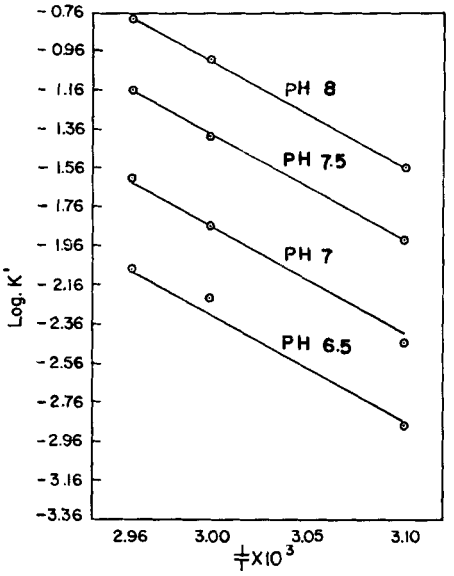


Fig. 7—Plot of log K' versus 1/T showing the temperature dependence of the rate of hydrolysis of glutethimide at various pH's.

procedure, *i.e.*, ion-exchange separation of glutethimide from EPG.

Although the ionic strength in 0.4 *M* phosphate buffers is beyond the limits of the Debye-Hückel limiting law (14), it nevertheless was desired to determine whether primary salt effects existed in the hydrolysis of glutethimide. The slight decrease in rate observed upon the addition of 1% potassium chloride (Fig. 4) cannot be unequivocally explained at this time. A plausible explanation may be that a "salting-out" effect on the slightly soluble glutethimide was brought about due to the increased electrolyte concentration.

The Arrhenius plot (Fig. 7) of log *k' versus* 1/*T* was found to give a straight line with a constant apparent energy of activation, E_a, of 25.86 Kcal. The apparent energy of activation, E_a, for the three temperatures, corrected for the ΔHi of water by the method of Harned and Hamer (15) are listed in Table I. The half-lives, $t_{1/2}$, of glutethimide at different pH were calculated for 25° by the Clausius-Clapeyron equation and are listed in Table II. A plot of log $t_{1/2}$ *versus* pH (Fig. 8) shows that below pH 5 glutethimide is very stable since at pH 1.0 and 5.0 the rate is independent of hydrogen ion concentration.

Mechanism of Hydrolysis—The hydrolysis of glutethimide probably involves a bimolecular (S_N2) mechanism (16) with direct attack by the hydroxyl ion being the rate-determining (slow step) step of hydrolysis as shown in Scheme I.

Bimolecular mechanisms have been known to be

TABLE I—ENERGY OF ACTIVATION CORRECTED FOR HEAT OF IONIZATION OF WATER[a]

50°—13.47 Kcal.
60°—13.92 Kcal.
65°—14.15 Kcal.

[a] Heats of ionization of water calculated according to Harned and Hamer (15) were found to be: 50°—12.39 Kcal., 60°—11.936 Kcal., and 65°—11.71 Kcal.

TABLE II—HALF-LIFE PERIODS, $t_{1/2}$, FOR THE HYDROLYSIS OF GLUTETHIMIDE AT DIFFERENT pH'S AND AT 25°

pH	$t_{1/2}$ (months)	log $t_{1/2}$[a]
8.0	1.02	0.008
7.5	2.43	0.386
7.0	6.82	0.840
6.5	22.10	1.345
6.0	43.40	1.637
5.0	351.00	2.546

[a] Calculated using the apparent activation energy, E_a, from the Arrhenius plot, and the specific rate constant at 25°.

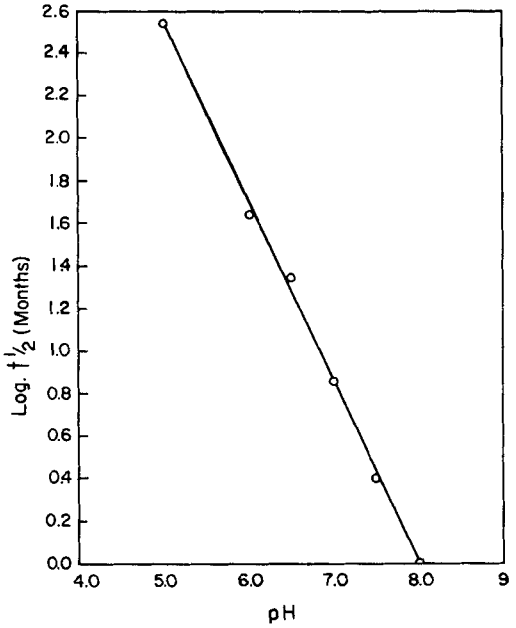
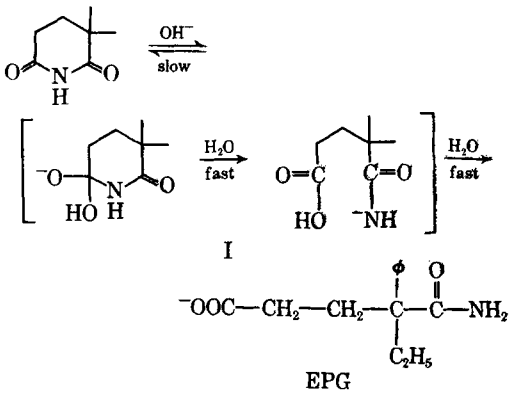


Fig. 8—Log $t_{1/2}$, the half-life of the reaction in months against pH at 25°.

subject to steric hindrance. The rear of the carbon where displacement of the C—N bond is occurring must be unhindered in order to permit facile entrance of the attacking hydroxyl group for the formation of the reaction intermediate (I). Therefore, the reaction may be expected to be sensitive to steric hindrance at the site of reaction but relatively insensitive to electronic effects.

Fischer-Hirschfelder models of glutethimide and of the postulated intermediate in the hydrolysis mechanism (I) were prepared. Steric hindrance due to the 2-ethyl-2-phenyl substituents appears to be quite effective on the 1-carbonyl carbon. The 5-carbonyl carbon is comparatively unhindered for attack by the hydroxyl ion, and 4-ethyl-4-phenyl glutaramic acid would be the main product from such an attack. Further hydrolysis to the corresponding dicarboxylic acid should occur only under adverse conditions due to the same steric retardation. This view is substantiated by a report (12) indicating that 40% potassium hydroxide is necessary for the amide cleavage.

No attempt was made to isolate the postulated degradation product, 4-ethyl-4-phenyl glutaramic acid (EPG), in the kinetic study. However, using thin-layer chromatography, EPG was found to be the degradation product of glutethimide. EPG



Scheme I

was prepared by refluxing a 5-Gm. sample of glutethimide in 50 ml. of a 10% sodium hydroxide solution for 2 hr. The solution was cooled and acidified with concentrated hydrochloric acid to a Congo Red end point. The precipitated solid was collected on a Büchner funnel and washed with deionized water. The compound recrystallized from 20% ethanol had a m.p. 156–157°.

Anal.—Calcd. for $C_{13}H_{17}NO_3$: C, 66.32; H, 7.28; N, 5.95. Found: C, 66.81; H, 7.39, N, 6.01.

A solution containing 100 mg./100 ml. of glutethimide, adjusted to pH 8.0 with phosphate buffer, was prepared. The solution was heated in a constant temperature bath adjusted to 65°. After 24 hr. the solution was cooled and a sample was spotted on a prepared thin-layer plate beside a sample taken from a solution containing ungraded glutethimide plus EPG. A methanol–acetone–triethanolamine solvent was used for development and Dragendorff's reagent for visualization. The sample taken from the glutethimide solution that had been heated for 24 hr. showed two spots which corresponded to the two spots obtained from the sample containing undegraded glutethimide and EPG.

Recent work on the hydrolysis kinetics of phthalimide and *ortho*-carboxyphthalimide (17) shows that a small region in the very low pH range exists where acid catalysis occurs at 100°. However, similar acid catalysis was unobserved in the pH ranges and temperatures employed in this study.

SUMMARY AND CONCLUSION

The degradation of glutethimide in buffer solution of pH's ranging from 5.0–8.0 at 65, 60, and 50° has been studied. The degradation of glutethimide in aqueous solutions appears to be a base-catalyzed reaction, since the contribution of ionized species to the reaction rate in the pH range studied is very small. The rate is first order with respect to the concentration of glutethimide.

The apparent energy of activation, E_a, for the degradation of glutethimide at pH 8.0 was found to be of the order of 25.86 Kcal. Corrections were made for the apparent energy of activation, E_a, for the heat of ionization of water in the temperature range studied.

The half-lives at 25° for glutethimide in aqueous solutions were determined from the calculated specific reaction constants for reactions at different pH's and at 25°. The $t_{1/2}$ of glutethimide in a

buffered solution of pH 5 was calculated to be 28.3 years at 25°. The use of water-soluble hydrochloride glutethimide is recommended due to high solubility and high stability.

A mechanism is proposed which involves direct attack by a hydroxyl ion on the unhindred carbonyl of the glutethimide, followed by cleavage of the ring to 4-ethyl-4-phenyl glutaramic acid.

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Keyphrases

Glutethimide degradation
Hydrolysis, gluthethimide—factors affecting
Half-life, glutethimide—variable pH
Degradation product—glutethimide
Anionic exchange resin—separation
TLC—identity

Some Pharmacological and Toxicological Properties of Several Phthalimidoaldehydes

By A. M. BURKMAN*, G. L. RINGHAM†, and M. H. WEINSWIG

Four phthalimidoaldehyde derivatives were prepared and several parameters of pharmacological activity were examined: acute toxicity, coordination deficit, ability to alter barbiturate-induced "sleep" in mice, provoke lacrimation and chromodacryorrhea in rats, and influence contractility of excised guinea pig ileum. Gamma-phthalimidobutyraldehyde and α-phthalimido-β-methylbutyraldehyde, the more toxic members of the group, significantly prolonged hexobarbital sleep time. The most intense lacrimatory effects were produced by α-phthalimido-β-methylbutyraldehyde while the most pronounced contractile response in isolated gut was provoked by phthalimidoacetaldehyde. The two latter responses were blocked by atropine premedication. None of the compounds produced chromodacryorrhea.

ALTHOUGH A variety of aldehyde derivatives and acyclic ketones have been examined for hypnotic activity since the introduction of chloral hydrate by Liebreich in 1869 (1), few remain prominent today as therapeutic agents. Most of these substances proved to be less desirable in terms of toxicity or effectiveness than other available hypnotics (2, 3) although chloral hydrate itself still occupies a preeminent position as a highly regarded and widely used soporific (4). In the search for and evaluation of aldehydic substances having potential hypnotic activity, attention was directed toward a group of phthalimidoaldehydes whose biological activity had never been investigated. Although the compounds to be described in this communication are known substances, that is, their syntheses have been reported (5–7), no pharmacological information is available from the literature. It was our intent to prepare and examine several phthalimidoaldehyde derivatives for gross CNS depressant activity. The nature of the responses displayed by animals during an initial toxicity screen would then dictate other pharmacological tests to be pursued.

Received March 3, 1967, from the Departments of Pharmacology and Pharmaceutical Chemistry, Butler University College of Pharmacy, Indianapolis, IN 46207
Accepted for publication February 6, 1968.
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METHODS

Synthesis—The phthalimidoaldehydes were all prepared using the Rosenmund reduction procedure

buffered solution of pH 5 was calculated to be 28.3 years at 25°. The use of water-soluble hydrochloride glutethimide is recommended due to high solubility and high stability.

A mechanism is proposed which involves direct attack by a hydroxyl ion on the unhindred carbonyl of the glutethimide, followed by cleavage of the ring to 4-ethyl-4-phenyl glutaramic acid.

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Keyphrases

Glutethimide degradation
Hydrolysis, gluthethimide—factors affecting
Half-life, glutethimide—variable pH
Degradation product—glutethimide
Anionic exchange resin—separation
TLC—identity

Some Pharmacological and Toxicological Properties of Several Phthalimidoaldehydes

By A. M. BURKMAN*, G. L. RINGHAM†, and M. H. WEINSWIG

Four phthalimidoaldehyde derivatives were prepared and several parameters of pharmacological activity were examined: acute toxicity, coordination deficit, ability to alter barbiturate-induced "sleep" in mice, provoke lacrimation and chromodacryorrhea in rats, and influence contractility of excised guinea pig ileum. Gamma-phthalimidobutyraldehyde and α-phthalimido-β-methylbutyraldehyde, the more toxic members of the group, significantly prolonged hexobarbital sleep time. The most intense lacrimatory effects were produced by α-phthalimido-β-methylbutyraldehyde while the most pronounced contractile response in isolated gut was provoked by phthalimidoacetaldehyde. The two latter responses were blocked by atropine premedication. None of the compounds produced chromodacryorrhea.

ALTHOUGH A variety of aldehyde derivatives and acyclic ketones have been examined for hypnotic activity since the introduction of chloral hydrate by Liebreich in 1869 (1), few remain prominent today as therapeutic agents. Most of these substances proved to be less desirable in terms of toxicity or effectiveness than other available hypnotics (2, 3) although chloral hydrate itself still occupies a preeminent position as a highly regarded and widely used soporific (4). In the search for and evaluation of aldehydic substances having potential hypnotic activity, attention was directed toward a group of phthalimidoaldehydes whose biological activity had never been investigated. Although the compounds to be described in this communication are known substances, that is, their syntheses have been reported (5–7), no pharmacological information is available from the literature. It was our intent to prepare and examine several phthalimidoaldehyde derivatives for gross CNS depressant activity. The nature of the responses displayed by animals during an initial toxicity screen would then dictate other pharmacological tests to be pursued.

Received March 3, 1967, from the Departments of Pharmacology and Pharmaceutical Chemistry, Butler University College of Pharmacy, Indianapolis, IN 46207
Accepted for publication February 6, 1968.
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METHODS

Synthesis—The phthalimidoaldehydes were all prepared using the Rosenmund reduction procedure

(8). This involved, in all cases, a three-step sequence of reactions in which (*a*) a phthalimido acid was formed by interaction of phthalic anhydride and one of four amino acids, (*b*) conversion of the acid to the acid chloride, and (*c*) reduction of the acid chloride to the aldehyde. Glycine, β-alanine, γ-aminobutyric acid, and valine were used as amino acid reactants and thus the following compounds were formed: phthalimidoacetaldehyde, β-phthalimidopropionaldehyde, γ-phthalimidobutyraldehyde, α-phthalimido-β-methylbutyraldehyde.

Preparation of Drugs for Biological Testing—The compounds, which were all sparingly soluble in water, were administered to animals as an aqueous suspension or, in the case of α-phthalimido-β-methylbutyraldehyde, an emulsion. The vehicle contained 0.25% methylcellulose, 1,500 cps. For the *in vitro* experiments, the drugs were dissolved in polyethylene glycol 200.

Acute Toxicity—Median lethal doses and their 95% confidence intervals were determined in female albino mice[1] using the intraperitoneal route of administration. For each drug three doses, six mice per dose, were used to estimate the position of the dose-lethality line (9).

Coordination Deficit—The method of Dunham and Miya (10) was employed to examine the drugs' ability to alter coordinated motor activity in mice. Animals were placed on a 2.5-cm. diameter horizontal rod rotating at a constant rate of 5 r.p.m. Mice that remained on the rod for 3 min. on each of two consecutive trials were selected for subsequent experimentation. Approximately 96% of the mice drawn from a stock colony met this criterion. Groups of six experimental mice were injected i.p. with doses of the compounds ranging from 50 to 400 mg./Kg. and reexamined for coordination deficit at intervals of 15 min. from injection until 3 hr. had elapsed and at 30-min. intervals thereafter until 6 hr. had elapsed. Animals that failed to remain on the rod *more* than once out of two consecutive trials were judged to suffer from a drug-induced neurological deficit. Twenty-four mice were used for each drug.

Potentiation of Barbiturate Sleep Time—For each of the phthalimidoaldehydes a fixed dose, equivalent to 1.06 mmoles/Kg., was administered to groups of mice. This dose, held constant to facilitate a comparison among drugs, represented no more than one-third the LD_{50} of the most toxic compound. Phthalimidoacetaldehyde was injected into seven groups of six mice. An eighth group of six, which served as a control, received only saline. Sodium hexobarbital, 120 mg./Kg., i.p., was administered to the groups at intervals of 0, 15, 30, 60, 90, 120, and 180 min. from aldehyde injection. "Sleep time" was recorded as that period during which the righting reflex was abolished. An animal was not credited with recovery until it was able to right itself twice within 15 sec. The results were subjected to an analysis of variance and Dunnett's test (11). This protocol was repeated for each aldehyde.

Lacrimation and Chromodacryorrhea—The method described by Malone *et al.* (12), was employed to detect and quantify muscarinic-like activity. Two female albino rats were used to test each of two dose levels of phthalimidoaldehyde. Saline was employed as a "blank" control and methacholine chloride, 10 mg./Kg., as a "positive" control. Lacrimation and chromodacryorrhea scores were assigned at 15-min. intervals for a period of 1 hr. following i.p. injection of the drugs. In subsequent experiments, the drugs' muscarinic activity was similarly evaluated in rats pretreated with atropine sulfate, 1 mg./Kg., i.p.

Contractility of Guinea Pig Ileum—Segments of terminal ileum were excised, suspended in aerated Tyrode's solution (13), and subjected to varying concentrations of the aldehydes which had been dissolved in appropriate amounts of polyethylene glycol 200. Smooth muscle activity was recorded electromechanically *via* an isotonic myograph. The glycol vehicle alone was included among the drugs tested. A single gut segment was used to evaluate the peripheral activity of *each* drug. The segments were initially challenged with two or three small doses of acetylcholine (ACH) chloride (0.001–0.004 mcg./ml. of bath) until stable, reproducible contractile responses were obtained. After the stabilization period, a dose of 0.004 mcg./ml. evoked a "standard" response (for each segment) against which aldehyde responses were subsequently compared. During the course of the experiment gut segments were periodically challenged with acetylcholine Cl, 0.002 mcg./ml. of bath, and atropine sulfate, 0.02 mcg./ml. of bath.

RESULTS AND DISCUSSION

Synthesis—The four phthalimidoaldehydes were successfully prepared in good yields and their melting points, along with those described in the literature, are summarized in Table I. The α-phthalimido-β-methylbutyraldehyde obtained as a viscous oil could not be crystallized. Similar difficulties were evidently encountered by others (7).

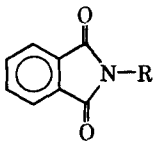
Biological Activity—Table II lists the 24-hr. LD_{50}'s of the four compounds along with their 95% confidence intervals. Phthalimidoacetaldehyde (PIA) and α-phthalimido-β-methylbutyraldehyde (APIM) were the least and most toxic compounds, respectively. The dose-lethality lines for β-phthalimidopropionaldehyde (BPIP) and γ-phthalimidobutyraldehyde (GPIB) were virtually superimpossible and thus their LD_{50}'s did not differ significantly. The dose-lethality lines for all four compounds, having a combined slope of approximately 16.8 probits/log unit, did not deviate significantly from parallelism.

Although PIA was least toxic in terms of lethal dose requirements, it was the only member of the series that elicited clonic convulsions in mice at high doses in the lethal range. Animals usually died in the midst of a convulsive seizure or during a period of extreme excitability which often followed convulsions. No other compound possessed convulsive activity. On the contrary, animals receiving drugs other than PIA usually lapsed into a gradually progressive depression terminating in death or slow recovery. Animals that survived remained markedly depressed for about 24 hr.

At sublethal concentrations each of the drugs, including PIA, was observed to suppress locomotor activity lasting for several hours without loss of the righting reflex. This apparent depressant activity was not reflected in any recognizable coordination deficit. When subjected to doses up to 400 mg./

[1] In this and all subsequent mouse experiments, female albino mice (Harlan ICR strain), 18–22 Gm. were employed.

TABLE I—PHTHALIMIDOALDEHYDES PREPARED FOR STUDY



Compd.	Code	R	Mol. Wt.	Experimental % Yield	Experimental M.P.[a]	Literature % Yield	Literature M.P.[a]
Phthalimidoacetaldehyde	PIA	—CH_2—CHO	189	87	110–112°	70	112–114°
β-Phthalimido-propionaldehyde	BPIP	—$(CH_2)_2$—CHO	203	88	114–116°	65	115–117°
γ-Phthalimido-butyraldehyde	GPIB	—$(CH_2)_3$—CHO	217	17	71–73°	91	72–73°
α-Phthalimido-β-methylbutyraldehyde	APIM	—CH(CHO)—CH(CH_3)(CH_3)	231	...	oil	...	oil

[a] Uncorrected.

TABLE II—ACUTE MEDIAN LETHAL DOSES OF PHTHALIMIDOALDEHYDES IN MICE

Compd.	Intraperitoneal LD_{50} and 95% Confidence Interval mg./Kg.	mmoles/Kg.
PIA	1725 (1531–1932)	9.1 (8.1–10.2
BPIP	1055 (942–1182)	5.2 (4.6–5.8)
GPIB	1055 (942–1182)	4.9 (4.3–5.5)
APIM	800 (721–888)	3.4 (3.0–3.7)

Kg., mice continued to successfully maintain their position on the rotating rod. At no time during the 6-hr. observation period did they fail the test.

In order to assess the subtler aspects of the drugs' CNS depressant action, the compounds' ability to alter hexobarbital-induced depression was investigated. The results are presented in Table III. Analysis revealed that using a fixed dose of 1.06 mmoles/Kg. of each drug only GPIB and APIM produced significant prolongation of sleep time. The dose employed was purposefully chosen so as not to exceed one-third the LD_{50} of the most toxic derivative (APIM). In spite of its relative insolubility significant hexobarbital potentiation was observed for GPIB even when the substance was administered at the same time as hexobarbital (0 premedication time). Although potentiating action could be demonstrated even when the hexobarbital injection was delayed for 120 min., GPIB's influences were apparently dissipated by 180 min. A significant but much less impressive potentiating effect was seen following APIM administration. Significant prolongation of hexobarbital sleep time could be demonstrated only after 60 min. had elapsed between APIM premedication and hexobarbital administration. This suggests that GPIB may be not only intrinsically more active than APIM, but more efficiently absorbed as well. The potentiating activities of GPIB and APIM are not, of course, necessarily reflections of direct CNS action. Their ability to influence hexobarbital sleep could be due to a wholly peripheral effect (*e.g.*, inhibition of barbiturate metabolizing enzymes). Furthermore, failure of the phthalimidoaldehydes to influence rotating rod performance clearly demonstrates that the compounds lack potent depressant activity. This, coupled with the fact that the depression of spontaneous locomotor activity may have been due to peritoneal irritation, leads to the inescapable suspicion that these compounds' central actions are weak at best.

At comparatively high doses the phthalimidoaldehydes were frequently observed to produce lacrimation, salivation, and flushing of the paw pads and ears. These observations prompted experiments designed to evaluate the drugs' cholinomimetic action *in vivo* and *in vitro*. Chromodacryorrhea, the secretion of porphyrin pigments by the Harderian gland (14) first described by Freud (15), was characteristically evoked by muscarinic stimulants. Although the response was readily provoked by methacholine, it was not elicited by any of the aldehydes (Table IV).

All compounds, except GPIB, increased lacrimatory responses and premedication of the animals with atropine successfully abolished these effects. APIM was responsible for the most intense lacrimation at a dose level of 2.1 mmoles/Kg. These cholinergic-like effects are not manifested until fairly high doses are attained and at these concentrations all animals were distinctly sedated.

TABLE III—INFLUENCE OF PHTHALIMIDOALDEHYDES ON HEXOBARBITAL SLEEP TIME IN MICE[a]

Compd.[b]	Control	Premedication Time, Min. 0	15	30	60	90	120	180
PIA	74 ± 10[c]	96 ± 14	85 ± 23	107 ± 12	92 ± 11	74 ± 13	71 ± 7	77 ± 7
BPIP	78 ± 13	64 ± 13	100 ± 24	97 ± 9	89 ± 10	83 ± 12	73 ± 9	57 ± 8
GPIB	51 ± 7	120 ± 22[d]	169 ± 22[d]	134 ± 19[d]	159 ± 24[d]	126 ± 20[d]	131 ± 6[d]	101 ± 16
APIM	72 ± 9	108 ± 15	111 ± 18	104 ± 14	141 ± 22[d]	109 ± 14	96 ± 11	87 ± 11

[a] Sodium hexobarbital, 120 mg./Kg., i.p. [b] Each compound was administered in fixed molar equivalent quantities (1.06 mmoles/Kg.). [c] Mean sleep time in min. ± standard error (6–10 mice/determination). [d] Significant at 5% level (1-tailed).

TABLE IV—LACRIMATION AND CHROMODACRYORRHEA RESPONSES

Compd. and Dose	Response[a]	Time from Injection, min. 15	30	45	60
Saline control	L	4.3[b]	3.3	2.3	2.8
	C	0	0	0	0
Methacholine Cl, 10 mg./Kg.	L	13.8	8.0	3.0	3.3
	C	5.8	0	0	0
Methacholine, Cl 10 mg./Kg. and	L	4.0	2.3	...	...
Atropine SO_4, 1 mg./Kg.	C	0	0	...	...
PIA, 400 mg./Kg. (2.1 mmoles)	L	8.8	5.0	5.5	3.0
	C	0	0	0	0
800 mg./Kg. (4.2 mmoles)	L	20.0	11.3	5.5	5.5
	C	0	0	0	0
PIA, 800 mg./Kg. and	L	10.5	7.5	...	...
Atropine SO_4, 1 mg./Kg.	C	0	0	...	...
BPIP, 300 mg./Kg. (1.4 mmoles)	L	6.3	6.0	3.3	1.5
	C	0	0	0	0
600 mg./Kg. (2.8 mmoles)	L	10.0	6.3	3.3	2.8
	C	0	0	0	0
BPIP, 600 mg./Kg. and	L	2.0	4.0	...	...
Atropine SO_4, 1 mg./Kg.	C	0	0	...	...
GPIB, 300 mg./Kg. (1.4 mmoles)	L	3.0	4.0	2.5	2.5
	C	0	0	0	0
600 mg./Kg. (2.8 mmoles)	L	3.8	3.8	2.8	3.0
	C	0	0	0	0
APIM, 300 mg./Kg. (1.3 mmoles)	L	9.3	2.3	3.8	2.5
	C	0	0	0	0
500 mg./Kg. (2.1 mmoles)	L	14.0	7.0	4.8	3.3
	C	0	0	0	0
APIM, 500 mg./Kg. and	L	6.8	6.3	...	...
Atropine SO_4, 1 mg./Kg.	C	0	0	...	...

[a] L-lacrimation; C-chromodacryorrhea. [b] Scores are in millimeters, the mean values derived from two albino rats; the higher the score the greater the intensity of response.

TABLE V—INFLUENCE OF PHTHALIMIDOALDEHYDES ON ISOLATED GUINEA PIG ILEUM

Ileum Segment[a]	ACH Response, mm.[b]	Drug	Dose, mmoles/ml. bath	Drug Response mm.	% ACH	CD50[c], mmoles/ml. bath
1	12	PIA	1.0×10^{-3}	14	1.17	
			8.0×10^{-4}	10	0.84	5.8×10^{-4}
			5.0×10^{-4}	4	0.33	
2	10	BPIP	1.0×10^{-3}	6	0.60	
			8.0×10^{-4}	4	0.40	8.9×10^{-4}
			5.0×10^{-4}	0.5	0.05	
3	16	GPIB	1.2×10^{-3}	10	0.62	
			1.0×10^{-3}	7.5	0.47	1.0×10^{-3}
			8.0×10^{-4}	5	0.32	
			5.0×10^{-4}	0	0	
4	10	APIM	1.0×10^{-3}	−20	−2.00	
			5.0×10^{-4}	−9.5	−0.95	...[d]

[a] Adjacent segments, each approximately 1 cm. in length, were cut from a 5-cm. section of terminal ileum. [b] The "standard" response evoked by acetylcholine chloride, 0.004 mcg./ml. of bath; in mm. of polygraph pen deflection. [c] Contractile dose 50: the interpolated dose of aldehyde producing a contractile response one half that evoked by the "standard" dose of ACH [d] Only depression of gut tone was observed.

PIA provoked the most intense contractile response in isolated gut (Table V). BPIP and GPIB were considerably less active as spasmogens while APIM produced only a sustained relaxation of ileal musculature. Both the stimulant and depressant effects were dose-dependent and inspection revealed that the log dose-percent response lines closely approached parallelism. A comparatively long onset time (about 20–25 sec.) was associated with the stimulant response while APIM's depressant action was seen within 3–5 sec. of drug administration. Contractions could be prevented by the prior introduction of atropine into the tissue bath. Responsiveness of depressed tissues to ACH remained essentially unaltered. Control vehicle, polyethylene glycol 200, produced no observable responses in the doses used.

SUMMARY

(*a*) Four phthalimidoaldehydes were prepared and examined for biological activity: phthalimidoacetaldehyde (PIA); β-phthalimidopropionaldehyde (BPIP); γ-phthalimidobutyraldehyde (GPIB), and α-phthalimido-β-methylbutyraldehyde (APIM).

(*b*) The order of decreasing i.p. toxicity in mice was APIM, BPIP (GPIB), PIA.

(*c*) PIA, alone among the aldehydes, produced hyperactivity and convulsions in mice when administered in lethal or near-lethal concentrations. At lower doses all compounds produced distinct sedation in both rats and mice. The sedative action expressed itself primarily as a depression of spontaneous locomotor activity. Sedated mice, however, did not exhibit any coordination deficit as

determined by their ability to maintain their position on a rotating rod.

(*d*) The most pronounced prolongation of hexobarbital sleep time in mice was produced by GPIB. Less impressive, but nonetheless significant, was the sleep potentiating effect of APIM. In equimolar concentrations neither PIA nor BPIP exhibited any influence on barbiturate depression.

(*e*) At comparatively high doses, the aldehydes were frequently observed to produce lacrimation, salivation, and flushing of the paw pads and ears, observations that prompted examination of the drugs for possible cholinomimetic activities.

(*f*) The estimated order of decreasing activity as lacrimatory stimulants in rats was APIM, BPIP (PIA), GPIB. This action was blocked by atropine premedication.

(*g*) None of the aldehydes provoked chromodacryorrhea in rats.

(*h*) PIA and BPIP and GPIB evoked short-lived tonic contractions in isolated guinea pig ileum that could be blocked by atropine. APIM produced only mild gut relaxation. There appears to be little relationship between the aldehydes' spasmogenic properties and their ability to stimulate lacrimation.

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Keyphrases

Phthalimidoaldehydes—synthesis, pharmacology
LD_{50} values—phthalimidoaldehydes
Locomotor activity—depressed
Hexobarbital sleep—prolonged
Lacrimatory responses—increased
Spasmogenic activity—evaluated

Thin-Layer Chromatography and IR Spectrophotometry of Commercial Sodium Sulfobromophthalein Solutions

By F. BARBIER and G. A. DEWEERDT

Techniques and results of analysis of some commercially available sulfobromophthalein (phenoltetrabromphthalein disulfonate) solutions by means of TLC and IR spectrophotometry are reported. The IR spectra of sulfobromophthalein and sulfobromophthalein-like compounds are presented and discussed. Three of the additional fractions found could be identified as phenoltetrabromphthalein and its mono- and trisulfonate derivatives. These impurities probably are due to imperfections in the synthesis of sulfobromophthalein. With but one exception they represent less than 1 percent of the total absorbance at 578 mμ. Such low concentrations do not interfere with the clinical use of sulfobromophthalein.

DESPITE THE WIDE use and established value of sulfobromophthalein (phenoltetrabromphthalein disulfonate) in the diagnosis of liver disease, little information is available on the composition of the dye solutions for intravenous use (1–5). The purpose of this study was to analyze some sulfobromophthalein solutions, commercially available in Belgium, with the aid of thin-layer chromatography (TLC) and IR spectrophotometry.

Received January 6, 1967, from the Policlinic of Internal Medicine, University of Ghent, Gent, Belgium.
Accepted for publication October 12, 1967.

METHODS

Sulfobromophthalein solutions for intravenous use and marketed by the following laboratories were analyzed: Vitarine Co., lot 9364; E. Merck, lots R493, R879, R959, and T959; Hynson, Westcott and Dunning, lots P_{23}, R_{96}, T_{47}, and U_{28}; and Simes, lot 3E29.

Thin-layer Chromatography—TLC was carried

determined by their ability to maintain their position on a rotating rod.

(*d*) The most pronounced prolongation of hexobarbital sleep time in mice was produced by GPIB. Less impressive, but nonetheless significant, was the sleep potentiating effect of APIM. In equimolar concentrations neither PIA nor BPIP exhibited any influence on barbiturate depression.

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Thin-Layer Chromatography and IR Spectrophotometry of Commercial Sodium Sulfobromophthalein Solutions

By F. BARBIER and G. A. DEWEERDT

Techniques and results of analysis of some commercially available sulfobromophthalein (phenoltetrabromphthalein disulfonate) solutions by means of TLC and IR spectrophotometry are reported. The IR spectra of sulfobromophthalein and sulfobromophthalein-like compounds are presented and discussed. Three of the additional fractions found could be identified as phenoltetrabromphthalein and its mono- and trisulfonate derivatives. These impurities probably are due to imperfections in the synthesis of sulfobromophthalein. With but one exception they represent less than 1 percent of the total absorbance at 578 mμ. Such low concentrations do not interfere with the clinical use of sulfobromophthalein.

DESPITE THE WIDE use and established value of sulfobromophthalein (phenoltetrabromphthalein disulfonate) in the diagnosis of liver disease, little information is available on the composition of the dye solutions for intravenous use (1–5). The purpose of this study was to analyze some sulfobromophthalein solutions, commercially available in Belgium, with the aid of thin-layer chromatography (TLC) and IR spectrophotometry.

Received January 6, 1967, from the Policlinic of Internal Medicine, University of Ghent, Gent, Belgium.
Accepted for publication October 12, 1967.

METHODS

Sulfobromophthalein solutions for intravenous use and marketed by the following laboratories were analyzed: Vitarine Co., lot 9364; E. Merck, lots R493, R879, R959, and T959; Hynson, Westcott and Dunning, lots P_{23}, R_{96}, T_{47}, and U_{28}; and Simes, lot 3E29.

Thin-layer Chromatography—TLC was carried

out on chromatoplates (20 × 20 cm.) prepared by the technique of Stahl (thickness of the absorptive layer of Silica Gel G, Merck, 250 μ) with two solvent systems: butanol–acetic acid–water (30:7.5:12.5 v/v) and butanol–propionic acid–water (30:20:7.5 v/v). When the solvent front reached a height of 10 cm., the plate was removed, dried in air, and developed in ammonia vapors. The fraction corresponding with phenoltetrabromphthalein disulfonate was located with bromsulfalein Fuka. [The other reference compounds (phenoltetrabromphthalein and its mono- and tetrasulfonate derivatives) were kindly provided by N. B. Javitt, New York, N.Y.] All these compounds develop a purple color on alkalization. The fractions obtained were scraped off the plate, eluted with 0.1 *N* NaOH, and centrifuged. The absorptions in the visible range of the spectrum (wavelength scan from 320 to 750 mμ) of each fraction were scanned on a Beckman DB recording spectrophotometer. The percentage of each fraction was estimated from its absorbance at 578 mμ.

IR Spectrophotometry—All IR spectra were obtained with a Perkin-Elmer NaCl Infracord using the potassium bromide pellet technique.

Prior to preparative TLC of the fast-running fractions, RI and RII, 400 ml. of sulfobromophthalein solution (Simes) was alkalized with 1 Gm. of KOH and extracted twice with butanol. To this extract HBr was added till the purple color disappeared. Next the extract was washed twice with distilled water, evaporated *in vacuo* until saturation, and chromatographed on MN Silica Gel N-HR/UV 254 (Macherey, Nagel and Co.) absorptive layers of 500-μ thickness. Fraction RI was isolated with butanol–acetic acid–water (12:4.5:5.5 v/v) and fraction RII with hexane–butanol (20:1 v/v). Both fractions were visualized under UV light (wavelength of 254 mμ), scraped off the plate, and eluted with ethanol.

Sufficient amounts of pure fraction SII were obtained from lot P23 of Hynson, Westcott and Dunning and lot 3E29 of Simes. The dye solutions were chromatographed on MN Silica Gel N-HR with *tert*-butanol–water–butanol (32:10:5 v/v), visualized with KOH spray, scraped off the plate, eluted with water, concentrated *in vacuo*, and finally filtrated on Sephadex G 25.

RESULTS

Commercial sulfobromophthalein solutions always proved to be separable into several fractions by TLC (Table I). After alkalization, a total of eight additional purple fractions was demonstrated. In both chromatographic systems the R_f of the fastest running fraction (RII) corresponds with phenoltetrabromphthalein, and that of the other fast-running fraction (RI) with phenoltetrabromphthalein monosulfonate. No fraction corresponding with phenoltetrabromphthalein tetrasulfonate was found.

Table II shows the percentages of the fractions found in the sulfobromophthalein solutions. With the exception of the sulfobromophthalein, Simes lot 3E29, the additional fractions always represent less than 1% of the total absorbance at 578 mμ.

The visible spectra of sulfobromophthalein and all additional fractions were indistinguishable; so this technique is unable to characterize the multiple fractions eluted from the chromatogram. Fortunately IR spectrophotometry gives more gratifying results.

The phthalein compounds studied differ by the number of Br atoms linked to the phthalide group, and the number and the localization of the sulfonate substituents on the hydroxyphenyl groups (Fig. 1).

Table I—Sulfobromophthalein-Like Fractions of Commercial Sulfobromophthalein Solutions Separated by TLC

	Phenol-tetra-brom-phthalein	Phenol-tetra-brom-phthalein Mono-sulfonate	Phenol-tetra-brom-phthalein Tetra-sulfonate	Mfr. A[a] R959	Mfr. A[a] R879	Mfr. A[a] R493	Mfr. A[a] T959	Mfr. B[b] 9364	Mfr. C[c] 3E29	Mfr. D[d] P 23	Mfr. D[d] R 96	Mfr. D[d] T 47	Mfr. D[d] U 28
RII	+	...	...	...	...	...	...	...	+	...	...	...	+
RI	...	+	...	+	+	+	+	+	+	+	+	+	+
Sulfobromo-phthalein	...	...	...	+	+	+	+	+	+	+	+	+	+
SI	...	...	...	...	...	...	...	...	+	+	...	...	+
SII	...	...	...	...	...	...	...	...	...	+	+	+	+
SIII	...	...	...	+	+	+	+	...	+	+	+	+	+
SIV	...	...	...	...	...	...	...	...	...	+	...	...	...
SV	...	...	...	...	...	...	...	...	+	+	...	...	...
SVI	...	...	...	...	...	...	...	...	...	+	...	...	...
SVII	...	...	+	...	...	...	...	...	...	...	...	...	...
No. of fractions				3	3	3	3	2	6	8	4	4	6

[a] Merck. [b] Vitarine. [c] Simes. [d] Hynson, Westcott & Dunning.

Table II—Percentage of Sulfobromophthalein-Like Fractions Found in Commercial Sulfobromophthalein Solutions

	Mfr. A[a] R959	Mfr. A[a] R879	Mfr. A[a] R493	Mfr. A[a] T959	Mfr. B[b] 9364	Mfr. C[c] 3E29	Mfr. D[d] P23	Mfr. D[d] R96	Mfr. D[d] T47	Mfr. D[d] U28
RII	...	...	...	...	...	0.0723	...	...	...	0.0009
RI	0.0012	0.0007	0.0008	0.0020	0.0026	3.8718	0.0618	0.0683	0.0223	0.5762
Sulfobromophthalein	99.9558	99.9990	99.9990	99.9956	99.9974	95.9318	99.9054	99.9280	99.9741	99.4203
SI	...	...	...	...	...	0.0445	0.0054	...	...	0.0009
SII	...	...	...	...	...	...	0.0032	0.0027	0.0009	0.0007
SIII	0.0030	0.0003	0.0002	0.0024	...	0.0796	0.0205	0.0010	0.0027	0.0010
SIV	...	...	...	...	...	...	0.0016	...	...	...
SV	...	...	...	...	...	Traces	0.0017	...	...	...
SVI	...	...	...	...	...	...	0.0004	...	...	...

[a] Merck. [b] Vitarine. [c] Simes. [d] Hynson, Westcott & Dunning.

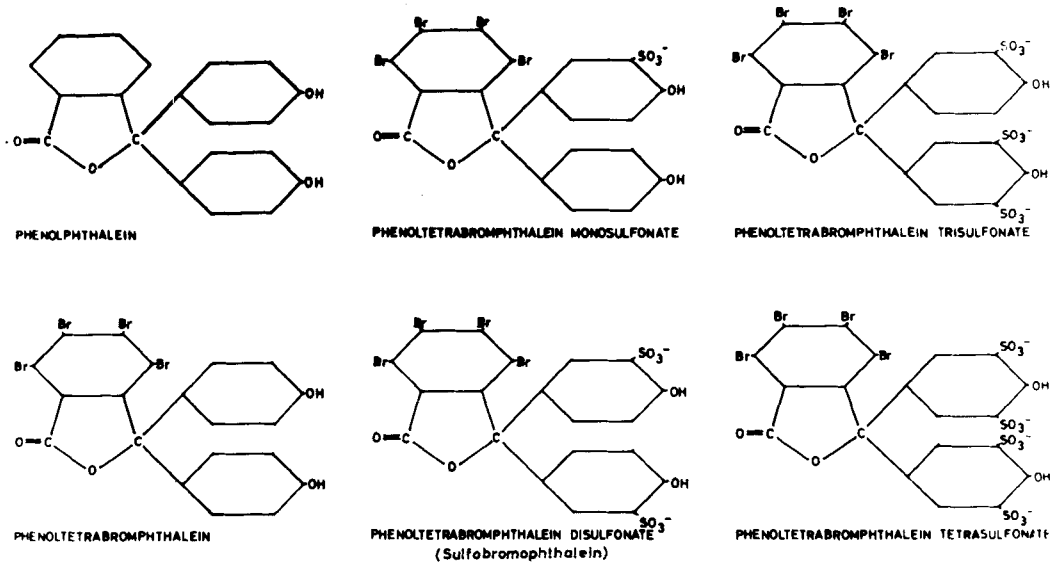

Fig. 1—Structural formulas of some of the phthalein dyes examined.

Phenolphthalein (Table III)—The band at 5.7 μ can be assigned to the —C=O group in the phthalide nucleus. This absorbance is present in all compounds examined and sufficiently individualized to provide reliable measurements of the concentration of the compound in the KBr pellet. Aromatic ring vibrations: in most cases three peaks are found (6.2, 6.6, and 6.9 μ); a fourth peak at 6.85 μ is perhaps due to the aromatic ring in the phthalide group. The strong 12-μ band arises from the adjacent pairs of H atoms on the phthalide group.

Phenoltetrabromphthalein (Fig. 2, Table III)—The halogenation has the following effects on the IR spectrum: absence of the band at 13.2 μ, and the mass increase induces a lowered vibrational frequency of the aromatic ring of the phthalide group and hence shifts its absorption from 6.85 μ toward longer wavelengths (7.4 μ).

Phenoltetrabromphthalein Monosulfonate (Fig. 2, Table III)—The introduction of one sulfonate substituent results in the following changes: (*a*) the band at 9.7 μ, which can be assigned to the sulfonate substituent, is very well individualized and provides a simple means of estimating the number of sulfonate substituents on comparing to the absorbance at 5.7 μ. (*b*) The site of the new substituent causes the loss of one adjacent pair of H atoms (decrease at 12 μ) which is replaced by one isolated H atom (weak absorption at 11 μ). (*c*) The mass increase in one hydroxyphenyl group lowers its frequency and shifts the aromatic ring absorption toward longer wavelengths. As only one group is involved, a reduplication of these bands results (6.6 and 6.7 μ; 6.9 and 7.1 μ).

Phenoltetrabromphthalein Disulfonate (Sulfobromophthalein) (Fig. 3, Table III)—The introduction of a second SO_3^- substituent restores the symmetry of the hydroxyphenyl groups. Hence, the reduplication of the aromatic ring peaks disappears and an absorption is now found only at 6.7 and 7.1 μ; the SO_3^- band at 9.7 μ doubles in comparison to the absorbance at 5.7 μ, and another adjacent H pair is replaced by an isolated H atom (further decrease at 12 μ, increase at 11 μ).

Phenoltetrabromphthalein Tetrasulfonate (Fig. 3, Table III)—Two more SO_3^- substitutents are introduced, resulting in a more pronounced shift of the aromatic ring peaks toward 6.9 μ (and perhaps 7.4 μ?); once more a doubled absorbance at 9.7 μ, four SO_3^- substituents now being present in one molecule; and since all adjacent H atoms are eliminated, the absorption at 12 μ disappears while the intensity of the band at 11 μ is much enhanced.

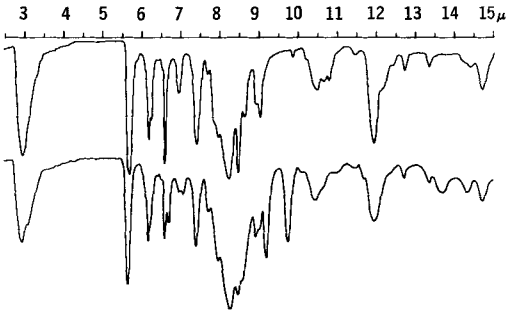

Fig. 2—Infrared spectra of phenoltetrabromphthalein (top curve), and phenoltetrabromphthalein monosulfonate (bottom curve).

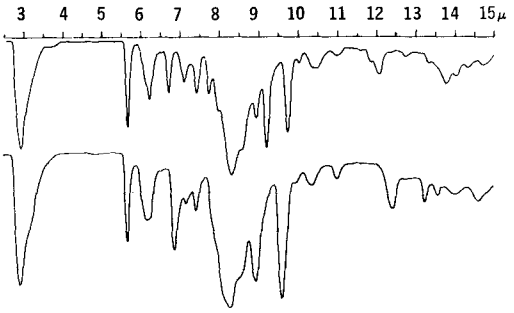

Fig. 3—Infrared spectra of phenoltetrabromphthalein disulfonate (sulfobromophthalein) (top curve) and phenoltetrabromphthalein tetrasulfonate (bottom curve).

TABLE III—MEAN INFRARED ABSORPTION BANDS OF SULFOBROMOPHTHALEIN-LIKE COMPOUNDS

	Phenolphthalein			Phenoltetrabromphthalein			Phenoltetrabromphthalein Monosulfonate			Phenoltetrabromphthalein Disulfonate			Phenoltetrabromphthalein Tetrasulfonate			Fraction RII			Fraction RI			Fraction Sulfobromophthalein			Fraction SIII		
	μ	cm.$^{-1}$	I	μ	cm.$^{-1}$	I	μ	cm.$^{-1}$	I	μ	cm.$^{-1}$	I	μ	cm.$^{-1}$	I	μ	cm.$^{-1}$	I	μ	cm.$^{-1}$	I	μ	cm.$^{-1}$	I	μ	cm.$^{-1}$	I
—O—	3.0	3350	5	3.0	3350	6	2.95	3400	5	2.9	3450	6	2.9	3450	6	2.95	3400	8	2.95	3400	6	2.95	3450	10	2.95	3400	7
—C=O	5.75	1740	*10*	5.7	1760	*10*	5.65	1770	*7*	5.65	1770	*6*	5.65	1770	*4*	5.72	1750	*10*	5.65	1770	*7*	5.65	1770	*8*	5.65	1770	*4*
	6.2	1620	5	6.2	1620	6	6.2	1620	4	6.2	1620	3	6.15	1630	2	6.2	1620	6	6.2	1620	5	6.2	1620	5	6.15	1625	sh
													6.25	1600	2										6.25	1600	7
	6.6	1520	8	6.6	1520	8	6.6	1515	4					...		6.6	1520	6	6.6	1515	4						
							6.7	1495	3	6.7	1490	3							6.7	1495	3	6.75	1480	5	6.75	1480	5
													6.85	1460	4										6.85	1460	5
	6.9	1440	4	6.9	1440	3	6.9	1440	2							6.9	1440	4	6.9	1440	3						
							7.1	1410	2	7.1	1410	2	7.15	1395	2				7.1	1410	3	7.15	1405	3	7.15	1400	sh
	6.85	1465	3																								
		...		7.4	1350	6	7.4	1350	5	7.4	1350	3	7.4	1355	2	7.4	1350	5	7.4	1350	5	7.4	1350	3	7.4	1350	sh
													8.9	1120	7										9.0	1115	8
—SO_3^-		...			...		9.15	1090	6	9.15	1090	7		...			...		9.2	1085	6	9.2	1085	7	9.15	1090	sh
		...			...		9.7	1030	*4*	9.7	1030	*6*	9.55	1045	*8*		...		9.7	1030	*4*	9.75	1025	*6*	9.7	1030	*7*
H		...			...		11.0	9.10	1	11.0	910	1	11.0	910	1		...		11.0	910	1	11.0	910	1	11.0	910	sh
H H	11.9	838	4	11.9	840	6	12.0	835	3	12.0	835	2		...		11.9	840	4	12.0	835	3	12.0	835	2	12.0	830	sh
H H H H	13.2	758	3		...			...			...			...			...			...			...			...	

Fraction RII (Table III)—The very low concentration of this compound resulted in tedious isolation and difficult purification. Two spectra were obtained but the purification was still incomplete. Nevertheless some conclusions were possible: (*a*) the phthalide group is present as a Br derivative (7.4 μ instead of 6.85 μ). A four-Br derivative is most likely, as the corresponding H atoms would give supplementary bands in the region 11–13 μ. (*b*) The hydroxyphenyl groups are symmetrical (6.6 and 6.9 μ); neither presence of SO_3^- groups (no absorption at 9.7 μ) nor of other substituents (strong band at 11.9 μ, no band at 11 μ). (*c*) The region 12–15 μ is identical with that of phenoltetrabromphthalein. Therefore it is concluded that compound RII is identical with phenoltetrabromphthalein.

Fraction RI (Table III)—A tetrabromphthalide group is present (7.4 μ). Asymmetry of the hydroxyphenyl groups (6.6 and 6.7 μ; 6.9 and 7.1 μ) related to the presence of one SO_3^- substituent (absorption at 9.7 μ) in one molecule (ratio of the absorbance at 5.65 μ to the absorbance at 9.7 μ) and a weak absorption at 11 μ suggest the replacement of one adjacent H pair by an isolated free H atom. The region 12–15 μ is identical with that of phenoltetrabromphthalein monosulfonate. It is concluded that fraction RI is identical with phenoltetrabromphthalein monosulfonate.

Fraction Sulfobromophthalein (Table III)—A tetrabromphthalide group is present (7.4 μ); hydroxyphenyl groups are symmetrical but shifted (6.75 and 7.15 μ); two SO_3^- substituents are present (ratio of the absorbance at 9.7 μ to the absorbance at 5.7 μ); adjacent pairs of H atoms are diminished but still present (12 μ); isolated free H atoms are present, indicating a substitution in the hydroxyphenyl groups (11.0 μ). The region 12–15 μ is identical with that of sulfobromophthalein. The conclusion is that fraction sulfobromophthalein is identical with phenoltetrabromphthalein disulfonate.

Fraction SIII (Table III)—The ratio of the absorbance at 9.7 μ (SO_3^- group) to the absorbance at 5.65 μ argues for the presence of three sulfonate substituents in one molecule. The number of Br atoms in the phthalide group cannot be clearly stated, as a (inconstant) band at 7.2 μ (NH_4^+?) obscures the region of 7.4 μ. The most probable number is four, as there is a little shoulder appearing at 7.4 μ. The band at 11 μ (isolated H atom in the hydroxyphenyl ring) is stronger, and the band at 12 μ (adjacent free H atoms) is weaker as compared to sulfobromophthalein. This argues for the presence of only two adjacent free H atoms in one molecule.

Phthalide groups with 2 adjacent H atoms as found in 3,6-dibromo-, 3,4-dibromo-, and 3,6-dichloroderivatives absorb at 11.6 μ (unpublished data). The absorbance of this band from the spectrum of fraction SIII suggests that the adjacent H atoms are situated on one of the hydroxyphenyl groups, which obviously contains only one of the sulfonate substituents. A similar type of hydroxyphenyl group is found in sulfobromophthalein and results in absorptions at 6.7 and 7.1 μ (shifted aromatic ring vibrations) and at 9.15 μ (SO_3^- substituent).

As a matter of course the other hydroxyphenyl group contains the two remaining sulfonate substituents and two free isolated H atoms. The latter type of hydroxyphenyl group is found in the tetrasulfonate derivative and results in absorptions at 6.85 and 7.15 μ (aromatic ring) and at 8.9 μ (SO_3^- substituent).

In fraction SII, the corresponding bands are found at 6.75 and 6.85 μ (aromatic ring); 7.1 and 7.15 μ, obscured (aromatic ring); 9.0 and 9.15 μ (SO_3^- group). This configuration is very well explained by the presence of both kinds of hydroxyphenyl groups in one molecule. Therefore this is most probably phenol (tetra ?) bromphthalein trisulfonate.

Fractions SI, SII, SIV, SV, and SVI were not obtained in sufficient amounts for IR analysis. Finally, the stability of aqueous solutions of phenoltetrabromphthalein and its mono-, di-, and tetrasulfonate derivatives was investigated. Chromatograms developed 1, 2, 3, and 4 months after preparing the solution revealed no additional fractions. Chromatograms of an aqueous solution of sulfobromophthalein (50 mg./ml.) autoclaved at 120° during 20 min. showed only one band, comparable in R_f to the standard dye.

DISCUSSION

TLC having a greater resolving power than paper and column chromatography successfully separates several sulfobromophthalein-like impurities in commercial sulfobromophthalein solutions. The chemical structure of two of the additional fractions was defined by IR spectrophotometry as phenoltetrabromphthalein and phenoltetrabromphthalein monosulfonate, whereas the third fraction has been tentatively identified as phenoltetrabromphthalein trisulfonate.

These investigations suggest that the additional fractions are due to imperfections in the synthesis of sulfobromophthalein. Because these impurities represent less than 1% of the total absorbance at 578 mμ in all but one of the sulfobromophthalein solutions for intravenous use examined, their influence on the disappearance rate of the dye from plasma must be negligible. Of paramount importance is the possible influence of these impurities on the number and composition of the sulfobromophthalein conjugates found in blood and bile. Indeed, Javitt (6, 7) demonstrated that phenolsulfodibromphthalein and phenolsulfotribromphthalein (8) are recovered mostly in bile, primarly as a glucuronide, and that conjugation of phenoltetrabromphthalein monosulfonate occurs mainly with glutathione although a glucuronide conjugate has also been found.

Phenoltetrabromphthalein tetrasulfonate and phenol 3,6-dibromphthalein disulfonate (9), on the other hand, formed no conjugates. At the present time no data are available about the fate of injected phenoltetrabromphthalein trisulfonate. Based on a considerable body of evidence (1–3, 10–12), it is now known that at least three of the four major ninhydrin-positive sulfobromophthalein compounds appearing in the bile consist of cleavage products and isomers of a conjugate of sulfobromophthalein and glutathione. In contrast to paper and column chromatography, TLC separates trace amounts of one to three additional sulfobromophthalein-like compounds in bile and blood of most patients (13).

It seems unlikely that these additional compounds have their origin in the tiny quantities of sulfobromophthalein-like impurities found in the dye solutions. Anyhow, free sulfobromophthalein and its major conjugates form the bulk of the dye appearing in the bile. Thus, the present investigation indicates that the small quantities of sulfobromophthalein-like impurities found in most commercial dye solutions for intravenous use do not interfere with the clinical use of the dye.

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Keyphrases

Sulfobromophthalein solutions—analysis
Impurities, sulfobromophthalein solution—identified
TLC—separation, identity
Colorimetric analysis
IR spectrophotometry—identity

Relationship Between Sputum Viscosity and Total Sialic Acid Content

By R. MUNIES, T. C. GRUBB, and R. E. CALIARI

Sputum samples collected from patients suffering from pulmonary diseases were hand homogenized, and the viscosity of the samples determined by measuring the flow time through a No. 100 Cannon-Fenske capillary viscometer at 37°. Total sialic acid content (free plus bound) was determined on an aliquot of each sample. In some patients producing an insufficient volume, samples from different patients were pooled and the data obtained treated separately. A sputum model was developed, and the viscosity and sialic acid content were determined in the same manner as the sputum. The data show a statistically significant linear relationship between viscosity and sialic acid concentration, until a point is reached where the acid content approaches a maximum and then plateaus, although the viscosity continues to increase.

VISCOSITY measurements of sputum have frequently been used for evaluating mucolytic or expectorant action of drugs. This measurement alone does not adequately describe the physicochemical properties of sputum. Measurements of adhesiveness and tensile strength of fibers should provide a more complete evaluation. However, reduction in viscosity is the prime objective to make the sputum more easily eliminated from the respiratory tract. Unfortunately "viscosity" is a term applied to homogeneous systems and is inappropriate for sputum, which is nonhomogeneous. Therefore, what is the appropriate measurement of viscosity to provide meaningful results?

Sputum viscosity has been measured in many different ways. Basch *et al.* (1) used a narrow glass tube and measured flow under pressure. However, Forbes and Wise (2) using the same technique could not obtain consistent readings on the same specimen. They, therefore, used a torque viscometer which measures viscosity by its dampening effect on the rotation of a metal cylinder suspended in it by a torsion wire. This instrument has limited use, because not less than 25 ml. is needed for each measurement and only fairly viscid samples can be measured accurately. In a study comparing the action of various expectorant drugs Simon and Harmon (3) blended the sputum specimens by vigorously stirring at

Received May 22, 1967, from the Vick Divisions Research & Development, Vick Chemical Company, Vick International Divisions of Richardson-Merrell Inc., Mount Vernon, NY 10553
Accepted for publication February 15, 1968.

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Relationship Between Sputum Viscosity and Total Sialic Acid Content

By R. MUNIES, T. C. GRUBB, and R. E. CALIARI

Sputum samples collected from patients suffering from pulmonary diseases were hand homogenized, and the viscosity of the samples determined by measuring the flow time through a No. 100 Cannon-Fenske capillary viscometer at 37°. Total sialic acid content (free plus bound) was determined on an aliquot of each sample. In some patients producing an insufficient volume, samples from different patients were pooled and the data obtained treated separately. A sputum model was developed, and the viscosity and sialic acid content were determined in the same manner as the sputum. The data show a statistically significant linear relationship between viscosity and sialic acid concentration, until a point is reached where the acid content approaches a maximum and then plateaus, although the viscosity continues to increase.

VISCOSITY measurements of sputum have frequently been used for evaluating mucolytic or expectorant action of drugs. This measurement alone does not adequately describe the physicochemical properties of sputum. Measurements of adhesiveness and tensile strength of fibers should provide a more complete evaluation. However, reduction in viscosity is the prime objective to make the sputum more easily eliminated from the respiratory tract. Unfortunately "viscosity" is a term applied to homogeneous systems and is inappropriate for sputum, which is nonhomogeneous. Therefore, what is the appropriate measurement of viscosity to provide meaningful results?

Sputum viscosity has been measured in many different ways. Basch *et al.* (1) used a narrow glass tube and measured flow under pressure. However, Forbes and Wise (2) using the same technique could not obtain consistent readings on the same specimen. They, therefore, used a torque viscometer which measures viscosity by its dampening effect on the rotation of a metal cylinder suspended in it by a torsion wire. This instrument has limited use, because not less than 25 ml. is needed for each measurement and only fairly viscid samples can be measured accurately. In a study comparing the action of various expectorant drugs Simon and Harmon (3) blended the sputum specimens by vigorously stirring at

Received May 22, 1967, from the Vick Divisions Research & Development, Vick Chemical Company, Vick International Divisions of Richardson-Merrell Inc., Mount Vernon, NY 10553
Accepted for publication February 15, 1968.

constant speed and measuring the viscosity in an Ostwald viscometer. In this study it was necessary to dilute some samples with water and extrapolate the viscosity to zero dilution.

Since sputum is non-Newtonian, some investigators feel any measurement of viscosity must be made with an instrument which can measure and vary the velocity gradient and tangential stress separately (4). Rotational viscosimeters fill this need but also have their shortcomings. A cone and plate rotational viscosimeter has the disadvantage that the small quantity of sputum which can be measured may not be representative. In addition, lumps in the sample may not always fill the gap between the cone and vessel floor, thus the values of corresponding tangential stresses are not comparable.

All current methods appear to have some shortcomings, however, if a positive relationship between viscosity and a common ingredient of sputum can be established, chemical assay of this component may then avoid this dilemma.

It is generally accepted that glycoproteins are mainly responsible for the physical properties of mucous secretions and that the terminal unit of the carbohydrate prosthetic groups is in many instances sialic acid (5). The naturally occurring sialic acids are substituted neuraminic acid derivatives (*N*-acetyl, *N*-glycol, *N,O,*-diacetylneuraminic acid). However, in man only the *N*-acetylneuraminic acid is found.

Sialomucins are regular components of the mucoid material covering the epithelial surfaces of the respiratory, digestive, and urogenital tracts. Their relationship to the viscosity of mucous secretions has been alluded to by several investigators. Atassi *et al.* (6) reported that the bronchial secretions from patients with chronic bronchitis contained increased levels of sialic acid. Hoskins and Zamcheck (7) tested the serous saliva and mucoid saliva from one subject and found that the fucose and sialic acid content relative to hexosamine was less in the thinner material. Sialic acid was also more abundant in mucoid saliva than in gastric juice from the same subject. This work is supported by additional evidence in which relatively high ratios of concentrations of sialic acid to fucose were found in jelly from pseudomucinous ovarian cysts compared to that from pseudomyxomatous cysts (8). In the former, the cyst contains a viscoelastic gel. Gibbons (9) found a significant difference in sialic acid concentration between estrus and pregnancy samples of bovine cervical mucin. The thick plasto-elastic gel secreted during pregnancy had a higher sialic acid content than the thinner material secreted at estrus suggesting that the content of this acid is one of the factors affecting the viscosity of mucin.

The work cited above provides a theoretical framework for the hypothesis that there is a correlation between the viscosity of mucus secretions and the carbohydrate complex, namely the sialomucins synthesized and discharged from the mucus-secreting glands. The authors are unaware of any previous study with a sufficient number of samples to determine if a statistically significant positive correlation exists. Therefore, the purpose of this study was to quantitate both variables (viscosity and sialic acid) simultaneously to determine if this relationship for sputum does exist.

EXPERIMENTAL

Sample Collection—Sputum was obtained from patients with bronchiectasis, bronchial asthma, or pulmonary emphysema with the majority of samples from the latter two conditions.[1] No attempt was made to identify sample with disease.

The method used to obtain these samples was a modification of that described by Bickerman *et al.* (10). Essentially this consisted of having patients inhale a mixture of 0.2 ml. of 2.25% racemic epinephrine[2] and 1.0 ml. of 0.25% phenylephrine hydrochloride. Following this the patients inhaled a warm normal saline aerosol generated in a high flow nebulizer by means of an air pump. All of the material expectorated, during and for 10 min. following the aerosol inhalation, was collected and immediately frozen. Mucoid material recovered in this manner is reported to be from the tracheobronchial passageway (10).

Viscosity Determination—The frozen samples were brought to ambient temperature and passed through a Logeman hand homogenizer with the gap adjusted to the smallest permissible size. The homogenate was warmed to 37° and 8.0 ml. was transferred to a No. 100 Cannon-Fenske capillary viscometer positioned in a 37° constant temperature water bath, and allowed to equilibrate for 12–20 min. before the flow time was measured. The viscometer was washed in a chromic acid solution at the beginning of each day of use and rinsed with water and acetone and air-dried between each viscosity measurement. Viscosity calculations were based on a distilled water standard. Samples from different patients producing insufficient volume for viscosity measurements were pooled.

Sialic Acid Assay—The analytical procedure employed for determining total sialic acid content (free plus bound) measured as *N*-acetylneuraminic acid was that of Warren (11). The method is based upon periodate oxidation of sialic acids to form β-formylpyruvic acid, which is combined with thiobarbituric acid to form a chromophore that is extracted in cyclohexanone, and measured spectrophotometrically. Though there is some interference from 2-deoxyribose present in sputum, this can be readily compensated for as described by Warren.

[1] The authors are indebted to Dr. Hylan A. Bickerman for supplying all clinical material used in this study.
[2] Trademarked as Vaponephrine.

The above assay only measured free sialic acids. Therefore, 1.0 ml. of each sputum homogenate (pooled and unpooled) was diluted with 1.0 ml. of distilled water and heated at 80° in 2.0 ml. 0.2 *N* H_2SO_4 for 1 hr. in order to hydrolyze the bound acids. Then 0.2 ml. of the hydrolyzate, representing 0.05 ml. of sputum was assayed for total sialic acid.

Sputum Model—A partially purified mucoprotein preparation derived from commercial hog gastric mucin was prepared in the manner described by Sheffner (12). Colloidal suspensions ranging from 0.25–2.50% were prepared from this tan spongy material and their viscosity and sialic acid content determined.

RESULTS AND DISCUSSION

The authors are of the opinion that capillary viscometry is well suited to this investigation. If a relationship exists between viscosity and sialic acid content, it is a reasonable assumption that, although the viscosity values reported here may differ from the numerical values obtained when using a different measuring technique, the comparative relationship would still be valid. Even the best rotation viscometry remains a semiexact method for measuring the viscosity of sputum, and it is far from suitable for the determination of absolute values.

Although homogenization alters the integrity of mucoproteins and mucopolysaccharides, the writers concluded that homogenization would still be acceptable for investigating the relationship between viscosity and sialic acid content.

It was also concluded that though the patients had a variety of diseases, were on different drug therapy, or may have had slightly different concentrations of saline in their bronchial passages, it was unlikely that these factors could influence the interpretation of the results.

Unpooled Sputum—The sialic acid content reported is in μmole per 0.1 ml. of sputum.

Table I is a summary of the data obtained on unpooled sputum. The data graphically depicted in Fig. 1 show that there is a linear relationship between viscosity and sialic acid concentration until a point is reached where the acid content appears to approach a maximum and then plateaus, although the viscosity continues to increase. A linear relationship is evident until a viscosity of approximately 2 cps. is reached. This linearity is supported by a correlation coefficient of 0.979 obtained from samples 1–10 whereas a much lower coefficient is obtained by using the data from all the samples. Unfortunately only a few samples are on the plateau portion of the curve, so the maximum sialic acid value can only be estimated to be between 0.11 and 0.12 μmole.

TABLE I—UNPOOLED SPUTUM SAMPLES

Sample No.	Viscosity, cps.	μ moles, Sialic Acid/ 0.1 ml.
1	0.818	0.012
2	1.089	0.021
3	1.125	0.036
4	1.186	0.028
5	1.311	0.029
6	1.322	0.042
7	1.398	0.048
8	1.914	0.079
9	2.022	0.075
10	2.131	0.092
11	2.326	0.075
12	2.739	0.110
13	3.700	0.109
14	5.453	0.118

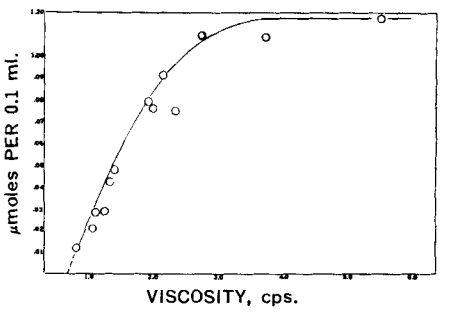

Fig. 1—Relationship between the viscosity and sialic acid concentration of unpooled sputum.

TABLE II—POOLED SPUTUM SAMPLES

Sample No.	Viscosity, cps.	μmoles, Sialic Acid/ 0.1 ml.
1	1.177	0.079
2	1.192	0.090
3	1.549	0.102
4	2.175	0.172
5	2.280	0.176
6	2.429	0.180
7	2.475	0.157
8	2.622	0.194
9	3.470	0.190
10	4.902	0.220
11	26.820	0.240

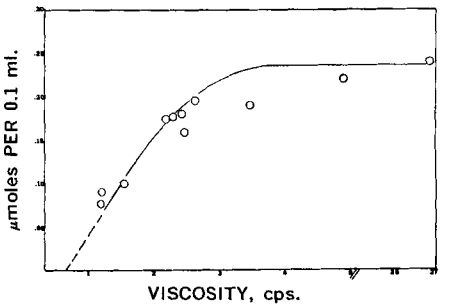

Fig. 2—Relationship between the viscosity and sialic acid concentration of pooled sputum.

It is significant that when the curve is extrapolated to the *X* axis, (that is, sialic acid equals 0.00 μmoles), the interscept is 0.70 cps.—the viscosity of water at 37°.

Pooled Sputum—There was a greater percentage of high viscosity sputums in the pooled specimens (Table II) than in the unpooled. Figure 2 shows that the relationship between viscosity and sialic acid is the same for pooled and unpooled sputum. A sample with an extremely high viscosity (26.82 cps.) is five and one-half times greater than its nearest neighbor, yet there is a difference of only 0.02 μmole in sialic acid content. Though there is only one sample on this high end of the viscosity scale, it does emphasize the leveling of the acid concentration with a concomitant rise in viscosity above

TABLE III—SPUTUM MODEL

% Muco-protein	Viscosity, cps.	μmoles, Sialic Acid/ 0.1 ml.
0.25	0.94	0.007
0.50	1.26	0.014
0.75	1.66	0.023
1.00	2.22	0.028
1.50	3.00	0.043
2.00	4.00	0.055
2.50	5.00	0.069

2 cps. The correlation coefficient for the lower portion of the curve is 0.988, again indicating a linear relationship. When the curve is extrapolated to the X axis, it too intersects at 0.70 cps.

The major difference between the pooled and unpooled data is the magnitude of the sialic acid concentration relative to the same viscosity. Though the shapes of the curves are quite similar, the sialic acid content of the pooled samples is higher, with the plateau value approximately double that of the unpooled.

Sputum Model—The authors developed a simulated biological model in which some of the variables could be controlled. Gastric mucin was first selected because it represents a crude source of mucoprotein; however, the sialic acid content of the material available was quite low, and the viscosity of a standard suspension was difficult to duplicate on a day-to-day basis. A partially purified mucoprotein substrate which met the practical conditions needed for the model was used (12). The data are tabulated in Table III.

Because this model is a series of uniform aqueous suspensions, the curve has been drawn so that it is forced to intersect the X axis (sialic acid = 0.000) at the viscosity of water at 37°. The similarity of this curve (Fig. 3) to the two mucous curves is evident. Although the sputum model does not closely simulate natural mucus because it utilizes a lyophilized, partially purified mucoprotein, which disperses readily in water, it lends considerable weight to the relationship under study.

While it is premature to attempt to quantitate the viscosity of sputum in terms of sialic acid content, it appears that within limits and with continuing research this will be possible. The plateau seen

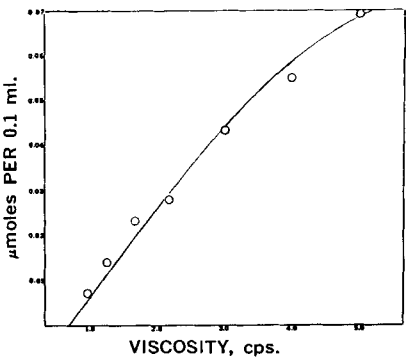

Fig. 3—Relationship between the viscosity and sialic acid concentration of sputum model.

in all three sets of data suggest, as expected, that there are other factors besides sialic acid affecting the viscosity of sputum.

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Keyphrases

Sputum viscosity
Sialic acid content—sputum viscosity
Capillary viscosimeter—analysis
Colorimetric analysis

Carbonate Ester Prodrugs of Salicylic Acid

Synthesis, Solubility Characteristics, *In Vitro* Enzymatic Hydrolysis Rates, and Blood Levels of Total Salicylate Following Oral Administration to Dogs

By L. W. DITTERT*, H. C. CALDWELL, T. ELLISON†, G. M. IRWIN, D. E. RIVARD, and J. V. SWINTOSKY*

The methods of synthesis, solubilities, and partition coefficients for the ethyl-, butyl-, hexyl-, and 2,2,2-trichloroethylcarbonate esters of salicylic acid are reported. The solubility and partitioning characteristics of the ethyl- and trichloroethylcarbonates were similar to those of aspirin, whereas the butyl- and hexylcarbonates were more lipid soluble than aspirin. The *in vitro* hydrolysis rates of the compounds were also determined. The hydrolysis of the carbonate esters of salicylic acid were only slightly accelerated by 2 percent human plasma, and aspirin hydrolysis was not accelerated at all by this enzyme system. In the pseudocholinesterase and α-chymotrypsin systems, the hydrolysis of the butyl- and hexylcarbonates were accelerated to a much greater degree than those of the ethylcarbonate and aspirin. The results suggest that carbonates with 4 or 6 carbon alkyl chains fit the active sites of these esterolytic enzymes better than a carbonate with a 2 carbon alkyl chain or an acetate ester. Aspirin and the butyl-, hexyl-, and trichloroethylcarbonates were administered orally to dogs and the plasma levels of total salicylate were followed for 8 hr. The resulting blood level curves were virtually superimposable; all peaked at about 2 hr. and fell off at about the same rate. These results suggest that the prodrug carbonate esters are as readily absorbed as aspirin despite their different aqueous and lipid solubilities and that all the drugs including aspirin are converted to a common form, *i.e.*, free salicylate, within 2–3 hr. after oral administration.

FECAL BLOOD loss studies (1, 2) have shown that approximately 70% of persons taking aspirin experienced occult gastric bleeding averaging 5 ml. of blood per day, and Kelly (3) has reported that patients taking aspirin for long periods of time may develop severe iron deficiency anemia due to fecal blood loss. The mechanism by which aspirin causes gastric hemorrhage is a matter of considerable controversy (4). There is little doubt, however, that at least part of the problem is due to local irritation of gastric mucosa (5).

It was the purpose of our studies to seek prodrug derivatives of salicylic acid which would be nonirritant, would be readily absorbed, and which would be rapidly hydrolyzed in blood and other tissues to release free salicylate in the human blood stream. It was reasoned that carbonate esters of salicylic acid which are readily hydrolyzed by dilute human plasma, but which are less soluble in water and more soluble in lipids than aspirin, might be distributed and absorbed over a broader area of the gastrointestinal tract and might be less irritating to the gastric mucosa than aspirin.

This paper presents the synthesis, solubility, and partitioning properties, and *in vitro* hydrolysis rates of four carbonate ester prodrugs of salicylic acid. It also presents the blood levels of total salicylate following oral administration to dogs of three of the carbonate esters compared with aspirin.

EXPERIMENTAL

Chemical Synthesis—Melting points were determined in capillary tubes on a Thomas-Hoover apparatus and are uncorrected. Reported yields are of pure compound.

The following general procedure for synthesizing the compounds was adapted from the method of preparation of the methylcarbonate reported by Fischer (6). To an ice-cooled mixture of 85 Gm. (0.61 mole) of salicylic acid and 155 ml. (1.22 moles) of dimethylaniline in 500 ml. of dry benzene, the appropriate chloroformate (0.61 mole) was added over a period of 15 min. The ice bath was removed, and the mixture was stirred at room temperature for 2 hr. and washed with six 100-ml. portions of 10% HCl and three 100-ml. portions of water. The benzene solution was dried over anhydrous magnesium sulfate, and the solvent was removed. The remaining material was purified by crystallization from the appropriate solvent system (see Table II) after treatment with activated charcoal.

Particle Size Reduction—The purified compounds were micronized by one or more passes

Received January 9, 1968, from Smith Kline & French Laboratories, Philadelphia, PA 19101

Accepted for publication February 2, 1968.

The authors wish to acknowledge the assistance of the following: Mr. Philip Goldman and Mr. Ralph Matz for preparing the compounds; Mrs. Elisabeth Rattie for assistance with assays, and Miss Margaret Carroll and staff for microanalysis.

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through a fluid energy mill (Trost Jet Mill, Helme Products, Inc., Helmetta, N. J.). This procedure produced particles in the 2–20 μ size range.

Solubilities—The saturation solubilities of the compounds at 37° were determined in 0.1 *N* HCl and spectral grade cyclohexane (Fischer) in rotating bottles utilizing an assembly that has been described previously (7). HCl (0.1 *N*) was used to assure that the compounds were completely unionized and to reduce the degree of hydrolysis of the compounds during equilibration. The aqueous samples were rotated for 6 hr., and the cyclohexane samples were rotated for 16 hr. (overnight). Samples of the clear supernatant saturated solutions were withdrawn into hypodermic syringes through Millipore filters held in Swinney filter adapters (Millipore Filter Corp.). The samples were analyzed spectrophotometrically.

Partition Coefficients—The partition coefficients of the compounds between cyclohexane and 0.1 *N* HCl at 25° were determined using a method and apparatus previously described (8). Since 0.1 *N* HCl was used as the aqueous phase in these experiments, the partition coefficients shown in Table III are "true partition coefficients" of the unionized compounds.

In Vitro Hydrolysis Rates—Half-lives for hydrolysis of the compounds at 37° in 0.1 *M* phosphate buffer with and without enzymes were determined by a spectrophotometric method previously described (9). Frozen human blood plasma (Type O^+) was obtained in approximately 100-ml. quantities from single donors through the Philadelphia Serum Exchange. Purified human pseudocholinesterase was obtained from Sigma Chemical Co., and α-chymotrypsin (salt free from EtOH) was obtained from Nutritional Biochemicals Co.

Blood Levels of Total Salicylate in Dogs—Purebred beagle dogs were used in a 4 × 4 crossover study with aspirin and the butyl- and trichloroethylcarbonates of salicylic acid. Each dog received an equimolar dose of drug per Kg. body weight according to Table I.

The drugs were administered *via* a stomach tube in single doses of finely ground powder suspended in 20 ml. of 0.5% tragacanth. Blood specimens were collected at 0, 15, 30, 60, 120, 240, 360, and 480 min. following drug administration, and total salicylate in the plasma was determined by the method of Cosmides, Stemler, and Miya (10).

Table I—Dosing Schedule

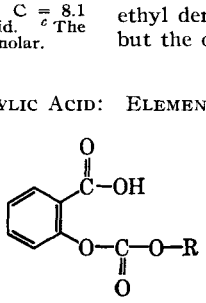
Compound	Mol. Wt.	Dose, mg./Kg.	Dosing Schedule[a] Day 1	4	8	11
Aspirin	180.16	75	A	B	C	D
Trichloroethyl[b]	313.54	130	B	C	D	A
Butyl[b]	238.24	90[c]	C	D	A	B
Hexyl[b]	266.30	111	D	A	B	C

[a] Dog weights: A = 12.8 Kg.; B = 8.6 Kg.; C = 8.1 Kg.; D = 12.0 Kg. [b] Carbonate of salicylic acid. [c] The dose of the butyl carbonate is 10% less than equimolar.

RESULTS AND DISCUSSION

The molecular weight, yields, melting points, elemental analyses, and recrystallization solvents for the four carbonate ester prodrugs of salicylic acid used in this study are shown in Table II. The ethyl derivative has been reported previously (11) but the others are new compounds. All are white

Table II—Carbonate Esters of Salicylic Acid: Elemental Analyses and Physical Properties



R	Formula	Mol. Wt.	Yield, %	M.p., °C.	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Recrystallization Solvent
Ethyl (C_2H_5—)	$C_{10}H_{10}O_5$	210.190	40	93.5–94.5[a]	57.14	57.21	4.80	4.77	Chloroform-hexane
n-Butyl (C_4H_9—)	$C_{12}H_{14}O_5$	238.244	36	79.5–80.5	60.50	60.36	5.92	5.83	Carbon tetrachloride
n-Hexyl (C_6H_{13}—)	$C_{14}H_{18}O_5$	266.298	54	79.5–80.5	63.15	63.20	6.81	6.73	Carbon tetrachloride
Trichloroethyl (Cl_3C—CH_2—)	$C_{10}H_7Cl_3O_5$	313.537	42	126–128	38.31	38.37	2.25	2.29	Isopropanol-hexane

[a] Literature melting point is 95° (11).

Table III—Aspirin and Carbonate Esters of Salicylic Acid: Solubilities, Partition Coefficients, and Hydrolysis Rates

Derivative	Solubilities in mg./ml., 37° 0.1 *N* HCl[a]	Solubilities in mg./ml., 37° Cyclohexane	Cyclohexane/0.1 *N* HCl Partition Coefficient, 25°[a]	Half-Lives for Hydrolysis, 37° Phosphate Buffer (0.1*M*) pH 7.4, hr.	Phosphate Buffer (0.1*M*) pH 12, min.	Enzyme in Phosphate Buffer (pH 7.4, 0.1*M*)[b] 2% Human Plasma, hr.	0.05% Human Pseudocholinesterase, hr.	0.05% α-Chymotrypsin, hr.
Ethyl	6.7	1.1	0.17	41	3.8	13.3 (3.1)	7.4 (5.5)	53 (—)
n-Butyl	2.8	2.7	1.0	29	4.5	11.7 (2.5)	1.23 (24)	1.38 (21)
n-Hexyl	0.28	5.6	20	15	4.8	8.2 (1.8)	1.33 (11)	0.25 (58)
Trichloroethyl	1.1	0.24	0.22	0.53	1.1	0.35 (1.5)	...	...
Aspirin	5.3	0.06	0.05	10	1.5	21 (—)	3.3 (3)	6.3 (1.6)

[a] 0.1 *N* HCl was used in these experiments to assure that the compounds were completely unionized in the aqueous phases and to reduce the degree of hydrolysis in the aqueous phase during equilibration. [b] The numbers in parentheses are $t_{1/2}$ buffer (pH 7.4)/$t_{1/2}$ enzyme ratios which represent the degree of enzymatic catalysis.

crystalline, somewhat waxy, solids in their pure forms.

The solubilities of the carbonate esters and aspirin in 0.1 *N* HCl and in cyclohexane at 37° are shown in Table III along with the corresponding cyclohexane–0.1 *N* HCl partition coefficients at 25°. In these studies, 0.1 *N* HCl was used in place of water to assure that the compounds would not hydrolyze appreciably during equilibration and that they were completely unionized in the aqueous phases. Thus, the solubilities shown in Table III are those of the unionized compounds and the partition coefficients are "true partition coefficients."

As expected, the solubilities of the aliphatic carbonate esters decrease in 0.1 *N* HCl and increase in cyclohexane with increasing chain length. The measured partition coefficients show the expected increase with increasing chain length. The trichloroethyl derivative is less soluble in cyclohexane and much less soluble in water than the ethyl derivative. It might have been expected that the cyclohexane solubility of the trichloroethyl derivative would be greater than it is, since trichloroethyl groups tend to be more lipophilic than ordinary ethyl groups, but the trichloroethylcarbonate of salicylic acid has a higher melting point than the other derivatives indicating a stronger crystal lattice which tends to reduce solubility in all solvents.

Predictions of the relative availabilities of the compounds for oral absorption based on the solubilities and partition coefficients shown in Table III must take into account the fact that they probably do not have precisely equivalent pKa values; therefore, at pH's near their pKa's, some of the compounds might show relatively higher or lower apparent partition coefficients than would be expected on the basis of the true partition coefficients shown in Table III. Also, because the compounds are weak acids, the aqueous solubilities will increase with increasing pH. However, the fact that solubilities shown in Table III are of a reasonable order of magnitude suggests that if the drugs are finely ground and properly wetted by water, they should all be readily absorbed following oral administration.

If the solubilities and partition coefficient of aspirin are compared with those of the carbonates, it might be predicted that the ethyl derivative would have gastrointestinal absorption characteristics similar to those of aspirin. In subsequent tests (12) it was found that the ethyl derivative was almost as irritating to the gastric mucosa of rats as aspirin itself. It would seem that salicylate derivatives with relatively high aqueous solubilities and relatively low lipid solubilities are more irritating to the gastric mucosa than those which are less soluble in water and more soluble in lipids.

Table III also shows enzymatic and nonenzymatic hydrolysis data for the four carbonate esters and aspirin. The aliphatic carbonates are more stable toward hydrolysis in buffer than aspirin. The trichloroethylcarbonate is less stable, probably because the chlorine atoms act as good "electron sinks" drawing electrons away from the carbonyl carbon and making it susceptible to nucleophilic attack.

In 2% human plasma in pH 7.4 buffer, the aliphatic carbonates and aspirin are very slowly hydrolyzed. The values for $t_{1/2}$ buffer/$t_{1/2}$ enzyme, shown in parentheses in Table III, represent the degree of enzymatic catalysis and show that the esterases of human plasma have very little catalytic effect on the hydrolysis of these compounds. Even though the hydrolysis of the trichloroethylcarbonate derivative is relatively rapid, the degree of enzymatic catalysis is practically nil. In 0.05% "purified" human pseudocholinesterase and 0.05% α-chymotrypsin (from bovine pancreas) the ethylcarbonate derivative and aspirin are hydrolyzed relatively slowly and the degree of enzymatic catalysis in each case is relatively small. On the other hand, the butyl- and hexylcarbonates are hydrolyzed relatively rapidly by these enzymes and the degree of enzymatic catalysis is considerably larger. These prodrugs, however, are not nearly as susceptible to enzymatic hydrolysis by these enzymes as is 4-acetamidophenyl 2,2,2-trichloroethyl carbonate. For this carbonate ester prodrug of acetaminophen, the $t_{1/2}$ buffer/$t_{1/2}$ enzyme values were 1680 for 0.05% human pseudocholinesterase and 646 for 0.05% α-chymotrypsin (12). Of all the enzymes studied, these two were the most powerful catalysts for the hydrolysis of carbonate esters of acetaminophen, but they are apparently much weaker catalysts for the hydrolysis of carbonate esters of salicylic acid.

Augustinsson and Nachmansohn (13) reported the presence of an aspirin esterase in human blood which is clearly different from the pseudocholinesterase of serum. Morgan and Truitt (14) studied the *in vitro* hydrolysis of aspirin in serum from various species of laboratory animals and humans, and they reported that the aspirin esterase activity of human blood is very weak. The esterases of human blood, having a somewhat greater affinity for carbonate esters, than for aspirin, hasten the hydrolysis of these esters.

The *in vitro* enzyme hydrolysis studies suggested that free salicylate might be released at different rates following oral administration of aspirin and the carbonate prodrugs, but there are many esterolytic enzymes and many sites in the body other than the blood stream where hydrolysis of these compounds might occur. To investigate the release rates and sites of hydrolysis and to study the influence of the physical properties of the carbonate prodrugs on their *in vivo* performance, the prodrugs and aspirin were administered orally to dogs, and the blood levels of salicylate were followed. The analytical method employed (10) converts the carbonate esters and aspirin to free salicylate, therefore, the blood levels were obtained in terms of total salicylate.

The average total salicylate plasma levels in dogs following oral administration of aspirin and the butyl-, hexyl-, and trichloroethylcarbonates of salicylic acid are shown in Fig. 1. The curves in Fig. 1 show that the drugs are nearly identical with respect to the plasma salicylate levels they produce in dogs. All curves reach a peak near 20 mg. % at 2 hr. and then fall off at about the same rate. The minimum and maximum individual blood levels for all drugs overlap at all time points.

These results suggest that the solubility and partitioning properties of these prodrug carbonate esters did not grossly affect their gastrointestinal absorption rates. A possible exception is the hexyl derivative which appears to have a somewhat slower absorption rate than the others. However, the absorption rate of this compound is apparently fast.

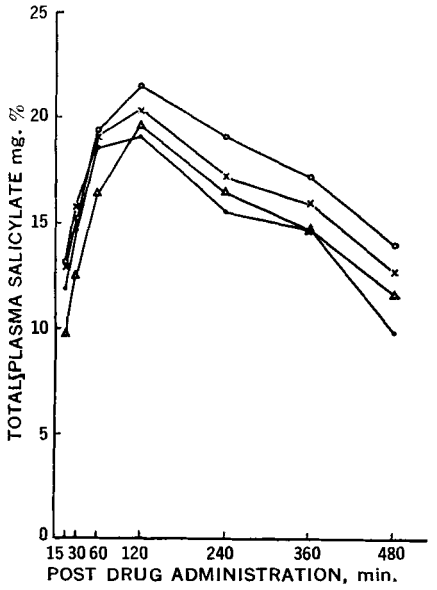


Fig. 1—Plot showing plasma levels of total salicylate in purebred beagle dogs following oral administration of equimolar doses of aspirin (.—., 75 mg./Kg.); trichloroethylcarbonate of salicylic acid (○—○, 130 mg./Kg.); butylcarbonate of salicylic acid (×—×, 90 mg./Kg.); and hexylcarbonate of salicylic acid (Δ—Δ, 111 mg./Kg.). The study was carried out according to a 4×4 crossover plan, and each point represents the average of four plasma levels. The minimum and maximum individual plasma levels overlapped for all drugs at all time points.

enough to catch up with the other carbonates and aspirin at the 2-hr. point. The fact that all the blood levels follow the same pattern and fall off at about the same rate suggests that beyond 2 or 3 hr., aspirin and the carbonate prodrugs of salicylic acid are all in the same form, that is, they have been completely converted to free salicylate. Thus, it would appear that aspirin and the three carbonates are rapidly converted to free salicylate *in vivo*, and it might be expected that the pharmacologic activities of the three carbonates would be very similar to those of aspirin.

Based on these findings, extensive pharmacologic evaluations of the butyl-, hexyl-, and trichloroethylcarbonates were undertaken. Future publications in this series will deal with their analgesic, antipyretic, and anti-inflammatory activities and their gastrointestinal irritation liabilities compared with aspirin.

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Keyphrases

Salicylic acid carbonate esters—prodrugs
Carbonate esters salicylic acid—synthesis
Blood levels—salicylate
Hydrolysis rates—*in vitro*
Solubility—salicylic acid carbonate esters

Dissolution of Salicylic Acid and Polyvinylpyrrolidone from Compressed Mixtures

By MILO GIBALDI and HOWARD WEINTRAUB

The present study was initiated to describe quantitatively the dissolution mechanism of a polyphase mixture of drug and water-soluble polymer. The release pattern of both salicylic acid and polyvinylpyrrolidone (PVP) was described quite well by the theoretical model for the dissolution of two noninteracting phases. The PVP dissolution rates from various mixtures, as determined by a weight-loss method, were in excellent agreement with a model describing dissolution of nonbarrier solid. The dissolution rates of salicylic acid over a wide composition range were essentially invariant but lower than the intrinsic dissolution rate of salicylic acid.

THERE IS considerable pharmaceutic interest in the dissolution of drugs from solid mixtures. The various mathematical theories and physical models presented in the reports of T. Higuchi (1–3) and the more recent efforts of W. I. Higuchi (4, 5) form the basis for much of the current research on diffusion-controlled dissolution phenomena.

A recent report from this laboratory was concerned with the dissolution of drugs dispersed in polyvinylpyrrolidone (6). The observed dissolution phenomenon was not consistent with the anticipated release rate of solute from a hydrophilic matrix (2). Hence, the present study was initiated to describe quantitatively the dissolution mechanism of a polyphase mixture of drug and polyvinylpyrrolidone (PVP).

The release pattern of both salicylic acid and the hydrophilic polymer from compressed mixtures was described quite well by the theoretical model for the dissolution of two noninteracting phases (5). These findings are of particular interest in that: (*a*) there is evidence of significant physical-chemical interaction between salicylic acid and PVP (7), (*b*) the PVP/salicylic acid solubility ratio probably exceeds the limit suggested by Higuchi (4), and one might anticipate that dissolution would adhere to the models developed for solute release from an inert matrix, and (*c*) a previous report by Lapidus and Lordi (8) demonstrated that the release of chlorpheniramine maleate from a compressed hydrophilic methylcellulose matrix follows the theoretical relationships proposed for solid drugs in solid matrices (2) rather than the models proposed for simple polyphase mixtures (5).

EXPERIMENTAL

Determination of Dissolution Rates—Salicylic acid and PVP,[1] each with a particle size <250 μ, were blended thoroughly, in various ratios, in a mortar. The blend was then compressed at 10,000 lb. load for 10 sec. on a laboratory model Carver hydraulic press using a 1.2 cm. (0.5-in.) flat-faced punch and die. Tablet thickness was about 5 mm.

Initial dissolution rates[2] were determined by the rotating disk method (9, 10) using 250 ml. of 0.1 *N* HCl at 37° and 100 r.p.m. One-milliliter samples were withdrawn at various times and diluted with 0.1 *N* HCl. Salicylic acid concentration was determined spectrophotometrically at 303 mμ using a Beckman DB-G recording spectrophotometer. Preliminary studies indicated no need to correct for PVP interference at this wavelength under the experimental conditions.

The initial dissolution rate of PVP was estimated by measuring weight loss of the tablet as a function of time, and correcting the weight loss for the previously observed dissolution rate of salicylic acid. Tablets of the desired composition were prepared as described above and the dissolution rate studies were run in a manner similar to that described for salicylic acid with the following exceptions. At selected time intervals the stirring was stopped, the Plexiglas holder was withdrawn from the dissolution medium, and the holder surface wiped with a tissue. The entire unit was placed in an oven at 45° for 5 min. and then immediately weighed on a Mettler H-15 balance. After weighing, the Plexiglas unit was returned to the dissolution apparatus and the procedure repeated at appropriate intervals.

Preliminary studies indicated that further drying for 1 hr. beyond the 5-min. period resulted in minimal weight loss (about 4 mg./hr.) as compared to the initial weight loss of residual water (about 130 mg./hr.). Moreover, the apparent retention of water after the 5-min. drying period was independent of the total length of time the disk was exposed to the dissolution medium. This additional dehydration essentially reflects the loss of the normal moisture content of the PVP rather than the penetration of dissolution fluids into the matrix. Drying of disks of pure PVP under identical conditions for 1 hr. showed a rate of loss of about 3 mg./hr. Hence, penetration of water into the tablet (beyond a surface phase) does not occur to a significant extent. These

Received December 26, 1967, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication February 14, 1968.

Supported in part by training grant GM-555-06 from the U. S. Public Health Service, Bethesda, Md.

[1] Plasdone K 29-32, average mol. wt. 40,000, was generously supplied by the General Aniline and Film Corporation, New York, N. Y.

[2] The term "initial dissolution rate" in this context indicates steady-state dissolution under essential sink conditions.

data also indicate that the weight-loss method provides a valid measure of the dissolution of PVP.

Solubility Studies—Salicylic acid solubility was determined in a series of aqueous solutions of PVP in 0.1 *N* HCl. In each case, 200 mg. of salicylic acid was added to 20 ml. of solution contained in 25-ml. culture tubes. The tubes were sealed, placed in a Metabolyte shaker (New Brunswick Scientific Co., N. J.), and equilibrated at 37°. The solutions were sampled periodically by means of a filter pipet, diluted, and assayed for total salicylate to determine equilibrium. Under the conditions of the study, PVP gave no spectral interference.

A limited study was conducted on the behavior of salicylic acid in PVP solution as a function of time according to the method of Bates *et al.* (11). A 2.5-Gm. quantity of salicylic acid was added to 250 ml. of 2% PVP solution in 0.1 *N* HCl at 37°. The system was stirred at 100 r.p.m. by means of a Teflon blade. Samples were withdrawn at various intervals and assayed for total salicylic acid.

RESULTS

The initial dissolution rates of both PVP and salicylic acid followed apparent zero-order kinetics in accord with the experimental conditions of constant dissolution surface area and apparent sink conditions. Representative plots at various compositions are shown in Figs. 1 and 2. The PVP data in Fig. 1 were corrected for an initial time lag to yield a zero intercept in each case.

The use of the weight-loss method manifested a lag of 2 to 3 min. before the occurrence of apparent zero-order kinetics. The initial time lag may reflect a surface hydration phenomenon (a presteady-state condition), and the mild drying employed between sampling was apparently insufficient to dehydrate completely the tablet surface, since the lag is noted initially but not between drying periods.

Figure 3 shows the dissolution rates of PVP and salicylic acid as a function of the composition of the biphasic mixture, as determined from the apparent zero-order dissolution rate plots. At each composition studied the dissolution rate of salicylic acid is essentially constant and somewhat lower than the intrinsic dissolution rate of salicylic acid. The dissolution rate of PVP was found to increase with increasing proportions of PVP in the mixture.

The results of the solubility studies at 37° are presented in Fig. 4. These findings must be considered preliminary in that an exceedingly long period was required for apparent equilibrium. The early sampling period provided results which indicated a decreased apparent solubility of salicylic acid at low PVP concentrations. Apparent solu-

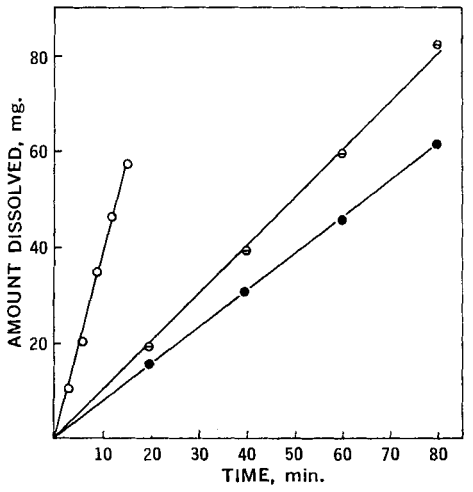

Fig. 1—Apparent zero-order dissolution rates of PVP from compressed mixtures of varying composition. Key: PVP:salicylic acid ratio, w/w. ○, 5:1; ⊖, 3:1; ●, 1:1. Each plot is corrected to origin.

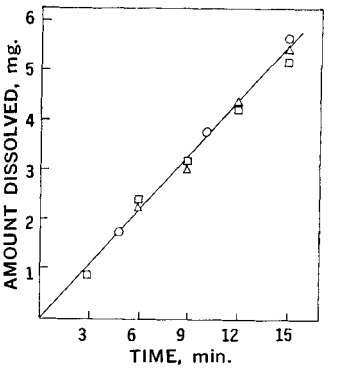

Fig. 2—Apparent zero-order dissolution rates of salicylic acid from compressed mixtures of varying composition. Key: PVP:salicylic acid ratio, w/w. □, 10:1; Δ, 3:1; ○, 1:2. Each plot is corrected to origin.

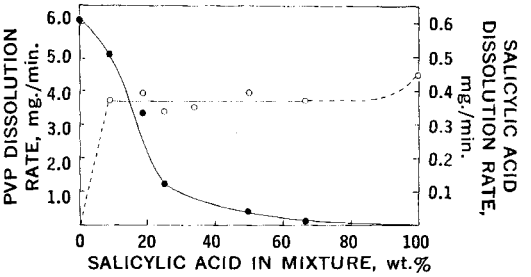

Fig. 3—Dissolution rates of salicylic acid and PVP as a function of mixture composition. Key: ○, salicylic acid; ●, PVP. Each data point represents the average of 2–4 determinations.

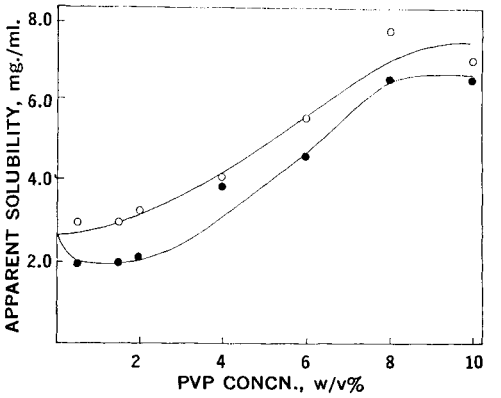

Fig. 4—Apparent solubility of salicylic acid in various concentrations of PVP in 0.1 N HCl. Key: ○, after 336 hr.; ●, after 48 hr.

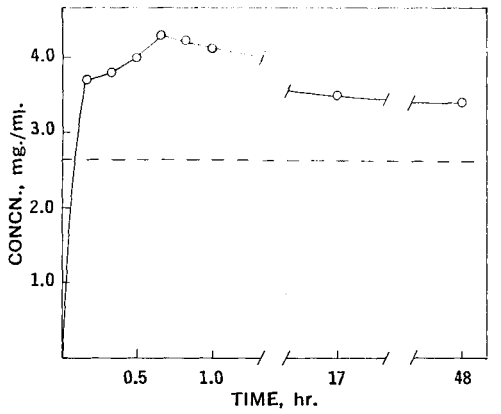

Fig. 5—Dissolution of salicylic acid in 2% PVP solution in 0.1 N HCl. Key: ———, intrinsic solubility of salicylic acid.

TABLE I—COMPOSITION OF "COMPLEXES" ISOLATED FROM 2% PVP SOLUTION AT 37°

Determination	Molar Ratio (PVP Repeating Unit/Salicylic Acid) Stirrer Solid	Bulk Solid
1	1.81	0.68
2	1.87	0.69

bility increased with time at each PVP level and "equilibrium" appeared to be established after about 2 weeks. The equilibrium solubility plot indicates a continuous increase in apparent solubility of salicylic acid as a function of PVP concentration up to about 8%. Twenty-four hours after incubation, no crystalline salicylic acid was observable in any tube. Each tube contained an amorphous, plastic precipitate.

The concentration of salicylic acid in 2% PVP solution as a function of time is shown in Fig. 5. A maximum occurs at about 40 min. and thereafter the concentration of drug in solution decreases. After 48 hr., stirring was stopped and two distinct amorphous solid phases were observed. No crystalline salicylic acid was noted. One phase was a transparent glass material concentrated about the stirring shaft (in the region of the system where the vortex had existed). The second phase was an opaque amorphous material occurring at the bottom of the flask. A sample of each solid phase was dried to constant weight and the stoichometry determined by chemical assay. The composition of each phase is reported in Table I.

DISCUSSION

The PVP and salicylic acid dissolution rate data were consistent with the models recently presented to describe the dissolution rate behavior of polyphase mixtures (5). The PVP data were in agreement with the simplest of these models which describes the dissolution of nonbarrier solid from a mixture of two noninteracting solid phases.

For a two-phase mixture of components A and B, Higuchi (5) has proposed the following model. Upon exposure to solvent, A and B dissolve initially at rates proportional to their solubilities and diffusion coefficients. If the ratio of amounts in the mixture, *i.e.*, N_A/N_B, exceeds the ratio of the product of diffusion coefficient (D) and solubility (C^0), *i.e.*, $D_AC^0_A/D_BC^0_B$, then after a short time, component B will become depleted from the solid–liquid interface region of the dissolving solid. Consequently, a surface layer of pure component A is formed.

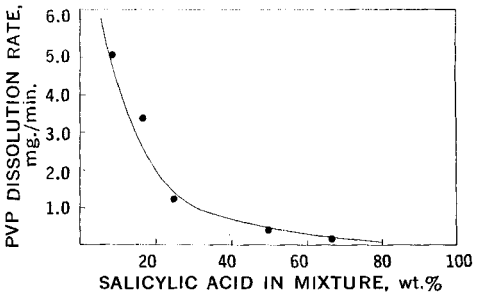

Fig. 6—Comparison of theoretical and experimental dissolution rates of PVP as a function of mixture composition. Key: ——, theoretical; ●, experimental.

Estimates of the magnitude of the ratio of diffusion coefficients and solubilities of PVP and salicylic acid (SA) indicate that $D_{SA}C^0_{SA}/D_{PVP}C^0_{PVP} < 0.1$. Hence, in mixtures with $N_{SA}/N_{PVP} > 0.1$, PVP would be expected to behave in a manner similar to component B described above.

Accordingly, when $N_A/N_B > (D_AC^0_A)/(D_BC^0_B)$, the dissolution rate of component B from the mixture is described by the following equation (6):

$$(DR)_B = \frac{N_B}{N_A}(DR)^0_A \qquad \text{(Eq. 1)}$$

where $(DR)^0_A$ is the intrinsic dissolution rate of component A.

Figure 6 shows a comparison of the theoretic dissolution curve of PVP *versus* mixture composition, as determined from Eq. 1 and the experimental data. Good agreement is observed despite the fact that solubility studies suggest significant interaction of PVP with salicylate. Apparently the formation of complex in the diffusion layer contributes little to the overall dissolution rate of PVP from the nonbarrier phase. The diffusion of the complex will be discussed in greater detail below.

The physical model employed to describe the PVP dissolution rate predicts that the dissolution rates of salicylic acid over the composition range studied should be constant and equal to the intrinsic dissolution rate of salicylic acid, since this component represents the barrier phase. As shown in Fig. 3, the dissolution rates of salicylic acid over a wide composition range is essentially invariant but somewhat lower than the intrinsic dissolution rate of salicylic acid. The regression line fitted to the six data points gave a slope of essentially zero ($\sim 10^{-4}$). The data provide no indication of the contribution of a salicylate–PVP complex to the dissolution process. In fact, the results are somewhat lower than those predicted by the noninteracting model.

Although the dissolution rate studies of various PVP–salicylic acid mixtures provide little evidence for the existence of solution interaction, the solubility data indicate definite interaction of PVP and salicylic acid. The nature of the complex is not clear at present but probably could be represented by PVP–$(SA)_n$ where n is a variable depend-

ent upon the PVP concentration and temperature. The maximum value of n is likely to be in the order of 360. This would be consistent with a complex containing one salicylic acid molecule bound to each vinylpyrrolidone repeating unit. Previous studies (12) of the interaction of phenol with PVP report a value for bound phenol of 0.94 moles $L.^{-1}$/equiv. of PVP $L.^{-1}$ corresponding to a 1:1 complex of phenol with the repeating unit.

Significant existence of a 1:1 complex with the vinylpyrrolidone repeating unit in the salicylic acid system is unlikely except at very low concentrations of PVP (<0.5%). The formation of the 1:1 complex may be limited by the poor solubility and precipitation of less substituted complexes at 37°. The initial increase in apparent solubility noted in Fig. 5 may be due to the formation of soluble complexes of PVP–$(SA)_n$ where n has a relatively low order of magnitude. It is likely that n increases with time and the resulting higher molecular weight complexes precipitate from solution.

The complex isolated from the stirrer shaft probably represents precipitated complex. The bulk complex was found to have a salicylic acid:vinylpyrrolidone repeating unit ratio exceeding unity, which suggests the possible physical inclusion of unbound, undissolved salicylic acid. The apparent absence of undissolved salicylic acid from the stirrer solid may reflect the turbulent conditions in the vortex which would minimize the presence of drug particles. Further studies are in progress to characterize more clearly the PVP–salicylic acid interaction.

Regardless of the nature of the complex which exists in the diffusion layer during dissolution, it would appear likely that the large molecular weight and presumably small diffusion coefficient of the complex precludes a significant contribution of this species to the diffusion process. Since the complex species is expected to contribute little to the dissolution phenomenon, the dissolution of PVP and salicylic acid from the biphasic mixture adheres to a simple model developed for two noninteracting phases rather than the more complex case where the phases demonstrate solution interaction.

The decreased dissolution rate of salicylic acid from the barrier phase of the mixtures, as compared to the intrinsic dissolution rate of salicylic acid may reflect the increased viscosity in the diffusion layer of the dissolving solid due to the presence of high concentrations of PVP. The diffusion coefficient and hence the dissolution rate of the drug is an inverse function of viscosity in the diffusion layer. The presence of PVP in the diffusion layer would, therefore, tend to decrease the dissolution rate of the drug. Alternatively, a film of a high-molecular weight PVP–salicylic acid complex may form at the interface and depress the salicylic acid dissolution rate while minimally affecting the more rapidly dissolving PVP.

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Keyphrases

Salicylic acid–PVP—compressed mixtures
Dissolution rates—salicylic acidP–VP
PVP determination—weight-loss method
UV spectrophotometry—analysis, salicylic acid
Solubility—salicylic acid–PVP

Effect of a Nonionic Surface-Active Polymer on Passage of Hydrocortisone Across Rat Intestine *In Vitro*

By WITOLD SASKI

Employing the Crane and Wilson everted sac technique, the passage of hydrocortisone cortisol—in micellar solutions of oxyethylated tertiary octylphenol polymethylene polymer (tyloxapol)—from the mucosal to the serosal side of the rat intestinal membrane was studied. It was determined that the mechanism by which cortisol passes across the membrane is that of diffusion. However, it was found that the relative rate of passage of cortisol dissolved in micellar solutions of tyloxapol is a function of concentration of the solubilizing agent used and is inversely proportional to the concentration of tyloxapol and viscosity of the solution tested.

THE SCIENCE of biopharmaceutics has been well advanced in recent years and excellent reviews on the subject have been published (1–5). However, the part surface-active agents may play in the passage of drugs across biological membranes is far from being understood at the present time. The use of surfactants in the formulation of solubilized systems in pharmacy is rather common, and their effect upon the absorption and biological activity of drugs is of paramount importance.

Originally described by McBain (6), solubilized systems are those in which the solubility of materials otherwise insoluble, or only poorly soluble, in a given medium is increased due to the prior presence of particles of colloidal dimensions, termed micelles. Solubilized systems in pharmacy were reviewed by Swarbrick (7) and Mulley (8). Reports concerning the effects of surface-active agents on drug absorption have been conflicting. As Levy (1) has indicated, enhancement as well as inhibition of the gastrointestinal absorption and the pharmacologic activity of drugs has been observed when amphiphilic compounds were added to a medication. These varied effects may have been due to such factors as the possible interaction with the drug and modification of the physical properties of the dosage form, or possible effects on the absorbing membrane. Recently, the effect of various concentrations of the nonionic surfactant, polysorbate 80,[1] on the absorption of a number of alcohols and barbiturates by goldfish has been studied (9). The absorption rate of the barbiturates was increased significantly in the presence of low concentrations of polysorbate 80, and decreased by higher concentrations of the surfactant. The absorption rate of the alcohols studied was not significantly affected by the surfactant. The retardation of barbiturate absorption at higher polysorbate 80 concentration was interpreted as being indicative of the absence of a dissociating effect of the biologic membrane on the drug–micelle complex. By kinetic analysis, the authors have shown that the modification of barbiturate absorption by polysorbate 80 represents the net effect of enhanced absorption and decreased thermodynamic activity of the drug due to micellar complexation. Kakemi *et al.* (10) reported a decrease of the rectal absorption of sulfonamides by addition of various nonionic surface-active agents, polysorbate 80 included. These workers ascribed the decrease to an entrapment of the drug in micelles. However, contrary to the notion that the only form in which the drug passes the membrane is free drug and not drug in micelle (11), the authors found that the drug in micelle was also absorbed to some extent, and therefore, an overall enhancement of drug absorption was obtained by solubilization. They presented a new model illustrating the proposed mechanism, and developed a formula mathematically expressing the absorption rate of the solubilized drug.

Steroids have been reported to be absorbed by diffusion (12), the more lipoidal the compounds, the greater the absorption rate; the more polar and water-soluble the compound, the less absorption rate. However, there has been no information available as to the effect of amphiphilic, preferably nonionic, compounds on the absorption of steroids. Kavanau (13) suggested that the amphiphilic agents may exert some striking effects by their penetration into the lipid phase of membranes with a consequent alteration of the lipid phase state, the membrane configuration, the rates of transformation of the membranes, and the ability of the membranes to transform between

Received April 14, 1967, from the College of Pharmacy University of Nebraska, Lincoln, NB 68508

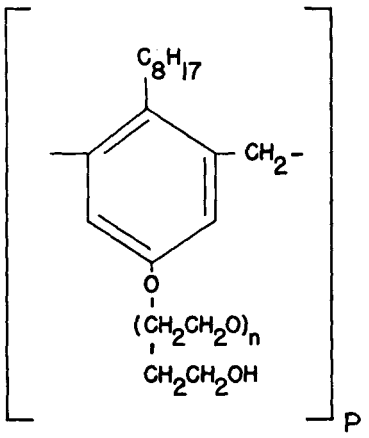
Accepted for publication November 15, 1967.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This investigation was supported in part by research grant AM 08576-02, from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service, Bethesda, Md.

The technical assistance of William H. Bulger in carrying out the experiments involving the everted sac technique and the cortisol assays is acknowledged.

[1] Trademarked as Tween 80, Atlas Chemical Industries, Wilmington, Del.



I

different configurations. The penetration of amphiphiles which loosen the liquid-condensed structure of the "bimolecular" disks probably would tend to lead to an increase in the pore size. In some cases, concluded Kavanau, these amphiphiles would tend to promote a localized, or perhaps even an extensive, transformation of membranes to the open configuration and a decreased stability of the lipid–lipoprotein–protein complex. Such compounds, of course, would tend to increase permeability.

The present author set out to investigate the effects a nonionic solubilizer would have on the absorption of poorly soluble corticosteroids. Cortisol (hydrocortisone) was chosen as a representative of the group and, as a solubilizing agent, an oxyethylated tertiary octylphenol polymethylene polymer,[2] better known under its generic name of tyloxapol (14). Tyloxapol was selected as the agent of choice on the basis of its record as practically nonhemolytic (15) and otherwise innocuous to the biologic membranes. It is used as a solubilizer in a sterile ophthalmic solution[3] of cortisol (2 mg./ml.) and in other pharmaceutical preparations. The structure of tyloxapol is shown in I: where $n \simeq 10$ and $p \simeq 4$, and the average molecular weight ($n = 10$, $p = 4$) is 2,812 (16).

The Crane and Wilson (17) everted sac technique seemed well suited for the investigation. Although this method does not give an estimate of the rate of absorption *in vivo*, it can provide useful information about the kinetics of the absorptive process, in addition to showing whether it is active or passive. Several established criteria (18) for adequate characterization of the mode of passage of substances across the biological membrane have been observed in this study. They were, the concentration-effect curve, the time-effect curve, the capacity of the system to transfer both with and against a concentration gradient, experimental conditions such as kind and quantity of added substances, surface tension, viscosity, pH, ionic strength, and verification of the viability or biological integrity of the membrane under the conditions of the study of transfer.

[2] Trademarked originally as Triton A-20, later renamed Triton WR-1339, Winthrop Laboratories, New York, N. Y.
[3] Trademarked as Optef, The Upjohn Co., Kalamazoo, Mich.

EXPERIMENTAL

Apparatus—Eberbach water bath shaker, Beckman DB spectrophotometer with Sargent SR recorder, Central Scientific Co. du Nouy 70545 tensiometer, Federal Pacific Electric Co. Wilhelmy plate tensiometer, Ostwald viscometer, Beckman Zeromatic pH meter, Burrell wrist-action model DD shaker, thermostatically controlled incubator, Crane and Wilson apparatus.

Materials and Reagents—Cortisol (Nutritional Biochemicals Corp., dried at 70° for 24 hr.); tyloxapol (obtained from Ruger Chemical Co., Inc.); triphenyltetrazolium Cl (TTC, Nutritional Biochemicals Corp.), freshly prepared 0.5% solution in aldehyde-free dehydrated ethanol; tetramethylammonium hydroxide 10% solution (Eastman Kodak), freshly diluted 1:10 with aldehyde-free dehydrated ethanol; 1,2-dichloroethane, purified; sodium chloride, reagent grade (Fisher Scientific Co.); benzopurpurin 4B, biological stain grade (Matheson Coleman and Bell).

Preparation of Solutions—Predetermined amounts of cortisol were dissolved in the aqueous solutions of tyloxapol at concentrations of this polymer varying from 0 to 10% w/v. Each solution was made isotonic with the body fluids by the addition of sodium chloride. The solutions were freshly prepared for each experiment using the Eberbach water bath shaker at 37°. The solubility of cortisol in aqueous solutions of tyloxapol has been reported previously (19).

Intestinal Absorption Study—Everted sac technique of Crane and Wilson (17) was used and further perfected in a few important details. Female Sprague-Dawley rats weighing about 250 Gm. were fasted for 24 hr. but had access to drinking water at all times. The animal was killed by a blow on the head and the abdomen cut open by a midline incision, care being taken not to damage the small intestine. The intestine was completely cut across in the region of the upper duodenum and at the ileocecal junction. A 20-ml. syringe cannula was then inserted into the upper end of the intestine and the lumen cleared of its contents by allowing oxygenated saline at room temperature to pass through under moderate pressure. The washed out length of intestine was removed from the animal by carefully stripping off the mesentery at the line of its attachment to the intestine. For the eversion of the intestine, a glass rod of an appropriate length and diameter with a slightly flattened end was used. The intestine was then sleeved onto the glass rod and everted. A tie made with thread at the end of the intestine segment helps the process considerably. Upon completion of eversion a loop was made of the distal end of the sac, and an additional tie using catgut was applied beneath the loop. These precautionary measures practically

exclude the possibility of a leakage. Two segments of approximately 13 cm. length each were obtained from the upper intestinal tract of each animal. Proximal ends of the sacs were attached to the cannula of the Crane and Wilson apparatus.

The sacs were filled with 1–2 ml. of oxygenated saline termed "serosal fluid." Each sac was suspended in 45 ml. of cortisol in saline or cortisol in tyloxapol–saline solution ("mucosal fluid") maintained at 37° and aerated continuously with a mixture of 95% oxygen and 5% carbon dioxide. At indicated times, the total serosal fluid was withdrawn by means of a syringe with a polyethylene cannula attached and the sac rinsed once with about 2 ml. of saline. A fresh 1–2-ml. portion of saline was then placed in the sac. Both serosal and mucosal fluids were assayed each time. Retention within the intestinal wall was low.

The object of everting the sac is twofold. Eversion, with slight distension of the sac, stretches the mucosa and improves its oxygenation. The small volume of fluid inside the sac causes passage of any solute from the mucosal to the serosal side producing a relatively large increase in its concentration in the serosal fluid. This facilitates the detection of transport against the concentration gradient (20).

Analytical Methods—The withdrawn serosal fluid and washing were combined in a separator, boric acid–sodium hydroxide buffer, 0.5 ml. per 5 ml. of solution was added (12), and the fluid extracted twice with 20 volumes of ethylene dichloride, using the Burrell wrist-action shaker for 5 min. each time. The extract was evaporated to dryness in a Petri dish in a stream of hot air. The residue was dissolved in aldehyde-free dehydrated ethanol, transferred to a 10-ml. volumetric flask, and brought up to volume with ethanol. This alcoholic solution was transferred to a 15-ml. capped test tube and 1 ml. of tetramethylammonium hydroxide 10% solution, freshly diluted 1:10 with aldehyde-free dehydrated ethanol, was added, followed by 1 ml. of 0.5% triphenyltetrazolium chloride solution in aldehyde-free dehydrated ethanol. The test tube, after gentle inverting to ensure a thorough mixing, was placed in a dark room (incubator set at 25°) for exactly 25 min. A respective blank solution treated in the identical way served as a control. Color, developed by the reduction of triphenyltetrazolium chloride to form the formazan in a solution buffered with tetramethylammonium hydroxide, was read in a Beckman DB spectrophotometer at 485 mμ. Cortisol concentration was calculated by reference to the respective standard Beer's law curve previously prepared. The effect of air, water, and the concentration of tetramethylammonium hydroxide, have been discussed by Johnson *et al.* (21) and their paper should be consulted to ensure the reproducibility of the tetrazolium assay.

Determination of the Critical Micelle Concentration (CMC) for Tyloxapol—Surface tension method employing a du Nouy tensiometer (22) and a Wilhelmy plate tensiometer (23), and a spectral absorption benzopurpurin 4B method (24) using a Beckman DB spectrophotometer were applied.

Determination of the Viscosity—Relative, absolute, and specific viscosities of the solutions of tyloxapol in physiologic saline at 37° were determined by means of an Ostwald viscometer in a constant-temperature water bath. Densities of the solutions were determined using a pycnometer.

RESULTS AND DISCUSSION

In preliminary experiments, it was found that the integrity of the rat intestine was not impaired by immersion of the everted sac in 1.0% tyloxapol oxygenated saline solution for 30–60 min. in a water bath at 37°. The sacs so treated were subsequently used in several experiments with the mucosal solution containing 0.10 and 0.20 mg./ml. solutions of cortisol in saline (no tyloxapol present). The results obtained were nearly identical with those obtained with the sacs which were not so treated. At rather elevated concentrations of tyloxapol, far above the critical micelle concentration (CMC) of the solubilizing agent, it was found that the relative rate of passage of cortisol is a function of concentration of the surface-active polymer and is inversely proportional to its concentration. This fact served as direct evidence that the membrane did not change so as to allow for the enhancement of passage of cortisol across it.

In an initial series of experiments, the concentration of 200 mcg. of cortisol per ml., that is below the limit of its water solubility, was used while the concentrations of tyloxapol were 0, 0.005, 1.0, 3.0, and 5.0% w/v. The rates of passage of cortisol were observed to be consistently a function of concentration of the surfactant and inversely proportional to its concentration.

The concentration of 0.005% w/v of tyloxapol was included to see whether this amount—below the CMC—would be of any consequence. No significant changes in the rate of passage of cortisol were observed at this concentration of the surfactant. It is conceivable that by means of more accurate methods, such as the use of labeled radioactive cortisol molecules, the effect of the minute amounts of the solubilizing agents could be evaluated more precisely.

The CMC of tyloxapol determined by surface tension measurements was found to be about 0.04 Gm./dl. However, employing the benzopurpurin 4B spectral absorption method, the first sharp inflection indicative of the CMC formation was detected at the concentration of 0.01 Gm./dl.

Detailed data are presented for the series of experiments involving cortisol actually solubilized. Concentration of 500 mcg./ml. was used throughout the series, the only variable being the concentration of tyloxapol, *i.e.*, 1.0, 2.0, 5.0, and 10.0% w/v. The pattern of diffusion of cortisol across the rat intestine is clearly demonstrated by the time-effect curve (Fig. 1) as a function of the concentration of tyloxapol. $C_{serosal}/C_{mucosal}$ on the ordinates denotes the concentration ratio of cortisol passed in a given time from the serosal to mucosal fluid. Number of runs with each particular concentration is indicated, and so are standard deviations for each time interval within each group of the runs, in Table I.

To determine the relative rates of passage, expression of the results in terms of Fick's law was used:

$$\frac{d(C_o - C_i)}{dt} = -P(C_o - C_i) \quad \text{(Eq. 1)}$$

where t is time, and P is permeability coefficient

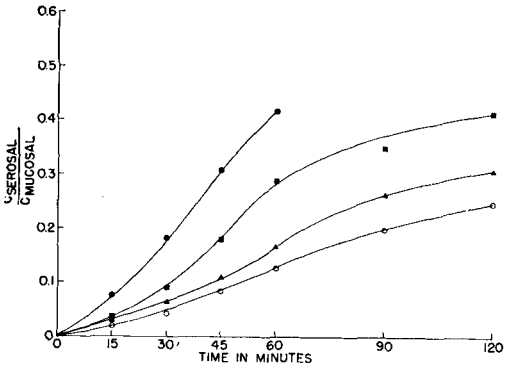


Fig. 1—Passage of solubilized cortisol (0.5 mg./ml.) across the rat intestine in vitro *as a function of the concentration of tyloxapol. Key: Tyloxapol % w/v—●, 1.0; ■, 2.0; ▲, 5.0; ○, 10.0.*

TABLE I—PASSAGE OF SOLUBILIZED CORTISOL (0.5 mg./ml.) ACROSS THE RAT INTESTINE *In Vitro* AS A FUNCTION OF THE CONCENTRATION OF TYLOXAPOL

Tyloxapol w/v, % Concn.	No. of Runs	Time, min.	Mean Ratio of Serosal to Mucosal Cortisol, Concn./ml.	S. D., ±
1.0	(3)	15	0.074	0.021
		30	0.179	0.078
		45	0.313	0.072
		60	0.433	0.078
2.0	(4)	15	0.029	0.010
		30	0.088	0.013
		45	0.178	0.024
		60	0.287	0.067
		90	0.349	0.050
		120	0.417	0.055
5.0	(3)	15	0.026	0.012
		30	0.060	0.009
		45	0.109	0.019
		60	0.165	0.024
		90	0.266	0.024
		120	0.307	0.016
10.0	(5)	15	0.023	0.006
		30	0.043	0.011
		45	0.081	0.024
		60	0.116	0.023
		90	0.184	0.055
		120	0.283	0.099

which is a measure of the relative ease with which the drug crosses the membrane. C_0 represents the drug concentration in the outside (mucosal) fluid, and C_i that in the inside (serosal) fluid. When C_o is made for all practical purposes constant by suspending the everted sac with a very small volume of saline in a relatively large volume of mucosal fluid, the equation may be integrated to give:

$$Pt = -\ln\left(1 - \frac{C_i}{C_o R}\right) \qquad \text{(Eq. 2)}$$

where R is the ratio C_i/C_o at the steady state (25). If R is assumed to be 1, on substituting the absorbed concentration ratios in the equation, and plotting the values of the right-hand side of the equation against time, straight lines are obtained (Fig. 2.). The permeability coefficient P (or the relative rate of penetration of cortisol) as a function of concentration of tyloxapol is given by the slope

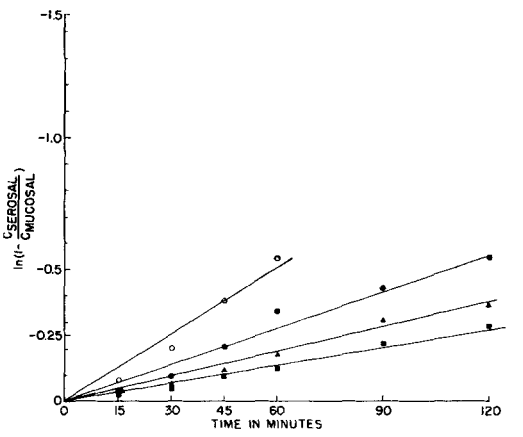


Fig. 2—The relative rates of passage of solubilized cortisol (0.50 mg./ml.) as a function of concentration of tyloxapol. Key: Tyloxapol % w/v—○, 1.0; ●, 2.0; ▲, 5.0; ■, 10.0.

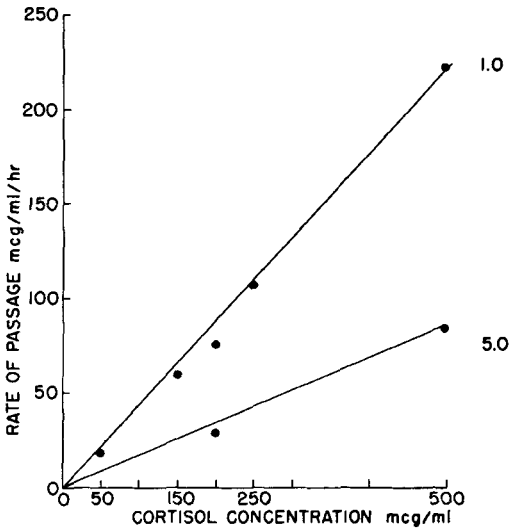


Fig. 3—Effect of concentration of the solubilizing agent on the amount of cortisol passed across the rat intestine in vitro *as a function of the initial concentration solubilized in 1.0 and 5.0 % tyloxapol solution after 1 hr. of incubation.*

of the respective line, $-\ln(1 - C_i/C_oR)/t$. The slopes vary dramatically depending on the concentration of the solubilizer present.

The cortisol concentration effect is illustrated in Fig. 3. It can be seen that the absolute amount of the drug absorbed is directly proportional to its initial concentration, in agreement with the concept of simple diffusion (26). However, it is evident that the amount of the drug absorbed is a function of concentration of the solubilizing agent. The higher concentration of tyloxapol, the lesser amount of cortisol absorbed. It is also evident that the fraction (ratio) of cortisol entering the sac is independent of the concentration of the drug in the outside solution. In other words, the amount passing across the rat intestine is directly proportional to the concentration gradient in accord with the law of simple diffusion. Indeed, it can be seen

from Fig. 3, that the C_i/C_o cortisol ratio in the case of 1.0% tyloxapol is consistently about 0.4 for all five cortisol concentrations, and in the case of 5.0% tyloxapol the ratio is about 0.15.

This series of experiments was carried out in such a way as to minimize variables inherent in using everted sacs from different animals. In one case, the concentrations of cortisol used were 50, 150, 250, and 500 mcg./ml., solubilized in 1.0% solution of tyloxapol, and the determination of cortisol which passed from the mucosal to the serosal side of the sac was consecutively made using the same sac at 1-hr. intervals. Upon repeating the experiment, employing a second segment obtained from the same animal, close results were obtained and averages of the two experiments plotted. A point corresponding to the 200 mcg./ml. concentration plotted on the 1.0% tyloxapol curve was derived from two other experiments involving another rat. Two separate experiments were carried out to obtain the data for the 5.0% tyloxapol curve.

Surface tension of the solutions used throughout the investigation did not seem to play any significant role in the process of cortisol across the rat intestine, differing by a fraction of 1 dyne/cm. in the 1.0–10.0% tyloxapol range (38.7–39.3 dynes/cm. at 21°). The rate of transfer of cortisol from the solutions containing no tyloxapol or just a minute amount of it (below the CMC) was the greatest.

The pH seemed to play no significant part either, although the solutions used were not adjusted to any specific pH. Those containing no tyloxapol were rather close to neutrality (pH 6.5 ± 0.2), while those with tyloxapol had a pH of about 5. The reasons for not adjusting the pH were twofold, cortisol is a nonionizing compound as is tyloxapol, thus, pH-partition theory does not apply. The other reason for not buffering the solutions was a reluctance to introduce ions that might conceivably alter the absorption rates. The ionic strength imparted by sodium chloride used for the sake of tonicity of the solutions was practically uniform throughout the investigation.

The Einstein equation (27) directly relates diffusion to viscosity of the diffusion medium:

$$D = \frac{RT}{N} \cdot \frac{1}{6\pi r\eta} \qquad \text{(Eq. 3)}$$

where D is the diffusion coefficient, N is Avogadro's number, R is the gas constant, T is the absolute temperature, r is the radius of the diffusing molecule, and η is the viscosity.

If it is assumed that except for viscosity all other factors are kept constant, the Eq. 3 may be written:

$$D = K \cdot \frac{1}{\eta} \qquad \text{(Eq. 4)}$$

Absolute, relative, and specific viscosities of 1.0, 2.0, 5.0, and 10.0% w/v of tyloxapol in saline solution at 37° were determined (Table II). When the logarithm of specific viscosity is plotted against tyloxapol concentration, a steep straight line is obtained. It is apparent that the diffusion coefficient of cortisol is inversely proportional to viscosity of the diffusion medium.

According to the views summarized by Levine and Pelican (18), only changes in the biological barrier can influence the rate of absorption of a particular molecular species of a drug transferred by passive diffusion. However, the rate of absorption of a species of a drug molecule which is transferred by facilitated diffusion may be increased or decreased by changes in, or on, the membrane, or by the presence of certain solutes. Consideration of the latter, as the present experiments would indicate, may have important consequences in the therapeutic use of a drug or combination of drugs administered orally. As Stein (28) pointed out, we have no clear idea as to the mechanism of action of facilitated diffusion as yet. At least in part, this is due to our ignorance of the detailed structure of the cell membrane and of the physical chemistry of such a system. In any event, active transport seems to have no place in the system studied since the attempts to detect a movement of solubilized cortisol against the concentration gradient (placing the solutions of the same concentration on both sides of the everted sac) failed.

Physicochemical study of micellar complexation of tyloxapol with cortisol and other steroids, which may throw more light on the mechanism of the passage of these compounds in micellar solutions across the biological membranes, is being carried out in this laboratory.

TABLE II—VISCOSITY OF TYLOXAPOL SOLUTIONS IN SALINE AT 37°

	Absolute, cps.	Relative	Specific
Water	0.6947	...	...
Tyloxapol 1.0% w/v	0.7139	1.0277	0.0277
Tyloxapol 2.0% w/v	0.7754	1.1161	0.1161
Tyloxapol 5.0% w/v	0.8967	1.2910	0.2910
Tyloxapol 10.0% w/v	1.3126	1.8895	0.8895

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Keyphrases

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Tyloxapol, effect—cortisol diffusion
Everted sac—technique
Colorimetric analysis
Diffusion rate—tyloxapol concentration function

Base-Catalyzed Hydrolysis of Flavins

Effect of Amines and the Particular Role of Position 3

By DONALD B. McCORMICK and WERNER FÖRY

The base-catalyzed hydrolysis of flavins was investigated in 50 percent water–methanol with excess sodium hydroxide or excess amines at different temperatures. Rate constants for the pseudo first-order cleavage of the pyrimidine portion of the isoalloxazine systems were measured and with sodium hydroxide shown to increase from lumiflavin to 3-ethyllumiflavin to 3-carboxyamido(phenylalanyl)lumiflavin. With amines, the size as well as basicity is of prime importance since the general order in efficacy of the nucleophile for hydrolysis of flavins is ethylamine > diethylamine > triethylamine > benzylamine > tributylamine. In general, alkylation of the 3-imino function of the flavin increases its lability because of inability to ionize and undergo resonance stabilization of the anionic tautomers. Moreover, the special case where a 3-carboxyamido function is present allows intramolecular polarization, *via* hydrogen bonding of the amide hydrogen to 4-carbonyl oxygen, which causes a marked increase in hydrolysis rate in sodium hydroxide. From the temperature dependencies of the rates of hydrolysis, the calculated values for energies of activation indicate the decrease expected upon 3-alkylation and further upon intramolecular enhancement with the 3-carboxyamido group. The calculated values for entropies of activation are large and negative in accord with considerable loss of degrees of freedom in the activated complexes formed in the bimolecular mechanism.

FLAVINS CAN undergo hydrolytic decomposition of their isoalloxazine nucleus, particularly in alkaline solution. Kuhn and Rudy (1) isolated urea and 1,2-dihydro-1,6,7-trimethyl-2-keto-3-quinoxaline carboxylic acid from the degradation of lumiflavin in alkaline medium. These results were confirmed by Surrey and Nachod (2) and extended by Svobodova (3) and Wada *et al.* (4) who noted several other unidentified products. Farrer and MacEwan (5) studied the kinetics for hydrolysis of riboflavin over a wide range of pH and showed the reaction to be general acid-base catalyzed. More recently Guttman and coworkers have examined the influence of complexing agents in decreasing the rates for hydrolysis of flavins (6–8) and the intermediates formed following cleavage of the pyrimidine portion of the isoalloxazine system (9–11).

Since most of the earlier studies on the hydrolysis of flavins involved only the use of hydroxyl ion as the attacking species, it seemed desirable to investigate more thoroughly the effects of size and basicity of other nucleophiles such as amines. Also the rate of hydrolysis of a 3-substituted flavin in aqueous sodium hydroxide had been observed to be faster than that of unsubstituted flavin (8), but the primary cause for the difference had not been reported. Furthermore, though such compounds as purines which complex with flavin by planar overlap of their ring systems (12–14) cause a decrease in rate of hydrolysis of flavin (6, 7), certain other types of molecular as-

Received January 2, 1968, from the Graduate School of Nutrition and Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, NY 14850
Accepted for publication February 15, 1968.
This investigation was supported in part by research grant AM-04585 from the National Institutes of Health and by funds from the State University of New York.

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The base-catalyzed hydrolysis of flavins was investigated in 50 percent water–methanol with excess sodium hydroxide or excess amines at different temperatures. Rate constants for the pseudo first-order cleavage of the pyrimidine portion of the isoalloxazine systems were measured and with sodium hydroxide shown to increase from lumiflavin to 3-ethyllumiflavin to 3-carboxyamido(phenylalanyl)lumiflavin. With amines, the size as well as basicity is of prime importance since the general order in efficacy of the nucleophile for hydrolysis of flavins is ethylamine > diethylamine > triethylamine > benzylamine > tributylamine. In general, alkylation of the 3-imino function of the flavin increases its lability because of inability to ionize and undergo resonance stabilization of the anionic tautomers. Moreover, the special case where a 3-carboxyamido function is present allows intramolecular polarization, *via* hydrogen bonding of the amide hydrogen to 4-carbonyl oxygen, which causes a marked increase in hydrolysis rate in sodium hydroxide. From the temperature dependencies of the rates of hydrolysis, the calculated values for energies of activation indicate the decrease expected upon 3-alkylation and further upon intramolecular enhancement with the 3-carboxyamido group. The calculated values for entropies of activation are large and negative in accord with considerable loss of degrees of freedom in the activated complexes formed in the bimolecular mechanism.

FLAVINS CAN undergo hydrolytic decomposition of their isoalloxazine nucleus, particularly in alkaline solution. Kuhn and Rudy (1) isolated urea and 1,2-dihydro-1,6,7-trimethyl-2-keto-3-quinoxaline carboxylic acid from the degradation of lumiflavin in alkaline medium. These results were confirmed by Surrey and Nachod (2) and extended by Svobodova (3) and Wada *et al.* (4) who noted several other unidentified products. Farrer and MacEwan (5) studied the kinetics for hydrolysis of riboflavin over a wide range of pH and showed the reaction to be general acid-base catalyzed. More recently Guttman and coworkers have examined the influence of complexing agents in decreasing the rates for hydrolysis of flavins (6–8) and the intermediates formed following cleavage of the pyrimidine portion of the isoalloxazine system (9–11).

Since most of the earlier studies on the hydrolysis of flavins involved only the use of hydroxyl ion as the attacking species, it seemed desirable to investigate more thoroughly the effects of size and basicity of other nucleophiles such as amines. Also the rate of hydrolysis of a 3-substituted flavin in aqueous sodium hydroxide had been observed to be faster than that of unsubstituted flavin (8), but the primary cause for the difference had not been reported. Furthermore, though such compounds as purines which complex with flavin by planar overlap of their ring systems (12–14) cause a decrease in rate of hydrolysis of flavin (6, 7), certain other types of molecular as-

Received January 2, 1968, from the Graduate School of Nutrition and Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, NY 14850
Accepted for publication February 15, 1968.
This investigation was supported in part by research grant AM-04585 from the National Institutes of Health and by funds from the State University of New York.

sociations could be expected to facilitate the hydrolysis rate.

The purposes of the present investigation were to circumscribe more thoroughly the efficacy of amines in the base-catalyzed hydrolysis of flavins, to ascertain the effect of substitution in position 3 of the flavin nucleus, and to indicate how intramolecular polarization can enhance the rate of hydrolysis.

Lumiflavin and its analogs were chosen as they are much less susceptible to photolytic decomposition (15) and because the absence of a polyhydroxy side chain precludes certain complicating behavior observed with riboflavin (7). A solution of 50% water–methanol was used as solvent to be compatible with solubility of both flavin and the larger aliphatic amines. The rate of loss of the flavin yellow color ar 450 mμ was used as a satisfactory index for cleavage of the pyrimidine portion of the flavins (6). Rate constants were determined for hydrolysis effected by sodium hydroxide as well as by a series of amines, the relative effectiveness of which is shown to be dependent upon size and basicity. Alkylation of position 3 in the flavin increases the rate for hydrolysis as resonance stabilization through tautomerization is lost. A further increase in rate is caused by interaction of amide-linked amino acid ester in this position due to polarization of flavin carbonyl 4 *via* hydrogen bonding with the amide function. The temperature dependency for hydrolysis in aqueous sodium hydroxide was measured for lumiflavin to compare with the energy of activation previously reported (6) and additionally for ethylamine-catalyzed hydrolyses of lumiflavin and its 3-substituted analogs the latter of which have lower values for activation energies. The entropies of activation then were calculated and found to be large and negative and thereby reflect greater loss of translational and rotational freedom in the activated complexes of 3-substituted flavins, especially with a 3-carboxyamido function.

EXPERIMENTAL

Materials—Lumiflavin (7,8,10-trimethylisoalloxazine) was synthesized according to Hemmerich (16). The 3-ethyl and 3-carboxymethyl derivatives were prepared following the general method of Hemmerich (17) by alkylation of lumiflavin in *N,N*-dimethylformamide with potassium carbonate and the appropriate iodide. The 3-carboxymethyllumiflavin was activated with *N,N'*-carbonyldiimidazole after the procedure of Staab *et al.* (18) to yield the flavin imidazolium amide which was then reacted with L-phenylalanine methyl ester according to the method of Anderson and Paul (19) to form the 3-carboxyamido(phenylalanyl)lumiflavin [3-carboxyamido-(L-phenylalanine methyl ester)-7,8,10-trimethylisoalloxazine].

Usual reagents and other chemicals were obtained from commercial sources.

Methods—Flavins were dissolved in methanol and diluted with water plus base such that the final concentrations of reactants in 25 ml. were 5×10^{-5} *M* flavin and base at a specified concentration, usually 0.1 *M*, in 50% (v/v) water–methanol. These aerobic solutions were heated at a specified temperature controlled by a rheostat and heating mantle or a Haake model F constant temperature circulator. Aliquots of 2 ml. were taken at intervals and the concentration of remaining flavin determined by measuring the absorbance at 450 mμ in a Beckman DU spectrophotometer.

Infrared spectra of flavin compounds were run in potassium bromide pellets (1 mg. flavin per 300 mg. KBr) with a Perkin-Elmer Infracord spectrometer set for slow scan.

Calculations—Under the conditions employed (excess base and water) for the pseudo first-order hydrolysis of the pyrimidinoid portion of the flavin ring system, the apparent rate constants, *k*, were determined by multiplying by 2.303 the initial slopes of lines obtained in plots of log A_{450} *versus* min. where

$$\log A_{450} = \left(\frac{k}{2.303}\right) t + \text{constant} \quad \text{(Eq. 1)}$$

The heats of activation, ΔHa, were calculated from the rate constants at different temperatures using the differential expression of the Arrhenius equation integrated between limits

$$\log \frac{k_2}{k_1} = \left(\frac{\Delta Ha}{2.303R}\right) \frac{T_2 - T_1}{T_2 T_1} \quad \text{(Eq. 2)}$$

The entropies of activation, ΔSa, were calculated from a rate constant at a particular temperature and the heat of activation, where from the Eyring equation

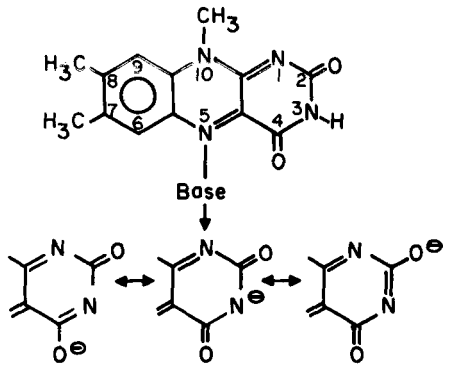
$$\Delta Sa = 2.303R \log \frac{kNh}{RT} + \frac{\Delta Ha}{T} \quad \text{(Eq. 3)}$$

RESULTS AND DISCUSSION

Rate constants for the sodium hydroxide-catalyzed hydrolysis of flavins are given in Table I. From the rate constants at 75 and 50° which indicate the thermal dependency for hydrolysis of lumiflavin, the energy (heat) of activation was calculated to be 20.2 Kcal. which is in good agreement with the value of 20.0 reported previously (6). The entropy of activation was calculated to be −16 e.u. (cal. deg.$^{-1}$) which is reasonable for a bimolecular reaction where decrease in entropy is expected in passing to an activated complex with loss of translational and rotational freedom before rupture of the flavin ring system. Alkylation of the 3-imino function of a flavin

TABLE I—PSEUDO FIRST-ORDER RATE CONSTANTS FOR THE HYDROLYSIS OF 5×10^{-5} *M* FLAVIN WITH 0.1 *M* SODIUM HYDROXIDE IN 50% WATER–METHANOL

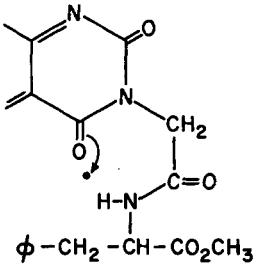
Flavin	°C.	*k*, Min.$^{-1}$
Lumiflavin	75	2.58×10^{-2}
	50	2.69×10^{-3}
3-Ethyllumiflavin	50	1.38×10^{-1}
3-Carboxyamido(phenylalanyl)-lumiflavin	5	1.02



Scheme I

leads to a considerable increase in the rate of hydrolysis as can be seen by comparing the rate constants for lumiflavin and its 3-ethyl derivative, both at 50°. Flavins unsubstituted in position 3 can undergo tautomerization between the 3-imino and 2- and 4-carbonyl functions. In the alkaline medium used for hydrolysis, the isoalloxazine system is ionized, as it is well above its pKa near 10 (20). Thus, as shown in Scheme I, lumiflavin exists as resonance-stabilized anionic forms in strongly basic solution and is less susceptible to nucleophilic attack.

Because the acidic proton in position 3 is lacking, ionization cannot occur with 3-alkylated flavins. The pyrimidine portion of their isoalloxazine systems is more electron deficient and hydrolysis resulting from nucleophilic attack occurs more readily. The exceedingly rapid rate for hydrolysis of 3-carboxyamido(phenylalanyl)lumiflavin in aqueous sodium hydroxide even at 5° must reflect an additional cause for lability. Not only is this 3-substituted flavin not able to form resonance-stabilized anions, but also space-filling molecular models indicate the possibility for intramolecular interaction of the flavin carbonyl functions with the amino acid group. As with lumiflavin and 3-ethyllumiflavin, increasing the concentration of sodium hydroxide increases the rate of hydrolysis of the 3-carboxyamido derivative in agreement with the bimolecular mechanism for hydrolysis. Hence, a unimolecular process such as would occur if the amide nitrogen served as the nucleophile can be excluded as a single-step mechanism. However, this does not exclude such a mechanism as hydrolysis of a function followed by an intramolecular attack by the generated nucleophile. As shown by the infrared spectra in Fig. 1, the band for the 4-carbonyl stretch vibration at 1710 cm.$^{-1}$ in lumiflavin and at 1700 cm.$^{-1}$ in 3-ethyllumiflavin is considerably diminished in the 3-carboxyamido derivative, while the amplitude of the 2-carbonyl band remains approximately the same near 1660 cm.$^{-1}$ for all three compounds. These assignments agree well with those reported earlier for lumiflavin and other derivatives (21). Therefore, it appears that the hydrogen of the amide function intramolecularly bonds to the 4-carbonyl oxygen and, as shown in the formula below, thereby polarizes this function in a manner favorable for hydrolysis.



The decomposition of lumiflavin as a function of time in 0.1 *M* bases at 75° is shown by the data in Fig. 2. A "hard" base such as sodium hydroxide is most effective in the base-catalyzed hydrolysis, but facile degradation is also effected with small aliphatic amines. Molecular size of the amine is quite important as ethylamine (pKb = 3.25) is somewhat less basic than diethylamine (pKb = 2.90), but the rate of hydrolysis with the former is somewhat greater. Further decrease in hydrolysis rate is seen with the larger triethylamine (pKb = 3.25) and more yet with tributylamine (pKb = 3.50). Also the latter is more basic than benzylamine (pKb = 4.70), but its greater size leads to a slightly decreased rate. Aniline (pKb = 9.42) is too weak a base and *N,N*-diethylaniline is too large to be effective. Thus the

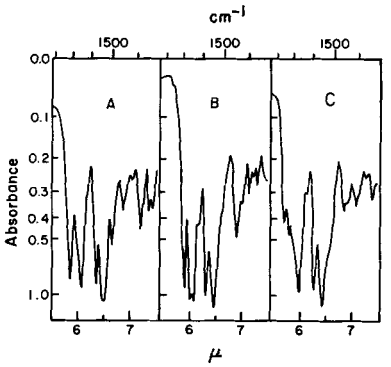


Fig. 1—Infrared spectra of flavins in the carbonyl absorption region. Key: A, lumiflavin; B, 3-ethyllumiflavin; C, 3-carboxyamids(phenylalanyl)lumiflavin.

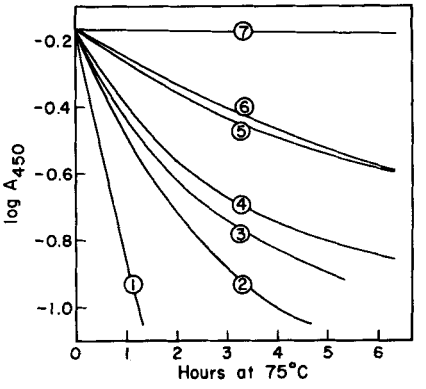


Fig. 2—The decomposition of lumiflavin in 50% water–methanol at 75° with 0.1 M bases. Key: sodium hydroxide; 2, ethylamine; 3, diethylamine; 4, triethylamine; 5, benzylamine; 6, tributylamine; 7, aniline or N,N-*diethylaniline.*

TABLE II—PSEUDO FIRST-ORDER RATE CONSTANTS FOR THE HYDROLYSIS OF 5×10^{-5} *M* FLAVIN WITH 0.1 *M* AMINES IN 50% WATER–METHANOL AT 75°

Flavin	Amine	k, Min.$^{-1}$ × 10^3
Lumiflavin	Ethyl-	11.5
	Diethyl-	10.1
	Triethyl-	7.94
	Tributyl-	2.99
	Benzyl-	3.38
3-Ethyllumiflavin	Ethyl-	16.3
	Diethyl-	10.8
	Triethyl-	15.4
	Tributyl-	2.07
	Benzyl-	2.17
3-Carboxyamido-(phenylalanyl)lumiflavin	Ethyl-	9.21
	Diethyl-	4.61
	Triethyl-	4.60
	Tributyl-	0.92
	Benzyl-	0.99

steric factors which limit the approach of an amine as nucleophile are of major importance even when basicity is favorable. As the hydrolysis proceeds, accumulation of intermediate breakdown products leads to deviation from pseudo first-order kinetics with a curving of lines after 1 or 2 hr.

Rate constants for the initial pseudo first-order amine-catalyzed hydrolysis of flavins are listed in Table II. As with lumiflavin, the relative effectiveness of the amine as nucleophile for hydrolysis of the 3-substituted flavins is markedly dependent upon size. An additional steric restriction imposed by the 3-alkyl group may be manifest, as a greater proportional decrease in hydrolysis rate is seen in going from the triethyl- to tributylamine with 3-ethyllumiflavin than with lumiflavin. Also as with sodium hydroxide, the rates for hydrolysis of 3-ethyllumiflavin with small amines is somewhat greater than found for hydrolysis of lumiflavin. As explained before, lack of the acidic hydrogen on position 3 of 3-alkyl flavins causes greater lability toward hydrolytic degradation. The absolute rates for amine-catalyzed hydrolysis of 3-carboxyamido(phenylalanyl)lumiflavin are lower than those for lumiflavin or its 3-ethyl analog.

The heats and entropies of activation for the hydrolysis of flavins with ethylamine are presented in Table III. Values of ΔHa and ΔSa for lumiflavin with this base are similar in magnitude to the respective values obtained with sodium hydroxide as given above. The energies (heats) of activation required for ethylamine-catalyzed hydrolysis of the 3-alkyl flavins are lower and their entropies of activation more negative than lumiflavin. Especially noteworthy is the very large ΔSa value calculated for 3-carboxyamido(phenylalanyl)lumiflavin. This appears to reflect the large orientation factors expected in this bulky, intramolecularly hydrogen-bonded compound upon forming an activated complex with the amine.

TABLE III—HEATS AND ENTROPIES OF ACTIVATION FOR THE HYDROLYSIS OF 5×10^{-5} *M* FLAVIN WITH 0.1 *M* ETHYLAMINE IN 50% WATER–METHANOL

Flavin	ΔHa, Kcal.	ΔSa, e.u.
Lumiflavin	22.2	−12
3-Ethyllumiflavin	16.1	−29
3-Carboxyamido-(phenylalanyl)lumiflavin	5.8	−60

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Keyphrases

Flavins—base catalyzed hydrolysis
Hydrolysis, flavins-amines, position 3 effect
Rate constants—flavin hydrolysis
Lumiflavin and derivatives—synthesis
UV spectrophotometry—analysis
IR spectrophotometry—structure

Quantitative Gas Chromatographic Determination of Methapyrilene Fumarate, Ephedrine Hydrochloride, and Codeine Phosphate in Syrup

By HAROLD J. WESSELMAN and WILLIAM L. KOCH

A method for determining methapyrilene fumarate, ephedrine hydrochloride, and codeine phosphate simultaneously by gas chromatography is described. Temperature programming plus the use of an internal standard provide a simple and rapid method for separating and determining the three components in a syrup. Precision and accuracy studies are included.

BROCHMANN-HANSSEN AND Svendsen (1) determined ephedrine by gas chromatography in 1962. At about the same time Fales and Pisano (2) reported the determination of ephedrine as did Parker, Fontan, and Kirk (3). The latter authors (4) also determined codeine as did Massingill and Hodgkins (5) and Mule (6). Later Fontan, Smith, and Kirk (7) and Mac Donald and Pflaum (8) were able to determine methapyrilene. All of these determinations were qualitative in nature.

The quantitative determination of methapyrilene was performed by Celeste and Turczan (9) while the quantitative determination of codeine was done by Schmerzler *et al.* (10).

While the syrup can be assayed using an infrared or a combination spectrophotometric and titrimetric procedure, these methods leave much to be desired as several interdependent correction factors must be applied to the infrared and ultraviolet absorbance values obtained from tediously prepared extracts. In the present method, a simplified extraction procedure is combined with gas chromatography to give a rapid assay.

EXPERIMENTAL

Equipment—A linear programmed-temperature gas chromatograph (F and M Scientific Corp., model 402), equipped with a flame-ionization detector, was used for the experimental work. The detector signal was fed to a Honeywell Electronic 16 1-mv. recorder with a chart speed of 15 in./hr. and a 1-sec. full scale response. Samples were injected with a 10-μl. Hamilton, No. 701, syringe.

Materials—Helium was used as a carrier gas, while electrolytic hydrogen and oxygen were used in the detector. The stationary phase was 3.8% Linde W-98 silicone gum applied by the solution technique of Horning *et al.* (11) to Diatoport S (80–100 mesh) and packed in dual borosilicate glass columns (91 cm. × 6.4 mm.). Chloroform (analytical reagent grade) was used to dissolve the free bases obtained from the commercially available methapyrilene fumarate, ephedrine hydrochloride, and codeine phosphate.

Operating Conditions—The column temperature was programmed from 145–255° at a heating rate of 10° per min. At the end of each programmed run, the column oven was cooled for exactly 10 min. and then equilibrated for exactly 10 min. at 145° before injecting the next sample. These times were selected to keep the assay within practical limits of time and precision. The helium flow rate was 55 ml. per min. with an inlet pressure of 40 psig. Oxygen and hydrogen flow rates were 300 and 35 ml./min., respectively. The sample injection port and the detector block were maintained at 285°. One-microliter injections of all samples were used throughout. The electrometer range was 10 with an attenuation of 256.

Quantitative Analysis—The internal standard technique of Ray (12) was used because of its accuracy. In this method amobarbital is employed as the internal standard.

A 10.0-ml. sample of the syrup is placed into a 125-ml. separator containing 15 ml. water. Then add 1.5 ml. 1:1 sodium hydroxide to make the mixture basic. Extract the mixture with four 25-ml. portions of chloroform and filter the extracts through anhydrous sodium sulfate into a 150-ml. beaker. Using a stream of dry air and a water bath, evaporate the chloroform to a volume of about 3 ml. Transfer quantitatively the extracts to a 10-ml. volumetric flask, add exactly 2.0 ml. of chloroform containing 20.0 mg. amobarbital, and dilute to volume with chloroform. In a like manner prepare a standard solution by extracting 20.0 mg. codeine phosphate, 10.0 mg. ephedrine hydrochloride, and 27.0 mg. methapyrilene fumarate dissolved in 25.0 ml. water.

Chromatograph the standard and the sample solutions and measure the peak heights of each com-

Received October 31, 1967, from the Analytical Development Department, Eli Lilly and Company, Indianapolis, IN 46206

Accepted for publication January 26, 1968.

ponent. Calculate the amounts of each component in the syrup by using the following formulas:

$$\frac{\text{standard amobarbital peak height/standard ephedrine peak height}}{\text{sample amobarbital peak height/sample ephedrine peak height}} \times 1.0 = \text{mg. ephedrine hydrochloride/ml. syrup}$$

$$\frac{\text{standard amobarbital peak height/standard methapyrilene peak height}}{\text{sample amobarbital peak height/sample methapyrilene peak height}} \times 2.7 = \text{mg. methapyrilene fumarate/ml. syrup}$$

$$\frac{\text{standard amobarbital peak height/standard codeine peak height}}{\text{sample amobarbital peak height/sample codeine peak height}} \times 2.0 = \text{mg. codeine phosphate/ml. syrup}$$

PRECISION AND ACCURACY

To determine the precision and accuracy of the method, a freshly prepared solution of the three components in distilled water was used as the standard solution. This solution was chromatographed five times and the peak height ratios for each component were averaged to obtain values which were then used in the calculations for the sample solution. The relative standard deviations for ephedrine, methapyrilene, and codeine are ±3.76, ±2.38, and ±2.02%, respectively. Since the ratios for the standard solution are fairly constant and reproducible, this solution need be chromatographed only two times when routine assays are performed. The sample solution was prepared by dissolving the same three components in a freshly made syrup blank. These studies were carried out in accordance with the suggestions of the Advisory Board of Analytical Chemistry (13) and the recommended nomenclature is used. Table I shows the results of these studies.

The precision of the method was also examined by having another analyst assay a production lot of the syrup. Each of five replicate extractions was chromatographed five times. The relative standard deviation values could probably be improved if a longer equilibration period is allowed before beginning the programmed temperature run. The results of this study are recorded in Table II.

Table I—Precision and Accuracy Study

	Ephedrine	Methapyrilene	Codeine
	Standard Solution, mg./ml.		
	1.00	2.70	2.00
	Sample Solution, mg./ml.		
$\bar{X}$	1.01	2.71	1.99
N	25	25	25
s	0.0669	0.0946	0.0425
RSD	±6.62%	±3.49%	±2.14%
Mean error	+0.01	+0.01	−0.01
Relative error	+1.00%	+0.37%	−0.50%

Table II—Precision Study of a Production Lot of Syrup of Methapyrilene Fumarate, Ephedrine Hydrochloride, and Codeine Phosphate

	Ephedrine Hydrochloride	Methapyrilene Fumarate	Codeine Phosphate
Theory, mg./ml.	1.00	2.90	2.00
N	25	25	25
$\bar{X}$	1.00	3.08	2.11
RSD	±7.76%	±4.78%	±4.93%

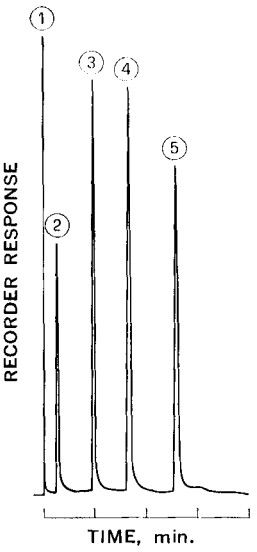

Fig. 1—Typical gas chromatogram for chloroform (1), ephedrine (2), amobarbital (3), methapyrilene (4), and codeine (5).

Table III—Analysis of Syrup of Methapyrilene Fumarate, Ephedrine Hydrochloride, and Codeine Phosphate

	Ephedrine Hydrochloride	Methapyrilene Fumarate	Codeine Phosphate
Theory mg./ml.	1.00	2.90	2.00
Lot No.			
A[a]	1.01	2.93	1.98
B[b]	1.05	2.85	2.05
C[b]	1.01	2.82	2.00
D[c]	1.01	2.92	2.00
E[c]	1.01	2.94	2.00

[a] Average of 10 determinations; RSD values similar to those of Table II. [b] Average of 5 determinations. [c] Average of 3 determinations.

The results indicate that while the precision is less than anticipated, it is better than conventional methods which are combinations of spectrophotometric and titrimetric procedures. This method has the important advantage of being specific for each component. Efforts are being made to increase the precision of the method by improving the temperature programming technique.

RESULTS AND DISCUSSION

A typical chromatogram of a mixture of methapyrilene, ephedrine, codeine, and amobarbital is shown in Fig. 1. Table III shows the results of the assay of five production lots of the syrup. The overall time for determining the standard and the sample amounts to approximately 1.5 hr.

CONCLUSION

A simplified method for determining methapyrilene fumarate, ephedrine hydrochloride, and codeine phosphate by means of gas chromatography is presented. The use of temperature programming plus an internal standard result in a good separation

of the three components which have widely spaced boiling points.

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Keyphrases

Methapyrilene fumarate, ephedrine HCl, codeine PO_4 syrup—analysis
Simultaneous determination
GLC—analysis
Amobarbital—internal standard

Quantitative Determination of Some Single and Multiple Component Drugs by Gas-Liquid Chromatography

By BOBBY R. RADER and EMMA S. ARANDA

A gas-liquid chromatographic procedure has been developed to separate and quantitate various drug mixtures. The samples are extracted by various techniques and determined by the use of a polar (4 percent cyclohexanedimethanol succinate) or a polar–nonpolar (1 percent cyclohexanedimethanol succinate plus 10 percent SE 52 silicone gum rubber) gas-liquid column. Retention data relative to pentobarbital are presented for 50 drug materials. Quantitative data are presented for 25 different drugs found in 19 commercial preparations, and for 7 synthetically prepared drug combinations. Recoveries ranged from 96 to 106 percent.

GAS-LIQUID CHROMATOGRAPHY (GLC) has been used to successfully separate antihistamines (1–5), barbiturates (6–11), and alkaloids (12–14) where several of the same class occur together. Many multiple component drugs, however, are not confined to a single class of ingredient (*e.g.*, barbiturates only) but have a variety of active ingredients. The purpose of this study was to find appropriate column materials for GLC which could be used to separate various classes of drugs in a single dosage form.

Previous reports on drug separation by GLC (2, 5, 11, 12) indicate that columns containing either polar or polar–nonpolar liquid phases give more symmetrical peaks for a larger number of drugs than nonpolar liquid phases. Peak symmetry is desirable for accurate quantitation of drugs. Two such columns were investigated in this study.

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Accepted for publication January 3, 1968.

Nineteen commercial drug preparations were analyzed. These drugs were in several dosage forms including tablets, capsules, liquids, and lotions. They contained from one to ten active ingredients each, but a maximum of six ingredients was analyzed in any one sample.

Seven synthetic drug mixtures were also analyzed. These mixtures were prepared, in most cases, with the concentration of active ingredients and excipients (starch, lactose, and magnesium stearate) equivalent to commercial drug preparations.

EXPERIMENTAL

Column Preparation

A mixed column of 1% HI-EFF-8BP (cyclohexanedimethanol succinate) + 10% SE 52 (a methylphenyl silicone gum rubber) on Gas Chrom Q (Column A) and a polar column of 4% HI-EFF-8BP on Gas Chrom Q (Column B) were prepared and conditioned in the following manner.

Column A—Into a 600-ml. beaker, 200 mg. of HI-EFF-8BP (Applied Science Laboratories) and

of the three components which have widely spaced boiling points.

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Keyphrases

Methapyrilene fumarate, ephedrine HCl, codeine PO_4 syrup—analysis
Simultaneous determination
GLC—analysis
Amobarbital—internal standard

Quantitative Determination of Some Single and Multiple Component Drugs by Gas-Liquid Chromatography

By BOBBY R. RADER and EMMA S. ARANDA

A gas-liquid chromatographic procedure has been developed to separate and quantitate various drug mixtures. The samples are extracted by various techniques and determined by the use of a polar (4 percent cyclohexanedimethanol succinate) or a polar–nonpolar (1 percent cyclohexanedimethanol succinate plus 10 percent SE 52 silicone gum rubber) gas-liquid column. Retention data relative to pentobarbital are presented for 50 drug materials. Quantitative data are presented for 25 different drugs found in 19 commercial preparations, and for 7 synthetically prepared drug combinations. Recoveries ranged from 96 to 106 percent.

GAS-LIQUID CHROMATOGRAPHY (GLC) has been used to successfully separate antihistamines (1–5), barbiturates (6–11), and alkaloids (12–14) where several of the same class occur together. Many multiple component drugs, however, are not confined to a single class of ingredient (*e.g.*, barbiturates only) but have a variety of active ingredients. The purpose of this study was to find appropriate column materials for GLC which could be used to separate various classes of drugs in a single dosage form.

Previous reports on drug separation by GLC (2, 5, 11, 12) indicate that columns containing either polar or polar–nonpolar liquid phases give more symmetrical peaks for a larger number of drugs than nonpolar liquid phases. Peak symmetry is desirable for accurate quantitation of drugs. Two such columns were investigated in this study.

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Seven synthetic drug mixtures were also analyzed. These mixtures were prepared, in most cases, with the concentration of active ingredients and excipients (starch, lactose, and magnesium stearate) equivalent to commercial drug preparations.

EXPERIMENTAL

Column Preparation

A mixed column of 1% HI-EFF-8BP (cyclohexanedimethanol succinate) + 10% SE 52 (a methylphenyl silicone gum rubber) on Gas Chrom Q (Column A) and a polar column of 4% HI-EFF-8BP on Gas Chrom Q (Column B) were prepared and conditioned in the following manner.

Column A—Into a 600-ml. beaker, 200 mg. of HI-EFF-8BP (Applied Science Laboratories) and

2.0 Gm. of SE 52 (Analabs, Inc.) were weighed, then dissolved in 350 ml. of benzene–toluene (1 + 2) by heating on the steam bath with mixing. To this solution, 20 Gm. of 80–100 mesh Gas Chrom Q (Applied Science Laboratories) was added.

Column B—Into a 600-ml. beaker, 800 mg. of HI-EFF-8BP was weighed, then dissolved in 350 ml. of benzene by heating on the steam bath with mixing. To the solution, 20 Gm. of 80–100 mesh Gas Chrom Q was added.

The solvent in each case was removed by evaporation on a steam bath with frequent stirring. The packing material was slowly added to a 1.8 m. × 63 mm. (6 ft. × 1/4 in. i.d.) glass coiled column, using vacuum and tapping. The columns were conditioned overnight at 250° with a flow rate of nitrogen of about 50 ml./min.

Apparatus

A Packard model 7621S gas chromatograph equipped with a hydrogen flame ionization detector and a 1-mv. recorder was used with the following conditions: Column temperature, 200–250°; inlet temperature, 280°; detector temperature, 250°; voltage, 250; carrier gas, nitrogen at 100 ml./min.; sensitivity, 1×10^{-7} to 1×10^{-10} amp. full scale.

Because of the concentration range of the components in some of the drugs, the concentrations in the sample solutions to be analyzed ranged from 0.2 to 3 mg./ml. Column temperatures and instrument sensitivity were varied to obtain adequate separation and response for quantitation.

Preparation of Standard Solution

A weighed amount of the drug to be determined was diluted with chloroform or methanol to approximate the same concentration as that in the sample solution (0.2–3 mg./ml.).

Barbiturates as sodium salts used as standards were extracted in the same manner as the sample.

Sample Preparations

For tablets, 20 tablets were weighed and powdered; then a portion was weighed for assay. For liquids, an aliquot was pipeted. For lotions, the density was determined and a sample was weighed for analysis.

Extraction Procedures

In the first stage of sample analysis, the active ingredients were extracted from the inert materials by one of three techniques: direct solution in an appropriate solvent, liquid-liquid extraction using a separator, or liquid-liquid partition column chromatography. The purpose of these procedures was not necessarily to separate one component from another but to isolate the active ingredients for subsequent chromatographic separation.

The following extraction procedures were used. (The ether and chloroform used in Procedures 3 and 4 were water-saturated just before use.)

Procedure 1—A sample aliquot was diluted to a suitable volume with chloroform or methanol and mixed.

Procedure 2—The solvent was added by pipet to an accurately weighed portion of the powdered sample. The sample was placed in an ultrasonic bath for 10 min. to aid solution, then mixed, and filtered.

Procedure 3—The sample was mixed with 3–4 ml. of 1 *N* NaOH and 4–5 Gm. of acid-washed diatomaceous earth[1] and transferred to a chromatographic column. The beaker and funnel were rinsed with 1 Gm. of acid-washed diatomaceous earth, which was also added to the column. The column was topped with a piece of glass wool, then eluted with one or two of the following: (*a*) 200–300 ml. of ether; (*b*) 200–300 ml. of chloroform; (*c*) 10 ml. of 10% acetic acid in chloroform, followed by 100 ml. of 1% acetic acid in chloroform; (*d*) 100 ml. of 1% triethylamine in chloroform; (*e*) 5 ml. of 20% triethylamine in chloroform, followed by 100 ml. of 1% triethylamine in chloroform.

Procedure 4—The sample was treated as in Procedure 3, except that water was used instead of 1 *N* NaOH.

Procedure 5—The sample was transferred to a separator, diluted to approximately 20 ml. with distilled water, and made either (*a*) basic with 1 *N* NaOH, or (*b*) acid with HCl and extracted 6–8 times with 25-ml. portions of chloroform.

All extracted sample solutions in Procedures 3, 4, and 5 were evaporated nearly to dryness, then dissolved in an appropriate solvent and transferred to a volumetric flask.

When multiple components in a drug were determined, the drug with the lowest concentration was determined first; then appropriate dilutions were made for assay of the other drugs present in greater concentrations.

The actual extraction method used for a given sample can be determined from Table I. For example, the extraction for a drug mixture (No. 7) of orphenadrine citrate, caffeine, and phenacetin (with aspirin) is listed in the table as *3 bd*. This sample was extracted on the NaOH–diatomaceous earth column, and eluted with solution *b* (chloroform), followed by solution *d* (1% triethylamine in chloroform).

Injection Technique

From 1–3 μl. of solvent was drawn into a 10-μl. syringe, followed by 1–3 μl. of air and then by 5 μl. of the sample solution. The plunger was drawn back, and the volume of sample solution was read and injected.

This technique allows the entire sample in the syringe to be injected, and eliminates the need for an internal standard (15).

Quantitation of Peaks

A number of methods can be used to quantitate chromatographic peaks (16). In this study, calculations were based on peak heights for drugs with retention times of less than 5 min., because it was difficult to accurately measure the width of sharp, narrow peaks. For peaks with retention times greater than 5 min., quantitation was based on peak area obtained by triangulation.

To obtain accurate results, an amount of standard was injected to produce a peak area within ±20% of sample peak area. Peak heights were between 20 and 90% of full-scale deflection.

DISCUSSION

The experimental conditions used were selected to allow the analysis of a variety of multiple component

[1] Celite, Johns-Manville Corp., New York, N. Y.

TABLE I—ANALYSIS OF COMMERCIAL AND SYNTHETIC DRUG PREPARATIONS

No.	Drugs Analyzed	Method of Extraction	Column Used	Amount Declared	% Found of Declared	Recovery of Synthetic Mix Equivalent to Sample	% Found of Declared by UV Method
1	Orphenadrine citrate	1	A	30 mg./ml.	96	...	100
2	Orphenadrine citrate	2	A	100 mg./tab.	98	...	99
3	Orphenadrine HCl	2	A	50 mg./tab.	103	...	...
4	Mephenesin	2	A	0.5 Gm./tab.	100	...	98
5	Pyrilamine maleate	2	A	50 mg./tab.	102	...	...
6	Mephenesin	3*ac*	B	300 mg./tab.	105	...	...
	Salicylamide			300 mg./tab.	93	...	...
7	Orphenadrine citrate	3*bd*	B	25 mg./tab.	98	101	...
	Caffeine			30 mg./tab.	95	98	...
	Phenacetin			160 mg./tab.	100	101	...
	(with aspirin)			225 mg./tab.	...	...	...
8	Phenindamine tartrate	2	A	25 mg./tab.	80	...	80
9	Caffeine	3*b*	B	30 mg./tab.	107	...	...
	Phenacetin			160 mg./tab.	102	...	...
	Chlorpheniramine maleate (with aspirin)			2 mg./tab.	114	...	...
10	Benzocaine	5*a*	B	0.5% soln	98	98	...
	Methapyrilene HCl			0.5% soln.	102	99	...
	Pyrilamine maleate			0.5% soln.	99	103	...
11	Salicylamide	3*ae*	A	3.5 gr./cap.	97	106	...
	Acetaminophen			2 gr./cap.	106	98	...
	Caffeine			0.5 gr./cap.	104	98	...
	Pheniramine maleate			12.2 mg./cap.	98	102	...
	Methapyrilene HCl			10.2 mg./cap.	98	102	...
	Pyrilamine maleate			12.2 mg./cap	91	102	...
12	Salicylamide	3*ae*	A	3.5 gr./cap.	106	...	...
	Acetaminophen			2 gr./cap.	112	...	...
	Caffeine			0.5 gr./cap	106	...	...
	Pheniramine maleate			6.25 mg./cap.	89	...	...
	Methapyrilene HCl			8.33 mg./cap.	97	...	...
	Pyrilamine maleate			8.33 mg./cap.	90	...	...
13[a]	Pheniramine maleate	3*be*	A	10 mg/tab.		103	...
	Methapyrilene HCl			15 mg./tab.		104	...
	Salicylamide			300 mg./tab.		101	...
14	Salicylamide	3*ae*	A	250 mg./tab.	104	...	...
	Acetaminophen			200 mg./tab.	89	...	...
	Caffeine			15 mg./tab.	93	...	...
	Chlorpheniramine maleate			2 mg./tab.	112	...	...
	Pyrilamine maleate			10 mg./tab.	98	...	...
15	Phenylpropanolamine HCl	1	A	12.5 mg./ml.	95	...	...
	Chlorpheniramine maleate			5 mg./ml.	104	...	...
16[a]	Phenobarbital	2	A	4.09 mg.	...	96	...
	Theophylline			20.44 mg.	...	100	...
	Glyceryl guaiacolate			20.42 mg.	...	103	...
17[a]	Caffeine	2	A	29.8 mg.	...	99	...
	Hexobarbital			100.6 mg.	...	102	...
	Phenacetin			200.2 mg.	...	98	...
	Dihydrocodeinone bitartrate			5.0 mg.	...	101	...
18	Sodium butabarbital	5*b*	B	15 mg./5 ml.	99	104	...
	Sodium pentobarbital			15 mg./5 ml.	103	103	...
	Sodium secobarbital			15 mg./5 ml.	105	103	...
	Sodium phenobarbital			15 mg./5 ml.	104	98	...
19	Dextromethorphan (and terpin hydrate elixir)	1	B	200 mg./100 ml.	103	...	...
20	Dextromethorphan (and terpin hydrate elixir)	1	B	11 mg./ml.	99	...	...
21	Diphenhydramine HCl	1	A	0.05% soln.	102	...	...
	Ephedrine sulfate (with sodium sulfacetamide and *dl*-deoxyephedrine)			0.5% soln.	97	...	...
22	Trifluoperazine	3*a*	A	2 mg./tab.	113	...	115

[a] Authentic samples only were analyzed.

drugs. Hence, the specified conditions may not be the optimum conditions for the analysis of a single drug material.

Different extraction procedures were necessary because of the variety of dosage forms and interferences from tablet excipients. A simple extraction with

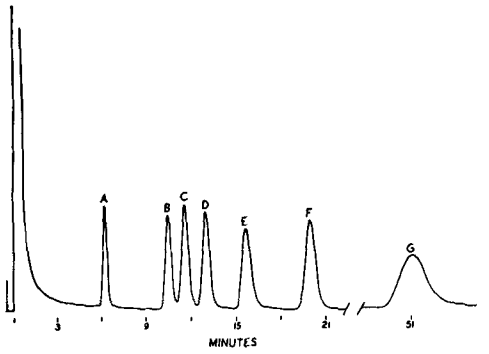

Fig. 1—Separation of barbiturates using a 1.8-m. (6-ft.) 1% HI-EFF-8BP + 10% SE 52 column on Gas Chrom Q 80–100 mesh at 220°. Key: A, *barbituric acid;* B, *butabarbital;* C, *amobarbital;* D, *pentobarbital;* E, *secobarbital;* F, *mephobarbital; and* G, *phenobarbital.*

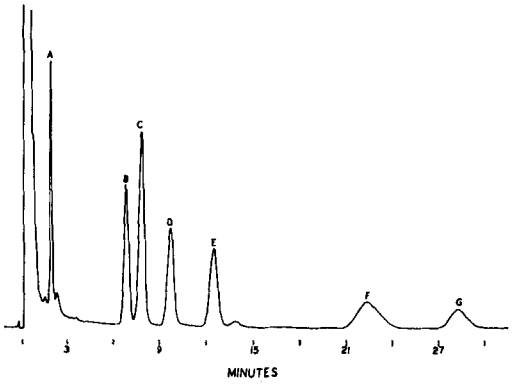

Fig. 2—Separation of antihistamines on 1.8-m. (6-ft.) 1% HI-EFF-8BP + 10% SE 52 column on Gas Chrom Q 80–100 mesh at 220°. Key: A, *phenylpropanolamine HCl;* B, *pheniramine maleate;* C, *diphenhydramine HCl;* D, *orphenadrine citrate;* E, *methapyrilene HCl;* F, *phenindamine tartrate; and* G, *pyrilamine maleate.*

chloroform or methanol is preferable when interferences are not encountered.

The 1% HI-EFF-8BP + 10% SE 52 column (Column A) is preferred for analysis since it gives sharper chromatographic peaks and can separate a greater variety of drugs. Figures 1, 2, and 3 show the separation of analgesics, barbiturates, and antihistamines. Figure 4 shows the separation of mixed classes of drugs by this polar–nonpolar type of column.

Retention times on the 4% HI-EFF-8BP column (Column B) in some cases are considerably different from those by the mixed column; thus, some drugs which are not separated by one can often be assayed and identified by the other. Figure 5 shows the separation of drugs by this column.

Relative retention times for 50 drugs were obtained and are listed in Table II. Some of these drugs were not determined quantitatively; they did, however, give good response and symmetrical peaks by one or both columns, indicating that quantitation would be possible.

No study was made of the stability of the drugs on the gas chromatographic columns used. This proce-

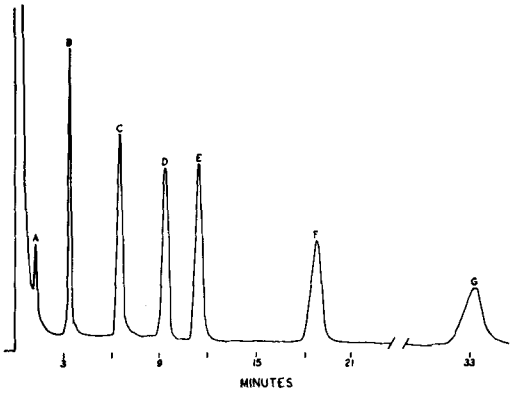

Fig. 3—Separation of some analgesics on 1.8-m. (6-ft.) 1% HI-EFF-8BP + 10% SE 52 column on Gas Chrom Q 80–100 mesh at 220°. Key: A, *aspirin;* B, *acetanilide;* C, *salicylamide;* D, *phenacetin;* E, *phenylsalicylate;* F, *caffeine; and* G, *acetaminophen.*

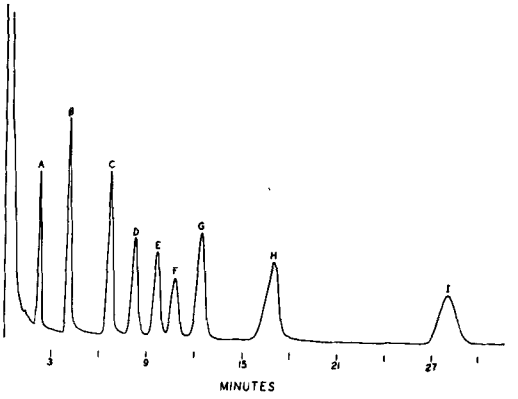

Fig. 4—Separation of mixed drugs using a 1.8-m. (6-ft.) 1% HI-EFF-8BP + 10% SE 52 column at 220°. Key: A, *acetanilide;* B, *salicylamide;* C, *phenacetin;* D, *amobarbital;* E, *caffeine;* F, *orphenadrine citrate;* G, *methapyrilene;* H, *acetaminophen;* I, *pyrilamine maleate.*

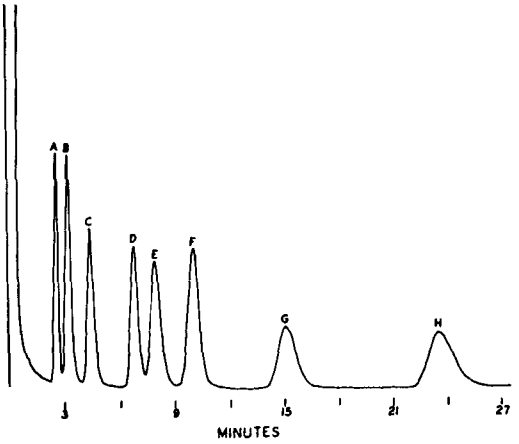

Fig. 5—Separation of mixed drugs using a 1.8-m. (6-ft.) 4% HI-EFF-8BP column on Gas Chrom Q 80–100 mesh at 210°. Key: A, *acetanilide;* B, *pheniramine maleate;* C, *orphenadrine citrate;* D, *salicylamide;* E, *methapyrilene HCl;* F, *phenacetin;* G, *amobarbital;* H, *pyrilamine maleate.*

TABLE II—RELATIVE RETENTION TIMES OF 50 DRUGS RELATIVE TO PENTOBARBITAL AT 220° C.

Name of Drug	Column A: 1% HI-EFF-8BP + SE 52	Column B: 4% HI-EFF-8BP
Acetanilide	0.26	0.19
Acetaminophen	1.86	2.63
Aminopyrine	1.27	0.62
Amobarbital	0.90	0.90
Antipyrine	1.35	1.01
Aspirin	0.11	...
Atropine sulfate	3.14	1.78
Barbituric acid	0.48	0.53
Benzocaine	0.46	0.33
Butabarbital	0.82	0.84
Caffeine	1.06	0.98
Chlorpheniramine maleate	1.43	0.49
Chlorpromazine	7.0	...
Codeine phosphate	5.23	4.00
Dextromethorphan	1.99	0.73
Diallylbarbital	0.69	...
Dihydrocodeinone bitartrate	6.22	5.79
Diphenhydramine HCl	0.83	0.24
Ephedrine sulfate	0.19	0.09
Glyceryl guaiacolate	0.62	0.55
Hexobarbital	1.24	0.88
Lidocaine	1.02	0.41
Lobeline HCl	0.86	0.42
Menadione	0.33	0.15
Mephenesin	0.48	0.33
Mephobarbital	1.51	1.32
Meprobamate	0.31	0.23
Methenamine	0.05	0.12
Methapyrilene HCl	1.36	0.51
Nicotinamide	0.38	0.40
Orphenadrine citrate	1.05	0.30
Pentobarbital	1.00	1.00
Phenacetin	0.73	0.60
Phenindamine tartrate	2.41	0.99
Pheniramine maleate	0.73	0.22
Phenobarbital	3.86	5.19
Phenylpropanolamine HCl	0.19	0.10
Phenylsalicylate	0.91	0.29
Pilocarpine	3.51	3.83
Procaine	1.98	1.42
Pyrilamine maleate	3.05	1.36
Salicylamide	0.44	0.44
Salicylic acid	0.25	0.25
Secobarbital	1.19	1.23
Tetracaine	3.47	1.90
Theophylline	4.00	...
Thenyldiamine	1.28	0.55
Theobromine	1.91	2.13
Thiamine	0.17	0.09
Trifluoperazine	10.6	...
Tripelennamine	1.36	0.44

dure is not recommended for the isolation of a pure drug prior to additional analysis (such as infrared or mass spectrophotometry).

CONCLUSION

The quantitative and qualitative determination of 25 different drugs in 19 commercial preparations and seven synthetic samples was accomplished by a fast, reasonably accurate method. The quantitative results of these analyses are shown in Table I. Recoveries from the synthetic samples ranged from 96 to 106%, with most recoveries between 98 and 103%. Analysis of commercial preparations containing one active ingredient by ultraviolet spectrophotometric methods showed good agreement with GLC results.

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Keyphrases

Quantitative determination—single, multiple components
Extraction—single, multiple component dosage forms
GLC—analysis
Retention times, 50 drugs—relative to pentobarbital

Automated Analysis of Tertiary Amines

By NORBERT R. KUZEL

An automated method for the analysis of tertiary amines has been developed using a basic automatic analyzer arrangement. The method is based on a modification of the familiar dye complex extraction method for amines. The method is simple and is not subject to adsorption of the dye on components of the analytical train. A new technique for automated extraction is also described as part of the method. The specificity of the method can be highly controlled by suggested changes in the dye reagent formulation enabling certain amines to be analyzed in the presence of other amines. The method has been designed for the analysis of solid samples.

A LARGE NUMBER of commercially useful compounds are tertiary amines. These compounds traditionally involve difficult and exacting assay procedures. A simple, general automated method for these compounds would be very desirable. It would be of even greater value if a controllable degree of specificity could be incorporated into the automated method since many of these compounds are produced or formulated as mixtures with other amines. The work described in this paper in large measure achieves these objectives.

The majority of published analytical methods for tertiary amines (1–13) are based on some modification of a dye complex formation with a halogenated acid dye as first observed by LaMer and Downes (14) and later supplemented by Griffiths (15). Prudhomme (11) was the first to utilize this principle as the basis of his method for the determination of quinine with eosin. Lehman and Aitken (8) used bromothymol blue to form their amine complex which was extracted into benzene and then reextracted into aqueous alkali. Brodie and Udenfriend (2) used methyl orange, and Cronheim and Ware (3) were the first to use bromocresol purple.

EXPERIMENTAL

Apparatus—The analytical system consisted of the following modules[1]: (*a*) automatic solid sampler; (*b*) proportioning pump; (*c*) photoelectric colorimeter (15-mm., tubular flow cell); (*d*) linearized recorder.

Preliminary Investigations—Since bromothymol blue is most often used in dye complex methods for tertiary amines, it was investigated for its suitability in an automated system. A basic automatic analyzer[2] with the previously mentioned modules was set up for evaluation. The analytical train was equipped with a Technicon glass-bead extractor and Acidflex tubing for all tubes exposed to chloroform. Bromothymol blue reagent was used at a pH of 7.8 and the reagent was extracted in the glass-bead extractor with chloroform. The reagent was decolorized in the system followed by a heavy build-up of yellow dye in the glass-bead extractor. The concentration of dye was increased until some blue color remained, but very high and variable blanks were observed. Other extraction solvents such as methylene chloride, ethylene dichloride, carbon tetrachloride, chlorobenzene, and mixtures of these with methanol, ethanol, propanol, butanol, acetonitrile, dioxane, and tetrahydrofuran were evaluated with very little improvement in performance. Coating the extractor with silicone offered only slight improvement. Mixtures of chloroform with ethanol showed the best response, but results continued to be variable and a pronounced base line drift still occurred.

Methyl orange at a pH of 5.0 and 8.0 was substituted for bromothymol blue with very little improvement. During these studies it was observed that as the dye solutions were made more acidic, dye retention decreased. Several halogenated sulfonphthalein dyes such as bromocresol green, bromophenol blue, bromochlorophenol blue, chlorophenol red, cresol red, iodophenol blue, metacresol purple, phenol red, and thymol blue were evaluated for their performance in acid solutions, and bromocresol purple was selected as the dye of choice. It was shown to have a high sensitivity to certain tertiary amines even in highly acid solutions and, because of its sensitivity, reextraction into alkali was considered unnecessary for most applications. Bromocresol purple also showed very little retention in the analytical train and the base line was only slightly elevated over that observed without reagent.

Reagents—(*a*) Bromocresol purple[3]; (*b*) dye solution—200 mg. of bromocresol purple dissolved in 1,000 ml. of 2% v/v glacial acetic acid; (*c*) Dow Antifoam B added to the wash solution pumped into the automatic sampler.[4]

Procedure—Fig. 1 shows a flow diagram of the

Received October 25, 1967, from the Analytical Research Department, Eli Lilly and Co., Indianapolis, IN 46206
Accepted for publication January 16, 1968.
Presented in part at the Technicon Symposium "Automation in Analytical Chemistry," New York, N. Y., October 17, 1966.
The author wishes to thank Mr. Charles Stevenson, Eli Lilly and Co., for his helpful suggestions.

[1] Technicon Instruments Corp., Chancey, N. Y.

[2] AutoAnalyzer, Technicon Corp.

[3] Eastman Corp., New York, N. Y.

[4] Solidprep Sampler, Technicon Corp.

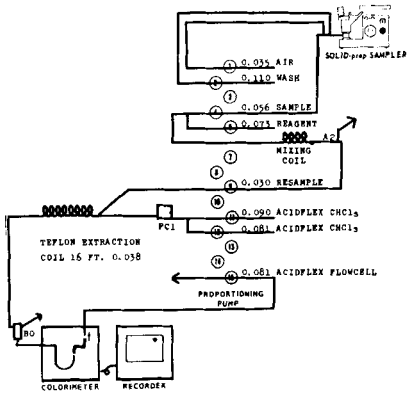

Fig. 1—Flow diagram of automated system for tertiary amine analysis.

analytical train including all tubing sizes. The automatic sampler was programmed to operate at 20 samples per hr. A 0.5 ml./min. sample is pumped from the automatic sampler and segmented with air. The sample stream is mixed with reagent and about 15% of the mixed stream is resampled and pumped back through the pump. Chloroform is pumped through Acidflex tubing into a PCI pulse chamber. The two phases are joined in a Teflon coil extractor and the aqueous dye complex is extracted with the chloroform. The phases are separated in a BO phase separator, and a portion of the chloroform extract is drawn through the flow cell by pumping from the cell. The extract is measured in the colorimeter equipped with 420-mμ filters and the absorbance recorded on a linearized recorder. A 10-roller chain is used on the proportioning pump to provide smooth flow of the pumped solutions. A small glass wool filter is placed in the chloroform line as it emerges from the pump to filter out shreds of Acidflex tubing that are produced in the proportioning pump. The system is started by pumping ethanol through the Acidflex tubing before any aqueous solutions are pumped. The ethanol is replaced by chloroform and after all the ethanol has been purged, the aqueous solutions are started. This technique has provided excellent base line stability and has prevented contamination of the flow cell with aqueous solutions. Dow Antifoam B is used in the wash solution to provide uniform air segmentation.

RESULTS AND DISCUSSION

The method was evaluated primarily for its application to the analysis of propoxyphene hydrochloride (16) (dextro)-4-dimethylamine-1,2-di-phenyl-3-methyl-2-propionoxybutane hydrochloride.

The response follows Beer's law over an absorbance range of 0–0.9 and relative standard deviations of an average of five observations are typically 0.5–1.2%. Figure 2 shows typical recordings of the response of the system to five replicate standards of propoxyphene hydrochloride. Inspection of the curve shows a stable base line, good reproducibility, symmetrical peaks, and no sample interaction. Comparative results between the automatic method and a manual nonaqueous titration method are shown in Table I. The results by the automatic method represent an average of

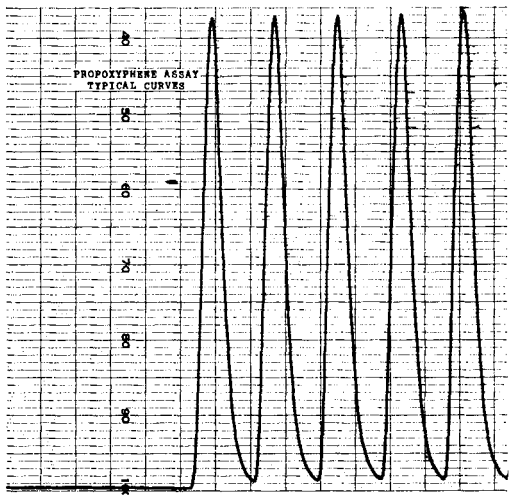

Fig. 2—Response of replicate standards of propoxyphene hydrochloride.

Table I—Comparative Data on Propoxyphene Hydrochloride Samples Between Manual and Automated Methods

Lot	Manual mg./dose	Automated mg./dose	
1	66.2	66.3	(20)
2	65.0	64.4	(20)
3	65.0	64.9	(20)
4	33.6	32.6	(10)
5	32.8	32.8	(10)
6	33.2	32.9	(10)

the indicated number of single-dose assays, while the manual method represents the result obtained on a composite sample of 10 doses. Considering that the same doses were not assayed by both methods, the correlation is very good. This data was obtained on multicomponent mixtures in both capsule and tablet formulations.

The method is shown using an automatic sampler since the majority of the tertiary amines analyzed in our laboratory are assayed as solid samples, primarily as multicomponent tablets or capsules. Tablets are placed directly in the cups of the automatic sampler and capsules are emptied into the cups and the empty capsules are discarded. Standard solutions of the amine are normally dissolved in the appropriate solvent and aliquots of these solutions are pipeted into the sample cups. A correction is made in the absorbance of the standard to account for the volume change resulting from the use of liquid standards. Sample size differences are accommodated by changing the amount of solvent delivered by the automatic sampler or by changing to an alternate size pump tube. Alternate pump tubes are placed in the proportionating pump manifold and tube connections are changed without opening the pump platen.

The concentration of the dye required in the reagent was determined by adjusting the concentration until linearity was obtained throughout the desired concentration range. An additional 50% excess of dye was used since large increases in dye concentration increase the reagent blank slightly. The acidity was adjusted for highest sensitivity to

propoxyphene hydrochloride. The acid concentration can be varied from 1.5 to 3% glacial acetic acid without significant change in response. Eastman bromocresol purple provides a much higher response to the amines tested than some of the other brands, but the reason for this difference in response was not investigated.

The automatic sampler was evaluated for its performance characteristics by analysis of potassium permanganate tablets. These tablets were selected since they provide a relatively stable, highly colored sample which can be traced throughout the system and their tablet weight is essentially equivalent to their content of potassium permanganate. Use of these tablets vividly emphasizes any localized problems in sampling, mixing, sample interaction, and wash characteristics. Hydroxylamine hydrochloride is run through the system occasionally to clean out any traces of manganese dioxide formed in the analytical train. This technique pointed up two serious problems in the use of the automatic sampler. Sample interaction was significant and solvent delivery was erratic. The source of the eratic solvent delivery was found to be due to faulty check valves in the sampler, thus allowing variable amounts of air to be delivered along with the solvent. This problem was solved by replacing the valves with check valves[5] which provide a very positive action and result in precise dispensing of solvent in the sampler. The conventional system of withdrawing a sample from the automatic sampler resulted in too large a sample interaction for precise work and a new sampling device was designed. This consisted of a small rectangular Lucite block with a 0.89-mm. (0.35-in.) hole drilled through its long axis and 2 side ports. The device was placed in the sample line adjacent to the blender compartment of the sampler. The side port nearest the sampler was connected to the wash pump tube. This tube is selected to provide at least twice as much wash volume as is withdrawn by the sample pump tube. During sampling the wash tube is bypassed and has no effect on the system. During the rinse part of the cycle the wash solution divides so that a portion flows into the sample pump tube and the remainder flows into the blender compartment of the sampler, effectively blocking off any leakage of sample from the blender. The other side port is connected to the air pump tube and all air segmentation occurs at this point during both sample and rinse cycles. With this arrangement a uniformly segmented stream, originating near the blender, is always pumped. Positive and precise sampling is obtained with no evidence of sample interaction.

The glass-bead extractor was found to provide inconsistent and erratic extraction in the described system. Base line drift was encountered and significant sample interaction was observed. Several other extractor designs were evaluated with only moderate improvement in results. One of the critical factors in automatic extractions is the method of introduction of the two phases. If one phase is allowed to lag or lead the other or if one phase is allowed to pool in the extractor, erratic results are obtained. An excellent, yet simple extractor was designed to eliminate these problems. It consisted of a coil of 0.96-mm. (0.38-in.) i.d. Teflon spaghetti tubing wound around a 2.54-cm. (1-in.) diameter cylinder. One end of the Teflon coil is simply connected to the pulse chamber containing chloroform by a short length of Acidflex tubing. A piece of 0.8-mm. (0.030-in.) o.d. stainless steel tubing is inserted into the side of the Acidflex tubing so that one end of the stainless steel tube enters the end of the Teflon coil extractor in a coaxial arrangement. The other end of the extractor coil is connected to a small volume phase separator by another short piece of Acidflex tubing. The aqueous phase is introduced through the stainless steel tube and is swept into the extraction coil by the chloroform. Because of the small diameter of the Teflon extraction tubing and the high velocities of flow, the aqueous phase is separated into small discrete segments and remains segmented throughout the entire extraction coil resulting in excellent sample separation. The aqueous stream is debubbled prior to introduction into the extractor and the aqueous phase in effect replaces the air bubble for segmentation. Since this extractor is free of violent agitation, essentially no emulsion formation has been observed. This allows the use of small volume phase separators, and clean phase separations are obtained as a result of the uniform floating off of the very small segments of the lighter phase as it exits from the extraction coil. Because of the segmentation, the length of the extraction coil can be adjusted for optimum extraction efficiency without affecting sample interaction.

SPECIFICITY

A limited investigation was made into the specificity of the method as described. About 100 amines and related compounds were evaluated for their response in the system. Some of the results are shown in Table II. As can be seen, the method in

TABLE II—RESPONSE OF COMPOUNDS TO AUTOMATED METHOD

Compound	Strong Response	Weak Response	No Response
Acetanilide	...	...	×
p-Aminobenzoic acid	...	...	×
4-Aminosalicylic acid	...	...	×
Amphetamine hydrochloride	...	×	...
Aniline	...	...	×
Atropine	×	...	...
Benzalkonium chloride	...	...	×
Benzamide	...	...	×
Benzylamine	...	×	...
n-Butylamine	...	×	...
Cetylpyridinium chloride	...	...	×
Codeine phosphate	×	...	...
Dimethylamino-benzaldehyde	...	...	×
Diphenylamine	...	...	×
Erythromycin	×	...	...
Ethylenediamine	...	...	×
Glycine	...	...	×
Procaine hydrochloride	×	...	...
Pyridine	×	...	...
Quinine hydrochloride	×	...	...
Sulfathiazole	...	...	×

[5] Circle Seal, 230T1—2pp Circle Seal Products Co., Inc. Pasadena, Calif.

TABLE III—MOLAR EXCITATION VALUES OBTAINED BY THE AUTOMATED METHOD IN RESPONSE TO VARIATIONS IN THE DYE REAGENT FORMULATION

Compound	pH 5.0 Citrate Buffer	Acetic Acid, 2%	Sulfuric Acid, 1%
Propoxyphene hydrochloride	10,600	15,600	10,900
Levopropoxyphene hydrochloride	10,700	15,700	10,800
Piperocaine hydrochloride	17,200	16,800	13,500
Quinine hydrochloride	12,400	16,400	190
Erythromycin base	11,600	15,200	7,800
Methapyrilene hydrochloride	12,000	12,750	neg.
Cyclomethycaine hydrochloride	1,970	8,950	4,180
Procaine hydrochloride	8,200	6,400	neg.
Amphetamine hydrochloride	1,070	760	17
2-Diethylaminoethanol	1,070	375	neg.
Benzylamine	730	200	neg.
n-Butylamine	170	60	neg.
Benzalkonium chloride	neg.	neg.	neg.

general responds to aromatic tertiary amines while showing very little or no response to primary and secondary amines or to amides. This response is quite different from that reported by Woods *et al.* (13) in their work with bromocresol purple which did not depend on extraction of the dye complex from acid solutions. The lack of response to quaternary ammonium salts was probably due to adsorption in the analytical train since a positive response was seen in a manual extraction procedure. Special techniques for prevention of adsorption in the analytical train might allow the method to be used for analysis of certain quaternary amines.

Some of the compounds which did show response were further evaluated for their response to changes in dye reagent formulation. Reagents were made up at a pH of 5.0 with citrate-phosphate buffer, pH 2.2 with 2% glacial acetic acid, and pH 1.2 with 1% sulfuric acid. Table III shows some of the results. The results are expressed as molar extinction values calculated from sample size, dilution in the automated system, and the absorbance obtained. Molar extinction values reported here are about 30% lower than those obtained by manual extraction and measurement in a suitable spectrophotometer. A very interesting spectrum of response was observed and considerable change in specificity was observed with changes in reagent.

ANALYTICAL POTENTIAL

The described method should prove very useful in the precise analysis of large numbers of tertiary amine samples such as might be encountered in single-dose analysis profiles of production lots of pharmaceutical formulations. Appropriate process samples, both solid and liquid, could also be analyzed in large numbers. Modifications of the dye reagent such as those indicated might allow analysis of the desired amine in the presence of other amines while still retaining satisfactory sensitivity. The described specificity could also be effectively utilized in manual adaptations of this method. The new extractor design provides the possibility of repetitive extractions or scrubbing with immiscible solvents without significant loss of sample separation or increase of sample interaction. Decreased emulsion problems could lead to an increased sensitivity in repetitive extraction procedures since this would allow a larger volume of the desired phase to be retained for the next step of analysis. All of these advantages could be utilized in the application of this method to analysis of natural products, feeds, or fermentation samples.

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Keyphrases

Tertiary amines—automated analysis
Automated analysis—dye complex extraction
Bromocresol purple—dye reagent
Diagram—automated analyzer
Colorimetric analysis

Urinary Excretion of Chlorpheniramine and Its *N*-Demethylated Metabolites in Man

By PETER KABASAKALIAN, MARILYN TAGGART, and EDWARD TOWNLEY

The *N*-demethylated metabolites of chlorpheniramine, *N*-desmonomethylchlorpheniramine, and *N*-desdimethylchlorpheniramine were found in the urine of males using a repetitive dosage schedule.

CAVALLITO, CHAFETZ, AND MILLER (1) studied the urinary excretion of chlorpheniramine[1] on four humans who orally took 10.2-mg. chlorpheniramine maleate in gelatin capsules. The total 24-hr. urinary excretions fluctuated from 12 to 57% of the ingested dose among the four subjects. Their analytical method, however, was nonspecific, and their high results were above the upper limits found by Beckett and Wilkinson (2).

Beckett and Wilkinson (2) studied the influence of urine pH and flow rate on the renal excretion of chlorpheniramine. They found that renal excretion rates vary with both urinary pH and the rate of urine flow. When an aqueous solution of 14.2 mg. of chlorpheniramine maleate was orally administered to male subjects, the excretion rates fluctuated from 4.5 to 11.5% in 24 hr. When the urine was maintained acid (pH 5.0 ± 0.5) by the administration of ammonium chloride, 20.0 to 26.5% was excreted, whereas when the urine was maintained alkaline (pH 8.0 ± 0.5) by the administration of sodium bicarbonate, 0.3 to 0.4% was excreted. They also found that under constant acid urine conditions a high urine flow rate increased the excretion rate. The volume-dependent fluctuations became negligible when (by water loading the subjects) the urine rate was maintained above 150 ml./hr.

Since the data of Beckett and Wilkinson (2) suggested that chlorpheniramine is extensively metabolized, its probable *N*-demethylated metabolites were sought in this work. The multiple-dosage regimen appeared to be ideally suited to the problem of locating these metabolites. All one had to do was to find urinary excretion products which increased, leveled off, and declined with the dosage regimen.

EXPERIMENTAL[2]

Protocol—A male subject under a normal diet with no urine pH or fluid intake control was used in this study.

Single-Dose Regimen—The subject orally took a single dose of 12 mg. of chlorpheniramine maleate in tablet form (three 4-mg. chlorpheniramine maleate tablets). Urine was collected normally for 5.5 days and combined into 12-hr. samples.

Multiple-Dose Regimen—The same subject orally took 4 mg. of chlorpheniramine maleate in tablet form daily for 29 days. Urine was collected normally for 39 days and combined into 12-hr. samples.

Analytical Methods—The urine was analyzed by the general method of Beckett and Wilkinson (2) which involved extraction with ether at pH 10, concentration, and subsequent gas chromatography on a 5% potassium hydroxide coated Anakrom ABS column[3] containing 3% polyethylene glycol 20M[4] as liquid phase and operating at 180°C. A 76.2-cm. (2.5-ft.) column exhibited 1,500 theoretical plates for chlorpheniramine.

Free chlorpheniramine had a retention time of 6.0 min. Quantitative determinations were made using a chlorpheniramine reference standard. Isolation by thin-layer chromatography on alumina (3) and subsequent mass spectroscopy (4) showed an *m/e* value of 58 for $CH_2{=}N(CH_3)_2$ indicative of a tertiary amine and *m/e* values of 203, 202, and 167 which are characteristic of the *p*-chlorobenzyl-2-pyridyl group. It did not form derivatives either with cyclopentanone or acetone using the procedure of Capella and Horning (5) or with acetic anhydride (6).

Metabolite X had a retention time of 10.8 min., identical to that of an authentic sample of *N*-desmonomethylchlorpheniramine. Quantitative determinations were made using the authentic sample as a calibration standard. Isolation by thin-layer chromatography on alumina (3) and mass spectroscopy (4) showed *X* to be a secondary amine with an *m/e* value of 44 for $CH_2{=}NH(CH_3)$ and the *m/e* values of 203, 202, and 167 of the *p*-chlorobenzyl-2-pyridyl group. It formed a derivative (retention time >30 min.) with cyclopentanone (indicative of primary and secondary amines), but none with acetone (indicative of the absence of a primary amine) using the procedure of Capella and Horning (5). It formed a derivative with acetic anhydride (6) (retention time >30 min.).

Metabolite Y had a retention time of 14.8 min., identical to that of an authentic sample of *N*-desdimethylchlorpheniramine. Quantitative determinations were made using an authentic sample as a calibration standard. Isolation by thin-layer chromatography on alumina (3) and subsequent mass spectroscopy (4) showed *Y* to be a primary amine with an *m/e* value of 30 for $CH_2{=}NH_2$ and the *m/e* values of 203, 202, and 167 of the *p*-chlorobenzyl-2-

Received April 10, 1967, from the Research and Development Division, Schering Corporation, Bloomfield, NJ 07003
Accepted for publication October 27, 1967.

[1] Chlorpheniramine is *dl*-2-{*p*-chloro-α-[2-(dimethylamino)-ethyl]benzyl} pyridine. It is marketed as Chlor-Trimeton by Schering Corporation, Bloomfield, N. J.

[2] All gas chromatograms were obtained on a F and M model 400 gas chromatograph with a flame-ionization detector and a Minneapolis Honeywell recorder. The mass spectra were obtained on a Consolidated Electrodynamics Corporation model CEC-103C mass spectrometer.

[3] Available from Analabs, Incorporated, Hamden, Conn.

[4] Marketed as Carbowax by Union Carbide Corporation, New York, N. Y.

TABLE I—URINARY EXCRETION OF FREE CHLORPHENIRAMINE BY SUBJECT WHO RECEIVED ONE DOSE OF THREE 4-mg. CHLORPHENIRAMINE MALEATE TABLETS

Time After Drug[a] Taken, Days	Combined 12-hr. Urine Volume, ml.	Chlorpheniramine[c] in 12-hr. Urine mg.
0.0	...[b]	...[d]
0.5	550	0.215
1.0	530	0.404
1.5	800	0.151
2.0	630	0.111
2.5	530	...
3.0	420	0.082
3.5	585	0.059
4.0	540	0.033
4.5	540	0.062
5.0	520	0.030
5.5	390	0.048

[a] Experiment started at 7:30 p.m. [b] Sample not collected. [c] Calculated as chlorpheniramine maleate. [d] No assay results obtained because of sample loss, *etc.*

pyridyl group. It formed derivatives, indicative of a primary amine, with cyclopentanone (retention time = 26 min.) and with acetone (retention time = 17 min.) using the procedure of Capella and Horning (5). It formed a derivative with acetic anhydride (6) (retention time >30 min.).

RESULTS AND DISCUSSION

Single-Dose Regimen—The urinary excretion of free chlorpheniramine by the subject on the single-dose regimen is shown in Table I.

The amount of unchanged chlorpheniramine excreted by the single subject in the first 24 hr. was 5.2% of the ingested dose. This is well within the range (4.5 to 11.5%) found by Beckett and Wilkinson (2) for several subjects who were not under pH or fluid intake control. The total estimated excretion of free chlorpheniramine was about 12% of the ingested dose.

Multiple-Dose Regimen—The urinary excretion of free chlorpheniramine by the subject in a multiple-dose regimen is listed in Table II.

The 12-hr. urinary excretion rate varies with time in a "saw-tooth" manner. The peaks and valleys increased with time until a "steady state" (repetitive 24-hr. excretion pattern) was reached. The minimum values or valleys occur prior to the repetitive drug ingestion and the peaks following ingestion. The gradually increasing pattern indicates that chlorpheniramine was building up in the body.

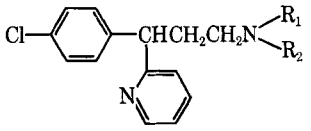
In the steady state, the input of the drug to the body should equal the output from the body. Large fluctuations were observed during the steady-state condition and were probably caused by either the variance in the daily fluid intake, urine pH, or the

TABLE II—URINARY EXCRETION OF FREE CHLORPHENIRAMINE AND ITS METABOLITES BY SUBJECT WHO DAILY RECEIVED ONE 4-mg. CHLORPHENIRAMINE MALEATE TABLET FROM DAY 0 TO DAY 28

Time,[a] Days	Combined Vol.[b] of 12-hr. Urine, ml.	Chlorpheniramine[c] Excreted in 12 hr. Urine, mg.	Metabolite *X*[d] Excreted in 12-hr. Urine, mg.	Metabolite *Y*[e] Excreted in 12-hr. Urine, mg.[f]	Time,[a] Days	Combined Vol.[b] of 12-hr. Urine, ml.	Chlorpheniramine[c] Excreted in 12-hr. Urine, mg.	Metabolite *X*[d] Excreted in 12-hr. Urine, mg.	Metabolite *Y*[e] Excreted in 12-hr. Urine, mg.[f]
0.0	...	...	...	...	19.5	655	0.268	0.365	0.182
0.5	595	0.211	0.036	...	20.0	290	0.136	0.141	0.070
1.0	340	0.085	0.049	...	20.5	420	0.345	0.304	0.189
1.5	570	0.173	0.087	...	21.0	305	0.213	0.257	0.080
2.0	450	0.066	0.057	...	21.5	475	0.309	0.366	0.120
2.5	610	0.215	0.091	0.019	22.0	400	0.061	0.099	0.041
3.0	395	0.143	0.134	0.017	22.5	485	0.274	0.311	0.126
3.5	380	0.304	0.258	...	23.0	450	0.198	0.330	0.120
4.0	480	0.144	0.271	...	23.5	570	0.295	0.333	0.098
4.5	570	0.317	0.564	...	24.0	420	0.162	0.276	0.107
5.0	420	0.187	0.134	...	24.5	370	0.574	0.262	0.076
5.5	665	0.260	0.293	0.055	25.0	385	0.291	0.216	0.070
6.0	430	0.158	0.211	0.016	25.5	405	0.478	0.336	0.182
6.5	530	0.292	0.212	...	26.0	...	...	...	...
7.0	...	...	...	...	26.5	500	0.574	0.279	0.096
7.5	310	0.364	0.325	0.101	27.0	590	0.148	0.185	0.068
8.0	420	0.139	0.250	0.035	27.5	345	0.387	0.192	0.108
8.5	515	0.264	0.266	...	28.0	490	0.194	0.218	0.093
9.0	400	0.281	0.220	...	28.5	365	0.366	0.236	0.117
9.5	620	0.274	0.210	...	29.0	400	0.241	0.208	0.102
10.0	375	0.187	0.260	...	29.5	375	0.203	0.199	0.142
10.5	540	0.357	0.147	...	30.0	355	0.123	0.085	0.069
11.0	315	0.141	0.081	...	30.5	325	0.057	0.152	0.103
11.5	485	0.412	0.316	...	31.0	395	0.032	0.092	0.036
12.0	260	0.159	0.214	...	31.5	420	0.035	0.099	0.073
12.5	510	0.378	0.417	...	32.0	305	0.020	0.070	0.059
13.0	425	0.165	0.253	...	32.5	550	0.017	0.071	0.062
13.5	605	0.330	0.370	...	33.0	455	0.013	0.043	0.032
14.0	...	...	...	...	33.5	670	0.011	0.038	0.043
14.5	405	0.207	0.285	0.113	34.0	820	0.006	0.017	0.014
15.0	405	0.120	0.228	0.091	34.5	590	0.005	0.029	0.032
15.5	390	0.215	0.273	0.109	35.0	...	...	...	...
16.0	510	0.066	0.177	0.062	35.5	260	0.003	0.014	0.038
16.5	455	0.196	0.377	0.188	36.0	340	0.002	0.009	0.022
17.0	305	0.139	0.257	0.117	36.5	610	0.003	0.007	0.021
17.5	460	0.286	0.322	0.154	37.0	380	0.008	0.004	0.006
18.0	515	0.201	0.210	0.056	37.5	550	0.004	0.003	0.007
18.5	490	0.346	0.304	0.151	38.0	350	0.003	0.002	0.004
19.0	295	0.182	0.232	0.121	38.5	745	0.006	0.002	0.006
					39.0	520	0.003	0.001	0.002

[a] Underscored days 4-mg. chlorpheniramine maleate tablet ingested; experiment started at 7:30 p.m. [b] ...sample not collected. [c] Reported as chlorpheniramine maleate. [d] Reported as *N*-desmonomethylchlorpheniramine maleate. [e] Reported as *N*-desdimethylchlorpheniramine maleate. [f] ... assay result not obtained.

TABLE III—STRUCTURAL FORMULA OF CHLORPHENIRAMINE AND ITS METABOLITES



Compd.	R_1	R_2
Chlorpheniramine	CH_3	CH_3
Metabolite *X*	CH_3	H
Metabolite *Y*	H	H

diurnal excretion pattern. The calculated *average* daily excretion rate was 0.502 mg., or 12.6% of the repeated 4-mg. chlorpheniramine dose. This compares favorably with the 12% that was estimated from the single-dose experiment.

On termination of the drug-intake regimen, the chlorpheniramine excretion rate decreased. From the total amount of free chlorpheniramine excreted after the last dose is taken on day 28.0, and using the average 0.502 mg. of chlorpheniramine excreted per 4-mg. dose, one can calculate the equivalent amount of chlorpheniramine maleate present in the subject immediately after the last dose. This calculation yields a figure of 9.3 mg. of chlorpheniramine maleate (equivalent to 2.4 tablets, each containing 4 mg. of chlorpheniramine maleate).

Metabolites X and Y—The *N*-dealkylation reaction, especially of monomethyl and dimethyl-substituted primary amines is a major pathway of drug metabolism (7). If these metabolites were present, the gas chromatographic method of Beckett and Wilkinson (2) should have revealed them, albeit with much higher retention times (6, 8) than the parent compounds. Unfortunately, in the single dose regimen, it is difficult to differentiate the desired metabolites from other constituents of urine. The metabolites, *X* and *Y*, which were found to be *N*-desmonomethylchlorpheniramine and *N*-desdimethylchlorpheniramine, respectively (Table III), built up, leveled off, and declined like chlorpheniramine but more slowly.

During the steady-state condition, an *average* of 0.510 mg. of *N*-desmonomethylchlorpheniramine and 0.216 mg. of *N*-desdimethylchlorpheniramine were excreted daily (13.2% and 5.8% of the repeated 4-mg. oral dose). Together with the free chlorpheniramine excreted daily, this accounts for 32% of the dose, leaving the fate of the remaining 68% still unaccounted for.

SUMMARY AND CONCLUSIONS

A repetitive dosage regimen using a commercially available pharmaceutical dosage of chlorpheniramine has been successfully used to identify two metabolites of chlorpheniramine, *N*-desmonomethylchlorpheniramine and *N*-desdimethylchlorpheniramine, in the urine of a single human subject.

ADDENDUM

Kamm and Van Loon (9) have recently reported the presence of the metabolites, *N*-desmonomethylchlorpheniramine and *N*-desdimethylchlorpheniramine, in the urine of the rat and the dog.

REFERENCES

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Keyphrases

Chlorpheniramine—urinary excretion
Metabolites, chlorpheniramine—isolated, identified
GLC—analysis, urine
TLC—separation
Mass spectroscopy—identity

A Micro-Extraction Technique with Compounds Isolated from Thin-Layer Chromatograms

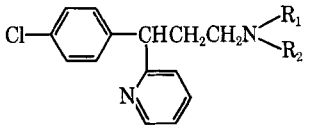
By ALMA L. HAYDEN, WILSON L. BRANNON, and NEUMAN R. CRAIG

A micro-apparatus is described for the extraction of drugs and related compounds from thin-layer or paper chromatograms by continuous elution with 1 ml. solvent. The sample, after extraction, is ground with KBr, mounted on a micro potassium halide plate, and placed in the center of a paper frame with a window area for infrared analysis. Spectra can be determined on 5- or 10-mcg. samples without beam condensers, ordinate expanders, or micro-dies.

RECENT INVESTIGATIONS in this laboratory have involved structure characterization and identification of microgram quantities of drugs and related materials by infrared spectroscopy. Most often, these have been polar compounds which have been isolated from thin-layer and paper chromatograms where impurities from the matrix and eluting solvent may interfere. To minimize these effects while

Received June 30, 1967, from the U. S. Department of Health, Education, and Welfare, Food and Drug Administration, Division of Pharmaceutical Chemistry, Spectrophotometric Research Branch, Washington, DC 20204
Accepted for publication October 5, 1967.

TABLE III—STRUCTURAL FORMULA OF CHLORPHENIRAMINE AND ITS METABOLITES



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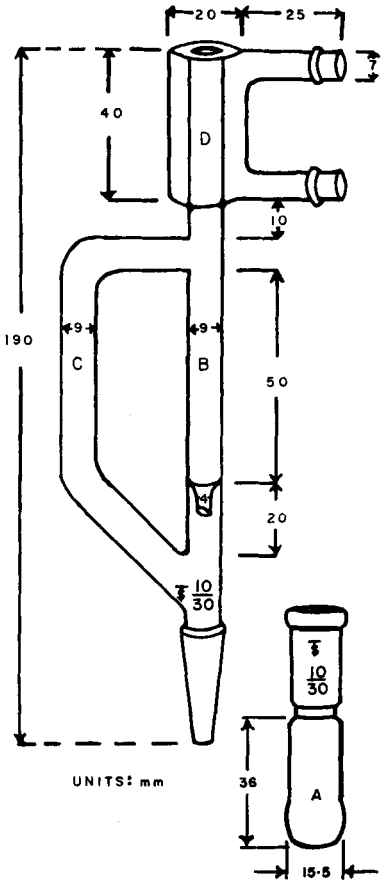

Fig. 1—A continuous extractor with flask (A), column (B), delivery tube (C), and condenser (D).

obtaining spectra on generally available equipment, the development of micro-sample handling techniques has been required.

The potassium halide disk technique can be easily modified, since the matrix is simply a means of mounting the sample. By localizing the compound in the center of the disk to approximate the final slit size of the beam, the required quantity of sample can be reduced significantly (1, 2). In this way, identifiable infrared spectra have been obtained in this laboratory without the aid of beam condensers or ordinate expanders on as little as 5 mcg. of a pure compound. As little as 10 mcg. of material isolated from a thin-layer chromatogram can be identified when this technique is used in combination with a continuous extractor (3). A further 5–10-fold reduction in sample size is possible if beam or signal intensifiers are used.

In previous reports of greater sample sensitivity (0.05–1.0 mcg.), other sampling and pelleting techniques were combined with beam condensers, ordinate expansion, and micro-dies (4–6). Bisset *et al.* (2) obtained similar results by using a reflecting microscope. The technique described here should be of particular interest when the above-mentioned accessories are not available.

The extraction apparatus shown in Fig. 1 operates efficiently with 1 ml. of solvent. The extractor consists of a boiling flask (A), a small column (B) with a side-arm delivery tube (C), and a condenser (D). The side-arm is wrapped with asbestos tape; the condenser is water or air cooled.

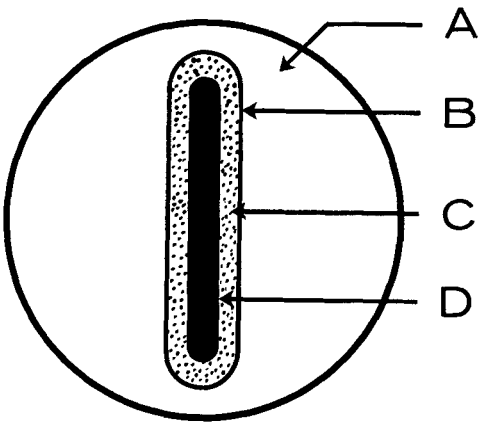

Fig. 2—Micro-plate in paper frame: A, frame; B, window; C, KBr periphery; D, KBr sample mixture.

In operation, the chromatographic zone or sample is placed in the column, which contains a pledget of glass wool. The zone may be prewashed with a solvent if desired. The apparatus is then assembled and about 1 ml. of the extracting solvent is poured through the column and collected in the flask. Single or mixed solvents may be used. The solvent is refluxed and the condensate continuously elutes the compound from the column. The cycle is continued until the extraction is completed. The extract or solution containing the compound is cooled and filtered through chloroform-washed fine filter paper or glass wool into a 50-mm. millite mortar. The filtrate is evaporated to dryness under nitrogen and the crystalline residue is dried in a vacuum over phosphorus pentoxide.

A pressed paper or cardboard frame[1] (12.7 mm. in diameter, 0.6 mm. in thickness) containing a 2 × 11 mm. window is placed on the anvil of a 0.5-in. potassium bromide die. The dry residue is ground by hand with 2 mg. of potassium halide, and the mixture is aligned vertically (1 × 10 mm.) in the center[2] of the window area of the paper frame. Approximately 25 mg. of potassium halide powder is used to rinse the mortar and then to fill in the remaining window area. The die is assembled and evacuated, and the micro-plate is pressed in the usual manner. After pressing, the micro-plate with paper frame (Fig. 2) is mounted in the micro-sample position of a double-beam spectrophotometer. Perkin-Elmer models 21 and 237 were used in this laboratory to record the spectra.

Figure 3 shows the spectra of 1-methylhydantoin: A is 10 mcg. isolated from a thin-layer chromatogram, B is 10 mcg. crystallized directly from chloroform. Both spectra were obtained on micro-plates. For comparison, a standard 0.5% disk (C) is included. Figure 4 shows a spectrum, obtained as described above, of 5 mcg. of 1-methylhydantoin recrystallized from chloroform. In the reference beam, an attenuator was used for the 10-mcg.

[1] Supplied as 13-mm. frames from Limit Research Corp., Darien, Connecticut.

[2] For use with a beam condenser, the mixture is pressed in a 1 × 1 mm. central area of the window.

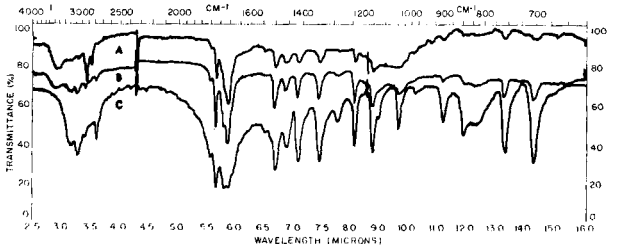

Fig. 3—Spectra of 1-methylhydantoin: 10 mcg. isolated from TLC (A), 10 mcg. crystallized from $CHCl_3$ (B), and a standard 0.5% disk (C).

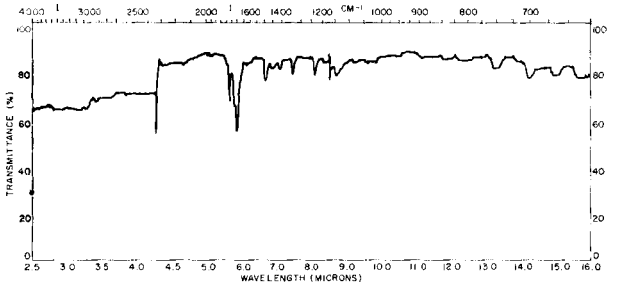

Fig. 4—Spectrum of 5 mcg. 1-methylhydantoin crystallized from $CHCl_3$.

spectra; a potassium bromide reference disk or plate was used with the 0.5% and 5-mcg. spectra. The spectra are essentially the same except for additional absorption in the C-H and 9 μ spectral regions of the sample from the thin-layer chromatogram. These spectra were recorded on a Perkin-Elmer model 237 grating spectrophotometer. Comparable, though less intense, spectra were examined in the micro-sample position of a NaCl prism spectrophotometer.

It must be emphasized that spectral identification of mcg. quantities of pure compounds depends on sample handling techniques as well as on crystal form and absorption characteristics of the molecule. In addition, the identification of materials isolated in microgram quantities from thin-layer and paper chromatograms requires adequate extraction with the inclusion of minimal solvent and substrate residues. By using the continuous extractor, microgram quantities of polar compounds can be isolated from thin-layer chromatograms with 1 ml. of solvent. However, the effects of solvent and substrate residues must be further reduced by washing glassware, thin-layer plates, and paper with the extracting solvent before use. In this application, the extraction was performed with freshly distilled, dried, and ethanol-stabilized chloroform. The extractions and disk preparations should be made on the same day. In this way, reproducible spectra of suitable intensity are obtained. Effects of excipient residues on the spectra can be further reduced by using a reference micro-plate prepared under the same conditions as the sample.

This technique has been used also in the identification of barbiturates, steroids, and other compounds isolated from thin-layer and paper chromatograms.

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Keyphrases

Micro—extraction from chromatograms
Diagram—extraction apparatus
Extraction technique—microgram quantities
IR spectrophotometry—identity

Osmotic Properties of Aqueous Solutions Containing Caffeine

By FRANK M. GOYAN and HANNA N. BORAZAN

An improved form of the thermoelectric osmometer developed by one of the authors has been used to study solutions of *p*-aminobenzoic acid, citric acid, sodium citrate, sodium salicylate, sodium benzoate, morphine hydrochloride, and papaverine hydrochloride alone and in combination with caffeine. The measured colligative property is plotted against concentration of caffeine. These data are described by two intersecting straight lines for each substance. The point of intersection occurs at different positions on the caffeine scale depending upon the nature and concentration of the complexing agent. The shift along the caffeine scale from the break in the pure caffeine data is divided by the concentration of the complexing agent to give a characteristic complexing capacity number. Osmotic coefficients may be calculated from tabulated slopes.

THE IDEA that the molecular forces related to the solvent effect of caffeine may be involved in attachments of biological significance was suggested by Weil-Malherbe (1). Higuchi and his colleagues (2–6) have established a relationship between the solubilizing action of caffeine and its tendency to form complexes and aggregates. The combination of these two ideas suggests that the instrumentation developed by Johnson and Goyan (7–9) may be adapted to the study of properties related to drug action.

A preliminary survey of the problem indicated the need for greater sensitivity and temperature stability. These improvements were approached on a developmental basis while measurements were in progress. The final outcome was an approximately 10-fold increase in sensitivity with considerable improvement in stability and reproducibility, compared to the same instrument before modification.

EXPERIMENTAL

The circuit and technique previously described (7–9) were not changed except that the regulated power supply (Leeds and Northrup part No. 099012) was installed in place of the battery and calibrating circuit of the Speedomax recorder. The slide wire of the recorder was connected to this power source through two 400-ohm resistors which served as end coils. A 100-ohm resistor was connected across the regulated power supply. The extemporaneous arrangement of 200-ohm precision resistors increased the sensitivity of the recorder from 10 mv. full scale to approximately 1 mv. full scale. Because of the fact that the recorder is used as a nul instrument and calibrated against the resistance box, this temporary arrangement was used throughout the course of the work. It is planned to install the recommended range card for future work.

Attempts to stabilize the temperature regulation and otherwise to improve the stability and reproducibility of the instrument continued throughout the course of the measurements. Manual adjustment and empirical uniformity of method had to be resorted to in the early phases. The following improvements are significant: (*a*) a 4-L. Dewar flask was substituted for the original 1-L. flask used to maintain water at constant temperature. (*b*) The Dewar flask was provided with an insulated metal cover to which was attached brass holders to keep the cells in a vertical position under the water. (*c*) The part of the cell extending above the cover was protected from draft by means of a plastic cylinder which was split to allow half to be removed temporarily while adding sample. (*d*) A thin glass rod less than 1 mm. in diameter was attached to the bottom of the platinum coil around the thermistor. This had the effect of maintaining nearly equal drops by drawing away excess sample. The rod extended to the lowest level of the platinum gauze. (*e*) Cells not adequately vented seemed to cause trouble. This was corrected in one case by sealing a glass tube just below the platinum gauze and bringing the open end of this tube up to the level of the top of the sample tube. (*f*) Temperature control was improved by the control of the air bath with a modified Melabs proportional temperature controller, using a thermistor sensing element. No attempt was made to heat or cool the water, except for initial manual adjustment. The stirring motor was operated from a constant voltage source. Once the correct differential between air and water was established, temperature drift was too slow to influence the readings when using the reference thermistor in the circuit. It was found necessary to improve the insulation of the air bath and to manipulate the droppers through holes draped with cloth.

Solutions were made on a molal basis using ordinary distilled water. The solid needed to make the most concentrated solution of a series was weighed out and the other concentrations made on a molal basis by dilution with the appropriate vehicle. Because of the concurrent study of the instrument it was necessary to discard preliminary results in many cases. However, all of the data included in this paper were taken after proper adjustments were made. All points fall within the circles shown on the figures. USP or equivalent quality chemicals obtained from reliable sources were used without further purification.

RESULTS

All results obtained were plotted with ΔR as ordinate and molal concentration as abscissa. It was found that intersecting straight lines could be used to express all data given in Table I within the limit of experimental error. These intersecting straight lines are of special interest because it was discovered that the point of intersection shifted as a function of the nature and concentration of the

Received August 16, 1967, from the University of California School of Pharmacy, San Francisco Medical Center, San Francisco, CA 94122

Accepted for publication October 13, 1967.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

This investigation was supported in part by Academic Senate research grant, University of California.

TABLE I—DATA FALLING ON TWO INTERSECTING STRAIGHT LINES

Variable	Vehicle	Intercept, ohms	First Slope dR/dM	Intersection M	Second Slope dR/dM
Caffeine	Citric acid, 0.1 M	108.9	835	0.0250	553
Caffeine	Morphine HCl, 0.03 M	52.3	658	0.0825	320
Caffeine	PABA, 0.005 M	4.2	743	0.0276	528
Caffeine	PABA, 0.010 M	8.2	659	0.0331	498
Caffeine	PABA, 0.015 M	12.7	605	0.0365	445
Caffeine	Papaverine HCl, 0.03 M	39.4	458	0.0832	130
Caffeine	Sodium benzoate, 0.06 M	106.8	554	0.0807	325
Caffeine	Sodium citrate 0.01 M	29.8	880	0.0214	554
Caffeine	Sodium salicylate, 0.02 M	35.3	579	0.0808	330
Caffeine	Water	0.0	880	0.0232	558
Morphine HCl	Water	0.0	1760	0.0267	1481
Papaverine HCl	Water	0.0	1760	0.0120	1034

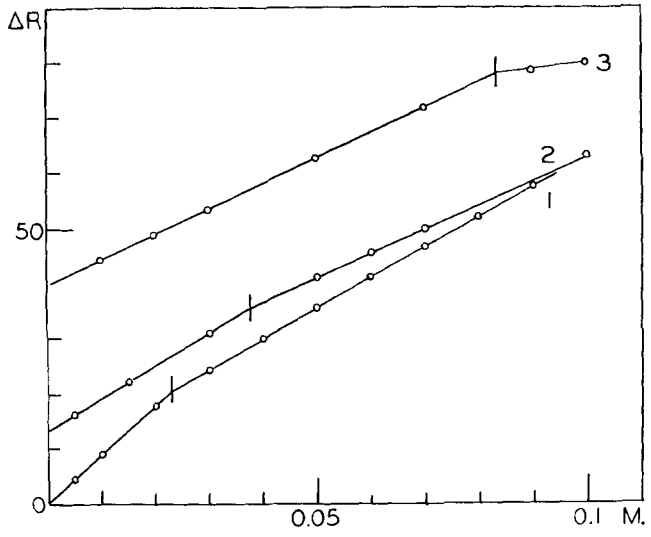

Fig. 1—Plot of measured differences in resistance in ohms plotted against molal concentration of caffeine in the following vehicles: 1, water; 2, 0.015 M *PABA; 3, 0.03* M *papaverine hydrochloride. The intersection of two straight lines is marked by a short vertical line.*

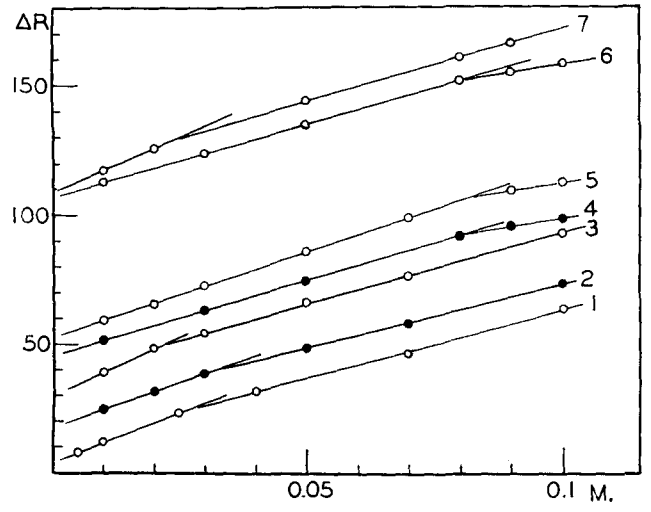

Fig. 2—Similar to Fig. 1 except that points represented by solid disks were raised 10 ohms in order to avoid crowding. Numbers refer to the following vehicles: 1, 0.005 M *PABA; 2, 0.01* M *PABA; 3, 0.01* M *sodium citrate; 4, 0.02* M *sodium salicylate; 5, 0.03* M *morphine hydrochloride; 6, 0.06* M *sodium benzoate; 7, 0.1* M *citric acid.*

compound used to prepare the vehicle. For these reasons, all slopes and intercepts shown graphically were checked by the method of least squares.

The same data are displayed in Figs. 1 and 2 to show the shift in the point of intersection of the two lines for caffeine. This difference between the intersection in water and in the solution under study is divided by the concentration of the vehicle solute to yield the complexing capacity number shown in Table II. It is difficult to estimate the accuracy of these values, but it is almost certain that the last figure shown in the column of calculated values is not significant. The close approach to simple integers suggests that these integers are in

TABLE II—COMPLEXING CAPACITY NUMBERS WITH CAFFEINE

Vehicle	Calculated from Table I	Nearest Integer	Increased Solubility Ref.
Citric acid, 0.1 *M*	0.02	0	(10)
Morphine HCl, 0.03 *M*	1.98	2	...
PABA			
0.005 *M*	0.88	1	(4, 5)
0.010 *M*	0.99		
0.015 *M*	0.89		
Papaverine HCl, 0.03 *M*	2.00	2	...
Sodium benzoate, 0.06 *M*	0.96	1	(2, 10)
Sodium citrate, 0.01 *M*	−0.18	0	...
Sodium salicylate, 0.02 *M*	2.88	3	(3, 10)

necessary to state complexing capacity numbers as integers. It is quite possible that the decimal fractions given in the first column of Table II have some significance. The important conclusion is that these numbers do exist and can be measured.

It is a well-known fact that a true colligative property of sucrose plotted against concentration does not fall exactly on a straight line (11). The treatment of the present data was indicated by interest in the complexing numbers and by the fact that the authors are not yet in a position to state absolute values based upon empirical corrections. However, all of the slopes given may be used to calculate apparent osmotic coefficients for pure substances at a concentration of 0.1 *M* by the equation:

$$\phi = \frac{S_1 C_1 + S_2(0.1 - C_1)}{88} \qquad \text{(Eq. 1)}$$

where ϕ is the osmotic coefficient, S_1 is the first slope, C_1 is the concentration at the intersection, and S_2 is the second slope of Table I. Similar equations may be derived for other concentrations. Comparison of the present work with the work of others shows some discrepancies not easily explained. Blake and Harris (12), working near the freezing point of water, have assumed that caffeine behaves as an ideal solute. Several papers combined (13–15) indicate that the osmotic coefficient of caffeine is about 0.65 as compared to a value of 0.72 calculated by Eq. 1.

TABLE III—DATA FALLING ON SINGLE STRAIGHT LINES

Variable	Vehicle	Intercept, ohms	Slope dR/dM	Last Point *M*	Number of Points
Benzoic acid	Water	0.0	880	0.025	5
Citric acid	Water	2.3	1000	0.20	6
Citric acid	Caffeine 0.01 *M*	8.8	1012	0.10	5
Citric acid	Caffeine 0.1 *M*	61.5	1075	0.10	4
PABA	Water	0.0	873	0.03	5
Salicylic acid	Water	0.0	880	0.015	3
Sodium benzoate	Water	0.0	1760	0.05	3
Sodium citrate	Water	4.2	2621	0.10	4
Sodium citrate	Caffeine 0.02 *M*	21.6	2629	0.10	4
Sodium salicylate	Water	0.0	1760	0.10	4
Sucrose	Water	0.1	882	0.20	6

some way related to the stoichiometry of complex formation.

A few other systems were studied in the hope that they might throw some light on the meaning of these complexing capacity numbers. These data are shown in Table III. The straight lines represented by slope and intercept pass through all of the measured points within the limit of experimental error. The intercepts are not experimental points. The experimental value for zero concentration is always zero because of the practice of using a drop of pure water to establish a base resistance reading before substituting drops of solution. In every case a 0.01 *M* or lower concentration was included in the series.

DISCUSSION OF RESULTS

The discovery of caffeine complexing numbers reported in this paper present new information about a few compounds. Emery and Wright (10) report that citric acid is a more effective solubilizing agent for caffeine than is sulfuric acid. The value of zero for the caffeine complexing number for citric acid and for sodium citrate shown in Table II indicates that the interaction with citric acid implied by the experiments of Emery and Wright is different from interaction with compounds of Table II known to have specific biological activity. It is not

The present work adds a new dimension to the study of caffeine complexes and is in agreement with the work of Donbrow and Jan (16) concerning the absence of aggregates in solutions of caffeine more dilute than 0.02 *M*. However, something like a critical micelle concentration may be found at 0.023 *M*. The complexing capacity number of unity for sodium benzoate may be regarded as being in agreement with Donbrow and Jan. It is interesting to speculate that the higher complexing capacity numbers found for morphine, papaverine, and sodium salicylate are in some way related to biological activity.

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Osmotic coefficient—calculated, equation
Thermoelectric osmometer—improved

Triterpenic Constituents of *Lepechinia chamaedryoides*

By MARIO SILVA

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From plant material collected in November (spring), ursolic acid (4) was obtained which gave a methyl ester with a rather peculiar melting point (105°). Therefore, it was characterized through several derivatives. Betulinic acid could be detected by thin-layer chromatography only as its methyl ester.

These different results appear to be a seasonal variation of the triterpenic constituents.

EXPERIMENTAL[1]

Isolation of Betulinic Acid—Stems and leaves of *Lepechinia chamaedryoides* collected in March near Concepción, were dried at 80° with air circulation, and 2100 Gm. of the dried and ground plant was extracted in a Soxhlet apparatus with petroleum ether (b.p. 65–75°) to exhaustion. This solution was concentrated to yield 109 Gm. of a dark green product. This product was crystallized several times from ethanol to yield 11 Gm. of a crude acid m.p. 286°.

Betulinic Acid Methyl Ester—This ester was prepared by diazomethane treatment in ether solution; crystallized from ethanol as colorless crystals with m.p. 221°, $[\alpha]_D$ +4.4° (chloroform, c 0.44), $\nu_{max.}^{Nujol}$ 3610, 1695, and 1637 cm.$^{-1}$, $\nu_{max.}^{CHCl_3}$ 1724, 1647 cm.$^{-1}$.

Anal.—Calcd. for $C_{31}H_{50}O_3$: C, 79.10; H, 10.71. Found: C, 79.34; H, 10.69.

Betulin Diacetate—The betulinic acid methyl ester on $LiAlH_4$ reduction using ether as solvent gave an noncarbonilic compound which on pyridine–acetic anhydride acetylation at room temperature gave a diacetate (5), m.p. 222°, $[\alpha]_D$ + 28.7° (chloroform, c 0.48), $\nu_{max.}^{Nujol}$ 1732, 1640, and 1250 cm.$^{-1}$, τ = 7.9 (two acetates).

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Isolation of Ursolic Acid—Stems and leaves of *Lepechinia chamaedryoides*, collected in November (spring) near Concepción, were dried at 80° with air circulation, and 4611 Gm. of the dried and ground plant was extracted in a Soxhlet apparatus with benzene to exhaustion. This solution was concentrated to yield 253 Gm. of a dark green product, and 150 Gm. of this product was chromatographed over alumina grade III to give crude ursolic acid m.p. 250°, $[\alpha]_D$ + 52.8° (pyridine, c 0.35).

Ursolic Acid Acetate—Treatment of the acid with pyridine–acetic anhydride gave an acetate, m.p. 248°,

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Keyphrases

Triterpenic constituents—*Lepechinia chamaedryoides*
Betulinic acid—summer plant growth
Ursolic acid—spring plant growth
TLC—identity
Optical rotation—identity
NMR spectrometry—identity
IR spectrophotometry—structure
UV spectrophotometry—structure

Coumarins VII. The Coumarins of *Lomatium nuttallii*

By KUO-HSIUNG LEE and T. O. SOINE

The ether extract of the root of *Lomatium nuttallii* (A. Gray) Macbr. has been examined and has provided a number of known coumarins. These are osthol, jatamansin, pteryxin, and lomatin. The ether extract also contained a new coumarin, provisionally named *nuttallin*, which has been identified as the senecioate ester of lomatin. The methanol extract yielded the glycosidic coumarin, columbianin.

INTEREST in naturally occurring agents with potential physiological activity has led the authors, in the past, to consider the *Lomatium* genus. At least one representative of this genus has been employed extensively by the American Indians as a therapeutic agent (1) without, however, any firm rationale for such usage. Our previous examinations have encompassed *L. columbianum* Math. and Const. (2). *L. suksdorfii* (Wats.) Coulter and Rose (3), and *L. dissectum* var. *multifidum* (Nutt.) Math. and Const. (4) and it has been determined that coumarins are the principal chemical type present. That coumarins can be responsible for a variety of physiological activities is well known (5). A continuing investigation of this genus has led the authors to examine the roots of *L. nuttallii* (A. Gray) Macbr. and has resulted in the isolation of a number of known coumarins. Specifically, osthol (I), lomatin angelate (II) [*i.e.*, jatamansin (6) or selinidin (7)] pteryxin (III), lomatin (IV),[1] and columbianin (V) (2, 8) have been isolated and identified. The presence of a new coumarin, lomatin senecioate (VI), provisionally named *nuttallin*, has been demonstrated. Naturally occurring nuttallin has proved to be elusive as far as isolation is concerned but its synthesis has been easily achieved following its identification in the NMR spectrum of the jatamansin residues. The fact that the spectrum of the mixture corresponded exactly for jatamansin (*i.e.*, lomatin angelate) was expected and it was not surprising that the peaks of any contaminant as closely related as a senecioate to an angelate of the same alcohol would coincide except for the specific protonic differences in the acid portion of the ester. These differences were observed as the singlets at τ 8.13 (3H), 7.84 (3H), and 4.34 (1H) which are the typical signals for the senecioyl group (9). The synthetic senecioate of the optically active natural lomatin had the NMR spectrum predicted from the previous observations on the crude mixture, *i.e.*, it peaks at τ 8.64 (6H, s.), 8.12 (3H, s.), 7.85 (3H, s.), 4.37 (1H, s.), 6.90 (2H, t.), 4.87 (1H, t.), 3.83 (1H, d.), 3.25 (1H, d.), 2.77 (1H, d.), and 2.42 (1H, d.). With the exception of the peaks noted above the spectrum is virtually identical with that of jatamansin. Curiously, nuttallin does not crystallize, a circumstance that is analogous to a similar situation with regard to the angelate (VII) and senecioate (VIII) esters of (−)-3′-hydroxy-3′,4′-dihydroxanthyletin. In this case, however, Lemmich *et al.* (10), in spite of extensive purification, were unable to secure VII in a crystalline form whereas Hata and Sano (9) had little difficulty in obtaining crystalline VIII (*i.e.*, decursin). The reasons for such anomalous behavior are not apparent.

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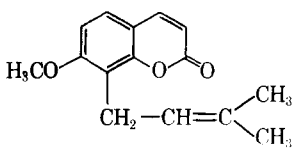

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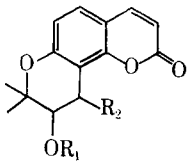
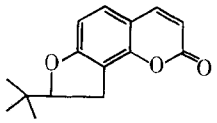
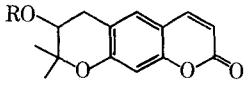


II, R_1 = angeloyl; R_2 = H
III, R_1 = acetyl; R_2 = angeloyloxy
IV, R_1 = H; R_2 = H
VI, R_1 = senecioyl; R_2 = H



O-β-D-gentiobiosyl



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checked for accuracy against a set of standard samples and are uncorrected.

Ultraviolet spectra were determined on a Bausch and Lomb Spectronic 505 recording spectrophotometer.

Infrared spectra were determined on a Perkin-Elmer 237B grating infrared spectrophotometer.

Values of [α] were determined on a Perkin-Elmer 141 polarimeter.

Nuclear magnetic resonance (NMR) spectra were determined on a Varian Associates A-60 instrument in $CDCl_3$ using tetramethylsilane (TMS) as the internal standard. The authors are indebted to the School of Chemistry, University of Minnesota, for these determinations.

Silica gel for column chromatography refers to Baker Analyst No. 3405 activated at 120° and silica gel for thin-layer chromatography refers to Silica Gel G supplied by Brinkmann Instruments, Inc., Great Neck, N. J. activated at the same temperature.

Material[2]—*Lomatium nuttallii* (A. Gray) Macbr. is a species belonging to the *Umbelliferae* family of plants. It is known by the following pseudonyms: *Seseli nuttallii* (Gray), *Peucedanum graveolens* (Wats.), *P. kingii* (Wats.), *Cynamarathrum nuttallii* (Gray), and *Cogswellia nuttallii* (Gray) Jones. The present investigation was confined entirely to the roots of the plant which had been collected and identified in the summer of 1956 at an elevation of 8800 ft. in St. Charles Canyon, Bear Lake County, Idaho. The roots, which had been stored in a deep-freeze refrigerator, were reduced to a powder suitable for extraction in a Jacobsen laboratory grinder designed to keep frictional heat at a minimum.

Ether Extract—The dried and ground root (500 Gm.) was extracted with diethyl ether for several hours until only a faint fluorescence was evident in the menstruum when spotted on filter paper and examined under ultraviolet light. The marc was dried and reserved for methanol extraction (*q.v.*). Upon removal of the ethereal solvent a viscous oil remained. This oil was dissolved in 90% methanol, defatted with petroleum ether, and evaporated to give a residue weighing 30 Gm. A portion (15 Gm.) of this residue was chromatographed on silica gel (450 Gm.) impregnated with 10% of water. The column was successively eluted with benzene, benzene–chloroform, chloroform, chloroform–ethyl acetate, ethyl acetate, and ethyl acetate–methanol. Nine hundred and forty eight 20-ml. fractions were collected and the composition of the fractions determined by examining thin-layer chromatograms under ultraviolet light. The first benzene eluate (fractions 1–25) yielded only nonfluorescent, yellow-colored oily substances and was not investigated further. The subsequent fractions (26–184) contained a single spot having a purple-blue fluorescence under ultraviolet light and fractions 185–239 (0.6 Gm.) appeared to be a mixture of fluorescent compounds.

The benzene–chloroform mixture eluted the following fractions all of which contained a mixture of fluorescent compounds, *i.e.*, fractions 240–486, 487–498, 499–512, 513–524, 525–537, and 538–572.

Chloroform was used to elute fractions 573–763 which also appeared to be a mixture of fluorescent compounds.

The chloroform–ethyl acetate mixture eluted only traces of fluorescent substances in fractions 764–779 and 780–787. The same was true of the ethyl acetate and ethyl acetate–methanol fractions (788–948) and these fractions were not investigated further.

Isolation of Coumarins from the Ether Extract—The fractions containing fluorescent materials were worked up to yield the following:

(*a*) *Osthol*—The residue (4.1 Gm.) resulting from the removal of solvent from fractions 28–184 was recrystallized from a small amount of ether to provide fine, colorless needles, m.p. 81–82°; infrared spectrum (KBr disk): 3000, 2950, 2820 (triplet of aromatic OCH_3), 1721 (α-pyrone C=O), 1606, 1498, 1460 (aromatic C=C), 1563 (aromatic ring with conjugated C=C), 1272, 1245, 1120, 1085 (asymmetric and symmetric stretching of =C—O—C), and 820 cm.$^{-1}$ (1,2,3,4-aromatic substitution). The melting point of this compound was undepressed on admixture with an authentic sample of osthol and their infrared spectra were identical.

(*b*) *Jatamansin*—a blue-fluorescent oily substance (4.8 Gm.) was obtained from fractions 240–486. Thin-layer chromatography on silica gel using different solvent systems gave only one spot with the

[2] Collected and initially identified by Dr. T. G. Call, California State Polytechnic College, San Luis Obispo, California, with confirmation of the identification by Dr. Lincoln Constance, Botany Department, University of California, Berkeley. A voucher specimen has been placed in the herbarium of the Botany Department, University of Minnesota, Minneapolis.

same R_f value as that of jatamansin.[3] The oily substance (3.0 Gm.) was dissolved in a small amount of benzene and, after adding a few drops of cyclohexane, was kept in the refrigerator for 1 week. The white, crystalline semisolid that separated was removed by filtration and washed with cyclohexane to yield white crystals (83 mg.), m.p. 85–93°. Recrystallization from ether–cyclohexane provided colorless prisms (61 mg.), m.p. 90–92°; $[\alpha]_D^{25}$ −17.8° (c 1.0, $CHCl_3$); infrared spectrum (Nujol mull): 1724 (α-pyrone C=O), 1610, 1490 (aromatic C=C), 1245, 1147, 1119 (asymmetric and symmetric stretching of =C—O—C), and 847 cm.$^{-1}$ (1,2,3,4-aromatic substitution). It failed to depress the melting point of an authentic sample of jatamansin on admixture and the infrared spectra were identical.

After the isolation of jatamansin, the mother liquor was rechromatographed on silica gel impregnated with 3% of water using benzene, benzene–chloroform, and chloroform–ethyl acetate as eluants, successively. No further separations were realized and, similarly, thin-layer chromatography on silica gel failed to show any spots other than the one ascribable to jatamansin. The oily residue had an infrared spectrum quite similar to that of jatamansin but differed in minor details. The NMR spectrum agreed with that of jatamansin (6) but, in addition, had superimposed on it singlets at τ 8.13 (3H), 7.84 (3H), and 4.34 (1H). Further treatment of this fraction is deferred to a later portion of this paper (*q.v.*).

(*c*) *Pteryxin*—The orange-colored oily substance (1.66 Gm.) obtained from fractions 499–512 was rechromatographed on silica gel (50 Gm.) impregnated with 5% of water. Elution with chloroform furnished a colorless oily residue which was seeded with one small crystal of pteryxin[4] and the whole then kept in a refrigerator for 5 days. The colorless, large tetragonal crystals which formed were collected and twice recrystallized from skellysolve-B to give 21 mg. of white crystals, m.p. 81–83° undepressed by admixture with pteryxin; $[\alpha]_D^{21}$ −1.8° (c 0.69, $CHCl_3$): infrared spectrum (KBr disk): 1745 (α-pyrone C=O), 1615, 1495 (aromatic C=C), 1235, 1145 (asymmetric and symmetric stretching of =C—O—C), and 837 cm.$^{-1}$ (1,2,3,4-aromatic substitution). The infrared spectrum of this compound was identical to that of pteryxin.

(*d*) *Lomatin*—The reddish-orange oil obtained from the chloroform fractions (573–763) weighed 0.89 Gm. and was rechromatographed on silica gel (40 Gm.) impregnated with 5% of water. Elution with chloroform yielded a fraction having a single spot with the same R_f value as lomatin on examination by silica gel thin-layer chromatography. This fraction was dissolved in acetone and brought to turbidity with skellysolve-B at which time it was seeded with one small crystal of lomatin and kept in the refrigerator for 3 days. The resulting crystalline deposit was filtered and washed with benzene. Recrystallization from skellysolve-B–acetone mixture yielded colorless crystals (11.4 mg.), m.p. 179–181°; $[\alpha]_D^{28.5}$ +46.4 (c 0.44, C_2H_5OH). A mixed melting point with authentic lomatin showed no depression and the infrared spectra were identical.

Methanol Extract—The marc from the ether extraction was dried in air and extracted with methanol until a portion of the extract failed to give an appreciable residue when evaporated to dryness (7 days). This extract was allowed to concentrate spontaneously and deposited crystalline material as the volume decreased. The crystalline material was removed by filtration, rinsed with methanol, and dried, m.p. 270–275°. The filtrate and washings from these crystals was allowed to dry completely resulting in a thick reddish-brown syrupy residue. Paper chromatographic examination of this residue showed only one blue-fluorescent spot corresponding to that for the crystalline material. It was not examined further.

The crystalline material was recrystallized from hot methanol, m.p. 274–275° and showed no depression of melting point on admixture with authentic columbianin.[5] Similarly, an acetate prepared in the conventional manner, m.p. 220–222° failed to depress the melting point of authentic columbianin acetate.[5]

Hydrolysis of the Jatamansin Mother Liquors—The mother liquor, after removal of the crystalline jatamansin, was evaporated to yield a viscous, oily residue. This residue (360 mg.) was dissolved in 3 ml. of dioxane and 11 ml. of 1 *N* potassium hydroxide and left overnight at room temperature. Examination of the mixture by thin-layer chromatography showed that the faster moving fluorescent ester spot had disappeared almost completely in favor of a much slower moving fluorescent alcohol spot. The reaction mixture was diluted with an equal amount of water and acidified with 10% sulfuric acid. After standing for 45 min. it was adjusted to pH 8 with 10% sodium carbonate solution and finally extracted with chloroform. The chloroform extract, after drying and evaporation, was dissolved in benzene and chromatographed on silica gel (10 Gm.) impregnated with 5% of water. Initial elution with a mixture of chloroform–benzene (1:9) yielded a colorless, viscous oily substance (25 mg.) which had an infrared spectrum virtually identical with the starting material. Subsequent elution with chloroform afforded a crystalline material which upon recrystallization from acetone–skellysolve-B formed colorless needles (140 mg.), m.p. 180–181°; infrared spectrum (KBr disk): 3470 (free OH), 1700 (C=O), 1600, and 1484 (aromatic C=C), 1281 and 1075 cm.$^{-1}$ (OH deformation and C—O stretching). This compound showed no depression in melting point on admixture with authentic lomatin and the infrared spectra were identical. No other fluorescent materials were eluted by the use of ethyl acetate.

The aqueous phase (pH 8) was acidified with 10% sulfuric acid and extracted with ether. After drying and evaporation, the oily residue (*ca.* 110 mg.) was treated with dicyclohexylethylamine (250 mg.) and converted to the *p*-phenylphenacyl ester form according to the method of Stodola (11). The resulting white solid product (130 mg.) was then chromatographed on silica gel (10 Gm.) impregnated with 10% of water. The eluant was benzene–skellysolve-B (2:1) to which increasing amounts

[3] Kindly supplied by Dr. S. C. Bhattacharyya, National Chemical Laboratory, Poona, India.

[4] Obtained from the studies reported in *Reference 3*.

[5] Obtained in these laboratories from the studies reported in *Reference 2*.

of benzene were added. Unreacted *p*-phenylphenacyl bromide, a mixture of *p*-phenylphenacyl angelate and senecioate and *p*-phenylphenacyl senecioate (31 mg.), m.p. 136°, were obtained successively. The identity of the senecioate ester was established by thin-layer chromatography, infrared spectroscopy, and mixed melting point determination with an authentic sample of *p*-phenylphenacyl senecioate.

Synthesis of Lomatin Senecioate—Lomatin (200 mg.) was suspended in dry benzene (5 ml.). The suspension was added slowly to a solution of senecioyl chloride (b.p. 67°/39 mm. Hg) (230 mg.) in dry benzene (3 ml.). The mixture, protected by a calcium chloride drying tube, was refluxed on a steam bath for 16 hr. The reaction mixture was then cooled and washed with 5% sodium bicarbonate solution followed by distilled water. The benzene solution was dried over anhydrous magnesium sulfate, filtered, and the solvent removed under reduced pressure to yield a colorless, glassy substance (230 mg.). All attempts to crystallize the ester were unsuccessful. Thin-layer chromatography, however, showed only one spot with the same R_f value as noted for jatamansin and fractions 240–486. This compound was then further purified by silica gel chromatography and dried in a high vacuum to yield an amorphous, very hygroscopic substance with m.p. 58 ~ 62° $[\alpha]_D^{20}$ +75.0 (c 1.0, $CHCl_3$); ultraviolet spectrum: $\lambda_{max.}$ 326, 256, 246, and 219 mμ (log $\epsilon_{max.}$ 4.16, 3.73, 3.83, and 4.53, respectively); infrared spectrum (in $CHCl_3$) was almost identical with fractions 240–486, *i.e.*: 1724 (α-pyrone C=O), 1610, 1490 (aromatic C=C), 1140, 1115 (asymmetric and symmetric stretching of =C—O—C), and 834 cm.$^{-1}$ (1,2,3,4-aromatic substitution).

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Keyphrases

Coumarins—*Lomatium nuttallii*
Lomatium nuttallii—coumarins, isolated, identified
Nuttallin—new coumarin, isolated
Column chromatography—separation
TLC—identity
UV spectrophotometry—structure
IR spectrophotometry—structure
Optical rotation—identity
NMR spectrometry—identity

Gas-Liquid Chromatographic Determination of Dienestrol in the Presence of Methyltestosterone

By FRED L. FRICKE, STEPHEN M. WALTERS, and WILLIAM T. LAMPKIN

A gas chromatographic procedure has been developed for the determination of dienestrol in the presence of methyltestosterone. Dienestrol was extracted from tablets and quantitatively determined by gas chromatography of its bis-(trimethylsilyl)ether. The bis-(trimethylsilyl)ether of alizarin was used as an internal standard. Preliminary experiments indicate that the method is applicable in the presence of other androgens and corticosteroids.

THIS STUDY describes a rapid, specific, gas-liquid chromatographic procedure for the determination of dienestrol in the form of its bis(trimethylsilyl)ether derivative, with bis-(trimethylsilyl)acetamide as the silylating agent.

A number of analytical procedures have been reported for the determination of dienestrol. The USP XVI utilizes the Folin-Denis reagent in a colorimetric procedure. Cocking (1) also developed a colorimetric method based on a reaction of dienestrol with bromine in acetic acid, whereas Malpress (2) formed a polynitro derivative and measured the orange color produced. A method for the determination of dienestrol in biological samples, utilizing the formation and subsequent titration of the maleic acid adduct, was proposed by Smith (3). Gottlieb (4) formed nitrosophenols from various synthetic estrogens including dienestrol and measured the colors produced, while Gry (5) determined the nitroso derivative of dienestrol polarographically. Summa and Graham (6) determined dienestrol both by a polarograpic method, which is the basis of the NF XII assay, and by measurement of a yellow color [first observed by Banes (7)] that is formed with ultraviolet irradiation of dienestrol. In applications of gas chromatography as the determina-

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THIS STUDY describes a rapid, specific, gas-liquid chromatographic procedure for the determination of dienestrol in the form of its bis(trimethylsilyl)ether derivative, with bis-(trimethylsilyl)acetamide as the silylating agent.

A number of analytical procedures have been reported for the determination of dienestrol. The USP XVI utilizes the Folin-Denis reagent in a colorimetric procedure. Cocking (1) also developed a colorimetric method based on a reaction of dienestrol with bromine in acetic acid, whereas Malpress (2) formed a polynitro derivative and measured the orange color produced. A method for the determination of dienestrol in biological samples, utilizing the formation and subsequent titration of the maleic acid adduct, was proposed by Smith (3). Gottlieb (4) formed nitrosophenols from various synthetic estrogens including dienestrol and measured the colors produced, while Gry (5) determined the nitroso derivative of dienestrol polarographically. Summa and Graham (6) determined dienestrol both by a polarograpic method, which is the basis of the NF XII assay, and by measurement of a yellow color [first observed by Banes (7)] that is formed with ultraviolet irradiation of dienestrol. In applications of gas chromatography as the determina-

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tive step, McGregor *et al.* (8) formed the dimethyl ether, and Luukainen *et al.* (9) prepared the trimethylsilyl ethers of steroids.

Advantages of the trimethylsilyl ethers are their easy and quantitative formation and the decrease in adsorption effects on the column. The trimethylsilyl ether derivatives have normally been prepared by the reaction of dienestrol with hexamethyldisilazane and trimethylchlorosilane in pyridine. However, complete formation of the derivative with these reagents often requires prolonged heating and removal of ammonia. Another disadvantage is that amine hydrochlorides are produced. Klebe *et al.* (10) have found that bis-(trimethylsilyl)acetamide as prepared by Birkofer *et al.* (11) is a superior silylating agent, in many respects, to the previously used reagents.

EXPERIMENTAL

Apparatus—Gas chromatograph, Packard series 7800 with flame-ionization detector and Minneapolis Honeywell recorder.

GLC Column—Glass coiled (1.8 m. × 4 mm.) packed with 5% SE-30 on Gas Chrom Q, 80/100 mesh. The column was conditioned for 8 hr. with a nitrogen flow of 60 ml./min.

Operating Conditions—Column temperature 230°; detector temperature 255°; injection port temperature 255°; hydrogen flow 90 ml./min.; air flow 750 ml./min.; nitrogen flow 100 ml./min.; sensitivity, 1×10^{-9} amp.

Reagents—Bis-(*trimethylsilyl*)*acetamide*[1]—This reagent must be stored under nitrogen to prevent destruction by atmospheric moisture. A dry box is convenient for storage.

Internal Standard Solution—Place 150 mg. of alizarin in a 50-ml. volumetric flask. Add approximately 40 ml. of ethyl acetate and shake until most of the alizarin is dissolved. Transfer to a dry box and add 2 ml. of bis-(trimethylsilyl)acetamide. Remove and heat at 60° for 5 min., cool, dilute to volume with ethyl acetate, and mix well.

Standard Dienestrol Solution—Place 4.0 mg. of NF dienestrol reference standard in a 10-ml. volumetric flask and dissolve in a small portion of ethyl acetate. Add 2.0 ml. of internal standard solution. Transfer to a dry box and add 10 drops of bis-(trimethylsilyl)acetamide. Remove the solution from the dry box and dilute to volume with ethyl acetate.

Extraction Procedure—Weigh not less than 20 tablets and grind to a fine powder. Weigh accurately a portion of the powder sufficient to give about 2 mg. of dienestrol for tablets containing 0.25 mg./tablet (or about 4 mg. for tablets containing more than 0.25 mg. of dienestrol/tablet) into a 250-ml. conical flask. Add 50 ml. of acetone, place on a hot plate with a magnetic stirrer, and boil gently with stirring for 10 min.; cool and let settle. Decant through a funnel fitted with a glass wool plug into a 250-ml. beaker. Repeat the extraction two more times with 50-ml. portions of acetone, combining the extracts in the 250-ml. beaker. Evaporate the extract on a steam bath with the aid of a current of dry air to approximately 10 ml. Transfer to a 50-ml. beaker with several small portions of acetone, using a stirring rod to break up any precipitate. Evaporate this extract just to dryness. Transfer the residue with several small portions of ethyl acetate to a 5-ml. volumetric flask if it contains 2 mg. of dienestrol (or to a 10-ml. volumetric flask if it contains 4 mg.). Then add 1.0 ml. of internal standard solution to the 5-ml. volumetric flask (or 2.0 ml. if a 10-ml. volumetric flask is used).

Etherification Step—Place the volumetric flask inside a nitrogen dry box and add an excess (about 0.5–1.0 ml.) of bis-(trimethylsilyl)acetamide. Mix

[1] Available from Aldrich Chemical Co., Milwaukee, Wis.

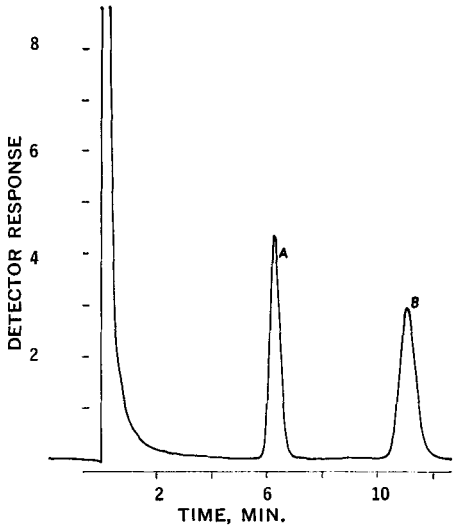

Fig. 1—A typical chromatogram. Key: A, bis-(trimethylsilyl)ether of dienestrol; B, bis-(trimethylsilyl)-ether of alizarin.

TABLE I—PRECISION DATA FOR THE CALIBRATION CURVE

Concn. of Dienestrol, mg./ml.	R			Mean	σ
0.1016	0.246	0.241	0.248	0.246	± 0.007
	0.244	0.250			
0.2032	0.497	0.495	0.491	0.494	± 0.003
	0.490	0.498			
0.4068	0.990	0.985	0.986	0.998	± 0.005
	0.987	0.990			
0.5080	1.249	1.251	1.245	1.247	± 0.008
	1.241	1.250			

TABLE II—RECOVERY STUDIES OF DIENESTROL ADDED TO A SIMULATED TABLET MIXTURE

Added, mg.	Recovered, mg.	% Recovery
3.95	4.01	102
4.08	4.10	100
3.97	3.97	100
4.59	4.55	99.1
4.13	4.06	98.3
4.06	4.20	103
4.10	4.13	101
3.92	3.92	100
2.21	2.19	99.1
2.29	2.24	97.8

Av. = 100% Standard deviation = 1.6

and remove from the dry box. (*Note*: If the volume is above the calibration mark, the excess may be removed with the aid of heat and a current of nitrogen.) Inject 6–7 μl. of this solution into the gas chromatograph. Make duplicate injections of both sample and standard.

Calculations—The peaks due to the bis-(trimethylsilyl)ethers of dienestrol and alizarin have retention times of approximately 6.6 min. and 11.1 min. (See Fig. 1.) Peak area is determined by multiplying the peak height times the width at half height.

The ratio of the peak areas is given by:

$$R = \frac{\text{area of bis-(trimethylsilyl)ether of dienestrol}}{\text{area of bis-(trimethylsilyl)ether of alizarin}}$$

mg. of dienestrol/tablet =

$$\frac{\text{R for sample} \times \text{standard concn.} \times \text{diln. factor} \times \text{av. wt. tablet}}{\text{R for standard} \times \text{wt. sample}}$$

RESULTS AND DISCUSSION

Table I shows the average peak area ratios (R) of the bis-(trimethylsilyl)ether of dienestrol to the bis-(trimethylsilyl)ether of alizarin of five injections of four standard solutions. These values indicate that dienestrol is linear over the concentration range used.

Simulated tablet mixtures containing all excipients including methyltestosterone and known quantities of dienestrol were taken through the entire procedure. These mixtures were chromatographed in duplicate. The results are given in Table II. These data indicate that the standard deviation for the method is 1.6. Table III shows results of the analyses of marketed products containing methyltestosterone and dienestrol.

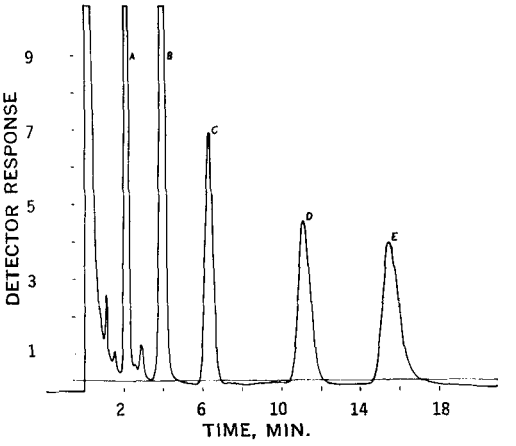

Fig. 2—A typical chromatogram of a sample. Key: A, (trimethylsilyl) ester of palmilic acid; B, (trimethylsilyl) ester of stearic acid; C, bis-(trimethylsilyl)ether of dienestrol; D, bis-(trimethylsilyl)ether of dienestrol; D, bis-(trimethylsilyl)ether of alizarin; E, methyltestosterone.

TABLE III—ANALYSIS OF DIENESTROL IN MARKETED PRODUCTS

Dienestrol found, % of Label Claim	
Preparation A: 0.5 mg./Tablet	Preparation B: 0.25 mg./Tablet
96.4	101
95.6	98.4
96.2	101
94.2	99.6
Av. 95.6	Av. 100
Std. Dev. 1.0	Std. Dev. 1.3

TABLE IV—PRELIMINARY STUDY OF SIMULATED TABLET MIXTURES CONTAINING ANDROGENS AND CORTICOSTEROIDS

Compound	Dienestrol Added, Mg.	% Recovery (Av. of 4)	Retention Time, min.
Corticosteroid			
Betamethasone acetate	3.84	98.5	133
Prednisone	3.84	99.4	13,31
Silylated prednisone	...	...	70
Prednisolone	3.84	100.0	20,44
Silylated prednisolone	...	...	107
Hydrocortisone	3.84	99.3	18,40
Silylated hydrocortisone	...	...	97
Desoxycortisone acetate	3.84	97.8	48
Androgen			
Testosterone	4.05	98.5	11.5
Silylated testosterone	...	...	14
Dromostanolone propionate	4.05	98.6	19

A typical chromatogram of a silylated mixture of stearates, dienestrol, alizarin, and methyltestosterone is shown in Fig. 2.

Preliminary experiments using simulated tablet mixtures indicate that the method is applicable in the presence of other androgens, corticosteroids, and stearates, which cause interference in many of the existing methods. The recovery data for dienestrol and the retention times of the various compounds are shown in Table IV.

The bis-(trimethylsilyl)ether of diethylstilbestrol has the same retention time as the bis-(trimethylsilyl)ether of dienestrol, but partially degraded upon injection in the chromatograph. Barbiturates were found to interfere in the silylation reaction.

In view of the results for both simulated mixtures and commercial samples, the proposed procedure appears to be suitable for determination of dienestrol in the presence of methyltestosterone.

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Keyphrases

Dienestrol analysis
Bis-(trimethylsilyl) ether, dienestrol—derivative
GLC—analysis
Bis-(trimethylsilyl) ether, alizarin—internal standard
Analysis—dienestrol–methyltestosterone combination

New Developments in Antibiotic Interference Thresholds of Microbial Assays

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Interference thresholds have been determined for a number of antibiotics in the microbial assays of specific antibiotics. Two aspects were considered in order to facilitate the quantitative analysis of mixtures of antibiotics: the "interference threshold," which is the concentration of an antibiotic causing a significant interference in the presence of another antibiotic in a given assay, and the "sensitivity threshold," which is the concentration of an antibiotic causing a response in the assay for a different antibiotic.

In 1957, Arret *et al.* (1) described the problems encountered in assaying pharmaceutical preparations in which two or more antibiotics are combined, and proposed solutions to these problems. If the test organism used for the assay of one antibiotic (*a*) is not affected by a second antibiotic (*b*), the assay for (*a*) is uncomplicated. But if the test organism is affected by (*b*), erroneously high or low values for (*a*) may be obtained. Consequently, methods for eliminating the effect of (*b*) were developed. In general, one of the following five methods was used: (*1*) inactivating by biological or chemical means; (*2*) using a test organism which is sensitive to (*a*) and relatively resistant to (*b*); (*3*) artificially making the organism resistant to (*b*); (*4*) separating antibiotics by differential solubility techniques; or (*5*) compensating for the presence of (*b*) by adding it to every solution of (*a*) used for the standard curve.

The analyst needs definite quantitative knowledge of the interfering effects of various antibiotics in certain widely used assay procedures. He must be aware of such effects if confidence is to be maintained in the specificity of an assay.

Since the earlier publication (1), new antibiotics have been discovered, and combinations of these antibiotics together with the older ones make it necessary to update this publication. As before, all the pertinent data are in tabular form for easy reference.

The data obtained from this work simplify the problems of assaying antibiotic combinations Although combinations of two antibiotics have specifically been considered here, the approach can be used as a general guide to the assay of combinations of three or more antibiotics.

Received September 21, 1967, from the Division of Antibiotics and Insulin Certification, Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Washington, DC 20204
Accepted for publication November 6, 1967.

EXPERIMENTAL

The effects of 15 antibiotics were determined by assaying them according to the official microbiological assay methods found in the *Code of Federal Regulations* (2). The following antibiotics (assay organisms are given in parentheses) were studied: Bacitracin[1] (*Sarcina subflava*), chloramphenicol (*Escherichia coli*), colistin (*Bordetella bronchiseptica*), erythromycin (*Sarcina lutea*), kanamycin (*Staphylococcus aureus*), neomycin (*S. aureus*, *S. epidermidis*) oleandomycin (*S. epidermidis*), paromomycin (*S. epidermidis*), penicillin (*S. aureus*), polymyxin (*B. bronchiseptica*), streptomycin (*Klebsiella pneumoniae*, *Bacillus subtilis*), tetracycline (*S. aureus*), and viomycin (*K. pneumoniae*).

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TABLE I—INTERFERENCE THRESHOLDS: CONCENTRATION OF ANTIBIOTIC *B* INTERFERING IN GIVEN ASSAY METHOD WHEN MIXED WITH THE REFERENCE CONCENTRATION OF ANTIBIOTIC *A*. ANTIBIOTIC *A*: ASSAY ORGANISM USED IN EACH METHOD[a] AND REFERENCE CONCENTRATION OF *A* PRESENT

Second Antibiotic Present (*B*)	Bacitracin 1 unit/ml. *S. subflava*	Chloramphenicol 2.5 mcg./ml. *E. coli*	Colistin 1 mcg./ml. *B. bronchiseptica*	Erythromycin 1 mcg./ml. *S. lutea*	Kanamycin 5 mcg./ml. *S. aureus*	Neomycin 10 mcg./ml. *S. aureus*	Neomycin 1 mcg./ml. *S. epidermidis*	Oleandomycin 5 mcg./ml. *S. epidermidis*	Paromomycin 1 mcg./ml. *S. epidermidis*	Penicillin 1 unit/ml. *S. aureus*	Polymyxin 10 units/ml. *B. bronchiseptica*	Streptomycin 30 mcg./ml. *K. pneumoniae*	Streptomycin 1 mcg./ml. *B. subtilis*	Tetracycline 0.24 mcg./ml. *S. aureus*	Viomycin 100 mcg./ml. *K. pneumoniae*
Bacitracin, units/ml.	...	...	100	...	4	...	...	0.3	70	...	...	...	...	...	75
Chloramphenicol, mcg./ml.	...	...	9	...	3[b]	...	...	5	20	...	...	...	...	...	0.2
Colistin, mcg./ml.	390	0.1	...	>500	450	150	7	290	90	>500	0.07	0.7	51	15	0.3
Cycloserine, mcg./ml.	97	130	0.5	...	90	210	250[b]	>500	>500	90	0.3	140	45[b]	20	100
Erythromycin, mcg./ml.	...	...	40	...	0.2[b]	...	...	0.4	0.8	...	...	...	...	...	0.5
Kanamycin, mcg./ml.	8	3	>500	400	...	2	0.3	3	0.3	>500	0.1[b]	3	0.2	3	1
Neomycin, mcg./ml.	...	...	>500	...	0.5	...	...	4	0.4	...	...	...	...	...	0.6
Novobiocin, mcg./ml.	0.3	6	20	10	4	2	2	0.7	0.7	4	200	0.9	9	0.2	2
Oleandomycin, mcg./ml.	3	3	270	0.4	0.3[b]	3	1	...	2	72	190	4	0.3[b]	0.1	2
Paromomycin, mcg./ml.	250	3	40[b]	140	0.5	0.9	0.2	2	...	>500	0.5[b]	3	0.2	2	0.9
Penicillin, units/ml.	...	...	0.2[b]	...	0.4	...	...	90	85	...	...	...	...	...	95
Polymyxin, units/ml.	...	...	8	...	>500	...	...	>500	>500	...	...	...	...	...	3
Streptomycin, mcg./ml.	...	...	60[b]	...	2	...	...	290	110	...	...	...	...	...	3
Tetracycline, mcg./ml.	...	...	3	...	1	...	...	125	50	...	...	...	...	...	0.07
Viomycin, mcg./ml.	36	5	0.1[b]	>500	20	60	3	25	10	>500	0.5[b]	20	0.8[b]	60	...

[a] All assays are plate methods except chloramphenicol, streptomycin *K. pneumoniae*, tetracycline, and viomycin. Streptomycin and dihydrostreptomycin may be considered as acting identically. To find how bacitracin interferes with the *S. aureus* assay for kanamycin, find "bacitracin" in the left-hand column headed "Second Antibiotic Present" and follow that row horizontally across to the column headed "Kanamycin, 5 mcg./ml., *S. aureus*." The figure 4.0 units/ml. indicates that samples containing bacitracin–kanamycin ratios of less than 4:5 (units:mcg.) could be assayed accurately for kanamycin by this method. [b] Indicates a depression rather than an enhancement of activity.

TABLE II—SENSITIVITY THRESHOLDS: CONCENTRATION OF ANTIBIOTIC ALONE[a] PRODUCING MINIMUM MEASURABLE GROWTH INHIBITION IN GIVEN ASSAY METHOD

Antibiotic Tested	Bacitracin *S. subflava*	Chloramphenicol *E. coli*	Colistin *B. bronchiseptica*	Erythromycin *S. lutea*	Kanamycin *S. aureus*	Neomycin *S. aureus*	Neomycin *S. epidermidis*	Oleandomycin *S. epidermidis*	Paromomycin *S. epidermidis*	Penicillin *S. aureus*	Polymyxin *B. bronchiseptica*	Streptomycin *K. pneumoniae*	Streptomycin *B. subtilis*	Tetracycline *S. aureus*	Viomycin *K. pneumoniae*
Bacitracin, units/ml.	...	...	15	...	7	...	...	20	9	...	...	...	...	...	110
Chloramphenicol, mcg./ml.	...	...	10	...	>500	...	...	6	7	...	...	...	...	...	0.3
Colistin, mcg./ml.	91	0.2	...	90	110	130	2	2	5	>500	0.08	1	7	2	0.5
Cycloserine, mcg./ml.	51	65	0.3	200	100	80	240	220	210	32	0.3	70	400	25	75
Erythromycin mcg./ml.	...	...	80	...	0.3	...	...	0.05	0.3	...	...	...	...	...	1
Kanamycin, mcg./ml.	3	2	120	25	...	2	0.4	0.5	0.3	1	120	5	0.2	2	2
Neomycin, mcg./ml.	...	...	70	...	0.7	...	...	0.09	0.07	...	...	...	...	...	2
Novobiocin, mcg./ml.	0.2	3	20	3	0.6	1	0.3	0.5	0.4	0.2	20	0.8	20	0.2	0.7
Oleandomycin, mcg./ml.	3	4	>500	0.2	0.6	0.5	0.6	...	2	7	150	4	1	0.06	3
Paromomycin, mcg./ml.	6	2	400	8	0.6	0.9	0.06	0.4	...	18	400	6	0.2	0.7	2
Penicillin, units/ml.	...	...	>500	...	0.2	...	...	>500	180	...	...	...	...	...	75
Polymyxin, units/ml.	...	...	1	...	400	...	...	25	35	...	...	...	...	...	6
Streptomycin, mcg./ml.	...	...	>500	...	2	...	...	480	>500	...	...	...	...	...	4
Tetracycline, mcg./ml.	...	...	1	...	0.3	...	...	>500	410	...	...	...	...	...	0.04
Viomycin, mcg./ml.	191	4	>500	170	40	30	2	9	5	200	>500	30	3	30	...

[a] All assays are plate methods, except chloramphenicol, streptomycin-*K. pneumoniae*, tetracycline, and viomycin. Streptomycin and dihydrostreptomycin may be considered as acting identically. To find the amount of bacitracin alone which produces an inhibitory zone on *S. aureus* kanamycin plates, find "bacitracin" in the left-hand column headed "Antibiotic Tested" and follow that row horizontally across to the column headed "Kanamycin, *S. aureus*." The fig. "7" indicates that bacitracin alone (7 units/ml.) produces a minimum measurable inhibitory zone.

bination with the reference concentration of (*a*), gave a relative potency [as compared to the reference concentration of (*a*)] of greater than 110% or less than 90% has been termed the "interference threshold." These limits were selected because the 95% confidence range of an average microbiological assay is ±10%. Any assay within this range of theoretical is considered normal variation and any assay beyond this range is considered significant.

The lowest concentration of (*b*) alone which caused a measurable response in the assay procedure of (*a*) is called the "sensitivity threshold."

The interference thresholds and the sensitivity thresholds for the antibiotics and methods tested are given in Tables I and II, respectively.

DISCUSSION

The information given in Table I makes it possible to predict whether the assay of an antibiotic will be complicated by interference from a second antibiotic included in the formulation, which then must be eliminated.

Table II describes the effects of single antibiotics on various assays. The information given in this table can be applied in numerous ways, such as: (*i*) a guide to determine if interference can be expected from different antibiotics in a given assay procedure; (*ii*) a guide to the specificity of the given assay procedure; (*iii*) a means of qualitative identification of unknowns; and (*iv*) selection of an alternative organism for various antibiotics and combinations of them. In dealing with the latter, caution must be exercised, because in many cases the zones are not as clear and well defined as those usually obtained with the original method.

It was noted in many cases that (*b*) alone produced inhibition in the assay for (*a*) at a much lower concentration than that which caused interference when combined with the reference concentration of (*a*). For example (see Table II), 200 mcg./ml. of viomycin will cause inhibition in the penicillin assay without penicillin, but (Table I) more than 500 mcg./ml. of viomycin is necessary to cause interference in the assay with penicillin present. This is due to the fact that assay conditions may be more favorable to (*a*) so that they mask the effect of (*b*). Factors such as pH and ionic strength of the solvent, composition of the nutrient medium, and incubation temperature are significant. Therefore, the data should not be construed to indicate any synergistic or antagonistic relationships.

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Keyphrases

Antibiotic combinations—microbial analysis

Interference threshold—antibiotics

Sensitivity threshold—antibiotics

Analysis specificity—interference elimination

Conversion of Griseophenone A to (±)-Dehydrogriseofulvin in the Presence of Horseradish Peroxidase and Hydrogen Peroxide

By ALVIN SEGAL and ELMORE H. TAYLOR

The horseradish peroxidase catalyzed conversion of griseophenone A (I) to (±)-dehydrogriseofulvin (V) has been demonstrated. The results support a one-electron oxidative coupling mechanism previously proposed.

Barton and Cohen (1) first suggested that in the biosynthesis of the antibiotic (+)-griseofulvin (VII) (Scheme I), the chlorobenzophenone (I) could conceivably be converted to the spiran, (−)-dehydrogriseofulvin (V), *via* a 1-electron oxidative coupling mechanism proceeding through the formation of the intermediate diradical (III). Chemical synthesis of (±)-V using 1-electron oxidizing agents supports this hypothesis (2–4). The final step was the stereospecific enzymatic reduction of (−)-V to form (+)-VII (5–7).

(+)-Griseofulvin (VII) was first isolated from the mycelium of *Penicillium griseofulvum* (8). It was subsequently shown to be a metabolic product of many species of *Penicillia* (9).

The enzyme peroxidase has been demonstrated to be present in species of *Penicillia* (10), and has been implicated in the biosynthesis of fungal metabolites such as the ergot alkaloids (11). The peroxidase catalyzed O—C oxidative coupling of phenols has been reported (12). In addition, 1-electron transfer mechanisms have been proposed for coupled oxidations involving peroxidase (13). On the basis of this information the authors decided to investigate the possibility of converting I to V *via* peroxidase catalysis.

Received September 18, 1967, from the Department of Pharmacognosy, College of Pharmacy, University of Tennessee, Memphis, TN 38103

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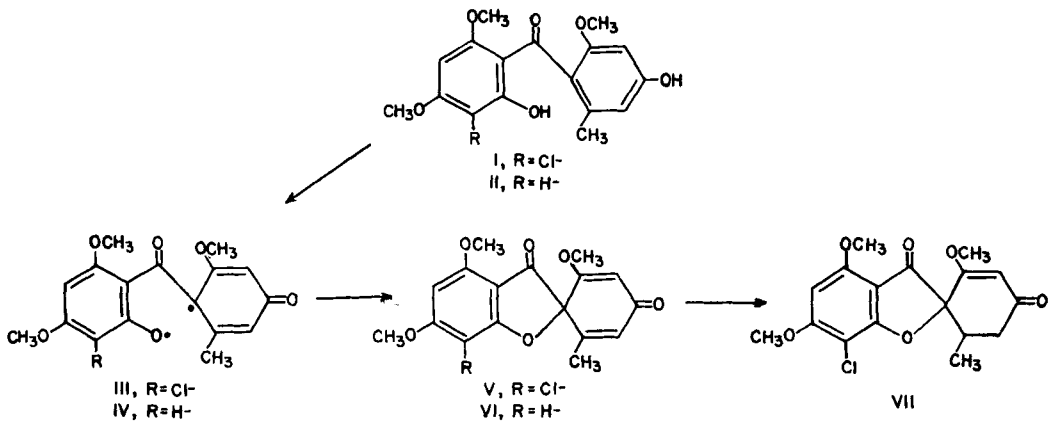


Scheme I

EXPERIMENTAL

Melting points were determined on a Leitz Wetzlar apparatus. Infrared spectra were determined in KBr disks using a Perkin-Elmer model 137 Infracord spectrophotometer. Ultraviolet spectra were determined in 95% ethyl alcohol using a Perkin-Elmer model 202 ultraviolet-visible spectrophotometer. NMR spectra were determined in deuteriochloroform solutions with tetramethylsilane as internal standard using a Varian A-60A spectrometer. Optical rotations were measured in acetone solutions using a Carl Zeiss polarimeter. Analytical and preparative thin-layer chromatography (TLC) were performed on plates covered with Silica Gel HF_{254} (Merck). The staining system used was 5% potassium carbonate–0.2% potassium permanganate. Solvent systems used were (*a*) butyl acetate (technical)–acetone, 4:1, and (*b*) chloroform–methanol, 98:2. Column chromatography was performed using Florisil (100–200 mesh) (Fisher Scientific Co., St. Louis, Mo.). Micro-analysis was performed by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

(−)-Dehydrogriseofulvin (V)—This substance was prepared essentially following the method of Taub (4). A suspension of 20 Gm. of (+)-VII (m.p. 219–220.5°; pure by TLC analysis using solvent systems *a* and *b*) and 40 Gm. of selenium dioxide in 1.2 L. of *tert*-butanol was refluxed for 65 hr. The hot reaction mixture was filtered through diatomaceous earth[1] and the solvent evaporated at 45° *in vacuo* to yield a red oil. TLC analysis of the reaction mixture with solvent system *a* revealed a series of spots from the origin to the solvent front, indicating a complex mixture, and containing VII (R_f 0.49). The reaction products were suspended in 200 ml. of benzene and chromatographed on a column of Florisil (453 Gm., 77 × 3.7 cm.). The column was eluted with benzene, mixtures of benzene and chloroform, chloroform, and mixtures containing V and VII were then eluted from the column with chloroform–methanol (1–3%). A series of yellow solids (11 Gm.) which remained after evaporation of solvent melted between 260–265°. TLC analysis of the solids with solvent system *a* revealed the presence of V (R_f 0.47) and VII (R_f 0.49), with solvent system *b* the presence of V (R_f 0.65) and VII (R_f 0.68). The presence of VII could be detected (before permanganate oxidation of the plates) by its brilliant blue fluorescence under ultraviolet irradiation. An attempt was made to purify V using preparative TLC (plates 0.5 mm. thick). The product obtained was a white powder which was recrystallized from chloroform–ether and then benzene to yield white crystals, m.p. 276–279° with phase change at 262–263°; $[\alpha]_D^{25}$ −30° (c 0.5); $\lambda_{max.}$ infl. 230 mμ (ε, 25,000); 292 mμ (ε, 35,000); infl. 320 mμ (ε, 7000). [Lit. (4) m.p. 276–279° with phase change at 260°; $[\alpha]_D$ −31.6° (acetone); $\lambda_{max.}$ infl. 230 mμ (ε, 25,000); 292 mμ (ε, 33,000); infl. 320 mμ (ε, 6800).] The infrared spectrum was essentially identical to that reported for (±)-V (14).

Anal.—Calcd. for $C_{17}H_{15}ClO_6$: C, 58.21; H, 4.31. Found: C, 58.31; H, 4.52.

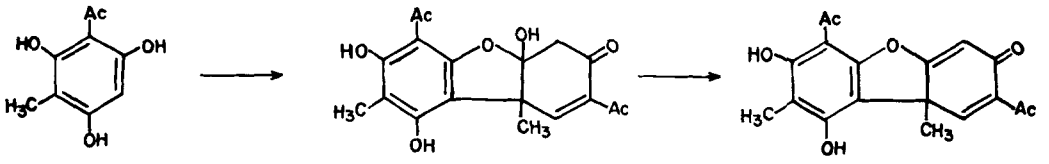
TLC analysis of the purified V (m.p. 277–279°) with solvent system *a* revealed the presence of a single spot (R_f 0.47, V) after permanganate oxidation; ultraviolet irradiation revealed a faint blue fluorescent spot of R_f 0.49 indicating a trace amount of VII. Analysis with solvent system *b* revealed the presence of a single spot (R_f 0.65, V) and ultraviolet irradiation revealed a faint blue fluorescent spot of R_f 0.68, again indicating the presence of a trace amount of VII.

Griseophenone A (I)—The method of Taub (4) was used to convert 800 mg. of (−)-V (m.p. 260–265°) to 280 mg. of I, using the zinc dust–acetic acid procedure. The yellow crystalline product melted at 212–214°; $\lambda_{max.}$ 297 mμ (ε, 18,800); 332 mμ (ε, 6300). [Lit. (4) m.p. 210–212°; $\lambda_{max.}$ 297 mμ (ε, 18,500); 332 mμ (ε, 6200).]

TLC analysis of I with solvent system *a* revealed a single spot of R_f 0.34.

(±)-Dehydrogriseofulvin (V)-From I by Enzymatic Conversion—Griseophenone A (I) (100 mg., 285 μmoles) was stirred in 2 L. of phosphate buffer (pH 7.6) containing 15 ml. of H_2O_2 (0.3%) and 100 mg. of horseradish peroxidase (200 units per mg., Nutritional Biochemical Corp., Cleveland, Ohio). The mixture was left stationary in the dark at 25° for 18 hr. and then extracted with chloroform. The chloroform extract was then concentrated to about 100 ml. and washed twice with 10 ml. of cold 2% KOH to remove 50 mg. of unreacted I. The chloroform solution was then washed with water until neutral and evaporated to dryness. The residue (40 mg.) was dissolved in acetone and streaked on three thick (0.5 mm.) TLC plates which

[1] Celite marketed by Johns-Manville Products Corp., New York, N. Y.



Scheme II

were developed with solvent system *b*. The zone corresponding in location to V was eluted with chloroform–methanol (10%) and evaporation of solvent yielded 29 mg. of a white powder. Recrystallization from benzene yielded 7 mg. of white crystals, m.p. 287–289°. [Lit. (4) m.p. 288–290°.] $[\alpha]_D^{25}$ 0° (c 0.5). The infrared, NMR, and ultraviolet spectra of (±)-V and (−)-V were congruent and (±)-V and (−)-V had identical mobilities by co-chromatography on TLC plates with solvent systems *a* and *b*, (±)-V exhibiting a single spot by ultraviolet irradiation and permanganate oxidation.

RESULTS

(+)-Griseofulvin (VII) was converted to (−)-V by selenium dioxide dehydrogenation and (−)-V was converted to I by reductive cleavage in acetic acid using powdered zinc. Griseophenone A (I) was then converted to (±)-V in a phosphate buffer (pH 7.6) containing horseradish peroxidase and hydrogen peroxide. The reaction did not take place in the absence of peroxidase and/or hydrogen peroxide. The infrared, NMR, and ultraviolet spectra of (−)-V, prepared by chemical means from (+)-VII, and (±)-V, prepared enzymatically from I, were congruent and their mobilities were identical on TLC.

DISCUSSION

The formation of (±)-V was not unexpected as Fales (12) has reported the horseradish peroxidase catalyzed conversion of methylacetylphloroglucinol (VII) (Scheme II), to the racemates, benzofuran derivative (IX), and usnic acid (X). Fales in fact reports the formation only of racemates from oxidative couplings in peroxidase systems.

The formation of racemic dehydrogriseofulvin (V) suggests the possibility that another enzyme(s) (including other peroxidases) could be involved in the *in vivo* conversion of I to (−)-V. On the other hand, it is possible that *in vivo*, peroxidase catalyzes the conversion of II to an optically active VI *via* IV, and that VI is then chlorinated to form (+)-VII. While considerable evidence exists that chlorination of II occurs in the biosynthesis of (+)-VII (15), nevertheless it has been shown that chlorination of a spiran can occur as in the biosynthesis of geodin (16).

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Keyphrases

Griseophenone A—conversion to (±)-dehydrogriseofulvin
Peroxidase, horseradish, hydrogen peroxide—catalyzed conversion
Chromatography, TLC, column—separation, identity
IR spectrophotometry—structure
UV spectrophotometry—structure
NMR spectroscopy—structure
Optical rotation—identity

More Convenient Syntheses of o-Azidobenzaldehyde and 2-Substituted Indazoles

By THOMAS J. SCHWAN* and CHARLES S. DAVIS†

Improved syntheses of o-aminobenzaldoxime, o-azidobenzaldehyde, and 2-substituted indazoles are described. An unequivocal synthesis of 2-(2-dimethylaminoethyl)indazole is reported.

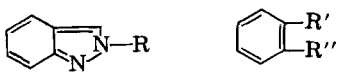
Because a series of 2-substituted indazoles (I) was desired for pharmacological evaluation, synthetic routes to these compounds were investigated. Recently there was reported the synthesis of 2-arylindazoles by the thermal decomposition in polychlorobenzenes of *o*-azidobenzylidene amines (II), which were prepared by condensation of *o*-azidobenzaldehyde (III) with the appropriate amines (1). The preparation of III by nitrous acid treatment of *o*-aminobenzaldoxime (IV) was previously reported by Bamberger and Demuth (2). Although Bamberger readily obtained IV from the aminoaldehyde (V) the synthesis of V from the nitroaldehyde (VI) required voluminous quantities of reactants and solvents. Hence, a route to large quantities of IV was sought.

Hydrogenation of *o*-nitrobenzaldoxime (VII) to IV without concomitant reduction of the oxime function was conveniently effected with 5% palladium-on-carbon in 70% yield.

Although difficulty was initially encountered in converting IV to III, it was found that III could more easily be isolated by extraction rather than by the steam distillation method reported previously (2).

Condensation of III with aniline or 2-dimethylaminoethylamine in dimethylformamide gave the intermediate Schiff base II which was not isolated by being thermally decomposed to the indazole I in refluxing dimethylformamide. This route to I allows the reaction mixture to be poured into water; the product is then easily isolated by filtration or extraction. Hence the use of water-insoluble, nonvolatile polychlorobenzenes is avoided; furthermore, isolation of the Schiff base is unnecessary.

The dimethylaminoethyl derivative (*Ib*), thus obtained, confirmed the structural assignment of Jao and Shchukina (3), who synthesized *Ib* and its 1-substituted isomer by the equivocal method of alkylating indazole with 2-dimethylaminoethyl chloride.



Ia R = C_6H_5
Ib R = $(CH_3)_2N(CH_2)_2$

II R′ = CH=N—R, R″ = N_3
III R′ = CHO R″ = N_3
IV R′ = CH=NOH, R″ = NH_2
V R′ = CHO, R″ = NH_2
VI R′ = CHO, R″ = NO_2
VII R′ = CH=NOH, R″ = NO_2

EXPERIMENTAL[1]

o-Aminobenzaldoxime (IV)—A mixture containing 97 Gm. (0.58 mole) of VII (4), 22 Gm. of 5% palladium-on-carbon (50% moisture), and 1600 ml. of ethanol was stirred with hydrogen at atmospheric pressure for 30 min., the temperature being maintained between 25 and 35° during the hydrogenation. Removal of the catalyst followed by evaporation of the solvent gave the crude product. Recrystallization from toluene gave 63 Gm. (79%) of IV, m.p. 136–136.5° [lit. (2) m.p. 132–133°].

o-Azidobenzaldehyde (III)—To a cooled stirred suspension of 4.08 Gm. (0.03 mole) of IV in 30 ml. of concentrated hydrochloric acid was added a solution of 2.28 Gm. (0.033 mole) of sodium nitrite in 4 ml. of water at 0–5°. To the solution was added 25 ml. of water followed by 60 ml. of 25% aqueous sodium hydroxide at 15–20°. The mixture was stored at room temperature for 16 hr., extracted with ether (3 × 75 ml.), the extracts dried ($MgSO_4$), and concentrated to dryness to give the crude product. Crystallization from hexane gave 2.35 Gm. (53%) of III, m.p. 29–34° [lit. (2) m.p. 35–37°].

2-Phenylindazole (Ia)—A solution of 1.47 Gm. (0.01 mole) of III, 1.03 Gm. (0.011 mole) of aniline, 0.1 Gm. of glacial acetic acid, and 20 ml. of dimethylformamide was stirred at 90–100° for 2 hr. and then refluxed for 30 min. After the mixture was cooled and poured into 400 Gm. of ice water, the product was collected and recrystallized from hexane to give 1.08 Gm. (56%) of *Ia*, m.p. 81–83° [lit. (1) m.p. 82–83°].

2-(2-Dimethylaminoethyl)indazole Dihydrochloride (Ib)—A solution of 3.11 Gm. (0.021 mole) of III, 2.21 Gm. (0.024 mole) of 2-dimethylaminoethylamine, 0.1 Gm. of glacial acetic acid, and 20 ml. of dimethylformamide was stirred at 90–100° for 2 hr. and then refluxed for 45 min. After the mixture was poured into 400 Gm. of ice water, the resulting solution was extracted with ethyl acetate (3 × 100 ml.), the extracts dried ($MgSO_4$), and concentrated to dryness *in vacuo*. Treatment of the free base with methanolic hydrogen chloride gave 2.63 Gm. (48%) of white crystals. Recrystallization from methanol gave the analytical sample, m.p. 181–191° [lit. (3) m.p. 171–181°]; the ultraviolet spectrum was identical to that reported for *Ib* (3).

Anal.—Calcd. for $C_{11}H_{15}N_3 \cdot 2HCl$: C, 50.39; H, 6.54; Cl, 27.04; N, 16.20. Found: C, 50.01; H, 6.63; Cl, 26.89; N, 16.00.

The *picrate* was prepared quantitatively from *Ib* and picric acid in ethanol; m.p. 241–243° dec. [lit. (3) 235° dec.].

Received October 2, 1967, from the Chemistry Division, The Norwich Pharmacal Company, Norwich, NY 13815
Accepted for publication November 30, 1967.
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[1] All melting points are corrected. Elemental analyses were performed by Grant Gustin and Marvin Tefft.

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Keyphrases

o-Azidobenzaldehyde—synthesis
2-Substituted indazoles—synthesis
Palladium-on-carbon—hydrogenation
Condensation reactions

Solubilization of Aminopteridines

By MILTON LAPIDUS

Aqueous solutions of a series of aminopteridines (5–10 mg./ml.) stable at physiologic pH's were prepared either by salt formation with hydrochloric acid or by complex formation with 10 percent deoxyribonucleic acid. Deoxyribonucleic acid was superior to ribonucleic acid, crystalline bovine serum albumin, human serum albumin fraction V, or gum acacia in solubilizing 4,7-diamino-2(*p*-chlorophenyl)-*N*-(2-diethylaminoethyl)-6-pteridinecarboxamide at pH 7. Potentiometric titration detected complex formation between deoxyribonucleic acid and the above aminopteridine.

In the course of preparing injectable solutions of a number of aminopteridines it was observed that very few in the series under investigation were appreciably soluble in water. The general lack of aqueous solubility of substituted pteridines has been reported as being due to substituted polar groups (OH, SH, and NH_2) (1). Pteridine (unsubstituted) was reported as being highly soluble (1 part in 7.5 parts of water at 20°).

Although classic salt formation has been used successfully to solubilize aliphatic amines, this method has limited applicability to weakly basic aminopteridines. The apparent acid solubility of triamterene (2,4,7-triamino-6-phenylpteridine) is reversed as the pH is raised to 7 (2).

Complex formation has been reported to increase the aqueous solubility of small molecules (3–5). Speculation that deoxyribonucleic acid, ribonucleic acid, serum proteins, and gum acacia may form aminopteridine complexes with enhanced solubility characteristics stimulated this study.

EXPERIMENTAL

The aminopteridines studied were synthesized by Osdene *et al.* (6, 7). Deoxyribonucleic acid-sodium (DNA-Na), ribonucleic acid-sodium (RNA-Na), and crystalline bovine serum albumin (BSA) were purchased from Nutritional Biochemical Corp., Cleveland, Ohio; human serum albumin (HA) fraction V from Pentex, Inc., Kankakee, Ill.; and USP gum acacia from S. B. Penick and Co., New York, N. Y. The standard reagents used were the best grades commercially available.

Analysis of Wy-4029—Wy-4029 [4,7-diamino-2-(*p* - chlorophenyl) - *N* - (2 - diethylaminoethyl) - 6-pteridinecarboxamide] in 0.1 *N* sulfuric acid fluoresces at 445 mμ when activated at 390 mμ. Using the Aminco Bowman spectrophotofluorometer, a linear response was obtained in the concentration range of 2–9 mcg./ml.

pH of Complete Solubility and Solubility Reversal—To 25 mg. of each of the aminopteridines suspended in 50 ml. of water increments of 1 *N* HCl were added until the soluble end point was reached. The pH was monitored with a Beckman pH meter, and pH equilibrium was established after the addition of each increment of acid. Back titration with 0.1 *N* sodium hydroxide resulted in the end point of solubility reversal.

pH Solubility Profile of Wy-4029—To a series of 500-mg. samples of Wy-4029 suspended in 20 ml. of water were added varying amounts of concentrated hydrochloric acid to obtain a constant pH of 3, 4, 5, 6, or 7. The contents of each beaker was stirred for 1 hr. at 25°. The mixtures were then filtered through a 0.45-μ Millipore filter (the standard method subsequently used) and the concentration of Wy-4029 in the filtrate was determined.

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Received October 27, 1967, from the Research Division, Wyeth Laboratories, Inc., Philadelphia, PA 19101
Accepted for publication January 11, 1968.
The author acknowledges the technical assistance of Mr. D. D. Austin.

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triturated in a Potter-Elvehjem homogenizer for 5 min., stirred an additional 2 hr., adjusted to pH 7, filtered, and the concentrations of Wy-4029 determined.

Effect of Initial pH of the Macromolecule Solution on Solubility of Wy-4029—To 20 ml. of 5% solutions of DNA-Na, RNA-Na, gum acacia, and BSA adjusted with 1 *N* hydrochloric acid or 1 *N* sodium hydroxide to pH's varying from 2 to 8 was added an excess (100–400 mg.) of Wy-4029. The mixtures were triturated in a Potter-Elvehjem homogenizer for 5 min., stirred an additional 2 hr., adjusted to pH 7, filtered, and the concentrations of Wy-4029 determined.

Preparation of Wy-4029–DNA Solution—This solution as prepared by adding 10 Gm. of DNA-Na to 90 ml. of water, adjusting to pH 3.5 with dilute hydrochloric acid, making up to 100 ml., and clarifying by centrifugation at 6,000 r.p.m. for 15 min. Wy-4029 (1 Gm.) was added and complete solubility was attained by trituration in a Potter-Elvehjem glass homogenizer. Each prepared solution was vigorously stirred and slowly adjusted to pH 7 with dilute sodium hydroxide, then either sterile-filtered through a 0.45-μ Millipore filter and stored in ampules or lyophilized and stored as a powder. This powder was readily reconstituted by the addition of water.

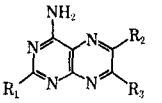
RESULTS AND DISCUSSION

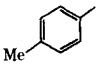
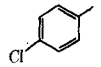
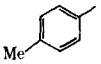
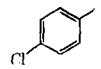
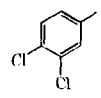
The aminopteridines investigated were all solubilized by classic salt formation (0.5 mg./ml.) with 0.1 *N* hydrochloric acid. By allowing pH equilibrium after the addition of each increment of acid, it was possible to arrive at the pH at which aqueous solubility was achieved. The compounds varied considerably in solubility pH (Table I). The individual values probably reflect the relative basicity: the weaker this basicity, the greater the difficulty of forming classic salts and the lower the pH required for solubilization.

Reversal of the apparent acid solubility of the aminopteridines, accomplished by the addition of sufficient 0.1 *N* sodium hydroxide, also occurred over a rather wide pH range (Table I). A number of aminopteridines remained soluble at 0.5 mg./ml. and pH 7.4, and for these compounds it was predicted that higher concentrations would be stable. Solutions of aminopteridines (10 mg./ml.) were then prepared by solubilization with hydrochloric acid and adjustment to pH 7.4 with alkali (Table I, footnote *c*).

To determine the effect of DNA on aminopteridine solubility, a 1% solution of DNA-Na (pH 2.5) containing 0.5 mg./ml. of an aminopteridine in solution was titrated with 0.1 *N* sodium hydroxide until solubility reversal occurred. In every example the rever-

TABLE I—EFFECT OF DNA ON "EXTENDING" pH SOLUBILITY OF AMINOPTERIDINES



Wy-	R_1	R_2	R_3	Soluble HCl Salt,[a] pH	Reversal of Salt Solubility, pH	Reversal of DNA Solubility,[b] pH
1841[c]		—CONHCH$_2$CH$_2$CH$_2$NEt$_2$	—NH$_2$	4.4	9.1	10.1
1843[c]		—CONHCH$_2$CH$_2$NEt$_2$	—NH$_2$	4.2	8.4	—
3519[d]	—NH$_2$		—NH$_2$	4.4	5.4	9.9
3648[d]		—CONHCH$_2$CHCH$_3$HMe$_2$	—NH$_2$	3.9	7.7	9.0
3665[c]		—CONHCH$_2$CH$_2$CH$_2$NEt$_2$	—NH$_2$	4.6	8.5	9.9
3873[c]		—CONHCH$_2$CH$_2$CH$_2$NMe$_2$	—NH$_2$	5.2	9.1	9.8
3876[d]		—CONHCH$_2$CH$_2$NMe$_2$	—NH$_2$	4.8	7.9	9.3
4027[c]	—NHCH$_2$CH$_2$CH$_2$NEt$_2$	—CONHCH$_2$CH$_2$CH$_2$NEt$_2$	—NH$_2$	8.5	10.8	—
4029[e]		—CONHCH$_2$CH$_2$NEt$_2$	—NH$_2$	4.1	7.7	9.4
4196[d]		—CONHCH$_2$CH$_2$NEt$_2$	—NH$_2$	4.3	7.0	9.0

(*Continued on next page*)

TABLE I—(*Continued*)

Wy-	R_1	R_2	R_3	Soluble HCl Salt,[a] pH	Reversal of Salt Solubility, pH	Reversal of DNA Solubility,[b] pH
4276[c]		—CONHCH$_2$CH$_2$NH$_2$	—NH$_2$	5.2	>10.1	—
4437[c]		—CONHCH$_2$(CH$_2$)$_2$CH$_2$NMe$_2$	—NH$_2$	7.6	9.2	—
4923[d]	Me	—CONHCH$_2$CH$_2$CH$_2$NEt$_2$	—NH$_2$	4.4	8.5	9.6
5120[c]		—CONHCH$_2$CH$_2$NEt$_2$	—NHCH$_2$CH$_2$NEt$_2$	8.0	8.8	9.8
5121[c]		—CONHCH$_2$(CH$_2$)$_2$CH$_2$NEt$_2$	—NH$_2$	4.2	9.3	10.3
5250[c]		—CONHCH$_2$(CH$_2$)$_4$CH$_2$NMe$_2$	—NH$_2$	3.6	8.5	10.5
5256[f]		—CONHCH$_2$CH$_2$OMe	—NHCH$_2$CH$_2$OMe	2.5	6.1	6.9
5330[c]		—CONHCH$_2$(CH$_2$)$_3$CH$_2$NMe$_2$	—NH$_2$	4.9	8.4	10.0
5365[d]		—CONHCH$_2$(CH$_2$)$_3$CH$_2$NEt$_2$	—NH$_2$	3.2	8.7	9.9
5588[c]		—CONHCH$_2$CH$_2$NEt$_2$	—NH$_2$	4.8	8.5	9.7
6520[e]	Cl	—CONHCH$_2$CH$_2$NEt$_2$	—NH$_2$	5.0	7.6	9.6
7037[c]	Me	—CONHCH$_2$CH$_2$NEt$_2$	—NH$_2$	4.6	8.3	—
7038[c]	Cl	—CONHCH$_2$(CH$_2$)$_2$CH$_2$NMe$_2$	—NH$_2$	5.6	8.5	10.4

[a] Aminopteridine, 25 mg./50 ml. water. [b] Aminopteridine, 25 mg./50 ml. of a 1% solution of DNA-Na. [c] Solubilized by salt formation, 10 mg./ml. pH 7.4. [d] Solubilized by complex formation, 5 mg./ml. in 10% DNA, pH 7.0. [e] Solubilized by complex formation, 10 mg./ml. in 10% DNA, pH 7.4. [f] Solubilized by complex formation, 5 mg./ml. in 10% DNA, pH 6.5.

sal occurred at a higher pH than had previously been determined for solutions prepared by classic salt formation (Table I). The 0.7–4.5 unit upward extension of the pH solubility range suggested the concomitant utility of complex formation by DNA as well as other macromolecules for preparing stable solutions of aminopteridines at higher concentrations than was previously possible. As shown in Table I, some aminopteridines cannot be solubilized at 10 mg./ml. and pH 7.4, probably because they are weakly basic and do not form stable hydrochlorides. Wy-4029, representative of this group, has limited solubility at pH 7 (Fig. 1). The concentration of Wy-4029 in solution was increased considerably by complex formation with macromolecules. Solutions of DNA-Na (pH 5), RNA-Na (pH 2.8), HA fraction V (pH 4.9), BSA (pH 5.1), and gum acacia (pH 4.4) at their respective natural pH's were effective in solubilizing Wy-4029 and in maintaining concentrations at pH 7 previously not considered possible (Fig. 2). Since the pH of the macromolecule solution is a variable in determining the amount of aminopteridine solubilized, a pH-solubility profile study of 5% solutions of DNA-Na, RNA-Na, BSA, and gum acacia was made. For each of the macromolecules an optimum pH for maximum solubility of Wy-4029 was determined (Fig. 3). The anticipated superiority of DNA-Na in solubilizing Wy-4029 was confirmed.

The potentiometric evidence of complex formation between Wy-4029 and DNA-Na was provided by three distinct alkali titration curves (Fig. 4) (8). Complex formation existed up to approximately pH 9. DNA-Na maintained the solubility of Wy-4029 above the pH at which solubility by salt formation failed.

A solution of Wy-4029 solubilized in 10% DNA-Na at pH 3.5 (10 mg./ml.) and then adjusted to pH 7.4 was found to be stable at 4° and 25° for a minimum of 3 months. A lyophilized powder prepared from a freshly made solution was readily reconstituted in water.

A toxicity evaluation of the aminopteridines and aminopteridine–DNA complexes (1 mg./mouse) demonstrated the protective effect of DNA in preventing skin lesions in mice at the subcutaneous site

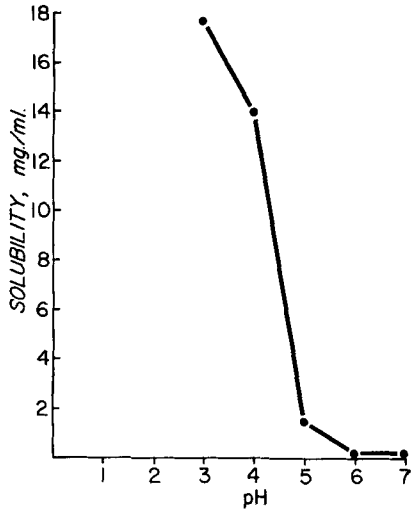


Fig. 1—pH Solubility profile of Wy-4029 in presence of hydrochloric acid.

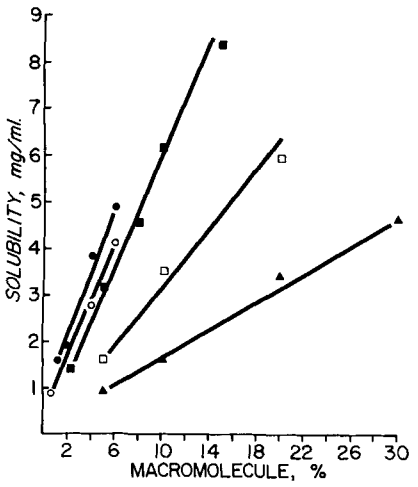


Fig. 2—Solubility at pH 7 of Wy-4029 complexed with each of five macromolecules. Complexes of Wy-4029. Key: ●, DNA-Na, (pH 5); ○, RNA-Na, (pH 2.8); ■, human albumin fraction V (pH 4.9); □, bovine serum albumin (pH 5.1); and ▲, gum acacia (pH 4.4). Wy-4029 was solubilized initially at the given natural pH (shown in parentheses) of each of the macromolecules.

of injection. Dissection of the subcutaneous site failed to reveal the depot of aminopteridine usually found after injecting an aqueous solution. It seems reasonable to assume that transport of the aminopteridine away from the site of injection is accelerated when it is complexed with DNA.

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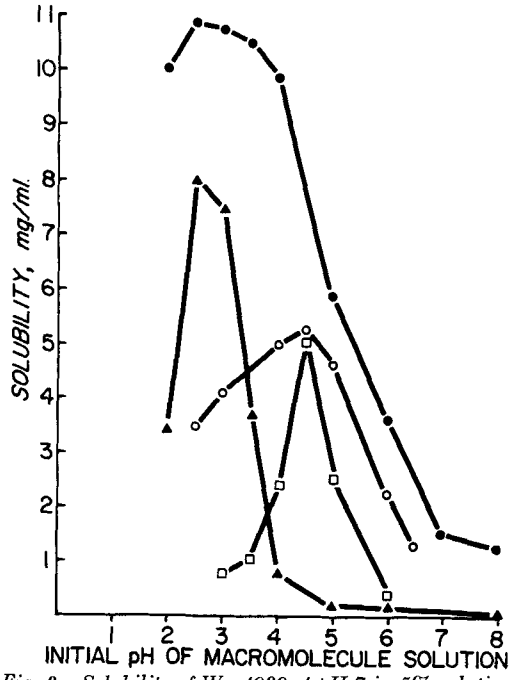


Fig. 3—Solubility of Wy-4029 at pH 7 in 5% solution of each of four macromolecules as a function of the solubilization pH. Key: ●, DNA-Na; ○, RNA-Na; ▲, gum acacia; and □, bovine serum albumin.

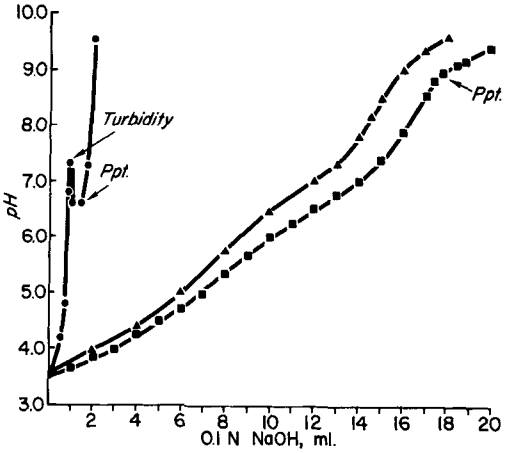


Fig. 4—Potentiometric detection of complex formation (25°). Key: ●, Wy-4029 (50 mg./100 ml.); ▲, DNA-Na (1 Gm./100 ml.); ■, Wy-4029 (50 mg./100 ml.) plus DNA-Na (1 Gm./100 ml.).

Keyphrases

Aminopteridines—solubilization
Complex formation—DNA–aminopteridines
Macromolecules—solubilizing effects
pH—Solubility profile
Toxicity—DNA–aminopteridines

Gas Chromatographic Determination of Anagestone Acetate in Fertility Control Tablets

By ARVIN P. SHROFF and R. E. HUETTEMANN

A gas-liquid chromatographic technique has been developed for the determination of anagestone acetate in tablets. A 3 percent DEGS column gave good resolution between the peaks of anagestone acetate and internal standard. The accuracy and the precision of the method are 1.97 and 0.052, respectively. Anagestone acetate does not undergo any alteration after 18 months of storage.

THE INCREASING awareness of the population "explosion" has stimulated the development of a number of oral contraceptive agents. Estrogen-progestin combinations are widely used for this purpose. Of the seven U. S. companies which offer eleven different dosage forms, the choice of estrogen is between either 17-α-ethynylestradiol (EE) or 17-α-ethynylestradiol 3-methylether (MEE). The progestational agents, however, vary in their chemical structure but they fall into two main classes. The members of one class are characterized by the absence of the C-19 methyl group and are designated as "19-nor" compounds. The other group is characterized by modification of the basic progesterone structure with different types of substitutions to form the so-called "substituted" progestins.

Estrogens in the presence of some of these progestins have been assayed by colorimetric (1, 2), ultraviolet (3), spectrofluorometric (4), and gas-liquid chromatographic (3, 5, 6) methods.

Similarly, the progestational agents of the "19-nor" and "substituted" progestin classes have been quantitated by various analytical techniques. For example, Bastow quantitated 17-α-hydroxy-19-nor-17-pregn-5(10)-en-20-yn-3-one (norethynodrel) with an ultraviolet spectrophotometric technique (7) and France and co-workers used GLC for 6-α-methyl-6-dehydro-17α-acetoxyprogesterone (6).

In the present investigation we describe the gas-liquid chromatographic determination of a new "substituted" progestin 3-desoxy-17 α-acetoxy-6-α-methylpregn-4-en-20-one(I) (anagestone acetate), in the presence of MEE (II). The determination was carried out on a 3% DEGS coated column using MEE as an internal standard. The steroids do not undergo any decomposition on the stainless steel column when proper conditions are maintained and MEE can be separated without prior acylation or trimethylsilyl etherification.

EXPERIMENTAL

Apparatus and Operational Parameters—An F&M model 1609 instrument equipped with a hydrogen flame-ionization detector and modified injection port was employed. The modified injection port was made by F&M Scientific to eliminate the potential for inlet sample splitting. The recorder was a Minneapolis Honeywell "Electronik" with a 1-mv. full-scale deflection and was operated at a chart speed of 15 in./hr. A coiled stainless steel column 39.4 in. long and 1/4 in. i.d. packed with 3% DEGS on Gas Chrom Z (100–120 mesh) was used. The column packing was prepared by the evaporation technique. The packed column was conditioned at 215° for 24 hr. with nitrogen flowing at 63 ml./min. All samples were injected by means of a 10-μl. Hamilton syringe. The operational parameters were: column temperature 215°; inlet-port temperature 235°, and detector temperature 250°. Nitrogen was used as a carrier gas flowing at 65 ml./min. Oxygen and hydrogen were maintained at about 400 ml./min. and 45 ml./min., respectively.

Standard MEE Solution—Weigh accurately 300 mg. of reference standard MEE (E 1%, 1 cm. 60–65) into a 100-ml. volumetric flask and dilute to mark with methylene chloride.

Standard Anagestone Acetate Solution—Place exactly 100 mg. of anagestone acetate into a 100-ml. volumetric flask and dilute to mark with methylene chloride.

Preparation of a Typical Standard Curve—Pipet into three different vials 1.0 ml., 2.0 ml., and 3.0 ml., of the standard anagestone acetate solution and evaporate to dryness on a steam bath and under a stream of nitrogen. Dissolve each residue with 1.0 ml. of standard MEE solution (internal standard) and inject approximately 2 μl. into the gas chromatograph. Adjust the attenuator to 320 and elute for 20 min. The first major peak is attributed to anagestone acetate and the second major peak to MEE. The areas are calculated, after drawing a tangent to both minima of each peak, by multiplying the height of the peak by the width at one-half the peak height. A plot of the ratios of areas under anagestone acetate and MEE *versus* the weight of anagestone acetate is obtained and a best straight line drawn. All the points on the curve are obtained in triplicate.

Analysis of Tablets—Two tablets (2 mg. anagestone acetate and 80 mcg. MEE per tablet) equivalent to 4.0 mg. of anagestone acetate are placed in a 21 × 50-mm. vial and treated with 2.0 ml. of distilled water and 2.0 ml. of cyclohexane. The vial is clamped to a Burrell wrist-action type shaker and allowed to shake vigorously for 0.5 hr., then centrifuged for 1 min. and 1.0 ml. of the upper phase is pipeted into another 21 × 50-mm. vial. This solution is evaporated on a steam bath and under a stream of nitrogen. The residue thus obtained is redissolved in 1.0 ml. of standard MEE solution (internal standard) and a 2-μl. injection made into the gas chromatograph. The attenuation and calculations are similar to "preparation of typical standard curve." The amount of anagestone acetate is obtained from the standard curve.

DISCUSSION AND RESULTS

Theoretically each combination tablet contains 2 mg. of anagestone acetate and 80 mcg. of MEE along with other excipients of the tablet. The anagestone acetate present is 25 times in excess of the MEE.

The efficiency of using MEE as an internal stan-

Received November 17, 1967, from the Analytical Research Group, Division of Organic Chemistry, Ortho Pharmaceutical Corporation, Raritan, NJ 08869
Accepted for publication January 8, 1968.

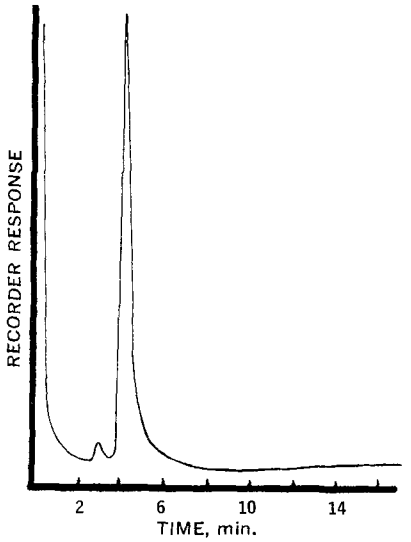


Fig. 1—Gas chromatogram of an extract from combination tablet showing dihydro derivative and anagestone acetate.

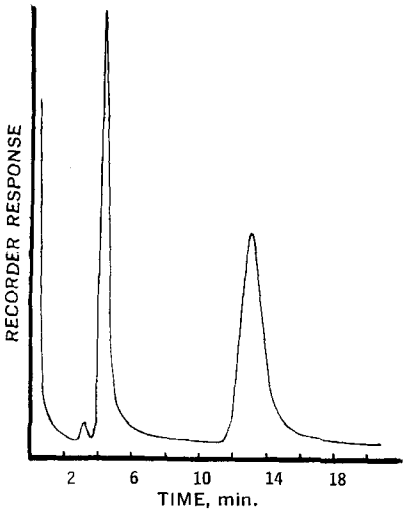
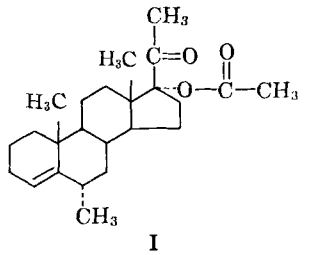


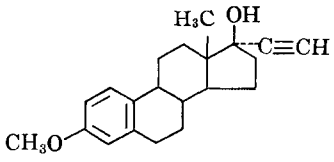
Fig. 2—A typical gas chromatogram of a tablet extract to which internal standard (MEE) has been added.

dard was checked by extracting two tablets with water–cyclohexane and following the procedure outlined in the experimental section but omitting the internal standard. A typical gas chromatogram of an extract from combination tablets is shown in Fig. 1. The small peak preceding the major anagestone acetate peak is attributed to a dihydro derivative of anagestone acetate (a synthetic by-product). The peak response for MEE (see Fig. 2 for retention time) was negligible, if any, with the attenuation used. The gas chromatogram of material extracted from tablets to which the internal standard (MEE) was added is shown in Fig. 2. The internal standard does not interfere with detection of anagestone acetate and the resolution between the peaks is good.

The MEE showed no decomposition to estrone methyl ether on a freshly prepared column. However, if the column packing was not prepared properly, then after a few days and, at times, after conditioning the column decomposition became apparent. The peak for estrone methyl ether appeared between anagestone acetate and MEE (Fig. 3) and it was found that this decomposition did not vary with time. Thus, by preparing a fresh calibration curve the column can be used effectively. Routinely we have found the method to be independent of the volume of the injected sample and independent of small variations in column temperature and gas flow.



I
Anagestone Acetate



II
MEE

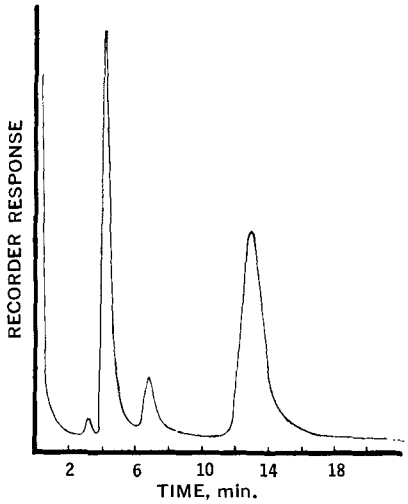


Fig. 3—Gas chromatogram showing the decomposition of MEE to estrone methylether.

The ratio of peak areas obtained for different concentrations of anagestone acetate in the standard solutions is given in Table I. A plot of these data

TABLE I—CONCENTRATION *Versus* RATIO OF PEAK AREAS

No.	Anagestone Acetate	Ratio of Peak Areas[a]
1	1.0	0.282
2	2.0	0.590
3	3.0	0.879

[a] Average of three determinations.

TABLE II—ACCURACY DATA FOR THE GLC METHOD

No.	Mg. Added	Mg. Found[a]	% Recovery ($\bar{x}$)[b]
1	2.0	1.95	97.5
2	2.0	1.96	98.0
3	2.0	2.02	101.0
4	2.0	2.04	102.0
5	2.0	2.01	100.5

[a] $\sigma = \pm 1.97\%$. [b] $\bar{x} = 99.8$.

TABLE III—REPRODUCIBILITY DATA FOR THE ASSAY METHOD ON FRESH TABLETS[a]

No.	Mg. Found (x)	$(\bar{x} - x)^2$
1	2.04	0.0009
2	1.96	0.0025
3	1.96	0.0025
4	2.08	0.0049
5	2.01	0.0000

$\sigma = \pm 0.052$

[a] Each tablet contains theoretical amount of 2 mg. of anagestone acetate.

is a straight line (standard curve). The accuracy of the GLC method was obtained from the data in Table II. The known and found values along with the sigma value (1.97) show that the analysis can be performed satisfactorily. The precision ($\sigma = 0.052$) studies were carried out on fresh tablets and indicated (Table III) the method was sensitive and can be adopted for routine analysis.

Several batches of tablets stored at various temperature stations for different lengths of time were assayed. The results, recorded in Table IV, demonstrate that over a period of 18 months, anagestone acetate does not undergo any alteration.

TABLE IV—ASSAY DATA ON COMBINATION TABLETS[a] FOR ANAGESTONE ACETATE

No.	Storage Conditions[b]	Mg. Anagestone Acetate per Tablet	% Recovery
1	Fresh	1.96	98.0
2	RT, 3 weeks	2.03	101.5
3	RT, 1 month	1.98	99.0
4	RT, 2 months	2.05	102.5
5	RT, 15 months	2.00	100.0
6	RT, 16 months	1.97	98.5
7	47°, 15 months	2.02	101.0
8	37°, 18 months	2.01	100.5
9	50°, 3 months	2.04	102.0

[a] Each tablet contains theoretically 2.0 mg. of anagestone acetate. [b] RT refers to room temperature.

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Keyphrases

17-α-ethynylestradiol 3-methylether—anagestone acetate tablets
Anagestone acetate—analysis
GLC—analysis

The Analgesic, Hypothermic, and Depressant Activities of Some *N*-Substituted α-5,9-Dimethyl-6,7-benzomorphans

By M. MAY, L. CZONCHA, D. R. GARRISON, and D. J. TRIGGLE

A series of *N*-substituted α-5,9-dimethyl-6,7-benzomorphans bearing *N*-2-hydroxyalkyl and *N*-2-bromoalkyl substituents have been synthesized and their analgesic, hypothermic, and depressant activities determined. The analgesic activities were not outstanding but the *N*-2-bromoethyl- and the *N*-2-bromopropyl derivatives produced prolonged hypothermia and depression. Possible mechanisms of these prolonged actions are discussed.

It is generally assumed that narcotic analgesics produce their pharmacological effects by interaction at a specific analgesic receptor (or receptors). Extensive work by Beckett and co-workers (1, 2) has resulted in the publication of a tentative description of the analgesic receptor. This description has recently been critically discussed by Portoghese (3) who points out that the analgesic receptor may be more flexible in its binding capability than Beckett originally assumed. Nevertheless, both Beckett and Portoghese assume that one of the principal binding sites at the analgesic receptor is an anionic grouping which is capable of binding, *via* an ion–ion interaction, a protonated amino group of the analgesic molecule.

An anionic binding site (a carboxylate or phosphate anion) is also assumed to be an important fea-

Received February 9, 1967, from the Department of Biochemical Pharmacology, School of Pharmacy, and The Theoretical Biology Center, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication January 24, 1968.

This work was supported by grants from the United States Health Foundation of Western New York (G-65-UB-3) and the U. S. Public Health Service (GM11603) with supplemental assistance from NASA (grant No. NGR-33-015-016).

TABLE II—ACCURACY DATA FOR THE GLC METHOD

No.	Mg. Added	Mg. Found[a]	% Recovery ($\bar{x}$)[b]
1	2.0	1.95	97.5
2	2.0	1.96	98.0
3	2.0	2.02	101.0
4	2.0	2.04	102.0
5	2.0	2.01	100.5

[a] $\sigma = \pm 1.97\%$. [b] $\bar{x} = 99.8$.

TABLE III—REPRODUCIBILITY DATA FOR THE ASSAY METHOD ON FRESH TABLETS[a]

No.	Mg. Found (x)	$(\bar{x} - x)^2$
1	2.04	0.0009
2	1.96	0.0025
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4	2.08	0.0049
5	2.01	0.0000

$\sigma = \pm 0.052$

[a] Each tablet contains theoretical amount of 2 mg. of anagestone acetate.

is a straight line (standard curve). The accuracy of the GLC method was obtained from the data in Table II. The known and found values along with the sigma value (1.97) show that the analysis can be performed satisfactorily. The precision ($\sigma = 0.052$) studies were carried out on fresh tablets and indicated (Table III) the method was sensitive and can be adopted for routine analysis.

Several batches of tablets stored at various temperature stations for different lengths of time were assayed. The results, recorded in Table IV, demonstrate that over a period of 18 months, anagestone acetate does not undergo any alteration.

TABLE IV—ASSAY DATA ON COMBINATION TABLETS[a] FOR ANAGESTONE ACETATE

No.	Storage Conditions[b]	Mg. Anagestone Acetate per Tablet	% Recovery
1	Fresh	1.96	98.0
2	RT, 3 weeks	2.03	101.5
3	RT, 1 month	1.98	99.0
4	RT, 2 months	2.05	102.5
5	RT, 15 months	2.00	100.0
6	RT, 16 months	1.97	98.5
7	47°, 15 months	2.02	101.0
8	37°, 18 months	2.01	100.5
9	50°, 3 months	2.04	102.0

[a] Each tablet contains theoretically 2.0 mg. of anagestone acetate. [b] RT refers to room temperature.

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Keyphrases

17-α-ethynylestradiol 3-methylether—anagestone acetate tablets
Anagestone acetate—analysis
GLC—analysis

The Analgesic, Hypothermic, and Depressant Activities of Some *N*-Substituted α-5,9-Dimethyl-6,7-benzomorphans

By M. MAY, L. CZONCHA, D. R. GARRISON, and D. J. TRIGGLE

A series of *N*-substituted α-5,9-dimethyl-6,7-benzomorphans bearing *N*-2-hydroxyalkyl and *N*-2-bromoalkyl substituents have been synthesized and their analgesic, hypothermic, and depressant activities determined. The analgesic activities were not outstanding but the *N*-2-bromoethyl- and the *N*-2-bromopropyl derivatives produced prolonged hypothermia and depression. Possible mechanisms of these prolonged actions are discussed.

It is generally assumed that narcotic analgesics produce their pharmacological effects by interaction at a specific analgesic receptor (or receptors). Extensive work by Beckett and co-workers (1, 2) has resulted in the publication of a tentative description of the analgesic receptor. This description has recently been critically discussed by Portoghese (3) who points out that the analgesic receptor may be more flexible in its binding capability than Beckett originally assumed. Nevertheless, both Beckett and Portoghese assume that one of the principal binding sites at the analgesic receptor is an anionic grouping which is capable of binding, *via* an ion–ion interaction, a protonated amino group of the analgesic molecule.

An anionic binding site (a carboxylate or phosphate anion) is also assumed to be an important fea-

Received February 9, 1967, from the Department of Biochemical Pharmacology, School of Pharmacy, and The Theoretical Biology Center, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication January 24, 1968.

This work was supported by grants from the United States Health Foundation of Western New York (G-65-UB-3) and the U. S. Public Health Service (GM11603) with supplemental assistance from NASA (grant No. NGR-33-015-016).

ture of the structure and function of the adrenergic and cholinergic receptors (4–6), and since it has proved possible to design antagonists which act at these receptors by forming covalent bonds to these anionic sites (4, 7, 8), it appeared of interest to extend this approach to the design of compounds that might interact irreversibly at the narcotic analgesic receptors. This approach, if successful, would yield information valuable in attempted identification of the receptor site(s) and, additionally, might furnish compounds of considerable therapeutic importance.

In this initial approach, the compounds selected for study were *N*-(2-bromoethyl) derivatives (I*b*–III*b*) of α-5,9-dimethyl-6,7-benzomorphan,

I(*a*) X = OH, R = H, (*b*) X = Br, R = H
II(*a*) X = OH, R = CH_3, (*b*) X = Br, R = CH_3
III(*a*) X = OH, R = Ph, (*b*) X = Br, R = Ph

together with their corresponding nonalkylating parent compounds (I*a*–III*a*). *N*-Methyl-α-5,9-dimethyl-6,7-benzomorphan was employed as an appropriate "standard" analgesic compound (7).

EXPERIMENTAL

Chemistry—Melting points were recorded on a Thomas-Kofler hot stage and are corrected. Analyses are by Dr. A. E. Bernhardt, Mulheim, West Germany.

***N* - Methyl - α - 5,9 - dimethyl - 6,7 - benzomorphan**—This was prepared by the method of May and Fry (9). The hydrochloride salt had m.p. 204° [lit. [9] m.p. 203–204°]. The hydrobromide salt was recrystallized from acetone and had m.p. 188–190°.

Anal.—Calcd. for $C_{15}H_{22}BrN$: C, 60.83; H, 7.48; Br, 26.97. Found: C, 60.91; H, 7.22; Br, 27.11.

***N* - (2 - Hydroxyethyl) - α - 5,9 - dimethyl - 6,7-benzomorphan Hydrobromide (Ia)**—To α-5,9-dimethyl-6,7-benzomorphan (10) (4.1 Gm., 0.02 mole) in methanol (20 ml.) was added ethylene oxide (1.3 Gm., 0.03 mole) and the solution was heated to 100° in a steel bomb for 6 hr. On cooling the solution was saturated with hydrogen bromide gas, evaporated under reduced pressure, and the residue dissolved in dry acetone. On cooling crystals of I*a* (74%) were deposited with m.p. 210–212°.

Anal.—Calcd. for $C_{16}H_{22}BrNO$: C, 58.9; H, 7.42; Br, 24.49; N, 4.29. Found: C, 59.21; H, 7.17; Br, 24.41; N, 4.51.

***N* - (2 - Bromoethyl) - α - 5,9 - dimethyl - 6,7-benzomorphan Hydrobromide (Ib)**—I*a* (3.3 Gm., 0.01 mole) suspended and stirred in chloroform (20 ml.) at 0° was treated dropwise with a solution of phosphorus tribromide (3.0 Gm., 0.011 mole) in chloroform (10 ml.). The mixture was then refluxed for 4 hr., evaporated under reduced pressure, and the orange residue extracted with boiling acetone (3 × 15 ml.) and filtered through diatomaceous earth. On cooling the filtrate yielded crystals of I*b*, (82%) which were recrystallized (acetone) to a constant m.p. of 195°.

Anal.—Calcd. for $C_{16}H_{23}Br_2N$: C, 49.38; H, 5.96; Br, 41.1. Found: C, 49.61; H, 5.71; Br, 40.83.

***N* - (2 - Hydroxypropyl) - α - 5,9 - dimethyl - 6,7-benzomorphan Hydrobromide (IIa)**—This was prepared from α-5,9-dimethyl-6,7-benzomorphan and propylene oxide using the procedure described for I*a*. The product had m.p. 208–215° (82%).

Anal.—Calcd. for $C_{17}H_{26}BrNO$: C, 59.99; H, 7.7; Br, 23.48; N, 4.11. Found: C, 60.15; H, 7.31; Br, 23.52; N, 3.98.

***N* - (2 - Hydroxy - 2 - phenylethyl) - α - 5,9-dimethyl-6,7-benzomorphan Hydrobromide (IIIa)**—This was prepared similarly from α-5,9-dimethyl-6,7-benzomorphan and 1,2-epoxyethylbenzene. The product had m.p. 225–235° (92%).

Anal.—Calcd. for $C_{22}H_{28}BrNO$: C, 65.63; H, 7.02; Br, 19.86. Found: C, 65.82; H, 6.77; Br, 19.76.

***N* - (2 - Bromopropyl) - α - 5,9-dimethyl - 6,7-benzomorphan Hydrobromide (IIb)**—This was prepared from II*a* and phosphorus tribromide using the procedure described under I*b*. The product had m.p. 180–190° (78%).

Anal.—Calcd. for $C_{17}H_{25}Br_2N$; C, 50.61; H, 6.25; Br, 39.64; N, 3.47. Found: C, 50.79; H, 6.51; Br, 39.34; N, 3.50.

***N* - (2 - Bromo - 2 - phenylethyl) - α - 5,9 - dimethyl-6,7-benzomorphan Hydrobromide (IIIb)**—This was prepared similarly from III*a* and phosphorus tribromide. The product had m.p. 168–175° (89%).

Anal.—Calcd. for $C_{22}H_{27}Br_2N$: C, 56.76; H, 5.85; Br, 34.34; N, 3.01. Found: C, 56.41; H, 6.13; Br, 34.33; N, 3.21.

PHARMACOLOGY

Analgesic activity (Table I) was determined using the Eddy-Leimbach modification (11) of the hot-plate method devised by Woolfe and Macdonald (12). Compounds were administered subcutaneously in 0.1 ml. of water. Swiss albino male mice (weight 20–30 Gm.) were employed, 10 animals each dose, and an increase of 10 sec. or more in reaction time from the pretreatment time was taken as an analgesic response (see under *Discussion*).

Spontaneous motor activity (Table II) was mea-

TABLE I—ANALGESIC ACTIVITIES OF *N*-SUBSTITUTED α-5,9-DIMETHYL-6,7-BENZOMORPHANS

Compd.	ED_{50}[a]	Time of Onset, min.	Duration, min.
Standard[b]	30	11	28
I*a*	100[c]	45	30
I*b*	28	80	180
II*a*	48	16	20
II*b*	30	140	180
III*a*	400[d]	30	20
III*b*	inactive at 400		

[a] Dose in mg./Kg. which produced an apparent analgesic response in 50% of the animals. [b] *N*-Methyl-α-5,9-dimethyl-6,7-benzomorphan. Murphy, Ager, and May (13) report the ED_{50} (as hydrochloride) as 27.3 mg./Kg. [c] Only 40% of the animals affected. [d] Only 30% of the animals affected.

TABLE II—DEPRESSANT ACTIVITIES OF *N*-SUBSTITUTED α-5,9-DIMETHYL-6,7-BENZOMORPHANS

Compd.	Dose,[a] mg./Kg.	Locomotor Activity,[b] —Time After Injection, %— 15–30 min.	30–60 min.	1,455–1,470 min.
Water	(control)	100	100	100
I*b*	30	87	16	100
II*b*	30	100	17	24
Standard	30	189	251	17
St. + I*b*[c]	30 + 30	337	12	...
St. + II*b*[c]	30 + 30	92	20	...

[a] Single subcutaneous injection on first day (time zero). [b] Locomotor activity expressed as % of control activity. [c] In the pretreatment studies standard (St.) was administered 30 min. before I*b* or II*b*.

TABLE III—EFFECTS OF *N*-SUBSTITUTED α-5,9-DIMETHYL-6,7-BENZOMORPHANS ON MICE RECTAL TEMPERATURES

Compd.	Dose,[a] mg./Kg.	Rectal Temperature, Time After Injection, min. 0	30	60	90	120	180	240	24 hr
Dist. water	...	37.3	37.3	37.2	37.3	37.0	...	...	...
Standard	70	38.4	36.5	37.4	38.3	38.5	...	...	...
Ia	100	37.3	37.3	37.1	37.1	37.1	...	...	...
Ib	40	37.1	35.6	34.6[b]	33.9	33.5	33.9	...	37.1
IIa	80	37.4	36.2	36.2	36.3	37.0	...	...	...
IIb	50	37.5	34.6	33.2[b]	33.0	32.3	31.7[b]	32.9	35.7
St. + *1b*[c]	70 + 40	38.1	36.2	34.5	31.9[b]	30.3	...	...	...
St. + *IIb*[c]	70 + 50	38.2	35.8	33.7	31.2[b]	29.1	...	...	...

[a] 5 animals per dose level. [b] *P* value for significance of difference from standard <0.01. [c] Compound administered 0.5 hr. after standard.

sured using a Lehigh Valley Electronics activity cage (model 1497). The compounds were administered subcutaneously to male mice which were placed immediately in the cage and the locomotor activity determined from the fifteenth to the thirtieth and from the thirtieth to the sixtieth min. Locomotor activity was again determined 24 hr. later. Control animals were treated similarly but received only an equivalent amount of injected water. Locomotor activity of treated mice is expressed as a percentage of the activity of control mice.

Rectal temperatures (Table III) were taken with an Electric Universal thermometer using male mice. Control animals received an equivalent injection of water only. A statistical analysis revealed the significance of the difference in temperature between treated and control mice. In some experiments the effects of prior administration of *N*-methyl-α-5,9-dimethyl-6,7-benzomorphan on the hypothermic and depressant activities of *Ib* and *IIb* were determined. These results are summarized in the bottom columns of Tables II and III.

DISCUSSION

The analgesic activities of compounds I–III listed in Table I are not outstanding, but it is relevant to note that the differences in each pair of compounds (*a* and *b*) are such as to preclude the possibility that hydrolysis of the 2-bromoethylamine to the 2-hydroxyethylamine is a prerequisite for analgesic activity. However, it is not entirely clear to what extent the effects of *Ib* and *IIb* listed in Table I are due to analgesic or to depressant actions since mice receiving these compounds were found to be profoundly depressed. *Ib* and *IIb* were shown to produce a severe reduction in the locomotor activity of mice (Table II) which, in the case of *IIb*, was still pronounced 24 hr. after administration. None of the other compounds listed in Table I produced depression, indeed, *N*-methyl-α-5,9-dimethyl-6,7-benzomorphan (standard) produced an increase in locomotor activity. These results are in general agreement with the data of Table I which show that compounds *Ia*, *IIa*, and *IIIa* are closely similar to *N*-methyl-α-5,9-dimethyl-6,7-benzomorphan with respect to times of onset and duration of action and thus probably are exhibiting a genuine analgesic effect, whereas *Ib* and *IIb* exhibit a delayed onset and prolonged duration of "analgesic action" which may, in fact, be attributed to depression rather than to true analgesia.

The prolonged depressant actions of *Ib* and *IIb* were also accompanied by severe and prolonged decreases in body temperature (Table III). The onset and duration of the hypothermia paralleled approximately the depressant actions. It is particularly interesting to note that only compounds *Ib* and *IIb* produced hypothermia and that the other compounds listed in Table I had no significant effect on body temperature.

The mechanisms by which *Ib* and *IIb* produce their prolonged effects remain to be elucidated. Since the alcohols (*Ia* and *IIa*) corresponding to *Ib* and *IIb* are devoid of both depressant and hypothermic activities, it is possible that the prolonged duration of pharmacological activity of *Ib* and *IIb* is due to alkylation, by the derived ethyleniminium ions, of specific sites in the CNS concerned with motor and temperature regulation. In order for this to occur, these compounds must enter the CNS: it is pertinent in this connection to note that *IIIb*, which will cyclize very rapidly to a quaternary ethyleniminium ion (14) probably incapable of entering the CNS, is devoid of pharmacological activity. Compounds *Ib* and *IIb* will be significantly less reactive (14) and thus may enter the CNS before cyclization to the ethyleniminium ion takes place. Alternatively, *Ib* and *IIb* may be slowly metabolized to an active product which is neither rapidly metabolized nor excreted and hence produce prolonged activity. McMahon *et al.* (15) have demonstrated, for example, that α-(−)-acetylmethadol is slowly metabolized to the active product α-(−)-noracetylmethadol the analgesic effects of which are fairly prolonged. However, the present work does not serve to distinguish either of these possibilities.

The pretreatment studies (Tables II and III, bottom columns) offer some evidence that the spectrum of activities of *N*-methyl-α,5,9-dimethyl-6,7-benzomorphan and *Ib* and *IIb* are different, since the depressant and hypothermic effects were not reduced by prior administration of the standard compound, as might have been expected had all three compounds acted at a common receptor site.

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Keyphrases

α-5,9-dimethyl-6,7-benzomorphans, *N*-substituted—synthesis
Pharmacological screening—5,9-dimethyl-6,7-benzomorphans
Analgesic activity
Depressant activity
Hypothermic activity

Titration of Residual Moisture in Lyophilized Vials

By EDGAR E. THEIMER and JOSEPH J. PAVELEK

Karl Fischer titration of residual water in lyophilized vials can be performed very simply, without specialized equipment, and gives results which are more reliable than loss-on-drying methods.

IN THE determination of moisture, the Karl Fischer (K.F.) titrimetric method presents many advantages over the weight-loss-on-drying method. The literature abounds with comparative studies, most of which favor the K.F. method. Levy, Murtaugh, and Rosenblatt (1) titrated water in penicillin vials, assuming a zero blank. Wiberley (2) stressed the use of micro-equipment and introduced some improvements intended to broaden the use of the K.F. reagent. Burger and Polderman (3) used similar technique in some precise work on effect of lyophilizing conditions on residual water, using mannitol, *etc.* For lyophilized proteinaceous preparations, Sobel (4) suggested a colorimetric procedure, and Alajos (5) devised a special two-chambered vial for extraction of water and subsequent K.F. titration, to prevent direct contact of K.F. reagent with the protein.

Figure 1 depicts a vial containing a lyophilized mixture of 100 mg. of mannitol and 20 mg. of acetylcholine chloride.[1] It was desired to set up a specification limiting the water content of these vials in production batches. The primary aim of this study was to devise a simple quantitative test for water, of reasonable precision, without the use of specialized apparatus, and as free as possible from sources of error.

The first test method used involved drying in a vacuum oven to constant weight. Since it seemed likely that the rubber center seal might lose some weight during this treatment, similar blank vials were simultaneously treated and a correction applied. After further trials, it became evident that this correction value was variable, sometimes becoming as large as 0.6 mg. Also, it was found difficult to prevent weight gain between drying and weighing. Another difficulty was the need for microweighing technique in handling relatively large vials. In addition, there was some evidence that slight differences in drying temperature gave different results. In order to deal with these difficulties, various rather awkward dodges were devised, including weighing the vials while still hot, withdrawing and discarding the center seal, and pairing of samples with individual blank vials, but eventually a different water determination method was sought.

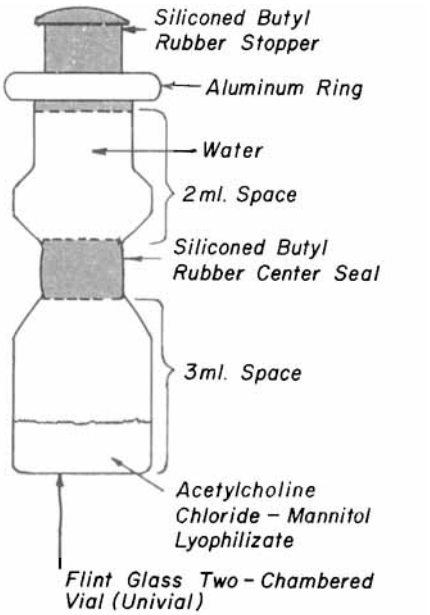

Fig. 1—Vial containing lyophilized mixture of 100 mg. mannitol and 20 mg. acetylcholine chloride.

Received October 18, 1967, from the Analytical Section, Research & Development Division, Smith, Miller & Patch, Inc., New Brunswick, NJ 08902
Accepted for publication December 18, 1967.
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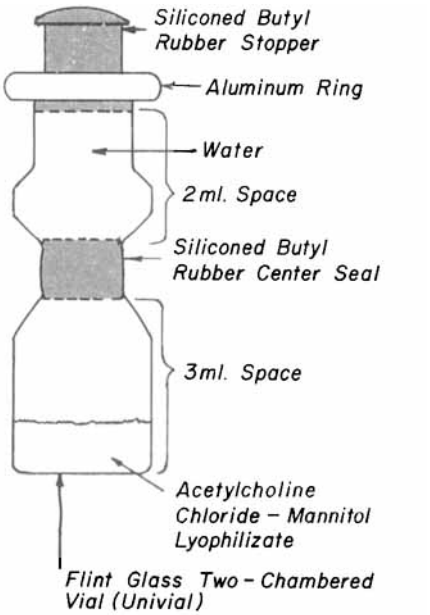

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The technique in the following method is simple and probably free from important sources of error. It does not involve the use of a magnetic stirrer, titrating vessel with special cover, or humidity-controlled work chamber, nor special drying of the diluent, methanol. In the case dealt with here, with the white background afforded by the methanol-insoluble mannitol, the end point can be detected visually when strong K.F. reagent is used and no electrometric equipment is needed. By using the vial as titrating vessel, it is possible to avoid sample transfer and exposure to the atmosphere. Also, the vial being designed to allow penetration of the stopper with a hypodermic needle for withdrawing the contents without exposure to the atmosphere, a hypodermic needle can be similarly used for addition of K.F. reagent. These advantages are important because the amount of water determined is less than 1 mg. and the sample is hygroscopic.

The actual amount of water in the methanol and the "handling blank" involved in the manipulations need not be determined directly, since they cancel out automatically.

TITRIMETRIC DETERMINATION OF WATER IN FREEZE-DRIED ACETYLCHOLINE CHLORIDE-MANNITOL

Reagents—*Methanol*—Rinse adequately an automatic buret equipped with a silica-gel drying tube, with absolute or anhydrous grade methanol; then fill it with methanol. Various brands were used in this work. A.C.S. anhydrous methanol "low in acetone" (Matheson, Coleman, and Bell) was found to give the lowest blank.

Water in Methanol—Rinse adequately a 100-ml. volumetric flask with a needle-penetrable stopper, using methanol from the same container. Add exactly 0.1 ml. water. Add methanol to volume.

Karl Fischer Reagent—Use the stabilized reagent available commercially with each ml. equivalent to approximately 5 mg. water. K.F. Reagent, single solution, stabilized (Fisher Scientific Co.) was used.

Protect all liquids from atmospheric moisture as much as possible.

Procedure—Preparation of vials and stoppers for blanks and standards. Obtain 10–20 vials similar to the sample vials, with siliconed stoppers and center-seals. Remove metal rings. Discard water in the top chambers. Push center seals through, using a metal rod or similar tool. Clean vials and stoppers by rinsing, as necessary, with water, acetone, and an air stream. Place them in a 105–110° oven for 1 hr. Remove the vials and stoppers from the oven, and stopper the vials at once. Store in a desiccator until used.

Preparation of Samples—Remove stoppers and metal rings. Pour water out from top chamber. Dry stoppers and top chambers with acetone and a stream of air. Place vials and stoppers in 105–110° oven for 1 hr. Remove, and at once insert oven-dried stoppers. Store in a desiccator until used.

Titration of Blanks—Remove stopper from vial, quickly add 2.0 ml. of methanol from buret and replace stopper. Release pressure by puncturing stopper with breather needle. Remove needle. Shake well. Titrate with K.F. reagent from a 1-ml. tuberculin syringe and needle. Repeat until precision confidence is established.

Titration of Standards—With a calibrated syringe and needle, withdraw water in methanol through the stopper, first rinsing the syringe several times, then measuring 2.0 ml. Remove stopper from Univial and quickly add the 2.0 ml. water in methanol and replace stopper. Insert breather needle. Shake well. Titrate with K.F. reagent from 1-ml. tuberculin syringe and needle. Repeat until precision confidence is established.

Titration of Samples—Remove stopper, quickly push center seal through, using a suitable instrument, add 2.0 ml. methanol from buret and replace stopper. Release pressure by puncturing stopper with breather needle. Remove needle. Shake well. Titrate with K.F. reagent from 1-ml. tuberculin syringe and needle. Fading of amber color is due to slow extraction of the last bit of water from the crystals by K.F. reagent. The end point should be stable for 15 sec.

Calculation—Sample titration = V_{sam}, blank = V_B, standard = V_{stan}. Then the weight of water in the lower chamber of the sample is:

$$\frac{V_{sam} - V_B}{V_{stan} - V_B} \times 2 \text{ mg.}$$

DISCUSSION AND RESULTS

The blank titer combines K.F. reagent comsumption due to: (*a*) water in the methanol reagent. (*b*) moisture unavoidably introduced in the handling.

Although some attempts were made to use as blanks vials containing oven-dried samples, the values showed unsatisfactory reproducibility. It was felt that any difference in titer between such blanks and the empty-vial blanks eventually adopted was due to the handling errors caused by the extreme hygroscopicity of acetylcholine chloride.

In order to justify the use of empty-vial blanks it was necessary to determine whether completely dry acetylcholine chloride or mannitol consume K.F. reagent. Two-gram samples of each were oven-dried to constant weight in flasks, closed with rubber-stoppers fitted with a short needle-penetrable rubber core, and cooled in a desiccator. Twenty-five milliliters of methanol was added quickly by pipet and the flasks were titrated with K.F. reagent. An empty flask was similarly treated, as a blank.

It was found that mannitol was easily dried at 105° to give zero net titer, and that acetylcholine chloride could be dried to a negligibly small or zero net titer by heating at 110° for 16 hr., although 105° was not hot enough to drive off the last 0.1% moisture in some cases even after 4 days heating.

In order to study the effect of the drying temperature used in preparing blanks, the following experiment was run. Two groups of samples, all from the same batch, were prepared:

(*A*) Top chamber dried with acetone, an air-stream, and 10 min. warming close under an infrared lamp. (Infra-red Economy Heater, Fisher Scientific Co.).

(*B*) Top chamber dried with acetone, an air stream, and heating entire vial at 110° for 3 hr.

The vials were then stoppered with oven-dried stoppers, and titrated in the usual way. The titration values are shown in Table I. The following conclusions may be drawn from this experiment.

(*a*) The brief warming of the top chamber of the Group A vials is sufficient to dry them, even though the center seal has been in contact with water for a

Table I—Reagent Needed to Titrate Samples Subjected to Varying Pretreatment, ml.

A	B
0.23	0.24
0.24	0.25
0.28	0.30
0.25	0.29
0.23	0.22
0.28	0.24
0.25	0.23
0.27	0.24
0.24	0.30

authors found that a lyophilized B complex containing 10 mg. riboflavin could be titrated to an easily identified end point despite the color in the sample. Other types of sample have not been tested but it would seem that the method is applicable to any parenteral-type vial containing lyophilized or powdered sample low in water content, unless it is deeply colored or contains a substance which interferes with the titration, such as ascorbic acid.

Simple vials do not require the preliminary preparation described for the double-chambered vial, but can be titrated directly.

Table II—Determination of Water in Freeze-dried Acetylcholine Chloride–Mannitol Mixture

Blanks	Standards	Samples			
		Lot X		Lot Y	
Titer, ml.	Titer, ml.	Titer, ml.	H_2O, mg.	Titer, ml.	H_2O, mg.
0.07	0.45	0.22	0.77	0.23	0.83
0.07	0.44	0.28	1.09	0.18	0.56
0.08	0.45	0.21	0.72	0.17	0.50
0.07	0.45	0.38	1.63	0.19	0.61
0.09	0.45	0.22	0.77	0.25	0.93
Av. = 0.076	Av. = 0.448	0.20	0.66	0.17	0.50
		0.23	0.83	0.21	0.72

long time and might be expected to have absorbed some water not easily driven off.

(*b*) There is no significant change in K.F. titer even at 110° for 3 hr., either through volatilization of water through the center seal or through chemical reaction.

For simplicity, oven heating was adopted for the procedure.

Table II shows some results obtained by the proposed method, the values for water in samples of Lot X and Y being calculated using the average blank and standard titers.

A recalibrated pipet was used in preparing the water in methanol standard. A weighed quantity of standard hydrated sodium acetate might be considered as an alternate.

Applicability to Other Types of Sample—The

CONCLUSION

It is believed that the proposed titrimetric procedure is the method of choice for determining water in certain applicable cases.

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Keyphrases

Lyophilized substances—moisture in vials
Vials—moisture determination
Karl Fischer titration—analysis
Methanol—reagent

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By GREGORY T. SINNER* and LEE C. SCHRAMM†

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Species of Claviceps have been found as parasites of nearly all members of the grass family (1).

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name which was never widely accepted. In 1959 Pantidou (4) published a complete description of wild rice ergot and proposed the presently accepted binomial, *Claviceps zizaniae* (Fyles) Pantidou, again as a species distinct from the common rye ergot *C. purpurea* (Fries) Tulasne. The consideration as a separate species results from discrete differences in physical characteristics of the sclerotia, and from the apparent host specificity of *C. zizaniae*.

Wild rice abounds in Minnesota and Wisconsin. Infestation of the natural stands of wild rice with *C. zizaniae* is common. The availability of sclerotia of wild rice ergot from local sources prompted the present phytochemical investigation for moisture, fat, and alkaloid content. An isolate of this fungus was grown in agitated submerged liquid culture, and the culture filtrate was examined for alkaloids.

EXPERIMENTAL

Sclerotia of wild rice ergot were collected from central Minnesota, near Fergus Falls, and from northern Wisconsin, near Spooner. These sclerotia corresponded to the description of Pantidou (4). The average size was 12 mm. in length (4–16 mm.) and 5 mm. in width, externally pink to black in color and having a brilliant purple fracture.

Moisture Determination—The sclerotia were ground in a Wiley Mill to a coarse powder. Moisture content was accomplished by determining weight loss after drying at 108° to constant weight. The average weight loss on drying was 4.6%.

Fat Determination—Samples of powdered wild rice ergot dried at 108° were extracted with petroleum ether by continuous extraction in a Soxhlet apparatus for 12 hr. Fat content was determined by loss of weight during extraction. The average fat content was 32% by weight.

Alkaloid Determination—The methods of Silber and Schulze (5) and Michelon and Kelleher (6) were used for the detection of alkaloids in wild rice ergot and on the filtrates of 7-day-old cultures of *C. zizaniae*.

Colorimetric analysis of the alkaloid content of wild rice ergot indicated the presence of alkaloids, but in a concentration of approximately 0.1 mg. %, the lower limit of detection of the analytical procedure. Water-soluble alkaloids appear to be absent in wild rice ergot, although they may have escaped detection because of a limited quantity of sample available for study. No indication of water-soluble alkaloids was observed chromatographically.

Investigation of Liquid Cultures—Individual sclerotia were sterilized by submersion in the following solutions for 30 sec. each: benzalkonium[1] chloride (1:500); sterile distilled water; 95% alcohol; 70% alcohol; 50% alcohol; sterile distilled water. After sterilization and rinsing, each sclerotium was peeled with a sterile scalpel; a small cubical portion (approximately 3 mm. on a side) was placed on a sterile agar slant of medium prepared according to Brady and Tyler (7). The slants were incubated at 26°; small portions of the mycelium were transferred to new medium monthly. Liquid cultures were prepared from 7-day-old slants by transferring a small portion of mycelium to 100 ml. of a sterile liquid medium modified slightly from Arcamone's "medium A" or preculture medium (8) consisting of 4% mannitol, 1% succinic acid, 0.1% neopeptone, 0.1% KH_2PO_4, 0.03% $MgSO_4$, and tap water, contained in a 500-ml. conical flask. The liquid cultures were incubated in a constant temperature room at 26° on a rotary shaker (300 r.p.m., 2.54-cm. diameter rotation). Filtrates of agitated submerged cultures of *C. zizaniae* were assumed not to contain ergot alkaloids since there was only a very weak reaction of the filtrate with the PDAB reagent. The color developed was not intense enough to be detectable in the analytical procedure.

Chromatographic Examination—Attempted identification of the alkaloids was accomplished on Silica Gel G thin-layer plates using two solvent systems comprised of benzene–ethanol (1:1) and (1:0.6) and on Alumina G thin-layer plates using solvents composed of chloroform, chloroform–*n*-butanol (10:1), and *n*-butanol. Each chromatoplate was examined under ultraviolet light to detect fluorescent substances, then sprayed with 0.2% para-dimethylaminobenzaldehyde in 18% HCl (PDAB) as a detection reagent.

On the silica gel plates, two alkaloid spots were observed. Although both yielded a blue fluorescence in ultraviolet light, they were readily differentiated on the basis of their reaction with the PDAB spray reagent. The alkaloid with the lower R_f value yielded a blue color with PDAB, while the other alkaloid gave a purple color with the reagent. The R_f values in the benzene–ethanol (1:1) system were 0.79 and 0.87; for the benzene–ethanol (1:0.6) system these values were 0.72 and 0.80.

On alumina plates, only one spot was observed. This spot also fluoresced blue and formed a purple or blue color with PDAB. The R_f value in the three solvent systems ranged from 0.93 to 0.96. Although other attempts were made to identify these alkaloids chromatographically, the results were inconclusive.

[1] Zephiran chloride, Winthrop Laboratories, New York, N. Y.

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Keyphrases

Ergot—wild rice fungus
Wild rice, *Zizania aquatica*—phytochemical properties
Cultures, liquid—*Claviceps zizaniae*
TLC—identity

Interpretation of the Kinetics of Salicylic Acid Elimination

Sir:

Wagner (1) contends that the data relating to the elimination of salicylate can be interpreted on the basis of first-order kinetics. The present communication presents evidence which indicates that the application of Wagner's theoretical considerations to these particular aspects is not acceptable. It also reaffirms the validity and application of certain equations which Wagner (1) has claimed to be erroneous.

It was first suggested by Cummings (2) in 1963 that when the plasma salicylate level exceeds a certain value, the formation of salicyluric acid closely approaches a zero-order process. Evidence has been presented by Cummings and Martin (3–6) to support the view that the elimination of salicylate in man cannot be completely described by first-order kinetics, and that above a certain drug level its elimination can be better described in terms of simultaneous first-order and zero-order processes. It may be pointed out that Wagner makes no reference to the publication of Bedford, Cummings, and Martin (5). A similar interpretation of experimental results has been offered by Levy (7) and Nelson, Hanano, and Levy (8).

The evidence for this interpretation can be considered as follows.

1. The fraction of the dose of aspirin which is eliminated and excreted in the urine at certain specified times shortly after dosage is almost constant for doses of 160, 240, and 320 mg., but at higher dosage the fraction of the dose excreted at these times becomes progressively less (5). This is explicable on the basis that the elimination of salicylate is not first order when the dose of aspirin exceeds 320 mg., when it is assumed that the absorption step is not rate limiting.

Furthermore, the percentage of the dose which is eliminated as salicyluric acid is less after the larger doses than after the small doses (5). This suggests that the rate of formation of salicyluric acid is not always proportional to the amount of salicylic acid in the body.

2. When the decline of the serum salicylate concentration and of the rate of excretion of salicyluric acid in urine was followed for about 40 hr. after loading doses of aspirin—1 Gm. every 6 hr. for 36 hr. (5, 6): (*a*) Plots of these values against time could not be described as log-linear for 20 hr. or more after the final dose. (*b*) The rate of excretion of salicyluric acid remained almost constant between the sixth and the 17th hr. after the final dose. (*c*) These plots ultimately tended to become log-linear with a slope similar to that of corresponding plots obtained after a low single dose of aspirin. (*d*) The amount of salicylic acid remaining in the body at a time when elimination approaches a first-order rate was calculated to be 150–300 mg. (5).

If an explanation of these findings based on the continuing absorption and distribution of salicylic acid over a period of 20 hr. is not accepted, the experimental findings provide a strong indication that the formation of salicyluric acid closely approaches a zero-order rate during the period between the sixth and the 17th hr.

Wagner was able to demonstrate in his model (Example 1) that first-order kinetics can give rise to a plateau in the excretion rate plot from the third to the sixth hour. Such an explanation cannot, however, be considered sufficient to account for a plateau extending over a period of 11 hr.

3. The area under the salicylate plasma level *versus* time plot appears to be much larger when aspirin is rapidly absorbed than when the equivalent dose is slowly absorbed (3, 4), although the total urinary recovery of drug after 24 hr. is the same in both instances (9). The maximum plasma salicylate level obtained when aspirin was rapidly absorbed is almost double that obtained when aspirin was slowly absorbed, yet the maximum rates of excretion of total salicylate in urine were almost identical (3, 4). This suggests that the elimination of salicylate is relatively slower at the higher drug level which results when absorption is rapid, assuming that the total amount of aspirin absorbed within the period of the study, *viz.*, 7 hr., is the same in both instances.

4. There is a considerable difference in the maximum plasma salicylate level obtained after a single 1-Gm. dose of aspirin and after multiple 1-Gm. doses of aspirin. Thus, in a group of individuals, a single 1-Gm. dose gave an average maximum observed value of 5.1 mg./100 ml. (10), whereas after seven doses each of 1 Gm. administered six hourly, the average maximum observed value was 18.1 mg./100 ml. (4).

An attempted interpretation of salicylate elim-

ination based on first-order kinetics gives a half-life equal to 2–3 hr., as calculated from the slope of the terminal log-linear section of the plasma level plot or urinary excretion plot after a small (0.3 Gm.) dose of aspirin (5). Wagner (1) makes use of a similar value. On this basis a 6-hr. multiple-dosage schedule corresponds to a dosage interval which is equal to 2–3 half-lives of the drug, and this would give a plasma salicylate level only marginally greater than that obtained with a single dose. The plasma levels obtained, however, are at least three times greater. This suggests that the half-life of salicylate which pertains after multiple 1-Gm. doses is certainly much longer than 2–3 hr. and would appear to be nearer 10 hr.

It is submitted that the cumulative evidence refutes Wagner's (1) contention that the elimination of salicylate can be interpreted solely on the basis of a first-order kinetic model. It is considered that the experimental observations can be better described by a model based on simultaneous first-order and zero-order elimination as postulated by Cummings and Martin (3–6) and by Levy (7, 8).

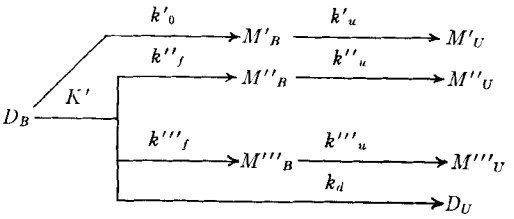
Wagner (1) also states that Cummings and Martin (11) have incorrectly used the Butler equation (12). This is not so. The considerations of Cummings and Martin (11), as those of Butler (12), were stated to apply to a substance, in this instance a metabolite, which was eliminated only by excretion, and in this case the elimination rate constant and the excretion rate constant are one and the same.

Wagner (1) states that the equation presented by Cummings, Martin, and Park (13), with respect to simultaneous first-order and zero-order drug elimination, is erroneous. The equation appears to be perfectly valid and its derivation in a more general form, is given below.

RATE OF EXCRETION OF TOTAL DRUG IN URINE WHEN ONE METABOLITE IS FORMED AT A ZERO-ORDER RATE

The kinetics of the excretion of total drug in urine is considered with respect to a model drug which is entirely eliminated by excretion in the urine and by metabolism to three metabolites (M', M'', M'''); the metabolites are eliminated solely by excretion in the urine. Thus, "total drug in urine" is defined as the sum of the amounts of drug and metabolite excreted and this will ultimately be equivalent to the amount of drug absorbed. One metabolite, M', is formed at a zero-order rate, otherwise all the processes of drug elimination and metabolite excretion are first order.

The processes of drug elimination may be represented as follows:



These processes are described by the following equations in which:

D_0	= The amount of drug in the body at a time when the process of absorption can be considered to be complete, this is designated $t = 0$
D_B	= The amount of drug in the body at time t
M_0	= The amount of metabolite in the body at a time $t = 0$
M_B	= The amount of metabolite in the body at time t
D_U	= The amount of drug excreted in the urine at time t
M_U	= The amount of metabolite excreted in the urine at time t
k'_0	= The zero-order rate constant for the formation of metabolite M'
k''_f, k'''_f	= The first-order rate constants for the formation of the metabolites M'' and M''', respectively
k_d	= The first-order rate constant for the excretion of drug
k'_u, k''_u, k'''_u	= The first-order rate constant for the excretion of the metabolites M', M'', and M''', respectively
K'	= $k_d + k''_f + k'''_f$

Thus,

$$-\frac{dD_B}{dt} = K'D_B + k'_0 \qquad \text{(Eq. 1)}$$

and integration between $t = 0$ and $t = t$ gives:

$$D_B = [D_0 + k'_0/K']e^{-K't} - k'_0/K' \qquad \text{(Eq. 2)}$$

Also,

$$M'_B = \frac{k'_0}{k'_u}[1 - e^{-k'_u t}] + M'_0 e^{-k'_u t} \qquad \text{(Eq. 3)}$$

and

$$M''_B = \frac{k''_f}{k''_u - K'}[D_0 + k'_0/K'][e^{-K't} - e^{-k''_u t}] - \frac{k''_f k'_0}{k''_u K'}[1 - e^{-k''_u t}] + M''_0 e^{-k''_u t} \qquad \text{(Eq. 4)}$$

Substitution of k'''_f and k'''_u for k''_f and k''_u in Eq. 4 gives the corresponding equation for M'''_B.

The rate of excretion of drug and of drug metabolites in urine is given by:

$$\frac{dD_u}{dt} = k_d D_B; \quad \frac{dM'_u}{dt} = k'_u M'_B; \quad \frac{dM''_u}{dt} = k''_u M''_B; \quad \frac{dM'''_u}{dt} = k'''_u M'''_B.$$

The rate of excretion of total drug in urine is ob-

tained by summing the individual rates, so that:

$$\frac{d[D_u + M_u]}{dt} = \frac{dD_u}{dt} + \frac{dM'_u}{dt} + \frac{dM''_u}{dt} + \frac{dM'''_u}{dt}$$

where

$$M_u = M'_u + M''_u + M'''_u$$

Then,

$$\frac{d[D_u + M_u]}{dt} = \left[k_d + \frac{k''_f k''_u}{k''_u - K'} + \frac{k'''_f k'''_u}{k'''_u - K'}\right] \times [D_0 + k'_0/K']e^{-K't} - \left[\frac{k''_f k''_u}{k''_u - K'} e^{-k''_u t} + \frac{k'''_f k'''_u}{k'''_u - K'} e^{-k'''_u t}\right][D_0 + k'_0/K'] + \frac{k'_0}{K'}[k''_f e^{-k''_u t} + k'''_f e^{-k'''_u t} - K'e^{-k'_u t}] - \frac{k'_0}{K'}[k_d + k''_f + k'''_f - K'] + k'_u M'_0 e^{-k'_u t} + k''_u M''_0 e^{-k''_u t} + k'''_u M'''_0 e^{-k'''_u t} \quad \text{(Eq. 5)}$$

The term $[k_d + k''_f + k'''_f - K'] = 0$, since by definition $K' = k_d + k''_f + k'''_f$, therefore only exponential terms remain.

Equation 5 is thus of the form:

$$\frac{d[D_u + M_u]}{dt} = Ae^{-K't} + Be^{-k'_u t} + Ce^{-k''_u t} + De^{-k'''_u t} \quad \text{(Eq. 6)}$$

where A, B, C, and D are constants.

When all k_u are large relative to K' and when t is large, all the $e^{-k_u t}$ terms become very small relative to the $e^{-K't}$ term and so Eq. 6 approaches:

$$\frac{d[D_u + M_u]}{dt} = Ae^{-K't} \quad \text{(Eq. 7)}$$

This equation may then be written:

$$\ln \frac{d[D_u + M_u]}{dt} = \ln [\text{const.}] - K't \quad \text{(Eq. 8)}$$

which is the equation of a straight line having a slope equal to $-K'$.

A plot of the log rate of excretion of total drug against time ultimately exhibits a linear section with a slope equal to the sum of the rate constants which govern the first-order processes of drug elimination when a zero-order process is occurring simultaneously.

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Received June 22, 1967.
Accepted for publication February 6, 1968.

Keyphrases

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Elimination kinetics—salicylic acid
Zero, first-order process—salicylic acid elimination

Deterioration of Nitroglycerin Tablets

Sir:

The effect of packaging and storage conditions on drug product stability is a subject of growing interest and concern (1). We wish to report here our investigations on the relationship between certain novel packaging materials and the deterioration of nitroglycerin tablets.

FDA recently found that a batch of nitroglycerin tablets sealed in aluminum foil was grossly subpotent. The faulty product had been prepared by individually wrapping 0.43-mg. ($^1/_{150}$-grain) nitroglycerin tablets in strips of aluminum foil to which a thin film of polyethylene had been laminated. Each tablet was placed between the polyethylene surfaces of two strips, and the ends were heat-sealed to provide a 25 × 15 mm. cell.

Assays of individual tablets showed that they contained 0–10% of the declared quantity of nitroglycerin (2). However, when the packaging material in one cell was extracted with isooctane, the extract contained 0.36 mg. of nitroglycerin [determined colorimetrically with phenoldisul-

tained by summing the individual rates, so that:

$$\frac{d[D_u + M_u]}{dt} = \frac{dD_u}{dt} + \frac{dM'_u}{dt} + \frac{dM''_u}{dt} + \frac{dM'''_u}{dt}$$

where

$$M_u = M'_u + M''_u + M'''_u$$

Then,

$$\frac{d[D_u + M_u]}{dt} = \left[k_d + \frac{k''_f k''_u}{k''_u - K'} + \frac{k'''_f k'''_u}{k'''_u - K'}\right] \times [D_0 + k'_0/K']e^{-K't} - \left[\frac{k''_f k''_u}{k''_u - K'} e^{-k''_u t} + \frac{k'''_f k'''_u}{k'''_u - K'} e^{-k'''_u t}\right][D_0 + k'_0/K'] + \frac{k'_0}{K'}[k''_f e^{-k''_u t} + k'''_f e^{-k'''_u t} - K'e^{-k'_u t}] - \frac{k'_0}{K'}[k_d + k''_f + k'''_f - K'] + k'_u M'_0 e^{-k'_u t} + k''_u M''_0 e^{-k''_u t} + k'''_u M'''_0 e^{-k'''_u t} \quad \text{(Eq. 5)}$$

The term $[k_d + k''_f + k'''_f - K'] = 0$, since by definition $K' = k_d + k''_f + k'''_f$, therefore only exponential terms remain.

Equation 5 is thus of the form:

$$\frac{d[D_u + M_u]}{dt} = Ae^{-K't} + Be^{-k'_u t} + Ce^{-k''_u t} + De^{-k'''_u t} \quad \text{(Eq. 6)}$$

where A, B, C, and D are constants.

When all k_u are large relative to K' and when t is large, all the $e^{-k_u t}$ terms become very small relative to the $e^{-K't}$ term and so Eq. 6 approaches:

$$\frac{d[D_u + M_u]}{dt} = Ae^{-K't} \quad \text{(Eq. 7)}$$

This equation may then be written:

$$\ln \frac{d[D_u + M_u]}{dt} = \ln [\text{const.}] - K't \quad \text{(Eq. 8)}$$

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Assays of individual tablets showed that they contained 0–10% of the declared quantity of nitroglycerin (2). However, when the packaging material in one cell was extracted with isooctane, the extract contained 0.36 mg. of nitroglycerin [determined colorimetrically with phenoldisul-

fonic acid (3)] or 84% of the declared quantity. The carbon disulfide extract of another cell exhibited the infrared absorption spectrum of nitroglycerin, when compared to a blank consisting of a carbon disulfide solution of extractives from the laminating adhesive.

Our explanation of these findings is based upon three observed facts: the low but appreciable volatility of nitroglycerin at room temperature; the permeability of plastics, such as polyethylene, polypropylene, and polyvinyl chloride to nitroglycerin vapor; and the solubility of nitroglycerin in the adhesive used to bond the polyethylene to the aluminum foil.

When nitroglycerin tablets are placed in an open container, the drug substance evaporates. If the tablets are sealed in an envelope of plastic film, evaporation may be retarded, but it proceeds to eventual depletion if the packet remains exposed in an open vessel. By contrast, our investigations have shown that when nitroglycerin tablets are stored in a tightly stoppered glass container they retain essentially the same concentrations of active ingredient for prolonged periods. Within such a closed system, evaporation away from the dosage form proceeds only until the comparatively low partial pressure of nitroglycerin in the available space is equal to the vapor pressure of the compound in the tablets, and a dynamic equilibrium is achieved. If the bottle is opened intermittently to remove tablets, nitroglycerin vapors escape, but the loss is usually so small that changes in tablet composition are negligible.

Although the sealed aluminum package comprising the deteriorated product under discussion also represents a closed system, an additional operating factor prevents establishment of a stabilizing dynamic equilibrium. Because nitroglycerin is soluble in the laminating adhesive, the vapors that escape from the surface of the tablet and diffuse through the polyethylene are continuously removed from the atmosphere by incorporation into the film of adhesive. Thus the flow of nitroglycerin molecules is unidirectional away from the tablet and all of the drug migrates to the packaging material.

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Received December 11, 1967.
Accepted for publication February 23, 1968.

Keyphrases

Nitroglycerin tablets
Deterioration—nitroglycerin tablets
Aluminum foil package—tablet stability
Plastic film package—tablet stability

JOURNAL OF Pharmaceutical Sciences

June 1968 volume 57, number 6

Review Article

The Binding of Drugs by Plasma Proteins

By MARVIN C. MEYER* and DAVID E. GUTTMAN†

GOLDSTEIN'S CLASSIC REVIEW (1) of interactions between drugs and proteins, which was published in 1949, surveyed and summarized a rather large literature and, in addition, clearly and lucidly emphasized the potential importance of protein binding with respect to the behavior of drugs. His review undoubtedly contributed to an increased awareness of the significance of protein binding and stimulated many investigators to study various aspects of the phenomenon. A large number of publications, describing such studies, has appeared in the 18 years since his review. All, either directly or indirectly, resulted from the desire to answer one or more of the five fundamental guideline questions posed by Scatchard *et al.* (2): "How many? How tightly? Where? Why? What of it?" Thus, many of the reported studies were physical-chemical in nature, utilized synthetic *in vitro* systems, and were oriented to the determination of the maximum number of: (*a*) small molecules which could be reversibly bound by a protein molecule, (*b*) the association constant or constants and other thermodynamic parameters which characterized the reversible association, (*c*) the chemical and conformational nature of the binding sites on the protein molecule, and (*d*) the nature of the intermolecular forces which were responsible for interaction. Other studies, by necessity of a more qualitative nature, attempted to experimentally and/or theoretically assess the importance of protein binding as it relates to the actions and uses of specific drugs.

Excellent general discussions of such studies have appeared. For example, principles and concepts fundamental to an understanding of the phenomenon were discussed by Foster (3), Weber (4), Klotz (5), Edsall and Wyman (6). Brodie and Hogben (7), in a review of physicochemical factors in drug action, discussed reversibility of binding, binding forces, and possible therapeutic consequences resulting from drug–protein interactions. Van Os *et al.* (8) briefly considered the influence of protein binding on drug excretion, drug concentration in tissue fluids, therapeutic activity, and toxicity of drugs. Several chapters, which were devoted to this topic, appeared in a book edited by Binns (9). Brodie (10), for example, cited the importance of binding on the kinetics of drug penetration through biological membranes. He also reviewed the concept of protein–drug complexes functioning as "storehouses" for drug in the body, effectively buffering the level of free drug in the biological fluids. Thorp (11) considered some general aspects of the binding phenomenon and noted that the majority of drug interactions with albumin appear to involve a single binding site on the protein. He speculated that rates of association and dissociations of drug–protein complexes are probably sufficiently great so as to not hinder drug transport from the plasma. The proceedings of a symposium entitled "The Transport Function of Proteins" edited by Desgrez and De Traverse (12) contains a number of chapters

Received from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

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which are directly related to important aspects of protein–small molecule interactions. Bennhold (13), for example, summarized studies pertaining to the transport of dyes. Guillot (14) reviewed the nature of intermolecular forces responsible for binding. Presentations by Vannotti *et al.* (15) and by Salvatore *et al.* (16) dealt with the binding of thyroid hormones. Polonovski (17), Desgrez (18), Raoul (19), and Lathe (20) discussed the role of plasma proteins in the transport of lipids, corticosteroids, vitamins, and bilirubin. Brodie (21) reviewed possible therapeutic implications of drug–protein interactions and considered "drug buffering," the absorption, distribution, and excretion of highly bound drugs, saturation of binding sites, and the displacement of drugs and endogenous substances by the concomitant administration of certain drugs. Truhaut (22) contributed a review of the interactions between a variety of toxic compounds and plasma proteins. Serum inactivation of penicillins due to protein binding has been recognized for many years and has been the subject of many investigations. Warren (23) has summarized much of the current literature and has attempted the difficult task of relating and correlating binding behavior with the clinical performances of a number of penicillins.

Scope of the Present Review—It did seem desirable to supplement the works cited above with a current review of drug–plasma protein interactions. Because of the extensiveness of the literature in this field, the objectives of a review required limitations. It was decided, therefore, to briefly summarize, in tabular form similar to that employed by Goldstein, some important observations for many of the studies reported during the past 18 years. Supplemental to this tabulation, this report will attempt to review some of the newer experimental techniques which have been employed to investigate protein–small molecule interactions, and the methods used to present and to treat experimental data. Additional emphasis will be directed, by reference to pertinent and representative studies, to recent advances which have been made in understanding, evaluating, and predicting the extent to which drug–protein interactions can influence the distribution of drug in the body and the removal of drug from the body. Finally, attention will be drawn to the many studies which have been concerned with the use of agents which compete with a particular small molecule for binding sites on the protein and can, thus, cause an inhibition or diminution of binding.

Tabulations—Studies which have been specifically concerned with the binding, by plasma proteins, of therapeutic agents or endogenous substances of physiologic importance are summarized in Table I. The tabulation is self-explanatory and reflects the current and continuing interest in the interactions of drugs, metabolites, and naturally occurring compounds with plasma proteins. Table II refers, without commentary, to a number of studies which have employed dyes as the substrates in binding experiments. This class of compounds will not be considered in detail and the table is merely presented as a reference to current work for those interested in such systems. While the majority of dyes included in Table II are not of therapeutic importance, they serve as useful tools in general studies of the binding process and such studies have provided insights into the mechanisms and sites of interaction.

References to the many studies dealing with the binding of steroids have been omitted from Table I because of the availability of several recent and extensive reviews of this topic (24–34). The binding of steroids by proteins is the subject of considerable current interest which stems from the possible involvement of binding in events which are of physiological and pharmacological importance. For example, the apparent absence of the manifestations of Cushing's syndrome during pregnancy, when the corticosteroid level is elevated, has been attributed to an increase in plasma protein binding (35). Evidence has been presented by a number of groups to support the existence of a specialized plasma protein, transcortin, which has a high affinity but low capacity for corticosteroids. It has been speculated (21) that many of the important nonsteroidal anti-inflammatory agents may function *in vivo* by displacing bound endogenous corticosteroid. It has also been reported (36, 37) that the administration of oral contraceptives can result in a marked increase in the fractions of cortisol and aldosterone which are bound in the plasma.

In addition to the many studies which have been considered by authors of the various review articles, the results of a number of recent investigations have appeared and are of interest. Akasu *et al.* (38) demonstrated that dehydroepiandrosterone is highly bound. Several workers have studied the binding of free (39) and conjugated estrogens (40, 41) to serum proteins. Pearlman and Crepy (42) utilized gel filtration to measure the binding of testosterone. Kroneberg and Stoepel (43), using the isolated frog heart, determined that the presence of protein inhibited the digitalis-like activity of a prednisolone derivative. Davidson *et al.* (44) determined the binding characteristics of aldosterone and spirolactones. Hydrocortisone binding to rat and guinea pig pro-

TABLE I—STUDIES INVOLVING INTERACTIONS BETWEEN PROTEINS AND DRUGS OR ENDOGENOUS SUBSTANCES

Substance	Protein Studied[a]	Method[b]	Remarks[c]	Ref.[d]
Antibiotics				
I. *Penicillins*				
A. Studies of one drug				
Dicloxacillin	S(H)	DF	B = 97%.	272
Benzylpenicillin	S(H)	B	B = 20–40%.	273
	S(B,R)	D	B = 35–40% (4°-pH 6.2).	274
	S(B,H)	D	B = 35–40% (4°-pH 6.8–7.0).	275
Methylchlorophenylisoxazolylpenicillin	S(H)	D	B = 62% (4°).	276
Penicillin	S,A(H)	D	—	277
	A(H)	D,BF	No binding at therapeutic levels. Binding at higher levels.	278
	A(H)	B	Binding inhibited biological activity.	190
Penicillin G-(piperidine salt)	S(H)	EP(^{35}S)	1° binding to albumin.	279
Penicillin G	S(H,D)	D	B = 34%(H), 14%(D). Displaced by probenecid.	210
	A(GP,H,E)	B	Antigenicity measured.	280
	S(H)	EP(^{35}S)	1° binding to albumin.	281
K-Penicillin G	S	D	0.94U bound/mg. albumin. 1° binding to albumin.	282
	S(D,R)	BF	Determined serum and CSF levels. Displaced by probenecid.	178
B. Studies of two drugs				
Na-penicillin G,V	S(D)	D	B = 25%(pen.G), 34%(pen.V). Probenecid caused no displacement.	209
Penicillin & mycerin	PF	EG	Penicillin binding greater than that of mycerin.	283
Oxacillin & methicillin	S(R)	—	B = 84–90%(oxacillin), 33–51%(methicillin).	284
Aminobenzyl & phenoxypropyl penicillins	S(E)	—	—	285
C. Studies of three or more drugs				
Syncillin, penicillins G,V	S(D)	D	B = 40%(Syn.), 28%(G), 36%(V).	286
3 Penicillin derivatives	S(H)	D,Dc,GF	Reversibility of binding observed.	287
	S,T(R),S(H)	BF	Distribution determined after i.v. administration. Orthocresotinic acid used as displacing agent.	177
	S(G,P,O,E,S)	U	—	288
	S(H)	D	Determined *in vitro* activity and studied displacement by sulfonamides, salicylate, and penicillins.	207
	S(H)	U,B	B = 87%(oxacillin), 63%(phenethicillin), 22%(methicillin).	289
4 Penicillin derivatives	S(H,R), A(H)	D	Over 250 compounds tested for displacing activity.	156, 157
	S(H)	EP	1° binding to A & G(α).	290
	S,L(D,H)	U,BF	Binding and distribution determined in blood and lymph.	174
	S(H)	BF,D	Binding studied after i.v. administration.	291
5 Penicillin derivatives	S(E)	D	n = 1 for 4 of 5 derivatives studied.	292
	S(H)	U		123
7 Penicillin derivatives	S,L(D)	U	Concentration of unbound drug similar in plasma and lymph.	175
	S(H,B)	D	1° binding to albumin, binding reversed by dilution.	293
	S(H,M)	—	—	294
	S,L(D)	BF	Lymph concentration always lower than plasma concentration.	173
8 Penicillin derivatives	S(H)	U,D	Finding decreased *in vitro* activity. Displacement by sulfonamides.	295
9 Penicillin derivatives	S(H), A(B)	B	Minimum inhibitory concentrations determined in serum and broth.	191
	S,CSF,T(R)	BF	B = 30–95% in serum. CSF and brain levels determined.	296
Series of several penicillins	S(H)	B	Binding reduced biological activity.	297
	S	—	Determined biological activity, distribution, and excretion.	298
	S(H)	U,D	Determined urinary excretion.	192
	S(H)	B	Binding increased with increasing mol. wt. of side chain.	299
	A(B)	D,S	Determined Δ F°.	300
	A,G(α)(B)	NMR	Phenyl group implicated in binding.	160
II. *Tetracycline(TC) and Derivatives*				
A. Studies of one drug				
Na *N*-methyllolchloro	S	—	Binding similar to other 7-chloro. derivatives.	301
Demethylchlor-(DMC)	S(H)	D,DF	—	302
Oxy-	S	P	—	303
	S(H)	D	B = 27%, renal clearance was determined.	304
Chlor-	S(H)	U	B = 70% at therapeutic levels. 1° binding to albumin.	262
B. Studies of two drugs				
DMC & TC	S(H)	B	Binding decreased *in vitro* activity.	305
2 TC salts	S(M)	—	—	306
Methacycline derivative & TC	S(H)	D	Half-life, volume of distribution, and clearance determined after i.v. administration.	263
DMC,TC, & derivative	S(H)	U,B	Blood levels studied after oral administration.	307
C. Studies of three or more TC's				
3 TC's	A,G(β,γ)	E	Binding due to several serum fractions.	308
	S(H,D)	D	—	309
	S,T(R)	BF	Binding reversible with dilution.	310
	S(H)	E	—	311

(*Continued on next page.*)

TABLE I—(*Continued.*)

Substance	Protein Studied[a]	Method[b]	Remarks[c]	Ref.[d]
	S(B,H)	EP	Derivatives ranked by degree of association.	312
	S(H,B)	D,T	—	313
4 TC derivatives	S(H)	U	—	314
5 TC derivatives	S(P)	D	1° binding to albumin.	315
6 TC derivatives	S(D,H)	D,U	B = 22–95%, excretion rate highest for least-bound derivative.	316
III. *Miscellaneous Antibiotics*				
Novobiocin	S	D,E	1° binding to albumin.	317
	S(H,B,R)	D	B = 75%(R), 64%(H), 62%(B) at drug concentration of 3 units/ml.	318
	S	B	Serum decreased biological activity *in vitro*.	319
	S(D,B)	D	B = 10%.	320
Streptomycin	A,G(γ)(B)	D	Also studied binding of large organic cations.	321
Spiramycin	A,G(γ)(B,R,M)	E	Tissue distribution determined.	322
Puromycin	A(B),S,G(γ)(H)	D	B = 5–10%, 1° binding to albumin.	323
Antimycin A	A(H,B,E)	B	Succinoxidase bioassay used to detect binding.	324
d,l-Chloramphenicol	S(H)	EP,D	1° binding to albumin. *d* form bound more extensively than *l* form.	325
IV. *Studies with More Than One Class of Antibiotics*				
3 Antibiotics	S	—	—	326
5 Antibiotics	PF(H)	U	—	327
8 Antibiotics	S	D	—	328
	S(H,R,Rt)	E	—	329
10 Antibiotics	PF(H,R)	E,P	Determined distribution into pleural cavity.	179, 180
12 Antibiotics	S(H)	D,GF,B	Also determined sulfonamide binding.	141
14 Antibiotics	S(H)	D,B	Used Freundlich isotherm in treating data.	330
17 Antibiotics	S(E,S,R,B,H)	U	Competition with sulfonamides, phenylbutazone, and salicylate.	163
	S(H)	E	Some association with α-globulin.	331
	S(B,D,Rt,M)	U	Reported % binding and concentration in liver.	332
	S,A(H)	D,B	Determined structure-binding relationships.	333
Sulfonamides				
I. *Studies of One Sulfa*				
Sulfadiazine, 5-ethyl	A(H)	U	*n* and *k* decreased in presence of tolbutamide.	334
Sulfamethoxypyridazines	A,G(γ)(B)	ST,D	B = 60%(3% alb.), determined *in vitro* acetylation rate.	250
	S(R)	BF	Binding decreased activity. Excretion rate not related to binding.	335
	S	—	B = 34%.	258
	S(H)	E,GF	$n = 70$, $k = 6.85 \times 10^3$. Also determined $\Delta F°$, $\Delta H°$, $\Delta S°$.	140
Sulfaethoxypyridazine	S(B)	D	B = 74–98%.	336
5-Methylsulfadiazine	S(R)	E,D(^{35}S)	B = 80%, 1° binding to albumin.	337
2-Sulfanilamido-5-ethylpyrimidine	S(H)	BF	B = 81%; clearance 2.1 ml./min.	338
Dimethoxysulfanilamidodiazine	S	BF	Determined plasma levels.	339
Sulfaethidole	A(H)	D	Variety of drugs displaced bound sulfa.	206
Sulfaethylthiadiazole	A(B)	D	Determined binding inhibition by tetracyclines.	205
	S(H)	D,U	—	340
	S,A(B,R)	BF,D	Studied displacement by 30 drugs.	204
Sulfathiazole	A(B)	ST	Studied stabilization of proteins to X-rays.	341
Sulfamethoxydiazine	S,T	BF	Determined distribution between blood and other body fluids.	342
II. *Studies of Two Sulfas*				
Sulfamethoxypyrazine, sulfamethazine	S(O,P)	BF	Blood levels determined.	343
Sulfathiazole, sulfamethazine	S(D)	EP	No clear association noted.	344
Sulfaproxyline, sulfamerazine	S(R)	E,D	B = 38–48%(Sp), 0–3%(Sm).	345
Sulfamoxole, sulfamethoxine	S(H)	D	Both bound similarly.	346
Sulfametopyrazine, sulfamethoxypyridazine	S(H)	BF	—	259
2 Sulfapyrazine derivatives	S	BF	Determined plasma and urine levels.	347
2 Sulfanilamido derivatives	S(H)	BF	Compared normal and renal patients.	348
III. *Studies of Three or More Sulfas*				
3 Long-acting sulfonamides	S(H)	D,BF	Activity not related to binding.	196
3 Sulfonamides	S(H)	U	Only unbound fraction biologically active.	194
	S(H)	BF	Binding had no effect on activity, but authors used large serum dilutions.	195
	S(H)	BF	Binding high, excretion rate low, acetylation low.	256
	S	—	Concluded that binding important in drug distribution.	349
3 Sulfonamides & N^4 glycosides	S(R)	D	Glycosides binding similar to that of bases.	350
3 Sulfonamides & 3 derivatives	S(H)	D,BF	B = 19–57%, blood levels determined.	351
4 Sulfonamides	S(H)	D	Biological activity and acetylation dependent on unbound drug.	249
	S(H)	—	Determined protein & RBC binding.	352
	S(10 species)	D,B	Determined species differences in binding. Phenylbutazone displaced bound drug.	197
	S(H)	E,D	Determined excretion rate and displacement by diethylbarbiturate.	253
4 S-containing compounds	A(B)	D	—	353
4 Sulfonamides & acetylated	S(R,Rt,D)	D,BF	Acetylated derivatives bound similarly to	354

(*Continued on next page.*)

TABLE I—(*Continued.*)

Substance	Protein Studied[a]	Method[b]	Remarks[c]	Ref.[d]
derivatives			parent compounds.	
5 Sulfonamides	S(H,O,S,P,E,T)	D	Human serum possessed highest affinity, turkey serum had least.	355
	S(R)	BF,U	Determined blood and tissue levels, excretion rate and volume of distribution after injection.	356
	A(B)	D	Determined n & k at pH 4.9–8.	357
	PF(H)	EP	1° binding to γ-globulin.	358
6 Sulfonamides	S(H)	U,UC	B = 62–96%.	359
	A(B)	D	Data fit Langmuir isotherm.	360
	S(H)	U	—	361
7 Sulfonamides	S	U,D,DF	B = 63% to over 90%.	362
	S(Rt)	D,BF	Data fit Langmuir isotherm; *in vivo* and *in vitro* studies reported.	184
8 Sulfonamides	S(H)	D	n = 1.56 to 3.26; kd = 6.1 to 848.6 μmoles/L.	363
14 Sulfonamides	S,A	D,U	Discusses types of binding and pharmacodynamic effects.	193
19 Sulfonamides	S(R)	U	Discusses binding, distribution, and excretion.	183
21 Sulfonamides & 6 metabolites	S(H)	U	Binding resulted in decreased glucuronidation, increased acetylation.	364
35 Sulfonamides	S(H)	U	Only unbound drug found to be active.	194
Series of several sulfonamides	S(H)	D	Discusses structure-binding correlation.	365
	A(H)	OR	Tertiary structure of albumin altered by binding.	366
	A,S(H)	D	Determined temperature and concentration effects.	367
	S(H)	U	Correlated binding, ionization, and metabolism.	251
	S,A(H)	UC,GF,D	Good agreement between experimental methods.	368
	S,A,G(γ)(H)	D	Influence of pH and sulfa structure studied.	369
	A(B)	NMR	—	159
	S(B)	U	Ultrafiltration discussed.	370
	S(H)	UC	Compared results with dialysis and ultrafiltration.	371
	S	—	Tripiraphen displaced bound sulfa.	372
Several pyridazine sulfonamides	S(H,M,Rt)	U	B = 60–90%.	373
Series of long-acting sulfonamides	S(H,R,Rt,B,D,M)	D,U,E,	Data fit Freundlich isotherm; determined activity, tissue distribution, and excretion.	182
	S(M,D,B,R,Rt,H)	D	Elimination rate independent of binding, activity, and tissue distribution dependent on binding.	181
	S(H)	U	Noted dependency of kidney elimination on binding.	255
	S	BF	Slow elimination due to protein binding.	254
	S(H)	BF	Factors affecting renal excretion discussed.	374
	S,A,G(γ)(H)	D,U	—	375
	S(M)	D,BF	Determined blood, tissue, and CSF levels.	376
	A(H,B)	—	n's reported.	
	G(γ)(H)	D	—	377
		Barbiturates		
Phenobarbital	A(B,H)	D	B = 0%(4% alb.), pH independent 6.1–8.6.	378
Pentobarbital	S(D)A(H)	(^{14}C)	Studied influence of binding on peritoneal dialysis in dogs.	186
	S(Rt)	B	Radiocontrast media and dextran potentiated barbiturate activity.	212
Thiopental	A(B)	D	n = 5, k = 12,000 (8° C., pH 7.4). EDTA decreased n and k.	379
	S(R)A(H,B)	U	Correlated sleeping time and binding.	380
Thiopentone & buthalitone	S(H)	D,B	Determined binding, partition coefficients, and plasma levels.	381
3 Barbituric acids	S(H)	U	Determined % bound and clearance.	382
4 Barbiturates	S(H)	GF(^{14}C)	Studied effect of urea and SO_4.	145
7 Barbiturates	S(H)	D	B = 46–81%.	383
Series of barbiturates	S(R)A(B)	U	B = 5–65%. Determined effects of pH, salts, and temperature. Reported n and k.	384
		Miscellaneous Drugs		
I. *Anti-inflammatory Agents, Analgesics, and Antipyretics*				
A. Salicylates	S(H)	D,EP	Compared binding behavior *in vitro* and *in vivo*. Studied effects of heparin & thyroid hormone.	237
	A(H)	D	B = 66–82%, n = 2.7–3.4(5°, pH 7.4).	385
	S(H,R)	D,B	Measured febrile response in rabbit.	198
	S,PF(B)	D	—	386
	S(R,B,H)	U	Binding and excretion rate compared.	387
	A(B)	D	Determined structure-binding correlations.	388
	A(B)	D	Measured competition by methyl orange.	389
	S	F	1° binding to albumin.	390
	A(B)	D	Studied 27 benzoate derivatives. Reported n, k, % binding, and pH effects.	211
B. *p*-Aminosalicylic acid	A(B)	D	Varied pH and ionic strength.	391
	S(H)	U	B = 58–73%	392
C. Na salicylate & morphiline sal.	S(H)	BF	Determined blood level and excretion rates with and without PABA.	393
D. Several nonsteroidal drugs	PF(B,H)	ST	Anti-inflammatory activity related to binding.	213

(*Continued on next page.*

TABLE I—(*Continued.*)

Substance	Protein Studied[a]	Method[b]	Remarks[c]	Ref.[d]
E. Other misc. drugs				
Morphine	S(H,D)	U	Suggested tolerance and prolonged blood levels related to binding.	264
Phenylbutazone	S	D	B = 99%.	394
	S(H)	D,U	n = 1.	395
	S		Measured association *in vivo* and *in vitro*.	396
	S(H)	D,SP	Loose binding, not displaced by salicylamide.	397
	S(H)	D		398
p-Hydroxyphenylbutazone	S(H)	D	Binding behavior similar to phenylbutazone.	399
Colchicine	S	SP	—	400
Probenecid	PF(H)	D	Reported % binding and half-lives after oral and i.v. doses.	401
	S(D)	U	Data fit Freundlich isotherm. B = 70–80%. Determined blood levels after injection.	402
Several anti-inflamm. drugs	A(B)	SP	Determined displacement of pyridoxal PO_4 and trinitrobenzaldehyde.	216
	S(B)G(H)	ST	Measured stability of protein to UV, heat, urea, and formalin denaturation.	403
II. *Tranquilizers and Antidepressants*				
A. Phenothiazines				
Chlorpromazine	S(R)	E	1° binding to albumin and globulins.	404
Phenothiazone & thional	A(B)	SP,D	Detn. n's and k's for oxidation products of phenothiazine.	405
Chlorpromazine & imipramine	S(D)	D,E	B = 31–36%, pH 8.6.	406
4 Phenothiazines	A	GF	Correlated antihistaminic activity with degree of binding.	200
B. Dicarbamates				
Meprobamate & 3 other derivatives	S(H)	D(^{14}C)	Correlated % binding with partition coefficients.	407
III. *Diuretics*				
Chlorthiazide	S(H)A(B)	D	Caused increased binding of pempidine.	247
Meralluride Na	S(H)	D	B = 90%(1:1000 concn.) and 50%(1:100 concn.).	408
Sulfamoyl-anthranilic acid derivative	S(E)	D	—	409
Ethacrynic acid	A(B)	D,EP(^{14}C)	n_1 = 4, n_2 = 12 (26°, pH 7.4.).	410
Acetazolamide	S(R)	D	B = 10–35%.	411
IV. *Antimalarials*				
Chloroquine	S(H)	E	1° binding to albumin.	412
	A(B)	SP	B = 33%, determined pH effect.	413
Acridine & quinoline derivative	S(B)	SP	Derived equations for spectral analysis.	414
V. *Antineoplastics*				
Methotrexate	S(H,D,M)	U(^{3}H)	B = 50%, determined excretion rate after i.v. dose.	415
	S,A(B)	U(^{3}H)	B = 50% BSA, displaced by PABA, sulfas, salicylates.	226
Melphalan	S(H)	U,D	B = 50–60%.	416
Pluramycin	PF(H)	C,E,UC	Bound drug less toxic.	202
2 Nitrogen mustards	S(H)	U	Data fit Freundlich isotherm.	417
3 Nitrogen mustards	S(H)	U,ST	Hydrolysis rate decreased in serum. Strong binding of 2 derivatives.	418
VI. *Anticoagulants*				
Heparin	A	E,T,B	Also studied suramin binding.	419
	A(B)	E	Also studied suramin binding.	420
	A(B)	E	1:2 heparin–alb. complex.	421
Bishydroxycoumarin	PF	D	B = 99%, half-life is dose-dependent.	261
Ethyl biscoumacetate	S(H)A(H)	D	B = 90%.	422
Warfarin Na	S,A(H)	B,BF	Displacement by phenylbutazone caused increase in activity.	222
	S(H)	B,BF	Hemorrhagic episodes with co-administration of phenylbutazone.	223
	S(H)		Clotting time increased with co-administration of oxyphenbutazone.	224
	S(H)	E,D	n_1 = 1, $k = 1.54 \times 10^6$, also calculated $\Delta F°$, $\Delta H°$, $\Delta S°$. Bound to albumin.	423
VII. *Hypoglycemic Agents*				
Glycodiazine	S(R,Rt,D)	B,BF(^{3}H)	B = 81–98%.	424
Tolbutamide	S,A(H,B)	U,B	Determined n and k, 5-ethyl sulfadiazine increased activity.	217
	S(H)	D,B,BF	Determined displacement by sulfa and phenylbutazone.	219
Chlorpropamide	S(H)	B	ASA increased hypoglycemic effect.	425
	S(H)	D(^{35}S)	% bound increased with increasing concentration.	426
	S(H)	D	B = 80%.	151
	S(H)	D(^{35}S)	B = 80%.	150
Phenethylbiguanide	A,G(B)	D	No significant interaction.	427
4 Sulfonylureas	A(B,H,D,E,P,R)	D,U	Noted species differences in binding. Salicylate displaced bound drug.	218
VIII. *Cardiovascular Drugs*				
Digitoxin	S(Rt)A(B)	D,U	Also determined binding of metabolites.	428
	S(Rt)	E(^{3}H)	Binding capacity = 0.01 Gm./mg. protein.	148
	PF(H,Rt)	EG(^{14}C)	1° binding to albumin fraction.	149
Several digitalis derivatives	A,G(H)	UC	Determined k and $\Delta F°$. Discussed binding forces.	429
Quinidine	A(H)	D	n = 3 (pH 10); ϵ-amino and carboxyl groups involved. Determined pH effects.	430
	A,G(H)	D	n = 1 (pH 7.4) $k = 7.7 \times 10^3$. Displaced by other quinoline drugs.	431

(*Continued on next page.*)

TABLE I—(*Continued.*)

	Substance	Protein Studied[a]	Method[b]	Remarks[c]	Ref.[d]
		A(H)	D	n = 1, k = 0.2 to 0.8 × 10^4 at pH 5.5–8. n = 3, k = 1.1 × 10^4 at pH 10.	432
	Procainamide	S(D)	D	B = 15%; calculated that most of drug localized in tissue.	433
IX.	*Anesthetics*				
	Procaine	S(D,R)	U	Data fit Freundlich isotherm. Determined effects of citrate, NaF, and PABA.	434
	Procaine & 3 other local anesthetics	A,G(H)	D	n = 2, k varies with drug. 1° binding to albumin.	435
	3 Anesthetic gases	A(H)	S	Albumin increases solubilities of gases.	436
	14 Anesthetic gases	A(B)	OR	Correlated high activity with high degree of binding.	437
X.	*Radiopaque Agents*				
		S(H)	E,A(^{131}I)	Used normal and diseased plasma.	438
		S(Rt)		Studied 4 compounds ranked in order of binding.	439
XI.	*Plasma Extenders*				
	Dextran	A(H)	E	Determined effect on binding of age and mol. wt. of dextran.	440
		PF(H)	E	1° binding to albumin.	441
	PVP	S	E	Mol. wt. 33,000 bound; mol. wt. 12,500 not bound.	442
XII.	*Parasympatholytics*				
	Atropine	S(H)	U,UC	Calcd. 50% of therapeutic dose bound *in vivo*.	443
		S,G(H,R)	EP,D	—	444
		A(B)	B,U,SP	N = 20 (pH 6), n = 100 (pH 8). Albumin decreased anticholinergic activity.	201
		A(B)	U,D	Determined effect of pH and buffers. Displaced by choline and acetylcholine.	248
XIII.	*Anti-infectives*				
	Furazolidone & nitrofurantoin	S(B,H)	E	1° binding to albumin.	445
	5 Nitrofurans	PF(B)	—	—	446
	20 Nitrofurans	S(Rt)	U	No correlation between binding and pharmacological effects.	447
XIV.	*Tuberculostatics*				
	Isoniazid	S(H)	(^{14}C)E	No apparent binding noted.	147
		S	PG	—	448
		PF(B,H)	U,E	PAS, streptomycin bound, but not isoniazid.	449
	Sulzolin	S(H)	U	B = 16%.	450
XV.	*Misc. Studies and Studies with Drugs of Various Classes*				
	Rivanol	A(B)	E,D,P	n = 17, k = 150.	451
	Disulfuram & diethyldithiocarbamate	S(H)	GF	Suggested formation of disulfide bond with disulfuram.	452
	d-Tubocurarine	S(H)	BF,D,E	Used plasma from normal and refractory patients.	199
	Epinephrine & norepinephrine	S(H)	BF	Epinephrine highly bound.	453
	Several basic amines	S	SP	Studied ephedrine, amphetamine, and adrenalin.	454
	Xanthine derivatives	A(B)	D	Determined % bound, n, k, and structural requirements for binding.	455
		A(B,H)	SP	Determined effects of temperatures, pH, and modified protein structure.	456
	Several alkaloids & mercurials	A,G	E	—	457
	3 Drugs	S(Rt)	Dc	37°. Studied thiopental, acetazolamide, and iproniazid.	458
	5 Drugs	S	D	Studied sulfadimethoxine, chlorpropamide, phenbutamide, acepromaxine, and dimethylbebeerine.	459
			Vitamins		
	Vitamin B_{12}	S(H)	E,B	B = 93%, 1° binding to α_1-globulin.	460
		S(H,B)	E	Determined effect of pH. 1° binding to α-globulin.	461, 462
		S(var.)	C(^{60}Co)	Used 11 animal species. 1° binding to albumin.	154
		S(H)	D,C(^{57}Co)	Noted several binding sites.	155
		S(H)	—	Binding by transcobalamin studied.	463
		S(H)	C	Hydroxycobalamin and cyano derivative bound by transcobalamin.	464
		S(H)	C	Normal serum saturated *in vivo*.	465
		S(H)	Ad	Bound vitamin resists charcoal adsorption.	466
		S(H,D,R,F,C)	D	n = greater than one.	467
		S(var.)	U,E	Studied binding in 7 animal species.	468
		S(H)	C	Noted two classes of binding sites.	469
	Vitamin B_{12}	S(H)	EP,C	Studied normal and leukemic serum.	470
		S(H)	Dc	Endogenous B_{12} bound greater than exogenous B_{12}.	471
		S(H)	E	1° binding to α-globulin.	472
		S(H)	B	Noted a limited binding capacity.	473
		S(H)	D	Used normal and leukemic serum. 1° binding to β-globulin.	474
		S(H)	EG	Used normal and leukemic serum.	475
		S	E,A	1° binding to γ-globulin.	476
		S(H)	E	1° binding to α-globulin.	477
		S(H)	D	1° binding to γ-globulin, trypsin, and pepsin displaced bound vitamin.	478
		S(H)	EP	1° bound to α_2-globulin.	479
		S(H,C)	E	Measured effect of sialidase on binding.	480
		S(H)	E	*In vivo* 1° binding to α_1-globulin. *In vitro* 1° binding to α_1 and α_2-globulin.	481

(*Continued on next page.*)

TABLE I—(*Continued.*)

Substance	Protein Studied[a]	Method[b]	Remarks[c]	Ref.[d]
	S,CSF	E,A(^{58}Co)	Also measured binding of ^{46}Ca.	153
	S(H)	D(^{60}Co)	Compared normal and leukemic serum.	482
	S(H)	GF	Compared normal and leukemic serum.	483
Hydroxocobalamin	ST	—	Binding to serum protein greater than to liver tissue.	484
Vitamin A	S(R)	BF,E	1° binding to albumin.	485
Vitamin D_2	S(H)	E(^{14}C)	1° binding to α-globulin and albumin.	486
Biotin	S(H)	EP(^{14}C)	1° binding to albumin.	152
Vitamin D_3	S(D)	P,UC,BF(^{3}H)	Noted strong binding *in vivo* and *in vitro*.	487
Tocopherol	A(B)	Pt	—	125
	S	B	Bound vitamin apparently had same activity as unbound.	488
Several fat-soluble vitamins	S(H)	E,D	Vitamin A bound 90%, other vitamins tested bound 10%.	489
13 Vitamins	PF(H)		Determined distribution of vitamins between globulin, albumin, and other proteins.	490
		Endogenous Substances		
Insulin	S(H,Rt,GP)	—	Tolbutamide displaced bound insulin.	246
	S(H)	E(^{131}I)	Binding to a specific insulin antibody reported.	491
	S(H)	E(^{131}I)	Used serum from normal and diseased patients. 1° binding to albumin and globulin.	492
	S(H)	E	Bound insulin found not to be active.	493
	S	Ad	Measured differential adsorption to Cellulose.	494
Glucagon	S(H)	(^{131}I)	Measured effect of pH and growth hormone.	495
Growth hormone	S(H)	E	1° bound to α_2-globulin.	496
Vasopressin	S(H)	B	Pressor activity decreased in presence of protein.	497
Serotonin	S(H)	B	$k = 6.5 \times 10^2$. Activity decreased by albumin. Measured effects of pH, Cu, and Zn.	498
Hyaluronic acid	A(B)	E,UC	—	499
Urate	S(H)	GF	Saturation of protein observed in hyperuricemic patients.	500
	S(H)	GF	Saturation at 10 mg.% urate with 50 mg.% HSA.	138
Bile acids	PF(H)	D	Detn. k and n. Studied effect of pH and structure.	501
Palmitic acid	A(B)	(^{14}C)	Measured uptake of acid by rat epididymal fat pads.	502
Pyridoxal-5-PO_4	A(B)	SP	$n_1 = 1$, $n_2 = 1.9$, $n_3 = 2.5$, $k_1 = 10^6$, $k_2 = 10^5$, $k_3 = 7.8 \times 10^2$.	503
18 Amino acids	S(H)	—	—	504
Indole analogs	A(H)	D	Determined effect of fatty acids, $\Delta F°$, $\Delta H°$, $\Delta S°$.	505
	A(H)	D	Determined effect of pH, salts, and fatty acids.	119
	A(H)	D	Determined effect of pH, salts, and fatty acids.	506
Tryptophan	A(H,B)	D	Reported n and k, effect of pH, fatty acids, and competing compounds.	507
	S(H)	U,UC	$n = 1$, B = 75%, k reported with and without competitors.	508
	S(H)	U,D	Calculated $\Delta F°$, $\Delta H°$, $\Delta S°$.	509
	S(H)	D	Used D and L isomers.	510
Acetyl tryptophan and skatol	A(B)	D	Studied effect of KCl, dioxane, temperature, pH, urea, and glycine.	511
Phosphatidylserine	A(H)	T	$n_1 = 2$, $n_2 = 30$; $k_1 = 2 \times 10^5$, $k_2 = 1.3 \times 10^3$.	512
Bilirubin	A(H)	B	Measured oxidative phosphorylation in brain and liver mitochondria.	513
	S		pH effect determined, 1° association with albumin.	514
	A,G(H)	SP,EP	1° association with albumin, none with globulin.	515
	A(B,H)	SP	Bound to extent of 20 mg./Gm. protein.	516
	PF	E	1° association with albumin. Association with globulins occurred at high concentration.	517
	S(H)	EG	Only albumin binding found in hyperbilirubinemic serum.	518
	A(H)		Studied peritoneal dialysis.	187
	S(H)	D,UC	B = 98%, $n = 10$, also studied metabolites.	519
	S(H)	SP	Displaced by sulfa, salicylates, pH, caffeine Na benzoate.	229
	S(H)A(B)	SP,U,D	Bound form displaced by salicylates and sulfas.	228
	S(H)	BF	Studied 40 infants with neonatal jaundice. Bilirubin displaced by heme, salicylates, and sulfonamides.	227
	S(H)	BF	Compared ability of drug to displace bilirubin in infants.	232
	S(H)	BF	Studied effect of sulfisoxazole on bilirubin level in infants.	230
Bilirubin	A,S(H)	U,GF	$n = 2$ for HSA, displaced by sulfonamides.	231
	S(D,H)	U,D,UC,E	1° bound to albumin, measured excretion rate.	520

[a] Type of protein: A, albumin; CSF, cerebral spinal fluid; G, globulin; L, lymph; PF, plasma fractions; S, serum or whole plasma; T, tissues. Animal species in parentheses: B, bovine; C, chicken; D, dog; E, horse; F, frog; G, goat; GP,

(*Continued on next page.*)

TABLE I—(*Continued.*)

guinea pig; H, human; M, mouse; O, ox; P, pig; R, rabbit; Rt, rat; S, sheep; T, turkey. [b] A, autoradiography; Ad, adsorption; B, assay for biological activity; BF, assay of biological fluids for drug; C, column chromatography; D, dialysis; Dc, continuous flow dialysis; DF, diffusion; E, electrophoresis; EG, gel electrophoresis; EP, paper electrophoresis; F, fluorescence; GF, gel filtration; NMR, nuclear magnetic resonance spectroscopy; OR, optical rotation; P, precipitation; PG, polarography; Pt, partitioning; S, solubility; SP, spectrophotometric; ST, stabilization of protein or drug; T, turbidimetric analysis; U, ultrafiltration; UC, ultracentrifugation. [c] Includes experimental conditions, observations, and conclusions derived from the various studies. B = percentage of drug which was bound; 1° = protein involved in primary interaction; n = number of binding sites on the protein; k = binding association constant; k_{dp} = binding dissociation constant; U = drug concentration in units. [d] Grouped by class of compound investigated.

TABLE II—SUMMARY OF STUDIES INVOLVING PROTEIN BINDING OF VARIOUS DYES

Dyes	Ref.
Azo Dyes	
Methyl orange	(117, 122, 521–534)
Methyl red	(535)
Trypan blue	(536–538)
Trypan red	
Congo red	(534, 539–543)
Evans blue	(124, 544, 545)
Miscellaneous azo dyes	(91, 116, 265, 546–556)
Phthalein Dyes	
Phenol red	(557–562)
Bromophenol blue	(563–567)
Bromocresol green	(565, 568–570)
Bromothymol blue	(571)
Sulfobromophthalein	(137, 572–574)
Fluorescein	(575)
Miscellaneous dyes and studies involving several classes of dyes	(576–595)

tein was measured by several investigators (45, 46). Kripalani and Sorby (47) measured albumin binding of cortisol and its degradation products and have emphasized the necessity of determining the extent of steroid degradation when equilibrium dialysis and other methods requiring extended periods of time are employed to detect and quantitate binding behavior. Blair *et al.* (48) and Beisel *et al.* (49) studied the binding and renal excretion of cortisol and the glucuronides of 17-hydroxy-corticosteroids. A number of other investigations employing electrophoresis (50–52), ultracentrifugation (53), ultrafiltration (54–56), and biochemical assays (54, 57) have dealt with the binding of corticosteroids to plasma proteins of man, rat, cow, and a variety of other animal species.

Similarly, the binding of thyroid hormones by proteins has been the subject of comprehensive reviews (58–66) and references to this topic were also omitted from Table I. Such interactions are thought to be of consequence in the distribution, biotransformation, elimination, and activity of the hormones. Specialized plasma proteins with high affinities for thyroxine have been identified and classified. Of possible therapeutic importance is the observation that 2,4-dinitrophenol, salicylate (58, 59), trypan blue (58), organic dyes, and diphenylhydantoin (67) can inhibit the binding of thyroxine by some proteins. An interesting study (68) showed that ethylchlorophenoxyisobutyrate can apparently increase the binding capacity of serum proteins for triiodothyronine. More recent studies, which were not included in the cited review articles, utilized a variety of techniques including fluorescence (69), dialysis (69, 70), and electrophoresis (71–77) to demonstrate binding. Other studies were concerned with the effect of subject age (78), sex (79), thyroidectomy (80), pH, and buffers (81) on the plasma binding of thyroid hormones.

A number of studies dealt with the interactions of plasma proteins with compounds which are not of therapeutic significance, and illustrate the ability of albumin, in particular, to interact with a seemingly limitless variety of structures. Many surfactants (82–92, 3), for example, have been studied in this respect. Ethanol (93), various polyelectrolytes (94), polymers (95), fatty acids (96–98), buffer components (99–101), thio acids (102), quinone imides (103), acetamide (104, 105), octane and dodecane derivatives (106), *p*-aminobenzene (107), phosphoric esters (108), aromatic acids (109), benzpyrene (110), and a variety of other compounds have been shown to reversibly combine with plasma proteins. In addition, many inorganic anions and cations were observed to interact with albumin (111–113, 2). It has been demonstrated that the presence of inorganic ions can influence drug protein interactions by forming a bridge between protein and small molecule (114, 115), by competing for available binding sites (116–119), by altering the configuration of the macromolecule or inducing changes in ionization characteristics of the protein (2, 120).

New Experimental Methods — Classical methods, employed to detect, determine, and study binding characteristics, such as equilibrium dialysis, ultrafiltration, ultracentrifugation, and electrophoresis have been discussed by a number of authors (1, 6, 121). Although most of the studies tabulated in Tables I and II utilized these methods, modifications and some relatively new approaches were also described. Stein (122) described a rapid, nonequilibrium dialytic method which was shown to yield, for the protein binding of methyl orange, results which were comparable to those obtained by equilibrium methods. Bennett and Kirby (123) developed a vacuum ultrafiltration cell which was used to investigate the

binding of penicillins. Differences in the polarographic behavior of bound and free substrate have been demonstrated and applied to the determination of binding parameters as illustrated by the studies of Markus and Baumberger (124) with Evans blue. The interactions of protein with methyl orange (117), fatty acids (97), tocopherol (125), testosterone (118, 126–128), and other steroids (129) were examined by determining the influence of protein concentration on the apparent partition coefficient of the small molecule. Gel filtration has, in recent years, been extensively used to detect the binding of small molecules. Techniques and theory applicable to this method have been presented by several authors (130–134). Such procedures employ beads of cross-linked polysaccharides which are water insoluble but which undergo extensive swelling when in contact with water. Macromolecules such as proteins and protein complexes are unable, because of size, to penetrate into the internal volume of the gel matrix while small molecules do penetrate. The method is capable of yielding data similar to that obtainable from dialytic techniques without concomitant problems of membrane binding, Donnan effects, and time considerations. However, difficulties can be encountered. For example, many small molecules can interact with the gel material resulting, from mass law considerations, in dissociation of the protein complex (135, 136). In addition, when gel-filtration chromatography is used to demonstrate binding, the elution step results in dilution of the binding system and possible significant dissociation of the complex. The technique has been employed to study the binding by plasma proteins of a variety of compounds such as dyes (137), uric acid (138), antibiotics and sulfonamides (139–141), corticosteroids (142–144), and barbiturates (145).

Improved sensitivities have been realized with many of the classical techniques through the use of isotopically labeled small molecules. The ease of analysis afforded by this approach has permitted *in vitro* studies at concentration levels comparable to those encountered *in vivo*. This subject was recently reviewed by Cohen *et al.* (146). Illustrative of the utility of this approach are studies which employed isoniazid-^{14}C (147), ^{3}H or digitoxin-^{14}C (148, 149), chlorpropamide-^{35}S (150), testosterone-^{14}C (151), biotin-^{14}C (152), vitamin B_{12} ^{57}Co-, ^{58}Co-, ^{60}Co- (153–155), penicillins-^{14}C (156, 157), and progesterone-^{14}C (158).

Recent studies have established the value of nuclear magnetic resonance spectroscopy as a tool for studying protein–small molecule interactions and for assessing the extent to which various functional groups on the small molecule participate in the interaction. Jardetsky and Wade-Jardetsky (159) and Fisher and Jardetzky (160), for example, studied the binding of sulfonamides and penicillins by determining NMR spectra of the compounds in the presence and absence of albumin. They observed that relaxation rates for certain protons changed in the presence of protein and were able to conclude that the *p*-aminobenzene sulfonamide moiety of sulfonamides and the phenyl ring of penicillin were the functional groups which participated in the interaction.

The majority of reported studies were concerned with a characterization of binding behavior at equilibrium. A limited number of studies were also conducted to assess the kinetics of binding. Robbins *et al.* (161) investigated the rate of interaction of thyroxine with serum protein. They employed a rapid dialysis technique which consisted of placing a thin piece of lens paper, which was saturated with a solution of drug or drug and protein, directly on a dialysis membrane. It was shown that the association and dissociation reactions were complete within 2 min. A more sophisticated method was also employed and consisted of determining the rate of quenching of the fluorescence of albumin caused by the interaction with thyroxine. Association was determined to be complete within 150 msec. The dissociation process was resolved into two steps with half-lives of 0.1 sec. and 7 sec. Froese *et al.* (162) employed a temperature-jump technique to investigate the kinetics of interaction between albumin and two azo dyes. The reaction system was perturbed by about 10° within 1 μsec. and the readjustment of the system to equilibrium conditions at the higher temperature was followed spectrophotometrically. Rate constants for association were found to be 0.36×10^{6} and 2.1×10^{6} mole^{-1} sec.$^{-1}$. Rolinson and Sutherland (163) attempted to investigate the kinetics of penicillin binding using dialysis and ultrafiltration. They found that equilibrium was attained rapidly and that the rate of attainment was too fast to investigate by these methods. Although there is general agreement that, in most systems, rates of association and dissociation are very rapid, there is a paucity of quantitative information and a need for additional studies in this area.

Treatment and Presentation of Experimental Data—Most investigators have been concerned with protein–small molecule systems at equilibrium and with the determination of degree of binding as a function of compositional and environmental variables. A number of methods for treating and presenting the results of such investi-

gations are commonly used. The reciprocal plot and the Scatchard plot of experimental data are employed frequently and are directly derived from mass-law considerations. It can be shown (6) from such considerations that reversible binding is described by the familiar equation:

$$r = \sum_{i=1}^{i} \frac{n_i k_i D_f}{1 + k_i D_f} \qquad \text{(Eq. 1)}$$

where

r = moles of small molecule bound to total moles of protein in the system.
n_i = number of binding sites in the i'th class of sites.
k_i = intrinsic association constant for the binding of small molecule by sites in the i'th class.
D_f = concentration of unbound small molecule.

Equation 1 assumes that activities can be represented by concentrations, that all sites within a class are equivalent in binding affinity, and that all sites are mutually independent. For a single class of sites:

$$r = \frac{nkD_f}{1 + kD_f} \qquad \text{(Eq. 2)}$$

Equation 2 can be rearranged to yield:

$$1/r = 1/n + 1/nk\,D_f \qquad \text{(Eq. 3)}$$

which is the basis for the reciprocal plot in which $1/r$ is plotted as a function of $1/D_f$. Estimations of n and k are made from the slope and intercept values of such a plot. The Scatchard plot is based on a different rearrangement of Eq. 2;

$$\frac{r}{D_f} = nk - rk \qquad \text{(Eq. 4)}$$

A plot is made of r/D_f as a function of r; extrapolation to abscissa and ordinate allows estimation of n and nk. Curvature of such plots is usually indicative of the existence of more than one class of sites, *i.e.*,

$$r = \frac{n_1 k_1 D_f}{1 + k_1 D_f} + \frac{n_2 k_2 D_f}{1 + k_2 D_f} + \cdots + \frac{n_i k_i D_f}{1 + k_i D_f} \qquad \text{(Eq. 5)}$$

Graphical treatment of data by means of the reciprocal plot heavily weights those experimental points which are obtained at low D_f and can, therefore, lead to misinterpretations concerning behaviors at high D_f. The Scatchard plot does not suffer from this disadvantage and is the graphical method of choice. Extrapolation of a Scatchard plot curve, obtained when more than one class of sites are responsible for binding, yields $\Sigma n_i k_i$ and Σn_i. Various curve-fitting techniques can be employed to estimate individual n and k values. Hart (164) has recently discussed the analysis of such curves and showed that n and k values for each class of site can be evaluated by solving a set of simultaneous linear equations. For a system containing i classes of sites, $2i$ experimental points are required to generate $2i$ linear equations which can be solved for the i sets of n and k.

Reciprocal and Scatchard plots cannot be constructed if the nature and amount of protein in the experimental system is not known, as would be the case, for example, in determining the binding characteristics of plasma. Many authors have presented the results of such determinations in terms of "percent bound" or "fraction bound." This way of describing binding behavior is so commonly used that it is pertinent to repeat Goldstein's criticism (1): "The statement that a given fraction is bound in plasma is meaningless, unless qualified by an indication of the unbound drug concentration at equilibrium." The relationship between "fraction bound," β, and the fundamental parameters of binding, n and k, for the case of a single class of binding sites, is:

$$\beta = \frac{1}{1 + D_f/n\,P_t + 1/nk\,P_t} \qquad \text{(Eq. 6)}$$

where, P_t = total molar concentration of protein.

It is apparent that "fraction bound" exhibits a dependency on the concentration of free drug and the concentration of protein. Thus, "fraction bound" is a useful characterization of intrinsic binding strength only when D_f/nP_t approaches zero. Under such conditions, β approaches a maximum value and

$$nk\,P_t = \frac{\beta}{1 - \beta} \qquad \text{(Eq. 7)}$$

It should also be noted in this respect that experimental techniques which perturb the protein–small molecule system by dilution prior to the determination of the fraction of drug which is bound can grossly underestimate the true extent of binding. Saris (165) has presented data related to sulfonamide binding which illustrates this potential source of error. Similarly, Oppenheimer and Surks (166) have shown that when the association constant is large, such as in thyroxine-plasma systems, "fraction bound" is unaffected by reasonable dilution.

Krüger-Thiemer *et al.* (167) have discussed mathematical problems associated with studies on the binding of drugs in plasma samples. They showed that plasma binding, if due to a single plasma protein with a single set of sites, can be characterized by two parameters, according to the equation:

$$D_t = D_f\left(w + \frac{\beta' p}{D_f + k_{dp}}\right) \qquad \text{(Eq. 8)}$$

where

D_t = total molar concentration of drug in the plasma
D_f = molar concentration of unbound drug in the plasma
w = volume fraction of water in the plasma
β' = maximum binding capacity of plasma for drug in μmoles/Gm.
k_{dp} = intrinsic dissociation constant for the drug–protein complex
p = protein concentration in Gm./L.

They demonstrated the application of three equations, first published by Woolf (168), for linearizing data and for the graphical determination of β' and k_{dp}:

$$\frac{\beta' p}{D_b} - \frac{k_{dp}}{D_f} = 1, \qquad \text{(Eq. 9)}$$

$$D_b = \beta' p - k_{dp}\frac{D_b}{D_f}, \qquad \text{(Eq. 10)}$$

$$D_f = \beta' p \frac{D_f}{D_b} - k_{dp} \qquad \text{(Eq. 11)}$$

where D_b = concentration of bound drug. They also described the application of a digital computer to the estimation of β' and k_{dp} from experimental data and illustrated their approach with studies on three sulfa drugs. The binding behaviors of two of these could be adequately described by Eq. 8. The third drug exhibited a more complex behavior which could be explained by expanding Eq. 8 to include a term for a second binding process.

Sandberg *et al.* (169) and Rosenthal (170) have also discussed treatment of binding results when the nature and concentration of the protein is not known. They have recommended a Scatchard-type plot based on a rearrangement of Eq. 2:

$$\frac{D_b}{D_f} = nkP_t - kD_b, \qquad \text{(Eq. 12)}$$

which will also be recognized as a slightly modified form of Eq. 10. A plot of D_b/D_f as a function of D_b is independent of protein concentration and allows estimation of nkP_t, nP_t, and k from ordinate intercept, abscissa intercept, and slope. If more than one protein or more than one class of binding site on a single protein are involved in the binding, curvature of the plot results. This plot can also be used to provide a useful display of data from which can be determined the concentrations of all drug-containing species at any total or free drug concentration.

Hansch *et al.* (171) discussed a novel type of data treatment which was designed to provide an insight to the nature of intermolecular forces responsible for binding. They studied the binding by albumin of phenol and 18 substituted phenols and determined in each case the concentration, C, required to yield a 1:1 complex with the protein. They also determined the partition coefficient of each compound between octanol and water and calculated a substituent constant, π, which was defined as:

$$\pi = \log P_X/P_H, \qquad \text{(Eq. 13)}$$

where

P_X = partition coefficient of a phenol derivative
P_H = partition coefficient of phenol.

A semi-log plot of $1/C$, which is proportional to the complex association constant, *versus* π was found to be linear. The authors concluded that, mechanistically, binding closely paralleled the transfer of phenol from water to octanol, that binding was rather nonspecific, and was best described as being due to hydrophobic bonding.

Influence of Protein Binding on Drug Distribution—There is general consensus that, because macromolecules and macromolecular complexes pass across membranes only with difficulty, protein binding in plasma can influence the distributional pattern of a drug in the body. The extent to which this influence is manifest and significant has been the subject of a number of studies. Martin (172) has provided, by means of a simple model, a quantitative visualization of the potential effects of plasma binding on drug distribution. His treatment admirably serves as a basis for a discussion of this topic. His approach to this evaluation was based on the following assumptions: (*a*) drug in the body is distributed into two aqueous compartments—plasma and a compartment composed of the remaining body water, (*b*) unbound drug is in equilibrium in the two compartments, (*c*) binding of drug occurs only in the plasma and results from the formation of 1:1 complexes with albumin, (*d*) for a 70-Kg. man, the plasma volume is 3 L. and the total volume of water is 42 L., (*e*) the molar concentration of albumin in the plasma is 5×10^{-4}. He then conducted calculations related to the binding of four hypothetical drugs whose interactions with albumin could be characterized by association constants (k) of 10^4, 10^5, 10^6, and 10^7 L. mole^{-1}, respectively. For each drug he assumed a concentration of unbound drug, D_f, and calculated, first, the total concentration of drug in plasma, D_t, by an equation directly derivable from Eq. 6:

$$D_t = D_f\left(1 + \frac{P_t}{1/k + D_f}\right) \qquad \text{(Eq. 14)}$$

He then calculated for each value of D_f: (*a*) amount of drug in body $= 39\,D_f + 3\,D_t$; (*b*) percent of drug, free in body $= 42\,D_f \times 100/(39\,D_f + 3\,D_t)$; and (*c*) percent of drug in plasma $= 3\,D_t \times 100/(39\,D_f + 3\,D_t)$.

The results of such calculations were presented in graphical form such as illustrated by Figs. 1 and 2. It is apparent from these representative theoretical curves that binding can influence drug distribution in the body and that the magnitude of the effect will depend both on the strength of association and the dosage of drug. For example, a strongly bound drug such as that with a k of 1×10^7 will, at low dosage levels, be concentrated primarily in the plasma compartment. However, at higher dosage levels, the fraction of drug in the plasma will be markedly reduced. Martin also emphasized another characteristic attributable to the plasma binding of drugs with a high affinity for proteins; that there is a dosage range within which small increases in dose result in relatively large increases in the amount of drug in the body which is not bound. He noted that this behavior can have interesting manifestations on dose-response characteristics and pharmacokinetic properties of such drugs. The treatment additionally emphasizes that binding to the plasma proteins will have an appreciable effect on drug distribution only if the strength of binding is quite large ($k > 10^4$). Martin (172) showed, to illustrate this point, that with a drug having a k of 10^4, the "fraction bound" in plasma can be 83.4% but, nevertheless, 73% of the total dose is present in the body in the unbound form. Martin's model is a highly simplified representation of what might indeed be a highly complex distributional pattern involving binding to tissue proteins, partitioning into fatty compartments, the unavailability of certain aqueous compartments, *etc.* Nevertheless, he clearly demonstrates that plasma-protein binding can be an important determinant in drug distribution for some drugs but that for many others, it might not be of significance in spite of *in vitro* demonstrations that interaction occurs to produce a seemingly high "fraction bound."

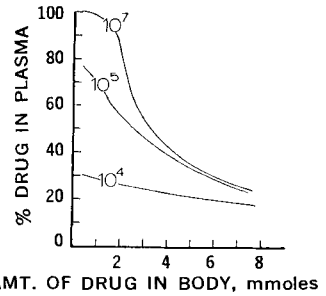

Fig. 1—A plot, based on theoretical considerations, illustrating the potential influence of plasma binding on the distribution of drug between plasma and other aqueous compartments in the body. Each curve represents a different value of k. *After Martin (172).*

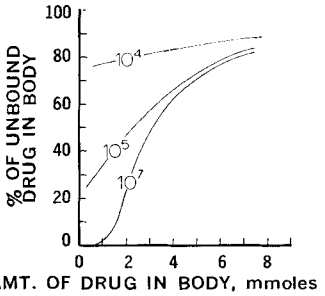

Fig. 2—A plot, based on theoretical considerations, illustrating the potential influence of plasma binding on the distribution of drug between bound and unbound forms in the body. Each curve represents a different value of k. *After Martin (172).*

A number of recent experimental studies have demonstrated, in a qualitative sense, that plasma binding can influence drug distribution. Verwey and co-workers (173–175) developed a method for sampling lymph by cannulation of the peripheral lymphatics of dogs. They noted that examination of peripheral lymph is useful in drug distribution studies since it is derived directly from interstitial fluid. They studied, in dogs, the distribution of seven penicillins between plasma and lymph and showed that for six of the seven analogs, plasma concentrations were significantly higher than those found in the lymph. The degree of binding in plasma and in lymph was determined and it was demonstrated that unbound drug was in equilibrium between the two fluids. Their results supported the conclusions of Rolinson (176) who considered theoretical aspects of protein binding as it relates to the distribution of penicillins and emphasized that only unbound drug is free to diffuse from plasma. Similar conclusions were made by Kunin (177) who showed with a series of penicillins that as the degree of protein binding decreased, localization of antibiotic in brain, muscle, lung, and heart of rabbits increased. He also showed that the co-administration of an agent capable of displacing protein-bound drug increased the amount of antibiotic which localized in the tissues.

Fishman (178) found that probenicid, when co-administered with penicillin, resulted in a higher level of antibiotic in the cerebral spinal fluid of the dog. He postulated that this effect was due, in part, to the displacement of protein-bound antibiotic by probenicid. Additional studies of this nature will be discussed in a subsequent section. Kharchenko (179, 180) also conducted studies on the distribution of antibiotics in the rabbit. He showed that when human serum was injected into the pleural cavity, distribution into this compartment occurred to a much greater extent than when saline controls were injected. Johnson *et*

al. (150) reported on the distribution of chlorpropamide-^{35}S in a patient with edema. They determined that the plasma concentration of drug was two to six times higher than that in the edematous fluid. Scholtan (181, 182) showed that a relationship existed between the degree of binding of sulfonamides and tissue distribution, in several species of animals. Rieder (183) also found a correlation between binding parameters and sulfonamide distribution in the rabbit and reported that the concentration of unbound drug in the plasma provides a reasonable estimate of the concentration of unbound drug in extravascular fluids. Ruiz-Torres and Meinig (184) reported, however, that no such correlation existed in their studies on the distribution of sulfonamides in the rat.

An interesting application of the influence of drug–protein interactions on drug distribution is illustrated by studies in which albumin was employed as an additive to the fluid used for reducing, by peritoneal dialysis, body levels of toxic substances. Campion and North (185) treated, in this way, dogs that were intoxicated with phenobarbital, amobarbital, and secobarbital and reported that the dialysis fluid containing albumin was more efficient than a control fluid. Bourne *et al.* (186) utilizing pentobarbital-intoxicated dogs, found that only a slightly greater efficiency was achieved with the technique. Grollman and Odell (187) observed that inclusion of albumin in dialysis fluid increased the amount of bilirubin which could be removed by a factor of 25. Other authors have noted enhanced removal, by this method, of salicylates from dogs (188) and barbiturates from humans (189).

Competitive Inhibition of Plasma Binding—A number of investigations have clearly demonstrated that the pharmacological response to a drug can be diminished as a result of interaction to form nondiffusable protein complexes. It is pertinent to briefly review some of these studies and to then examine the possibility of eliciting, either intentionally or unintentionally, a more pronounced response through the administration of agents which competitively inhibit binding.

Antibiotics and anti-infective agents have been intensely investigated in this respect. Plasma inactivation of penicillins, cephalosporins, and other antibiotics is a well-known phenomenon (190–192). Rolinson and Sutherland (163), for example, studied the plasma binding of 17 antibiotics and found that *in vitro* activity was inversely proportional to the observed extent of binding. Evidence supporting the premise that only unbound sulfonamides are antibacterially active was accumulated by Witzgall and Boyens (193) and Krüger-Thiemer *et al.* (194). Other workers (195, 196) failed to observe this effect, but it should be noted that their experimental methods employed rather large dilutions of plasma samples with concomitant pertubation of binding equilibria. Anton (197) also demonstrated an inverse correlation between bacteriostatic activity and extent of binding of sulfonamides.

Reynolds and Cluff (198) have shown that the antipyretic activity of sodium salicylate in rabbits was decreased when albumin was coadministered. Aladjenoff *et al.* (199) observed that plasma levels of *d*-tubocurarine were unusually high in patients who were refractory to the drug. They also found that the drug, when preincubated with albumin, lacked a curarizing effect in dogs. They suggested that the highly bound drug was unable to diffuse from the plasma to the neuromuscular junction. Jindrova *et al.* (200) evaluated four phenothiazine derivatives and determined that those which were least bound exhibited the greatest antihistaminic activity. Oroszlan and Maengwyn-Davies (201) found that the cholinolytic activity of atropine, on the isolated rat colon, was inhibited by the presence of albumin. Ogawara *et al.* (202) showed that a pluromycin A–albumin complex was less toxic than pluromycin A, and that the complex had a greater antitumor activity. Several investigators (54, 57, 203) reported that the presence of protein interfered with the participation of various steroids in biochemical processes.

Reversal of this type of drug "inactivation" by the addition of a competitive inhibitor to the binding system has been considered by a number of workers and attempts to evaluate such a possibility, *in vitro* and *in vivo*, have been reported. The theoretical foundations for this approach can be readily demonstrated. If drug, D, and competitor, C, for example, form 1:1 protein complexes and compete for the same binding site on the macromolecule, it can be shown that:

$$\frac{D_f}{D_t} = \frac{1 + k_D D_f + k_c C_f}{1 + k_D P_t + k_D D_f + k_c C_f}, \quad \text{(Eq. 15)}$$

where

k_D = association constant for the drug–protein complex
k_c = association constant for the competitor–protein complex
C_f = concentration of unbound inhibitor.

It is readily apparent, from Eq. 15, that the effectiveness of an inhibitor in "freeing" drug from combination with protein will depend on its concentration and affinity for the protein. It is il-

lustrative to assess quantitative aspects of such drug "displacement" by the construction of drug distribution curves in the same manner as was previously discussed. The results of such a treatment for a strongly bound drug ($k_D = 10^7$) is shown in Fig. 3. These theoretical curves were generated from Eq. 15 by the calculations and assumptions that were employed by Martin (172) and which were discussed previously. Various values of k_eC_f were assumed in making these calculations. It is obvious from examination of the curves that the influence of a competitive inhibitor on the fraction of drug which is free in the body can be quite pronounced and that this influence will be especially significant at lower dosage levels of the drug. It is also apparent that, in order to displace a strongly bound drug by this mechanism, an inhibitor must be present in the system at a relatively high concentration or have an affinity for the protein which is significantly greater than that exhibited by the drug.

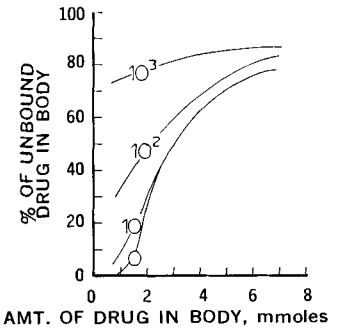

Fig. 3—A plot, derived from theoretical considerations, illustrating the potential influence of competitive inhibitors of plasma binding on the distribution of drug between unbound and bound forms in the body. See text for the assumptions made in deriving the relationship. Each curve represents a different value of k_eC_f.

Anton's studies (197, 204) have been frequently cited as an example of this approach to the possible potentiation of the pharmacological activity of a strongly bound drug. He was concerned with the inhibition, *in vitro* and *in vivo*, of sulfonamide binding. He demonstrated that phenylbutazone, sulfinpyrazine, ethyl biscoumarate, and iophenoxic acid were, among many compounds evaluated, effective in displacing protein-bound sulfonamides and that the antibacterial activity of a sulfonamide in the presence of albumin was markedly increased by the presence of a displacing agent. He showed quite dramatically that administration of sulfinpyrazone to rats which were dosed with sulfaethylthiadiazole or sulfamethoxypyridazine resulted in a precipitous decline in the total plasma concentration of sulfonamide but an increase in the concentration of unbound drug. Concomitant with this was an increase in the concentration of sulfonamide in tissues. Studies with other sulfonamides showed that displacing agents altered tissue and plasma concentrations of only those which were reasonably strongly bound. Genazzani *et al.* (205) determined that various tetracycline derivatives competed with sulfaethylthiazole for binding sites on albumin. Bahal (206) found that the binding of sulfaethylthiazole was modified by the presence of salicylate. His attempts to evaluate *in vivo* displacement of bound sulfonamide and the resulting effect on excretion rate was complicated by the observation that the agents which were effective in displacing the sulfonamide also retarded tubular secretion of the drug.

Kunin (156, 157, 207, 208) has published the results of exhaustive studies in which over 250 compounds, representing a wide variety of structures, were evaluated for their ability to displace protein-bound penicillin G, penicillin V, and other derivatives. Three compounds—sulfamethoxypyridazine, sulfaethylthiadiazole, and acetylsalicylic acid—were found to be effective and were employed in studies with human volunteers to determine their effects *in vivo*. The results indicated that it was possible to reduce serum binding of penicillins in man by this approach. The administration of binding inhibitors tended to lower the total serum concentration and to increase the concentration of unbound antibiotic in the serum. His results demonstrated that the relative effectiveness of an inhibitor is defined by both the relative concentrations and relative affinities for the protein of drug and inhibitor. Thus, administration of sulfaethylthiadiazole was effective in reducing the binding of the moderately bound penicillin V from approximately 81 to 65%. However, the binding of strongly bound dicloxacillin was reduced only slightly from 98.3 to 97.7%. Kunin discussed the possible clinical implications of reversing, by even a small amount, the binding of a strongly bound drug. He also noted that attainment of enhanced clinical activity by displacement might be realized only with difficulty because of the large doses of inhibitors required and the often limited degree of displacement which may be achieved in man. Rolinson and Sutherland (163), Anderson *et al.* (209), and Fishman (210) have also reported the results of *in vitro* investigations on the competitive inhibition of penicillin binding.

Other studies in this general area include those of Davison and Smith (211) who observed that a variety of benzoates and salicylates are bound to the same sites on albumin. Lasser *et al.* (212) found that the administration of a highly bound radiopaque agent caused potentiation of pento-

barbital hypnosis which they attributed to the displacement of the barbiturate from protein complexes. Brodie (21) reported a correlation between the activity of nonsteroidal anti-inflammatory agents and their ability to displace bound corticosterone. Mizushima and Suzuki (213) similarly noted some correlation between the antirheumatic activity of nonsteroidal drugs and their ability to interact with various serum protein fractions. Florini and Buyske (214), however, concluded from their investigations that such compounds did not appear to function *via* displacement of bound hydrocortisone. Other studies have been concerned with the displacement of aldosterone by spirolactones and other steroids (44), and the mutual competition between cortisol, corticosterone, and progesterone (55), as well as estrone (215) for plasma protein binding sites. Skidmore and Whitehouse (216) have suggested that displacement of protein-bound pyridoxal phosphate or 2,4,6,-trinitrobenzaldehyde might be useful as an approach to the screening of anti-inflammatory activity. Buttner and Portwich (217) observed that 5-ethylsulfadiazine and tolbutamide mutually displaced each other from albumin binding sites. Concomitant administration of the two drugs resulted in enhanced clinical activity. Wishinsky *et al.* (218) measured the binding of four sulfonylurea drugs and found that addition of salicylate to the binding system resulted in a substantial increase of unbound drug. The occurrence of hypoglycemic episodes was reported with a patient who was receiving both tolbutamide and sulfaphenazole (219). It was explained, however, as resulting not from drug displacement but rather from an increased half-life for tolbutamide due to inhibition of a carboxylation process.

A general discussion of possible therapeutic difficulties which might be encountered in the concomitant administration of drugs has been presented by MacGregor (220) and Brodie (221). Several reports have been concerned with possible hazards resulting from the displacement of bound anticoagulants. Aggeler *et al.* (222) observed that phenylbutazone can decrease warfarin plasma concentrations 35 to 60%, decrease biological half-time 20 to 60%, and increase anticoagulant effects 40 to 80% in normal patients and 250% in warfarin-resistant patients. Other clinical reports (223, 224) noted a marked increase in prothrombin time as a result of the co-administration of sodium warfarin and phenylbutazone or oxyphenbutazone. Solomon and Schrogie (225) demonstrated by *in vitro* studies that a variety of agents were capable of displacing protein-bound warfarin. Reference has been made to the fact that methotrexate (226), which is administered in near toxic doses, can be displaced by salicylates and certain sulfonamides.

Consequences resulting from the displacement by drugs of protein-bound endogenous compounds have also been considered and studied. Bilirubin is an important example since its displacement can result in toxic manifestations in infants and in individuals with impaired bilirubin conjugating mechanisms. Odell (227, 228) showed that salicylates and sulfonamides, in concentrations which are encountered clinically, could displace bilirubin. He reviewed (229) the clinical implications of such an event. Kantor *et al.* (230) and Josephson and Furst (231) also considered displacement of this compound by the administration of sulfonamides to premature infants with kernicterus or to mothers before and during labor. Silverman *et al.* (232) compared the mortality rate of premature infants who received either penicillin and sulfisoxazole, or tetracycline. Their data suggested that kernicterus was more prevalent in the population which received the sulfonamide.

Christensen (233) has studied the effect of several benzoates and salicylates on thyroxine binding and has attempted to relate physiological and toxicological effects to the displacement of bound hormone. Yamada *et al.* (234) have concluded that a prime effect of diazo dyes, such as trypan blue, on the thyroid occurs due to displacement of thyroxine from plasma proteins and subsequent inhibition of TSH release. Numerous studies have shown that salicylate (235–237), hydantoin derivatives (238, 239), penicillins (239, 240), thyroxine analogs (241), dinitrophenol (242–244), and novobiocin (245) were capable of displacing bound thyroxine. In addition, studies on the binding of insulin (246) indicate that tolbutamide was able to liberate protein-bound insulin in guinea pig serum.

Of interest are two studies which indicate binding enhancement induced by the presence of a second compound. Thus, Dollery *et al.* (247) noted that pempidine, a ganglionic blocking agent, did not interact with bovine serum albumin. However, when chlorothiazide was added to the system, considerable binding was apparent. It was speculated that chlorothiazide induced a structural alteration in the protein which resulted in a configuration capable of binding pempidine. Similarly, acetylcholine was found to increase binding of atropine to albumin in systems buffered at pH values from 5 to 6 (248).

Protein Binding and Pharmacokinetic Behavior—The influence of protein binding on the time course of drug in the body has been con-

sidered by a number of authors. Many qualitative and semiquantitative observations have indicated that binding can be an important parameter in the pharmacokinetic characterization of a drug. *In vitro* studies have, for example, established that the addition of protein to isolated biochemical and physiological preparations can result in a decreased rate of substrate disappearance. Thus, Newbould and Kilpatrick (249) showed that the rate of acetylation of two long-acting sulfonamides in a perfused rabbit liver preparation was decreased when plasma was added to the perfusion fluid. They determined that the observed rate depended on the concentration of unbound drug. Similarly, Anton and Boyle (250) showed that, in an *in vitro* enzyme system, albumin interfered with the acetylation of sulfamethoxypyridazine. Wiseman and Nelson (251) reported a rank-order correlation between the rates of *in vivo* metabolism of a number of sulfonamides and their degree of protein binding. A number of other studies (252–256) have suggested that an interdependence does exist between the rates of elimination of sulfonamides and degree of plasma binding. Rieder (183), however, did not observe a correlation between binding parameters and rate of disappearance from plasma or rate of renal excretion for the various sulfonamides which he studied. Similar results were obtained by Scholtan (181, 182) and by others (257–259). Rieder, in his discussion, points out that only when elimination proceeds primarily *via* glomerular filtration and without significant tubular secretion and reabsorption should binding be anticipated to exert an appreciable effect on the rate of elimination of a drug.

Other observations pertinent to this topic include those of Bennhold (260). He noted that the rate of elimination of congo red from patients with depressed albumin blood levels (0.1 to 0.2% of normal) was approximately 60% more rapid than with normal subjects. Upon infusion of human albumin, the elimination rate of the dye was depressed to near normal levels. Weiner *et al.* (261) have suggested that the slow biotransformation and negligible kidney elimination of dicoumarol is a result of the high degree of protein binding of this drug. The prolonged blood levels of chlortetracycline[1] were explained by Sirota and Saltzman (262) as being due to protein binding since the renal excretion of the drug was found to be solely a process of glomerular filtration. Kunin (263) has similarly related the prolonged plasma levels, slow kidney clearance, and low apparent volume of distribution of methacycline to its degree of binding. Nadeau and Subolewski (264) have suggested that extended blood levels and tolerance to morphine in dogs may be related to binding. Priestly and O'Reilly (265) reported a qualitative correlation between the biliary excretion rates of four azo dyes and their relative degrees of binding to liver homogenates and blood proteins. Beisel *et al.* (266), from their studies on cortisol elimination, concluded that plasma binding provides a reservoir of readily available steroid which is protected from excretion and metabolism. Other workers (267–269) studying different steroidal compounds have made similar conclusions.

Krüger-Thiemer and his associates, who have made outstanding contributions to the quantitative understanding of the influence of protein binding on the behavior of drugs, recently (270) discussed pharmacokinetic models which focus attention on the involvement of the binding phenomenon. One model, which was discussed in detail, depicted a drug as being distributed and in rapid equilibrium between two compartments, plasma water of volume V_1, and residual body water of volume V_3. Protein binding occurred only in the plasma compartment and the rate of renal excretion or elimination, characterized by the rate constant k', was proportional to the concentration of unbound drug in the plasma water. A rate equation analogous to the following was derived:

$$-\frac{d \ln Dt}{dt} = \frac{k'}{\left(1 + \frac{nP_t}{k_{dp} + D_f}\right)\left(V_1 + \frac{V_3}{1 + \frac{k_{dp} n P_t}{(k_{dp} + D_f)^2}}\right)} \quad \text{(Eq. 16)}$$

It should be noted that for the purposes of consistency in this paper, Eq. 16 was derived on the assumption that drug binding resulted from the interaction of drug with a specific plasma protein having n equivalent sites, and is, therefore, slightly different from that presented by Krüger-Thiemer *et al.* (270). Examination of the equation leads to the conclusion that, in such a case, a semi-log plot of D_t (plasma concentration, determined as the sum of bound and unbound drug) *versus* time will not be linear but will be characterized by a diminishing steepness of slope as time proceeds. They similarly showed that:

$$-\frac{d \ln D_f}{dt} = \frac{k'}{V_3 + V_1\left(1 + \frac{k_{dp} n P_t}{(k_{dp} + D_f)^2}\right)} \quad \text{(Eq. 17)}$$

Equation 17 predicts nonlinearity of a semi-log

[1] Trademarked as Aureomycin, Lederle Labs., Pearl River, N. Y.

plot of D_f (the concentration of unbound drug in the plasma) as a function of time. Moreover, the slope of such a curve will be initially greater than the corresponding D_t curve and will change more rapidly with time.

The model was examined by Krüger-Thiemer *et al.* (270) using computer generated data for various values of k_{dp}. In addition, blood level data obtained from a patient who was administered a long-acting sulfonamide, sulfaorthodimethoxine, were evaluated and found to be consistent with the behavior predicted by the model.

Martin (271) also considered theoretical aspects of the kinetics of elimination of drugs possessing high affinities for plasma proteins. He discussed a model in which drug was distributed between residual water and plasma. Binding occurred to form a 1:1 complex and drug disappearance proceeded at a rate which was directly proportional to the concentration of free drug. He did not present a rigorous mathematical analysis of the model but by approximate graphical methods was able to show that the rate of decline of drug concentration in the plasma underestimates the rate of drug elimination from the body. His calculations led to a conclusion that, because of protein binding, it could be possible to obtain a nonlinear semi-logarithmic plot of plasma concentration *versus* time in which slope values increase as time proceeds. It would seem, however, that this could not be the case and that this result was artifactually generated by his method of estimating plasma levels of drug. This model is similar to that discussed by Krüger-Thiemer *et al.* (270) and should exhibit the same type of behavior as they described.

Conclusions—The binding of drugs by plasma proteins continues to be a subject of active research interest and has attracted the attention of investigators from diverse disciplines. Research in this field is dipolar in character, consisting of, on the one hand, studies oriented to a fundamental physical-chemical understanding of the phenomenon and, on the other, investigations directed toward evaluation and quantitation of possible clinical manifestations and implications. Substantial progress has been made in both general areas.

While the majority of the reported *in vitro* studies have been semi-quantitative in nature, in recent years an increasing number have been conducted to yield a quantitative characterization of fundamental binding parameters. Analytically, the newer techniques of gel filtration, nuclear magnetic resonance spectroscopy, and fluorescence quenching provide the means of rapidly detecting binding, elucidating active sites involved in binding, and estimating the rates of interaction.

Considerable *in vitro* and *in vivo* evidence has been accumulated to demonstrate that plasma-protein binding can influence the distributional, pharmacological, and pharmacokinetic properties of certain drugs. In addition, theoretical contributions have been made which are of value in assessing the possible magnitudes of such influences. An impression, gained from the literature, is that there appears to be a tendency to overemphasize the general importance of the binding phenomenon in the behavior of drugs in the body. Evidence exists that only in the case of highly bound agents will binding be important in a practical sense. Many workers, in attempting to extrapolate *in vitro* data to *in vivo* expectations, tend to lose sight of the fact that the plasma comprises a relatively small fraction of the total volume available for drug distribution and that protein–drug complexes of rather extraordinary stability must be formed to substantially reduce the amount of drug that exists in the body in the active, diffusible, unbound form. A number of important drugs do, however, fall in the category of "strongly bound" and these serve as examples which emphasize the need to at least consider protein binding as a necessary parameter in the characterization of drug behavior.

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New Method for Calculating the Intrinsic Absorption Rate of Drugs

By J. C. K. LOO and S. RIEGELMAN

If conceiving the body to be a single compartment is correct, calculations based on this presumption should result in an exact estimate of the rate of appearance of a drug into the blood when administered intravenously at a precisely known rate. In order to test this hypothesis selected drugs were administered intravenously at known logarithmic and linear rates of infusion, thereby mimicking first- and zero-order absorption conditions. It is shown that methods based on the single-compartment concept do not result in acceptable estimates of the absorption rates. Not only do these methods lead to an incorrect rate constant but occasionally allow incorrect assignment of the order of the process. A new equation is presented, presuming the drug distributes between a central and one peripheral compartment, which allows one to calculate the rate of absorption. The equation results in an accurate estimate of the known rates of infusion (absorption) for the drugs studied to date.

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Accepted for publication January 23, 1968.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
This research was supported in part by a grant-in-aid from the research funds of the Academic Senate, San Francisco Division, University of California.

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ment, the slope of the blood level time curve at time, t, and the apparent elimination rate constant of the drug from the volume of distribution which is assumed to be constant and independent of time and dose administered. A number of other methods based on the same model have been proposed. These include the blood level maximum and time of maximum methods of Dost (2), and the ordinate intercept and total area methods of Krüger-Thiemer and Diller (3). Each of these methods requires one to make a basic presumption that the drug is being absorbed by a first-order process starting immediately after the drug is administered.

In 1960 Nelson (4) proposed a method utilizing the first and second derivatives of the urinary excretion data as a function of time. Later, in 1963, Wagner and Nelson (5) published what has become the most commonly used methods for estimating the apparent absorption rate of drugs utilizing either urinary or blood data. These methods have gained wide acceptance since they do not require prior estimate of the volume of distribution and they place no limitations on the order of the absorption rate process.

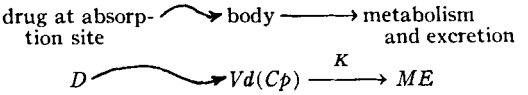
Several methods have been proposed by different workers which utilize the complete i.v. curve in conjunction with the oral absorption curve. The method of Scholer and Code (6) published in 1954 is an involved and extensive numerical estimation. The method of Silverman and Burgen published in 1961 (7) involves a curve follower and analog computer to deconvolute the blood data relative to a known i.v. curve. Recently Rescigno and Segre (8) have published a numerical rather than a computer method of deconvoluting the blood data. In 1965 Stelmach *et al.* (9) proposed an analog computer program which included the release of the drug from a dosage form prior to the absorption step. Their program initially was presented on a single-compartmental model. Their procedure was based on the presumption that one had previously defined the elimination rate constant, and the absorption rate constant of the drug administrated orally in solution.

In spite of all the available methods and the large number of estimates of the apparent absorption rate of different drugs by a variety of available methods, the literature[1] apparently contains no comparisons of these estimates with known input rates obtained from individual test subjects or animals.

The purpose of this paper is to present some experimental blood level data following infusion at known input rates. It will then be shown that the methods based on the single-compartmental model result in grossly inaccurate estimates of the known input rates and can lead to error and occasionally false interpretations of the results. Further, a new equation will be derived which allows one to use rather simple numerical procedures to estimate the true input rates.

THEORY

The Single-Compartmental Model—The single-compartmental model is based on the assumption that the dose absorbed is distributed into what is conceived to be a single compartment and is eliminated from it by first-order processes of metabolism and excretion. This is represented schematically below:



D is the amount of the original dose remaining at the absorption site, Cp is the concentration of the drug in the blood, Vd is the apparent volume of distribution[2] based on the presumption that the body behaves as a single compartment, ME represents the combined amount of drug metabolized and excreted by simultaneous first-order processes, K is the first-order rate constant for the elimination of the drug from the body and is equal to the sum of the individual rates of metabolism and excretion. On the basis of material balance, the amount of the intact drug reaching the fluids of distribution is taken to be the amount of drug absorbed at any time $(A)_t$. This is in effect the *net* influx of intact drug which has transferred into the blood stream. This is set equal to the amount of the drug in the body compartment plus the amount eliminated by all processes. Wagner and Nelson derived their absorption equations on the basis of this material balance. Since the rates of metabolism and excretion are often found to be directly proportional to the blood level, they proposed that the amount of the drug eliminated up to time t_n could be evaluated by $KV\int_{t_0}^{t_n} Cpdt$. Therefore, integration of the differential mass balance equation between the limits of t_0 and t_n, resulted in what has become known as the Wagner-Nelson (W-N) absorption equation for blood data:

$$(A/Vd)_{t_n} = Cp_{t_n} + K\int_{t_0}^{t_n} Cpdt \quad \text{(Eq. 1)}$$

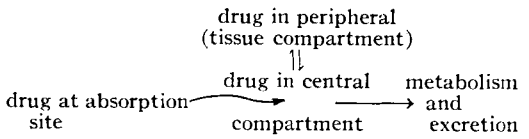
With Eq. 1, one attempts to evaluate the amount absorbed (expressed in concentration units) as being the sum of the concentration in the body as represented by the plasma concentration plus the second terms which is equal to the amount eliminated (expressed in concentration units). When all of the drug is absorbed, the value of $(A/Vd)_\infty$ will approach

[1] Wagner (10) utilized averaged data for 24 subjects obtained from Fig. 1 of Jopikii and Turpeinen's (11) data on the administration of i.v. infusions of glucose. Although a good fit was apparently obtained, the original data of Jopikii from these 24 subjects had been corrected for a varying basal glucose level. Further, the postinfusion elimination constant was different from the elimination constant during the infusion.

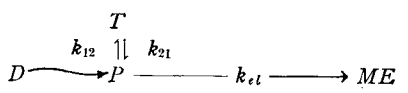
[2] In the nomenclature of the earlier papers of this series, this apparent volume of distribution is more correctly specified as Vd_{area}(11).

an asymptotic value representing the estimated total amount of the drug absorbed. Since the apparent volume of distribution may not be known, it is necessary to express the data in terms of the percent remaining to be absorbed. These data are frequently interpreted in terms of pseudo-zero and pseudo-first-order rates. Occasionally more complicated interpretation of the results is attempted (10).

The Two-Compartmental Open-System Model—Riegelman, Loo, and Rowland have recently proposed that it is mathematically and physiologically more acceptable to conceive the body to be a two-compartmental open system (12). Such a model is shown in the following schematic:



or in appropriate symbols:



P is the amount of the intact drug in the central compartment, which includes the blood, fluids, and tissues which, in effect, spontaneously equilibrate with the blood; T is the amount of intact drug in the peripheral (tissue) compartment; Vp is the volume of the central compartment; k_{12} and k_{21} are the first-order distribution rate constants out of and back into the central compartment from the peripheral compartment, and k_{el} is the sum of the (presumed) first-order rate constants for the simultaneous processes of metabolism and excretion. The above two-compartmental model results in the following general equation describing the blood level time curve after a single i.v. injection:

$$Cp = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 2)}$$

where at $t = 0$, $Cp^{\circ} = A + B$, and α and β are hybrid rate constants each influenced by *all* the individual rate processes. Because of this, Riegelman *et al.* (12) proposed that α and β be called the *fast and slow disposition* rate constants, respectively. The relationships between these disposition rate constants and the individual rate constants of this model have been presented in earlier publications. (12, 13). In the following presentation, it will be presumed that the accurate data have been obtained at appropriat : times and analyzed for the rate constants, k_{12}, k_{21}, and k_{el}.

It is convenient to convert all of the drug amounts represented in the model to concentration terms by dividing by Vp, whereupon they become Cp, Ct, and Cme, respectively. The amount metabolized and excreted up to time t_n, expressed in concentration units becomes:

$$(Cme)_{t_n} = k_{el} \int_{t_0}^{t_n} Cpdt \qquad \text{(Eq. 3)}$$

Integration of the differential mass balance equation between the limits of t_0 and t_n results in:

$$(A/Vp)_{t_n} = Cp_{t_n} + k_{el} \int_{t_0}^{t_n} Cpdt + Ct_{t_n} \qquad \text{(Eq. 4)}$$

This is in effect a modified Wagner-Nelson equation for the two-compartmental model; however, before Eq. 4 can be used to estimate the absorption rate of a drug, it is necessary for the tissue compartment concentration term to be expressed in measurable quantities. The differential equation representing the rate of change of this compartment with time is:

$$dCt/dt = -k_{21}Ct + k_{12}Cp \qquad \text{(Eq. 5)}$$

Since k_{12} and k_{21} can be estimated from the blood data, if Cp could be expressed as a function of time, the solution of Eq. 5 would be feasible. If one assumes that between the two data point times t_n and t_{n-1} the blood level data can be approximated by a straight-line segment, it is possible to substitute for Cp_{t_n} on the basis of the equation for a straight line:

$$Cp_{t_n} = Cp_{t_{n-1}} + M \cdot \Delta t \qquad \text{(Eq. 6)}$$

where

$$\Delta t = t_n - t_{n-1}\,; \quad \Delta Cp = Cp_{t_n} - Cp_{t_{n-1}}$$

and

$$M = \Delta Cp/\Delta t$$

whereupon we may substitute for Cp_{t_n} in Eq. 5 the equation can be integrated by Laplace transforms and the second term simplified by a two-term Taylor approximation:[3]

$$Ct_{t_n} = \frac{k_{12}}{k_{21}} Cp_{t_{n-1}}[1 - e^{-k_{21}\Delta t}] + k_{12}\frac{\Delta Cp\ \Delta t}{2} + Ct_{t_{n-1}}e^{-k_{21}\Delta t} \qquad \text{(Eq. 7)}$$

The total absorption equation based on the two-compartmental model can be obtained by substituting for $(Ct)_{t_n}$ in Eq. 4.

$$(A/Vp)_{t_n} = Cp_{t_n} + k_{el} \int_{t_0}^{t_n} Cpdt + \frac{k_{12}}{k_{21}} Cp_{t_{n-1}}[1 - e^{-k_{21}\Delta t}] + \frac{k_{12}\Delta Cp\Delta t}{2} + Ct_{t_{n-1}}\, e^{-k_{21}\Delta t} \qquad \text{(Eq. 8)}$$

As with the W-N equation, Eq. 1, the second term including the integral is evaluated by progressive summing of the area under the plasma concentration time curve from t_0 to t_n, usually using the trapezoidal rule for estimating the areas. Since the concentration of drug in the tissues is usually zero at the start of the study, when evaluating the tissue concentration at t_1 it is appropriate to set the last term in Eq. 8 equal to zero.[4] At each subsequent time, t_n, the tissue term must be solved by a method of successive substitution of the tissue concentration from the previous time interval.[5]

EXPERIMENTAL

The *N*-methylglucamine salt of acetylsalicylic acid (ASA) was prepared by mixing equimolar amounts of the base and acid. A 650-mg. dose of ASA was prepared in 10 ml. of solution. The solution was bacteriologically filtered and injected into

[3] A detailed derivation of Eq. 7 is given in the appendix.
[4] As with the use of Eq. 1, it may be necessary to make a time shift to correct for a delay in absorption.
[5] In order to facilitate the use of this complete absorption equation, a detailed analysis of sample data is included in Tables I and II under *Appendix*.

the antecubital vein by rapid i.v. injection. Blood samples were taken from the opposite arm at 1, 2, 4, 6, 8, 10, 15, 20, 25, 30, 45, 60, 75, 90, 120, 150, 180 min., and at hourly time periods for 8 hr. The samples were assayed for ASA by the specific gas chromatographic method of Rowland and Riegelman (13). Several weeks later the experiment was repeated using a Harvard infusion pump (model 975). Since this pump has a series of gears at constant ratio to one another, it was possible to establish a logarithmic rate of infusion by shifting the gears at constant time intervals. *In vitro* experiments showed the pump to produce logarithmic results through 97% of the injection, whereupon the final 3% were injected without further gear change. Different rates of infusion could be generated by merely modifying the time intervals for the gear changes. In order to keep the sampling as near as possible to the true log curve, the blood samples were taken at or near to the time of the gear changes. All experiments reported herein were done on male tests subjects.

Griseofulvin was injected into adult male volunteers in a specially prepared solution of griseofulvin dissolved in polyethylene glycol 300. The solution was not injected directly but by diluting it into a rapidly flowing normal saline solution, which was being administered by intravenous drip over a 3-min. period. Several weeks later the drug was administered over a 2-hr. constant infusion into the same test subject. The plasma samples were assayed for intact drug by a modification of the method of Bedford *et al.* (14).

An AEI model TR48 analog computer and a Varian Associates 11 × 17-in. X-Y recorder was used to study the models.

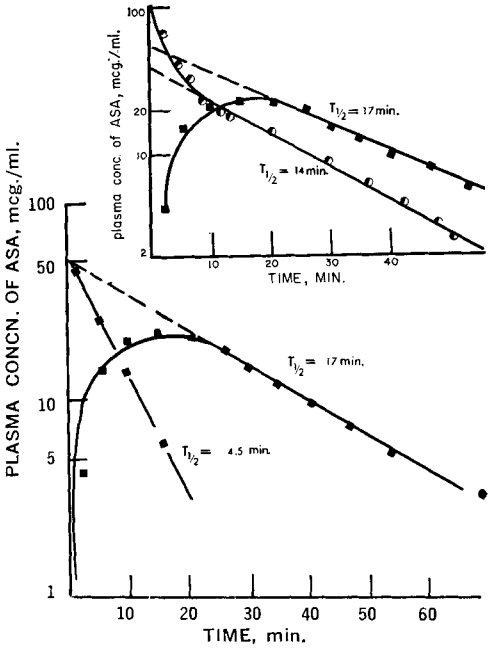


Fig. 1—Blood level of ASA after logarithmic infusion of 650 mg. into subject S at first-order rate of 0.0693 min.$^{-1}$ ($t_{1/2}$ = 10.0 min.) Infusion ceased at 64 min. Insert (Fig. 1a)—The superimposed blood curves after log infusion (■) and single i.v. injection (◐) of 650 mg. ASA.

RESULTS AND DISCUSSION

Figure 1 represents the data obtained when ASA was administered intravenously under logarithmic infusion conditions to a test subject. The data appear to be completely compatible with the concept of the body exhibiting the properties of a single compartment since the up curve could be said to represent the infusion period while the down curve then could represent the disappearance of the drug from the body by a single rate constant process. Indeed, a theoretical curve could be drawn through all the data points, except the 1-min. point, using the following equation where the rate constants were obtained by graphical estimation:

$$Cp = 0.53(e^{-0.041t} - e^{-0.15t})$$

The insert curves show the logarithmic infusion data superimposed on the rapid (10-sec.) i.v. injection data. The logarithmic infusion was continued for 64 min. Therefore, not only is absorption taking place at the same time as the drug is distributing into the peripheral tissue compartment, but it continues well beyond the peak in the blood curve. Due to the relative rates of drug infusion and loss from the body, the apparent disappearance curve changes. The observed disposition half life, $0.693/\beta$, for the i.v. curve is 14 min., while due to the continued influence of the infusion (absorption) process, the disposition half-life from the simulated logarithmic absorption data becomes 17 min. Changes in half-life such as these have been occasionally proposed to be due to changes in the elimination rate during the test period. Wagner (11) has also emphasized that the absorption process continues far beyond $t_{max.}$, the time of the maximum blood level.

Figure 2*a* represents the amount absorbed, divided by the appropriate volume constants *versus* time plots for Eqs. 1 and 8. The convergence on a false early asymptote with Eq. 1 is illustrated. Figure 2*b* includes plot of the log percent remaining to be absorbed *versus* time plot of the same data. The ASA was infused at a rate such that the half-life was precisely 10.0 min. Analysis of the data using Eq. 1 results in an estimate of the half-life for absorption in error by several orders of magnitude. In contrast, calculations based on Eq. 8 almost exactly duplicate the known infusion data. When 3% or less remains to be infused, the infusion pump no longer produces a log infusion. It is in this region that the calculated data diverges from the theoretical curve.

Figure 3 contains data for a second test subject administered ASA by a rapid i.v. and log infusion at a precise rate equal to a half-life of 6.0 min. The i.v. data are for a 650-mg. and a 325-mg. dose of the drug. While there is some small amount of experimental error, if the two curves are normalized, they are superimposable and therefore result in identical distribution and elimination rate constants and volume of distribution. Again there is a slight increase in disposition half-life in the log infusion (absorption curve). Additional assay data beyond the time indicated were obtained out to the limit of the assay method (0.4 mcg./ml.) and all three curves show mono-exponential decay. Figure 4 includes semilog and linear plots of the percent remaining to be absorbed plots. Once again the complete absorp-

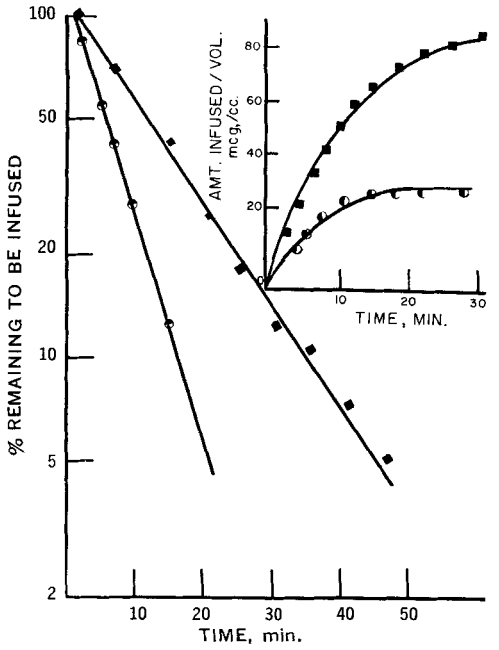


Fig. 2—The percent ASA remaining to be infused as determined by Eq. 1 (◐) and Eq. 8 (■). The latter data exactly coincide with the actual infusion rate ($t_{1/2}$ = 10.0 min.). Insert (Fig. 2a)—Corresponding amount infused divided by the volume constant intrinsic to the model, Eq. 1 (◐) and Eq. 8 (■).

tion equation predicts the known infusion rate, while it is difficult to draw a good curve through the W-N plot. When comparing Figs. 2, 4, and 6 it may appear that the magnitude of error in the Wagner-Nelson method is large only because of the infusion rates used and the selection of a rapidly metabolized drug such as ASA. That this is not so is seen where a PACE TR48 analog computer was programmed for distribution and elimination of griseofulvin found in the test subject reported in Figs. 5 and 6. Figure 7 includes curves illustrating the W-N plots which would have been found for this compound at selected first-order absorption rates. The disposition half-life was set at 9.5 hr. while the half-lives of the absorption processes were adjusted from 1.8 to 7 hr. When the input rates are large relative to the disposition rate constants, then the W-N calculation may result in an apparent first-order plot. However, the relative error in the estimated half-life varies with each experimental situation. The intermediate input rates result in concave descending-type curves to which it is difficult to assign a half-life. Usually the initial data points are emphasized in these analyses. However, it is important to note that the curves approach the theoretical half-lives near the end of the absorption process. Unfortunately, this is the portion of the data where the experimental error would be the largest. It would appear that if a given drug were made up as different formulations resulting in different rates of absorption, analysis of the blood data by Eq. 1 would apparently result in a varying error of estimation of their true absorption rates. The data for the theoretical input with a half-life of 3.8 hr. are replotted in Fig. 8 where the method of residuals is utilized to separate out the fast absorption component from the slower step. The appropriate bi-exponential equation for such a plot is:

$$\text{percent unabsorbed} = 0.45e^{-0.77t} + 0.55e^{0.183t}$$

It can be interpreted to indicate 45% of the drug was

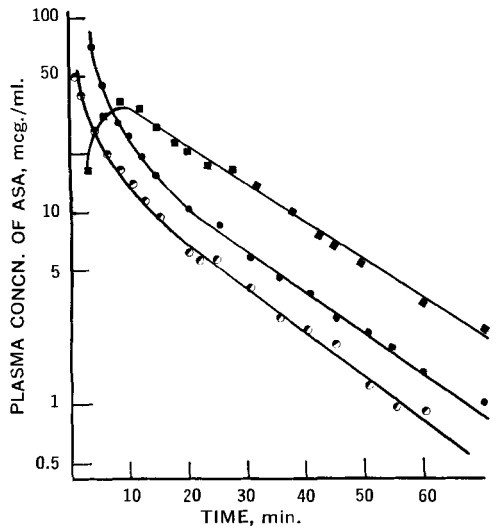


Fig. 3—Blood levels of ASA of subject P after single i.v. injection of 325 mg. (◐) and 650 mg. (●), and after a logarithmic infusion of 650 mg. ASA (■) at 0.115 min.$^{-1}$ ($t^1/_2$ = 6.0 min.).

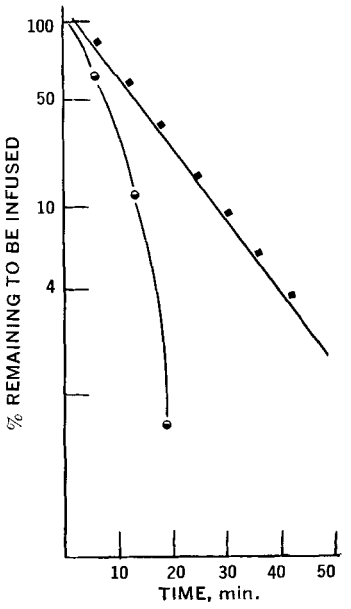


Fig. 4—The calculated percent ASA remaining to be infused as estimated by Eq. 1 (◐) and Eq. 8 (■). The latter is a good estimate of the actual infusion rate ($t_{1/2}$ = 6.0 min.).

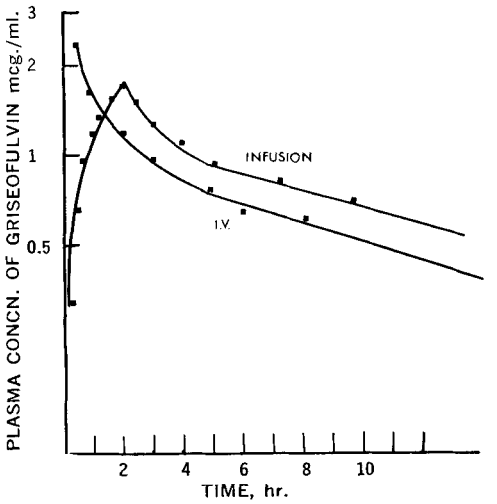


Fig. 5—The blood level of griseofulvin after a 2-hr. constant infusion at a rate of 12.8 mg./min. and after a single intravenous injection of 142 mg.

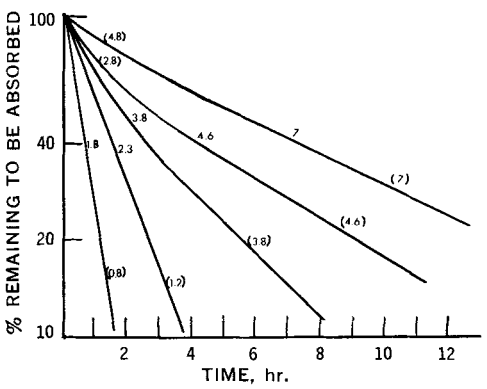


Fig. 7—Analog computer simulated plots of the percent remaining to be absorbed computed in accordance with Eq. 1. The values for the rate constants of the two-compartmental model are: $k_{12} = 0.29$ hr.$^{-1}$; $k_{21} = 0.31$ hr.$^{-1}$; $k_{el} = 0.16$ hr.$^{-1}$. The absorption half-life was changed for each plot and is indicated on each curve. The half-life values in parentheses are those which may be estimated from each appropriate linear segment.

absorbed at a half-life of 0.9 hr. while 55% was absorbed at a half-life of 3.8 hr. These are, of course, figments of the numerical method.

Using other appropriate distribution and elimination constants, the A/V *versus* time curves for the W-N method were obtained. It was noted that when the absorption rate is large relative to the rate of disappearance from the body that some of these curves exhibit a pronounced maxima. In their original publication Wagner and Nelson allude to this problem by indicating that the A/V value may reach a maximum. It appears that both the bi-exponential and the occasional appearance of maxima are artifacts of the numerical method. In Figs. 2*b* and 4, it can be seen from the percent unabsorbed plots that the use of Eq. 1 results in an underestimation of the time at which absorption ceases. This may cause difficulty in defining the release characteristics of prolonged-action dosage forms. It would appear virtually impossible to separate the perturbation of the absorption data caused by the use of the W-N equation from the complexities introduced by the two different absorption rates.

The absorption process of a drug is undoubtedly

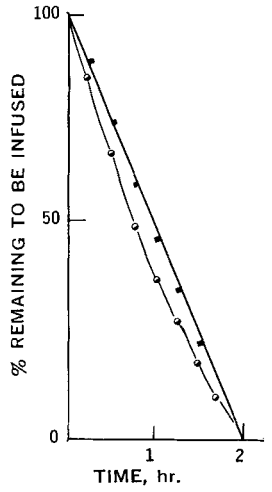


Fig. 6—The calculated percent griseofulvin remaining to be infused after a 2-hr. constant infusion. The curves are based on calculation using Eq. 1 (◐) and Eq. 8 (■); the latter is an excellent fit of the actual infusion rate curve.

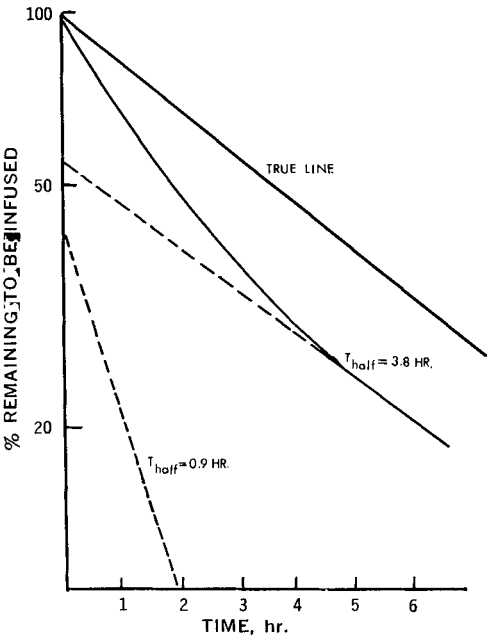


Fig. 8—An enlargement of the data given in Fig. 7 in which the input absorption half-life was set at 3.8 hr. The middle curve represents the data that would be obtained from Eq. 1. The lower curve is obtained from the middle curve by the method of residuals

highly complex. It is probable that each dose differs in its rate of passage down the gastrointestinal tract as well as in the rate at which the drug is released from its dosage form. If the new equation allows us to obtain valid data on such absorption rate processes, as it now appears, then it may allow some interpretation of the influence of the site of absorption on the rate process. This has been proposed as a significant contributing factor to the results obtained from the oral administration of griseofulvin to man. It appears that a significant fraction of the total griseofulvin is absorbed in a short interval of time which may coincide with the transfer of the drug through the duodenum. This is followed by a prolonged slow absorption for many additional hours (15). This small amount of secondary absorption causes a profound change in the apparent rate of disappearance of the drug from the blood. It would have been impossible to make any quantitative analysis of the absorption process of griseofulvin without the availability of i.v. data on this drug (15) along with this new method of analysis. The present authors have emphasized in earlier publications of this series (12, 13) that the plasma concentration time curves for virtually all drugs require utilization of a multi-exponential equation such as represented by Eq. 2. This is well recognized by many workers. However, until an i.v. dose is administered, with blood taken at proper sampling intervals, it is impossible to assess the contribution of the first term, Ae^{-at}, to the early phase of the drug disposition. The W-N equation is derived on the presumption that this first term is negligible. The modified absorption equation does not make this presumption. It is based only on the following two assumptions: (*a*) that the i.v. blood curve can be defined by a set of first-order differential equations, and (*b*) that the values of the rate constants are independent of dosage level and time. The data presented in this study have been shown to comply with those restrictions within the dosage ranges studied.

The two-compartmental model derivation has resulted in three important changes in the W-N equation. First, the very important peripheral tissue compartmental term Ct has been included. Second, the model forces one to distinguish between the rate constant for the disappearance of the compound from the body and the specific elimination rate constant, k_{el}. The former constant is a hybrid constant and is a complex function of the distribution rate constants k_{12} and k_{21} as well as the elimination rate constant, k_{el}. In most drugs the elimination rate constant, k_{el}, calculated on the multicompartmental model is two to fivefold larger than the slowest disposition rate constant. However, with drugs such as digitoxin, it may be as much as 40 times larger than the slowest disposition rate constant. Third, the volume constant used to convert the amounts into concentrations is found to be the volume of the central compartment, Vp, rather than the apparent total volume of distribution Vd_{area} (11). The effect of the first two modifications influences the shape of the A/V *versus* time plot, and results in evidence for a longer period for the absorption before the asymptotic value is reached. The third term merely changes the absolute value of the A/V.

It should be noted that the new absorption equation, derived on a two-compartmental open-system model, can be expanded to include additional peripheral compartments when necessary. For example, if the i.v. data indicates that a tri-exponential equation is required to describe the total curve, it would be necessary to expand the mammillary model to two peripheral compartments. One would then estimate the distribution rate constants for each compartment (13, 16). Finally, during an absorption study of such a drug one would have to estimate the tissue concentration in each peripheral compartment at each data point time by using Eq. 7 written to include the unique distribution rate constants of each compartment.

One criticism which might be made against the new equation is the dependency it places upon the evaluation of the distribution and elimination rate constants from previously administered i.v. dose. Undoubtedly there will be instances when these constants will change with continued use of the drug and the absorption test should be conducted as soon as possible after the i.v. experiment. Further, the effect of possible changes in the elimination rates, such as might be caused by induction of drug metabolizing enzymes can best be evaluated on the basis of the multicompartmental model. It is believed, however, that some of the variation in the disposition rate constant reported by some observers is due to the variable influence of the absorption rate process on the disposition rate constant. This can be seen in Fig. 9 by comparing the i.v. and oral curves which have been generated on theoretical data derived in the appendix and in experimental data shown in Figs. 3 and 4.

Absorption Analysis Based on Urinary Excretion Data—A relatively large number of the analyses of absorption rates published to date have been made using the W-N equation derived for the analysis of urinary data, which is:

$$f(A)_{t_n} = \frac{1}{k}\left(\frac{d\,Ae}{dt}\right)_{t_n} + (Ae)_{t_n} \qquad \text{(Eq. 9)}$$

$(Ae)_{t_n}$ is the cumulative amount excreted up to time t_n and $(dAe/dt)_{t_n}$ is the rate of excretion estimated from the differential data from t_{n-1} to t_n, and f is the fraction of the administered drug excreted intact. The first term on the right-hand side represents the amount of the absorbed dose remaining in the body and the second term is to represent the amount eliminated by all processes.

Application of the two-compartmental open-system model to urinary data requires insertion of a term for the tissue compartment. The resultant equation is:

$$f(A)_{t_n} = k_{el}\left[\left(\frac{dAe}{dt}\right) + Ae\right]_{t_n} + T_n \qquad \text{(Eq. 10)}$$

where

$$T_n = \frac{1}{k_{el}}\left[\left(\frac{dAe}{dt}\right)_{t_{n-1}} \cdot \frac{k_{12}}{k_{21}}\left(1 - e^{-k_{21}\Delta t}\right)\right] + \frac{1}{k_{el}}\left[\left(\frac{dAe}{dt}\right)_{t_n} - \left(\frac{dAe}{dt}\right)_{t_{n-1}}\right]\frac{k_{12}\Delta t}{2} + T_{n-1}e^{-k_{21}\Delta t}$$

It is immediately apparent that the application of this equation to urinary excretion data will be difficult. With some drugs, such as amines, it is extremely difficult to detect the level of these com-

pounds in the blood without recourse to radioactive tracers while they may be conveniently and more simply followed by urinary excretion methods. Nevertheless, to apply Eq. 10, it will be necessary to define the distribution and elimination rate constants from the urinary data after an i.v. injection. This will require urine samples at short intervals in the first few hours or may at times necessitate collection of the samples by catheterization. If urine samples are taken at sufficiently frequent intervals, under conditions of controlled pH, the urinary excretion data should also result in a bi-exponential curve, which upon being normalized may be analyzed for the appropriate distribution and elimination rate constants.

Alternate Methods for Analyzing Blood Data—The alternative analytical methods for estimating absorption rates utilizing both i.v. and oral data published by earlier authors have not been utilized in pharmacokinetic literature on drug absorption. The method of Scholer and Code was utilized by these authors in studying the oral absorption of D_2O (6) and later by Graham *et al.* (17) in their study of the absorption of ^{28}Mg. This method is a valid, but extremely tedious method of analyzing for the absorption rate of the compound. The method utilizes the multi-exponential equation representing the fate of the compound in the body (obtained in a prior i.v. experiment) to estimate numerically the fate of each increment of dose at each data point. This method, therefore, necessitates a large number of repetitive calculations, *i.e.*, N^2, where N is the number of data points during the interval where absorption is taking place. In contrast, Eq. 9 requires one calculation per data point.

The method of Silverman and Burgen and the method of Recigno and Segre each directly utilize the multi-exponential equation representing the fate of the drug in the body after an i.v. dose. In these methods the absorption data are compared to the curve of the i.v. dose and an amount remaining to be absorbed is obtained by a mathematical procedure of deconvolution of the oral absorption data. The method of Silverman and Burgen utilizes an analog computer while the latter method is a numerical procedure, which also may be carried out by utilizing a digital computer (18).

The method proposed in this paper utilizing Eq. 8 differs in an important characteristic from these alternative methods. While requiring prior information as to the multi-exponential fate of the drug in the body, a two- or three-compartmental open-system model is defined and the distribution and elimination constants are evaluated. The method does not require comparison with the i.v. curve itself but utilizes the distribution and elimination rate constants in the equation. It therefore allows one to carry out this calculation using a simple desk calculator. It also affords an excellent picture of the fate of the drug within the body at all times.

APPENDIX

A Derivation of Eq. 7—As noted in the text, Eq. 6 is the differential equation representing the rate of change of the tissue concentration in terms of the two variables, Ct and Cp. It is necessary to substitute for the Cp at time t_n in terms of the prior blood value Cp at time t_{n-1}. This can be done if we presume that the plasma concentration time curve is linear over this short interval so that the equation for the straight line applies. This is represented as Eq. 6 in the text. Equation 5 can be written as:

$$\frac{dCt}{d\tau} = -k_{21}Ct + k_{12}Cp + k_{12}M\tau \quad \text{(Eq. 1}a\text{)}$$

where $\tau = t_n - t_{n-1}$.

In order to integrate Eq. 1a, we convert to Laplace notation, with $\overline{Ct}$ referring to the Laplace transform of Ct, with the initial conditions set at $(Ct)_{t_{n-1}}$ and S is the Laplace operator notation:

$$\overline{Ct}S - Ct_{t_{n-1}} = -k_{21}\,\overline{Ct} + \frac{k_{12}\,Cp}{S}\,t_{n-1} + \frac{k_{12}M}{S^2} \quad \text{(Eq. 2}a\text{)}$$

Rearranging we have:

$$\overline{Ct}(S + k_{21}) = Ct_{t_{n-1}} + \frac{k_{12}Cp}{S}\,t_{n-1} + \frac{k_{12}M}{S^2} \quad \text{(Eq. 3}a\text{)}$$

or

$$\overline{Ct} = \frac{Ct}{S + k_{21}}\,t_{n-1} + \frac{k_{12}Cp}{S(S + k_{21})}\,t_{n-1} + \frac{k_{21}M}{S^2(S + k_{21})} \quad \text{(Eq. 4}a\text{)}$$

The inverse transform of Eq. 4a is

$$Ct_{t_n} = Ct_{t_{n-1}}e^{-k_{21}\Delta t} + \frac{k_{12}}{k_{21}}\,Cpt_{n-1}(1 - 3^{-k_{21}\Delta t}) + \frac{k_{12}}{(k_{21})^2}\,\frac{\Delta Cp}{\Delta t}\,(e^{-k_{21}\Delta t} + k_{21}\Delta t - 1) \quad \text{(Eq. 5}a\text{)}$$

where Δt is substituted for τ and $\Delta Cp/\Delta t$ for M. The third term of Eq. 5a can be reduced by substituting for the exponential component in terms of a two-term Taylor expansion, namely, $e^{-x} = 1 - x + x^2/2$, or

$$\frac{k_{12}}{(k_{21})^2}\,\frac{\Delta Cp}{\Delta t}\,(e^{-k_{21}\Delta t} + k_{21}\Delta t - 1) = \frac{k_{12}}{k_{21}}\,2\,\frac{\Delta Cp}{\Delta t}\left(1 - k_{21}\Delta t + \frac{(k_{21})^2(\Delta t)^2}{2} + k_{21}\Delta t - 1\right) \quad \text{(Eq. 6}a\text{)}$$

which reduces to $k_{12}\Delta Cp\Delta t/2$.

Substituting into Eq. 5a we arrive at the form given as Eq. 7 in the above text:

$$Ct_{t_n} = Ct_{t_{n-1}}e^{-k_{21}\Delta t} + \frac{k_{21}}{k_{12}}\,Cpt_{n-1}\,(1 - e^{-k_{21}\Delta t}) + \frac{k_{12}\Delta Cp\Delta t}{2} \quad \text{(Eq. 7)}$$

Sample Calculation Using Eq. 7—The following model will be utilized:

$$D \xrightarrow[k_a = 0.41\ \text{hr.}^{-1}]{} P \xrightarrow[k_{el} = 0.16\ \text{hr.}^{-1}]{} ME$$

$$P \underset{k_{21} = 0.31\ \text{hr.}^{-1}}{\overset{k_{12} = 0.29\ \text{hr.}^{-1}}{\rightleftarrows}} T$$

This results in a disposition rate constant, β, of 0.0725 hr.$^{-1}$ ($t_{1/2}$ = 9.5 hr.) after sufficient time for the absorption process to be complete. However, Table I includes data points for Cp at various times up to 15 hr., generated with the use of the TR-48 analog computer. These data are plotted in Fig. 9.

TABLE I—CALCULATIONS OF *Ct* VALUES[a]

1	2	3	4	5	6	7	8	9	10	11	12
Cp_{t_n}	$(t)_{t_n}$	ΔCp	Δt	$(k_{12}\Delta Cp\,\Delta t)/2$	$Cp_{t_{n-1}}$	$k_{12}/k_{21}(1-e^{-k_{21}\Delta t})$	$Cp_{t_{n-1}}k_{12}/k_{21}\times(1-e^{-k_{21}\Delta t})$	$Ct_{t_{n-1}}$	$e^{-k_{21}\Delta t}$	$Ct_{t_{n-1}}e^{-k_{21}\Delta t}$	$Ct_n = 5+8+11$
3.00	0.5	3.0	0.5	0.218	0	0.134	0	0	0.857	0	0.218
5.20	1.0	2.2	0.5	0.160	3.00	0.134	0.402	0.218	0.857	0.187	0.749
6.50	1.5	1.3	0.5	0.094	5.20	0.134	0.697	0.749	0.857	0.642	1.433
7.30	2.0	0.8	0.5	0.058	6.50	0.134	0.871	1.433	0.857	1.228	2.157
7.60	2.5	0.3	0.5	0.022	7.30	0.134	0.978	2.157	0.857	1.849	2.849
7.75	3.0	0.15	0.5	0.011	7.60	0.134	1.018	2.849	0.857	2.442	3.471
7.70	3.5	0.05	0.5	0.004	7.75	0.134	1.039	3.471	0.857	2.976	4.019
7.60	4.0	−0.10	0.5	−0.007	7.70	0.134	1.032	4.019	0.857	3.444	4.469
7.10	5.0	−0.50	1.0	−0.073	7.60	0.250	1.900	4.469	0.733	3.276	5.103
6.60	6.0	−0.50	1.0	−0.073	7.10	0.250	1.775	5.103	0.733	3.740	5.442
6.00	7.0	−0.60	1.0	−0.087	6.60	0.250	1.650	5.442	0.733	3.989	5.552
5.10	9.0	−0.90	2.0	−0.261	6.00	0.432	2.592	5.552	0.538	2.987	5.318
4.40	11.0	−0.70	2.0	−0.203	5.10	0.432	2.203	5.318	0.538	2.861	4.861
3.30	15.0	−1.10	4.0	−0.638	4.40	0.720	3.168	4.861	0.280	1.361	3.891

[a] Calculated using the following rate constants: $k_{12} = 0.29$ hr.$^{-1}$; $k_{21} = 0.31$ hr.$^{-1}$; $k_{el} = 0.16$ hr.$^{-1}$; $\beta = .0725$ hr.$^{-1}$; $Ct_n = k_{12}\Delta Cp\,\Delta t/2 + k_{12}/k_{21}Cp_{t_{n-1}}(1-e^{-k_{21}\Delta t}) + Ct_{t_{n-1}}e^{-k_{21}\Delta t}$.

Fig. 9—Simulated data for a theoretical drug with distribution and elimination characteristics given in Fig. 7 and used in calculation for Tables I and II. Note the slight divergence of the slopes of the i.v. and the oral curves. The straight line indicates the log percent remaining to be absorbed ($t_{1/2}$ = *1.8 hr.*).

It is to be noted that the data points beyond the fourth hour appear to lie on a straight line even though 20% of the drug remains to be absorbed ($t_{1/2}$ for the absorption = 1.8 hr.). One would usually define this slope as the elimination rate constant; more appropriately in the context of the nomenclature of this paper it is called the "apparent" disposition rate constant with a half-life of 8.9 hr. (β = 0.078 hr.$^{-1}$). It is to be noted that the value is not identical with the half-life for the true disposition rate process, *i.e.*, 9.5 hr. since the absorption and the fast disposition component, α, are still contributing to the observed value. Columns 3 through 12 include the steps required in the numerical calculation of the values for Ct_{t_n} utilizing Eq. 7. The accuracy of the estimates of *Ct* can be checked in this instance since the analog computer can generate the tissue concentration curve. The solid line in Fig. 10 increases the computer generated curve and the superimposed open circles indicate the calculated values as listed in Table I, Column 12. The excellent fit is readily apparent.

The estimation of the absorption rate constant from the data utilizing Eq. 7 is given in Table II. The appropriate steps are those used in the original W-N equation, in which the area under the blood curve between each set of data points is estimated by the trapezoidal rule. These are listed in Column 3. These values are progressively summed from t_o to t_n in Column 4, and the cumulative sum of these estimates are multiplied by the known elimination rate constant k_{el}. The A/Vp values are then calculated from the sum of Columns 2 + 5 + 6. These data can be converted into the percent unabsorbed as indicated.

Table II also includes the calculation of the

TABLE II—CALCULATIONS OF PERCENTAGE UNABSORBED VALUES[a]

1	2	3	4	5	6	7	8	9	10	11	12	13
Time, hr.	Cp_{t_n}	$\int_{n-1}^{t_n} Cpdt$	$\int_0^{t_n} Cpdt$	$k_{el}\int_0^{t_n} Cpdt$	Clt_n	$A/Vp = 2+5+6$	% A/Vp	100% − A/Vp%	$\beta\int_0^{t_n} Cpdt$	$A/Vd = 2+10$	% A/Vd	100 − % A/Vd
0.5	3.00	0.750	0.750	0.120	0.218	3.338	16.6	83.4	0.054	3.045	32.5	67.5
1.0	5.20	2.050	2.800	0.448	0.749	6.397	31.8	68.2	0.203	5.403	57.5	42.5
1.5	6.50	2.925	5.725	0.916	1.433	8.849	44.0	56.0	0.415	6.915	73.6	26.4
2.0	7.30	3.450	9.175	1.468	2.157	10.925	54.3	45.7	0.665	7.965	84.7	15.3
2.5	7.60	3.725	12.900	2.064	2.849	12.513	62.2	37.8	0.935	8.535	90.8	9.2
3.0	7.75	3.838	16.738	2.678	3.471	13.889	69.1	30.9	1.214	8.964	95.4	4.4
3.5	7.70	3.863	20.601	3.296	4.019	15.015	74.6	25.4	1.494	9.194	97.8	2.2
4.0	7.60	3.825	24.426	3.908	4.469	15.977	79.4	20.6	1.771	9.371	99.7	0.3
5.0	7.10	7.350	31.726	5.084	5.103	17.287	85.9	14.1	2.300	9.400	100.0	0
6.0	6.60	6.850	38.626	6.180	5.442	18.222	90.6	9.4	2.800	9.400		
7.0	6.00	6.300	44.926	7.188	5.552	18.740	93.1	6.9	3.257	9.257		
9.0	5.10	11.100	56.026	8.964	5.318	19.382	96.3	3.7	4.062	9.162		
11.0	4.40	9.500	65.526	10.484	4.861	19.745	98.1	1.9	4.751	9.151		
15.0	3.30	15.400	80.926	12.948	3.891	20.139	100.0	0	5.867	9.167		

[a] Columns 1-9: $A/Vp = Cp_{t_n} + k_{el}\int_0^{t_n} Cpdt + Clt_n$; $Clt_n = k_{12}\Delta Cp\Delta t/2 + k_{12}/k_{21}\, Cp_{t_{n-1}}(1 - e^{-k_{21}\Delta t}) + Clt_{n-1}e^{-k_{21}\Delta t}$. Columns 10-13: $A/Vd = Cp_{t_n} + \beta\int_0^{t_n} Cpdt$.

Fig. 10—Simulated plots of data taken from Table II simulating the blood level (Column 2; ●), tissue level (Column 6; ○), and drug elimination (Column 5; ■). The solid lines are the computer simulated curves. The insert includes the percent remaining to be absorbed plots (Column 9; ●) calculated using Eq. 8 and Eq. 1 (Column 13; □).

"apparent" percent unabsorbed according to the W-N equation. This is listed in Columns 10 through 13. Figure 10 includes the semilog plots of the percent unabsorbed, according to the two methods of calculation, *i.e.*, Columns 9 *versus* 13. It is noted that the calculations based on Eq. 8 (Column 9) result in an exact prediction of the known absorption half-life of 1.8 hr. while the W-N equation (Column 13) leads to a prediction that the half-life for the absorption process is approximately 0.8 hr.

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Keyphrases

Absorption rate, intrinsic—drugs
Drug absorption, distribution, elimination—models
Body considered—one, two compartments
Compartments, two *vs.* one—drug absorption
Kinetic equations—drug absorption
Acetylsalicylic acid salt—test compound
Griseofulvin—test compound

Effect of Topical Vehicle Composition on the *In Vitro* Release of Fluocinolone Acetonide and its Acetate Ester

By B. J. POULSEN, E. YOUNG, V. COQUILLA, and M. KATZ

A model was developed to test certain concepts regarding the *in vitro* release of steroids from topical vehicles. The steroids studied were fluocinolone acetonide and fluocinolone acetonide acetate. The vehicles selected for the study were propylene glycol–water mixtures gelled with Carbopol 934. Isopropyl myristate was used as the receptor phase for the diffusing steroids. Assay of the quantity of the steroids in the receptor phase was simplified by the use of radioactive compounds. The study indicated that the important factors influencing the release of steroid into the receptor phase were the solubility in the vehicle and the partition coefficient of the steroid between the vehicle and the receptor phase. Maximum release was achieved by altering the propylene glycol content of the vehicles.

OCCLUSIVE DRESSINGS have been successfully used to extend the therapeutic effectiveness of topical corticosteroids (1–3). This enhanced activity has been attributed to improved penetration of the skin by the drug (1). This indicates that, in certain instances, the development of formulations that increase drug penetration would be desirable.

There are two general approaches to the problem. One is to include in the vehicle agents which affect the barrier function of the epidermis so as to promote penetration of the therapeutic compound (4–7). The other approach is to alter the physical characteristics of the vehicle and thus affect the diffusion of the drug from the vehicle into the skin. As a preliminary to evaluating this latter effect of vehicles on skin penetration, the *in vitro* release of steroids from model vehicles was studied.

Higuchi (8) suggested the following equivalent relationships could be used to approximate the penetration of the barrier phase of the skin by a drug dissolved in a topical vehicle.

$$\frac{dQ}{dt} = (P.C.)C_v\frac{DA}{L} \qquad \text{(Eq. 1)}$$

$$\frac{dQ}{dt} = \frac{a_v}{\gamma_s}\frac{DA}{L} \qquad \text{(Eq. 2)}$$

The terms in these equations are defined as follows:

dQ/dt = steady rate of penetration
$(P.C.)$ = the effective partition coefficient of drug between skin barrier and vehicle
C_v = concentration of drug in the vehicle
D = the effective average diffusivity of the drug in the skin barrier
A = cross-sectional area of the application site
L = effective thickness of the skin barrier
a_v = thermodynamic activity of the drug in the vehicle

Received April 28, 1967, from Syntex Research, Standford Industrial Park, Palo Alto, CA 94304
Accepted for publication November 13, 1967.

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γ_s = effective activity coefficient of the drug in the skin barrier

If Eqs. 1 and 2 are considered with the objective of altering the penetration of a topically applied drug, it becomes apparent that only certain terms are readily susceptible to manipulation by the formulator. These are the partition coefficient (*P.C.*) and drug concentration (C_v) terms of Eq. 1 and, equivalently, the activity term (a_v) of Eq. 2.

Relationships between partition coefficients of various compounds and skin penetration have been reported (9–13). For our purposes, the partition coefficient can be defined as

$$P.C. = \frac{\gamma_v}{\gamma_s} \qquad \text{(Eq. 3)}$$

where γ_v and γ_s are the activity coefficients of the drug in the vehicle and skin, respectively. While it may be necessary to regard the activity coefficient of the drug in the skin barrier phase as an unknown and unalterable value, γ_v can be modified simply by altering the composition of the vehicle. Higuchi (8) has reported, for instance, that the activity coefficient of Sarin is approximately 1,500 times greater in perfluorotributylamine than in *m*-cresol. Katz and Shaikh (9) have previously reported the correlation of solubility and partition coefficient to topical corticosteroid therapeutic activity as measured by the Stoughton-McKenzie vasoconstrictor test.

It is evident that increasing the thermodynamic activity of a topically applied drug by altering vehicle composition might be expected to enhance its rate of penetration. It is, however, also necessary to consider what can be accomplished with a specific drug relative to the concentrations actually required in therapy. In practical terms, the saturation solubility in the vehicle places an upper limit on the activity that can be achieved for the penetrant (8). In this study, a simple model system was devised to test the effect of vehicle composition on the release of therapeutic concentrations of two topically active corticosteroids.

EXPERIMENTAL

Materials—Carbopol 934,[1] diisopropanolamine,[2] isopropyl myristate,[3] propylene glycol,[4] toluene (scintillation grade),[5] PPO,[6] POPOP,[6] fluocinolone acetonide,[7] and fluocinolone acetonide acetate[8] were used as received. In the release experiments, ^{14}C-labeled (acetonide label) fluocinolone acetonide and fluocinolone acetonide acetate were used as provided by the Institute of Steroid Chemistry, Syntex Research, Palo Alto, California.

Preparation of Vehicles—The propylene glycol and propylene glycol–water mixtures used as the donor phase were gelled by the addition of 1% w/w Carbopol 934 resin. The Carbopol was neutralized by the addition of an equal weight of diisopropanolamine. The steroids were incorporated into the vehicles by dissolving the steroid in the propylene glycol portion of the vehicle. The Carbopol resin was dispersed in water or a portion of the propylene glycol with the aid of an Eppenbach Homo-Mixer and added to the steroid solution. Diisopropanolamine was then added to gel the mixture. The final pH of the preparations ranged from 6–7. Freshly prepared formulations were used in the release studies.

Since viscosity of a preparation could be a factor affecting release, viscosity determinations were made on gels containing from 1 to 100% propylene glycol. The measurements were made with a Brookfield model RVT viscometer using the Helipath stand. Determinations made at room temperature, using the TC spindle at 5 r.p.m., indicated that a slight decrease in viscosity occurred as the propylene glycol content of the gels was increased. This decrease amounted to approximately a 20% reduction in viscosity over the propylene glycol concentration range from 1 to 100%.

Determination of Release Rates—The containers used to support the gels in this study were Petri dishes 100 mm. in diameter and 5 mm. deep. The dish was completely filled with the vehicle and the excess removed with the edge of a spatula to produce an even, uniform surface of constant dimensions. The weight of vehicle required to fill the dish was approximately 30 Gm. The dish containing the vehicle was placed in the bottom of a 1,000-ml. beaker, and the beaker was partially immersed in a large water bath at 37°. Two-hundred milliliters of isopropyl myristate previously equilibrated to 37° was carefully layered over the vehicle to begin the experiment. A large stirring blade of a width approximately equal to the diameter of the Petri dish was employed to produce agitation. The bottom edge of the stirring blade was positioned just above the surface of the gel. Six experiments could be run simultaneously in the same water bath by the use of a multiple-blade Phipps-Bird laboratory stirrer. It was determined experimentally that the release rates were sensitive to the rate of stirring up to approximately 30 r.p.m. Accordingly, the stirring rate was maintained at 40 r.p.m. Samples of isopropyl myristate were withdrawn for assay at intervals over a 24-hr. period.

Solubility Determinations—The solubilities of fluocinolone acetonide and fluocinolone acetonide acetate in propylene glycol–water mixtures were determined by two methods. In the first, excess steroid was rotated in sealed 10-ml. ampuls in a constant-temperature bath at 25°. Samples were withdrawn for chemical analysis after 2 weeks. In the second method, excess steroid was heated to 80°,

[1] Supplied by B. F. Goodrich Co., Cleveland, Ohio.
[2] Union Carbide Chemicals Co., New York, N. Y.
[3] Deltyl-Extra by Givaudan Co., Clifton, N. J.
[4] Union Carbide Chemicals Co., New York, N. Y.
[5] Toluene (scintillation and fluorometric grade) from Matheson, Coleman & Bell, Div. Matheson Co., Inc., Norwood, Ohio.
[6] Arapahoe Chemicals, Div. Syntex Corp., Boulder, Colorado.
[7] 6α,9α-Difluoro-16α hydroxyprednisolone 16,17-acetonide (Synalar), Syntex Laboratories, Inc., Palo Alto, California.
[8] 6α,9α-Difluoro-16α hydroxyprednisolone 16,17-acetonide 21-acetate, Syntex Laboratories, Inc., Palo Alto, California.

with rapid stirring for a few minutes, and the resulting mixture sealed into 10-ml. ampuls and rotated in the constant-temperature bath at 25° for 24 hr. prior to analysis. Equivalent results were obtained by the two methods.

The samples were assayed by withdrawing 5 ml. of solution, filtering through a Millipore filter (type GS, mean pore diameter 0.22 ± 0.02 μ), diluting with water, extracting the steroid with chloroform, evaporating the chloroform extract to dryness, taking up the steroid with ethanol, and assaying a suitable dilution of the alcoholic solution by the blue tetrazolium colorimetric assay.

Partition Coefficient Determinations—One-tenth milligram of steroid per ml. was dissolved in isopropyl myristate. Twenty-five milliliters of this solution was added to an equal volume of the various propylene glycol–water mixtures and equilibrated at 37°. After equilibrium, the phases were separated and assayed individually for steroid content. Partition coefficients were calculated as the ratio of the steroid concentration in the isopropyl myristate to that in the glycol–water phase.

Radiochemical Assays—The specific activities of the radiochemicals used in the study were 0.20 μc./mg. for fluocinolone acetonide-^{14}C and 0.10 μc./mg. for fluocinolone acetonide acetate-^{14}C. Samples of the isopropyl myristate receptor phase were assayed for steroid by counting in a Packard Tri-Carb liquid scintillation counter. Two-milliliter samples were mixed directly with 15 ml. of scintillation fluid consisting of 5 Gm. of PPO (2,5-diphenyloxazol), and 0.1 Gm. of POPOP (1,4-bis-2-[4-methyl-5-phenyloxazolyl]benzene) diluted to 1 L. with scintillation grade toluene. Internal standardizations were used to evaluate quenching of the samples. Ordinarily, quenching was found to be negligible and no quenching correction was required. Since steroids of low specific activity were used in the study, samples were counted for a total of 10 min. Total steroid release was calculated from the specific activities of the isotopes.

Confirmation that the radioactive assay measured only intact steroid was accomplished as follows: Samples of isopropyl myristate were withdrawn following release experiments of 24-hr. duration. The steroid was extracted from the isopropyl myristate and spotted from chloroform solution on silica gel TLC plates. The plates were developed in chloroform–butyl acetate–acetone (2:2:1) and dried. The plates were then scanned for radioactivity in a Vanguard model 880 dual-channel autoscanner equipped for thin-layer plates. It was found that essentially all the radioactivity was concentrated in a plate region corresponding to the correct R_f value for the steroid in question.

RESULTS

The solubilities of fluocinolone acetonide and the 21-acetate ester in propylene glycol–water mixtures at 25° are shown in Fig. 1. Partition coefficients for the two steroids between isopropyl myristate and various propylene glycol–water mixtures were determined at 37° and are shown in Fig. 2. Since the composition of the isopropyl myristate remained essentially unchanged during the release experiments, the partition coefficients are a reasonable approximation of the relative activity coefficients for the drug in the vehicles.

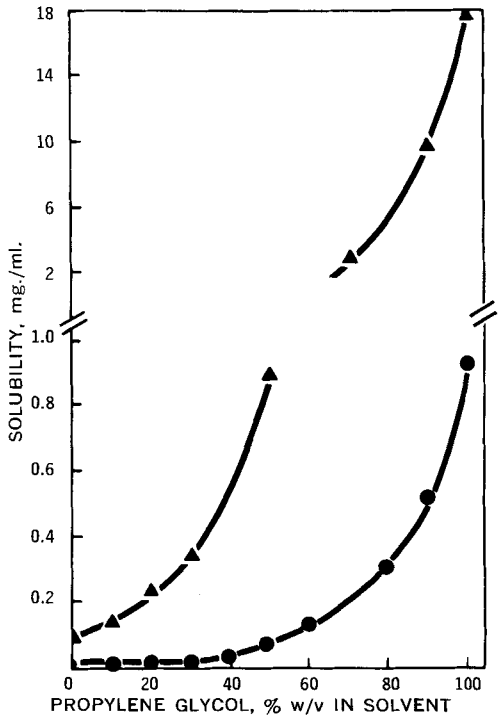

Fig. 1—Solubility of fluocinolone acetonide and its acetate ester in propylene glycol–water mixtures at 25°. Key: ▲, fluocinolone acetonide; ●, fluocinolone acetonide acetate.

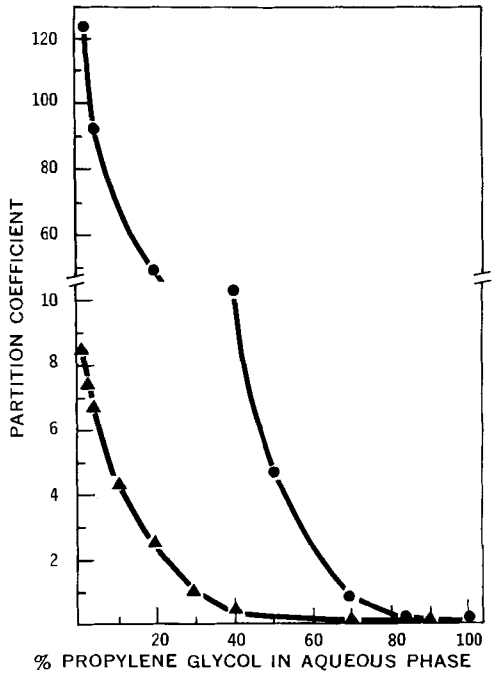

Fig. 2—Partition coefficients for fluocinolone acetonide and its acetate ester between propylene glycol–water mixtures and isopropyl myristate at 37°. Key: ▲, fluocinolone acetonide; ●, fluocinolone acetonide acetate.

Figure 3 is a plot of the total release of fluocinolone acetonide after 2, 6, and 12 hr. at 37° from gels containing various concentrations of propylene glycol. Optimal release for 0.025% fluocinolone acetonide preparations occurred with gels containing approximately 20% propylene glycol. As seen in Fig. 1 this concentration of propylene glycol is only slightly less than the minimum amount estimated to be required to completely solubilize 0.025% fluocinolone acetonide in the vehicle. At lower concentrations of propylene glycol, precipitated steroid appeared in the gel. Although reduced release rates were observed with gels containing mostly undissolved steroid, the poorest release was actually obtained with gels containing very high concentrations of propylene glycol.

Somewhat different results were found for the less soluble fluocinolone acetonide acetate (Fig. 4). The solubility data obtained for the acetate ester (Fig. 1) indicated that about 75% propylene glycol would be required to solubilize this compound at a concentration of 0.025%. Maximum release at 37° occurred from vehicles containing approximately 70% propylene glycol. In contrast to fluocinolone acetonide, the poorest release occurred from gels containing low propylene glycol concentrations in which a relatively large proportion of the steroid was present as undissolved material.

Figure 5 compares the release of the two steroids from several of the vehicles tested over a 24-hr. period. Figure 6 gives a comparison of percent steroid released after 6 hr. from vehicles containing 0.01, 0.025, and 0.1% fluocinolone acetonide.

These data are reasonably consistent with the observation that maximum release for various steroid concentrations in the vehicles studied occurred from gels containing the minimal amount of propylene glycol required to dissolve the compound. Figure 6 also shows the result of the increase in the activity

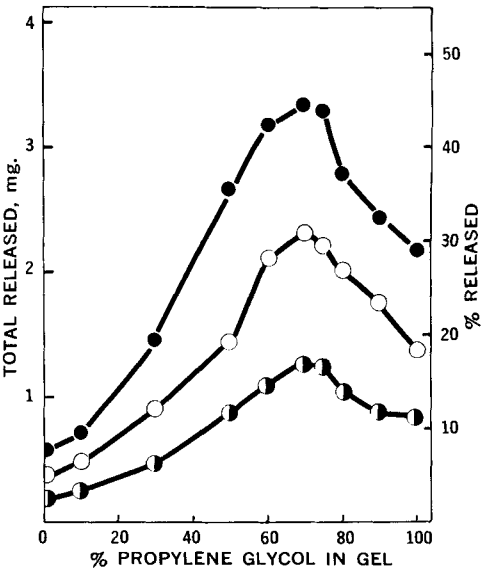

Fig. 4—Effect of propylene glycol concentration on the release of 0.025% fluocinolone acetonide acetate from propylene glycol–water gels at 37°. Key: release after— ◑, 2 hr.; ○, 6 hr.; ●, 12 hr.

coefficient that could be achieved at the lower (0.01 and 0.025%) fluocinolone acetonide concentrations. While total release was greater for the higher concentrations, the percent released over a 6-hr. time period was much greater for the 0.01% steroid concentration.

DISCUSSION

A number of studies describing the release of drugs from topical vehicles are reported in the literature. When the vehicle and receptor phase are mutually

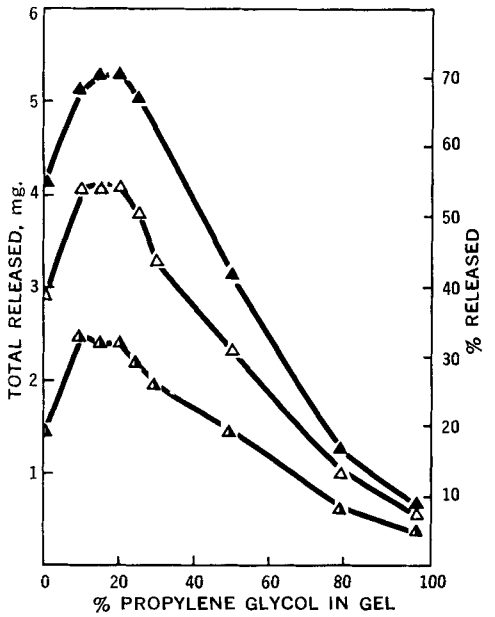

Fig. 3—Effect of propylene glycol concentration on the release of 0.025% fluocinolone acetonide from propylene glycol–water gels at 37°. Key: release after— △, 2 hr.; △, 6 hr.; ▲, 12 hr.

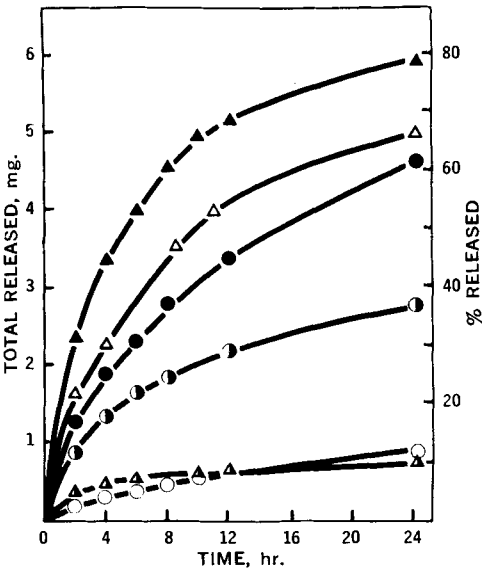

Fig. 5—Effect of propylene glycol concentration on the release of 0.025% steroid from Carbopol gels. Key: concentration of propylene glycol in fluocinolone acetonide gels—△, 1%; ▲, 20%; ◭, 100%; concentration in fluocinolone acetonide acetate gels—○, 1%; ●, 70%; ◑, 100%.

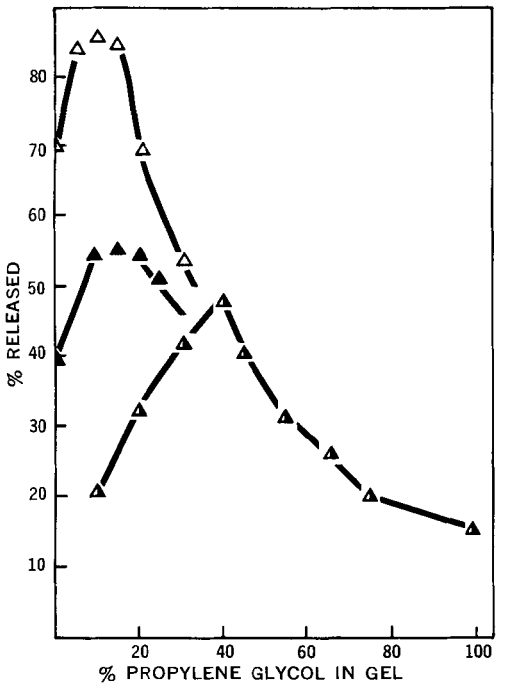

Fig. 6—Comparison of release of 0.01%, 0.025%, and 0.1% fluocinolone acetonide from propylene glycol–water gels after 6 hr. at 37°. Key: % steroid—△, 0.01%; ▲, 0.025%; ◭, 0.1%.

miscible, physical separation of the two phases is necessary. A dialysis membrane has frequently been used for this purpose (14–18). In this study, isopropyl myristate was employed instead of water as the receptor phase to more closely simulate skin lipids or skin itself. Since isopropyl myristate is immiscible with water and propylene glycol, it was not necessary to introduce an artificial membrane to separate it from the vehicles used in this study. The solubilities of fluocinolone acetonide (0.9 mg./ml.) and fluocinolone acetonide acetate (0.2 mg./ml.). at 37° were adequate for this lipid-like material to be used as the receptor phase.

Propylene glycol–water mixtures gelled with Carbopol 934 were selected as vehicles because steroid solubility could be readily altered by varying the concentration of propylene glycol. Solubility data for the two steroids in propylene glycol–water mixtures were determined at 25° (Fig. 1) and used to estimate the solubility in these same mixtures gelled with Carbopol. Microscopic examination of the vehicles indicated that addition of the gelling agent resulted in negligible changes in the solubility of the steroids in the glycol–water mixtures.

Partition coefficients were determined between glycol–water mixtures and isopropyl myristate (Fig. 2) and, as indicated by Eq. 3, were useful as an index of the relative activity coefficients for the steroids in these vehicles. This information was particularly valuable in estimating the potential effect of changes in the steroid concentrations in these vehicles on the release rate.

The possibility exists that molecular interactions between the steroids and the carboxyvinyl polymer gelling agent were a factor affecting the release rates. In addition, the solubility data for the steroids in glycol–water mixtures were obtained at room temperature (25°), whereas the release experiments were conducted at 37°. Consequently, steroid solubility in the gels under the conditions of the experiments was probably slightly greater than shown in Fig. 1. This may account in part for the observation that maximum release of 0.1% fluocinolone acetonide (Fig. 6) occurred from vehicles containing somewhat less propylene glycol than required to completely dissolve the steroid at room temperature. For these reasons, the solubility and partition coefficient data developed with the glycol–water mixtures were used only as an aid in interpreting the release data.

Maximum release for a given concentration of either fluocinolone acetonide or fluocinolone acetonide acetate was obtained from vehicles containing approximately the minimum amount of propylene glycol necessary to dissolve the steroid completely. Excess propylene glycol increased the affinity of the vehicle for the steroid and resulted in a decrease in steroid release. When insufficient propylene glycol was present to dissolve all the steroid, then diffusion into the receptor phase became dissolution rate limited and the release rate was reduced. As might be expected, the latter effect was much more pronounced with the less soluble compound, fluocinolone acetonide acetate.

The results obtained in this study clearly demonstrate the dependence of steroid release on the physical properties of both steroid and vehicle. For steroid concentrations of 0.025%, a vehicle containing 20% propylene glycol releases approximately 70% of available fluocinolone acetonide but only 15% of the acetate ester after 12 hr. Likewise, a vehicle containing 70% propylene glycol releases 45% of the acetate ester and only 25% of fluocinolone acetonide. Clearly, a vehicle that provides good release of one drug may be a poor vehicle for another closely related compound. The results obtained with different concentrations of the same steroid are of particular interest (Fig. 6). A much higher percent release over a given time interval could be obtained from 0.01% fluocinolone acetonide gels than from vehicles containing higher concentrations of steroid. In this case, higher partition coefficients were obtained with the lower steroid concentration while completely solubilizing the steroid. The obvious implication is that vehicle composition can be varied not only to improve release of different steroids but also specific concentrations of a given steroid.

Release studies have long been used as one criterion for judging the possible effect of vehicle on drug availability from topical formulations. There are obvious limitations of such methods, and conclusions must be drawn with great caution. Certainly the partition coefficient for a steroid between vehicle and isopropyl myristate will not duplicate that between vehicle and human skin. But, regardless of the actual value of the partition coefficient for a penetrant present on the surface of the skin, it must be subject to its environment. If that environment is a pharmaceutical vehicle, the composition of that vehicle should affect the passage of penetrant into an adjacent phase whether that phase is sebum or skin tissue. The degree to which this is actually true, and its clinical implications, must ultimately be established by correlation to *in vivo* studies using the same topical formulations.

SUMMARY

Isopropyl myristate was used as the receptor phase to evaluate the release of fluocinolone acetonide and its acetate ester from gelled propylene glycol–water vehicles. Steroid release was found to be a function of its (*a*) concentration, (*b*) solubility in the vehicles, and (*c*) partition coefficient between vehicles and receptor phase. Release was reduced if a vehicle contained cosolvent in excess of that required to dissolve the steroid. Release could be improved not only for the different steroids, but also for different concentrations of the same steroid, by use of the proper vehicle.

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Keyphrases

Vehicles, topical—steroid release
Propylene glycol–water–Carbopol 934—vehicles
Fluocinolone acetonide and its acetate–test compounds
Radioactive compounds—release rates
Liquid scintillation counting—analysis
Solubility—propylene glycol–water
Partition coefficient—isopropyl myristate–propylene glycol system

Euphorbia esula L. (*Euphorbiaceae*) I

Preliminary Phytochemical and Biological Evaluation

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Accepted for publication February 13, 1968.

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* Recipient of an Alexander von Humboldt Stiftung research fellowship.

SUMMARY

Isopropyl myristate was used as the receptor phase to evaluate the release of fluocinolone acetonide and its acetate ester from gelled propylene glycol–water vehicles. Steroid release was found to be a function of its (*a*) concentration, (*b*) solubility in the vehicles, and (*c*) partition coefficient between vehicles and receptor phase. Release was reduced if a vehicle contained cosolvent in excess of that required to dissolve the steroid. Release could be improved not only for the different steroids, but also for different concentrations of the same steroid, by use of the proper vehicle.

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(*E. ingens*, *E. bupleurifolia*, *E. clavarioides*, *E. gorgonis*) (6), and experimentally, extracts of *Euphorbia amygdaloides* (7), *E. drummondii* (8), *E. marginata* (9), *E. pilulifera* (8), and *E. resinifera* (8) have been reported to induce tumor remissions in laboratory animals.

Interest was piqued by the limited phytochemical reports on *Euphorbia esula*, and by the interesting biological activity reported for this and related species of this genus; therefore, an investigation of this plant was initiated.

EXPERIMENTAL

Plant Material—Plant material used in this investigation was collected in Greensburg, Pa., during May and August 1964. Whole, flowering plants were uprooted, air-dried, separated into roots and aerial parts, and milled to a coarse powder. Voucher specimens were prepared and authenticated as *Euphorbia esula* L. (*Euphorbiaceae*).[1] Specimens representing the collections have been deposited in the herbaria of Carnegie Museum, and in the Department of Pharmacognosy, University of Pittsburgh, Pittsburgh, Pa.

Extraction and Fractionation—Only the aerial parts were used in this study. A total of 27.57 Kg. of plant material was extracted continuously with skellysolve B (*n*-hexane) for 72 hr. in a Lloyd extractor. Concentration of this extract *in vacuo* to about 4 L. yielded, on standing overnight, 116 Gm. of a gray powder (Fraction *A*). The mother liquor remaining from the removal of Fraction *A* was reduced to dryness to yield 410 Gm. of residue (Fraction *B*).

The skellysolve B-exhausted plant material was again continuously extracted with methanol for 72 hr. in a Lloyd extractor. This extract was concentrated to about 4 L., 4 L. of distilled water was added, and the mixture concentrated *in vacuo* until all traces of methanol were removed. Lyophilization of the methanol-free mixture yielded 1,688 Gm. of dark-green, dry powder (Fraction *C*) (Scheme I).

Isolation of Hexacosanol-(1) from Fraction A—Thin-layer chromatography of Fraction *A* on Silica Gel G plates, using a developing solvent composed of *n*-hexane–ethyl acetate (75:25), revealed that the fraction consisted mainly of a single spot at R_f 0.56, with small amounts of a substance traveling at or near the solvent front. The detecting reagent was 70% sulfuric acid, applied as a thin spray, followed by heating the treated plates at 120° for 10–15 min.

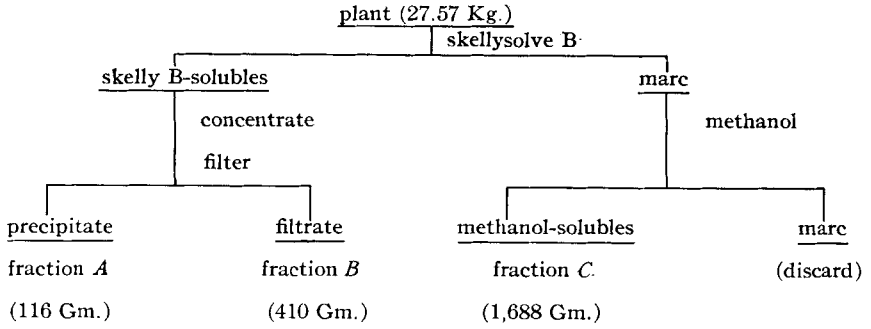
A small amount of Fraction *A* was purified by boiling it with acetone, filtering, and cooling the filtrate to room temperature. An amorphous precipitate formed which was removed, dissolved in hot chloroform, boiled for several minutes with decolorizing charcoal, and the mixture filtered. This filtrate, on standing at room temperature, produced a copious white precipitate which was removed and dried in a desiccator at room temperature *in vacuo* for 48 hr. Thin-layer chromatography of this material, as previously described, indicated a homogeneous one-spot material. The sample was identified as hexacosanol-(1) on the basis of its having melting point 76.5–77° (cap., uncorr.) [lit. m.p. 79.5–79.8° (10)], and a specific rotation $[\alpha]_D^{23}$ + 0° (c 1.0, $CHCl_3$).

Anal.—Calcd. for $C_{26}H_{54}O$: C, 81.60; H, 14.22. Found: C, 81.86; H, 14.00.

An infrared spectrum (KBr) was identical with that of an authentic sample of hexacosanol-(1), and a mixed melting point of the isolated material and the authentic sample was not depressed.

Saponification and Separation of Fraction B—An aliquot (195 Gm.) of Fraction *B* was dissolved in about 1 L. of 95% ethanol, 30 Gm. of KOH added, and the mixture refluxed for 1.5 hr. Removal of the ethanol was accomplished by means of a flash evaporator and the residue remaining was mixed with 1 L. of distilled water. This mixture was then extracted several times with petroleum ether (b.p. 30–60°), the combined petroleum ether extracts (about 3 L.) washed with distilled water, and the aqueous phase discarded. Attempts to reduce the petroleum ether extract to dryness, *in vacuo*, yielded a thick oil. This oil was mixed with about 1 L. of acetone, heated on a steam bath, and filtered while hot to remove 11 Gm. of a black tar (Fraction *B*-1). Cooling the filtrate to room temperature resulted in the deposition of a gray, amorphous precipitate, which was removed by filtration, air-dried, and labeled Fraction *B*-2 (4 Gm.). The Fraction *B*-2 mother liquor was concentrated to about 500 ml. and formed, on standing at room temperature, a copious precipitate which weighed 30 Gm. (Fraction *B*-3). Concentration of the mother liquor remaining from the isolation of

[1] Dr. Leroy K. Henry, Carnegie Museum Herbarium, Pittsburgh, Pa.



Fractionation Scheme for Euphorbia esula aerial parts
Scheme I

Fraction *B*-3, to about 250 ml. yielded 27 Gm. of an additional precipitate (Fraction *B*-4). This mother liquor was reduced to dryness, about 1 L. of *n*-hexane was added, the mixture heated to boiling, and then filtered while still hot. Concentration of the filtrate (*n*-hexane-solubles) to dryness yielded 32 Gm. of Fraction *B*-5. The filtration residue (*n*-hexane-insoluble material), which represented a considerable weight of material, was mixed with about 1 L. of acetone, the mixture heated to boiling, and filtered while hot. Evaporation of the filtrate to dryness yielded 55 Gm. of acetone soluble material (Fraction *B*-6). The acetone-insoluble residue (Fraction *B*-7). weighed 3 Gm.

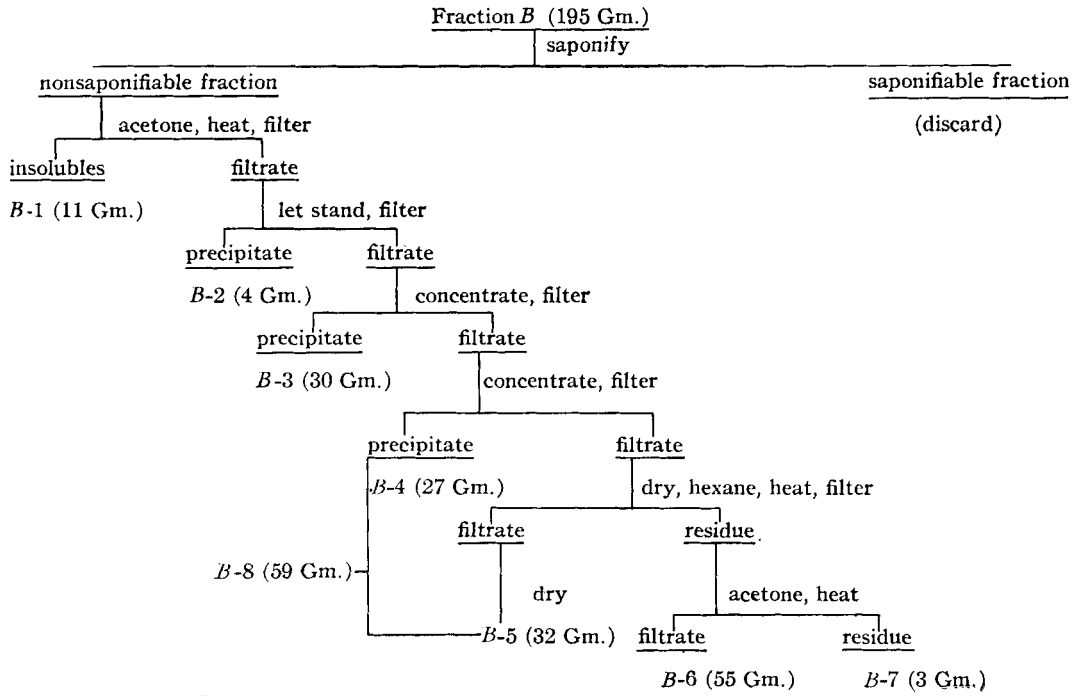
Thin-layer chromatography of Fractions *B*-1 to *B*-7 was accomplished on silica gel G plates, utilizing a solvent system of *n*-heptane–benzene–ethanol (50:50:0.5), and revelation of spots was effected by spraying each plate with 70% sulfuric acid, followed by heating at 120° for 15 min. Fractions *B*-1, *B*-2, and *B*-3 were found to exhibit identical chromatographic patterns. Each exhibited a major spot at R_f 0.11 and trace amounts of *n*-alkanes migrating at or near the solvent front. The R_f 0.11 spot in each fraction corresponded to hexacosanol-(1) in this and in several other solvent systems; therefore, no further studies were made on these fractions.

Fractions *B*-4 and *B*-5, however, showed the presence of several spots, giving characteristic color reactions suggestive of the presence of triterpenes and/or sterols (11), in addition to hexacosanol-(1) and *n*-alkanes. Since these two fractions were almost identical in migration patterns and chromogenic response of the resolved components to the spray reagent, they were combined and marked as Fraction *B*-8 (59 Gm.). Fractions *B*-6 and *B*-7 exhibited dissimilar chromatographic patterns and therefore were not combined (Scheme II).

Investigation of Fraction B-8—The fraction (59 Gm.) was dissolved in a minimum volume of hot petroleum ether (b.p. 30–60°) and this solution held overnight at room temperature. Crystals which formed were removed and recrystallized from petroleum ether in a similar manner yielding 0.194 Gm. of material having a melting point of 75–77° (cap., uncorr.). Comparison of this isolate with hexacosanol-(1) by means of mixture melting point, optical rotation, infrared absorption spectrum, and thin-layer chromatography, showed them to be identical.

After removal of the hexacosanol-(1), the fraction was taken to dryness, dissolved in a minimum volume of *n*-hexane, and added to the top of a column (5.5 × 55 cm.) of Woelm, neutral, activity grade I alumina, packed as an *n*-hexane slurry. Elution was initiated with *n*-hexane, followed by *n*-hexane–benzene (1:1), benzene, ether, and methanol. One hundred-milliliter fractions were collected, reduced to about 10 ml., and subjected to thin-layer chromatography as previously described, using a solvent system of *n*-heptane–benzene–ethanol (50:50:0.5). Fractions were grouped according to the eluting solvent used, taken to dryness, and crystallizing attempts initiated with the usual solvents. These data are presented in Table I.

Isolation of *n*-Nonacosane and *n*-Hentriacontane—Fractions 1–41 were combined, taken to dryness, and dissolved in a minimum volume of *n*-hexane. Chilling produced a material which deposited as shiny plates when dried *in vacuo* at room temperature for 24 hr. This isolate was weighed (0.685 Gm.) and found to have a m.p. of 67–68° (cap., uncorr.). The sample exhibited no ultraviolet absorption spectrum and had optical rotation $[\alpha]_D^{24} \pm 0°$ (c, 1.25, $CHCl_3$). An infrared absorption spectrum (KBr) showed typical absorption peaks suggestive only of *n*-alkanes, with an absence of OH absorption. The



Fractionation Scheme for Euphorbia esula Aerial Parts, Fraction B
Scheme II

TABLE I—CHROMATOGRAPHIC SEPARATION OF FRACTION *B*-8[a]

Fraction,[b] No.	Eluent	Isolated Compd.	Yield, Gm.	Crystn. Solvent
1–41	*n*-Hexane	*n*-Alkane mixture	0.685	*n*-Hexane
42–57	*n*-Hexane–benzene (1:1)	...	...	...
58–152	Benzene	Hexacosanol-(1)	0.331	Benzene
153–193	Ether	Hexacosanol-(1)	0.031	Methanol
194–210	Methanol	...	...	...

[a] On Woelm, neutral, activity grade I alumina. [b] Each fraction was 100 ml.

sample was analyzed by gas chromatography, using the conditions described in an earlier work (12), and was found to be composed of the following *n*-alkanes: *n*-pentacosane (<1%), *n*-hexacosane (<1.0%), *n*-heptacosane (<1%), *n*-octacosane (<1%), *n*-nonacosane (31%), *n*-triacontane (2%), *n*-hentriacontane (53%), *n*-dotriacontane (2%), and *n*-tritriacontane (7%). An additional sample of this *n*-alkane mixture was subjected to a preparative gas chromatographic separation and the major peaks, corresponding to the C_{29} and C_{31} *n*-alkanes, were collected. Melting point determinations (cap., uncorr.) on the two isolates showed m.p. 63.0–63.5° for the C_{29} peak isolate, and 67.5–68.0° for the C_{31} peak isolate, which corresponded to m.p. 63.4–63.7° and 67.5–68.5° reported in the literature for *n*-nonacosane and *n*-hentriacontane, respectively (10).

Direct crystallization procedures performed on the other column chromatographic fractions yielded only hexacosanol-(1) (Table I).

Separation of Fraction B-8 (58-152)—After removal of the hexacosanol-(1), obtained by direct crystallization procedures, the material eluted by benzene during the column chromatography of Fraction *B*-8 (column Fractions 58–152) was dried, dissolved in a minimum volume of benzene, and rechromatographed on this column with benzene as the initial eluent. Again, 100-ml. fractions were collected, each fraction concentrated to about 10 ml., and monitored by means of TLC using *n*-heptane–benzene–ethanol (50:50:0.5) as previously described. Based on changes in the TLC patterns, eluents were increased in polarity and fractions were pooled as indicated in Table II. Combined Fractions 69–83 were taken to dryness, dissolved in hot methanol, and the resulting solution chilled. After 24 hr. crystals formed which, after they were harvested and dried *in vacuo* in an Abderholden pistol over boiling methanol for 24 hr., weighed 0.247 Gm., and melted at 112–113.5° (Kofler, uncorr.). Thin-layer chromatography of the sample, as previously described, utilizing *n*-heptane–benzene–ethanol (50:50:2.0) as the developing solvent, showed it to be a homogeneous one-spot material, exhibiting an R_f of 0.19, which was identical to the R_f of a reference sample of 24-methylenecycloartanol.[2]

Identification of 24-Methylenecycloartanol—The isolate having m.p. 112–113.5° [lit. m.p. 118–119° (13)] exhibited an optical rotation of $[\alpha]_D^{24}$ + 47.9° (c 1.087, $CHCl_3$) [lit. $[\alpha]_D^{14.5}$ + 48.8° (c 0.26, $CHCl_3$) (13)], and an infrared absorption spectrum (KBr) that was identical with that of reference 24-methylenecycloartanol[2] (Fig. 1).

Anal.—Calcd. for $C_{31}H_{52}O$: C, 84.48; H, 11.89. Found: C, 84.03; H, 11.86.

Acetylation of the isolate was accomplished with acetic anhydride in pyridine in the usual manner, and the acetate crystallized from ethanol to give needles having m.p. 105–106° [lit. m.p. 110–111° (13)], and optical rotation $[\alpha]_D^{24}$ + 56.8° (c 1.467, $CHCl_3$), lit. $[\alpha]_D^{18.5}$ + 56.6° (c 0.49, $CHCl_3$) (13).

Anal.—Calcd. for $C_{33}H_{54}O_2$: C, 82.10; H, 11.27; $OCOCH_3$, 10.96. Found: C, 81.60, 81.68; H, 11.20, 11.42; $OCOCH_3$, 10.95.

Isolation of β-Sitosterol—Fractions 84-128 (12.7 Gm.) (Table II) from the column chromatographic separation of the combined Fractions 58–152 obtained from the column chromatography of Fraction *B*-8 would not yield crystals on direct crystallization attempts from the usual solvents. Therefore, 9.5 Gm. of the fraction was dried, dissolved in 40 ml. of *n*-hexane, applied to the top of a glass chromatographic column (5.5 × 49 cm.) containing an *n*-hexane slurry of silicic acid (Kieselgel-Merck, 0.05–0.20 mm.), which had previously been activated by

TABLE II—COLUMN CHROMATOGRAPHIC SEPARATION OF FRACTION *B*-8(58–152)(58 Gm.)[a]

Fraction,[b] No.	Eluent	Combined Fraction No.	Fraction Wt., Gm.	Isolated Compd.	Yield, Gm.
1–33	Benzene	1–38	1.3	...	...
34–96	Benzene–ether (9:1)	39–68	6.8	...	...
	Benzene–ether (9:1)	69–83	6.7	24-Methylenecycloartanol	0.247
	Benzene–ether (9:1)	84–128	12.7	...	...
97–113	Benzene–ether (8:2)	...			
114–125	Benzene–ether (1:1)	...			
126–136	Methanol	129–136	1.8	...	...

[a] On Woelm, neutral, activity grade I alumina. [b] Each fraction was 100 ml.

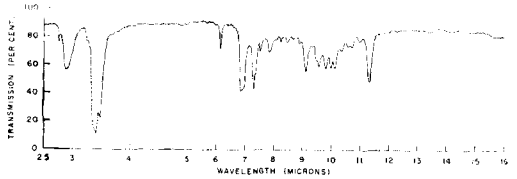


Fig. 1—Infrared absorption spectrum of 24-methylenecycloartanol (KBr).

[2] Reference samples of 24-methylenecycloartanol were supplied through the courtesy of Drs. Wesley Cocker, A. N. Starratt, and G. Ponsinet.

heating at 110° for 24 hr., and elution was initiated with *n*-hexane. Fractions (100 ml.) were collected, grouped on the basis of TLC behavior as previously described, and on the basis of shifts in TLC patterns, eluents were changed as indicated in Table III. Fractions 17–22 were combined, taken to dryness, and the residue dissolved in a minimum volume of hot methanol. A small amount of decolorizing charcoal was added, the mixture boiled for a few minutes, filtered until clear, and refrigerated for 24 hr. Crystals that formed were removed and, after drying *in vacuo* over boiling methanol for 24 hr., were shown to be one-spot material (R_f 0.09) on TLC as previously described, using the solvent system *n*-heptane–benzene–ethanol (50:50:2.0). This isolate was found identical in its chromatographic behavior, in several solvent systems, with β-sitosterol. The yield was 0.319 Gm. and the crystals had m.p. 133–135° [lit. m.p. for β-sitosterol 137–138° (14, 15)]. Optical rotation $[\alpha]_D^{22}$ −22.58° (c 0.80, $CHCl_3$), lit. $[\alpha]_D$ −37.8° (c 1.2, $CHCl_3$) (16).

Anal.—Calcd. for $C_{29}H_{50}O$: C, 83.99; H, 12.15. Found: C, 84.02, 83.92; H, 12.14, 11.75.

The infrared absorption spectrum was identical in all respects with that of a reference sample of β-sitosterol.[3] Acetylation of a small sample of the isolated product with acetic anhydride in pyridine in the usual manner produced an acetate which, after crystallization from ethanol, gave needles having m.p. 124–125° [lit. m.p. for β-sitosterol acetate 126–128° (17)].

Anal.—Calcd. for $C_{31}H_{52}O_2$: C, 81.52; H, 11.48; $OCOCH_3$, 11.48. Found: C, 81.25, 81.14; H, 11.28, 11.36; $OCOCH_3$, 10.62.

An infrared spectrum of this acetate was identical with that of an acetate prepared as described above from authentic β-sitosterol.

Fractions 23–26 yielded 0.125 Gm. of crystals from chloroform–methanol that were identical in all respects with the β-sitosterol isolated from Fractions 17–22.

Treatment of Fractions 11–14, in methanol, with decolorizing charcoal, yielded 0.065 Gm. of crystals that were identical with the 24-methylenecycloartanol previously isolated from Fractions 69–83 from the column chromatographic separation of Fraction *B*-8 (58–152). Crystals could not be induced to form from the remaining fractions, either before or after acetylation.

Separation of Flavonoids—Fraction C (500 Gm.) was dissolved in about 2 L. of distilled water with the aid of heat, the resulting solution concentrated on a steam bath to about 1 L., and then refrigerated for 24 hr. During this time a green scum separated, which was removed by filtration, to give a clear brown solution. This solution was extracted several times with chloroform, which on drying yielded Fraction *C*-1 (6.78 Gm.). The aqueous phase remaining from the chloroform extraction was then extracted several times with diethyl ether, which, following evaporation of the ether and drying, yielded 2.97 Gm. of Fraction *C*-2. Fractions *C*-3 (19.50 Gm.) was obtained by extracting the aqueous phase several times with ethyl acetate. Finally, the aqueous phase was extracted several times with ethyl acetate–methanol (9:1) to yield 5.93 Gm. of Fraction *C*-4. Thin-layer chromatography of Fractions *C*-1, *C*-2, *C*-3, and *C*-4 on polyamide plates, using methanol as the developing solvent, and followed by spraying each plate with an aqueous saturated solution of lead subacetate, revealed the presence of several fluorescent and visible spots in each fraction that were suggestive of the presence of flavonoids (18). Work is now in progress toward the isolation and identification of the flavonoid constituents in each of these fractions.

Detection of Alkaloids—A 20-Gm. sample of Fraction *C* was dissolved in methanol, mixed with 250 ml. of 5% hydrochloric acid, and the mixture was steam distilled *in vacuo* to remove the methanol and convert any alkaloids present to hydrochloride salts. This mixture was then filtered, and the filtrate was made basic to litmus with 28% ammonium hydroxide solution. Three 500-ml. and six 250-ml. chloroform extractions of the basic solution were pooled, dried over anhydrous sodium sulfate, and filtered. This filtrate was then taken to dryness to yield 300 mg. of residue, which was designated as the tertiary alkaloid fraction.

The alkaline mother liquor was acidified with 1.0 *N* hydrochloric acid, refrigerated overnight, and then filtered. This solution was next concentrated by the addition of methanol and evaporation *in vacuo* using a flash evaporator. During the concentration procedure, copious amounts of a white crystalline material separated (about 18 Gm. was collected), which was shown to be ammonium chloride. The extract was then taken to dryness, treated with hot acetone, and filtered to remove the quaternary bases. This treatment failed to remove a large quantity of residual crystalline material, which was presumed to be ammonium chloride. The acetone solution was designated as the quaternary alkaloid fraction.

TABLE III—COLUMN CHROMATOGRAPHIC SEPARATION OF FRACTIONS 84–128 (9.5 Gm.)[a]

Fraction,[b] No.	Eluent	Fraction Wt., Gm.	Isolated Compd.	Yield, Gm.
1–3	*n*-Hexane	0.15	...	...
4–10	*n*-Hexane–benzene (9:1)	0.20	...	...
11–14	*n*-Hexane–benzene (1:1)	1.10	24-Methylenecycloartanol	0.065
15–16	Benzene	5.01	...	...
17–22	Benzene–ether (9:1)	2.80	β-Sitosterol	0.319
23–26	Ether	0.30	β-Sitosterol	0.125
27–32	Methanol	0.10	...	...

[a] On silicic acid (Kieselgel-Merck, activated, 0.05–0.20 mm.). [b] Each fraction was 200 ml.

The tertiary alkaloid fraction (300 mg.) was dissolved in 15 ml. of chloroform. Ten milliliters of this solution was mixed with 10 ml. of 5% hydrochloric acid solution, the mixture was shaken, and the acidic portions removed. Two-milliliter aliquots of this acidic extract were placed in each of four test tubes, and three drops of the following reagents were added

[3] A reference sample of β-sitosterol was supplied by Dr. G. H. Constantine, Jr.

TABLE IV—THIN-LAYER CHROMATOGRAPHY OF *E. esula* ALKALOID FRACTIONS[a]

Solvent System	R_f Values of Resolved Alkaloids: Tertiary Fraction	Quaternary Fraction	Control[b]
n-Propanol–NH_4OH–water (2:1:1)	0.87	0.95, 0.88, 0.73	0.88
Ethyl methyl ketone–methanol–10% NH_4OH (6:3:1)	0.27, 0.17	0.85, 0.15	0.22
Methanol–NH_4OH(99:1)	0.85, 0.05	...	0.25

[a] On Silica Gel G plates (250 μ). [b] *l*-Hyoscyamine.

separately to the contents of each tube: Valser's reagent, Mayer's reagent, phosphotungstic acid, Bouchardat's reagent. A moderate precipitate was observed in each of the four tubes after addition of the reagents, indicative of the presence of tertiary alkaloids.

Twenty milliliters of 5% hydrochloric acid solution was added to about one-half of the quaternary alkaloid fraction in acetone, and the acetone removed by means of a flash evaporator. The resulting solution was divided equally between four test tubes and the alkaloid test reagents indicated above were added. A moderate precipitate was observed in each of the tubes, indicative of the presence of quaternary alkaloids.

In order to confirm these initial qualitative tests for alkaloids, both the tertiary and quaternary alkaloid fractions were subjected to thin-layer chromatography on Silica Gel G plates (250 μ) and several different solvent systems.

Because of the reported presence of tropane alkaloids in *Euphorbia* species (19), hyoscyamine was used as a control. The dried chromatograms were sprayed with modified Dragendorff's reagent as a means of detecting the alkaloid components. These results are presented in Table IV.

Biological Activity Screening—A defatted ethanol extract was prepared from *Euphorbia esula* aerial parts as previously described (20). This extract was evaluated for antitumor, antibiotic, and mouse behavior activity, using the protocols described in an earlier work (20).[4] The extract tested by these methods had no antitumor activity at the doses indicated in each of the following tumor systems: adenocarcinoma 755 (30 mg./Kg.), mecca lymphosarcoma (30 mg./Kg.), myeloma X-5563 (30 mg./Kg.), C-1498 leukemia (30 mg./Kg.), and the P-1534 leukemia (7.5 and 15 mg./Kg.). Using different tumors and methods (21),[5] the extract was inactive against the P-1798 lymphosarcoma (31.3–250 mg./Kg.), the Lewis lung carcinoma (31.3–250 mg./Kg.), and the sarcoma 180 (250 mg./Kg.), and had an $ED_{50} > 1.0 \times 10^2$ mcg./ml. (inactive) against Eagle's 9 KB human epidermoid carcinoma of the nasopharynx in cell culture.

The extract was devoid of antibiotic activity against the bacterial and fungal pathogens and saprophytes, the plant pathogens and material-degrading organisms, and the algae and protozoa employed as test organisms in the evaluation (20).[4] At doses ranging from 5–400 mg./Kg., i.p., the extract produced only a weak central nervous system depressant effect in mice (20).[4]

[4] Extracts were evaluated through the courtesy of Eli Lilly and Company, Indianapolis, Ind.

[5] Extracts were evaluated for antitumor activity through the Cancer Chemotherapy National Service Center (CCNSC), National Cancer Institute, National Institutes of Health, Bethesda, MD 20014. The procedures were those described in *Cancer Chemotherapy Rept.*, **25**, 1 (1962).

DISCUSSION

Whole plant extracts of *Euphorbia esula* L. were previously reported to be devoid of hemolytic saponins, flavonoids, alkaloids, and tannins (4). However, this investigation has shown that several flavonoids are present in the aerial parts of this plant, and at least two tertiary and three quaternary alkaloids have been shown to be present by means of thin-layer chromatography. It is interesting to note that at least 50 species of the genus *Euphorbia* have been tested for the presence of alkaloids, and 25 of them have been recorded as alkaloid-positive. However, it has only been recently that Hart *et al.* succeeded in isolating the first crystalline alkaloid, (+)-9-aza-1-methylbicyclo[3.3.1]nonan-3-one, from the genus *Euphorbia* (*E. atoto*) (19).

Details for the extraction and fractionation of *E. esula* aerial parts have been presented, together with the first report on the isolation of 24-methylenecycloartanol, β-sitosterol, hexacosanol-(1), *n*-nonacosane, and *n*-hentriacontane from this plant.

Hexacosanol-(1) was previously reported isolated from *E. pilulifera* (22), and *E. cyparissias* (14, 23), but not from closely related *E. esula* or any other *Euphorbia* species. The *n*-alkanol, octocosanol-(1), was reported isolated from *E. jolkini* (24) and from *E. regis-jubae* (25); tetracosanol-(1) from *E. watanabei* (15); triacontanol-(1) from *E. hirta* (26), and docosanol-(1) from *E. calculata* (27), but none of these later *n*-alkanols were encountered in the present investigation.

β-Sitosterol has been isolated for the first time from *E. esula*, but it is known to occur in *E. adenochlora* (16), *E. cyparissias* (14), *E. hirta* (26), *E. hirta* var. *procumbens* (28), *E. lathyris* (29), *E. maculata* (25), *E. watanabei* (15), and *E. pulcherrima* (30).

The tetracyclic triterpene, 24-methylenecycloartanol, which is a probable intermediate in the biosynthesis of phytosterols (31), was also isolated for the first time from *E. esula*, as the major constituent of the triterpene fraction. Its presence in other *Euphorbia* species appears to be restricted to *E. cyparissias* (14). On the basis of interpretation of thin-layer chromatograms of the several *E. esula* nonsaponifiable fractions separated in this investigation, it is evident that several triterpenes are present in this plant. However, all attempts to crystallize them from several column chromatographic separations, either before or after acetylation, were unsuccessful.

The finding of the *n*-alkanes having composition C_{25} to C_{33} in *E. esula*, with *n*-nonacosane and *n*-hentriacontane being present in the highest concentrations, is consistent with the studies of Eglinton *et al.* (32) who investigated the *n*-alkanes of *E. peplus*, *E. balsamifera*, *E. atropurpura*, *E. regis-jubae*, *E. aphylla*, and *E. bourgaeana*, and studied their distribution in this and in other genera.

To date, *n*-nonacosane has been reported isolated from *E. adenochlora* (16) and *E. watanabei* (15, 33); *n*-triacontane from *E. pilulifera* (34), and *E. antisiphilitica* (34); *n*-hentriacontane from *E. pilulifera* (22), *E. lathyris* (29, 33), *E. antisiphilitica* (35), *E. candelilla* var. *luxurians* (36), *E. hirta* (26), *E. hirta* var. *procumbens* (28), *E. humifosa* (22) and *E. thymifolia* (35), and *n*-dotriacontane from *E. antisiphilitica* (35). However, these identifications must all be considered as equivocal, since it has been shown that most reports on the isolation of *n*-alkanes from plants have been erroneous (37, 38). Indeed, in most cases an investigator bases his identity of an *n*-alkane almost completely on melting point, elemental analysis and/or solubility characteristics. It has been shown recently that the substance reported as *n*-nonacosane from *E. watanabei* (15) was, in effect, a complex *n*-alkane mixture having *n*-hentriacontane as the major component (33). Similarly, the *n*-hentriacontane isolated from *E. lathyris* (29) was shown to be a mixture containing only about 53% of this compound, together with varying amounts of six other *n*-alkanes (33).

SUMMARY

(*a*) β-Sitosterol, 24-methylenecycloartanol, hexacosanol-(1) (ceryl alcohol), *n*-nonacosane, and *n*-hentriacontane have been isolated and are reported for the first time from the aerial parts of *Euphorbia esula*.

(*b*) The *n*-alkanes having constitution C_{25} to C_{33} were shown by means of gas chromatography.

(*c*) In addition to flavonoids, small amounts of tertiary, as well as quaternary alkaloids, were shown to be present in the aerial parts of *E. esula*.

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Keyphrases

Euphorbia esula
Phytochemistry—aerial parts, *Euphorbia esula*
Pharmacology—ethanol extract, *Euphorbia esula*
Column chromatography—separation
TLC—separation, identity
Optical rotation—identity
GLC—identity
IR—structure

Opium Alkaloids VII

Isolation of a New Benzylisoquinoline Alkaloid. Synthesis and NMR Studies of Papaveroline Trimethyl Ethers

By EINAR BROCHMANN-HANSSEN and KENTARO HIRAI*

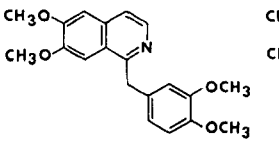
A new phenolic alkaloid was isolated from opium and named palaudine. It was characterized spectroscopically as 1-(3′-hydroxy-4′-methoxybenzyl)-6,7-dimethoxyisoquinoline and the structure confirmed by synthesis. The other three papaveroline trimethyl ethers were also synthesized and their NMR spectra investigated.

1-BENZYLISOQUINOLINE ALKALOIDS containing a hydrogenated heterocyclic ring (1,2,3,4-tetrahydro) are widely distributed in several plant families. On the other hand, very few fully aromatic benzylisoquinolines have been isolated from natural sources, the best known being the opium alkaloid papaverine (I). Investigation of the minor alkaloids of opium has now revealed a new alkaloid of this type. It was isolated from the phenolic alkaloid fraction of powdered opium and purified by preparative thin-layer chromatography on silica gel and by column chromatography on alumina and crystallized from ethanol, m.p. 175–176°. The mass spectrum showed a molecular ion with mass *m/e* 325. The M-1 peak (*m/e* 324) was of greater intensity than the peak of the parent ion. The M-15 peak (*m/e* 310) was also quite pronounced, while the M-31 peak (*m/e* 294) was small. The UV spectrum (in methanol) had maxima at 239, 280, 313.5, and 326.5 mμ and minima at 262, 306.5, and 320 mμ, and was very similar to that of papaverine (1). The NMR spectrum in deuteriochloroform revealed three methoxyl groups resonating at τ 6.01, 612, and 6.19, corresponding to three of the four methoxyl group signals of papaverine. A 2-proton signal at τ 5.51 corresponded to the methylene group signal of papaverine. These spectroscopic data suggested that the new alkaloid had the aromatic benzylisoquinoline structure and a substitution pattern similar to papaverine, *i.e.*, *O*-desmethylpapaverine = papaveroline trimethyl ether. The alkaloid gave a positive test with Gibb's reagent, indicating that it might be unsubstituted in the *para* position to the phenolic hydroxyl group (II). This structure was confirmed by unambiguous synthesis as outlined in Scheme I.

β - Methoxy - β - (3,4 - dimethoxyphenyl)-ethylamine (III) was prepared according to Rosenmund *et al.* (2) by reduction of 1-methoxy-1-(3,4-dimethoxyphenyl)-2-nitroethane with lithium aluminum hydride. The acid chloride (IV) was synthesized from 3-benzyloxy-4-methoxybenzaldehyde *via* the azlactone as described by Robinson and Sugasawa (3). Schotten-Baumann reaction gave the amide (V) which lost methanol and water by treatment with phosphorus oxychloride in toluene.

Acid hydrolysis of VI gave the phenolic base II, which was crystallized from ethanol, m.p. 176°. No melting point depression was observed when mixed with the natural alkaloid. The natural and the synthetic compounds had the same R_f values on silica gel in two different solvent systems (4), and had identical NMR, UV, and IR spectra. Since the new opium alkaloid is closely related to papaverine and has the same pattern of oxygenated substituents as laudanidine, the authors propose to name it palaudine.

Pohl and Wiegrabe (5) have reported the NMR spectrum of papaverine, but the chemical shifts of the individual methoxyl groups have not been established. In order to make these assignments, the three remaining papaveroline trimethyl ethers were synthesized by the same route described for II and illustrated in Scheme I. In this way were obtained 1-(3′,4′-dimethoxybenzyl)-6-methoxy-7-hydroxyisoquinoline (VII), m.p. 163–165°, 1-(3′-methoxy - 4′ - hydroxybenzyl) - 6,7- dimethoxyisoquinoline (VIII), m.p. 165°, and 1 (3′-4′-dimethoxybenzyl) - 6- hydroxy - 7 - methoxyisoquinoline (IX), m.p. 165–167°. Billek (6) has previously synthesized VII and IX by catalytic dehydrogenation of the corresponding 1,2,3,4-tetrahydro derivative. These melting points are in good agreement with his. Boucherle and Alary



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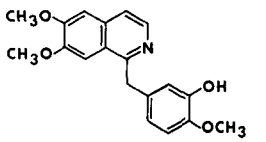

II

Received December 4, 1967, from the Department of Pharmaceutical Chemistry, University of California School of Pharmacy, San Francisco, CA 94122

Accepted for publication February 7, 1968.

This work was supported by a research grant (MH 03487-08) from the National Institutes of Health, Bethesda, Md.

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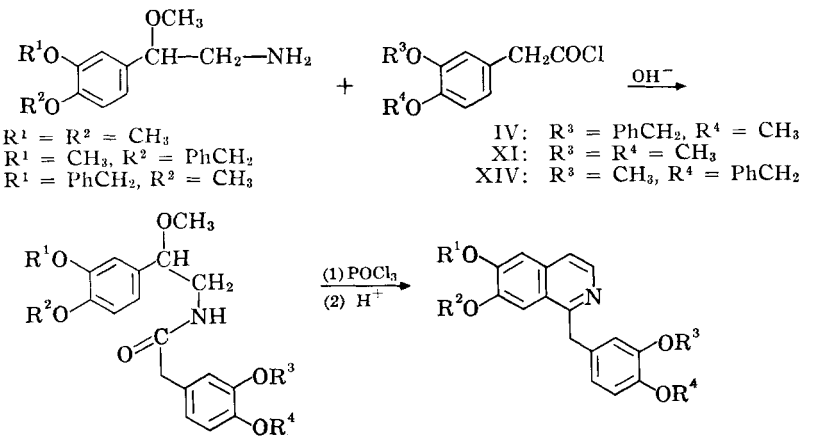

III: $R^1 = R^2 = CH_3$
X: $R^1 = CH_3$, $R^2 = PhCH_2$
XVII: $R^1 = PhCH_2$, $R^2 = CH_3$

IV: $R^3 = PhCH_2$, $R^4 = CH_3$
XI: $R^3 = R^4 = CH_3$
XIV: $R^3 = CH_3$, $R^4 = PhCH_2$

V: $R^1 = R^2 = R^4 = CH_3$, $R^3 = PhCH_2$
XII: $R^1 = R^3 = R^4 = CH_3$, $R^2 = PhCH_2$
XV: $R^1 = R^2 = R^3 = CH_3$, $R^4 = PhCH_2$
XVIII: $R^1 = PhCH_2$, $R^2 = R^3 = R^4 = CH_3$

VI: $R^1 = R^2 = R^4 = CH_3$, $R^3 = PhCH_2$
II: $R^1 = R^2 = R^4 = CH_3$, $R^3 = H$
XIII: $R^1 = R^3 = R^4 = CH_3$, $R^2 = PhCH_2$
VII: $R^1 = R^3 = R^4 = CH_3$, $R^2 = H$
XVI: $R^1 = R^2 = R^3 = CH_3$, $R^4 = PhCH_2$
VIII: $R^1 = R^2 = R^3 = CH_3$, $R^4 = H$
XIX: $R^1 = PhCH_2$, $R^2 = R^3 = R^4 = CH_3$
IX: $R^1 = H$, $R^2 = R^3 = R^4 = CH_3$

Scheme I

(7) have reported obtaining IX by heating of papaverine hydrochloride. However, their product decomposed without melting at 245°, thus leaving some uncertainty as to its identity.

The NMR spectrum of papaverine in deuteriochloroform showed methoxyl groups resonating at τ 6.01, 6.10, 6.18, and 6.23. The methoxyl signal at τ 6.23 was absent from II and could, therefore, be assigned to the 3′-position. Similarly, by comparing the methoxyl signals of papaverine with those of the other papaveroline trimethyl ethers, the chemical shift assignment could be made as shown in Table I.

TABLE I—CHEMICAL SHIFTS (τ) IN DEUTERIOCHLOROFORM

Compd.	6-OMe	7-OMe	4′-OMe	3′-OMe	—CH₂—
I (papaverine)	6.01	6.10	6.18	6.23	5.47
II (palaudine)	6.01	6.12	6.19	...	5.51
VII	5.98	...	6.23	6.28	5.55
VIII	6.00	6.09	...	6.24	5.47
IX	...	6.08	6.18	6.24	5.44

EXPERIMENTAL

The melting points were measured with a Kofler hot-stage micro melting point apparatus and are uncorrected. Ultraviolet spectra were obtained in ethanol solutions with a Cary model 11 spectrophotometer. Infrared spectra were determined in potassium bromide with a Beckman IR-9 spectrophotometer. Nuclear magnetic resonance spectra were measured in deuteriochloroform with a Varian Associates model A-60A nuclear magnetic resonance spectrometer. Tetramethylsilane was used as the internal standard. The mass spectrum was obtained with a Varian M-66 mass spectrometer. The elemental analyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, California.

Isolation of Palaudine from Opium—Powdered opium of Indian origin was extracted and the major alkaloid groups separated by liquid–liquid extraction as described in a previous communication (8). The phenolic alkaloid fraction was subjected to preparative thin-layer chromatography on silica gel (9). A broad band having a high R_f value in chloroform–methanol (9:1) consisted of a mixture which was chromatographed on a column of neutral alumina, Merck (activity 4) with chloroform. Twenty-milliliter fractions were collected with an automatic fraction collector. One of the fractions obtained in this way was again subjected to preparative thin-layer chromatography on silica gel with chloroform–methanol (9:1) and gave an alkaloid (7 mg.) which was purified by crystallization from ethanol, m.p. 175–176°. It produced a single spot by thin-layer chromatography in two different solvent systems (4) and gave a single peak by gas chromatography on a polyester column (3% HIEFF 8B, 4 ft., 220°) with a retention time of 162 min. A grayish blue spot was produced on the TLC plate with Gibb's reagent. UV spectrum: $\lambda_{max.}$ at 239, 280, 313.5, and 326.5 mμ (log ϵ, 4.78, 3.89, 3.70, 3.73, respectively); $\lambda_{min.}$ at 262, 306.5, and 319 mμ (log ϵ, 3.81, 3.59, 3.65, respectively). IR spectrum: $\nu_{max.}$ at 3430, 3000, 2868, 2817, 1620, 1589, 1569, 1513, 1478, 1463, 1450, 1432, 1420, 1385, 1350, 1323, 1273, 1235, 1203, 1160, 1130, 1080, 1050, 1030, 988, 961, 921, 894, 860, 823, 810, 788, 761, 652, 600, 565, 560, 500 and 400 cm.$^{-1}$ Mass spectrum: peaks at *m/e* 325 (M), 324 (M-1), 310 (M-15).

Syntheses of—N-(3′-benzyloxy-4′-methoxyphenylacetyl)-β-methoxy-β-(3,4-dimethoxyphenyl)ethylamine(V)—3-Benzyloxy-4-methoxyphenylacetic acid (0.45 Gm.) (3) was refluxed with

0.8 ml. of thionyl chloride in 8 ml. of chloroform for 2 hr. The reaction mixture was concentrated *in vacuo*. Excess of thionyl chloride was removed by repeated addition and evaporation of anhydrous toluene. The oily residue of acid chloride (IV) was dissolved in 10 ml. of anhydrous ether and added dropwise, with stirring and cooling in an ice bath, to a mixture of 10 ml. sodium hydroxide (10%) and 10 ml. of an ether solution of β-methoxy-β-(3,4-dimethoxyphenyl)-ethylamine (III) (2) prepared from 0.52 Gm. of the oxalate. After the addition was completed, stirring was continued for 1 hr. at 0°. The amide crystallized, was filtered off, washed with cold water, and dried. Yield 0.90 Gm. Recrystallization from ethanol gave colorless needles, m.p. 115–116°. The NMR spectrum showed the following proton resonances: Singlet at τ 6.82 for β-methoxyl group, singlet at τ 6.13 for two aromatic methoxyl groups, singlet at τ 6.52 for —CH_2—CO—, and singlet at τ 4.85 for the benzyloxy methylene protons.

Anal.—Calcd. for $C_{27}H_{31}NO_6$: C, 69.66; H, 6.71; N, 3.01. Found: C, 69.45; H, 6.75; N, 2.92.

1 - (3' - Hydroxy - 4' - methoxybenzyl) - 6,7 - dimethoxyisoquinoline (II)—A mixture of 0.436 Gm. of V, 1.2 ml. of phosphorus oxychloride, and 6 ml. of toluene was heated under reflux for 1.5 hr. The reaction mixture was concentrated *in vacuo* to give an oil of 1-(3'-benzyloxy-4'-methoxybenzyl)-6,7-dimethoxyisoquinoline (VI) hydrochloride. The crude product was refluxed with a mixture of 4 ml. of 18% hydrochloric acid and 2 ml. of ethanol for 2 hr. The solution was concentrated *in vacuo*, made strongly alkaline with 10% sodium hydroxide solution, and washed once with chloroform. Ammonium chloride was added to a pH of about 10 and the liberated phenol extracted with chloroform. The chloroform extract was dried over anhydrous magnesium sulfate and evaporated to dryness *in vacuo* to a brown oil (0.1 Gm.), which crystallized on addition of a small amount of ethanol. Recrystallization from ethanol gave colorless prisms, m.p. 176°. The sample gave no melting point depression in mixture with palaudine isolated from opium. The UV, IR and NMR spectra corresponded to those described for palaudine.

Anal.—Calcd. for $C_{19}H_{19}NO_4$: C, 70.14; H, 5.89; N, 4.31. Found: C, 69.91; H, 5.89; N, 4.19.

N - (3',4' - Dimethoxyphenylacetyl) - β - methoxy-β - (3 - methoxy - 4 - benzyloxyphenyl)ethylamine (XII)—The acid chloride (XI) was prepared from 0.25 Gm. of 3,4-dimethoxyphenylacetic acid as described for IV. It was dissolved in 10 ml. of benzene and added dropwise and with stirring and cooling to a mixture of 10 ml. of 1 *N* sodium hydroxide and 10 ml. of a benzene solution of β-methoxy-β-(3-methoxy-4-benzyloxyphenyl)-ethylamine (X) (10) obtained from 0.5 Gm. of the hydrochloride. After 2 hr. of stirring, the benzene layer was separated and washed with water, then with dilute hydrochloric acid and again with water. The benzene layer was dried with anhydrous magnesium sulfate and evaporated *in vacuo* to give the amide (XII) as a brown oil (0.7 Gm.). It was purified by chromatography on a column of neutral alumina, Merck (activity 4) with chloroform. The colorless syrup obtained after evaporation of the solvent gave a single spot by thin-layer chromatography on silica gel with chloroform–methanol (9:1). The NMR spectrum showed the following signals: singlet at τ 6.13 for two aromatic methoxyl groups, singlet at τ 6.52 for —CH_2—CO— and singlet at τ 4.87 for the benzyloxy methylene protons.

1 - (3',4' - Dimethoxybenzyl) - 6 - methoxy - 7-benzyloxyisoquinoline (XIII)—A mixture of 0.5 Gm. of the amide (XII), 1.5 ml. of phosphorus oxychloride, and 6 ml. of toluene was refluxed for 1.5 hr. After cooling, petroleum ether was added, and a dark brown oil separated. The petroleum ether layer was decanted off, and the residual oil dissolved in methanolic hydrochloric acid and poured into cold 1 *N* sodium hydroxide solution. The mixture was extracted with chloroform, the extract dried over anhydrous magnesium sulfate, and the chloroform removed under reduced pressure. The brown, oily residue was purified by preparative thin-layer chromatography on silica gel with chloroform–methanol (9:1) to give a colorless oil. NMR spectrum: singlets at τ 6.23, 6.18, and 6.01 for three aromatic methoxyl groups, singlet at τ 5.55 for the methylene substituent in position-1, singlet at τ 4.82 for the benzyloxy methylene and doublet at τ 1.65 for the C-3 proton (J = 6 c.p.s.).

1 - (3',4' - Dimethoxybenzyl) - 6 - methoxy - 7-hydroxyisoquinoline (VII)—A solution of XIII (0.095 Gm.) in 2 ml. of ethanol was mixed with 1.5 ml. of hydrochloric acid (38%) and refluxed for 3 hr. The reaction mixture was evaporated to dryness *in vacuo*, the residue dissolved in water, and the solution washed once with chloroform. After addition of sodium hydroxide and ammonium chloride to pH about 10, the solution was extracted with chloroform, the extracts dried over anhydrous magnesium sulfate, and evaporated under reduced pressure. The residual oil crystallized on treatment with ethanol. Recrystallization from ethanol gave pale brown prisms, m.p. 163–165° [lit. 165–167° (6)]. The substance gave a single spot by thin-layer chromatography on silica gel with chloroform–methanol (9:1).

Anal.—Calcd. for $C_{19}H_{19}NO_4$: C, 70.14; H, 5.89; N, 4.31. Found: C, 70.06; H, 5.97; N, 4.45. The NMR signals are given in Table I.

N - (3' - Methoxy - 4' - benzyloxyphenylacetyl)-β - methoxy - β - (3,4-dimethoxyphenyl)ethylamine (XV)—The acid chloride (XIV) was prepared from 0.7 Gm. of 3-methoxy-4-benzyloxyphenylacetic acid (11) and reacted with β-methoxy-β-(3,4-dimethoxyphenyl)ethylamine (III) (from 0.8 Gm. of oxalate) as described for the synthesis of V. The amide which separated (1.3 Gm.) was recrystallized from ethanol to give colorless needles, m.p. 117–118°.

Anal.—Calcd. for $C_{27}H_{31}NO_6$: C, 69.66; H, 6.71; N, 3.01. Found: C, 69.54; H, 6.60; N, 3.15. The NMR spectrum gave the following proton resonances: singlet at τ 6.83 for the β-methoxyl group, singlet at τ 6.12 for two aromatic methoxyl groups, singlet at τ 6.50 for —CH_2—CO— and a singlet at τ 4.83 for the benzyloxy methylene protons.

1 - (3' - Methoxy - 4' - benzyloxy) - 6,7 - dimethoxyisoquinoline (XVI)—Cyclization of XV (0.6 Gm.) was carried out with phosphorus oxychloride in toluene as described above. The reaction mixture was concentrated *in vacuo*, made alkaline with sodium hydroxide solution, and extracted with chloroform. The chloroform extract was dried and evaporated to a brown oil (0.48 Gm.). The NMR

spectrum showed the following signals: singlets at τ 7.27, 6.12, 5.98 corresponding to three aromatic methoxyl groups, singlet at τ 4.89 for the benzyloxy methylene protons and doublets at τ 1.60 and 2.52 (J = 5.5 c.p.s.) for the C-3 and C-4 protons.

1 - (3' - Methoxy - 4' - hydroxybenzyl) - 6,7 - dimethoxyisoquinoline (VIII)—Hydrolysis of XVI (0.4 Gm.) with ethanolic hydrochloric acid was carried out as described for VII, and the isolated oil (0.1 Gm.) crystallized on standing. Recrystallization from ethanol gave pale tan-colored prisms, m.p. 165° [lit. 165° (6)]. The substance gave a single spot by thin-layer chromatography on silica gel with chloroform–methanol (9:1). The NMR signals are recorded in Table I.

Anal.—Calcd. for $C_{19}H_{19}NO_4$: C, 70.14; H, 5.89; N, 4.31. Found: C, 69.87; H, 5.87; N, 4.62.

N-(3',4'-Dimethoxyphenylacetyl)-β-methoxy-β-benzyloxy-4-methoxyphenyl)ethylamine (XVIII)—A benzene solution of the acid chloride (XI), prepared from 0.35 Gm. of acid, was added dropwise with stirring and cooling to a flask containing 10 ml. of sodium hydroxide solution (10%) and 10 ml. of a benzene solution of β-methoxy-β-(3-benzyloxy-4-methoxyphenyl)ethylamine (XVII), prepared from 0.6 Gm. of oxalate. After 2 hr. in an ice bath, the benzene layer was separated, washed with water, then with dilute hydrochloric acid and again with water, dried and evaporated *in vacuo*. The residual oil (0.7 Gm.) showed the following NMR signals: singlet at τ 6.90 for the β-methoxyl group, singlet at τ 6.12 for three aromatic methoxyl groups, singlets at τ 6.52 for the —CH_2CO— group, and at τ 4.87 for the benzyloxy methylene protons.

1 - (3',4' - Dimethoxybenzyl) - 6 - benzyloxy - 7-methoxyisoquinoline (XIX)—Cyclization of XVIII (0.6 Gm.) was carried out as described for XIII. The product was a brown oil (0.45 Gm.) which gave only a single spot by thin-layer chromatography on silica gel with chloroform–methanol (9:1). The NMR spectrum gave the following signals: singlets at τ 6.23, 6.18, and 6.10 corresponding to three aromatic methoxyl groups, singlet at τ 5.47 for the methylene substituent in position 1, and a singlet at τ 4.72 for the benzyloxy methylene protons.

1 - (3',4' - Dimethoxybenzyl) - 6 - hydroxy - 7-methoxyisoquinoline (IX)—The hydrolysis of XIX (0.45 Gm.) was carried out as described above, and the isolated oily compound (0.3 Gm.) purified by preparative thin-layer chromatography on silica gel with chloroform–methanol (9:1). This gave a crystalline substance which was recrystallized from ethanol as prisms having a light tan color, m.p. 165–167° [lit. 165° (6)]. The substance produced a single spot by thin-layer chromatography on silica gel. The NMR data are recorded in Table I.

Anal.—Calcd. for $C_{19}H_{19}NO_4$: C, 70.14; H, 5.89; N, 4.31. Found: C, 69.96; H, 5.93; N, 4.35.

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Keyphrases

Opium alkaloids—benzylisoquinoline isolated
Palaudine—isolated, structure confirmed
Column chromatography—separation
TLC—separation
Mass spectrometry—identity
NMR spectrometry—identity
UV spectrophotometry—structure
IR spectrophotometry—structure

Evaluation, Control, and Prediction of Drug Diffusion Through Polymeric Membranes I

Methods and Reproducibility of Steady-State Diffusion Studies

By EDWARD R. GARRETT and PRAMOD B. CHEMBURKAR

The equipment and methods for the study of drug diffusion through polymeric membranes are described. Among studies conducted on a given day, diffusion rates were reproducible within a coefficient of variation of 3.2 percent. Variation in ionic strength from 0.1–0.4 and the variations in hydrostatic head studied had no significant effect on the rates of diffusion of 4′-aminopropiophenone from 6.8 phosphate buffer through silastic membrane into 0.12 *N* HCl. Silastic membrane is shown to be impermeable to phosphate buffer and hydrogen and chloride ions.

THE POSSIBLE mechanisms for the passage of nutrients and drugs across a succession of membranes in the living organism have been explained on their postulated similarity to the transport or diffusion processes in artificial membranes (1–5).

Diffusion is characterized by the tendency of molecules to migrate from a region of high concentration to a region of lower concentration, but is more rigorously stated with respect to chemical potentials or activities rather than concentrations. The membrane is an imperfect barrier separating two fluids and hinders free diffusion of a substance in an isotropic medium.

Dialysis is due to sieve action and is largely dependent on the molecular weight of the diffusate and the viscosity of the solvent in accordance with the Sutherland and Einstein equation (6). Dialysis membranes may be considered as heterogeneous barriers in the sense that they possess pores. The ease of transport is generally a measure of the probability of a solvated molecule entering and diffusing through these pores. There is little selectivity in the separation of two closely related molecules except when their size is approximately that of the size of the pore (7). In general, the solvent as well as the solute is transported; membranes which allow salt transport are permeable to water (8).

Many polymeric membranes, such as copolymers based on polyoxyethylene glycols and polyethylene terephthalate, act as homogeneous barriers to free diffusion (7). Transport may be generally dependent on the relative adsorption of the molecules diffusing to the face of the membrane and solubility of these molecules in the membrane. Lyman and co-workers (7, 9, 10) have prepared synthetic membranes with the express intent of endowing them with specific characteristics which would transfer substances by an adsorption and solubility mechanism and not by sieve action. Variation in copolymer ratios of the synthetic membranes variably affected the transfer rates of glucose and urea. These observations were consistent with postulates of different degrees of association or partitioning with the membranes (11).

Studies on the diffusion of drugs from solution through polymeric substances that cannot be considered as dialysis have been limited. Johnson (12) has studied the diffusion of drugs in cross-linked thiolated gelatin and has observed that both ionic and nonionic drugs diffuse in accordance with Fick's law, which implies dialysis. No rigorous dependence of diffusivity on molecular weight can be concluded among the compounds studied.

The extensive use of polymeric materials for packaging purposes in the pharmaceutical industry has led to the investigation of polymer-drug interactions (13–18). It has been concluded from pH-sorption studies that undissociated acids interact with the basic amide groups of the polyamide nylon through hydrogen bonding. The rate-determining step was stated to be the diffusion of the solute within the polymer. The number of binding sites are finite and Langmuir or Freundlich adsorption models are applicable. Decreased solvent polarity decreased the extent of sorption.

Model systems for the kinetics of drug transport through lipoidal barriers have been studied recently. A series of nonpolar liquids have been selected as models of living membranes between aqueous phases (19–22). The purpose was to obtain values which could be employed to cor-

Received December 18, 1967, from the College of Pharmacy, University of Florida, Gainesville, FL 32601

Accepted for publication February 14, 1968.

Presented in part to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

Abstracted in part from a thesis submitted by P. B. Chemburkar to the University of Florida, in partial fulfillment of Doctor of Philosophy degree requirements.

The authors are grateful to the Dow Corning Center for Aid to Medical Research, Midland, Mich., for their supplies of the polymeric membranes.

relate the structures, solubilities, and partition coefficients of drug molecules with their ability to pass through different membranes.

The studies reported in this series on the diffusion and permeation of drugs through polymeric membranes from various solvent systems were initiated to establish and test model systems with solid membranes as homogeneous barriers. These may be considered a more realistic simulation of drug transport through solid lipoidal barriers. The purpose was also to quantify those parameters necessary for the evaluation, control, and prediction of drug diffusion through polymeric membranes. This is of practical importance in the light of recent pharmaceutical interest in the applications of such phenomena to the design of new dosage forms (7, 9, 10), particularly with reference to silicone rubber or silastic membranes (23, 24).

This first paper of the series describes the apparatus and techniques used to study steady-state and quasi-steady-state diffusion through such a lipid-like polymeric membrane and statistically evaluates the reproducibility of the former.

EXPERIMENTAL

Materials—The silastic medical grade sheeting (H-0169, H-0293), a dimethyl-siloxane polymer (25) was obtained from the Dow Corning Center for aid to Medical Research, Midland, Michigan. They were available in labeled thicknesses of 3, 5,10, and 20 mil. The actual thickness was obtained from the difference of measurements between two sheets of hard-surfaced paper and of the thickness of the two sheets alone. A micrometer screw with an accuracy of 0.1 mil was used at 10 equally spaced sites on seven different 2.54 cm. × 2.54 cm. square patches.

The 4′-aminopropiophenone, m.p. 139–140.5°, lit. value 140° (26), was purchased from Eastman Organic Chemicals, Rochester, New York. All other materials were of analytical reagent grade.

Analytical Methods—The absorbance of 4′-aminopropiophenone, 307 mμ, ϵ = 16,016 was measured in pH 6.8 phosphate buffer solutions against an appropriate blank with a Beckman model DU spectrophotometer in standard 10-mm. light path silica cells at 24.0 ± 1.0°.

Steady-State Diffusion Cells, Assembly, and Procedure—The steady-state diffusion cell designed was a modification of the cell used by Lyman *et al.* (10). An exploded schematic view of the diffusion cell is shown in Fig. 1. It consisted of two stainless steel plates (6.35 cm. × 5.1 cm. × 3.8 mm.), a glass T joint, two silicone rubber gaskets (2.57 cm. i.d. × 2.95 cm. o.d. × 0.16 cm. thick), and a set of four stainless steel nuts and bolts.

Each of the stainless steel plates had a hole in the center, 2.57 cm. in diameter. Around this hole there was a border 0.43 cm. in width from the perimeter of the hole and recessed 2 mm. into one face of the stainless steel plate. The silicone gaskets fitted into the recessed borders around the holes. The two plates had four holes in four corners for the stainless steel bolts.

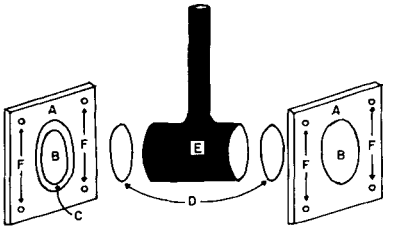

Fig. 1—Diffusion cell used for steady-state diffusion. Key: A, stainless steel plates (6.35 cm. × 5.1 cm. × 3.2 mm.), B, hole in stainless steel plate (2.57 cm. in diameter); C, recessed border around hole B (0.43 cm. in width from the perimeter of the hole and recessed 2 mm. into the stainless steel plate); D, silicone rubber gaskets (2.57 cm. i.d.; 2.95 cm. o.d.; 0.16 cm. thick); E, glass T joint with horizontal glass cylinder (2.6 cm. in diameter, 3 cm. long) and vertical stem (0.8 cm. in diameter and 11 cm. long); F, holes for stainless steel bolts.

The glass T joint consisted of a 3 cm. long hollow glass cylinder 2.60 cm. in diameter joined in its center at right angles on one side to an 11 cm. long glass tube 0.8 cm. in diameter to form a hollow T-shaped device. The two annular edges of the glass cylinder fitted into the recessed borders against the gaskets in the plates.

A 5.1 cm. × 5.1 cm. square of membrane under study (after washing with distilled water and drying in air) was positioned between the recessed border of each of the plates and a silicone rubber gasket. The open ends of the glass cylinder were then fitted into the recessed borders on the face of the silicone rubber gaskets. The whole assembly was held in position by a set of four nuts and bolts passing through the holes in the corners of the plates. The volume of the assembled cell was approximately 22 ml. and the total area of the two membranes available for diffusion was 10.4 cm.^{2}.

The diffusion cell was filled with 20 ml. of the buffered solution of the drug under study and was placed in either a 200 (or 400) ml. beaker which contained either 120 (or 200) ml. of solution into which the drug was to diffuse. The level in the cell was maintained about 3.88 cm. above the horizontal portion of the inverted T joint. This was effected by pumping the drug solution from a 2-L. reservoir through one channel of a Durrum 12-channel Dial-A-Pump (Durrum Instrument Corp., Palo Alto, California) at a rate of 25–30 ml./min. to a tube opening at the diametric center of the horizontal portion of the inverted glass T joint of the assembled diffusion cell. Another pump channel removed the solution back to the reservoir at a rate of 35–40 ml./min. through a tube whose opening was positioned in the stem of the cell at the level of the solution in the beaker (Fig. 2). The net results were to provide excellent agitation of the solution in the cell, maintain a constant level at the positioning of the opening of the effluent tube, renew the cell's contents every few min., and maintain a constant concentration therein (Fig. 2).

The beaker was covered with a Parafilm membrane (American Can Company, Neenah, Wisconsin) taped to the sides of the beaker with a hole in it

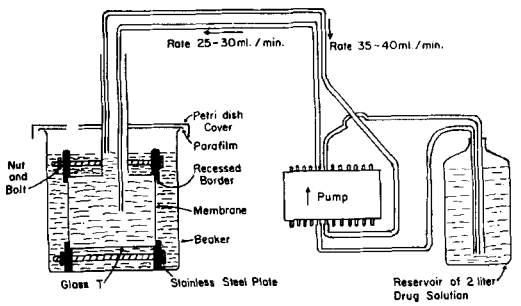

Fig. 2—Diffusion apparatus and pump assembly for steady-state diffusion studies.

for the stem of the diffusion cell. Over this was placed a plastic Petri dish with one hole for the stem and another for sample removal. This construction immobilized the diffusion cell in the beaker and minimized evaporation or contamination of the solution in the beaker (Fig. 2). The beakers with their respective diffusion cells were fitted rigidly in a metal rack inserted in the water contained in the thermostatted shaker bath (American Instrument Co., Silver Spring, Maryland) which was operated at 108 strokes per min. of 3.8 cm.

Prior to initiating any study, the beaker and its contents and the reservoir and its contents were equilibrated at the temperature of the study for about 10 hr.

At the end of each experiment, the volume and pH of the solutions in the beakers and the diffusion cells were measured. The pH values did not change throughout the course of an experiment for all studies.

Reproducibility of Steady-State Diffusion Studies—The reproducibility of diffusion studies was tested by studying the diffusion of 4′-aminopropiophenone, PAPP, from 22.0 ml. of its saturated solution in pH 6.53 ± 0.02 phosphate buffer through 3 mil silastic membrane into 184 ml. of 0.12 *N* HCl at 37.3° ± 0.1°. The cell solution contained excess undissolved PAPP and additional agitation was afforded by bubbling nitrogen through the cell. Samples (1.00 ml.) of the beaker solution of phosphate buffer were removed every hr. for 8–10 hr. Each sample, diluted with 4.00 ml. of pH 6.8 phosphate buffer (μ = 0.3) was measured at 307 mμ. Five separate experiments were performed on each of 5 days. Typical data for one such day are given in Table I.

Effect of Hydrostatic Pressure on Steady-State Diffusion—Solutions of PAPP ($1.70 \times 10^{-3} M$) in pH 6.8 phosphate buffer were circulated into four diffusion cells. The suction tubes of the channels returning the solutions from the diffusion cells to the reservoir were adjusted at four different levels (see Fig. 3) in the stem of the diffusion cell. The levels of the tubes were 0, 1.3 cm., 2.54 cm., and 7.62 cm. above the level of the solution in the beaker. The higher rate of pumping the solution from the cell to the reservoir over that of pumping the solution from the reservoir to the cell, kept the level in the cell constant at the level of the suction tube orifice. The rate of diffusion was monitored by spectrophotometric analysis of the samples from the beaker.

Effect of Ionic Strength on Steady-State Diffusion—A 100-ml. solution of 0.4 *N* HCl containing 2.5 Gm. of PAPP was prepared. Twenty-milliliter aliquots of this solution were added to 170, 330, 500, and 670 ml. of pH 6.8 phosphate buffer with an ionic strength of μ = 1.2. The solutions were then diluted with distilled water to 2 L. to obtain solutions of PAPP of the same concentrations in phosphate buffer but with ionic strengths of 0.102, 0.198, 0.300, and 0.402, respectively. The pH values of these solutions were 6.69, 6.73, 6.75, and 6.78, respectively. These solutions were then circulated in four diffusion cells to study the diffusion of PAPP through silastic membrane into 200 ml. of 0.12 *N* HCl at 23.0°.

TABLE I—STEADY-STATE DIFFUSION OF 4′-AMINOPROPIOPHENONE FROM 22.0 ML. SATURATED SOLUTION ($2.36 \times 10^{-3} M$) IN pH 6.5 PHOSPHATE BUFFER THROUGH 3-MIL SILASTIC MEMBRANE INTO 184 ML. OF 0.12 *N* HCl AT 37.3°[a]

Hr.	Absorbance[a] at 307 mμ in HCl Solution 1	2	3	4	5
0	0.007	0.004	0.008	0.014	0.006
1.00	0.134	0.141	0.116	0.121	0.112
2.00	0.234	0.224	0.232	0.207	0.209
3.00	0.357	0.319	0.340	0.287	0.319
4.00	0.478	0.428	0.460	0.395	0.433
5.00	0.560	0.515	0.532	0.470	0.505
6.00	0.663	0.602	0.653	0.559	0.612
8.00	0.886	0.815	0.875	0.722	0.825

[a] The desorbing sample solutions surrounding the cell were diluted 1:5 with pH 6.5 phosphate buffer before measurement of absorbance at 307 mμ.

Permeability of Silastic Membrane to Phosphate Buffer Salts and Hydrochloric Acid—A steady-state diffusion cell was assembled with 5-mil thick silastic membrane in position. It was filled with about 20 ml. of distilled water. The cell was kept in a 400-ml. beaker containing about 250 ml. of pH 6.5 phosphate buffer (μ = 1.2). A sample of distilled water from the cell was tested after 15 hr. for the presence of PO_4^{3-} by the ammonium molybdate test (27).

Another diffusion cell filled with 20 ml. distilled water was kept in a beaker containing 250 ml. of 0.1 *N* HCl solution. The distilled water inside the cell was tested for the presence of chloride ions after 11 hr. by the silver nitrate test (27). The pH of the distilled water was also measured.

Quasi-Steady-State Diffusion Cell, Assembly, and

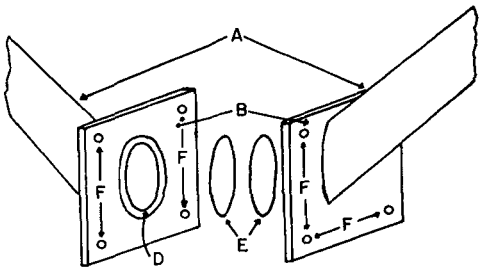

Fig. 3—Diffusion cell used for quasi-steady-state diffusion. Key: A, stainless steel cylinders (3.81 cm. in diameter, 17.8 cm. long); B, stainless steel plates (6.35 cm. × 6.35 cm. × 3.2 mm.); C, hole in stainless steel plate (3.1 cm. in diameter); D, recessed border around hole C (0.8 cm. in width from perimeter of the hole and recessed 2 mm. into the stainless steel plate); E, silicone rubber gaskets (3.1 cm. i.d.; 3.8 cm. o.d.; 3 mm. thick); F, holes for stainless steel bolts.

Procedures—The quasi-steady-state diffusion cell (Fig. 3) consisted of two stainless steel plates (6.35 cm. × 6.35 cm. × 3.2 mm.). Each of these plates was welded to one end of a stainless steel tube (3.81 cm. in diameter and 17.8 cm. long) at an angle of 45°. Each plate had a hole in the center (3.1 cm. in diameter) with a circular border 0.8 cm. in width from the perimeter of the hole and recessed 2 mm. into the face of the plate. The plates had four holes in the four corners for the stainless steel bolts. Two silicone rubber gaskets (3.1 cm. i.d. × 3.8 cm. o.d. × 3 mm. thick) fitted into the recessed border around the holes in the plates. A square of the membrane under study after washing with distilled water and drying in air was clamped between the two silicone rubber gaskets placed in the recessed borders in the plates. The plates were then clamped together with four stainless steel nuts and bolts. The diffusion cell when assembled formed a V-shaped device and held 150 ml. of solution in each of the tubes. The area of the membrane available for the diffusion was 7.55 cm.^{2}.

The solutions in the arms of the quasi-steady-state diffusion cell were agitated by the thermostated shaker bath in which the cell was immersed and wired to the shaker tray.

The cell was initially equilibrated in the thermostated shaker bath for about 8–10 hr. with 100 ml. of the appropriate buffer solution in both arms. Subsequently these solutions were removed by suction and a previously thermally equilibrated 50 ml. or 100 ml. solution of fresh appropriate buffer was added to one arm and an equal volume of thermally equilibrated solution of the drug in the buffer was added to the other arm. The open ends of the arms were covered with Parafilm. At regular intervals, samples from both arms of the diffusion cell were removed and spectrophotometrically analyzed for drug against the appropriate blanks until the differences in absorbances became small. The volume and pH of the solutions in both arms were measured at the end of the experiment.

RESULTS AND DISCUSSION

Impermeability of Silastic Membranes to Salts and Acid—The ammonium molybdate reagent solution (28) gave a yellow colored solution when mixed with an equal volume of 4.8×10^{-4} *M* solution of phosphate buffer. A faintly yellow colored solution was obtained with 9.60×10^{-6} *M* phosphate buffer solution. The sample from the diffusion cell kept for 15 hr. in a beaker containing phosphate buffer did not yield a precipitate or a colored solution with this reagent. It was therefore concluded that phosphate buffer salts do not diffuse significantly through silastic membrane.

A white turbid solution was obtained when 4×10^{-5} *M* solution of HCl was acidified with nitric acid and an equal volume of 10% silver nitrate solution was added. A sample from the diffusion cell kept for 11 hr. in a beaker containing 0.1 *N* HCl did not yield a turbid solution when treated similarly. Also, the 6.45 pH of the solution in the cell was constant for the time interval. It was therefore concluded that HCl does not diffuse significantly through silastic membrane.

Reproducibility of Steady-State Diffusion Studies—An example of the typical data obtained from a steady-state diffusion study of saturated solutions of PAPP in 6.5 phosphate buffer through a 3-mil silastic membrane into 200 ml. of 0.12 *N* HCl (where the concentration of the diffusing species, uncharged PAPP, is effectively zero) is given in Table I for five different cells run simultaneously on 1 day at 37.5°. The absorbances at 307 mμ of PAPP in the desorbing solution were monitored as a function of time, and when plotted against time are straight lines that appear to have zero intercepts.

The data of Table I were statistically analyzed (28–30) to determine linear regression against time, and it was concluded that the hypothesis that the intercepts were zero could not be rejected at the 5% level. The coefficient of variation for values among the slopes of the 5 replicate studies (Table I) conducted on 1 day was 7.0%.

Studies of five replicate diffusion experiments that resulted in data similar to Table I were repeated on each of 5 days. The slopes of the linear plots of concentration of the desorbing solution *versus* time and the analysis of variance (28–30) of these slopes are given in Table II. There was no significant difference between the slopes on the same day and between the slopes on different days. The estimates of variances were: for error, $\sigma_{E^2} = 0.0029$; for replicates, $\sigma_{R^2} = 0.000609$; and for days, $\sigma_{D^2} = 0.0042$. The coefficient of variation for all slopes was 12% and for the means of the slopes on differ-

TABLE II—REPRODUCIBILITY OF 4′-AMINOPROPIOPHENONE DIFFUSION THROUGH SILASTIC MEMBRANE[a]

	Slopes of Concentration of Desorbing Solution *Versus* Time[b]				
Days	1	2	3	4	5
1	0.491	0.482	0.467	0.404	0.425
2	0.531	0.476	0.517	0.444	0.496
3	0.695	0.452	0.501	0.518	0.506
4	0.537	0.483	0.434	0.471	0.509
5	0.509	0.493	0.550	0.592	0.422

Analysis of Variance

Source of Variation	*df*	*SS*	Mean Square	*F* Ratio	Components of Variance
Replicates (*R*)	4	0.021305	0.005326	1.84 N.S.[c]	$\sigma_{E^2} + 4\,\sigma_{R^2}$
Days (*D*)	4	0.018229	0.004557	1.58 N.S.[c]	$\sigma_{E^2} + 4\sigma_{D^2}$
Error (*E*)	16	0.046222	0.0028888		σ_{E^2}
Total	24	0.085756			

[a] Steady-state diffusion of 4′-aminopropiophenone from saturated solution (2.35×10^{-3}*M*) in phosphate buffer through 3-mil silastic membrane into 200 ml. of 0.12 *N* HCl at 37.5° where in the latter solution the PAPP is fully protonated. [b] Moles of PAPP diffused per hr. would be 0.200 time these slope values which are in moles/L./hr. [c] N.S.—not significant.

ent days was 5.3%. This would include the inherent errors of the measurements and the variations among the membranes.

In subsequent experiments where nitrogen purging was not used, the variability among repetitive experiments was even less. For example, in a similar steady-state diffusion experiment at 23.0° through 3-mil silastic membrane where the PAPP concentration was 1.79×10^{-3} moles/L., the ionic strengths and the slopes of plots of concentration in desorbing solution *versus* time in 10^5 moles/L./hr. were: 0.102; 1.25; 0.198; 1.24; 0.300; 1.30; 0.402; 1.31. The coefficient of variation of the slopes was 3.2%, well within the 7.0% calculated for the variation in slopes of the five replicated nitrogen purged diffusion studies conducted in 1 day. These data showed that ionic strength had no significant effect on steady-state diffusion of PAPP through silastic membranes.

Another example is a similar steady-state diffusion experiment at 25.0° through 3-mil silastic membrane where the PAPP concentration was 1.70×10^{-3} moles/L. The number of inches the level of the solution in the cell was above that of the desorbing solution in the beaker (Fig. 3) and the slopes of the plots of concentration in desorbing solution *versus* time in 10^5 moles/L./hr. were: 0.00 in.; 1.42; 0.50 in.; 1.46; 1.00 in.; 1.39; 3.00 in.; 1.45. The coefficient of variation of the slopes was 1.5%. These data showed that hydrostatic pressure exerted by variation of the levels in the diffusion cell within the limits studied had no significant effect on the rate of diffusion of PAPP through silastic membrane.

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Keyphrases

Polymeric membranes—drug diffusion
4′-Aminopropiophenone—diffusion
Diffusion cell, steady state—diagram
Hydrostatic pressure—steady-state diffusion effect
Ionic strength—steady-state diffusion effect
Silastic membranes—permeability
Quasi-steady-state diffusion cell—diagram

Evaluation, Control, and Prediction of Drug Diffusion Through Polymeric Membranes II

Diffusion of Aminophenones Through Silastic Membranes: A Test of the pH-Partition Hypothesis

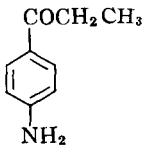
By EDWARD R. GARRETT and PRAMOD B. CHEMBURKAR

The steady-state and quasi-steady-state diffusions of 4′-aminopropiophenone, 4′-aminoacetophenone, and 3′-aminoacetophenone through various thicknesses of silastic membrane have been studied as functions of temperature, concentration, pH, and various ethanol–water compositions of diffusing and desorbing solutions. The membranes are impermeable to the protonated compounds, and the pKa values can be obtained from the pH profile of the apparent diffusion constants. The applicability of Fick's law was confirmed and the saturation of the membrane and/or limitations of numbers of binding sites are not rate determining. The transport of drug across the membrane is consistent with the partitioning from the diffusing solution into the membrane, diffusion within the membrane, and subsequent partitioning in the desorbing solution. Equations have been established on the basis of an assumed constancy of an intrinsic diffusion constant within the membrane to predict the transport of drugs through membranes separating different solvents where the drug would have different activities and have been experimentally verified. Satisfactory correlation between the apparent diffusion constants from steady-state diffusion studies and the partition coefficient in chloroform–water solutions has been observed for the several compounds. The apparent diffusion constant of 4′-aminopropiophenone from various ethanol–water solutions is linearly related to the reciprocal of its solubility in these solutions.

THE STUDIES reported here on the diffusion and permeation of drugs through polymeric membranes from various solvent systems were initiated to establish and test model systems with solid membranes as homogeneous barriers (1). These may be considered a more realistic simulation of drug transport through solid lipoidal barriers. The purpose was also to quantify those parameters necessary for the evaluation, control, and prediction of drug diffusion through polymeric membranes.

It has been implied that adsorption on surfaces of such polymeric membranes may be rate determining (2–4), that the number of surface binding sites may be finite, that the capacity of the membrane is related to its total number of internal binding sites (5–10), and that diffusion through aqueous diffusion layers on each side of the lipoidal membrane may be rate determining (11). If any but the last of these are so, the diffusion of drugs should not be linearly dependent on the concentration of the diffusing solution. As this concentration is increased, the rates of diffusion should approach a maximum value when adsorption or binding sites are limited. If desorption is rate determining, the rate of diffusion should become independent of membrane thickness as the concentration of the diffusing solutions is increased. These implications demand experimental evaluation to rigorously test the applicability of Fick's laws to diffusion through homogeneous polymer membranes.

This paper (part II of this series) considers the detailed study of the diffusion and permeation of the aminophenones, with specific reference to the antiradiation drug 4′-aminopropiophenone (PAPP), through silastic (silicone rubber) membranes.



4′-aminopropiophenone (PAPP)

EXPERIMENTAL

Materials—The silastic medical grade sheeting (H-0169, H-0293), a dimethyl-siloxane polymer (12), was obtained from the Dow Corning Center for aid to Medical Research, Midland, Mich. It was available in labeled thicknesses of 3, 5, 10, and 20 mil and the actual thicknesses were measured (1).

The following compounds were purchased from

Received January 12, 1968, from the College of Pharmacy, University of Florida, Gainesville, FL 32601

Accepted for publication February 14, 1968.

Presented in part to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

Abstracted in part from a thesis submitted by P. B. Chemburkar to the University of Florida in partial fulfillment of Doctor of Philosophy degree requirements.

The authors are grateful to the Dow Corning Center for Aid to Medical Research, Midland, Michigan, for their supplies of the polymeric membranes.

Eastman Organic Chemicals, Rochester, N.Y.: 4′-aminopropiophenone, m.p. 139–140.5° [lit. value 140° (13)]; 4′-aminoacetophenone, m.p. 105–106° [lit. value 106° (13)]; 3′-aminoacetophenone, m.p. 97–99° [lit. value 99° (13)]. All other materials were of analytical reagent grade.

Analytical Methods—The absorbances of the aminophenones were measured in pH 6.8 phosphate buffer solutions against appropriate blanks with a Beckman model DU spectrophotometer in standard 10 mm. light path silica cells at 24 ± 1°. The compound, wavelength used, and molar absorptivity were, respectively: 4′-aminopropiophenone, 307 mμ, ϵ = 16,016; 4′-aminoacetophenone, 312 mμ, ϵ = 13,700; 3′-aminoacetophenone, 327 mμ, ϵ = 1,840. Adherence to Beer's law was confirmed by readings of diluted solutions of a known molarity.

The pKa′ of 100 ml. of 8.56×10^{-5} *M* 4′-aminopropiophenone was determined spectrophotometrically. The pH of the solution was measured on the Beckman expanded scale pH meter standardized with pH 4.0 and 7.0 standard Beckman buffer solutions. An ultraviolet spectrum of this solution was obtained against a distilled water blank on the Cary recording spectrophotometer, model 15. The sample solution in the cell was returned to the bulk solution, and a drop of concentrated HCl was added and the solution stirred. The pH of the solution was measured and the spectra determined again. This procedure was repeated and the absorbances at 307 mμ were plotted against the pH of the solutions. The pH value corresponding to the midpoint of the resultant sigmoidal curve was the half neutralization point taken as the pKa′ of 4′-aminopropiophenone and was 2.42.

Ethanol in aqueous solutions was quantitatively determined with an F & M model 700 gas chromatograph equipped with a flame-ionization detector. The column was a 3.05 cm. (1.2 in.) × 6.4 mm. o.d. stainless steel column packed with 20% Carbowax 20 M on 60–80 mesh Chromosorb W maintained isothermally at 50°. The temperature of the detector was 250° and that of the injection port was 150°. The helium carrier gas was used at a pressure of 30 psig. while hydrogen and air for the flame were used at 10 and 40 psig., respectively. Samples of 5 μl. of aqueous ethanol were injected without any prior treatment. The peak heights were measured and the concentration of the ethanol in the sample was obtained from a calibration curve prepared from ethanol solutions of known concentrations.

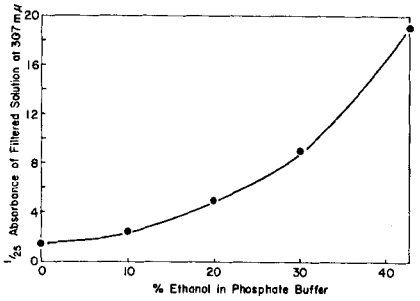


Fig. 1—Solubility of 4′-aminopropiophenone in 6.8 phosphate buffer at 25.0° plotted as absorbance of filtered saturated solutions at 307 mμ, ϵ = 16,016, against the percentage of ethanol in the phosphate buffer.

Solubility Studies—Saturated solutions of the compounds were prepared in pH 6.8 phosphate buffer (ionic strength of 0.3) at 50° and equilibrated at 37.5° in a thermostated shaker bath. The saturated solutions of 4′-aminopropiophenone in pH 6.8 phosphate buffer containing 0, 10, 20, 30, and 40% ethanol by volume were prepared by vigorously shaking the solutions at room temperature in the presence of excess solids and allowed to equilibrate at 25° in the thermostated shaker bath for 48 hr.

The solutions were filtered by suction through electrode isolation tubes which are fitted with a finely porous fritted-glass membrane (E. H. Sargent & Co., Chicago, Ill.). Aliquots of the filtered solution were appropriately diluted with pH 6.8 phosphate buffer and spectrophotometrically analyzed against appropriate blanks. The solubility of 4′-aminopropiophenone in phosphate buffer is shown as a function of ethanol concentration in Fig. 1.

Partition Coefficients—Aqueous pH 6.8 phosphate buffer and chloroform were saturated with respect to each other. The mixtures were separated by a separator and subsequent centrifugation at 3,200 r.p.m. for 5 min. An approximately 5×10^{-4} *M* solution of the compound was prepared in the phosphate buffer presaturated with chloroform. One milliliter of this solution was diluted appropriately with phosphate buffer and spectrophotometrically analyzed (at an absorbance 0.5–0.7) against a blank treated similarly. Five milliliters of the phosphate solution and 5.0 ml. of the chloroform presaturated with aqueous phosphate buffer in a capped stoppered vial were mixed on a Vortex Jr. mixer for 3 min., centrifuged for 3 min. at 3,200 r.p.m., and 1.00 ml. of the aqueous phase was removed, diluted, and spectrophotometrically analyzed. The ratio of the differences in absorbances of the aqueous layer before and after partitioning to the absorbance of the aqueous layer after partitioning was taken as the partition coefficient of the drug in the $CHCl_3$–phosphate buffer system.

Steady-State Diffusion Studies—The equipment and methods used have been described in detail in the prior paper of this series (1).

Effect of Thickness of Membrane on Steady-State Diffusion—Silastic membrane was available in four different thicknesses of 3, 5, 10, and 20 mil. The diffusion of a 1.517×10^{-3} *M* PAPP through these four thicknesses into 200 ml. of 0.12 *N* HCl was studied at 24.9°. A solution of PAPP in pH 6.8 phosphate buffer from one reservoir containing about 8 L of the solution was circulated through four cells, each one fitted with a silastic membrane of different thickness. The samples of 0.12 *N* HCl from the beakers were analyzed as a function of time to monitor the rates of diffusion of PAPP (Fig. 2).

Effect of Temperature on Steady-State Diffusion—The diffusion of PAPP from its solution in pH 6.8 phosphate buffer through 3-mil thick silastic membrane into 200 ml. of 0.12 *N* HCl was studied at seven different temperatures: 24.75°, 24.90°, 30.40°, 31.25°, 33.60°, 37.50°, and 41.0° ± 0.25°. At each temperature the diffusion was studied at four or more concentrations of PAPP in the phosphate buffer solutions. The same diffusion cells were used without changing the membranes to avoid any variation in membrane thickness and in area of the membrane available for diffusion.

Effect of pH on Steady-State Diffusion of PAPP—

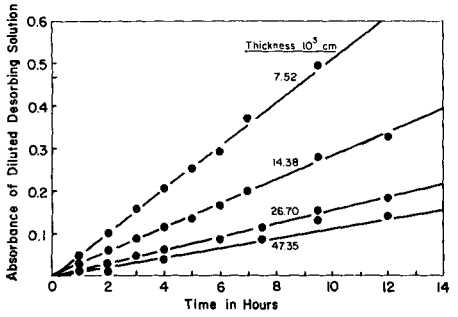


Fig. 2—Diffusion of 4′-aminopropiophenone (C_2 = 1.52×10^{-3} M) *from pH 6.8 phosphate buffer through silastic membranes of different thicknesses at 24.9°. The absorbance at 307 mμ of the desorbing 200 ml. of 0.12* M *HCl solution was monitored as a function of time after a 1:5 dilution with pH 6.8 phosphate buffer.*

The solutions of PAPP were prepared in 0.001 *N*, 0.01 *N*, and 0.1 *N* HCl and pH 3.48, 4.38, and 5.47 acetate buffer and pH 6.70 phosphate buffer. The steady-state diffusion of PAPP from these solutions through 3-mil thick silastic membrane into 200 ml. of 0.12 *N* HCl was studied at 25.0°. The pH values of the solutions were noted before and after the diffusion experiments and were observed to be unchanged. The absorbance of PAPP in the HCl solution in the beaker was measured as a function of time after 1:5 dilution with phosphate buffer. The absorbances of the solutions in the reservoirs, used for the circulation into the diffusion cells, were measured after appropriate dilutions with phosphate buffer.

Steady-State Diffusion of 4′-Aminoacetophenone and 3′-Aminoacetophenone—The diffusion of these two drugs through a 3-mil silastic membrane from their solutions in pH 6.8 phosphate buffer into 120 ml. of 0.12 *N* HCl was studied at 25.0° and 37.5°. The concentrations of the drugs diffusing into HCl were measured spectophotometrically after 1:5 dilutions with pH 6.8 phosphate buffer.

Effect of Alcohols on Steady-State Diffusion of PAPP—The effect of ethanol on the steady-state diffusion of PAPP through 3-mil silastic membrane at 25° was studied by varying the percentages of ethanol in the phosphate buffer (pH 6.8, aqueous) diffusion cell and in the outside desorbing solutions (0.12 *N* HCl). The percentage v/v of ethanol in both solutions and the concentrations of PAPP diffused are given in Table I.

The diffusion of PAPP from its saturated solutions in 0, 10, 20, and 30% v/v ethanol–water through 3-mil silastic membrane into 0.1 *N* HCl containing equivalent percentages of ethanol was studied at 24.3° (Table I). The same diffusion apparatus (Figs. 1 and 2 of *Reference 1*) was also used in the quasi-steady-state diffusion studies of PAPP from 0, 10, 20, and 30% ethanol–water solutions of phosphate buffer into 0, 10, 20, and 30% ethanol–water solutions of phosphate buffer in various combinations. The volume of the diffusing solution in the cell was 0.500 L. and that of the desorbing solution 0.120 L. The concentrations of the desorbing solution were spectrophotometrically monitored as a function of time.

Phosphate buffer (pH 6.8) solutions were prepared 2.0 *M* in ethyl, propyl, isopropyl, and *tert*-butyl alcohol and each contained 0.25 mg./ml. of PAPP. The steady-state diffusion of PAPP from each solution through 3-mil silastic membrane into 200 ml. of 0.12 *N* HCl was measured as a function of time.

TABLE I—APPARENT DIFFUSION CONSTANTS[a] OF 4′-AMINOPROPIOPHENONE[b] AS A FUNCTION OF ETHANOL CONCENTBATION AT 24.60°

% Ethanol in Phosphate Buffer Diffusing Solution	% Ethanol in 0.12 *N* HCl Desorbing Solution	Concn. of PAPP, 10^3 *M*	Apparent Diffusion Constant,[a] 10^{10}L./sec.-cm.
	Set A		
0.0	0.0	1.216	3.49
7.5	7.5	1.402	2.27
15.0	15.0	1.445	1.88
22.5	22.5	1.655	0.97
30.0	30.0	1.561	0.80
37.5	37.5	1.445	0.64
45.0	45.0	1.467	0.46
	Set B		
0	0	1.31	3.53
0	10	1.31	4.02
0	20	1.31	3.92
0	30	1.31	3.92
0	40	1.31	3.92
0	50	1.31	4.10
	Set C		
18	0	1.87	1.84
18	0	2.43	1.93
18	0	2.78	1.96
18	0	3.28	1.85
	Set D		
In water	0.1*N* HCl		
0	0	2.40	3.22
10	10	3.98	2.74
30	30	9.13	1.01
50	50	36.2	0.33

[a] The apparent diffusion constant was obtained from steady-state diffusion studies from ethanol–water phosphate buffer (6.8 pH aqueous) diffusing solutions of constant concentration C_2 through a silastic membrane of thickness X = 7.52×10^{-3} cm. or 3.0 mil and available surface area S = 10.4 cm.² into a desorbing ethanol–water 0.12 *M* HCl solution of a volume V = 0.200 L. The concentrations, C_1, of the desorbing solution where the diffusing species was entirely protonated were monitored spectrophotometrically as a function of time, t, and the obtained slope C_1/t of the plotted data which passed through the origin was used to obtain the apparent diffusion constant, $D = (C_1/t)(XV_1)/SC_2$. [b] The solubility of PAPP in ethanol–water phosphate buffer interpolated from a plot of solubilities *versus* % ethanol were: 10^3 *M*, % ethanol; 2.33, 0.0; 3.15, 7.5; 5.63, 15.0; 9.30, 22.5; 14.43, 30.0; 25.20, 37.5.

Effect of Ethanol on Silastic Membrane—Three steady-state diffusion cells were filled with 0, 10, and 30% ethanolic phosphate buffer solutions and kept in 0.12 *N* HCl solutions containing the same concentrations of ethanol. After 15 hr. the cells were emptied and washed with water, and the steady-state diffusion of PAPP from one solution of PAPP in phosphate buffer circulated in all three cells was studied in the absence of ethanol, inside or outside the cell.

Diffusion of Ethanol Through Silastic Membrane—The steady-state diffusion of ethanol through 3-mil silastic membrane into 200 ml. of 0.12 *N* HCl was studied at 24.6° from absolute ethanol, from phosphate buffer solutions of 0.0, 1.90, 3.01, and 5.06 $\times 10^{-3}$ *M* PAPP containing 18% ethanol, and from phosphate buffer solutions containing 10, 20, 30, 40, and 50% ethanol.

Absolute ethanol was circulated into a diffusion

TABLE II—APPARENT DIFFUSION CONSTANTS[a] OF 4′-AMINOPROPIOPHENONE THROUGH SILASTIC MEMBRANE AT VARIOUS TEMPERATURES

°C.	Concn., C_2, PAPP in Diffusing Solution, 10^4 M	C_1/t Concn. Increase of Desorbing Solution,[b] 10^6 M/L./hr.	$C_1/t\ C_2$ Specific Rate of Concn. Increase of Desorbing Solution,[c] 10^3 hr.$^{-1}$	Diffusion Constant, D[a], 10^{10} L./cm.-sec.
25.74	3.31	2.47	8.30	3.33
	7.21	5.59		
	9.48	7.74		
	11.37	9.12		
	13.99	11.23		
	16.45	13.30		
24.90	3.62	2.98	8.70	3.49
	9.80	8.18		
	12.10	10.23		
	18.30	15.77		
30.40	3.73	3.68	9.00	3.62
	9.21	9.68		
	10.93	10.20		
	16.71	15.30		
31.25	3.65	3.93	10.10	4.06
	9.33	9.43		
	13.74	14.10		
	18.24	21.20		
33.60	3.13	3.75	11.0	4.42
	9.96	11.20		
	14.67	16.50		
	19.11	21.20		
37.50	3.62	4.18	12.00	4.82
	9.37	10.40		
	13.49	16.70		
	17.73	21.20		
41.0	3.64	5.87	14.0	5.62
	9.68	14.40		
	13.99	20.00		
	18.79	27.20		

[a] The apparent diffusion constant, D, was obtained from steady-state diffusion studies from pH 6.5 phosphate buffer diffusing solutions of constant concentrations, C_2, through silastic membrane of thickness, $X = 7.52 \times 10^{-3}$ cm. or 3.0 mil and available surface area, $S = 10.4$ cm.^{2}, into a desorbing 0.12 M HCl solution of a volume $V_1 = 0.200$ L. so that D(L./cm.-sec.) = $(C_1/C_2t)\ (XV_1)/S$, where C_1/t and C_1/C_2t values are obtained as given in the subsequent footnotes. [b] The concentrations C_1 of the desorbing solution, where the diffusing species was entirely protonated, were monitored spectrophotometrically as a function of time, t, in hr., and these values are the obtained slopes, C_1/t in moles/L. of desorbing solution per hr., of the plotted data which passed through the origin. These values represent the slopes of C_1/t *vs.* C_2, where C_2 is the concentration of PAPP in the phosphate buffer diffusing solutions and the slopes of these plots are C_1/tC_2 in hr.$^{-1}$, the rate of increase in concentration of the desorbing solution per unit concentration of diffusing solution per hr.

cell fitted with a 3-mil silastic membrane. About 0.5-ml. samples of 0.12 N HCl were removed at hourly intervals, and 5 μl. of each of these samples was injected into a gas chromatograph for vapor phase analysis of ethanol. The percent ethanol present in the samples was calculated from a calibration curve.

Quasi-Steady-State Diffusion Studies—The quasi-steady-state diffusion cell and the methods previously described (1) were used to study the diffusion of PAPP from 50 and 100 ml. of pH 6.8 phosphate buffer through 5-mil silastic membrane into equal volumes of the same buffer at 25°.

RESULTS

Effect of Drug Concentration on Rate of Diffusion—The slopes, C_1/t, of the plots of concentration of PAPP in the 200 ml. of 0.12 M HCl desorbing solution after steady-state diffusion through 3-mil silastic membrane *versus* time are listed in Table II with the temperatures and the appropriate concentrations, C_2 of PAPP in the pH 6.5 phosphate buffer diffusing solution. Typical plots of these slopes, C_1/t in moles/L./hr., against the respective concentrations, C_2 in moles/L., of the diffusing solutions are shown in Fig. 3. The slopes of these plots, C_1/tC_2 in hr.$^{-1}$, are the specific rates of concentration increase in the desorbing solution per unit concentration of diffusing solution through 3-mil silastic membranes and are also given in Table II. The linear dependence of these diffusion rates, C_1/t, for the same membrane area, thickness, and volumes of diffusing and desorbing solutions on concentration of PAPP in the diffusing solution unequivocally demonstrates that the rate of diffusion through silastic membrane is directly proportional to the concentration of the diffusing drug.

Effect of Membrane Thickness on Rate of Diffusion—The plots of absorbance, $A = \epsilon C_1$ of PAPP in 1:5 diluted samples of the 200 ml. of 0.12 M HCl of the desorbing solution after steady-state diffusion *versus* time, t, for various thicknesses of silastic membrane are given in Fig. 2. The concentration increases with time, C_1/t, derived from the slopes of these plots are listed in Table III as well as the derived specific rates of diffusion (the rates of diffusion in moles of PAPP per cm.^{2} of membrane per sec. per mole/L., C_2, of diffusing solution). When these derived specific rates of diffusion, D/X, are plotted against the reciprocal of the thickness of the membranes, a linear relation is obtained (Fig. 4) with a slope of $3.76 \pm 0.19 \times 10^{-10}$ L./sec.-cm., the apparent diffusion constant D for the diffusion of PAPP through silastic membrane at 24.9°.

Effect of Temperature on Rate of Diffusion—The rates of steady-state diffusion of constant concentrations, C_2 of PAPP decrease with decreasing temperature (Fig. 3) through 3-mil silastic membrane and all are linearly dependent on the concentration, C_2, of the diffusing solution. The slopes, C_1/t, of the PAPP concentrations, C_1, of 200 ml. of 0.12 M HCl desorbing solution as a function of time, t, are given for the various studied temperatures in Table II. The derived specific rates, C_1/tC_2, of concentration increase of desorbing solution, per mole/L., C_2, of diffusing solution as well as the apparent diffusion constants, D, at the various tem-

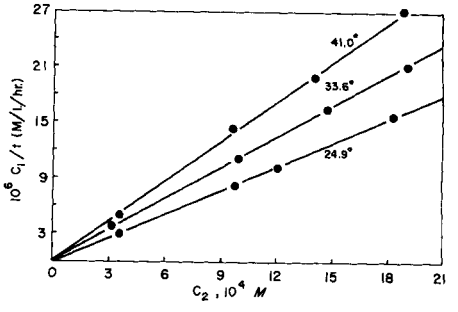


Fig. 3—Rates, C_1/t, of concentration increase in 200 ml. of 0.12 M HCl desorbing solution as a function of concentrations of 4′-aminopropiophenone in pH 6.5 phosphate buffer solution diffusing through 3.0-mil silastic membrane at several temperatures.

TABLE III—DIFFUSION OF 4′-AMINOPROPIOPHENONE AS A FUNCTION OF SILASTIC MEMBRANE THICKNESS AT 24.9°

Labeled thickness (mil)	3	5	10	20
Measured thickness (mil)[a] (10^3 cm.)	2.96 ± 0.05 7.52	5.66 ± 0.09 14.38	10.51 ± 0.10 26.70	18.64 ± 0.06 47.35
Coeff. of variation, %[b]	6.42	6.36	3.62	1.34
Slopes of concn. of desorbing solution *vs.* time, C_1/t($10^5 M$/L./hr.)[c]	1.436	0.749	0.406	0.250
Specific rates of diffusion, D/X (10^8L./sec.-cm.^{2})[d]	5.06	2.64	1.43	0.88

[a]Mean thickness and 95% confidence limits of the mean. [b]Standard deviation divided by the mean value. [c]The concentration, C_1, of the 0.200 L., V_1, of 0.12 *M* HCl desorbing solution was spectrophotometrically monitored as a function of time in hr. and these values are the slopes C_1/t of these linear plots with zero intercepts. [d]The specific rates of diffusion are the rates of diffusion in moles of PAPP per cm.^{2} of membrane, S = 10.40 cm.^{2}, per sec. per mole/L. of diffusing solution, C_2. The conditions were steady-state and the concentration of the diffusing solution was maintained constant, $C_2 = 1.517 \times 10^{-3}$ *M*, and the concentration of the diffusing unprotonated species in the acid desorbing solution was effectively zero. Thus, specific diffusion rate = $D/X = (C_1/t)(V_1/C_2S)$.

peratures are also listed in Table II. The logarithm of the apparent diffusion constants, D, are plotted against the reciprocal of the absolute temperatures, T, in Fig. 5 in accordance with the expression

$$\log D = \log (D)_0 - (\Delta E_a/2.303)(1/T) \quad \text{(Eq. 1)}$$

The estimated activation energies of diffusion through silastic membrane, ΔE_a, as obtained from the slope of these plots, are listed in Table IV for the various compounds. In addition, the determined partition coefficients and solubilities are also given.

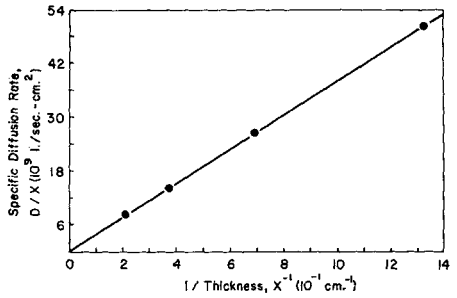


Fig. 4—Effect of thickness, X, of silastic membrane on the specific rates of diffusion, D/X, in moles of PAPP per cm.^{2} of membrane per sec. per mole/L. of phosphate buffer diffusing solution, $C_2 = 1.517 \times 10^{-3}$ M, into 0.12 M HCl desorbing solution at 24.9°.

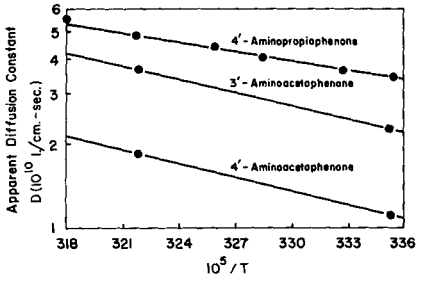


Fig. 5—Arrhenius plots of the logarithm of the apparent diffusion constants, log D, against the reciprocal of the absolute temperature, 1/T, for the diffusion of aminophenones through 3-mil silastic membranes from phosphate buffer solution.

Effect of pH on Rate of Diffusion—The apparent diffusion constants for the steady-state diffusion of PAPP through 2.43-mil silastic membrane into 0.12 *M* HCl from buffered solutions of various pH values with maintained concentrations, C_2, are listed in Table V and are plotted against pH in Fig. 6. The apparent diffusion constant, D, approaches an asymptotic value of 3.66×10^{-10} L./cm./sec. at high pH values and approaches zero at pH values less than one. The pH value corresponding to half the asymptotic value of 1.83×10^{-10} is 2.45, consistent with the experimentally determined spectrophotometric pKa′ of 2.41.

Effect of Alcohols on the Steady-State Diffusion of 4′-Aminopropiophenone (PAPP)—The plots of absorbance, $A = \epsilon C_1$, of PAPP in 1:5 diluted samples of the 200 ml. of 0.12 *M* HCl of the desorbing

TABLE IV—PARTITION COEFFICIENTS,[a] SOLUBILITIES,[b] APPARENT DIFFUSION CONSTANTS,[c] AND ACTIVATION ENERGIES OF DIFFUSION[d] OF AMINOPHENONES

Compd.	4′-Amino-aceto-phenone	3′-Amino-aceto-phenone	4′-Amino-propio-phenone
Partition[a] coefficient at 25.0°	15.97	53.30	134.4
Solubility[b] at 37.5° ($10^3 M$)	24.8	52.2	2.36
Apparent diffusion constants, D^c 10^{10}L./cm.-sec.			
25.0°	1.10	2.21	3.42
37.5°	1.84	3.62	4.82
Activation[d] energy (ΔEa) Kcal./mole	7.55	7.49	4.90

[a] Concentration in chloroform/concentration in pH 6.8 phosphate buffer solution at 25.0°. [b] Solubility in pH 6.8 phosphate buffer at 37.5°. [c] The apparent diffusion constants, D, were obtained from steady-state diffusion studies of maintained concentrations, C_2, in the pH 6.8 phosphate buffer diffusing solution through silastic membrane of thickness X = 7.52 × 10^{-3} cm. or 3.0 mil and available surface area S = 10.4 cm.^{2} into a desorbing 0.12 *M* HCl solution of a volume V_1 = 0.200 L. for PAPP and V_1 = 0.120 L. for the other compounds. The concentration increase of the desorbing solution C_1/t, was spectrally measured as a function of time so that D(L./cm.-sec.) = $(C_1/t)(XV_1/C_2S)$. [d] Calculated from slopes according to plots of the expression: $\log D = \log D - \Delta Ea/2.303\,RT$.

TABLE V—APPARENT DIFFUSION CONSTANTS[a] OF 4′-AMINOPROPIOPHENONE AS A FUNCTION OF pH AT 25.0°

pH	Concn. of PAPP, $C_2 10^3$ M	Apparent[a] Diffusion Constant, 10^{10} L./cm.-sec.
1.30[b]	1.685	0.21
2.00[b]	1.655	1.01
2.45[b]	1.639	1.88
3.48[c]	1.592	2.71
4.38[c]	1.59	3.25
5.47[c]	1.670	3.61
6.70[d]	3.310	3.66

[a] The linear concentration, C_1, increases of the V_1 = 0.200 L. of 0.12 M HCl desorbing solution were spectrally monitored as a function of time, t, and the C/t values obtained from the slope. The diffusion was studied from maintained concentrations, C_2, of the specified buffer solutions through X = 6.09 × 10^{-3} cm. or 2.43-mil silastic membrane with available surface area S = 10.4 cm.^{2}. The apparent diffusion constant was calculated from D(L./cm.-sec.) = $(C_1/t)(XV_1/C_2S)$. [b] HCl. [c] Acetate buffer. [d] Phosphate buffer.

solution after steady-state diffusions through silastic membrane *versus* time, t, for various percentages of ethanol in the diffusing solutions are given in Fig. 7. The derived apparent diffusion constant, D, from the slopes of such plots are given in Table I for various concentrations of ethanol of the diffusing solution and the desorbing solution.

The apparent diffusion constants for PAPP decreased with increasing ethanol concentrations of the phosphate buffered diffusing solution when the PAPP concentrations remained constant (Set A, Table I). Variation in the ethanol concentration of the 0.12 N HCl desorbing solution, where the concentration of the uncharged PAPP was effectively zero and where the diffusing solution had no ethanol, had no significant effect on the apparent diffusion constant (Set B, Table I). Variation in PAPP concentration of an 18% ethanol-containing diffusing solution showed that steady-state diffusion was a linear function of this concentration, and that the apparent diffusion constant (per unit of concentration of the diffusing solution) was truly constant (Set C, Table I).

The steady-state diffusion rates at 24.25° of saturated solutions of PAPP from aqueous saturated solutions with 0, 10, 20, and 30% ethanol through 3-mil silastic membrane into 0.1 N HCl with the same ethanolic concentration are given in Table I, Set D. The increased solubility of PAPP gave a decided increase in the overall rates of diffusion.

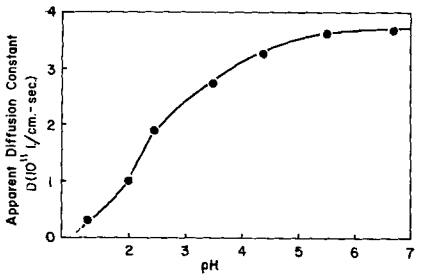


Fig. 6—Apparent diffusion constants for 4′-aminopropiophenone through silastic membrane into 0.12 M HCl desorbing solution at 25.0° against the pH of the diffusing PAPP solutions.

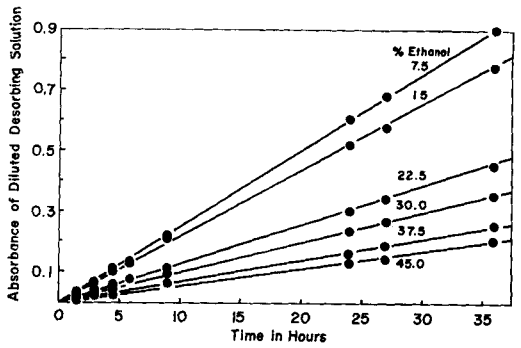


Fig. 7—Diffusion of 4′-aminopropiophenone (C_2 = 1.4 × 10^{-3} M) from various percentages of ethanol–water phosphate buffer (pH 6.8, aqueous) through 3.0-mil silastic membrane at 24.6°. The absorbance at 307 mμ of the desorbing 200 ml. of 0.12 M HCl solution of the same ethanol–water composition was monitored as a function of time after a 1:5 dilution with pH 6.8 phosphate buffer.

However, the apparent diffusion constants decreased with decreasing ethanol concentrations on both sides of the membrane.

The steady-state diffusion rates of PAPP concentrations increase in 200 ml. of 0.12 N HCl desorbing solution per unit concentration of PAPP in alcohol–water phosphate buffer diffusing solution (pH 6.8, aqueous) containing 2.0 M ethyl, propyl, isopropyl, and *tert*-butyl alcohol were 5.1, 6.0, 6.4, and 5.1 × 10^{-3} hr.$^{-1}$, respectively. It could not be concluded that the rates of diffusion of PAPP were significantly modified by variation among these alcohols.

The specific rates of steady-state diffusion of ethanol from phosphate buffer containing 18% ethanol were found to be independent of the PAPP concentration for concentrations up to 5 × 10^{-3} M (Table VI). The specific rates of diffusion of ethanol from phosphate buffer, *i.e.*, per unit of concentration in the diffusing solution, were independent of the concentration of ethanol (10–50%) in the diffusing solution (Table VI). The specific rates of diffusion of PAPP from phosphate buffer through silastic membrane pretreated with ethanol solutions of

TABLE VI—DIFFUSION OF ETHANOL AS A FUNCTION OF 4′-AMINOPROPIOPHENONE CONCENTRATION AT 24.6°

Concn. of Ethanol in Diffusing Solution, %	Concn. of PAPP in Diffusing Solution, 10^3 M	Slope of % Ethanol in Diffusing Solution *vs.* Time, %/hr.	Specific Rate[a] of Diffusion, 10^3 hr.$^{-1}$
18	0	0.032	1.77
18	1.90	0.032	1.77
18	3.01	0.032	1.77
18	5.06	0.032	1.77
10	0	0.0143	1.43
20	0	0.0273	1.37
30	0	0.0453	1.51
40	0	0.0633	1.53
50	0	0.0777	1.56

[a] Calculated for diffusion of ethanol through 3-mil silastic membrane by dividing slope or the plot of the percent ethanol in 200 ml. of 0.12 M HCl desorbing solution in ethanol–water phosphate buffer solution (pH 6.8, aqueous), where area of membrane, S = 10.4 cm.^{2} and thickness of membrane, X = 7.52 × 10^{-3} cm.

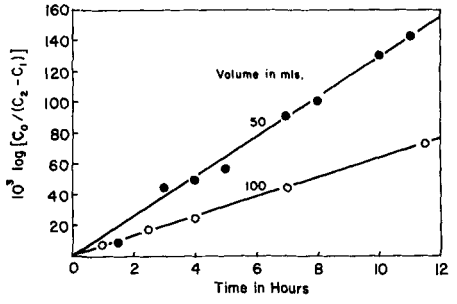


Fig. 8—Plots of log $[C_0/(C_2 - C_1)]$ *against time for quasi-steady-state diffusion of 4'-aminopropiophenone through 5-mil silastic membrane at 25.0°. The* C_2 *and* C_1 *are the concentrations of PAPP in equal volumes of the phosphate buffers of the diffusing solution and the desorbing solution, respectively, where* $C_2 = C_0$ *and* $C_1 = 0$ *at zero time.*

different concentrations were invariant. This indicated that the diffusing properties of these membranes are not altered by ethanol or that any alteration was reversible.

Quasi-Steady-State Diffusion Studies—Quasi-steady-state diffusion differs from steady-state diffusion in that (*a*) the concentrations of the diffusing and desorbing solutions are not held constant; (*b*) the concentration C_2 of the diffusing solution, initially C_0, decreases as the concentration C_1 of the desorbing solution increases; (*c*) as the values of both solutions approach the same equilibrium value with time. The plots of $\log[C_0/(C_2 - C_1)]$ *versus* time are linear for the quasi-steady-state diffusion of PAPP through 5-mil silastic membrane from and to pH 6.8 phosphate buffer at 25° (Fig. 8). The apparent diffusion constants were calculated from the slopes of these plots and were 3.93×10^{-10} and 3.94×10^{-10} L./cm.-sec. for 50-ml. and 100-ml. volumes of the solutions in the arms of the diffusion cell at 25°. These values agree with the estimated diffusion constant obtained from the various steady-state diffusion experiments, *i.e.*, $3.76 \pm 0.19 \times 10^{-10}$ L./cm.-sec. (Tables I, II, IV, V).

DISCUSSION

Fick's Law and Steady-State Diffusion—Fick's first law of diffusion (14–16) states that the rate, dA/dt, of diffusion of moles, A, per unit of time is proportional to the surface area of diffusion, S in cm.^{2}, and the concentration gradient (the rate of change of concentration C' with distance x in cm., dC'/dx) in an isotropic medium:

$$dA/dt = D'SdC'/dx \qquad \text{(Eq. 2)}$$

The experimental steady-state conditions specify that the concentration gradient, dC'/dx, is constant so that at any two planes in the isotropic medium where $x = 0$ at C_2' and $x = X$ at C_1'

$$dA/dt = D'S(C_2' - C_1')/X \qquad \text{(Eq. 3)}$$

In the specific case of diffusion through a membrane of thickness X cm. and surface area S cm.^{2} the steady-state Eq. 3 states that the rate is constant with time for constant concentrations C_2' and C_1', in the first and last monolayers, respectively. If the solutions of concentrations C_2'' and C_1'' of the diffusing species on the diffusion and desorption sides of the membrane, respectively, are in instantaneous equilibrium with their adjacent monolayers in the manner of a partition coefficient, *i.e.*,

$$C_2' = K_2C_2'' \qquad \text{(Eq. 4}a\text{)}$$

$$C_1' = K_1C_1'' \qquad \text{(Eq. 4}b\text{)}$$

the Eq. 3 can be rewritten as

$$dA/dt = D'S(K_2C_2'' - K_1C_1'')/X \qquad \text{(Eq. 5)}$$

Silastic membrane is impermeable to chloride, phosphate, and hydrogen ions (1). The additional fact that the estimated apparent diffusivities of PAPP decreased with increases in the fraction of the concentration that is charged (Fig. 6) confirms that this membrane acts as a lipid-like barrier, permeable only to uncharged species.

Thus the C_i'' terms in the diffusion Eq. 5 refer only to the concentrations of the unionized or uncharged drug in the solutions and are related to total concentrations, C_i by

$$C_i'' = f_iC_i \qquad \text{(Eq. 6)}$$

where f_i is the fraction of the drug that is unionized or uncharged and

$$f_i = K_a/([H^+] + K_a) \qquad \text{(Eq. 7}a\text{)}$$

for an amine such as PAPP whose conjugate acid has a dissociation constant K_a and

$$f_i = [H^+]/([H^+] + K_a) \qquad \text{(Eq. 7}b\text{)}$$

for an uncharged acid of dissociation constant K_a where $[H^+]$ is the hydrogen ion concentration.

The Eq. 5 can be rewritten as

$$dA/dt = D'S(K_2f_2C_2 - K_1f_1C_1)/X \qquad \text{(Eq. 8)}$$

For the special case where $K_1 = K_2 = K$,

$$dA/dt = D'KS(f_2C_2 - f_1C_1)/X = DS(f_2C_2 - f_1C_1)/X \qquad \text{(Eq. 9)}$$

where $D = D'K$ is the apparent diffusion constant or permeability constant for the system of transfer from a diffusing solution through a membrane of X cm. thickness and S cm.^{2} area into a desorbing solution.

In the steady-state diffusion studies of this investigation, the effective concentration $C_1'' = f_1C_1$ of the uncharged diffusing species of PAPP and its analogs was zero at the high acidities of the desorbing HCl solution since $f_1 = 0$ (Eqs. 6 and 7*a*). The concentration C_2'' of the uncharged species was maintained constant in the diffusing solution. The applicable Eq. 9 reduces in this special case to

$$dA/dt = DSf_2C_2/X \qquad \text{(Eq. 10)}$$

which integrates between the limits of zero time and time t to

$$A = C_1V_1 = DSf_2C_2t/X \qquad \text{(Eq. 11)}$$

where A is the amount permeating the membrane in time t. The desorbing solution of volume V_1 was analyzed for its total concentration of the drug, C_1, in moles/L., as a function of time so that plots of the concentration, C_1, *versus* time in seconds provide slopes proportional to the apparent diffusing constant, D, where

$$\text{Slope} = DSf_2C_2/V_1X \quad \text{(Eq. 12)}$$

$$D \text{ (L./cm.-sec.)} = D'K_2 = \text{slope} \cdot (XV_1)/(Sf_2C_2) \quad \text{(Eq. 13)}$$

The validities of Eqs. 11 and 12 have been experimentally verified. The stated conditions of steady-state diffusion give constant rates of diffusion with time (Figs. 2 and 7). The rate of steady-state diffusion of drug through silastic membrane is directly proportional to the concentration of drugs in the diffusing solution, C_2 (Table II, Fig. 3), and this rate is inversely proportional to the thickness of the membrane (Table III, Figs. 2 and 4).

The Eqs. 11 and 12 state that the rates of steady-state diffusion will be directly proportional to the fraction of unionized diffusing drugs and predict that these rates will be lowered to one-half their maxima when $[H^+] = K_a$ or pH = pKa (Eqs. 7, 11–13). This is confirmed by the data of Fig. 6 for PAPP, pKa = 2.42, where the apparent diffusion constants, D (Eq. 13) (actually f_2D) on the assumptions of $C_2'' = C_2$, $f_2 = 1$ are plotted against pH.

The Eqs. 9 and 13 assume that the apparent diffusion constant, D, for steady-state diffusion when $C_1'' = f_1C_1 = 0$ is directly proportional to the partition coefficient, K, of the drug between the diffusing solvent and the membrane. The partition coefficient is a ratio of the activities and/or solubilities (17) so that it may be postulated that

$$D = D'K = D'C_m/C_i = D'S_m\,(1/S_i) \quad \text{(Eq. 14)}$$

where C_m and C_i are the effective concentrations (or activities) of a compound in a membrane and its equilibrated solution, respectively; S_m and S_i are the solubilities in the membrane and its equilibrated solution, respectively.

If it is assumed that drugs in a homologous series have similar intrinsic diffusion constants, D', in the membrane and that the partition coefficients in a chloroform–water system, K_p, are proportional to those in the membrane–water system, K, *i.e.*, $K_p = \alpha K$ where α is a proportionality constant, the apparent diffusion constants, D, should increase with increases in K_p in accordance with Eq. 14. An approximately linear relationship does exist between the apparent diffusion constant, D, and the chloroform–phosphate buffer partition coefficient of the several compounds studied (Table IV).

$$D = 2.3 \times 10^{-12}\,K_p + 0.75 \times 10^{-10} \quad \text{(Eq. 15)}$$

A necessary condition for the plot of D *versus* $1/S_i$ to be linear is that the solubility in the membrane (S_m) is invariant with structural modification of the drug. The data of Table IV confirm the expectation that this is not necessarily so among several drugs.

However, when only the solvent is varied and the diffusion of one drug alone is studied, this necessary condition may be met. Experimental confirmation of Eq. 14 is obtained from the linearity of the plot (Fig. 9) of the apparent diffusion constants of PAPP, D (Table I) obtained from steady-state diffusion studies from ethanolic phosphate buffer solutions through silastic membrane into 0.12 *M* HCl desorbing solution against the reciprocal of the solubilities (Fig. 1) of PAPP, $1/S_i$, in these solutions. Regression analysis showed that the intercept is not significantly different from zero at the 5% level of significance. The slope of the plot of Fig. 9 implies

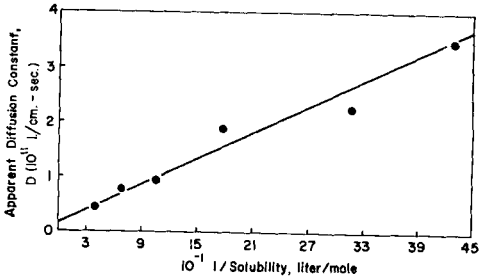


Fig. 9—Plots of apparent diffusion constant D *for the diffusion of 1.5 × 10⁻³M PAPP from various ethanol–water solutions of phosphate buffer (pH 6.8 aqueous) through 3.0-mil silastic membrane into 200 ml. of 0.12* M *HCl desorbing solution against the reciprocal of the solubility in these ethanol–water solutions.*

that the product of the intrinsic diffusion constant and the solubility in the membrane, $D'S_m$, is 8.35×10^{-13} moles/cm.-sec.

Quasi-Steady-State Diffusion—When the concentrations on both sides of a membrane are allowed to equilibrate with time and are monitored by the change of their concentrations with time, these rates can be expressed as (see Eq. 8)

$$dC_1/dt = D'S\,(K_2f_2C_2 - K_1f_1C_1)/XV_1 \quad \text{(Eq. 16}a\text{)}$$

$$dC_2/dt = -D'S\,(K_2f_2C_2 - K_1f_1C_1)/XV_2 \quad \text{(Eq. 16}b\text{)}$$

where V_1 and V_2 are the volumes of the desorbing and diffusing solutions, respectively. When the amounts entering and leaving the membrane are equal for all analytical purposes even though the rate of permeation changes with time as a function of the concentration gradient, this transport is termed "quasi-steady-state diffusion."

The stoichiometry of quasi-steady-state diffusion can be represented by

$$V_1C_1 + V_2C_2 = V_2C_0 \quad \text{(Eq. 17)}$$

and when the value of C_2 from Eq. 17 is substituted into Eq. 16*a* and the resultant expression integrated between the limits of $C_1 = 0$ at $t = 0$ and $C_1 = C_1$ at $t = t$,

$$\log\left\{\frac{K_2f_2C_0 - (K_2f_2V_1 + K_1f_1V_2)\,C_1/V_2}{K_2f_2C_0}\right\} = \frac{-(K_2f_2V_1 + K_1f_1V_2)D'S}{2.303X\,V_1V_2}\,t \quad \text{(Eq. 18)}$$

When both numerator and denominator within the logarithmic term is multiplied by D'.

$$\log\left\{\frac{D'K_2f_2C_0 - (D'K_2f_2V_1 + D'K_1f_1V_2)C_1/V_2}{D'K_2f_2C_0}\right\} = \frac{-(K_2D'V_1f_2 + K_1D'V_2f_1)S}{2.303X\,V_1V_2}\,t \quad \text{(Eq. 19)}$$

A similar expression in terms of C_0 and C_2 results when the value of C_1 from Eq. 12 is substituted in Eq. 16*b*.

If the thermodynamic activities of the drug in the diffusing and desorbing solutions are equal for the same solutions on both sides of the membrane, *i.e.*, $K_1 = K_2 = K$, and when the value of C_0 from Eq. 17 is considered, Eq. 19 reduces to

$$\log \left\{\frac{f_2C_0 - (f_2V_1 + f_1V_2)C_1/V_2}{f_2C_0}\right\} = \log \left\{\frac{f_2C_2 - f_1C_1}{f_2C_2}\right\} = \frac{-(f_2V_1 + f_1V_2)KD'S}{2.303\,XV_1V_2}\,t \quad \text{(Eq. 20)}$$

If $V_1 = V_2 = V$ and $f_2 = f_1 = f$, Eq. 20 reduces to

$$\log \left\{\frac{C_0 - 2C_1}{C_0}\right\} = \log \left\{\frac{C_2 - C_1}{C_1}\right\} = \frac{-2KD'fS}{2.303\,XV}\,t = \frac{-0.869DfS}{XV}\,t \quad \text{(Eq. 21)}$$

where $D = D'K$ is the apparent diffusion constant. This D value can be obtained from the slopes of linear plots of log $(C_2 - C_1)/C_0$ *versus* time for equal volumes of the same phosphate buffer solution in both arms of the quasi-steady-state apparatus (1) (Fig. 8). The linearity is consistent with the expectations of Eq. 21 for quasi-steady-state diffusion. The apparent diffusion constant, $D = 3.94 \times 10^{-10}$ L./cm.-sec., calculated from the slope of such plots, ($f = 1$) was consistent with the highly reproducible apparent diffusion constants 3.66×10^{-10} cm.^{2}/sec. (Table V) for uncharged PAPP obtained from steady-state diffusion data from phosphate buffer into HCl solution at the same temperature for the same silastic membrane.

Quasi-steady-state diffusion (Eq. 20) studies also produced estimates of the apparent diffusion constants $D = KD'$ for PAPP in various percent ethanol–water solutions of phosphate buffer, $f = 1$, diffusing into similar solutions when the volumes were unequal in both arms of the apparatus, $V_2 = 0.500$ L. and $V_1 = 0.120$ L. The obtained values as calculated from the slopes of plots in accordance with Eq. 20 are listed in Set A of Table VII. A new lot of silastic membrane was used in these studies.

Effects of Ethanol on PAPP Diffusion—The rates of steady-state diffusion of PAPP and thus the apparent diffusion constants through silastic membrane decrease with increased concentrations of ethanol in the diffusing solution (Set A, Table I) but are independent of ethanol concentration in the desorbing solution when the effective concentration of the transported drug is essentially zero (Set B, Table I). The specific rates of diffusion (with respect to unit concentrations) or the apparent diffusion constants of PAPP and ethanol from solutions of varying concentrations of PAPP in ethanolic phosphate buffer show negligible variation (Set C, Table I and Table VI). Thus, the diffusions of PAPP and ethanol through silastic appear to be independent processes. Those compounds do not compete or interact within the membrane or on the surfaces for sites. Apparently, the diffusion of the one does not significantly modify the intrinsic diffusivity constant, D', of the other within the membrane.

However, the apparent diffusivity constant of PAPP, $D = D'K$, is modified (Sets A and D, Table I) by varying ethanol concentrations in the diffusing solution. Its independence of the ethanol concentration of the desorbing solution (Set B, Table I), where the effective concentration of PAPP therein is zero ($f_1C_1 = 0, f_1 = 0$) in steady-state diffusion, is consistent with Eqs. 10–13 where the nature of the desorbing solution is not pertinent to the rates of transfer through the membrane. This phenomenon can be assigned to the change in the partition constant, K.

When the diffusing and desorbing solutions are of the same ethanolic concentrations ($K_1 = K_2 = K$) and pH values, ($f_1 = f_2 = f$) in quasi-steady-state diffusion, Eq. 20 is applicable. Thus, when the concentration data is appropriately plotted (Fig. 10) $D'K_i$ values can be obtained from the slope (Set A, Table VII where $f_1 = f_2 = 1$). In these cases the

TABLE VII—CONSTANTS FOR THE QUASI-STEADY-STATE DIFFUSION OF 4′-AMINOPROPIOPHENONE THROUGH, SILASTIC MEMBRANE FOR VARIOUS ETHANOL–WATER CONCENTRATIONS OF DIFFUSING (V_2) AND DESORBING (V_1) 6.8 pH PHOSPHATE BUFFER SOLUTIONS AT 25.3°

Set	% Ethanol in V_2	% Ethanol in V_1	10^{10} L./cm.-sec. $D'K_2$	10^{10} L./cm.-sec. $D'K_1$	$D'K_2V_1 + D'K_1V_2$ 10^{10} L.^{2}/cm.-sec. Obtained[c]	$D'K_2V_1 + D'K_1V_2$ 10^{10} L.^{2}/cm.-sec. Calcd.[d]
A[a]	0	0	2.405	2.405	1.48	1.49
	10	10	1.752	1.752	1.09	1.09
	20	20	1.237	1.237	0.77	0.77
	30	30	0.849	0.849	0.53	0.53
B[b]	0	10	2.405	1.752	1.15	1.16
	0	20	2.405	1.237	1.00	0.91
	0	30	2.405	0.849	0.81	0.71
C[b]	10	0	1.752	2.405	1.40	1.41
	10	20	1.752	1.237	0.93	0.83
	10	30	1.752	0.849	0.70	0.63
D[b]	20	0	1.237	2.405	1.45	1.35
	20	10	1.237	1.752	1.00	1.02
	20	30	1.237	0.849	0.60	0.57
E[b]	30	0	0.849	2.405	1.20	1.30
	30	10	0.849	1.752	0.91	0.98
	30	20	0.849	1.237	0.72	0.72

[a] In this case $D'K_1 = D'K_2 = D$ which are apparent diffusion constants obtained from the slope of the left-hand side of the following equation *vs.* time t, *i.e.*, $\ln C_0V_2/[C_0V_2 - C_1(V_1 + V_2)] = DSt(V_1 + V_2)/XV_1V_2$ where $V_2 = 0.500$ L., $V_1 = 0.120$ L., $S = 10.4$ cm.^{2}, the labeled thickness was 3 mil. [b] In these cases the $D'K_1$ values listed are the apparent diffusion constants DK' of Set A for diffusion from diffusing to desorbing solutions of the same ethanol–water composition of the desorbing solutions in these studies. The $D'K_2$ values listed are the apparent diffusion constants $D'K$ of Set A for diffusion from diffusing to desorbing solutions of the same ethanol–water composition of the diffusing solutions of these studies. [c] The $D'K_2V_1 + D'K_1V_2$ values are obtained from the slopes of the plots of left-hand term of the following equation *vs.* time, t, *i.e.*, $\ln [D'K_2C_0V_2/(D'K_2C_0V_2 - C_1(D'K_2V_1 + D'K_1V_2))] = (DK_2V_1 + D'K_1V_2)St/XV_1V_2$ where C_0, V_1, V_2, S, X are known values, the $D'K_1$ and $D'K_2$ values in logarithmic term are obtained from Set A, and C_1 is experimentally obtained. [d] The $D'K_2V_1 + D'K_1V_2$ values are calculated from the known V_1 and V_2 values and $D'K_2$ and $D'K_1$ values obtained from Set A.

ethanolic water membrane partitioning at the diffusing and desorbing surfaces would be equivalent and $K_1 = K_2 = K$.

If the partition hypothesis (Eq. 4) is correct, these $D'K_i$ values can be used to predict the quasi-steady-state diffusion when the solvents on both sides of the membranes are different and the $D'K_1$ and $D'K_2$ values are not equivalent. The appropriate $D'K_i$ values obtained (Set A, Table VII) for a given ethanol–water mixture can be used to construct the plots of log $[D'K_2C_0 - (D'K_2V_1 + D'K_1V_2)C_1/V_2]/D'K_2C_0$ *versus* time t in accordance with Eq. 19 where $f_1 = f_2 = 1$. The $D'K_2$ values are the apparent diffusion constants for quasi-steady-state diffusion when both solutions (Set A, Table VII) were of the same ethanol–water composition as the diffusing solution (Sets B–E, Table VII). The $D'K_1$ values are the apparent diffusion constants when both solutions (Set A, Table VII) were of the same ethanol–water composition as the desorbing solution (Sets B-E, Table VII). The C_0 (initial concentration of diffusing solution) and C_1 (concentration of desorbing solution at any time) values are experimentally known. The values of $(K_2D'V_1 + K_1D'V_2)$ can be obtained from the slopes of these plots (Fig. 10) in accordance with Eq. 19. These values are given in Sets B–E, Table VII where $V_2 = 0.500$ L. and $V_1 = 0.120$ L. and are in excellent agreement with those calculated from the K_2D' and K_1D' values obtained from the studies with the same ethanol–water composition in both solutions (Set A, Table VII).

Temperature Dependence of Diffusion Through Silastic Membrane—Estimates of the apparent activation energy of diffusion for the several drugs studied (Table IV) were made in accordance with

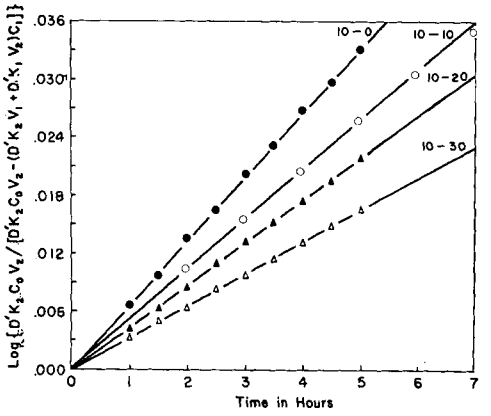


Fig. 10—Examples of plots of a logarithmic function of values against time, t, in accordance with Eq. 19 where $f_1 = f_2 = 1$ *for the diffusion of 4′-aminopropiophenone through silastic membrane from various % ethanol–water diffusing solutions into various % ethanol–water desorbing solutions. The representative plots are labeled accordingly. The slopes of these plots give the* $(K_2D'V_1 + K_1D'V_2)S/2.303XV_1V_2$ *values where* K_2 *and* K_1 *are the partition coefficients between the membrane and the diffusing and desorbing solutions, respectively, where* V_2 *and* V_1 *are the respective volumes, S is the area, X is the thickness of the membrane,* C_0 *is the initial concentration in the diffusing solution, and* C_1 *is the concentration in the desorbing solution as a function of time.*

Eq. 1 from the slopes of the plots of Fig. 5. Since the apparent diffusion constant, D, is a product of the intrinsic diffusion constant D' in the membrane and the partition constant, K in the steady-state diffusion system, it follows from Eq. 14 that

$$\log D' = \log P_D' - \Delta E_D'/2.303RT \quad \text{(Eq. 22)}$$

$$\log S_M = \log P_{S_M} - \Delta E_{S_M}/2.303RT \quad \text{(Eq. 23)}$$

$$\log S_i = \log P_{S_i} - \Delta E_{S_i}/2.303RT \quad \text{(Eq. 24)}$$

see that Eq. 1 may be reinterpreted as

$$\log D = (\log P_D' + \log P_{S_M} - \log P_{S_i}) - (\Delta E_D' + \Delta E_{S_M} - \Delta E_{S_i})/2.303RT \quad \text{(Eq. 25)}$$

and,

$$\Delta E_T = \Delta E_D' + (\Delta E_{S_M} - \Delta E_{S_i}) \quad \text{(Eq. 26)}$$

The apparent energy of diffusion is the sum of the true energy of diffusion inside the membrane and the differences between the heats of solution of the diffusing species in the membrane and the solvent phases. In general the values are approximately double the usual energies of diffusion in solution which are in the range of 3–5 Kcal./mole (18). However, these values are generally obtained for diffusion of large molecules through small molecule liquid solvents. Since the apparent energy of diffusion is perturbed by the heats of solution, changing the diffusing solvent and thus the accompanying heat of solution could increase or decrease the temperature coefficient of apparent diffusion.

Nonsteady-State Diffusion—The rate-determining factor in nonsteady-state transport through a membrane is the rate of diffusion in the membrane. In steady-state transport it is the constant concentration gradient alone and in quasi-steady-state transport both the concentration gradient and the rates of approach to equilibrium of both extramembrane phases are rate determining.

When a membrane is interposed between a solution of the penetrant and the solvent, the transportation of the penetrant is initially a nonsteady-state process and a "time lag" in transport results until the amount leaving the membrane equals the amount entering. This time lag has been used extensively (19–21) for the calculation of the diffusion constant in the nonsteady-state on the premise that a finite amount of time will be needed for a penetrant to traverse the thickness of the membrane before the attainment of a steady state (21). After this initial lag period, when the concentrations on both sides of the membrane are constant, the plot of the amount diffused *versus* time will be a straight line (Eq. 11) which when extrapolated will give an intercept θ on the time axis of

$$\theta = X^2/6D' \quad \text{(Eq. 27)}$$

Use of the authors' apparent diffusion constant, $D = 3.7 \times 10^{-10}$ L./cm.-sec., for the intrinsic diffusion constant D' in Eq. 27 for a membrane thickness $X = 7.52 \times 10^{-3}$ cm. would imply that the intercept of amounts diffused *versus* time would be 7 hr., whereas it is not statistically different from zero. This is consistent with our definition of D as $D'K$. If we assume an excessive error in the zero intercept of as much as 5 min., this would imply that the value of D' is greater than 10^{-8} L./cm.-sec. and that K is less than 10^{-2}.

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Keyphrases

Polymeric membranes—drug diffusion
Silastic membranes—aminophenones, diffusion
Diffusion, steady state—membrane, pH, temperature, ethanol effect
Aminophenones, concentration—diffusion rate
Quasi-steady-state diffusion
Nonsteady-state diffusion

Mechanistic Study of the Influence of Micelle Solubilization and Hydrodynamic Factors on the Dissolution Rate of Solid Drugs

By P. SINGH*, S. J. DESAI*, D. R. FLANAGAN, A. P. SIMONELLI, and W. I. HIGUCHI

The influence of micelle-drug solubilization on the dissolution rate has been investigated. The dissolution rates predicted by the diffusion layer model, the Danckwerts theory, and by the rotating disk theory were calculated and compared with experimental data obtained for the benzocaine–polysorbate 80 system. All the parameters involved were independently determined. The different conditions under which each of these theories is applicable have been discussed. Different kinds of dissolution experiments were designed to produce the conditions under which each theory would apply. The theoretical and experimental procedures involved in these studies provide a unique method for distinguishing mechanisms and should be useful in future studies, *e.g.*, in studies of the effects of agitation on mechanisms of interphase transport.

As PART of the authors' program on drug release rates it was decided to conduct experiments on the influence of micelle-drug solubilization on the dissolution rate behavior. There were two reasons for carrying out these studies. First, there has been relatively little work reported in the literature on the effects of solubilization by colloids upon the dissolution rate. Therefore, establishing a relationship between solubilization and dissolution rate appeared to be a worthwhile endeavor. Secondly, as will be seen, the different theories for dissolution rates based upon different hydrodynamic models predict significantly different rate relationships when a solubilizing agent is present. Experimental tests of such theories by other methods (1, 2) are extremely difficult, and, in the authors' opinion, past attempts have not led to convincing results. Thus meaningful information on the hydrodynamical aspects of the dissolution rate process might be obtained by the proposed method of study involving colloidal solubilizing agents.

Received September 20, 1967, from the College of Pharmacy, The University of Michigan, Ann Arbor, MI 48104
Accepted for publication February 21, 1968.
This research project was supported in part by a grant from the Parke, Davis and Company, Detroit, Michigan.

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THEORETICAL

Diffusion Layer Theory—Recently (3) Higuchi discussed the one-dimensional diffusion layer model for the dissolution rate of a pure solid in a stirred medium containing a colloidal solubilizing agent. The model assumes that equilibrium exists between the solid and the solution at the solid-solution interface, and that therefore the rate is controlled by the diffusion of the free and solubilized solute across an effectively stagnant liquid diffusion layer of thickness, h. Equilibrium between the free and the solubilized solute is assumed at every point in the diffusion layer.

These assumptions lead to the following equation for this model:

$$G = \frac{1}{h}\left(D_1C_1 + \sum_i D_iC_i\right) \qquad \text{(Eq. 1)}$$

Here G is the initial dissolution rate per cm.^{2} for the solid in the medium containing the solubilizing agent, D_1 is the diffusion coefficient of the free molecule in solution, C_1 is the contribution to the total solubility by the free solute, D_1 is the diffusion coefficient of the solubilized species, i, and S_i is the contribution of species, i, to the total solubility. Equation 1 reduces to

$$G = \frac{1}{h}(D_1C_1 + D_MC_M) \qquad \text{(Eq. 2)}$$

where the solubilization involves only a single solubilized species.

The Danckwerts Theory—The eddy diffusion model proposed by Danckwerts (4) assumes that fresh elements of liquid continually replace the aged ones at the solid-liquid interface. While at the interface, the elements absorb solute according to nonsteady-state diffusion laws. Thus the rate of dissolution may be visualized as being a function of the surface renewal rate and the diffusion rate.

When a colloidal solubilizing agent is present, the Danckwerts model gives

$$G = s^{1/2}\sqrt{(D_1C_1 + D_MC_M)(C_1 + C_M)} \qquad \text{(Eq. 3)}$$

Here s is the surface renewal rate constant, which is a function of the agitation rate, the viscosity and density of the medium, and the dimensions of the system. The derivation of Eq. 3 assumes that equilibrium between the free drug and the solubilized drug exists everywhere, and that the ratio of the free to solubilized drug is constant at all drug concentrations for a given concentration of the solubilizing agent.[1] Olander (5) has given the derivation for Eq. 3 although he presented it for the general situation that can be characterized by any equilibrium of the type, reactant $\overset{K}{\rightleftarrows}$ product.

It can be seen that Eq. 3 differs from Eq. 2 in the dependence of G upon the diffusion coefficients. The former predicts a square root dependence upon the effective diffusion coefficient, while the latter depends upon the first power of the effective diffusion coefficient. The effective diffusion coefficient can be written as:

$$D_{eff} = \frac{D_1C_1 + D_MC_M}{C_1 + C_M} \qquad \text{(Eq. 4)}$$

The Rotating Disk Theory—Levich (6) has provided a convective diffusion theory for the rate of mass transport to or from the face of a rotating disk. The equations are based upon Cochran's (7) exact solution to the hydrodynamic problem. As a result this situation represents one of the few instances where a physically well-defined picture can be presented mathematically.

The appropriate rotating disk equation for the dissolution rate of a solid in a medium containing a solubilizing agent is

$$G = 0.62(D_1C_1 + D_MC_M)^{2/3}\nu^{-1/6}\omega^{1/2}(C_1 + C_M)^{1/3} \qquad \text{(Eq. 5)}$$

where ν and ω are the viscosity of the medium and the angular velocity of the rotating disk, respectively. It is assumed, as was done in Eqs. 2 and 3, that equilibrium exists between the free and the solubilized drug and that the ratio of the two species is constant for all drug concentrations. Equation 5 differs from Eqs. 2 and 3 in that a two-thirds power dependence upon the effective diffusion coefficient is predicted.

The Noyes-Whitney Theory—This empirical theory simply predicts that the dissolution rate would be proportional to the total solubility. As the differences between the free drug and the solubilized drug diffusion coefficients are ignored, these parameters do not enter into this theory.

GENERAL CONSIDERATIONS IN THE DESIGN OF EXPERIMENTS

As mentioned earlier the object of these studies was twofold: first, to establish the role of colloid solubilization in dissolution rate processes and, secondly, to study the various theories that differ primarily in the assumptions regarding the interaction between hydrodynamics and molecular diffusion. In order to achieve these goals it was necessary to select a system that would provide a sufficiently large solubility increase and a sufficiently small D_M while not increasing the solvent viscosity and density appreciably with increasing solubilizing agent concentration. As will be seen, the system, benzocaine (ethyl *p*-aminobenzoate) in aqueous polysorbate 80,[2] generally satisfied these requirements and was therefore selected for this work.

Several hydrodynamic situations were selected. A tablet mounted in a beaker with a propeller stirrer, a rotating tablet in a disk mount, and a tablet mounted with no agitation were the three selections employed in the present study.

In addition to the dissolution rate experiments additional independent data were necessary for examining Eqs. 2, 3, and 5. Thus suitable solubility, diffusion coefficient, viscosity, and density experiments were considered and carried out.

EXPERIMENTAL

Dissolution Rates—Dissolution rates were carried out in both a propeller-driven stirrer apparatus and a rotating disk apparatus.

[1] Other experiments indicate that this assumption is approximately valid. In order to vigorously account for any deviations due to nonconstancy, more complex equations must be used, but should not otherwise affect our present interpretation.

[2] Marketed as Tween 80 by Atlas Chemical Industries, Wilmington, Delaware.

Propeller-Driven Stirrer Apparatus—The drug powder (<70 mesh) was compressed into a tablet in a 9.5-mm. ($^3/_8$-in.) die at 2,400 lb. using a Carver press[3] so that the tablet surface was flush with the die surface. The back of the die was then sealed with a cork stopper. The die was mounted directly in a Plexiglas holder in a water-jacketed beaker containing 400 ml. of solvent at 30°. Stirring was achieved by a Teflon-coated stirrer attached to a Hurst synchronous motor[4] mounted on the beaker cover. The stirrer was positioned so that the blade in its closest proximity to the tablet surface was 6 mm. away. The Plexiglas sample holder was also mounted on the cover so that (*a*) all components could be simultaneously introduced into the solvent as an entire unit, and (*b*) the hydrodynamics could be reproduced and maintained constant. Samples were withdrawn at known time intervals and spectrophotometrically assayed on the Perkin-Elmer Hitachi spectrophotometer at 286 mμ. These studies were conducted under stirring speeds of 50 r.p.m. and 150 r.p.m.

Rotating Disk Apparatus—The dissolution studies with the rotating disk were carried out by using an aluminum assembly similar to the one previously reported by Wood *et al.* (8). The powder was compressed in a 1.1-cm. ($^7/_{64}$-in.) die using a Carver press in the same manner as described before. The apparatus was attached to a Bodine motor[5] and started before immersion into the solvent. A 3-L. beaker, containing 950 ml. of solvent was immersed in a water bath maintained at 30°. The rotating disk and the Bodine motor were mounted on a beaker cover as before.

The diameter of the rotating disk was 4.5 cm. and that of the beaker was 15.5 cm.; the apparatus was maintained 5 cm. above the bottom of the beaker. The dissolution runs were conducted over a period of 30 min. at 60 and 300 r.p.m.

For the runs at 0 r.p.m. a different method was employed. The tablet was compressed in the die and the apparatus immersed in the solvent for a known period of time. Sampling was accomplished by removing the apparatus entirely from the solvent, stirring the solution, and removing a known volume for assay. The surface of the tablet was lightly dabbed with tissue to remove adhering solvent and the rest of the apparatus was also quickly dried. The entire apparatus was then immersed in fresh solvent with as little agitation as possible and timing started from zero. Essentially, each time the apparatus was immersed, the tablet was considered to be a new one. The release rate as a function of time was obtained by successively immersing the tablet for proportionally longer periods of time. The validity of this technique was proven by the reproducibility of the dissolution rates for the same solvent system using different tablets with different immersion times, showing that the release rate was independent of the past history of the tablet.

Diffusion Coefficient[6]—The method employed to determine the diffusion coefficients is essentially similar to the one reported earlier (9). The diffusion cell was modified by using a Gelman Versapor[7] filter in place of the porous sintered-glass disk. Also, the apparatus was not water-jacketed so that the two halves could be separated and the filter mounted between them. One Teflon gasket was placed on either side of the filter and the joints were clamped together. A step, 0.6 mm. deep, was made in one of the Teflon gaskets so that the filter could be fitted into it. Once the apparatus was assembled, this procedure insured that leakage from the filter was completely prevented. The filter had a diameter of 37 mm., pore size of 0.9 μ, and a thickness of 0.635 mm.

The filter was initially soaked in a 50:50 ethanol–distilled water solution for 1 day and then in distilled water for 2 days, to remove all soluble components present. Diffusion experiments were then carried out by a similar procedure to that described in *Ref.* 9. Since the diffusion cell was not water-jacketed, it was immersed in a water bath maintained at 30°. Into one compartment of the diffusion cell the aqueous solution of benzocaine in polysorbate 80 was added and the rate of diffusion into the other compartment containing the same polysorbate 80 concentration was determined.

The Versapor filter is made of glass fibers reinforced with epoxy, and its inherent strength enabled it to be used repeatedly without any change in surface area (not true of several other filters tried). In order to check possible changes that might occur in the filter, a benzoic acid standard was run between every two diffusion coefficient determinations.

Other Parameters—Solubility measurements were made as reported earlier (9). The viscosities of all solutions used in this study were measured at 30° using the Ostwald viscometer. The densities of these solutions were measured using a pycnometer.

RESULTS

Dissolution rates of benzocaine were determined in water and five concentrations of polysorbate 80 using both the propeller-driven stirrer and the rotating disk methods. The dissolution rate data obtained from the propeller-driven stirrer apparatus at 50 r.p.m. and 150 r.p.m. and that obtained from the rotating disk apparatus at 60 r.p.m. and 300 r.p.m. were highly reproducible, as can be seen by a typical set of data obtained at 150 r.p.m., shown in Fig. 1. These dissolution rates are tabulated in Column 3 of Table I. Figure 2, illustrating the dissolution rates obtained without stirring, shows, as expected, a larger experimental fluctuation between the data points of each curve. The resulting slopes, however, were in good agreement with theory. The rotating disk dissolution rates are tabulated in Table II.

The data from the diffusion rate experiments are plotted in Fig. 3. It should be pointed out here that each diffusion experiment had a positive intercept (since sampling was started only after steady-state had been reached) which has been subtracted in order to pass all lines through the origin, permitting better comparison. This procedure does not alter the slope of the line, and only the desired steady-state slope is needed in the calculations (9). These diffusion coefficients, along with the respective solubilities and relative viscosities, are presented in Table III.

[3] Carver Laboratory Press, Model B, Fred S. Carver, Inc., Summit, New Jersey.
[4] Model CA, Hurst Mfg. Corp., Princeton, Indiana.
[5] Type KYC-23RB, Bodine Electric Co., Chicago, Illinois.
[6] In diffusion coefficient determination experiments of this type, there is some uncertainty arising from the assumption that there is an effective diffusion layer adjacent to the membrane. Thus it is assumed that through this apparent stagnant film only molecular diffusion is important. It is estimated from calculations that the effect of some corrective transport should be small compared to the uncertainty in the overall experiment.
[7] Gelman Instrument Co., Ann Arbor, Michigan.

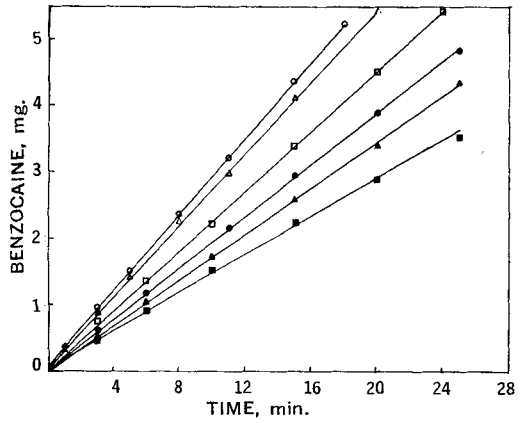

Fig. 1—Dissolution data for benzocaine in different concentrations of polysorbate 80 using the propeller-driven stirrer apparatus at a stirring speed of 150 r.p.m. Key: Polysorbate concn.—○, 6%; △, 4%; □, 2%; ●, 1%; ▲, 0.5%; ■, 0%.

Figure 4 illustrates the solubilization curve for benzocaine in polysorbate 80 solutions. The linearity obtained by plotting the amount solubilized against the concentration of polysorbate 80 suggests that the apparent individual micellar participation remains relatively constant over the entire range of surfactant concentrations.

Analysis of Data Obtained with Propeller-Driven Stirrer Apparatus—By using the definition for the effective diffusion coefficient, as given by Eq. 4, Eq. 2 can now be rewritten as

$$G = \frac{1}{h} D_{eff} C_T \qquad \text{(Eq. 6)}$$

where C_T is the total solubility. Similarly, Eq. 3 may be expressed as:

$$G = s^{1/2} D_{eff}^{1/2} C_T \qquad \text{(Eq. 7)}$$

The independently determined parameters listed in Table III can now be used to calculate the dissolution rates predicted by Eqs. 6 and 7 to test the diffusion layer and Danckwerts models, respectively. In order to eliminate the constants present in the two equations, the data were compared to the theories by taking the ratio of the dissolution rate in the surfactant solution to that in water.

Consequent to the above discussion, the final equation used to test the diffusion layer model is:

$$\frac{G'}{G_{H_2O}} = \frac{D_{eff} C_T}{D_1 C_1} \qquad \text{(Eq. 8)}$$

Similarly, for the Danckwerts model:

TABLE I—DISSOLUTION RATES (mg./min./cm.²) FOR THE BENZOCAINE–POLYSORBATE 80 SYSTEM USING THE PROPELLER-DRIVEN STIRRER APPARATUS

Polysorbate 80, % w/v	Stirrer Speed, r.p.m. 50	150
0	0.145	0.199
0.5	0.163	0.236
1.0	0.180	0.270
2.0	0.211	0.306
4.0	0.254	0.379
6.0	0.278	0.402

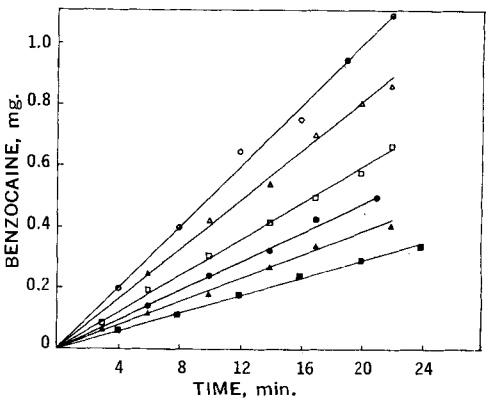

Fig. 2—Dissolution data for benzocaine in different concentrations of polysorbate 80 under nonstirring conditions. Key: polysorbate concn.—○, 6%; △, 4%; □, 2%; ●, 1%; ▲, 0.5%; ■, 0%.

TABLE II—DISSOLUTION RATES (mg./min./cm.²) FOR THE BENZOCAINE–POLYSORBATE 80 SYSTEM USING THE ROTATING DISK APPARATUS

Polysorbate 80, % w/v	Rotation Speed, r.p.m. 0	60	300	300/60
0	0.0151	0.105	0.223	2.12
0.5	0.0201	0.125	0.272	2.18
1.0	0.0242	0.145	0.312	2.14
2.0	0.0299	0.169	0.362	2.14
4.0	0.0415	0.199	0.415	2.08
6.0	0.0495	0.219	0.465	2.13

$$\frac{G'}{G_{H_2O}} = \left(\frac{D_{eff}}{D_1}\right)^{1/2} \frac{C_T}{C_1} \qquad \text{(Eq. 9)}$$

Here G' is the dissolution rate in the surfactant solution.

The theoretical ratio of rates predicted by Eqs. 8 and 9 have been drawn as smooth curves in Fig. 5. The graphical points illustrate the experimental ratio at both 50 r.p.m. and 150 r.p.m., as obtained from Table I. It can be readily seen that the diffusion layer theory gives a much closer fit than the Danckwerts model. Further examination of Fig. 5 reveals that the fit between the experimental data and the diffusion layer prediction is extremely good

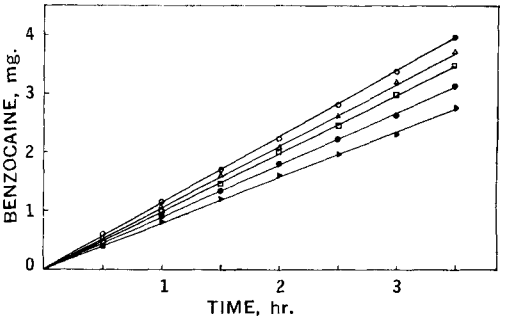

Fig. 3—Diffusion data for benzocaine in different concentrations of polysorbate 80. Key: Polysorbate concn.—○, 4%, 6%; △, 2%; □, 1%; ●, 0.5%; ▲, 0%.

TABLE III—PHYSICAL CONSTANTS DETERMINED FOR THE BENZOCAINE–POLYSORBATE 80 SYSTEM

Polysorbate 80, % w/v	C_T, Gm./ml. × 10^2	ν_{rel} Cps.	D ± σ[a] cm.²/sec. × 10^6
0	0.120	1.00	9.86 ± 0.19
0.5	0.184	1.04	7.61 ± 0.46
1.0	0.247	1.04	6.28 ± 0.33
2.0	0.371	1.06	4.53 ± 0.19
4.0	0.580	1.20	3.15 ± 0.39
6.0	0.810	1.35	2.31 ± 0.20

[a] The standard deviation was estimated to be 0.30, determined by considering all 48 experimental values.

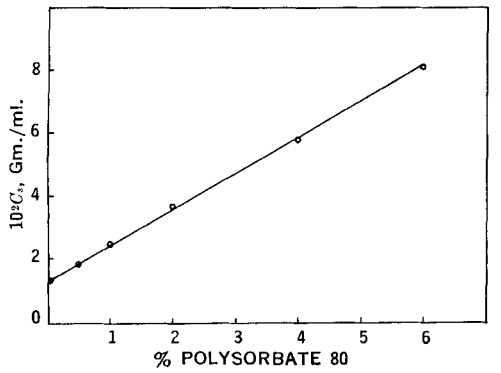

Fig. 4—Solubilization data for benzocaine in different concentrations of polysorbate 80.

at the lower concentrations of polysorbate 80. It was felt that the deviations of the data from the theory at the higher concentrations of polysorbate 80 may be due to some nonlaminar hydrodynamic condition near the tablet surface. In order to test this, a Plexiglas plate was attached to the front of the tablet surface at a distance of about 2 mm. This would ensure laminar solvent flow past the tablet surface. However, the dissolution rate ratios under these conditions were not significantly changed from those without the plate

Analysis of Data Obtained with No Agitation—In Fig. 6, a comparison of the theoretical curves has been made with the experimental dissolution rate data obtained by the rotating disk assembly un-

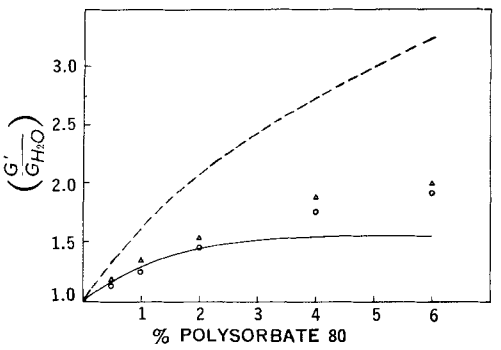

Fig. 5—Comparison of the theoretical ratio of dissolution rates as predicted by Eqs. 8 and 9 (smooth curves) and the experimental ratios obtained from the propeller-driven stirrer apparatus. Key: 0, 50 r.p.m.; △, 150 r.p.m.; – – –, Danckwerts model; —, diffusion layer model.

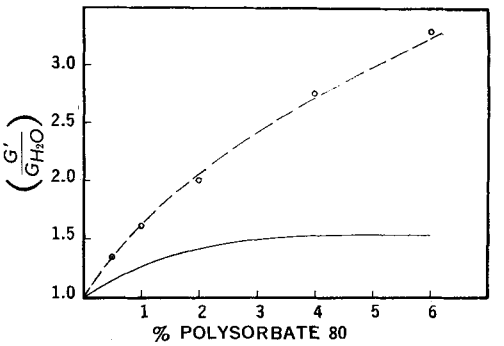

Fig. 6—Comparison of the theoretical ratio of dissolution rates as predicted by Eqs. 8 and 9 (smooth curves) and the experimental ratio obtained under nonstirring conditions. Key: – – –, Danckwerts model; ——, diffusion layer model.

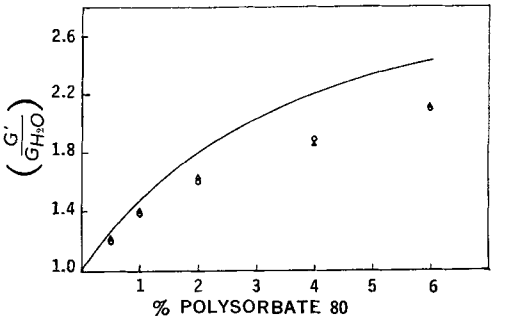

Fig. 7—Comparison of the theoretical ratio of dissolution rates as predicted by Eq. 10 (smooth curve) and the experimental ratios obtained from the rotating disk apparatus. Key: 0, 60 r.p.m.; △, 300 r.p.m.

der nonstirring conditions (see Column 2 of Table II). Here it is evident that the experimental data no longer fit the diffusion layer model but show excellent agreement with the Danckwerts model. It is of interest to point out that Gibaldi *et al.* (10) in their studies of the dissolution rates of benzoic acid in polyoxyethylene ether[8] solution have shown that under no forced convection conditions their data fit the Danckwerts predictions more closely than under stirring conditions.

Analysis of the Rotating Disk Data—Following the same general procedure as before, the fit of the rotating disk theory with the experimental dissolution data can be made by taking ratios of the dissolution rates. Then, under identical conditions of agitation, Eq. 5 becomes

$$\frac{G'}{G_{H_2O}} = \left(\frac{D_{eff}}{D_{H_2O}}\right)^{2/3} \frac{C_T}{C_1} \frac{1}{\nu_{rel}^{1/6}} \qquad \text{(Eq. 10)}$$

The theoretically predicted ratios of rates from Eq. 10 have been drawn as a smooth curve in Fig. 7. While it can be seen that the data are in approximate agreement with theory, the deviations observed may be significant.

DISCUSSION OF THE RESULTS

A further examination of the data with the theories can be made in the following manner. Since the

[8] Marketed as Brij, Atlas Chemical Industries, Wilmington, Del.

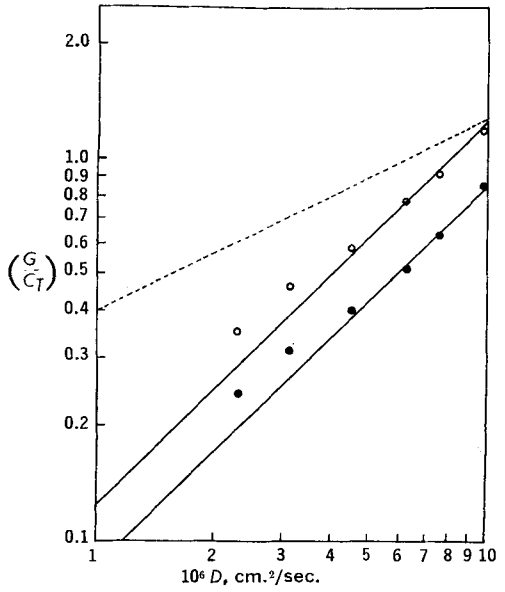

Fig. 8—Comparison of the theoretical slopes of 1.0 as predicted by Eq. 6 and the theoretical slope of 0.5 as predicted by Eq. 7 with experimental data. Key: - - -, 0.5; —, 1.0; ○, 150 r.p.m.; ●, 50 r.p.m.

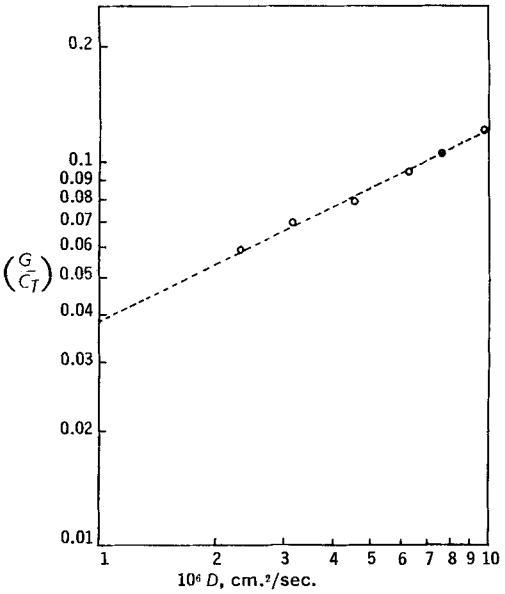

Fig. 9—Comparison of the theoretical slope of 0.5, 0 r.p.m., as predicted by Eq. 7 with experimental data.

main point of difference between these theories is in the exponent of the diffusion coefficient, a more stringent test can be made by plotting log (G/C_T) against log D_{eff}. By this means, the slope of the line in each case would be equal to the critical exponent, and would be a direct indication of the validity of the theory.

In Fig. 8, a theoretical line of slope = 0.5 (as predicted by the Danckwerts theory), and two theoretical lines of slope = 1.0 (as predicted by the diffusion layer model) have been drawn. The experimental data points for surfactant concentrations up to 2% (from Tables I and III) appear to fit a slope of one, which is in good agreement with the diffusion layer theory. The 4.0 and 6.0% polysorbate 80 points deviate from theory. Further studies are needed before an unambiguous reason for the deviations can be presented. However, it can be seen from Table III that the viscosities of the polysorbate 80 solutions begin to increase appreciably at 4.0%. As it has been assumed that viscosity changes are unimportant in comparing Eqs. 8 and 9 with data, the deviations of the 4.0 and 6.0% data from the diffusion layer theory may reside here.

Despite the deviations exhibited by the 4.0 and 6.0% polysorbate 80 data, the plot clearly shows the diffusion layer model best describing the situation with the propeller-driven stirrer apparatus. A least squares analysis was done on the data for both sets of experimental points. For the 50 r.p.m. data, by considering only the data up to 2.0% polysorbate 80, the slope of the line was calculated to be 0.965, whereas for the 150 r.p.m. data, the corresponding slope was 0.890. However, by considering all the data (up to 6.0% polysorbate 80), the slopes were 0.838 and 0.811, respectively.

A similar plot (Fig. 9) for the nonstirring dissolution rate experiment (from Tables II and III) shows that the experimental data give a slope of 0.5. This shows that the Danckwerts predicted dependence on the square root of the diffusion coefficient is applicable under conditions of no agitation.

Finally, a plot of log $G_\nu^{1/6}/C_T$ against log D_{eff} can be made for a test of the rotating disk theory (from Tables II and III). Figure 10 shows that the data for the 60 r.p.m. and 300 r.p.m. are in fair agreement with the predicted slope of 0.667, although somewhat higher—0.722 and 0.736, respectively. More work is needed to establish whether or not this

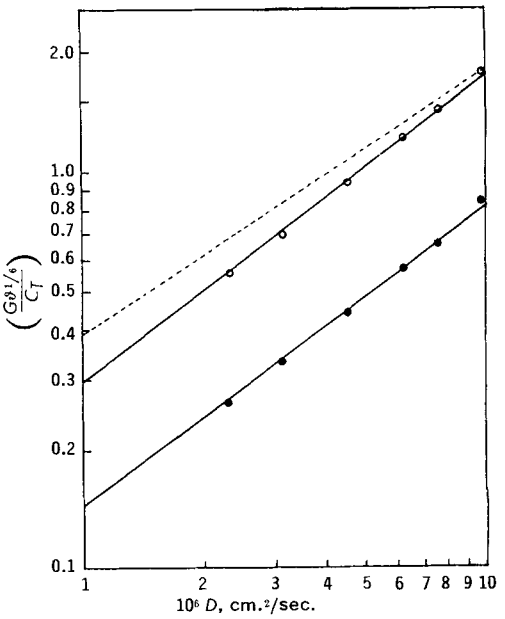

Fig. 10—Comparison of the theoretical slope of 0.667 as predicted by Eq. 5 with experimental data: Key: - - -, slope = 0.667; —○, slope = 0.736, 300 r.p.m.; —●, slope = 0.722, 60 r.p.m.

difference is really a breakdown of the theory. It is noteworthy that the stirring rate dependence obtained with the rotating disk apparatus (Column 5 of Table II) is very close to the theoretical square root prediction of 2.23.

PHARMACEUTICAL SIGNIFICANCE

These studies provide a firm quantitative basis for studying and establishing the particular mechanism of mass transport which is controlling a given dissolution experiment. The need for explicitly describing the hydrodynamics, under which such dissolution takes place also has been shown to be of prime importance. Although a limited number of examples were utilized, it is apparant that large differences in mechanisms may exist among different dissolution processes.

In view of the widespread interest in the use of dissolution studies as a basis for comparison of release profiles of different dosage forms and the resultant need for meaningful comparison of dissolution studies of independent workers, it becomes increasingly apparent that experimental conditions must be ei her standardized or taken into consideration. In addition the results of this study clearly indicate that the use of *in vitro* dissolution rates as a basis of drug availability must consider and account for the differences in the environmental conditions which exist in the respective *in vitro* and *in vivo* systems. As an example, it is very possible that an *in vitro* test in which the diffusion layer model may be operative could be used to draw conclusions regarding an *in vivo* system in which the Danckwerts model may be functioning. Such a possible system may be drug absorption when the rate of diffusion in the intestinal lumen is rate controlling.

These results also demonstrated that the effective diffusion coefficient can be radically altered with changes in components of the transport medium. Depending upon the kind and the intensity of the agitation, the dependence upon the effective diffusion coefficient may range from a one-half power to a first power. The conception of increased apparent solubility leading to an increased dissolution rate can be thus seen to be faulty if overextended. As a matter of fact, recent dissolution studies by Parrott (11) showed that dissolution rates did not increase linearly with an increased solubility and may be explained on the basis of a reduced effective diffusion coefficient.

Various biopharmaceutical transport systems which have been studied involve species of widely differing sizes, *viz.* molecules, micelles, colloidal particles, emulsion droplets, *etc.* In such situations the effective diffusion coefficient may range over several orders of magnitude and the importance of understanding the predominant transport mechanism becomes apparent.

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Keyphrases

Dissolution rates—solid drugs
Micellar solubilization effect—dissolution rate
Stirrer, propeller-driven—dissolution apparatus
Rotating disk—dissolution apparatus
Diffusion coefficients

Quantitative Determination of Volatile Components in Pressurized Aerosols by Gas Chromatography

By SHELDON COHEN

Gas chromatography has been used for analysis of aerosol propellants and active ingredients. The propellant analysis generally involved introduction of a previously vaporized sample into the gas chromatograph. Such methods become impractical when dealing with small aerosol packages such as those used for inhalation therapy. The purpose of this work was to develop methods for the accurate sampling and analysis of the propellants and cosolvents of such packages. These methods may also be extended to other volatile components of aerosol packages. Two sampling techniques were developed. These include the use of gas-tight sampling devices to either prepare solutions of the aerosol contents or to load a small-volume, high-pressure, liquid syringe. The volatile components were separated on a porous polystyrene bead column and the composition was calculated from peak areas.

PRESSURIZED AEROSOLS used for inhalation therapy generally are packaged in small containers fitted with metered valves. Accurate sampling of such packages and analysis of the volatile constituents, contained therein, are difficult, since the composition of a small volume of such mixtures in the liquid phase can change considerably by fractionating as it volatilizes. The aerosols may contain a wide boiling range of solvents, from propellant 12, b.p. −29.8°, to some of the glycols with boiling points in excess of 200°. To further complicate matters, the metered valve does not provide a convenient access to sample for such analyses.

Prior to the development of gas chromatography, aerosol propellants were analyzed by several methods such as mass spectrometry, infrared spectrophotometry, liquid density measurements, and vapor pressure determinations (1). Gas chromatography has been utilized for the past decade for the analysis of halogenated hydrocarbons such as used in aerosols (1, 2, 4, 7) and for the analysis of other ingredients in pharmaceutical aerosols (3, 6).

Percival (1) and Thonet (7) used variations of gas sampling systems to obtain samples of aerosol propellants in the vapor state prior to introduction into the gas chromatograph. The components were subsequently separated on di-*n*-octyl phthalate or alumina columns in the former work and on a di-*n*-butyl maleate column in the latter. The use of metered valves makes these methods impractical. There is also the possibility of condensation of the higher boiling constituents in the vapor sampling system and absorption of propellants by rubber "o" rings normally found in gas sampling valves.

Lysyj (4) used a parallel dual column system to analyze a mixture of air, carbon dioxide, propellant 11, propellant 12, and ethylene oxide used in a sterilizing process. The high boiling constituents were separated on a polyglycol column and the low boiling constituents on a dibenzyl ether column. Separate detectors were used to monitor each column simultaneously. The system was intended primarily as a rapid in-process control of a mixture in the gas phase.

Jenkins and Amburgey (2) chilled aerosol samples and dissolved them in large volumes of solvent. They used 0.5–0.8 Gm. of aerosol in 10 ml. of cyclohexane. The components were then separated on a decyl phthalate column. Such a system runs the risk of sample evaporation and fractionation during handling. If water is to be determined there is also the possibility of atmospheric water vapor condensing in the sample.

The methods, to be described here, were developed for the accurate sampling and analysis of the volatile constituents of small pressurized packages used in inhalation therapy. These methods take advantage of some of the newer hardware for handling samples and of porous polystyrene beads as column packing (5) for improved separations of aerosol components.

EXPERIMENTAL

Equipment—An F&M model 810-12 gas chromatograph using a thermal conductivity detector was used. The columns used were Porapak types Q, N, R, and S, 80/100 mesh packed in 2.5 m. (8 ft.) long, 5 mm. ($^3/_{16}$-in.) stainless steel columns. The column temperature was programmed starting at 120° and holding for 1 min. and then heating at 15°/min. to 240°, and holding as needed. The injection block temperature was 210°, and the de-

Received December 4, 1967, from the Riker Laboratories, Northridge, CA 91324

Accepted for publication March 19, 1968.

Presented at the Third Annual Western Regional Meeting of the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Los Angeles, Calif., October 1967.

The author wishes to thank Mr. T. H. Graves for his able assistance in preparing this manuscript.

tector temperature was 190°. The carrier gas used was helium at a flow rate of 65 ml./min. A 1-mv. Servo recorder equipped with a disk integrator was used.

Hamilton type 701N 10-μl. syringes were used to inject samples into the gas chromatograph in the solution method. A Pressure-Flo high-pressure liquid syringe (Precision Sampling Corp.) of 10 μl. capacity was used to inject samples in the direct method. The gas-tight syringes were Pressure-Lok (Precision Sampling Corp.). These come equipped with valves for containing pressurized samples and were used to prepare sample and standard solutions, and for loading the Pressure-Flo syringe. Sizes convenient for the amount of material being transferred should be used. (High-pressure materials are more easily handled in smaller syringes.) Plastic-coated aerosol bottles that could be fitted with crimp-on type multiple-dose stoppers were used as sample and standard solution containers.

Sampling—A commercial sampling system (Alltek Associates) was used for samples in metal containers. In this system a threaded plate is affixed to the metal container with hose clamps. Smaller hose clamps were substituted for those supplied with the system in order to accommodate the smaller containers. A threaded fitting, closed at one end with a replaceable rubber septum is engaged in the plate threads. The end of the fitting towards the container had a sharpened tube capable of puncturing the container and a rubber gasket around this tube to prevent leakage from the container after puncture. The fitting is twisted into the threads, thus puncturing the can and affixing to it the penetrable rubber septum, through which samples may be withdrawn with gas-tight syringes. In order to prevent septum rupture, it was found advisable to use a septum-retaining nut with an aperture smaller than that of the retaining nut supplied with the system.

For samples in glass containers, a sampling system (Fig. 1) was constructed that enabled the cap to be punctured with an 18-gauge hypodermic needle (C). Leakage at the puncture is prevented by a rubber washer (D). The hub of the needle is fitted with a small rubber septum (B) held in place by a male Luer-Lok plug (A) through which a 1.6 mm. ($^1/_{16}$-in.) hole has been drilled. The sample may then be withdrawn through this septum with a gas-tight syringe.

Samples that do not lend themselves to the use of these devices may be cooled to a temperature sufficiently low to minimize loss and then opened and transferred to a plastic-coated aerosol bottle cooled to the temperature of the sample. The bottle is immediately sealed with a multiple-dose cap through which a sample may be withdrawn with a gas-tight syringe. This operation is conveniently carried out inside an open chest-type freezer to minimize sample loss and moisture condensation. This last method runs a greater risk of sample change than the previous two.

Care must be taken to assure that a minimum change in composition is caused by the sampling procedure. Since the composition of the aerosol will change as it volatilizes, the system must be leak proof, a minimum volume of space should be added by the sampling system to the container, and the volume of space left by withdrawn samples must be considered.

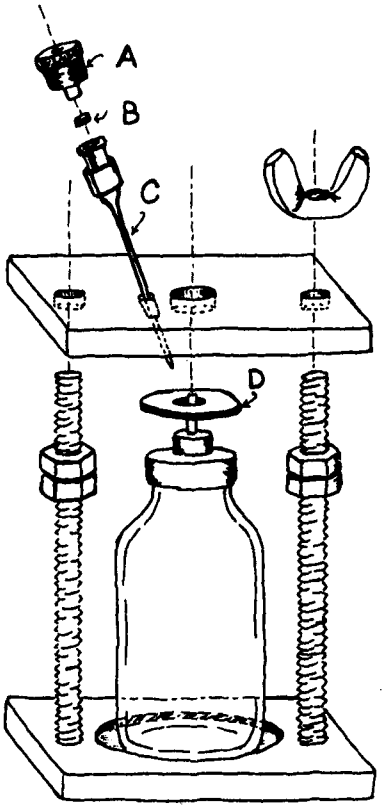

Fig. 1—Sampling system for glass containers.

Solution Method of Analysis—Ethylene glycol monomethyl ether, chloroform, or methylene chloride are suitable solvents for most samples. The choice may be determined by considering the retention time of the solvent relative to those of the aerosol components and by considering the other materials present in the solvent. For example, chloroform normally contains ethanol as a preservative and ethylene glycol monomethyl ether normally contains some water. Ethylene glycol monomethyl ether was found most convenient to use.

Twenty-five milliliters of the solvent is pipeted into a 60-ml. plastic-coated aerosol bottle. A multiple-dose cap is crimped in place and its safety seal removed. The 60-ml. bottle and its contents are weighed; 1.25–2.50 Gm. of the aerosol sample is drawn into a 2-ml. gas-tight syringe from the sample container, which has been prepared by one of the methods described under *Sampling*, and the syringe valve is shut. The sample is transferred from the gas-tight syringe, through the septum, into the tared 60-ml. bottle, the gross weight of the bottle is determined, and the weight of sample taken is calculated.

The standards are prepared in the same manner as the sample. All standard materials that boil near or below room temperature are handled in gas-tight syringes. Less volatile materials can be handled in ordinary syringes. Propellants for the standard solution may be stored in pressurized cylinders and transferred from an inverted cylinder to a gas-tight syringe through a rubber septum

fitted to the cylinder outlet. A 9.6 mm. (3/8 in.) diameter rubber septum can be fitted to 6.4 mm. (1/4-in.) flared tube fitting such as normally found on such cylinders. Alternately the propellants may be stored in the plastic-coated aerosol bottles sealed with multiple-dose caps. One solution may contain several standard components of known amounts. When preparing such a solution the least volatile materials should be added first. It is important that the standard solution contains the identical volume of solvent as the sample solution contains.

The sample solution, the standard solution, and several Hamilton syringes are placed in a freezer at −20°. Just prior to use, a solution and syringe are removed from the freezer, 5–8 μl. of the solution is immediately loaded into the syringe and injected into the gas chromatograph. The recorder chart drive is turned on and the temperature program is initiated simultaneously with the injection. The chromatograph is attenuated for maximum response on scale for each peak. Each component in the sample solution is run at the same attenuation as in the standard solution. A chromatogram of the solvent is run in the same manner as the other solutions. Care should be exercised to avoid bubbling the solutions in the Hamilton syringes. The injections should be made in a reproducible manner. Figure 2 illustrates a chromatogram obtained by this method.

From the integrator record the areas of each component of the sample solution and the standard solution are determined. The attenuation factor can be ignored for any component as long as it is the same in each chromatogram. The areas are corrected geometrically for any base line or integrator drift. The ratios of the area of each component to that of the solvent are calculated. These ratios are corrected if necessary for the blank solvent. For each individual component in the sample:

$$\% \text{ component (w/w)} = \frac{100\ (R_A)(W_S)}{(R_S)(SW)}$$

where, R_A = ratio of the area of the component peak to that of the solvent peak in the sample solution, R_S = above ratio in the standard solution, W_S = grams of component in the standard solution, SW = sample weight (Gm.) of the aerosol in the sample solution.

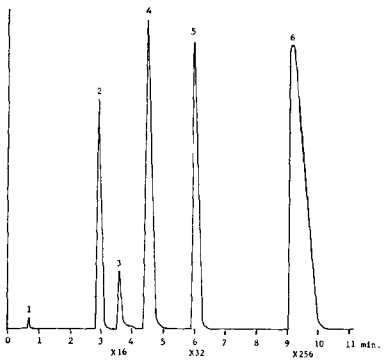

Fig. 2—Chromatogram of aerosol components on a Porapak R column. 1, air; 2, propellant 12; 3, water; 4, propellant 114; 5, ethanol; 6, ethylene glycol monomethyl ether.

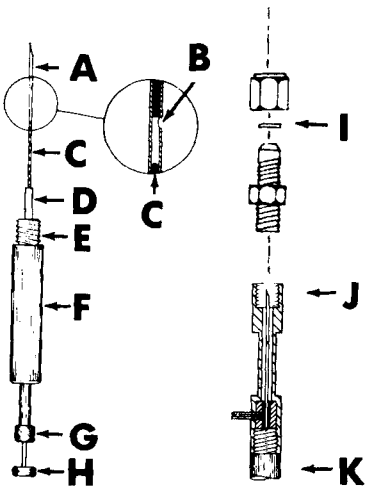

Fig. 3—Pressure-Flo syringe system.

Direct Method of Analysis—Samples analyzed by this method should be limited to those that will not impair the performance of the Pressure-Flo syringe with any residue. Using a 6.4-mm. flared tube to 3.2-mm. (1/8-in.)M.P.T. adapter (Fig. 3) a 6.9-mm. diameter rubber septum (I) is fitted to the sampling end (J) of the filling adapter supplied with the syringe. The retaining nut holding the septum in place should have a reduced aperture to prevent septum rupture. The Pressure-Flo syringe is fitted to the other end (K) of the adapter. The sample is injected through the rubber septum, into the adapter with a gas-tight syringe, preferably of 1.0 ml. capacity, Manual pressure is maintained on the plunger of the gas-tight syringe throughout the filling operation. The manufacturer recommends 25 p.s.i. pressure in excess of the sample vapor pressure to assure a reproducible sample. Unless elaborate equipment is available for determining this, the best approach is a few trial runs to develop an operator feel for the amount of pressure necessary.

The plunger handle (H) of the Pressure-Flo syringe is slowly pumped several times and left in the withdrawn position. This retracts the plunger (C) and allows the sample to enter the hollow needle through the port (B). If a volume of less than 10 μl. is desired using the 10-μl. syringe, a shim can be made so that the plunger is left in a reproducible partially withdrawn position. The needle handle (G) is now withdrawn, drawing the needle (A) into the sealing tube (D), thus entrapping the sample. The Pressure-Flo syringe is then removed from the adapter and a small Teflon sleeve is slipped over the sealing tube (D). The sleeve prevents the sealing tube from puncturing the injector septum of the chromatograph. The sealing tube should protrude slightly beyond the Teflon sleeve, enabling it to slightly penetrate the injector septum. The sealing tube is firmly butted against the injector septum and the needle handle (G) is rapidly depressed, driving the needle through the injector septum and allowing the sample to vaporize in the injection port. The plunger handle (H) is immediately depressed, pumped several times, and left in the depressed position to remove any residual vapor from the hollow needle. The needle handle (G) is with-

drawn, disengaging the syringe from the septum. The recorder chart drive is turned on and the temperature program is initiated simultaneously with the injection. The chromatogram is attenuated for maximum response, on scale, for each peak.

A mixture of the components found in the sample is accurately prepared. The composition should approximate that of the sample. This is done by weighing the components, in order of increasing volatility, into an evacuated plastic-coated aerosol bottle sealed with a multiple-dose cap. Gas-tight syringes are used where needed. To prevent composition changes in the standard mixture, the head space in the container should initially be limited to less than 10% of the liquid volume and should not exceed 40% as the standard mixture is used. This should prevent changes in composition from exceeding 0.5% relative. The standard mixture is treated in the same manner as the sample. The chromatogram is run at the same attenuations used for each component in the sample. Figure 4 illustrates a chromatogram obtained by this method.

From the chromatogram of the standard mixture, the relative detector response for each component is determined as compared to one arbitrarily selected for reference (that is, relative detector response = unity).

$$R = \frac{A/C}{A_r/C_r}$$

where, R = relative detector response for a given component, A = peak area for the component in the standard, C = percent composition of the component in the standard, A_r = peak area of the reference component in the standard, C_r = percent composition of the reference component in the standard. Then for each component in the sample:

$$\% \text{ composition} = 100\,\frac{A/R}{S}$$

where, S = sum of the ratios, A/R, for all the components in the sample. This calculation provides the composition in terms of the percent of the volatile portion of the sample.

DISCUSSION

Because of limitations of the Pressure-Flo syringe, only highly volatile systems could presently be analyzed by the direct method. The direct method offers applicability for rapid analysis and process control of systems which contain components of known identity. Samples containing suspended solids may be analyzed by the direct method if they can be filtered through a Swiney adapter from a gas-tight syringe. If extensive pretreatment is required, it may be better to use the solution method. The solution method offers general applicability for analysis of systems of unknown components having a wide boiling range. Nonvolatile solids in the sample would not be expected to interfere with the solution method, however, these would accumulate in the injection block of the gas chromatograph. This should be cleaned out at intervals, frequent enough to prevent interferences with the gas chromatograms due to adsorption and decomposition. The experience in this laboratory with ingredients such as epinephrine, isoproterenol, and phenylephrine has shown no complications with these when the injection block was cleaned at intervals of several days.

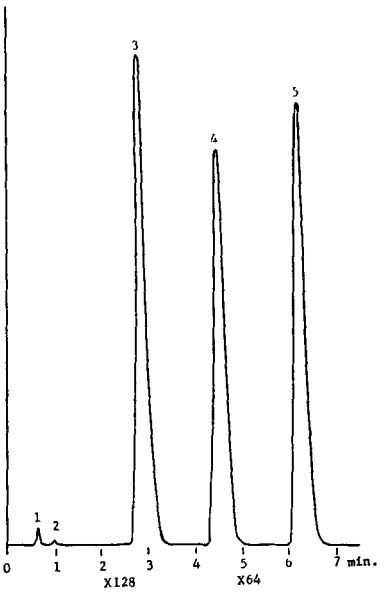

Fig. 4—Chromatogram of aerosol components on a Porapak Q column. 1, air; 2, water; 3–5 propellants 12, 114, and 11, respectively.

Both methods give results of comparable accuracy ±0.5% for most components in a multicomponent analysis. The results for several accurately prepared samples are given in Table I. Ethanol was found to give slightly higher errors up to +0.9%. When greater accuracy is desired for a selected component, it may be obtained by altering the system

TABLE I—ANALYSIS OF AEROSOL COMPONENTS

Components	% Composition (w/w) Taken	Found	Discrepancy
Solution Method			
Propellant 12	48.6 (50.6)	48.3	−0.3
Propellant 114	24.9 (24.1)	25.3	+0.4
Propellant 11	26.5 (25.2)	26.6	+0.1
Total	100.0	100.2	+0.2
Propellant 12	50.4	49.9	−0.5
Propellant 114	24.5	24.9	+0.4
Propellant 11	25.1	25.3	+0.2
Total	100.0	100.1	+0.1
Propellant 12	20.6	20.1	−0.5
Propellant 114	44.8	45.4	+0.6
Ethanol	32.6	33.5	+0.9
Water	2.0	2.1	+0.1
Total	100.0	101.1	+1.1
Propellant 12	20.6	20.1	−0.5
Propellant 114	44.9	44.9	0.0
Ethanol	32.5	33.2	+0.7
Water	2.0	2.1	+0.1
Total	100.0	100.3	+0.3
Direct Method			
Propellant 12	50.3	50.7	+0.4
Propellant 114	24.3	24.0	−0.3
Propellant 11	25.4	25.3	−0.1
Total	100.0	100.0	0.0

of analysis to obtain greater response and resolution for the selected component. The conditions for analysis of multicomponent systems are often necessarily compromises.

The parenthetical values given for the composition of the first example represent the actual amounts taken. Twenty grams of this solution was in a container that had 61 ml. of head space. The actual composition of the solution then in the container was calculated from physical data of the components.

The relative retention times (to that of propellant 11) for some of the components studied are given in Table II for several Porapak columns. The dimensions of the columns, temperature program, and other parameters are those described in the experimental portion. The values for the propellants are essentially the same for all columns. The values for the other components, which are more polar, vary with the columns. A column can be selected that will completely resolve most systems encountered. With a completely unknown system, the Type S column is useful, the only difficulty encountered being a very slight overlap of the propellant 12 and the water peaks. Type Q columns will not resolve propellant 114 and ethanol. Type R columns will only partially resolve propellant 11 and ethanol; type N columns will only barely resolve propellant 11 and ethanol and will also have a very slight overlap of the water and the propellant 114 peaks.

Other overlaps may be encountered if the column is overloaded causing broadening of a peak. The Porapak columns have very limited capacity and will display overloading by a lessening of the peak retention time and increased band width.

Table II—Relative Retention Times of Aerosol Components

Porapak Type	Q	R	S	N
Propellant 12	0.44	0.41	0.46	0.47
Propellant 114	0.74	0.69	0.74	0.74
Propellant 11	1.00	1.00	1.00	1.00
Propellant 113	1.25	1.26	1.25	1.21
Ethanol	0.74	0.96	0.89	1.03
Water	0.18	0.52	0.34	0.60
Ethylene glycol monomethyl ether	1.35	1.54	1.43	1.61

SUMMARY

Gas chromatography has been the method of choice in recent years for the analysis of aerosol propellants. The advent of the porous polystyrene beads has improved the separation and accuracy of these analyses. The methods presented here provide accurate and convenient sampling and analysis of small aerosol packaged pharmaceuticals. These methods may be extended to other sizes and products.

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Keyphrases

Aerosols—volatile components, analysis
Propellants—aerosol analysis
Diagram—sampling apparatus
Sampling techniques—aerosols
GLC—analysis

Bronchodilatory and Cardiovascular Effects of Metaproterenol

By L. DIAMOND*

Until recently, the methods which were available for evaluating bronchodilator drugs in the intact animal did not yield quantitative data which were primarily related to the smooth muscle tone of the airways. In the present study a technique was employed which yielded direct measurements of the total lung resistance to airflow. Since pulmonary resistance values depend directly on the dimensions of the airways, their magnitude is very sensitive to changes in the smooth muscle tone of the tracheobronchial tree. Metaproterenol [1-(3,5-dihydroxyphenyl)-2-isopropyl-aminoethanol sulfate], a new beta-adrenergic stimulant, inhibited the increase in total lung resistance evoked by histamine aerosol. The drug was effective in anesthetized, spontaneously breathing dogs when administered as a 2 percent aerosolized solution. A series of experiments was designed to compare the cardiovascular effects which followed inhalation of a therapeutic dose of metaproterenol with those which occurred following a similar dose of isoproterenol in anesthetized dogs. The positive chronotropic effect and the increase in pulse pressure were more pronounced after metaproterenol inhalation than after isoproterenol inhalation. However, the hypotensive effect of isoproterenol was somewhat greater than that of metaproterenol.

DRUGS CAPABLE of dilating the smooth musculature of the tracheobronchial tree have been employed extensively in the symptomatic treatment of obstructive diseases of the lungs. In recent years, oral inhalation has become a preferred means of administering bronchodilator drugs. Bronchospasmolytic agents administered *via* this route can provide rapid relief from acute episodes of bronchospasm.

The sympathomimetic amine, isoproterenol, is probably the most popular drug in use today for the management of asthma, emphysema, and related conditions. However, whether inhaled or given by some other route, isoproterenol has a limited duration of action and with repeated administration various undesirable systemic effects may develop, such as palpitations, tachycardia, hypotension, and central nervous system stimulation.

A new compound, purported to be as potent a bronchodilator as isoproterenol, but possessing a longer duration of action and producing fewer and milder side effects, has recently been made available. The new compound, metaproterenol,[1] differs chemically from isoproterenol by having the hydroxyl groups on the phenyl nucleus in the *meta* position. The general pharmacodynamic properties of metaproterenol were first studied by Engelhardt *et al.* (1) in 1961.

The method used by the latter workers, as well as many other investigators to assess the bronchodilatory activity of a compound in the intact animal, measures changes in the capacity of the respiratory tract as expressed by an increase or decrease in the volume of air taken up by the lungs under constant pressure inflation (2). This technique provides only a qualitative indication of the bronchiolar resistance. Moreover, it has been shown that at the usual frequencies of respiration this method is sensitive to changes in lung compliance but relatively less sensitive to changes in lung resistance (3).

The purpose of the present study was to obtain quantitative data relating to the bronchodilating efficacy of metaproterenol by utilizing direct determinations of the total lung resistance to airflow. A method similar to the one employed in this study has been previously used for the pharmacological evaluation of bronchodilator drugs by Familiar *et al.* (4). Since pulmonary resistance values depend directly on the caliber of the airways, their magnitude is very sensitive to changes in the smooth muscle tone of the tracheobronchial tree. The cardiovascular side effects which followed inhalation of a therapeutic dose of metaproterenol were also studied and compared with those which occurred after inhalation of a similar dose of isoproterenol.

EXPERIMENTAL

Ten mongrel dogs of either sex were anesthetized with an intraperitoneal injection of a solution containing 100 mg. of allobarbital, 400 mg. of urethan, and 400 mg. of monoethylurea in each ml. (0.6 ml./Kg.). The dogs were suitably restrained upon a surgical table and allowed to breathe spontaneously. A Murphy endotracheal tube was inserted into the trachea and secured there by inflation of its cuff. A

Received February 8, 1968, from the Department of Pharmacology, School of Pharmacy, University of Maryland, Baltimore, MD 21201

Accepted for publication March 8, 1968.

Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Miami meeting, May 1968.

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[1] Alupent, 1-(3,5-dihydroxyphenyl)-2-isopropyl-aminoethanol sulfate, brand of metaproterenol sulfate, Geigy Pharmaceuticals, Ardsley, N. Y.

cannula, consisting of an 18-gauge (3.8 cm., 1.5 in.) hypodermic needle attached to a length of polyethylene tubing, was introduced through one of the intercostal spaces into the intrapleural space. The intrapleural cannula was connected to one port of a Sanborn differential pressure transducer. A polyethylene tube leading from the endotracheal tube was connected to the other port of the transducer. This procedure permitted the pressure at the mouth to be subtracted from the intrapleural pressure to provide values for the transpulmonary pressure. A Sanborn pneumotachograph and differential strain gauge were used to measure the flow of air into and out of the lungs. A volume signal was obtained by electrical integration of the flow signal. The three parameters of pressure, flow, and volume were simultaneously recorded on a Sanborn direct-writing oscillograph.

Pulmonary Mechanics—The total lung resistance to airflow was calculated by selecting points along the ascending and descending limbs of the tidal volume record which represented points of equal inflation and deflation. Vertical lines were then drawn from these points through the pressure and flow curves. The difference in pressure and flow between the two points provided the data for the equation: pulmonary resistance = pressure difference in cm. H_2O/flow difference in L./sec. This method provides values which represent the average inspiratory and expiratory resistance near peak inspiratory and expiratory flows. Although the values thus obtained do not express the change of resistance with flow rate, they do closely approximate the average resistance during the respiratory cycle (5).

The compliance of the lungs was calculated from the following formula: pulmonary compliance = volume change in ml./pressure change in cm. H_2O. The necessary data were obtained by measuring volume and pressure changes at corresponding points of no airflow (beginning and end of inspiration) as seen from the flow-rate curve. The details of these methods have been described in an earlier report from this laboratory (6).

Evaluation of Bronchodilating Activity of Metaproterenol—Approximately 1 ml. of a 2% solution of histamine base was placed in a DeVilbiss No. 42 nebulizer which was modified with connectors and placed between the endotracheal tube and the pneumotachograph screen (Fig. 1). Control conditions were recorded and then histamine was administered by squeezing the hand bulb of the nebulizer at the outset of five consecutive inspirations. Immediately thereafter data for pulmonary mechanics calculations were recorded at 30-sec. to 2-min. intervals for a period of 5 min.

After allowing adequate time for the animals to recover from the bronchoconstricting effects of histamine, 10 inhalations of a 2% metaproterenol sulfate solution were administered. Two minutes were allowed for the drug to exert its activity after which time the respiratory patterns of the animals were again recorded. Histamine inhalation was then repeated, and the protection afforded by metaproterenol was evaluated by again measuring changes in pulmonary mechanical values over a 5-min. period.

Comparison of Cardiovascular Side Effects of Isoproterenol and Metaproterenol—A test group of 10 dogs was used to study and compare the cardiovascular side effects produced by inhalation of either isoproterenol or metaproterenol. Systemic blood

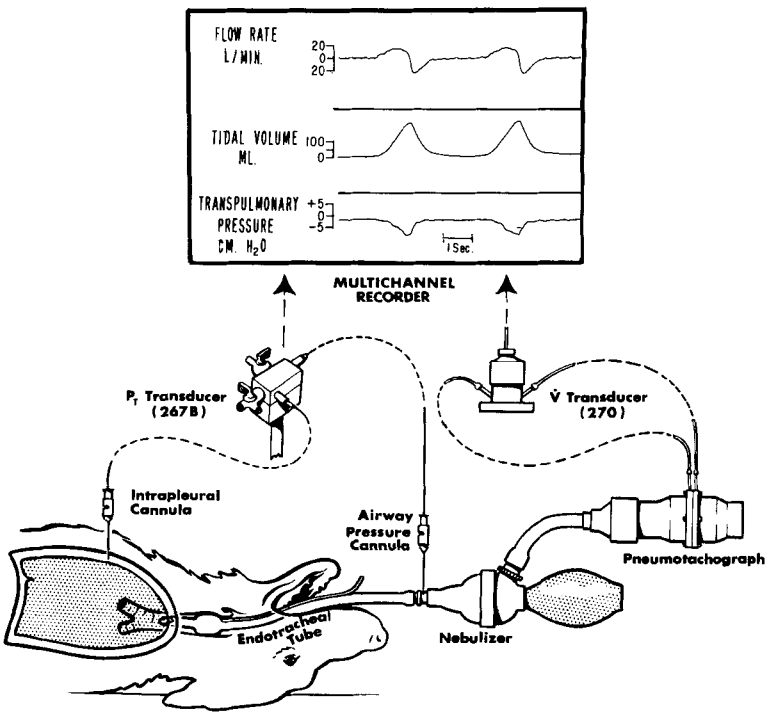

Fig. 1—Apparatus for administration of drug solutions and measurement of pulmonary mechanics.

pressure was measured from the abdominal aorta *via* the left femoral artery using a polyethylene catheter attached to a Sanborn pressure transducer. Pulse pressure was determined by subtracting the diastolic from the systolic pressure. Mean pressure was obtained by switching the pressure preamplifier to a filter circuit having a 1-sec. time constant. This procedure allowed the average value of the load on the transducer to be displayed on the oscillograph. A Sanborn high-gain preamplifier was used to record lead I of the electrocardiogram. Since the chart speed of the recorder was known, the heart rate was easily calculated.

Sufficient time was allowed for the animals' blood pressures to stabilize before control conditions were recorded. Each dog then received 10 inhalations of a 0.5% aqueous solution of isoproterenol hydrochloride. Drug-induced changes in the cardiovascular status of the animals were recorded at 1–3 min. intervals for a period of 15 min. After the isoproterenol effects had subsided, 10 inhalations of a 2% aqueous solution of metaproterenol sulfate were administered and the same data recording procedure repeated.

RESULTS AND DISCUSSION

The results of the first series of experiments, designed to evaluate the bronchodilator potential of metaproterenol, are presented in Tables I and II. The control value of the mean pulmonary resistance was 8.0 ± 0.4 (Table I). Following inhalation of histamine the mean pulmonary resistance increased sharply. The peak of the reaction usually occurred within 1 min. after administration of the spasmogen. Bronchoconstriction subsided in all of the test subjects within 20 min. at which time metaproterenol was administered. Two minutes thereafter the pulmonary resistance was again measured and the mean value found to be 9.4 ± 0.7. The latter value did not differ significantly from the initial control value ($p > 0.2$).

In each of the 10 experimental animals, metaproterenol afforded various degrees of protection

TABLE I—EFFECT OF HISTAMINE INHALATIONS ON PULMONARY RESISTANCE OF ANESTHETIZED, SPONTANEOUSLY BREATHING DOGS BEFORE AND AFTER TREATMENT WITH METAPROTERENOL

Dog	Sex	Wt., Kg.	Pulmonary Resistance (cm. H_2O/L./sec.)[a]						
			Drug	Control	Drug	30 sec.	1 min.	2 min.	3 min.	5 min.
1	F	12.2		8.4	Hist. (1)[b]	10.9	12.8	11.3	10.5	8.2
			Meta.	7.1	Hist. (2)[c]	8.4	9.7	9.3	9.4	7.4
2	M	6.7		8.3	Hist. (1)	16.7	21.8	19.6	18.2	14.4
			Meta.	10.6	Hist. (2)	11.1	12.1	11.6	10.8	11.3
3	F	6.0		8.7	Hist. (1)	31.2	31.1	28.4	23.6	19.5
			Meta.	12.9	Hist. (2)	13.4	15.8	16.1	16.4	16.4
4	M	10.0		6.3	Hist. (1)	13.9	14.1	12.8	11.6	9.9
			Meta.	5.3	Hist. (2)	6.5	6.0	7.1	7.9	7.7
5	F	12.2		8.2	Hist. (1)	ap[d]	23.1	19.3	15.6	13.6
			Meta.	9.8	Hist. (2)	11.3	11.1	11.1	10.6	10.4
6	F	11.4		7.6	Hist. (1)	9.4	11.2	12.8	12.7	11.3
			Meta.	8.6	Hist. (2)	10.4	11.5	11.4	11.0	9.6
7	F	11.4		9.6	Hist. (1)	ap[d]	ap[d]	ap[d]	62.2	31.2
			Meta.	13.0	Hist. (2)	16.4	16.9	15.9	15.6	12.8
8	F	9.0		5.8	Hist. (1)	ap[d]	60.0	57.1	48.0	38.3
			Meta.	9.1	Hist. (2)	9.8	10.8	11.0	9.4	9.8
9	F	10.0		9.8	Hist. (1)	ap[d]	51.7	28.0	33.1	41.6
			Meta.	9.9	Hist. (2)	8.4	11.9	10.3	9.3	10.0
10	M	13.6		7.7	Hist. (1)	13.1	15.8	15.9	14.4	11.9
			Meta.	7.9	Hist. (2)	8.3	9.2	9.1	8.8	8.6
Mean ± S.E.				8.0 ± 0.4	Hist. (1)	15.9 ± 2.9	26.8 ± 5.0	22.8 ± 4.5	25.0 ± 5.3	20.0 ± 3.1
Mean ± S.E.			Meta.	9.4 ± 0.7	Hist. (2)	10.4 ± 0.9	11.5 ± 0.9	11.3 ± 0.9	10.8 ± 0.9	10.4 ± 0.8

[a] Each value represents the average of 3 consecutive measurements. [b] Hist. (1) refers to the initial histamine administration. [c] Hist. (2) refers to the second administration of histamine to the same animal following treatment with metaproterenol. [d] ap denotes apnea.

TABLE II—EFFECT OF HISTAMINE INHALATIONS ON TIDAL VOLUME, RESPIRATORY RATE, MINUTE VOLUME, AND PULMONARY COMPLIANCE OF ANESTHETIZED, SPONTANEOUSLY BREATHING DOGS BEFORE AND AFTER TREATMENT WITH METAPROTERENOL

	Vol. (ml.)		Rate ($min.^{-1}$)		Min. Vol. (ml./min.)		Compliance (ml./cm. H_2O)	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Control	157	75–265	25	7–60	3393	1210–7800	38	23–52
Histamine administered								
30 sec.	69	32–110	75	15–108	4692	1650–3400	11	2–27
1 min.	72	30–143	78	18–125	4747	1920–10010	9	3–20
2 min.	89	37–164	72	18–120	5963	2448–13302	11	6–21
3 min.	98	39–190	59	18–95	6043	2574–12240	12	4–25
5 min.	100	40–156	48	9–80	4354	1848–12000	18	7–31
Meta.[a]	142	83–228	33	10–75	3898	2508–8025	35	15–78
Histamine administered								
30 sec.	125	56–256	53	18–100	5653	2800–10400	27	9–67
1 min.	126	63–240	57	18–103	6150	3950–10800	25	8–51
2 min.	137	75–260	55	20–100	6523	3720–11284	26	11–51
3 min.	144	74–260	53	18–90	6648	4512–10992	27	11–59
5 min.	130	64–261	51	15–84	5582	3000–8568	27	12–41

[a] The tabulated values were obtained 2 min. after metaproterenol had been administered.

against subsequent challenging doses of histamine. The percent protection at each of the measurement intervals was computed utilizing the following formula: $P = (I_c/R_c - I_t/R_t)/I_c/R_c \times 100$ where P is the percent protection, I_c is the increase in mean pulmonary resistance after histamine administration, R_c is the control mean pulmonary resistance before histamine had been given, I_t is the increase in mean pulmonary resistance after histamine administration to the metaproterenol treated dogs, and R_t is the mean pulmonary resistance 2 min. after the administration of metaproterenol. The percent protection values 30 sec., 1, 2, 3, and 5 min. after the second series of histamine inhalations were 89.2, 90.6, 89.2, 92.7, and 93.3%, respectively.

The effects of histamine inhalations on tidal volume, respiratory rate, minute volume, and pulmonary compliance, before and after treatment with metaproterenol, are summarized in Table II. The untreated animals experienced a decrease in tidal volume and pulmonary compliance and an increase in respiratory rate and minute volume following histamine. When the histamine inhalations were repeated in the metaproterenol-treated animals, the parameters again changed in the same direction but the magnitude of the changes differed. For instance, the maximum decrease in mean tidal volume of the unprotected animals was 88 ml., whereas the treated animals showed a maximum decrease of 17 ml. The mean respiratory rate of the unprotected animals increased from 25/min. to a maximum of 78/min., while the animals which had received metaproterenol experienced an increase of from 33/min. to a maximum of 57/min. The increase in minute volume of the untreated animals was only slightly less than the increase observed in the treated animals. The mean pulmonary compliance of the unprotected animals fell from the control value of 38 ml./cm. H_2O to a low of 9 ml./cm. H_2O while that of the treated animals fell from the control value of 35 ml./cm. H_2O to a low of 25 ml./cm. H_2O.

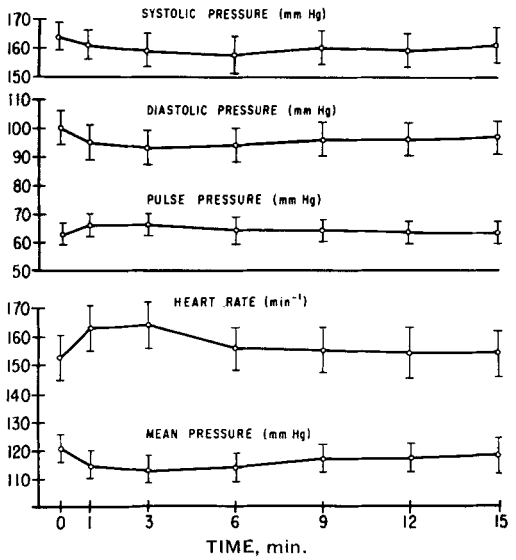

Fig. 2—Cardiovascular effects following 10 inhalations of isoproterenol hydrochloride (0.5%). The standard error is represented by vertical lines.

There are several possible explanations for the increase in respiratory frequency which was observed in both the unprotected and protected animals. Inhalation of histamine has been shown to reduce the systemic blood pressure of anesthetized dogs (7). The latter observation was confirmed by the present investigation. It is possible then that the respiratory stimulation was compensatory in nature and mediated by a reflex response evoked from baroreceptors in the carotid sinus and/or the aortic arch. In this connection it should be noted that in order for an inhaled drug to produce systemic effects it must first undergo transpulmonary absorption. Lovejoy and his co-workers (8) presented evidence to show that systemic effects frequently result after administering drugs from standard commercial nebulizers (such as the one used in this study) owing to their inherent property of delivering particles which are not exclusively submicronic. Robillard *et al.* (9) reported observing tachypnea and hypotension in human patients receiving carbachol from a commercial nebulizer. An increase in the respiratory rate of rabbits following carbachol inhalation was also observed by Karczewski and Dautrebande (10). These authors attributed the increase to the inhibiting effect of increased activity of pulmonary stretch receptors on the inspiratory center.

It is doubtful that the decrease in pulmonary compliance which followed histamine inhalation was caused by an actual lessening of the elastic qualities of the lungs. The changes in lung compliance followed the same time course and were proportional to the changes in total lung resistance. It is likely that histamine caused a marked constriction of several pulmonary units and as a consequence some alveoli collapsed due to surface forces. If such did occur, the decrease in compliance could be accounted for by atelectasis secondary to airway constriction.

The results of the experiments designed to compare the cardiovascular effects produced by inhalation of therapeutic doses of isoproterenol or metaproterenol are presented graphically in Figs. 2 and 3. Statistical analysis of the data was performed by using paired samples and the results were subjected to Student's analysis. A measured difference between control and postdrug values was considered to be significant and used to form conclusions only if $p < 0.05$.

Isoproterenol produced a significant drop in systolic blood pressure and a significant elevation of the heart rate at the 3-min. and 6-min. observation points. The diastolic and mean pressures were significantly depressed 1–6 min. after the drug had been administered. Isoproterenol had no significant effect upon pulse pressure. None of the cardiovascular responses to isoproterenol inhalation persisted for more than 6–9 min.

Metaproterenol produced a slight, insignificant increase in systolic blood pressure. The diastolic and mean pressures fell somewhat, but the decreases were significant only at the 9-min. postdrug administration time. The slight rise in systolic pressure coupled with the small decrease in diastolic pressure was sufficient to produce a significant increase in pulse pressure at each of the measurement intervals. The most pronounced cardiovascular effect of meta-

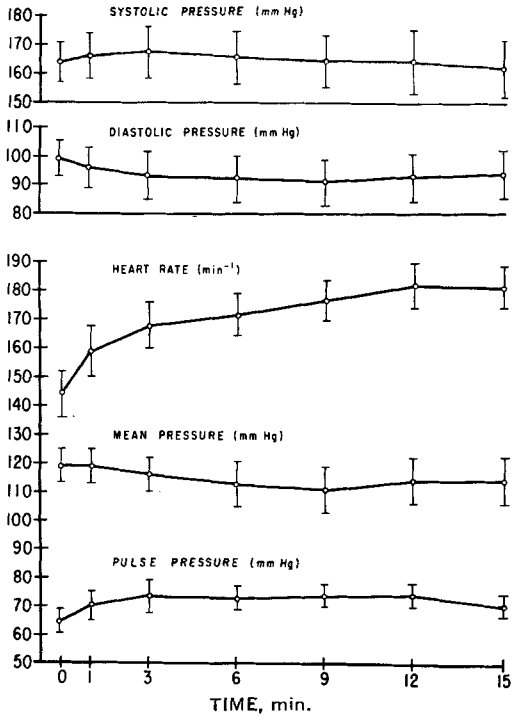

Fig. 3—Cardiovascular effects following 10 inhalations of metaproterenol sulfate (2%). The standard error is represented by vertical lines.

proterenol was observed on the heart rate which showed significant elevation at every observation time. Whereas the maximum increase in heart rate after isoproterenol inhalation was 7% and occurred 1 min. after the drug had been administered, the maximum increase following metaproterenol inhalation was 26% and occurred 12 min. after the drug had been administered.

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Keyphrases

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Pulmonary resistance, complicance—determined
Cardiovascular effects—metaproterenol
Histamine antagonism—metaproterenol

Effect of Deuterium Oxide on the Culturing of *Penicillium janczewskii* II

Isolation of Fully Deuterated Griseofulvin

By D. A. NONA, M. I. BLAKE, H. L. CRESPI, and J. J. KATZ

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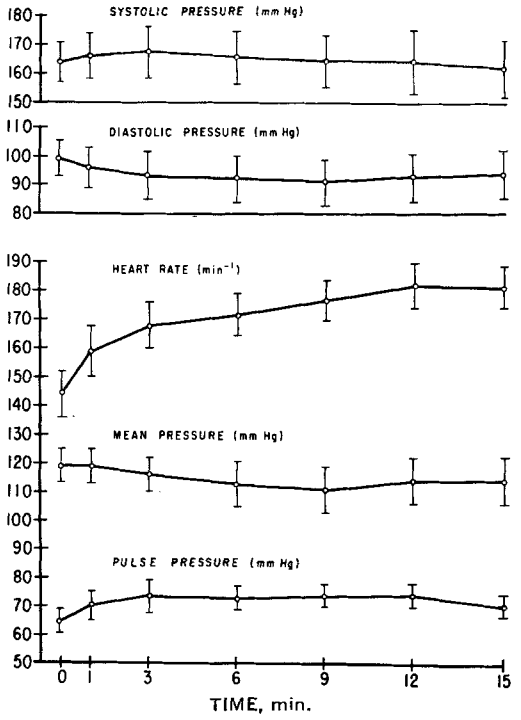
In a previous paper (1) the effect of heavy water both on the growth of the mold *Penicillium janczewskii* and on its production of antibiotic was reported. Nutritional requirements for optimal growth in D_2O and the general culture techniques were described in detail. Griseofulvin production was severely impaired when the organism was grown in pure D_2O media by the usual surface culture method, but the griseofulvin titer was improved when the D_2O culture medium was supplemented with vitamin B complex. In the present study a replacement culture method was employed to produce deuterated griseoful-

Received March 4, 1968 from the Department of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612, and the Chemistry Division, Argonne National Laboratory, Argonne, IL 60439
Accepted for publication March 21, 1968.
This investigation was supported by grant AI 06825-01 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and was performed in part under the auspices of the U. S. Atomic Energy Commission, Washington, D. C.



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vin. Deuterated antibiotic biosynthesized by the organism in replacement culture was isolated, purified, and characterized by chemical means.

EXPERIMENTAL

Replacement Culture Method—The griseofulvin-producing strain *Penicillium janczewskii* (NRRL 2301) was used throughout this study. Mycelial contents of shake cultures grown in an H_2O medium were used as starting tissue for preparative replacement cultures. After 7 days' growth the vegetative tissue from 60 ml. of an H_2O medium culture contained in a 250-ml. conical flask was separated from the medium by filtration. The tissue retained by the filter was resuspended in 75 ml. of sterile D_2O and the suspension was again filtered. This process was repeated twice. Then the mycelium was dispersed in 60 ml. of deuterated medium. The composition and preparation of the nutrient medium have been described previously (1). Fermentation was allowed to take place in 250-ml. conical flasks stoppered with cotton and sealed with aluminum foil. Cultures were returned to the shaker and incubation resumed for a period of 4–6 weeks. All manipulations involving exposed cultures were performed in a bacteriologic hood.

Surface cultures were prepared by inoculating 500 ml. of H_2O medium contained in 3-L. conical flasks with a spore suspension of the organism. Replacement was effected after a 7-day growth period. The water medium was removed from beneath the floating mat by introducing the tip of a sterile 2-ml. pipet below the mat. A section of Tygon tubing was attached to the other end of the pipet, and the medium was carefully siphoned away. The underside of the mat was washed with 100 ml. of sterile D_2O added by slow infusion through a reservoir joined to the pipet by Tygon tubing. After swirling several times, the D_2O wash was siphoned away and fresh sterile heavy water was added. This procedure was repeated twice. After the third washing, 500 ml. of deuterated replacement medium was slowly introduced under the mycelial mat with the aid of a pipet. The fluid flow rate was carefully controlled by adjustment of the reservoir height. For successful preparation of a replacement culture it was essential that the mycelial mat remain floating and that there be no liquid on the upper surface of the mat. In surface culture antibiotic production does not occur when the mat sinks into the medium.

Analysis for deuterium content was performed periodically throughout the fermentation period by the technique of Crespi and Katz (2). At no time was the final deuterium oxide concentration observed to be more than 2% below the initial concentration, indicating that little isotopic dilution had occurred.

Extraction Procedure—At harvest, the mycelium was separated from the fermentor broth by filtration, and both the mycelium and filtrate were extracted for griseofulvin. The filtrate was extracted with chloroform in a 1-L. liquid–liquid extractor for 76 hr. Preliminary studies indicated that this length of time was required for complete extraction of the antibiotic. The chloroform extract was reduced in volume to about 10 ml. on a flash evaporator. Antibiotic was recovered from the concentrate in pure form by preparative thin-layer chromatography.

The mycelium was treated according to the procedure of Oxford *et al.* (3). After drying in an oven at 100° for 24 hr., the mycelium was reduced to a fine powder in a mortar and transferred to an extraction thimble. The thimble was placed in the extractor of a Soxhlet continuous extraction apparatus. The powdered mycelium was extracted with ether for 76 hr. The ether extract was evaporated to dryness in a flash evaporator, the residue redissolved in chloroform, and products separated by preparative thin-layer chromatography.

Preparative Thin-Layer Chromatography—Silica Gel G preparative thin-layer plates, 500 μ in thickness, were prepared with a Desaga variable gauge applicator. Concentrated chloroform extracts were streaked across the plate at the origin (a line parallel to one side of the plate and 3.5 cm. from the edge) with an automatic applicator and streaker (Rodder Instruments, Los Altos, Calif.). All chromatograms were spotted with a chloroform solution of reference standard griseofulvin to serve as a control. Plates were placed in a standard developing tank which was lined with filter paper to ensure saturation of the chamber. The developing solvent consisted of ether–acetone, 3:2. The development time was about 25 min., which was the time required for the solvent front to reach the end of the plate. Since griseofulvin fluoresces strongly, its location on the plate was readily detected by scanning with an ultraviolet lamp. The fluorescent band having an R_f value of 0.62 was scraped from the chromatographic plate with a razor blade. Griseofulvin adsorbed to the Silica Gel G was isolated by shaking at 200 r.p.m. for 30 min., with a mixture of ether–acetone, 1:3; the extract was recovered by centrifugation. The extract after filtration was evaporated to dryness under a stream of nitrogen gas. The residue was dissolved in chloroform and rechromatogramed on a preparative thin-layer plate using as the developing solvent, ether–acetone, 4:1. Griseofulvin was removed from the plate as described previously. A cream-colored powder of purified griseofulvin was obtained.

Silica Gel Chromatopile Technique—As an alternate procedure for obtaining purified griseofulvin from the fermentor broth and mycelium, a preparative chromatopile technique was also employed. A cylindrical form was shaped by rolling a section of chromatography paper to a dimension of 14 cm. in length and 3 cm. in diameter at the base. The paper form was covered with aluminum foil and placed on end. A thick suspension of Silica Gel G (75 Gm. of gel in 35 ml. of water) was poured into the cylindrical mold. After 3 hr. the aluminum foil was removed and the chromatopile was allowed to air dry overnight. Finally, the paper mold was peeled away exposing a cylindrical bar of Silica Gel G.

A concentrated solution of griseofulvin in chloroform was placed in a 50-ml. evaporating dish. The base of the chromatopile was dipped into the solution. When the solvent front reached a height of about 1.5 cm., the chromatopile was removed, air dried, and returned to the evaporating dish. This process was continued until the entire chloroform concentrate had been adsorbed on the lower 1.5 cm. of the chromatopile. The band of sample at

the origin was developed by dipping into a solvent mixture of ether–acetone (3:2) contained in a 50-ml. evaporating dish. Development was continued until the griseofulvin band reached a height of about 4 cm. as determined by fluorescence under ultraviolet light. The chromatopile was then placed into the TLC developing tank containing developing solvent, and development was continued for approximately 25 min., after which it was removed and air dried. The band containing the griseofulvin was sliced from the column with a razor blade, reduced to a powder, and extracted as described previously.

Infrared Spectrum—A 0.5% solution of griseofulvin in chloroform was examined over the 4,000 to 650 cm.$^{-1}$ spectral region in a Beckman spectrophotometer, model IR 7, equipped with a 0.1-mm. ultramicro cavity cell.

Ultraviolet Absorption Spectrum—The ultraviolet absorption spectrum of a solution of griseofulvin in ethyl acetate was obtained with a Beckman DG-B grating spectrophotometer provided with a Sargent recorder (model SR).

Nuclear Magnetic Resonance Spectroscopy—NMR spectra of the griseofulvin samples prepared as 5% solutions in Silanar-C (deuteriochloroform with 1% TMS as internal standard; Merck Sharp and Dohme, Canada, Ltd.) were obtained on a Varian A-60 NMR spectrometer and a Varian HA-100 NMR spectrometer, equipped with a Varian C-1024 computer of average transients.

RESULTS AND DISCUSSION

Good growth does not necessarily imply good antibiotic production. It was noted, however, that good antibiotic production was always associated with good growth. The decreasing griseofulvin titers observed with increasing D_2O solvent concentrations were, for the most part, accompanied by inferior mycelial felts. A replacement culture method allows for the separate analysis of the effects of deuterium isotope replacement on vegetative growth and on griseofulvin production, and it provides a high degree of control over each phase. When the replacement medium consisted of pure D_2O and deuterio sugars, a fully deuterated antibiotic was obtained, and when pure D_2O and protio sugars were employed, the isolated griseofulvin was partially deuterated. Milligram amounts of fully deuterated griseofulvins were obtained from 500-ml. preparative replacement cultures. A comparison of antibiotic yields from varying culture conditions is listed in Table I. After the nutrient was replaced, the cultures were allowed to ferment for an additional 28 days. Both mycelium and fermentor broth were then extracted for antibiotic. Protio griseofulvin was not found to accumulate in mycelium grown in a protioculture before the fifteenth day of incubation. Therefore, if the protio medium is replaced with a deuterated medium before such a time, the mycelium originating from H_2O cultures may be extracted for deuterio griseofulvin at the conclusion of the fermentation period free of contamination with protio griseofulvin. The 50–60 days for maximum antibiotic production by direct fermentation can thereby be reduced to about 30 days by the replacement technique.

Since laboratory scale antibiotic production under the described conditions involves the isolation of small amounts of griseofulvin, the usual preparative procedures cited in the scientific and patent literature were not applicable in this study. A thin-layer chromatographic procedure was developed which served effectively to identify and separate the griseofulvin from its fermentation congeners. The method not only enabled the isolation of small quantities of pure griseofulvin, but was less tedious than solvent extraction and recrystallization techniques, and the small quantitites of griseofulvin produced were obtained in a chromatographically pure state. Purification of the extracts obtained from larger batches of mycelium and fermentor broth required at least five or six TLC plates. The large number of plates is needed because a good separation cannot be effected if the sample is very concentrated and the plates overloaded. The use of a chromatopile thus provided a second method for purification and had the advantage of adsorbing larger amounts of material without impairing separation.

TABLE I—GRISEOFULVIN PRODUCTION IN SURFACE CULTURE BY *P. janczewskii*

Medium	Growth Conditions	Fermentor Broth Titer, mcg./ml.	Recovery, mg./500 ml.
0% D_2O, protio sugars	Direct fermentation	110–120	13.0
99.6% D_2O, protio sugars and vitamins	Direct fermentation	40–50	4–6
99.6% D_2O, protio sugars	Replacement culture	45–50	4–6
99.6% D_2O, deuterio sugars and vitamins	Direct fermentation	45–50	5.2
99.6% D_2O, deuterio sugars	Replacement culture	45–50	4.6

Chloroform solutions of ordinary, partially deuterated, and fully deuterated griseofulvin prepared in this laboratory were characterized by infrared spectrometry. The fully deuterated griseofulvin samples were obtained by both direct fermentation and replacement culture with fully deuterated media. Partially deuterated griseofulvin was obtained from replacement cultures using pure D_2O and protio sugars. The spectra were compared with those obtained with chloroform solutions of authentic griseofulvin and spectral assignments were made according to the interpretation of Page and Staniforth (4).

Infrared spectra of protio griseofulvin isolated in this laboratory from H_2O cultures were identical to that of reference standard griseofulvin. The infrared spectra of chloroform solutions of fully deuterated griseofulvin were found to be identical to that of ordinary griseofulvin with one striking exception, namely, the replacement of the C—H absorption at 2,850 cm.$^{-1}$ by a C—D absorption at 2,120 cm.$^{-1}$. This is the usual isotope effect on the C—H stretch vibration, and the isotope shift confirms the incorporation of deuterium.

The NMR spectra of deuteriochloroform solutions of griseofulvin and selected analogs have been

examined and interpreted independently by Green *et al.* (5) and Arison *et al.* (6). These workers were able to assign each spectral line or group of lines to a particular proton or group of protons, and conversely, to correlate each proton with a particular line or group of lines. The spectra of partially deuterated compounds may be compared directly with the spectrum of the compound which contains only ordinary hydrogen, and in this way information can be obtained about the amount of deuterium present at each molecular site. The effects of the deuterons on the proton resonances may be observed indirectly, but the deuterons themselves will not contribute any signals to the recorded spectrum. Therefore, the situation that is created when the proton magnetic resonance bands are observed for partially deuterated griseofulvin is no different from that which is observed in the fully protiated griseofulvin. Since the NMR signal is proportional to the number of protons contributing to the resonance line in partially deuterated griseofulvin, the proton integral for a particular molecular position will be diminished by a factor which is proportional to the extent of proton replacement by deuterium. If all of the molecules in the collection that forms the sample are fully deuterated at a given position, no signal would be observed and the proton integral for the proton resonances of the position would be zero.

Fully Deuterated Griseofulvin—NMR spectra of griseofulvin samples obtained by direct fermentation and replacement cultures employing fully deuterated media showed a complete absence of resonance peaks at 6.15, 5.53, 4.04, 4.00, 3.63, 2.70, and 0.98 δ.[1] The only absorption peaks in the spectra were the TMS (standard reference signal) peak at 0, and a peak at 7.39 δ due to protons in the deuteriochloroform solvent. When the amplitude was adjusted to the upper limits of the Varian A-60, the noise level was understandably increased, but even so no absorption peaks were observed. These results indicate that the griseofulvin isolated under these fermentation conditions contains essentially no protons, and that there is complete isotopic replacement of hydrogen with deuterium.

High resolution spectra of the fully deuterated griseofulvin samples were then obtained on a Varian HA-100 spectrometer. The spectra obtained exhibited a complete absence of absorption peaks, confirming the spectral results obtained with the Varian A-60. Spectra were also obtained with a computer of average transients (C.A.T.). A readout after multiple scans showed only minor absorption in the C—CH_3 and CH_2—CH absorption region, and no other absorption peaks were observed. The proton resonance observed indicated the presence of some hydrogen in the molecule, but the amount was clearly very small. The origin of these protons is probably due to residual H_2O in the 99.6% D_2O used. From these NMR results it can be concluded that the griseofulvin produced is essentially fully deuterated.

Partially Deuterated Griseofulvin—The extent of deuterium incorporation into griseofulvin isolated from replacement cultures of partially deuterated media, composed of protioglucose (7%) and pure D_2O, was determined by NMR spectroscopy. The spectra obtained were compared directly to that for protio griseofulvin. Examination of the spectrum indicated that no significant chemical shift differences exist between ordinary and partially deuterated griseofulvin. Since the NMR signal integral is proportional to the number of protons contributing to the resonance line, the proton integral for a particular molecular position will be diminished by a factor which is proportional to the total number of nuclei contributing to the signal. By comparing the integrals for these areas to those of a standard signal by a known number of protons, the various regions of the spectrum were calibrated as to the number of protons which were represented.

Sample I was prepared by dissolving an exact amount of partially deuterated griseofulvin, which was obtained from replacement cultures, in exactly 300 μl. of $CDCl_3$ (Silinar-C.). The same quantity (accurately weighed) of ordinary hydrogen griseofulvin, isolated in this laboratory from H_2O cultures, was dissolved in the same volume of Silinar-C and designated as Sample II. The NMR spectra of the two samples were then obtained with a Varian A-60 spectrometer. A spectrum amplitude of 80 was required to obtain a readable spectrum with Sample I, whereas an amplitude of only 40 was needed for Sample II, reflecting the marked difference in proton content of the two samples. The proton content was quantitated by integrating the spectra. Because the volume of deuteriochloroform in each sample was exactly 300 μl., a comparison can be made of the area integrated under the absorption peak of deuteriochloroform arising from the small amount of residual $CHCl_3$ present in the $CDCl_3$. At an amplitude of 80 (Sample I) and 40 (Sample II) the area under the $CHCl_3$ absorption peak was represented by 15 units and 5 units, respectively. Therefore, if the griseofulvin molecule was fully protiated, the expected area under each absorption peak in the spectrum of Sample I (at an amplitude of 80) would be three times that of the spectrum of Sample II (at a spectrum amplitude of 40). Thus the areas under the resonance peaks can be normalized and the relative amounts of hydrogen at the various molecular positions in partially deuterated griseofulvin can be established.

Normalization shows that there is a considerable replacement of hydrogen by deuterium at various

Table II—Isotope Fractionation Factors for Partially Deuterated Griseofulvin Obtained by Biosynthesis in D_2O from Hydrogen Substrates

Resonance	Chemical Shift (δ, p.p.m.)	Partially Deuterated Griseofulvin—Expected Area[a] (Units)	Partially Deuterated Griseofulvin—Observed Area[a] (Units)	Isotope Fractionation Factor K_H/K_D
$CHCl_3$ (present in $CDCl_3$)	7.39	15	15	—
Aromatic *H*	6.15	54	11	0.26
Vinyl *H*	5.53	54	11	0.26
Aromatic OCH_3 (6-methoxyl)	4.04	75	17	0.30
Aromatic OCH_3 (4-methoxyl)	4.00	27	7	0.35
Vinyl OCH_3	3.63	42	10	0.31
CH_2—C*H*	2.70	54	18	0.50
C—CH_3	0.98	48	14	0.41

[a] Per proton, spectral amp. 80.

[1] Chemical shifts are given in parts per million relative to tetramethylsilane (TMS) with downfield shifts positive.

molecular positions in the partially deuterated griseofulvin (Table II), which results from the incorporation of deuterium during biosynthesis at the various proton sites in the molecule. The amount of deuterium incorporated varies with the particular proton site. From Table II it can be seen that replacement of hydrogen by deuterium is maximal at the aromatic and vinyl positions. These molecular sites apparently afford little opportunity for biosynthetic incorporation of proton from the protio substrate, and a maximum of solvent D_2O equilibration occurs at these loci. Decreasing amounts of isotopic substitution occur at the OCH_3, C—CH_3, and CH_2—CH positions, indicating that the protio sugars have a more extensive role in the biosynthetic mechanisms leading to the formation of these moieties. In addition, these sites probably have a decreased capability for equilibration with the solvent. The isotope fractionation factor K_H/K_D calculated as the average of all molecular positions in partially deuterated griseofulvin was found to be 0.36. This indicates a ratio of approximately one hydrogen atom for every three deuterium atoms in the molecule, and an average deuterium isotope replacement of about 75%.

CONCLUSIONS

Griseofulvin extracted from the mycelium and culture filtrates of *P. janczewskii* was purified by preparative thin-layer chromatography and characterized by visible, infrared and nuclear magnetic resonance spectra. Fully deuterated griseofulvin, the first fully deuterated antibiotic so far prepared, was obtained from organisms grown on a fully deuterated medium by direct fermentation. A yield of over 5 mg. was obtained from 500 ml. of culture medium. When a protio medium used for vegetative growth was replaced with a fully deuterated medium, fully deuterated griseofulvin was isolated. Approximately the same amount of antibiotic was obtained from 500 ml. of replacement medium. By this procedure the 50–60 days necessary for maximum deuterio griseofulvin production by direct fermentation could be reduced by about one-half. When the replacement medium was only partially deuterated (protio sugars and pure D_2O), an antibiotic was isolated which was deuterated in part. A yield of 4–6 mg. was obtained from 500 ml. of partially deuterated replacement medium.

Subsequent studies are concerned with the determination of the effect of deuterium on the antibiotic activity. If detoxification of griseofulvin requires rupture of carbon-to-hydrogen bonds it is expected that the deuterio analog should exhibit an enhanced activity since carbon-to-deuterium bonds are generally more stable. A statistical analysis of antifungal activity of deuterio griseofulvin will be the subject of the next report.

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Keyphrases

Penicillium janczewskii II—cultures
Deuterium oxide—*P. janczewskii II* cultures
Griseofulvin, fully deuterated—isolation
TLC—separation
Silica gel chromatohile—separation
NMR spectrometry—identity
IR spectrophotometry—structure
UV spectrophotometry—structure

Synthesis of 2-(*N*,*N*-Dialkylaminomethyl)-7-phenylthioindoles

By ROBERT L. DUNCAN, JR.* and JOHN ANDRAKO†

A series of 2-(*N*,*N*-dialkylaminomethyl)-7-phenylthioindoles was prepared. The Japp-Klingemann reaction followed by Fisher cyclization with polyphosphoric acid gave 7-phenylthioindole-2-carboxylic acid. The lithium aluminum hydride reduction of amides derived from this acid provided the corresponding aminomethyl indoles. Infrared and NMR data are reported.

As part of a program directed at the synthesis and pharmacological evaluation of dialkylaminoalkyl-7-substituted indoles, the authors wish to report the synthesis of a series of 2-(*N*,*N*-dialkylaminomethyl)-7-phenylthioindoles (VI). Relatively few 7-substituted indoles and indole derivatives have been reported and there is an almost total lack of pharmacological data for compounds of this type. Hiremath and Siddappa (1) reported the synthesis of 5-methoxy-7-

Received January 22, 1968, from the Department of Chemistry and Pharmaceutical Chemistry, School of Pharmacy, Medical College of Virginia, Richmond, VA 23219
Accepted for publication March 20, 1968.
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As part of a program directed at the synthesis and pharmacological evaluation of dialkylaminoalkyl-7-substituted indoles, the authors wish to report the synthesis of a series of 2-(*N*,*N*-dialkylaminomethyl)-7-phenylthioindoles (VI). Relatively few 7-substituted indoles and indole derivatives have been reported and there is an almost total lack of pharmacological data for compounds of this type. Hiremath and Siddappa (1) reported the synthesis of 5-methoxy-7-

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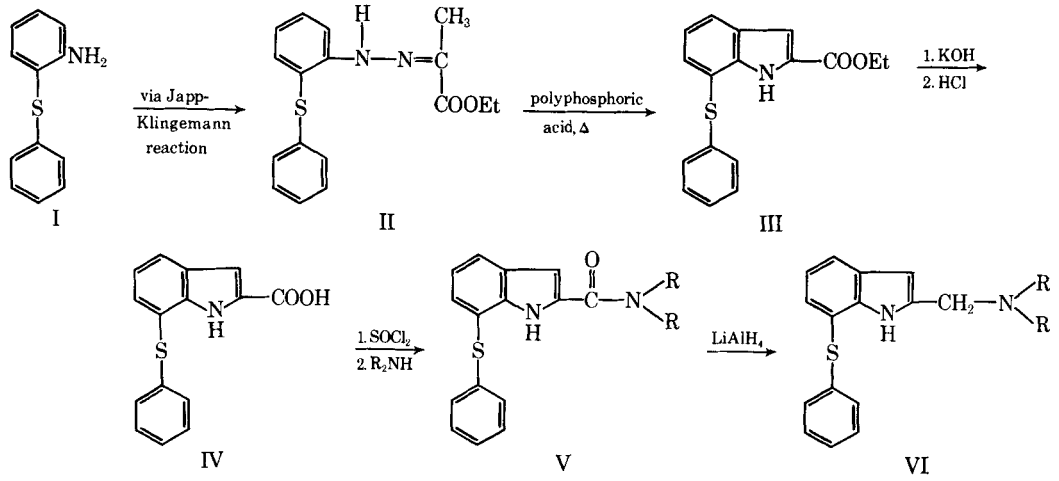
nitroindole, 5-methyl-7-nitroindole, and the corresponding aminoindoles as possible antiserotonin agents, but did not report any biological data for these compounds. The syntheses of a number of 2-dialkylaminomethylindole derivatives have been reported by Kornfeld (2), Yoneda, and co-workers (3) and others (4–6).

The 2-dialkylamino-7-phenylthioindoles are of particular interest since they may be related structurally to "open" phenothiazines.

DISCUSSION

The synthesis of the reported compounds was accomplished by the sequence of reactions outlined in Scheme I.



Scheme I

The *o*-(phenylthio)aniline (I) was obtained in good yield by reduction of *o*-(phenylthio)nitrobenzene which was prepared by treating sodium thiophenoxide with *o*-chloronitrobenzene. Diazotization of I and reaction of the resulting diazonium compound with ethyl 2-methylacetoacetate under Japp-Klingemann conditions yielded the desired ethyl pyruvate *o*-(phenylthio)-phenylhydrazone in about 50% yield. An attempt to obtain *o*-(phenylthio)phenylhydrazine by reduction of the diazonium compound of I with stannous chloride and hydrochloric acid was made. A solid was obtained which on the basis of its infrared absorption at 2260 cm.$^{-1}$ (N ≡ N) (7) appeared to be a stable tin double salt of diazotized II. No further characterization of the solid was made.

The indole ester, III, was prepared by cyclization of II using polyphosphoric acid. When the temperature of the reaction mixture was raised to 90° an exothermic reaction occurred. At this point the reaction was immediately quenched with ice water. If the reaction mixture was maintained at 90° for longer periods of time, extensive tar formation occurred, the yields of indole ester were lower, and it was more difficult to purify the small amounts of product which were isolated. Attempts to bring about the cyclization of II using a saturated ethanolic solution of hydrogen chloride or ethanolic zinc chloride were unsuccessful.

The 7-phenylthioindole-2-carboxylic acid, was obtained by hydrolysis of the ester (III) with potassium hydroxide followed by acidification with hydrochloric acid. The indole acid was converted to the acid chloride using thionyl chloride and was used without prior isolation to prepare the carboxamides, V*a*, *b*, *c*, *d*, described in Table I. Conversions of indole acids to indole acid chlorides have been reported (2, 8, 9).

The carboxamides were reduced with lithium aluminum hydride to the corresponding 2-(*N*,*N*-dialkylaminomethyl)-7-phenylthioindoles, VI*a*, *b*, *c*, *d*, indicated in Table II.

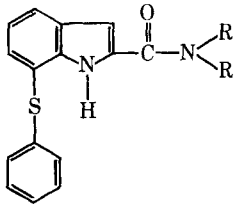
The infrared and NMR spectra are in agreement with the assigned structures at each step indicated in Scheme I.

EXPERIMENTAL[1]

o-(Phenylthio)nitrobenzene—To a sodium ethoxide solution prepared by adding slowly 20.7 Gm. (0.9 mole) of sodium metal to 300 ml. of absolute ethanol there was added in portions over a period of 20 min., 110 Gm. (1.0 mole) of benzenethiol followed by 157.6 Gm. (1.0 mole) of *o*-chloronitrobenzene.

[1] Melting points were obtained with a Thomas-Hoover capillary melting point apparatus and are uncorrected. All analyses were performed at the Medical College of Virginia unless otherwise indicated. Infrared spectra were obtained with a Beckman IR-8 spectrophotometer. NMR data were obtained with a Varian Associates model A60 NMR spectrometer through the courtesy of the A. H. Robins Co., Richmond, Va.

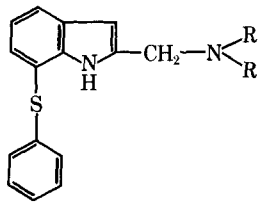
TABLE I—7-PHENYLTHIOINDOLE-2-CARBOXAMIDES



Compound	—N(R)R	M.p. °C.	Yield, %	Formula	Anal. Calcd.	Anal. Found
V *a*	—N(CH_3)$_2$	191–193[a]	82	$C_{17}H_{16}N_2OS$	C, 68.89 H, 5.44 N, 9.45	C, 68.56 H, 5.54 N, 8.9
V *b*	—N(C_2H_5)$_2$	168–169[a]	54	$C_{19}H_{20}N_2OS$	C, 70.34 H, 6.21 N, 8.64 S, 9.88	C, 69.84[b] H, 6.38[b] N, 8.52[b] S, 9.84[b]
V *c*	—N⬡	124–125.5[a]	64	$C_{20}H_{20}N_2OS$	C, 71.40 H, 5.99 N, 8.33 S, 9.53	C, 71.38[b] H, 5.98[b] N, 8.61[b] S, 9.46[b]
V *d*	—N⬡O	86–88[a]	59	$C_{19}H_{18}N_2O_2S$	C, 67.43 H, 5.36	C, 67.12 H, 5.61

[a] Recrystallized from methanol. [b] Analysis by Microtech Labs., Skokie, Ill.

TABLE II—2-(*N*,*N*-DIALKYLAMINOMETHYL)-7-PHENYLTHIOINDOLES



Compound	—N(R)R	M.p., °C.	Yield, %	Formula[b]	Anal. Calcd.	Anal. Found
VI*a*	—N(CH_3)$_2$	164–166[a]	74	$C_{17}H_{19}ClN_2S$	C, 64.03 H, 6.01 N, 8.70	C, 63.56 H, 6.18 N, 8.5
VI*b*	—N(C_2H_5)$_2$	120–122[a]	86	$C_{19}H_{23}ClN_2S$	C, 65.78 H, 6.68 N, 8.08	C, 65.49 H, 6.91 N, 7.9
VI*c*	—N⬡	207–208.5[a]	81	$C_{20}H_{33}Cl_3N_2S$	C, 66.92 H, 6.46 N, 7.81	C, 66.95 H, 6.90 N, 7.68
VI*d*	—N⬡O	187–188[a]	93	$C_{19}H_{21}ClN_2OS$	C, 63.23 H, 5.87 N, 7.76	C, 63.54 H, 6.33 N, 7.25

[a] Recrystallized from 2-butanone. [b] Hydrochlorides.

The mixture was allowed to reflux for 3.5 hr. After the addition of 500 ml. of water, the mixture was extracted with ether. The combined ethereal extracts were dried (anhydrous sodium sulfate) and concentrated. The solid residue thus obtained weighed 154.4 Gm. (74.2%) and after recrystalliza-

tion from ligroin melted at 77–79° [lit. 77° (10); 78–80° (11)].

o-(Phenylthio)aniline (I)—A solution of 43.1 Gm. (0.23 mole) of stannous chloride in 106 ml. of concentrated hydrochloric acid was added slowly to a stirred solution of 11.6 Gm. (0.05 mole) of *o*-(phenylthio)nitrobenzene. The solution was heated on a steam bath until the yellow color disappeared (2–3 hr.). The solution was cooled, carefully made basic with sodium hydroxide, and extracted with ether. The ethereal extracts were combined, dried (anhydrous sodium sulfate), and concentrated. The residual oil, upon standing in the freezer, crystallized and 7.1 Gm. (70%) of pale yellow crystals was obtained which melted at 34–36° [lit. 33° (11)]. The acetyl derivative melted at 84–86° [lit. 86° (10)].

Ethyl Pyruvate o-(Phenylthio)phenylhydrazone (II)—A solution of 60.4 Gm. (0.3 mole) of *o*-(phenylthio)aniline in 270 ml. of 6 *N* hydrochloric acid contained in a three-necked flask was cooled in an ice-salt bath and stirred. While maintaining the temperature of the reaction mixture at 0–5°, 22.1 Gm. (0.32 mole) of sodium nitrite in 60 ml. of water was added dropwise. The solution of the diazonium salt was maintained at 0° until it was used later. A solution of 44.6 Gm. (0.31 mole) of ethyl 2-methylacetoacetate in 300 ml. of ethanol was stirred and cooled to 0°, and 108 ml. of a 50% solution of potassium hydroxide was added with vigorous stirring. About 400 ml. of an ice–water mixture was added to the reaction mixture, and the previously prepared cold solution of the diazonium salt was introduced immediately. The red mixture was stirred for 16 hr. during which time it was allowed to come to room temperature. The aqueous reaction mixture was extracted with ether. The ethereal extracts were combined, and the ether was evaporated leaving a dark red oil which resisted all attempts at crystallization. The crude oil was distilled with care and the fraction boiling at 160–165°/0.05 mm. was collected, yielding 46.0 Gm. (49%) of the hydrazone ester. The infrared spectrum in chloroform showed $\nu_{max.}$ 3300 cm.$^{-1}$, 3220 cm.$^{-1}$, 1700 cm.$^{-1}$.

Anal.—Calcd. for $C_{17}H_{18}N_2O_2S$: C, 64.94; H, 5.77; N, 8.91. Found: C, 64.63; H, 5.73; N, 8.83.

Ethyl 7-(Phenylthio)indole-2-carboxylate (III)—A mixture of 45 Gm. (0.15 mole) of II and 120 Gm. of polyphosphoric acid was stirred in a 500-ml. conical flask equipped with a thermometer. The mixture was carefully heated to 90° whereupon an exothermic reaction occurred. At this point about 100 ml. of ice water was added immediately to the reaction mixture. When decomposition of the excess polyphosphoric acid was complete, the semi-solid ester was collected by filtration. The residue was dissolved in 100 ml. of ether, and the solution was dried and concentrated. A gummy residue which weighed 30.0 Gm. (71%) was obtained. Upon standing, crystals formed in the residue. After recrystallization from ethanol, the yellow crystals melted at 133–135°. The infrared spectrum (KBr disk) showed $\nu_{max.}$ 3350 cm.$^{-1}$, 1675 cm^{-1}.

Anal.—Calcd. for $C_{17}H_{15}NO_2S$: C, 68.66; H, 5.09; N, 4.71. Found: C, 68.71; H, 5.67; N, 4.25.

7-Phenylthioindole-2-carboxylic Acid (IV)—To a solution of 101 Gm. (0.34 mole) of III in 200 ml. of absolute ethanol was added an excess of aqueous potassium hydroxide solution prepared by dissolving 50 Gm. of potassium hydroxide in 50 ml. of water. The solution was refluxed for 5 hr. After standing 16 hr. the solution was diluted with 500 ml. of water and extracted with chloroform. The aqueous layer was made acidic with 5% hydrochloric acid solution, and a red oil, which crystallized when scratched with a glass rod, separated from solution. The solid was filtered with suction, washed with water, and air dried. The crude solid (75 Gm.) was dissolved in 100 ml. of a 5% sodium bicarbonate solution and the resulting solution was extracted with both chloroform and ether. The aqueous solution was treated with charcoal and warmed to remove any remaining organic solvent. The mixture was filtered and the filtrate made acidic with a 5% hydrochloric acid solution. The acid which precipitated was collected by filtration and dissolved in methanol. When water was added, a pale yellow solid separated which was removed by filtration and air dried. Decolorization with charcoal and recrystallization from methanol–water gave 60 Gm. (91%) of the crude acid which melted at 150–160°. Recrystallization from benzene gave a crystalline product which melted at 158.5–160.5°. The infrared spectrum (KBr disk) showed $\nu_{max.}$ 3410 cm.$^{-1}$, 1660 cm.$^{-1}$. The NMR spectrum (DMSO) showed only an aromatic multiplet, extending from 6.9–7.9 δ, an acid singlet at 12.3 δ, and a broad NH area in the region of 8.5 δ. The integration of 1:1:10 for the acid, NH, and aromatic areas, respectively, agreed with the assigned structure.

Anal.[2]—Calcd. for $C_{14}H_{11}NO_2S$: C, 66.89; H, 4.12; N, 5.20; S, 11.90. Found: C, 66.67; H, 4.16; N, 5.05; S, 11.86.

(N,N-Dimethyl) - 7 - phenylthioindole - 2 - carboxamide (Va)—A solution of 5.0 Gm. (0.019 mole) of IV in 50 ml. of thionyl chloride was stirred for 2 hr. at room temperature. The thionyl chloride was removed under vacuum, and the residue which remained was dissolved in 30 ml. of tetrahydrofuran. A solution of 1.6 Gm. (0.038 mole) of dimethylamine in tetrahydrofuran was added dropwise from a dropping funnel to the solution of the acid chloride. After 15 min. of stirring, the mixture was filtered and the filtrate was evaporated to dryness. The residue which remained was dissolved in 50 ml. of chloroform, and the chloroform solution was extracted successively with 15-ml. portions of 5% hydrochloric acid solution, 5% sodium hydroxide solution, and water. The chloroform layer was evaporated to dryness and the 4.5 Gm. (82%) of crude amide which remained was dissolved in hot methanol, decolorized with charcoal, and filtered. The white crystalline amide which separated melted at 191–193°. The infrared spectrum ($CHCl_3$) showed $\nu_{max.}$ 3440 cm.$^{-1}$, 1600 cm.$^{-1}$. The NMR spectrum ($CDCl_3$) showed a singlet at 3.07 δ (CH_3), a multiplet at 7.25 δ (aromatic), and a broad NH absorption band far downfield. Other data are in Table I.

(N,N - Diethyl) - 7 - phenylthioindole - 2 - carboxamide (Vb)—The procedure described for *Va* was followed. The infrared spectrum ($CHCl_3$) showed $\nu_{max.}$ 3440 cm.$^{-1}$, 1600 cm.$^{-1}$. The NMR spectrum ($CDCl_3$) showed a triplet at 1.1 δ (CH_3), a quartet at 3.5 δ (CH_2), and a multiplet at 7.25 δ (aromatic). Other data are in Table I.

1-(7-Phenylthio-2-indolylcarbonyl)piperidine (Vc)—The procedure described for *Va* was followed.

[2] Analyses by Microtech Laboratories, Skokie, Ill.

The infrared spectrum (KBr disk) showed $\nu_{max.}$ 3440 cm.$^{-1}$, 1600 cm.$^{-1}$. Other data are in Table I.

4 - (7 - Phenylthio - 2 - indolylcarbonyl)morpholine (Vd)—The procedure described for V*a* was followed. The infrared spectrum ($CHCl_3$) showed $\nu_{max.}$ 3440 cm.$^{-1}$, 1600 cm.$^{-1}$. Other data are in Table I.

2 - (N,N - Dimethylaminomethyl) - 7 - phenyl thioindole (VIa)—Lithium aluminum hydride, 540 mg. (0.015 mole) was added in portions to 75 ml. of tetrahydrofuran. The mixture was stirred for 15 min. A solution of 2.0 Gm. (0.007 mole) of (*N,N*-dimethyl)-7-phenylthioindole-2-carboxamide in 25 ml. of tetrahydrofuran was slowly added and the mixture was stirred for an additional 30 min. Water was added dropwise with caution until no visible reaction could be detected, and the mixture was stirred for an additional 15 min. After filtration and evaporation of the solvent, the oily residue which remained was dissolved in ether. The ether solution was extracted with 30 ml. of 5% hydrochloric acid solution and the amine hydrochloride separated as an oil. The hydrochloride and the acidic aqueous layer were combined and made basic with 5% sodium hydroxide solution. After extracting with ether, the ethereal extracts were dried. Addition of an excess of ethereal hydrogen chloride precipitated the white hydrochloride salt as a solid. Recrystallization from 2-butanone gave 1.6 Gm. (74%) of crystalline hydrochloride melting at 164–166°. The infrared spectrum of the free amine ($CHCl_3$) showed $\nu_{max.}$ 3440 cm.$^{-1}$. The NMR spectrum ($CDCl_3$) of the hydrochloride showed a singlet at 2.75 δ (CH_3), a singlet at 4.45 δ (CH_2), a singlet at 6.6 δ (3 position proton), a multiplet at 7.2 δ (aromatic), and the NH and HCl proton absorption bands far downfield. Other data are in Table II.

2-(N,N-Diethylaminomethyl)-7-phenylthioindole (VIb)—The procedure described for VI*a* was followed. The infrared spectrum of the free amine ($CHCl_3$) showed $\nu_{max.}$ 3450 cm.$^{-1}$. The NMR spectrum of the free amine ($CDCl_3$) showed a triplet at 0.9 δ (CH_3), a quartet at 2.45 δ (CH_2), a singlet at 3.6 δ (CH_2), a singlet at 6.3 δ (3 position proton), a multiplet at 7.2 δ (aromatic), and an NH absorption band far downfield. Other data are in Table II.

7-Phenylthio-2-piperidinomethylindole (VIc)—The procedure described for VI*a* was followed. The infrared spectrum of the hydrochloride ($CHCl_3$) showed a $\nu_{max.}$ 3225 cm.$^{-1}$ and a broad NH absorption band in the region 2630 cm.$^{-1}$ and 2120 cm.$^{-1}$. The NMR spectrum of the hydrochloride ($CDCl_3$) showed multiplets at 1.8, 2.8, and 3.2 δ (piperidinomethylenes), a doublet at 4.3 δ (CH_2), a singlet at 6.55 δ (3 position proton), a multiplet at 7.2 δ (aromatic), and the NH and HCl proton absorption bands far downfield. Other data are in Table II.

2-Morpholinomethyl-7-phenylthioindole (VId)—The procedure described for VI*a* was followed. The infrared spectrum of the hydrochloride ($CHCl_3$) showed $\nu_{max.}$ 3225 cm.$^{-1}$ and a broad NH absorption band in the region 2630 cm.$^{-1}$ and 2120 cm.$^{-1}$. The NMR spectrum of the hydrochloride ($CDCl_3$) showed multiplets at 3.15 and 3.9 δ (morpholino methylenes), a singlet at 4.4 δ (CH_2), a singlet at 6.6 δ (3 position proton), a multiplet at 7.2 δ (aromatic), and the NH and HCl proton absorption bands far downfield.

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Keyphrases

2-(*N,N*-Dialkylaminomethyl)-7-phenylthioindoles—synthesis
IR spectrophotometry—structure
NMR spectrometry—identity

Absorption Kinetics of Griseofulvin in Man

By M. ROWLAND, S. RIEGELMAN, and W. L. EPSTEIN

The distribution and elimination kinetics of griseofulvin have been examined in man. The drug was administered intravenously in polyethylene glycol 300 *via* a saline drip. The plasma-concentration time curve was satisfactorily described by a bi-exponential equation, the half-life for the first exponent was found to be 0.70–1.7 hr. and the second 9.5–21.0 hr. It was also shown that the area under the plasma-concentration time curve was proportional to the dose administered. Griseofulvin was then given orally and from the plasma concentration, the kinetics of the absorption of this drug were calculated. Absorption was found to occur up to 30 hr. after administration and 27–72.5 percent of the dose was absorbed.

SINCE ITS introduction in 1958 (1) griseofulvin still remains the only effective oral antifungal agent for the treatment of dermatophytic infections and much interest has been shown in the absorption of this drug. It was the evidence of Sharp and Tomich (2), that the oral toxicity in rats and mice was much lower than that following intraperitoneal administration, which first led to the suggestion that griseofulvin may be incompletely absorbed. This was confirmed by Davis *et al.* (3) who recovered 30–64% of an oral dose in the feces of rats. Numerous workers have since shown inefficient absorption of griseofulvin in man and have increased the degree of absorption by reduction of particle size (4–9), concomitant administration of fat (10, 11), and the preparation of a supersaturated solution of the drug in polyethylene glycol (PEG) 400 (12). However, only a change in the degree of absorption has been demonstrated and in no case has the actual amount of drug absorbed been assessed in man. The present paper reports a study of the kinetics of distribution, metabolism, and excretion following intravenous administration of griseofulvin in man. A standard dose of micronized griseofulvin was then given orally and both the percentage of drug absorbed and the kinetics of absorption were determined.

EXPERIMENTAL

Intravenous Study—A parenteral product of griseofulvin in PEG 300 (30 mg./ml.) was prepared as follows. Griseofulvin (recrystallized from benzene) was suspended in PEG 300 (Union Carbide), the suspension flushed with N_2, and autoclaved for 30 min. at 121°. Autoclaving resulted in solution of the griseofulvin in the PEG 300. The concentration of griseofulvin was determined by accurately weighing about 1 Gm. of the preparation and diluting to 100 ml. with spectroscopic grade ethanol. A 100-fold dilution of the ethanolic solution was prepared in distilled water (approximately 3 mcg. griseofulvin/ml.) and the absorbance measured at 292 mμ. The concentration of griseofulvin was calculated by reference to a standard curve of griseofulvin in 1% v/v ethanol in water (no absorbance at 292 mμ was observed with PEG 300 after autoclaving and assaying in the above manner).

Five healthy Caucasian male volunteers, 35 to 48 years of age, were used in the study. Each subject received griseofulvin intravenously, usually at two dose levels on occasions at least 1 week apart. The dose of griseofulvin 90–180 mg. (3–6 ml. PEG 300) was administered over a 3–6 min. period with a Harvard infusion pump (model 600-900) and diluted into a saline drip just before entering the vein. The drip was run in at 15 ml./min. so as to ensure that no precipitation of griseofulvin occurred in the vein. In one subject the drug was infused at a known constant rate for 2 hr., with the saline drip adjusted accordingly. The infusion pump was fitted with a 10-ml. glass syringe which had previously been calibrated so that the volume of PEG preparation administered was accurately known. Blood samples (5 ml.) were usually drawn at 0, 0.5, 1, 2, 3, 4, 5, 7.5, 10, 24, 28, 32, 36, and 48 hr. after drug administration. The sample was collected in a heparinized test tube, centrifuged, and the plasma stored at 4° until required for analysis.

Oral Study—The same volunteers as in the intravenous study were used. Each subject ingested one 0.5-Gm. micronized griseofulvin tablet (McNeil, Grifulvin, batch No. EL 9066) on an empty stomach with approximately 200 ml. water and no food was taken for at least 3 hr. Blood samples (5 ml.) were drawn at 0, 1, 2, 3, 4, 5, 6, 8, 11, 24, 28, 32, 36, and 48 and occasionally up to 72 hr. after drug administration. At least 1 month later the study was repeated in four subjects. The blood samples were collected and stored as in the intravenous study. All subjects had the same diet and were instructed to avoid drugs and food which might interfere with griseofulvin assay (especially salicylates).

Spectrophotofluorometric Assay of Griseofulvin—The plasma concentration of griseofulvin was determined by the method of Fischer and Riegelman (13) with the following modification. Plasma (0.5–1.0 ml.) was pipeted into a centrifuge tube containing 1.5 ml. distilled water, and extracted with 7 ml. distilled diethyl ether. A 5-ml. aliquot of the ether

Received August 28, 1967, from the Department of Pharmacy, School of Pharmacy and the Department of Dermatology, School of Medicine, University of California, San Francisco Medical Center, San Francisco, CA 94122

Accepted for publication November 14, 1967.

This work was supported by grant number AI-05241 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

M. Rowland is grateful to the Wellcome Foundation for a travel grant in connection with this study.

extract was pipeted into a weighing bottle, evaporated to dryness, and redissolved in 5 ml. of triple distilled water. After reading the fluorescence of the sample, activation wavelength 315 mμ (uncorrected) and fluorescent wavelength 450 mμ (uncorrected), 0.2 ml. of 6 *M* sulfuric acid was added to 2 ml. of the sample and the fluorescence again noted. The change in fluorescence before and after the addition of acid was used to calculate the concentration of griseofulvin. A standard curve was obtained by reading the fluorescence of known aqueous solutions of griseofulvin (0.01, 0.02, 0.04, 0.1, 0.2 mcg./ml.) before and after the addition of acid.

In the intravenous studies, it was found necessary to be able to determine levels as low as 0.1 mcg. griseofulvin/ml. plasma. With the previous assay, this was not possible as blank values equivalent to 0.2–0.3 mcg. griseofulvin/ml. plasma were frequently obtained. However, griseofulvin does not fluoresce in strongly acidic aqueous media (pH less than 1.0), whereas very little change occurred with blank plasma samples. This modification in the assay procedure enabled the blank reading to be reduced to less than 0.03 mcg./ml.

Calculation of the Absorption of Griseofulvin—The absorption of drug at various times was calculated by the method of Loo and Riegelman (14).

$$\text{Amount absorbed } A_t = VpCp + k_{13}Vp\int_0^t Cpdt + VpC_T \quad \text{(Eq. 1)}$$

$$\text{and percent of absorbed drug } \left(\frac{A_t}{A_\infty} \times 100\right) = \frac{\left[C_p + k_{13}\int_0^t Cpdt + C_T\right]}{k_{13}\int_0^\infty Cpdt} \times 100 \quad \text{(Eq. 2)}$$

where A_t is the cumulative amount of drug absorbed at time t, and Cp, C_T are the corresponding plasma concentration and tissue concentration (calculated and expressed relative to the plasma) at time t, while $\int_0^t Cpdt$ and $\int_0^\infty Cpdt$ are the areas under the concentration-time curve up to time t and infinity, respectively; k_{13} is the rate constant for metabolism and excretion and Vp is the volume of the central compartment. The equation is essentially one of mass balance in that the amount of drug absorbed at any time is the summation of that in the central compartment ($VpCp$), that eliminated ($k_{13}Vp\int_0^t Cpdt$) and that in the tissue compartment (VpC_T). As with the Wagner-Nelson method (15) the value of A_t/Vp is calculated until an asymptote (A_∞/Vp) is reached indicating that absorption has ceased. The total amount of drug absorbed was calculated by the following equation:

$$\text{percent administered dose absorbed} = \frac{\text{dose}_{\text{i.v.}}}{\text{dose}_{\text{oral}}} \times \frac{\left(\int_0^\infty Cpdt\right)_{\text{oral}}}{\left(\int_0^\infty Cpdt\right)_{\text{i.v.}}} \times 100 \quad \text{(Eq. 3)}$$

where $\left(\int_0^\infty Cpdt\right)_{\text{oral}}$ and $\left(\int_0^\infty Cpdt\right)_{\text{i.v.}}$ are the total areas under the plasma concentration-time curve which result from the oral and intravenous dose, respectively.

RESULTS AND DISCUSSION

Intravenous Study—Before the absorption kinetics of any drug can be adequately described, the pharmacokinetics following an intravenous dose of that drug must first be defined. In the case of griseofulvin, this presents a problem in that it is a neutral insoluble molecule (aqueous solubility 10 mcg./ml.) and one would require 10–20 L. of saline in order to give a 100–200 mg. dose. This difficulty may be overcome by administering griseofulvin in PEG 300 whereby a concentration of 30 mg./ml. is achieved. Although several of the low molecular weight polyethylene glycols were found to be suitable solvents for griseofulvin, PEG 300 was chosen owing to its low toxicity in man and its acceptance in several parenteral pharmaceutical products. As much as 80 ml. of this solvent has been given intravenously, over a 4-month period, without any reported ill effects (16). However, there is the problem of precipitation of griseofulvin in the vein if the PEG preparation were injected directly, and for this reason the product was given *via* a rapidly flowing intravenous saline drip.

In all the intravenous studies, it was found that the griseofulvin plasma level curve could be described by a bi-exponential equation (*e.g.*, Fig. 1). The initial, or distribution, phase had a half-life of approximately 1 hr. while the second or elimination phase had a half-life varying from 9.5 to 21.0 hr. (Table I). This bi-exponential curve can be interpreted in terms of a two-compartmental open system (17), as depicted in Fig. 2. From the bi-exponential equation, the distribution rate constants k_{12}, k_{21}, and the metabolism and excretion rate constant, k_{13}, were evaluated together with the sizes of the central (Vp) and peripheral (V_T) compartments (Table II). Since the urinary excretion of griseofulvin is negligi-

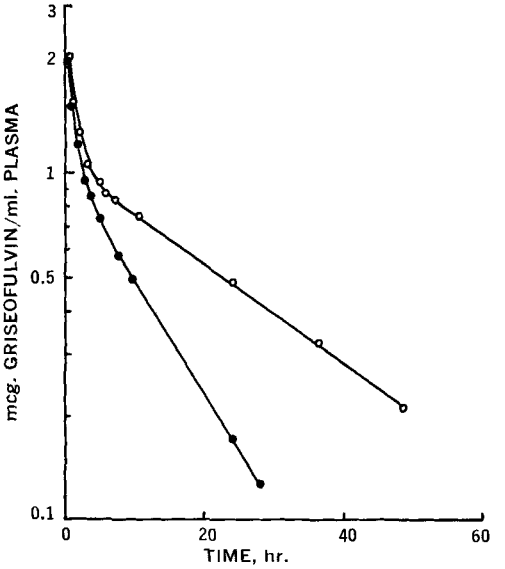

Fig. 1—Griseofulvin plasma levels following intravenous administration in man. Key: ○ subject A, 122 mg.; ●, subject C, 142 mg.

TABLE I—DATA OBTAINED FOLLOWING A SINGLE INTRAVENOUS INJECTION OF GRISEOFULVIN TO MAN

Subject[a]	Dose, mg.	Plasma Concn. Time Curve $Cp = Ae^{-\alpha t} + Be^{-\beta t}$	$0.693/\alpha$, hr.	$0.693/\beta$, hr.	Area, mcg. hr. ml.$^{-1}$	Area/ Dose
A	58	$0.75e^{-0.87t} + 0.56e^{-0.041t}$	0.80	17.0	14.7	0.254
	112	$1.60e^{-0.93t} + 1.06e^{-0.034t}$	0.75	21.0	33.7	0.276
B	142	$1.20e^{-0.60t} + 1.1e^{-0.045t}$	1.15	15.5	26.8	0.190
C	128	$1.35e^{-0.63t} + 0.93e^{-0.075t}$	1.10	9.3	14.5	0.113
	142	$1.55e^{-0.74t} + 1.0e^{-0.073t}$	0.95	9.5	15.8	0.111
D	99	$1.04e^{-0.70t} + 0.87e^{-0.039t}$	1.00	17.5	23.8	0.240
E	90	$0.90e^{-0.41t} + 0.62e^{-0.063t}$	1.70	11.0	12.1	0.134
	180	$1.7e^{-0.44t} + 1.1e^{-0.060t}$	1.55	11.5	22.1	0.123

[a] Subject weights: 73, 96, 73, 78, and 71 Kg., respectively.

TABLE II—VALUES OF CONSTANTS USED TO DEFINE THE PLASMA GRISEOFULVIN LEVELS FOLLOWING A SINGLE INTRAVENOUS INJECTION[a]

Subject	Dose, mg.	k_{12}, hr.$^{-1}$	k_{21}, hr.$^{-1}$	k_{13}, hr.$^{-1}$	Vp, ml.[b]	Vd_{ss}, ml.[c]
A	58	0.43	0.40	0.089	45,000	93,000
	112	0.49	0.39	0.080	46,000	102,000
B	142	0.25	0.31	0.085	62,000	112,000
C	128	0.27	0.30	0.154	56,000	107,000
	142	0.31	0.33	0.162	55,000	108,000
D	99	0.32	0.33	0.080	52,000	102,000
E	90	0.14	0.20	0.130	60,000	103,000
	180	0.17	0.21	0.130	64,000	116,000

[a] Calculations based on a 2-compartmental open system. [b] Vp = dose/A + B (see Table I). [c] $Vd_{ss} = Vp + V_T = Vp + k_{12}/k_{21}\ Vp$ (*Reference 21*).

ble, being less than 0.5% of the administered dose (18–20; personal observation), it may be ignored for the purpose of this model. Consequently the rate constant k_{13}, is almost exclusively associated with metabolism and it was found to have a twofold (0.08–0.16 hr.$^{-1}$) variation in this study (Table II).

In practice, there is probably at least a three-compartmental model, which would then give rise to a tri-exponential blood concentration-time curve. The first compartment into which griseofulvin distributes, depicted by a dotted circle in Fig. 2, can be considered to be the blood, liver, kidney, and other vascular rich tissues. This compartment is not seen, however, because the drug is infused over 3–6 min. so that griseofulvin can distribute into other tissues, such as muscle, skin and vascular rich fat tissue; consequently, the value of about 50,000 ml. for the central compartment (Vp) is much larger than one would have anticipated. However, as will be shown, absorption of griseofulvin is relatively slow compared to this initially expected, although not observed, distribution. Therefore, only a small error will occur in the calculation of the absorption of this drug by conceiving the body to be a two- instead of three-compartmental model. In contrast, since the observed apparent distribution phase is approximately 5 hr. following the i.v. dose (Fig. 1) any absorption rate calculations for griseofulvin must take this distribution phase into account, and therefore the model cannot be reduced further without introducing large errors.

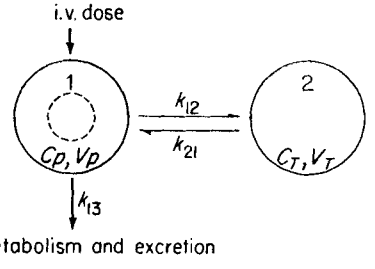

Fig. 2—Two-compartmental open model describing the distribution and elimination of griseofulvin in man. Key: 1, central compartment; 2, peripheral tissue compartment.

Regarding the second, or peripheral compartment, this can be considered to be the vascular poor fat tissue and is of significance owing to the high lipid solubility of griseofulvin. The size of this compartment is the same as that of the central compartment, so that the volume of distribution Vd_{ss} ($= Vp + V_T$; *Reference* 21) is approximately 100 L. indicating a marked extravascular concentration of this drug. It should be emphasized here, however, that the above model does not necessarily have any physiological meaning, but rather is the minimum model required to fit the data.

Another prior requirement to any quantitative absorption measurements from plasma level data is that the area under the plasma-concentration time curve must be proportional to the dose administered. This relationship was shown to hold with griseofulvin following intravenous doses of 90–180 mg. (Table I). In addition, the values of the rate constant k_{12}, k_{21}, and k_{13}, are essentially unchanged between studies showing that these parameters do not vary with time (Table II). These findings are contrary to that noted in rabbits when no relationship between an i.v. dose and the corresponding area could be established (13). The latter result may be explained by the very large interanimal

variation which these workers observed and the difficulty in using any rabbit repeatedly. In one study where they did manage to use an animal on three occasions, proportionality between dose and area appeared reasonably linear.

Oral Study—In the reported intravenous studies it was shown that, under the condition of administration, the data may be fitted by a two-compartmental open system model. Prior to proceeding to analyze data obtained after administering the dose orally, it was deemed essential to prove that the method of analyzing the resultant blood data will result in a true assessment of the absorption rate of griseofulvin. Therefore, griseofulvin was infused at a known zero-order rate (12.8 mg./min.) for 2 hr. From the values of the constants, k_{13}, k_{12}, and k_{21}, previously determined after a single rapid intravenous injection, the amount entering the body with time was calculated according to the method of Loo and Riegelman (14). The calculated absorption rate data are shown in Fig. 3 while in the insert are the plasma levels resulting from the single intravenous and zero-order infusion to that subject. The excellent fit between the known, 12.8 mg./min., and estimated, 12.4 mg./min. rate of infusion (absorption) of the drug shows that both the proposed model and the method of calculation for the absorption of this drug are acceptable.

Typical griseofulvin plasma levels following the oral dose of 0.5 Gm. micronized drug are shown in Figs. 4 and 5, together with their corresponding intravenous data. Without the intravenous study a long biological half-life might be calculated for this drug whereas in this study the levels are shown to be primarily the result of prolonged griseofulvin absorption. In fact, the dosage form exhibits the properties of a prolonged release product in that a plasma level is rapidly achieved and then maintained for 10–20 hr. Subsequent analysis of the area under the concentration time curves, following the oral and intravenous dose, shows that only 27–72% of the drug was absorbed (Table III). This not only demonstrates the variable and incomplete absorption of this drug but also illustrates one of the difficulties in trying to assess factors which might increase the extent of absorption. Obviously, the area under the plasma concentration-time curve for the person absorbing 72% drug can only increase by a further 39% whereas a 270% increase can occur with the person absorbing only 27%. Hence, the suggestion that the former was a poor responder to such factors could be misleading if, at the same time, the actual amount of drug absorbed was not determined. A further complication is that the absorption from

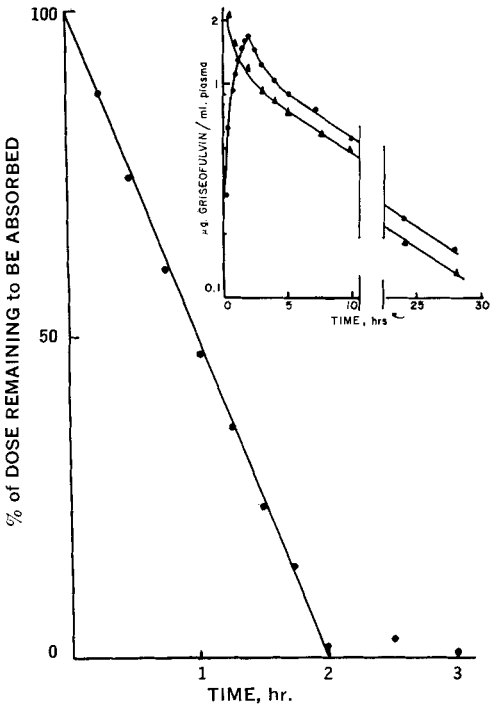

Fig. 3—Plot of the amount of griseofulvin remaining to be infused (absorbed) following a 2-hr. zero-order infusion (12.8 mg./min.) of drug in Subject C. Data calculated from plasma levels shown in insert. Key: ●, zero-order infusion; ▲, single intravenous injection (142 mg.).

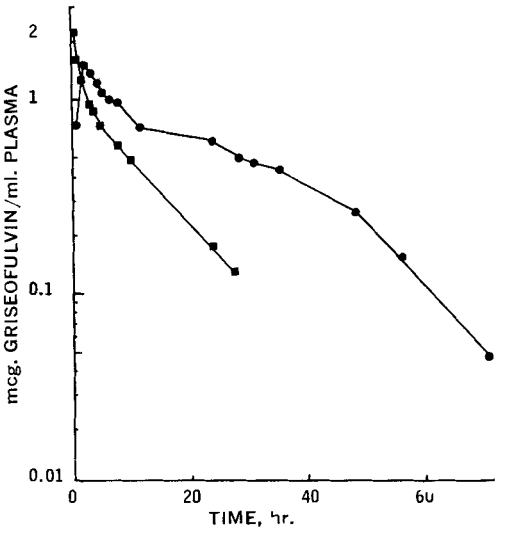

Fig. 4—Griseofulvin plasma levels following an oral dose of 0.5 Gm. micronized drug (●) and an intravenous injection, 142 mg. (■). Subject C.

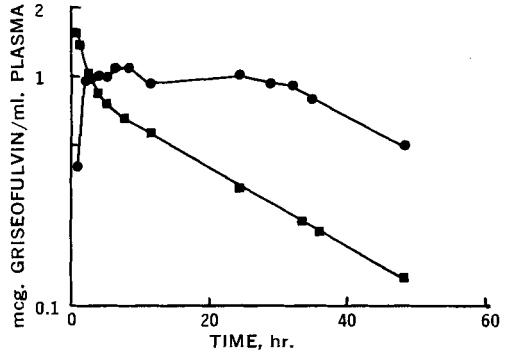

Fig. 5—Griseofulvin plasma levels following an oral dose of 0.5 Gm. micronized drug (●) and an intravenous injection, 99 mg. (■). Subject D.

TABLE III—AMOUNT OF GRISEOFULVIN ABSORBED AND THE CORRESPONDING AREA UNDER THE CONCENTRATION-TIME CURVE[a]

Subject	Date	Area, $\int_0^\infty Cpdt$	% Administered Dose Absorbed
A	12 May	52.7	38.5
B	12 May	58.5	62.4
	25 July	37.5	39.7
C	12 May	34.4	62.0
	25 July	32.4	58.0
D	12 May	58.4	48.5
	25 July	32.4	27.0
E	28 June	33.4	52.3
	25 July	46.0	72.5

[a] Following an oral dose of 0.5 Gm. micronized griseofulvin.

repeated doses of the same batch of griseofulvin is not reproducible resulting in large differences in some subjects (Table III).

Several workers have used the rationale that the area under the concentration time curve is a measure of "available griseofulvin" (4–6). This is a satisfactory response parameter only if a suitable crossover experiment is designed. However, Duncan *et al.* (6) noted a threefold variation in this parameter within a group of subjects on a standardized preparation of griseofulvin and inferred that this was due to absorption differences within this group. Obviously caution must be taken before relating area to absorption alone. In Table III both Subjects C and D gave an area value of 32.4 mcg. hr./ml. Whereas this indicates 58% drug absorption with the former, it only corresponds to 27% in the latter subject. The reason for this is clear from the following relationship:

$$\text{Area}\left(\int_0^\infty Cpdt\right) = \frac{\text{dose absorbed}}{k_{13}Vp} \quad \text{(Eq. 4)}$$

Therefore, a low area can be attributable to either poor absorption, fast metabolism (high k_{13}), or a high Vp value. With Subjects C and D, the volume Vp was essentially the same and the difference in amount absorbed for the same area is primarily due to the metabolic rate constant (k_{13}) being twice as large in Subject C as in Subject D (Table II). Consequently, within a given group on a single dose study differences in absorption can only be ascertained by reference to the intravenous dose.

Additional information was obtained by calculating the amount of griseofulvin absorbed at various times following drug ingestion (Fig. 6). From such data, it is seen that of the total drug absorbed approximately 50% occurs in the first 3–10 hr. Thereafter, absorption continues but much more slowly for a further 20–30 hr. suggesting that absorption probably takes place along the majority of the gastrointestinal tract.

Analysis for the absorption rate from the cumulative absorption plots demonstrates even more strikingly the rapid rate of absorption throughout the first 2–3 hr. following ingestion of griseofulvin (Fig. 7). After this period the absorption rate drops sharply even though only 7–28% of the drug has been absorbed. One possible explanation is that dissolution of the drug from griseofulvin particles may rate limit the absorption process in the upper part of the gut,

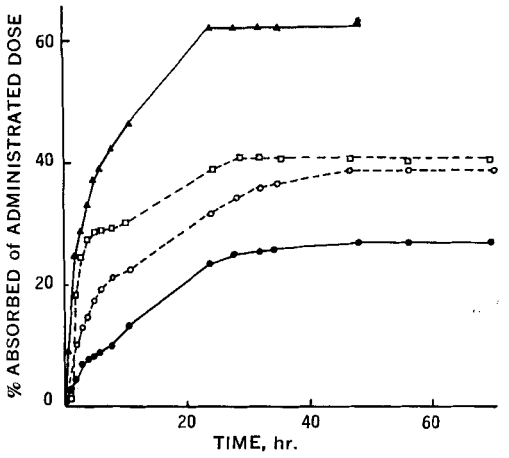

Fig. 6—Cumulative amount of griseofulvin absorbed following oral administration of 0.5 Gm. micronized griseofulvin. Key: ○, Subject A; □, Subject B; ▲, Subject C; ●, Subject D.

while further along intestinal absorption becomes the rate-limiting step. The finding by Davis *et al.* (3), that plasma griseofulvin levels fell while substantial amounts of griseofulvin was still present in the alimentary tract of rats, favors this hypothesis. Bedford *et al.* (18), suggested a simlar rationale for the absorption of this drug in cats. Alternately, the present results may have a physical rather than a biological explanation. Thus, it might be assumed that a certain fraction of the dose occurred as microfine readily dispersed particles which rapidly dissolved and was easily absorbed while a larger fraction was less readily available due to a much smaller surface for dissolution. Further studies are under way to distinguish between these two proposed explanations.

The present results confirm those of previous workers in showing that griseofulvin absorption is incomplete in man and was found to be 27–72% of the administered dose in this study. Absorption of

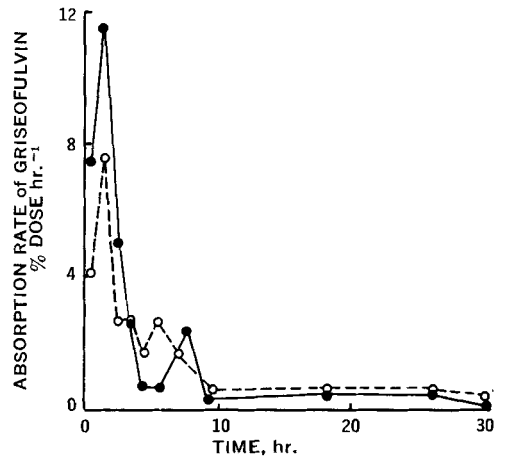

Fig. 7—Rate of absorption of griseofulvin after oral administration of 0.5 Gm. micronized drug to man. Key: ●, Subject C; ○, Subject D.

this drug was slow and whether it is due to physical or physiological factors has yet to be ascertained. Polyethylene glycol 300 has proved to be a useful solvent for griseofulvin and this and other low molecular weight polyethylene glycols might enable the pharmacokinetics of other insoluble neutral drugs to be evaluated in man.

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Keyphrases

Griseofulvin—absorption
Absorption kinetics—griseofulvin
Distribution, excretion—griseofulvin
Plasma—griseofulvin analysis
Fluorometry—analysis

Analgesic and Anti-Inflammatory Evaluation of Thymotic Acid and Certain Homologs

By FRED J. MAROZZI and MARVIN H. MALONE

Ortho-thymotic acid and nine homologs were submitted for general, analgesic, and antistress evaluations in the rat. While all appeared to be nonspecific central nervous system depressants, only 2-methyl-5-*tert*-butylsalicylic acid appeared to have significant analgesic ability in the rat tail flick test. 2-Hydroxy-4-methyl-5-isopropylbenzoic acid, 2-hydroxy-4-isopropyl-6-methylbenzoic acid, and 2-hydroxy-3-methyl-6-isopropylbenzoic aicd were superior to sodium salicylate and inferior to morphine sulfate in providing significant protection from stress induced by unilateral hind leg tourniquets. The latter two compounds were active in suppressing the acute inflammatory process produced by pedal injection of carrageenin, but were ineffective against chronic inflammation induced by the *Mycobacterium butyricum* adjuvant.

O'BRIEN AND Thoms (1) investigated the antipyretic and analgesic activity of the sodium salts of *o*- and *p*-thymotic acid in 1958. Von Kaulla (2) demonstrated in 1965 that *o*-thymotic acid possessed significant fibrinolytic activity. More recently, Rader and Wulf (3) investigated the fibrinolytic activity of nine homologs of *o*-thymotic acid on the human and cat fibrinolytic systems and were able to show that several compounds possessed significant fibrinolytic activity beyond that of *o*-thymotic acid. The present investigation is concerned with the analgesic and general evaluation of certain of these homologs along with *o*-thymotic acid, itself.

EXPERIMENTAL

The compounds shown in Table I were obtained in limited amounts from the Division of Medicinal Chemistry of the University of Connecticut. This group was selected so as to reveal possible structure–activity relationships. Table I also summarizes the fibrinolytic activity of the various homologs as determined by Rader and Wulf (3, 4).

Qualitative Screening—The hippocratic method of Malone and Robichaud (5) was used as a prelim-

Received September 18, 1967, from the Division of Pharmacology, Pharmacy Research Institute, University of Connecticut, Storrs, CT 06268
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The authors wish to thank Dr. Harvey R. Kaplan for his assistance in carrying out certain phases of this work.

this drug was slow and whether it is due to physical or physiological factors has yet to be ascertained. Polyethylene glycol 300 has proved to be a useful solvent for griseofulvin and this and other low molecular weight polyethylene glycols might enable the pharmacokinetics of other insoluble neutral drugs to be evaluated in man.

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Keyphrases

Griseofulvin—absorption
Absorption kinetics—griseofulvin
Distribution, excretion—griseofulvin
Plasma—griseofulvin analysis
Fluorometry—analysis

Analgesic and Anti-Inflammatory Evaluation of Thymotic Acid and Certain Homologs

By FRED J. MAROZZI and MARVIN H. MALONE

Ortho-thymotic acid and nine homologs were submitted for general, analgesic, and antistress evaluations in the rat. While all appeared to be nonspecific central nervous system depressants, only 2-methyl-5-*tert*-butylsalicylic acid appeared to have significant analgesic ability in the rat tail flick test. 2-Hydroxy-4-methyl-5-isopropylbenzoic acid, 2-hydroxy-4-isopropyl-6-methylbenzoic acid, and 2-hydroxy-3-methyl-6-isopropylbenzoic aicd were superior to sodium salicylate and inferior to morphine sulfate in providing significant protection from stress induced by unilateral hind leg tourniquets. The latter two compounds were active in suppressing the acute inflammatory process produced by pedal injection of carrageenin, but were ineffective against chronic inflammation induced by the *Mycobacterium butyricum* adjuvant.

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TABLE I—THYMOTIC ACID DERIVATIVES

Compd.	Chemical Name	Observed M.p., °C.	Fibrinolytic Concn., M[a]
I	2-Methyl-5-ethylsalicylic acid	162–163	>0.04
II	2-Methyl-5-isopropylsalicylic acid[b]	122–128	0.02
III	2-Methyl-5-*sec*-butylsalicylic acid	169	0.005
IV	2-Methyl-5-[2-(3-methyl-butyl)]salicylic acid	166–167	0.03
V	2-Methyl-5-*tert*-butylsalicylic acid	182–183	0.0075
VI	2-Methyl-5-*tert*-amylsalicylic acid	176	0.035
VII	2-Methyl-4-hydroxy-5-isopropylbenzoic acid[c]	156–160	>0.04
VIII	2-Hydroxy-4-isopropyl-6-methylbenzoic acid	115–122	0.02
IX	2-Hydroxy-4-methyl-5-isopropylbenzoic acid	141–144	0.03
X	2-Hydroxy-3-methyl-6-isopropylbenzoic acid[d]	142–145	0.035

[a] Concentration to obtain ≥ 80% dissolution of clot. [b] *Ortho*-thymotic acid. [c] *Para*-thymotic acid. [d] *Ortho*-carvacrotic acid.

inary gross observational screen of all test compounds. Adult albino Holtzman rats (320–500 Gm.) were injected intraperitoneally using at least three logarithmetically spaced dosage levels (at least one of which was lethal), and qualitative and semiquantitative symptomatology was observed and noted on a standardized work sheet (5) at the required times.

Tail Flick Studies—A conduction dolorimeter (Metro Scientific Inc., Long Island, N.Y.) was used to measure the rat tail flick response to a thermal stimulus. The apparatus was modified by the addition of a photo cell in series with a timer in order to provide more accurate recordings of reaction times. Adult albino rats of the Wistar strain (Steinhilber) weighing between 125–150 Gm. were used for the initial analgesic evaluation of each test compound. Each compound was tested on at least five animals per dosage level. All test compounds and the two reference standards (morphine sulfate and sodium salicylate) were injected intraperitoneally in 0.25% agar after determination of control reaction times. Reaction times were again determined at +15, 30, 45, 60, and 120 min. after injection. A reaction time of 20 sec. was considered indicative of 100% analgesia and any animal failing to respond within 20 sec. was manually removed from the dolorimeter. Percent analgesia was calculated for each animal according to the following formula:

$$\% \text{ analgesia} = \left[\frac{R_x - C_0}{20 - C_0}\right] 100$$

where R_x = reaction time in sec. of an individual rat at time x; C_0 = the control reaction time in seconds of the same individual at time 0. Due to the limited amounts of drug available, the compounds were screened for analgesic activity at only one dosage level (100 mg./Kg.). This dosage was considered to be sublethal and without major CNS effects considering the hippocratic screening data.

Hepatic Glutathione Depletion—The methods used were essentially those described by Cooper *et al.* (6). Adult male rats of the Wistar strain (Steinhilber) ranging in weight between 260–375 Gm. were injected intraperitoneally with the test compounds dissolved or suspended in 0.25% aqueous agar. One-half hour after this injection, unilateral right hind limb tourniquets were tied around the thigh muscle proximal to the hip joint using heavy suture thread (Dermalon skin sutures, size 3). Food and water were allowed *ad libitum*. Four hours after the application of the tourniquets the rats were sacrificed by cranio-vertebral dislocation and peripheral portions of the central lobe of the liver (approximately 2 Gm.) were excised immediately and frozen using dry ice. Sacrifice of all animals was arranged so as to occur between the hours of 3:30 and 7:30 p.m. to control for the diurnal variation in liver sulfhydryl content reported by Beck (7). Hepatic glutathione (GSH) levels were determined amperometrically according to the method described by Benesch and Benesch (8, 9). Filtrates of the liver samples were prepared by homogenizing 500 mg. wet weight of liver in 10 ml. of 2.5% sulfosalicylic acid, followed by centrifugation and filtration. Aliquots of each filtrate were then titrated using 0.0005 *N* silver nitrate and a rotating platinum electrode (10).

Acute Antiphlogistic Evaluation—Using methods similar to those described by Winter *et al.* (11), nonfasted male and female Wistar strain rats (Steinhilber) were randomized into 12 cages (2 males and 2 females per cage) to which the test treatments were randomly assigned. Volume changes in the rats' paws were recorded using a modified plethysmographic apparatus similar to that described by Harris and Spencer (12). All drugs were administered orally as aqueous suspensions in 0.25% agar to hydrated animals. One hour after drug administration, 0.05 ml. of a 1.0% suspension of carrageenin in sterile physiological saline was injected into the plantar aponeurosis of the left hind paw. Volume changes in the injected paw were recorded 3 hr. later. The antiphlogistic properties of the three compounds tested were compared statistically to a series of reference agents using Duncan's multiple-range test (13).

Chronic Anti-Inflammatory Activity—Young adult male albino rats (Steinhilber) were used in this study. Foot thickness was determined daily with a vernier caliper for both the injected and contralateral paws. The inflammatory state was induced by the injection of 0.05 ml. of a suspension of desiccated *Mycobacterium butyricum* cells in light liquid petrolatum prepared according to the method of Nuss (14, 15). The test drugs were administered orally daily (100 mg./Kg.) as aqueous suspensions in 0.25% agar for a period of 13 days after which drug treatment was discontinued. Measurements of body weight and foot thickness were continued for another 17 days. Both food and water were freely accessible throughout the test period, and consumption records were maintained. The percent inhibition of the inflammatory response on days 13 and 30 of the test period was calculated according to the following formula (16):

$$\% \text{ inhibition} = 100\left[\frac{1 - (a - x)}{(b - y)}\right]$$

where y = mean injected foot thickness of control rats before injection; b = mean injected foot thickness of control rats on a particular day (13th or 30th); x = mean injected foot thickness of treated rats before injection, and a = mean injected foot thickness of treated rats on a given day (13th or 30th). Thirty animals constituted the control group, and five rats were used for each of the test treatments.

RESULTS AND DISCUSSION

Qualitative Screening—All of the compounds tested were qualitatively similar in their gross pharmacologic effects. Dose-response patterns for decreased motor activity were seen as well as generalized central nervous system depression. The latter appeared to be nonspecific and was characterized by decreased spontaneous motor activity, ataxia, and decreased skeletal muscle tone. Analgesia was evaluated subjectively by observing an animal's response to sharp pressure applied to one of the hind paws by the operator's thumb nail. Analgesia, when noted, was accompanied by ataxia. Compound I differed from all others in that some delayed onset stimulatory activity was noted. *Para*-thymotic acid (VII), in addition to CNS depression, induced personality changes (fearful reactions) at all doses studied (56.2, 100, 177.8, 316.2 mg./Kg.). The following gross estimates of the intraperitoneal lethal dosages were made: compound VI and VII: $\leqslant 100$; compound V, VIII, IX, and X: $\leqslant 237$; compound II and IV: $\leqslant 316$; compound III: $\leqslant 562$; compound I: $\leqslant 1{,}000$ mg./Kg.

Tail Flick Studies—As shown in Table II, the rat tail flick evaluation indicated either a lack of or a very low order of analgesic activity for all compounds tested with the exception of morphine sulfate. This is consistent with the fact that thermal methods have proven generally unsatisfactory for the detection of nonnarcotic analgesics except when these drugs have been administered in very high doses.

Decreased reaction time from preinjection values for the 0.25% agar control animals is indicated by a tendency for "negative analgesia" as shown in Fig. 1.

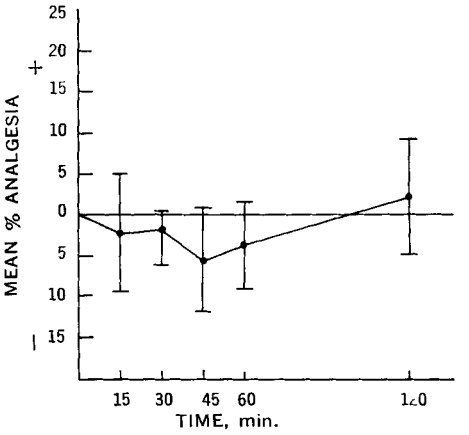

Fig. 1—Analgesic effect of 25 control rats receiving 0.15% aqueous agar solution intraperitoneally, 5 ml./Kg. I, 95% C.L.

This is indicative of a conditioning phenomenon whereby animals prematurely react to sensations of warmth rather than at the actual painful stimulus. Individual variation among control rats (as indicated by 95% C.L. in Fig. 1) was quite small and remained constant at the various times of measurement. In contrast, large variations were observed in responses after the test compounds. These large variances are consistent with similar results which have been reported in the literature (17). The very wide range of values which was seen following 10 mg./Kg. of morphine sulfate, a very potent analgesic, indicated that the extreme values encountered with the test homologs need not be considered aberrant. Of all the test compounds, only compound V may have some analgesic potential since significant analgesia was detected up through the 60-min. readings. In contrast, compound IV definitely appeared to facilitate the pain response, and compound III, likewise.

The study of O'Brien and Thoms (1) concluded that *o*- and *p*-thymotic acid possessed both analgesic and antipyretic activity. Using Davies' modification of the classical D'Amour-Smith technique (17), they reported sodium *o*-thymotate to possess 15 times the analgesic activity of sodium salicylate and

TABLE II—ANALGESIC SCREENING OF TEST COMPOUNDS AND REFERENCE ANALGESICS

Treatment	Mean % Analgesia +15	+30	+45	+60	+120 min.
Control[a]	−2	−2	−6	−4	+2
Morphine sulfate[b]	+68[c]	+86[c]	+88[c]	+80[c]	+87[c]
Sodium salicylate	+3	−4	−4	−5	−4
Sodium phenylbutazone	+1	−2	−2	−2	−1
I	−4	−7[d]	−7[d]	−5	−4
II	+1	−2	−4	−1	+2
III	+3	+11[c]	−6	−13[d]	−15[d]
IV	+8[c]	+7[c]	−15[d]	−20[d]	−25[d]
V	+21[c]	+45[c]	+36[c]	+34[c]	−5
VI	+3	−5	−1	−2	−6[d]
VII	−3	−6	−5	−4	−7[d]
VIII	−1	+5[c]	+1	−3	−2
IX	−4	−7[d]	−5	−5	−6[d]
X	−1	−9[d]	−8	−6	−6[d]

[a] The mean reaction times in seconds (observed range) for the 25 control animal are: preinjection: 4.58 (2.7–10.2); +15 min.: 4.46 (2.9–9.0); +30 min.: 4.41 (2.5–7.6); +45 min.: 3.94 (2.7–6.5); +60 min.: 4.22(2.4–5.7); and +120 min.: 3.81 (2.7–6.0). [b] The intraperitoneal dose of morphine sufate was 10 mg./Kg., while that for all of the other compounds was 100 mg./Kg. [c] Significant analgesia in that the mean percent analgesia is greater than the mean +2 standard errors for controls. [d] Significant facilitation of pain response in that the mean percent analgesia is less than the mean −2 standard errors for controls.

TABLE III—COMPARISON OF TEST TREATMENTS TO TOURNIQUET CONTROLS

Treatment	Dosage, mg./Kg.	N	Mean GSH Level, mg. %	Calcd. F	Significance Level, P
Tourniquet	—	10	145.5	—	—
No tourniquet	—	10	276.0	62.51	<0.001
Morphine sulfate	10	10	269.5	33.96	<0.001
Sodium salicylate	400	10	194.2	5.74	0.025–0.05
I	100	10	142.2	0.05	>0.50
II	100	9	168.1	2.05	0.10–0.20
III	100	10	158.8	0.61	0.25–0.50
IV	100	9	178.9	3.44	0.05–0.10
V	100	9	150.3	0.08	>0.50
VI	100	9	160.3	0.96	>0.50
VII	100	10	193.2	4.30	0.025–0.05
VIII	100	10	212.0	17.02	<0.001
IX	100	10	222.8	15.10	0.001–0.005
X	100	10	221.5	27.02	<0.001

one-tenth the activity of morphine sulfate. In the present study neither *o*- nor *p*-thymotic acid were found to possess significant analgesic activity. The discrepancy between these results and those of O'Brien and Thoms appears to lie in the manner chosen for the evaluation of the tail flick data.

Hepatic Glutathione Depletion—The effectiveness of unilateral hind leg tourniquets in inducing hepatic depletion of glutathione was clearly demonstrated. Statistical comparison of animals receiving 5 ml./Kg. of a 0.25% aqueous agar solution and no tourniquet (no pain) to animals receiving the same dose of agar and unilateral hind leg tourniquets for 4 hr. revealed a highly significant difference between the two means (Table III). In all comparisons, the variances of the control animal populations and the respective test animal populations were not significantly different at $p = 0.05$ (F test).

When the test compound means were compared to tourniquet means, morphine sulfate (10 mg./Kg.), sodium salicylate (400 mg./Kg.), and compounds VII, VIII, IX, and X (100 mg./Kg.) could be considered active ($p < 0.05$). Although tested only at one dosage level, the degree of effectiveness relative to each other can be estimated from the levels of significance presented in Table III. Morphine and compounds VIII, IX, and X all have definite protective capacity, as compared to the moderate activity of sodium salicylate and the slight but detectable effects of compound VII. Considering mg./Kg. dosage levels, compounds VIII, IX, and X are clearly more potent than sodium salicylate but less potent than morphine sulfate. The highly significant protection afforded by morphine indicated the nonspecificity of protection from hepatic GSH depletion as a biochemical metameter for nonnarcotic analgesia. Takesue and Miya (18) have reported protective activity with morphine sulfate (10 mg./Kg.) and phenobarbital (50 mg./Kg.), and concluded that protection from liver GSH depletion is not specific for the nonnarcotic analgesics. The finding that sodium salicylate was able to provide some protection from GSH depletion is significant inasmuch as this compound has been reported (18) as being capable of inducing hepatic GSH depletion (in small doses) in the absence of stress (tourniquets). This observation may be a reflection of the diurnal variation in rat sulfhydryl reported by Beck (7) in 1958.

The exact mechanisms whereby a drug may prevent stress-induced hepatic GSH depletion are at present unknown. It has been postulated that activation of the adrenal medulla under stress with resulting release of epinephrine and increased intermediary metabolism may be responsible for this phenomenon (19). This seems to be supported by Krimsky and Racker's finding that glutathione functions as a prosthetic group in glyceraldehyde-3-phosphate dehydrogenase (20), however, the mechanism (or mechanisms) by which a drug may interfere with the normal hepatic glutathione depletion induced by stress are not immediately accessible from current data and literature reports. Hepatic GSH depletion appears to be more indicative of biochemical alterations induced by stress rather than a true metameter of pain, however, the stress component in the human experience of pain cannot be neglected.

It is interesting to observe that compound V which appeared most active of the homologs in the rat tail flick evaluation (as well as equivocally active compounds III and IV) did not prevent stress-induced GHS depletion. Only compound VIII of the homologs appeared active by both techniques (equivocal: rat tail flick; highly significant: GSH depletion protection).

Acute Antiphlogistic Evaluation—Table IV shows a division of the various treatments employed in this study into subsets according to their relative activity at the administered doses. This Table indicates that while chlorpromazine, cryogenine, papain, compound VIII, and compound X were all significantly different from the controls at a dosage of 100 mg./Kg. orally ($p < 0.05$), indomethacin was clearly the most potent compound tested (5 mg./Kg.).

Chronic Anti-Inflammatory Activity—The results of a chronic anti-inflammatory screen of compounds VIII and X are presented graphically in Figs. 2 and 3. Compound IX was not tested by this procedure due to its lack of activity during the acute anti-inflammatory testing (Table IV) and general scarcity of the compound. Figure 2 indicates that compound VIII was of no benefit in reducing the inflammatory state and, in fact, appeared to aggravate the inflammatory response. Compound X (Fig. 3) did not affect the inflammatory state since the plotted test data closely parallel that of the large control population. Figure 4 depicts the anti-inflammatory response profile of acetylsalicylic acid, an agent of known effectiveness. None of the test agents significantly affected food and water consumption and rate of weight gain. The percentage inhibition of the increase in thickness of the injected foot of the animals treated with compound VIII was −10 and

TABLE IV—MULTIPLE-DRUG COMPARISONS OF ANTI-INFLAMMATORY ACTIVITY IN RATS WITH ACUTE CARRAGEENIN-INDUCED PEDAL EDEMA

Treatment	Oral Dosage, mg./Kg.	Mean Increase in Paw Volume, ml.	Significant Subsets[a]
Control	—	0.45(0.37–0.56)[b]	A
Control	—	0.44(0.39–0.54)	A
IX	100	0.46(0.38–0.64)	A
X	100	0.40(0.34–0.54)	B
VIII	100	0.36(0.31–0.40)	B
Papain	100	0.35(0.29–0.40)	B
Cryogenine[c]	100	0.27(0.19–0.36)	B
Indomethacin	5	0.22(0.18–0.31)	B
Chlorpromazine	100	0.21(0.05–0.33)	B

[a] The treatments effects represented by A and B differ significantly from each other ($p < 0.05$); however, the treatments within these subsets do not differ significantly ($p \geq 0.05$) from one another. [b] The figures within the parentheses represent the observed range of the four experimental values. [c] An alkaloid from *Heima salicifolia* Link and Otto recently shown to have anti-inflammatory potency (15).

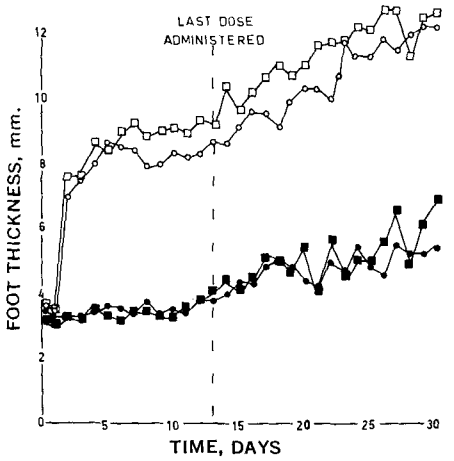

Fig. 2—Effect of 2 weeks of daily oral administration of 100 mg./Kg. of compound VIII on the development of adjuvant-induced polyarthritis. Key: ○, mean width of injected foot (controls); ●, mean width of contralateral foot (controls); □, mean width of injected foot (VIII); ■, mean width of contralateral foot (VIII).

−7% on Days 13 and 30, respectively. The calculated values for compound X were −0.3% on Day 13 and +1% on Day 30. The effectiveness of aspirin in inhibiting the inflammatory response in this study is indicated by a +33% inhibition on Day 13 and a +29% inhibition on Day 30.

SUMMARY

General hippocratic screening indicated that *o*-thymotic acid and nine homologs possessed some CNS depressant activity of a rather nonspecific nature. Rat tail flick data indicated a general lack of morphine-like analgesic activity, although 2-methyl-5-*tert*-butylsalicylic acid appeared to have some potential. Evidence obtained from hepatic glutathione depletion assays indicated that 2-hydroxy-4-isopropyl-6-methylbenzoic acid, 2-hydroxy-4-methyl-5-isopropylbenzoic acid, and 2-hydroxy-3-methyl-6-isopropylbenzoic acid may have some antistress effects being more potent than sodium salicylate and less potent than morphine sulfate in this regard. Only 2-hydroxy-4-isopropyl-6-methylbenzoic acid and 2-hydroxy-3-methyl-6-isopropylbenzoic acid were shown to have some acute anti-inflammatory activity, although their potency was clearly inferior

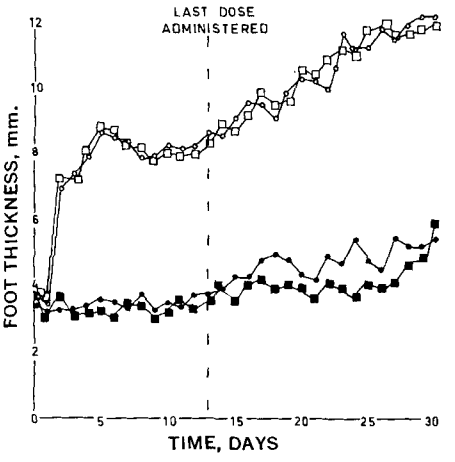

Fig. 3—Effects of 2 weeks of daily oral administration of 100 mg./Kg. of compound X on the development of adjuvant-induced polyarthritis. Key: ○, mean width of injected foot (controls); ●, mean width of contralateral foot (controls); □, mean width of injected foot (X); ■, mean width of contralateral foot (X).

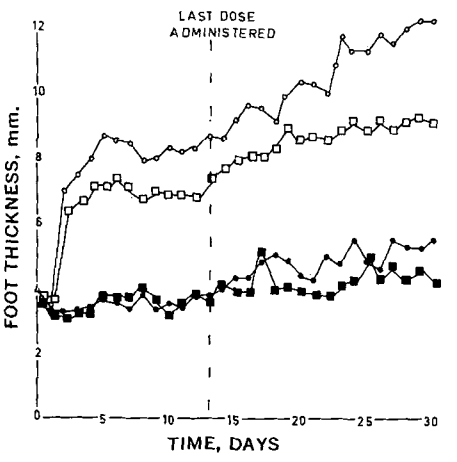

Fig. 4—Effects of 2 weeks of daily oral administration of 100 mg./Kg. of aspirin on the development of adjuvant-induced polyarthritis. Key: ○, mean width of injected foot (controls); ●, mean width of contralateral foot (controls); □, mean width of injected foot (aspirin); ■, mean width of contralateral foot (aspirin).

to that of indomethacin and they were without effect on the chronic inflammatory state induced by the mycobacterium adjuvant. No apparent structure-activity relationships could be deduced from the above studies.

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Keyphrases

Thymotic acid and homologs—activity
Analgesia rat tail flick method
Antiphlogistic activity—pedal carrageenin injection
Anti-inflammatory activity-*Mycobacterium butyricum* cell injection.
Hepatic glutathione depletion—hind leg tourniquet

Lag Time Before Essentially Constant Urinary Excretion Rate Is Attained

By JOHN G. WAGNER and JACK I. NORTHAM

If a substance is continuously infused intravenously at a constant rate, the plasma concentration will increase until it reaches an asymptotic concentration. If the urinary excretion rate of the substance is directly proportional to its plasma concentration, the urinary excretion rate will become essentially constant as the asymptotic plasma concentration is approached. Analogously, if a metabolite is formed at a constant rate, due to saturation of an enzyme system metabolizing the drug, the plasma concentration of the metabolite would be expected to approac hsome asymptotic concentration. If the urinary excretion rate of the metabolite is directly proportional to its plasma concentration, the urinary excretion rate of the metabolite would become essentially constant as the asymptotic plasma concentration is approached. Equations were derived to estimate the lag time between initiation of the maintained constant input rate to the plasma compartment and the time when, for all practical purposes, the asymptotic plasma concentration and the constant urinary excretion rate may be considered to have been reached for both the one- and two-compartment open models. The theoretical expectation is that, if the cumulative excretion curve is nearly linear, then there must be an appreciable negative intercept when the line is extrapolated back to zero time. It is theoretically impossible for a cumulative urinary excretion curve to be linear and the line extrapolate through the origin corresponding to zero excretion at zero time.

IF A SUBSTANCE is continuously infused intravenously at a constant rate the blood (serum or plasma) concentration will increase until it reaches an asymptotic value (1). However, as the curves of Rescigno and Segre (1) and Wagner and Nelson (2) illustrate, it requires an appreciable time to approach the asymptotic concentration. If the urinary excretion rate of the substance is directly proportional to its plasma concentration, then the urinary excretion rate will become essentially constant, and the cumulative urinary excretion curve will become essentially linear, as the asymptotic plasma concentration is approached.

Analogously, if a metabolite is formed at a constant rate, due to saturation of the enzyme system metabolizing the drug, the plasma concentration of the metabolite would be expected to approach some asymptotic value,

Received November 2, 1967, from the Upjohn Company, Kalamazoo, MI 49001
Accepted for publication February 13, 1968.

to that of indomethacin and they were without effect on the chronic inflammatory state induced by the mycobacterium adjuvant. No apparent structure-activity relationships could be deduced from the above studies.

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Lag Time Before Essentially Constant Urinary Excretion Rate Is Attained

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Analogously, if a metabolite is formed at a constant rate, due to saturation of the enzyme system metabolizing the drug, the plasma concentration of the metabolite would be expected to approach some asymptotic value,

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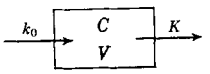
after a certain time. If the urinary excretion rate of the metabolite is directly proportional to its plasma concentration, then the urinary excretion rate of the metabolite will become essentially constant, and the cumulative urinary excretion curve will become essentially linear, as the asymptotic plasma concentration of the metabolite is approached. In the case of a metabolite some finite additional time would also be required for absorption of the drug and build up of blood concentration of unchanged drug which would be necessary before an enzyme system couid become saturated. This additional time would have to be added to the lag time between initiation of the constant (zero-order) formation rate and the time the asymptotic plasma concentration is approached, to estimate the total time required for the cumulative urinary excretion curve to become essentially linear.

The two most common pharmacokinetic models, which apply to the above situations, are the one- and two-compartment open models. One purpose of this report is to show how one may estimate for these models the time lag necessary between the start of the zero-order input into the plasma compartment and the time when, for all practical purposes, the asymptotic plasma concentration and the constant urinary excretion rate is attained. Another purpose is to show how the maximum excretion rate may be estimated before it is attained if the rate constant for elimination is known.

EXPERIMENTAL

One-Compartment Open Model

Intravenous Infusion at a Constant Rate—The model is shown in Scheme I. Here k_0 is the constant (zero-order) infusion rate with dimensions mass/time, V is the apparent volume of distribution, and C is the plasma concentration of the substance infused at time t, and K is the first-order rate constant for elimination by all processes. For this model the appropriate classical equation *during the infusion* is:



Scheme I

$$C = \frac{k_0}{VK}(1 - e^{-Kt}) \qquad \text{(Eq. 1)}$$

where t is the time from the start of the infusion. If urinary excretion rate is directly proportional to plasma concentration, then

$$dA_u/dt = fVKC \qquad \text{(Eq. 2)}$$

where dA_u/dt is the urinary excretion rate and f is the fraction of substance reaching the plasma compartment which is ultimately excreted in the urine. Substitution of Eq. 1 into Eq. 2 and simplifying gives

$$dA_u/dt = fk_0(1 - e^{-Kt}) \qquad \text{(Eq. 3)}$$

Let t_a be the time that the excretion rate reaches some proportion, p, of the maximum rate, fk_0. Then,

$$dA_u/dt = p.f.k_0 \qquad \text{(Eq. 4)}$$

when

$$e^{-Kt_a} = 1 - p \qquad \text{(Eq. 5)}$$

Taking logarithms of both sides of Eq. 5 we find

$$t_a \simeq 3/K \qquad \text{when } p = 0.95 \qquad \text{(Eq. 6)}$$

$$t_a \simeq 4.6/K \qquad \text{when } p = 0.99 \qquad \text{(Eq. 7)}$$

If one substitutes $\ln 2/t_{1/2}$ for K, where $t_{1/2}$ is the half-life, into Eq. 5, takes logarithms, and rearranges, one finds

$$t_a = -1.443 \ln (1 - p) \cdot t_{1/2} \qquad \text{(Eq. 8)}$$

Hence,

$$t_a/t_{1/2} = 4.322 \qquad \text{when } p = 0.95 \qquad \text{(Eq. 9)}$$

and,

$$t_a/t_{1/2} = 6.645 \qquad \text{when } p = 0.99 \qquad \text{(Eq. 10)}$$

Hence, the time, t_a, for the excretion rate to reach the same proportion, p, of the maximum rate, fk_0, and the plasma concentration to reach the same proportion, p, of the asymptotic value, k_0/VK, is directly proportional to the half-life of elimination if the one-compartment open model applies.

Integration of Eq. 3 yields

$$A_u = fk_0\left[t - \left(\frac{1 - e^{-Kt}}{K}\right)\right] \qquad \text{(Eq. 11)}$$

where A_u is the cumulative amount excreted in the urine to time t. Substitution of values for f, k_0, K, and various t values would yield a curve of A_u *versus* t by application of Eq. 11. If one chose two points, $(A_u^{T_2}, T_2)$ and $(A_u^{T_1}, T_1)$, on this curve, joined them with a straight line, then extrapolated the line back to time zero corresponding to an intercept $(A_u)_{t=0}$, then the slope, $\Delta A_u/\Delta t$, of the line may be shown to be

$$\frac{\Delta A_u}{\Delta t} = fk_0\left[1 - \left\{\frac{e^{-KT_1} - e^{-KT_2}}{K(T_2 - T_1)}\right\}\right] \qquad \text{(Eq. 12)}$$

The value of the intercept may be shown to be:

$$(A_u)_{t=0} = \frac{fk_0}{K}\left[\left(\frac{T_2e^{-KT_1} - T_1e^{-KT_2}}{T_2 - T_1}\right) - 1\right] \qquad \text{(Eq. 13)}$$

The equation of such a line will be:

$$\hat{A}_u = \left(\frac{\Delta A_u}{\Delta t}\right)t - (A_u)_{t=0} \qquad \text{(Eq. 14)}$$

The line will cross the time axis when $\hat{A}_u = 0$ at a point, $t_{(A_{u=0})}$, given by

$$t_{(A_{u=0})} = \frac{(T_2 - T_1) - (T_2e^{-KT_1} - T_1e^{-KT_2})}{K(T_2 - T_1) - (e^{-KT_1} - e^{-KT_2})} \qquad \text{(Eq. 15)}$$

Utilizing Eqs. 12 through 15 we can show that

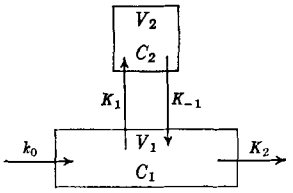
$$k_0 = \frac{(A_u)_{t=0}}{t_{(A_u=0)}\left[1 - \left\{\frac{e^{-KT_1} - e^{-KT_2}}{K(T_2 - T_1)}\right\}\right]} \quad \text{(Eq. 16)}$$

Hence, if an estimate of K is available, one may estimate the maximum excretion rate, fk_0, even from a markedly curved segment of the cumulative excretion plot.[1]

Metabolite Formation at a Constant Rate—If a metabolite is formed at a constant rate, then Scheme I may apply but the symbols have to be redefined. In this case k_0 would be the constant formation rate, V would be the volume of distribution, C the plasma concentration of the metabolite at time t, and K would be the first-order rate constant for elimination of the metabolite by all processes. If the metabolite was completely excreted in the urine (*i.e.*, $f = 1$), then K would be the first-order rate constant for urinary excretion of the metabolite. The same equations as above would apply.

Two-Compartment Open Model

Intravenous Infusion at a Constant Rate—The model is shown in Scheme II.



Scheme II

Here k_0 is the constant (zero-order) infusion rate with dimension mass/time; K_2 is the first-order rate constant for elimination of the substance infused by all processes; V_1 is the volume of the inner (plasma) compartment; and C_1 is the concentration of the substance infused in this compartment at time t; V_2 is the volume of the outer compartment, and C_2 is the concentration of the substance infused in this compartment at time t; K_1 is a first-order rate constant representing the instantaneous fraction of substance in the inner compartment being transferred to the outer compartment; and $K_{-1} = (V_1/V_2)K_1 = VK_1$, where $V = V_1/V_2$.

The solutions of the appropriate differential equations for this model were given by Gaudino (3). Rewritten in the nomenclature of Wagner and Northam (4) the equation for the concentration, C_1, is:

$$C_1 = \frac{k_0}{V_1K_2}\left[1 - \left(\frac{K_2 - \beta}{\alpha - \beta}\right)e^{-\alpha t} + \left(\frac{K_2 - \alpha}{\alpha - \beta}\right)e^{-\beta t}\right] \quad \text{(Eq. 17)}$$

where

$$\alpha = {}^1/_2[(K_1 + K_2 + K_{-1}) + \sqrt{(K_1 + K_2 + K_{-1})^2 - 4K_{-1}K_2}] \quad \text{(Eq. 18)}$$

[1] One may also estimate fk_0 with only one point on the curve by use of Eq. 11.

$$\beta = {}^1/_2[(K_1 + K_2 + K_{-1}) - \sqrt{(K_1 + K_2 + K_{-1})^2 - 4K_{-1}K_2}] \quad \text{(Eq. 19)}$$

If D is the total dose infused and T is the infusion time, then

$$k_0 = D/T \quad \text{(Eq. 20)}$$

It should be noted that $\alpha > \beta$, and Eq. 17 only applies when $t < T$. If urinary excretion rate is directly proportional to the concentration C_1, then:

$$dA_u/dt = fV_1K_2C_1 \quad \text{(Eq. 21)}$$

Substitution of Eq. 17 into Eq. 21 and simplifying yields

$$dA_u/dt = fk_0\left[1 - \left(\frac{K_2 - \beta}{\alpha - \beta}\right)e^{-\alpha t} + \left(\frac{K_2 - \alpha}{\alpha - \beta}\right)e^{-\beta t}\right] \quad \text{(Eq. 22)}$$

If t_a is the time required for the excretion rate to reach some proportion, p, of the maximum rate, fk_0, then

$$dA_u/dt = p.f.k_0 \quad \text{(Eq. 23)}$$

when

$$\left(\frac{K_2 - \beta}{\alpha - \beta}\right)e^{-\alpha t_a} - \left(\frac{K_2 - \alpha}{\alpha - \beta}\right)e^{-\beta t_a} = 1 - p \quad \text{(Eq. 24)}$$

Division by K_2 in Eq. 24 yields

$$(1 - \beta/K_2)e^{-\alpha/K_2(K_2t_a)} - (1 - \alpha/K_2)e^{-\beta/K_2(K_2t_a)} - (1 - p)\left(\frac{\alpha}{K_2} - \frac{\beta}{K_2}\right) = 0 \quad \text{(Eq. 25)}$$

For given V_1/V_2 and K_1/K_2 ratios, α/K_2 and β/K_2 remain constant, so that for a given p, the value of K_2t_a is unique. Based on Eqs. 18, 19, and 25 a digital computer program was written which allowed print-out of t_a and $t_a/t_{1/2}$, where $t_{1/2} = \ln 2/K_2$, for given values of V_1/V_2, K_1/K_2, K_2, and p. For $p = 0.95$ and 0.99, $K_1/K_2 = 0.1$, 0.25, 0.5, 1, 2, 5, 10, and 100 and $V_1/V_2 = 0.125$, 0.25, 0.5, 1, 2, 4, and 8, values of $t_a/t_{1/2}$ were estimated. Hence 112 values of $t_a/t_{1/2}$ were calculated and these values served as the basis for Figs. 1 and 2.

Integration of Eq. 22 yields

$$A_u = fk_0\left[t - \frac{(K_2 - \beta)}{\alpha(\alpha - \beta)}(1 - e^{-\alpha t}) + \frac{(K_2 - \alpha)}{\beta(\alpha - \beta)}(1 - e^{-\beta t})\right] \quad \text{(Eq. 26)}$$

One may plot a curve of A_u *versus* t by substituting appropriate values into Eq. 26. If one chose two points, $(A_u{}^{T_2}, T_2)$ and $(A_u{}^{T_1}, T_1)$, on this curve, joined them with a straight line, then extrapolated the line back to zero corresponding to the intercept, $(A_u)_{t=0}$, then the slope, $\Delta A_u/\Delta t$, of the line may be shown to be

$$\Delta A_u/\Delta t = fk_0\left[1 - \frac{1}{T_2 - T_1}\left\{\frac{K_2 - \beta}{\alpha(\alpha - \beta)}(e^{-\alpha T_1} - e^{-\alpha T_2}) - \frac{K_2 - \alpha}{\beta(\alpha - \beta)}(e^{-\beta T_1} - e^{-\beta T_2})\right\}\right] \quad \text{(Eq. 27)}$$

The value of the intercept may be shown to be

$$(A_u)_{t=0} = fk_0\left[\left(\frac{V+1}{V}\right)\left(\frac{1}{K_2}\right) - \frac{K_2-\beta}{\alpha(\alpha-\beta)}\left\{e^{-\alpha T_2} + \frac{T_2}{T_2-T_1}(e^{-\alpha T_1} - e^{-\alpha T_2})\right\} + \frac{K_2-\alpha}{\beta(\alpha-\beta)}\left\{e^{-\beta T_2} + \frac{T_2}{T_2-T_1}(e^{-\beta T_1} - e^{-\beta T_2})\right\}\right] \quad \text{(Eq. 28)}$$

The equation of the line is as given by Eq. 14 except the slope and intercept are defined by Eqs. 27 and 28. The line will cross the time axis at a point, $t(A_{u=0})$, given by

$$t(A_{u=0}) = \frac{\left(\frac{V+1}{V}\right)\left(\frac{1}{K_2}\right) - \frac{K_2-\beta}{\alpha(\alpha-\beta)}\left\{e^{-\alpha T_2} + \frac{T_2}{T_2-T_1}(e^{-\alpha T_1} - e^{-\alpha T_1})\right\} + \frac{K_2-\alpha}{\beta(\alpha-\beta)}\left\{e^{-\beta T_2} + \frac{T_2}{T_2-T_1} \times (e^{-\beta T_1} - e^{-\beta T_2})\right\}}{1 - \frac{1}{T_2-T_1}\left\{\frac{K_2-\beta}{\alpha(\alpha-\beta)}(e^{-\alpha T_1} - e^{-\alpha T_2}) - \frac{K_2-\alpha}{\beta(\alpha-\beta)}(e^{-\beta T_1} - e^{-\beta T_2})\right\}} \quad \text{(Eq. 29)}$$

Utilizing Eqs. 27 through 29 one can show that

$$fk_0 = \frac{(A_u)_{t=0}}{t(A_{u=0})\left[1 - \frac{1}{T_2-T_1}\left\{\frac{K_2-\beta}{\alpha(\alpha-\beta)}(e^{-\alpha T_1} - e^{-\alpha T_2}) - \frac{K_2-\alpha}{\beta(\alpha-\beta)}(e^{-\beta T_1} - e^{-\beta T_2})\right\}\right]} \quad \text{(Eq. 30)}$$

It should be noted that as T_1 and T_2 become large then

$$(A_u)_{t=0} \to fk_0\left(\frac{V+1}{V}\right)\left(\frac{1}{K_2}\right), \quad t(A_{u=0}) \to \left(\frac{V+1}{V}\right)\left(\frac{1}{K_2}\right), \Delta A_u/\Delta t \to fk_0$$

and

$$fk_0 \to (A_u)_{t=0}/t(A_{u=0})$$

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RESULTS

Table I gives values of the lag time, t_a, corresponding to different half-lives and rate constants for the one-compartment open model. The lag times listed were calculated with Eqs. 9 and 10.

Figures 1 and 2 are plots of the ratio $t_a/t_{1/2}$ *versus* K_1/K_2 on log-log scales for the two-compartment open model. Here $t_{1/2}$ is the half-life of elimination estimated from K_2 (*i.e.*, $t_{1/2} = \ln 2/K_2$). Figure 1 gives values of $t_a/t_{1/2}$ when $p = 0.95$, and Fig. 2 gives value of $t_a/t_{1/2}$ when $p = 0.99$. The values of t_a were estimated with an appropriate digital computer program based on Eqs. 18, 19, and 25.

Figure 3 has two examples illustrating use of Eqs. 11 through 16. Letting fk_0 be 1 unit/hr. and K be 1.0 hr.$^{-1}$, values of A_u corresponding to t = 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, and 4.6 hr. were calculated using Eq. 11; these correspond to the circled points in Fig. 3. Letting T_1 = 1.5 hr. and T_2 = 3.0 hr., a straight line was drawn joining the ($A_u{}^{T_2}$, T_2) and ($A_u{}^{T_1}$,T_1) points and the line extrapolated back to zero time; the equation of this line is $\hat{A}_u = 0.8845t - 0.6037$ corresponding to Eq. 14. Hence this line intersects the ordinate at $(A_u)_{t=0} = -0.6037$ and it crosses the time axis at the point $t(A_{u=0}) = 0.6825$ hr. The slope, $\Delta A_u/\Delta t$, estimated from these points, is 0.8845 indicating the excretion rate has reached only 88.45% of its maximum value. Using Eq. 16, however, the true maximum excretion rate of unity may be estimated. Letting T_1 = 3.0 hr. and T_2 = 4.6 hr., another straight line was drawn joining the points corresponding to these times, and the line was extrapolated back to the ordinate; the equation of this line is $\hat{A}_u = 0.9752t$

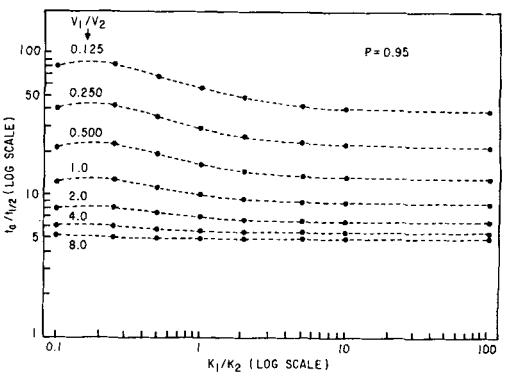

Fig. 1—Plot of the ratio $t_a/t_{1/2}$ versus *the ratio* K_1/K_2 *on a log-log scale for the two-compartment open model when* p = 0.95.

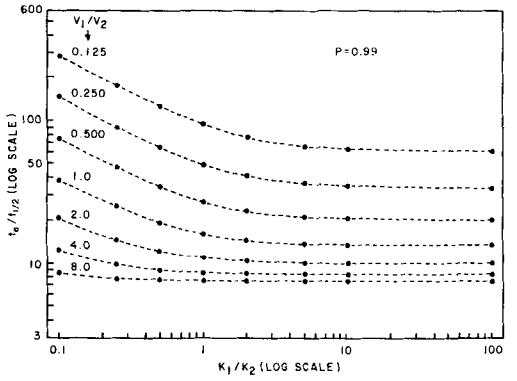

Fig. 2—Plot of the ratio $t_a/t_{1/2}$ *versus the ratio* K_1/K_2 *on a log-log scale for the two-compartment open model when* p = *0.99.*

— 0.8758 corresponding to Eq. 14. Hence this second line intersects the ordinate at $(A_u)_{t=0}$ = −0.8758 and it crosses the time axis at the point $t(A_{u=0})$ = 0.8981 hr. The slope, $\Delta A_u/\Delta t$, estimated from these points, is 0.9752 indicating that the excretion rate has reached 97.5% of its maximum value. Using Eq. 16 the time maximum excretion rate of unity may be estimated.

Figure 4 has two examples illustrating use of Eqs. 26 through 30. Letting fk_0 = 1 unit/hr., K_2 = 1 hr.$^{-1}$, K_1 = 5 hr.$^{-1}$, and V_1/V_2 = 2, use of Eqs. 18, 19, and 26 allowed calculation of the plotted points in the figure corresponding to the same times as in the one-compartment case. A straight line was drawn joining the points corresponding to 1.5 and 3.0 hr. and the line was extrapolated back to time zero; this line has the equation $\hat{A}_u$ = 0.7606 t − 0.5965. This line intersects the ordinate at $(A_u)_{t=0}$ = −0.5965 and it crosses the time axis at the point $t(A_{u=0})$ = 1.153 hr. The slope, $\Delta A_u/\Delta t$, estimated from these points, is 0.7606 indicating that the excretion rate has reached 76.06% of its maximum value. Using Eq. 30, however, the true maximum excretion rate of unity was estimated. A second straight line was drawn joining the points corresponding to 3.0 and 4.6 hr. and the line was extrapolated back to time zero; this line has the equation $\hat{A}_u$ = 0.9124t − 1.0518. This second line intersects the ordinate at $(A_u)_{t=0}$ = −1.0518 and it

TABLE I—VALUES OF THE LAG TIME, t_a, CORRESPONDING TO DIFFERENT HALF-LIVES, AND RATE CONSTANTS FOR THE ONE-COMPARTMENT OPEN MODEL

Half-Life, hr.	Rate Constant (K), hr.$^{-1}$	t_a, hr. p = 0.95[a]	t_a, hr. p = 0.99[b]
0.231	3.0	1.0	1.5
0.3465	2.0	1.5	2.3
0.693	1.0	3.0	4.6
1.386	0.5	6.0	9.2
2.77	0.25	12.0	18.4
4.62	0.15	20.0	31.0
6.93	0.10	30.0	46.0
13.86	0.05	60.0	92.0
69.3	0.01	300.0	460.0

[a] Values in this column calculated with Eq. 9. [b] Values in this column calculated with Eq. 10.

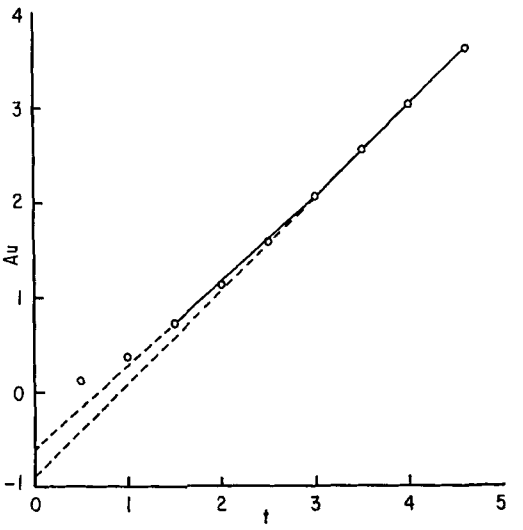

Fig. 3—Plot of the cumulative amount excreted in the urine (A_u) *against time* (t) *in hours for the one-compartment open model where* K = *1 hr.*$^{-1}$ *and* fk_0 = *1 unit/hr. The line joining the 1.5- and 3.0-hr. point has the equation* $\hat{A}_u$ = *0.8845*t − *0.6037; for this line* $(A_u)t_{=0}$ = −*0.6037,* $t(A_{u=0})$ = *0.6825 hr. and* $\Delta A_u/\Delta t$ = *0.8845. The line joining the 3.0- and 4.6-hr. points has the equation* $\hat{A}_u$ = *0.9752t* − *0.8758; for this line* $(A_u)t_{=0}$ = −*0.8758,* $t(A_{u=0})$ = *0.8981 hr. and* $\Delta A_u/\Delta t$ = *0.9752.*

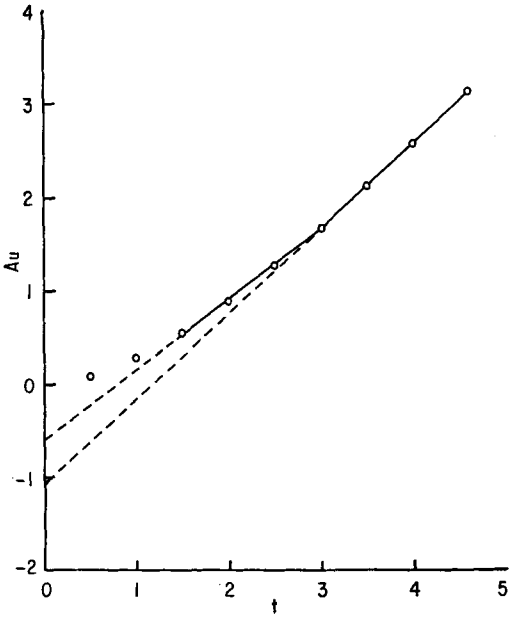

Fig. 4—Plot of the cumulative amount excreted in the urine (A_u) *against time* (t) *in hours for the two-compartment open model where* K_1 = *5 hr.*$^{-1}$, K_2 = *1 hr.*$^{-1}$, V_1/V_2 = *2, and* fk_0 = *1 unit/hr. The line joining the 1.5- and 3.0-hr. points has the equation* $\hat{A}_u$ = *0.7606*t − *0.5965; for this line* $(A_u)t_{=0}$ = −*0.5965,* $t(A_{u=0})$ = *0.7842 hr. and* $\Delta A_u/\Delta t$ = *0.7606. The line joining the 3.0- and 4.6-hr. points has the equation* $\hat{A}_u$ = *0.9124*t − *1.0518; for this line* $(A_u)t_{=0}$ = −*1.0518,* $t(A_{u=0})$ = *1.153 hr., and* $\Delta A_u/\Delta t$ = *0.9124.*

crosses the time axis at the point $t(A_{u=0}) = 1.153$ hr. The slope of 0.9124 indicates the excretion rate has reached 91.24% of its maximum value. Using Eq. 30 the true maximum excretion rate of unity may be estimated.

DISCUSSION

Cummings and Martin (5, 6) advanced theoretical considerations suggesting that the first-order rate constant for urinary excretion of a metabolite will not exceed 3 hr.$^{-1}$ and that it will probably not exceed 1 hr.$^{-1}$, even if the metabolite exhibits the maximum renal clearance. In light of this a maximum value of $K = 3$ hr.$^{-1}$ was used in Table I, and a value of $K = 1$ hr.$^{-1}$ was used in constructing Fig. 3 for the one-compartment open model. Analogously, a value of $K_2 = 1$ hr.$^{-1}$ was used in constructing Fig. 4 pertaining to the two-compartment open model.

Although an observed cumulative urinary excretion curve may *appear* linear before the theoretically required lag time for a given half-life of elimination, it would be wise to use the equations and figures in this report as a guide to the expectation of linearity of such curves. Cumulative urinary excretion curves are extremely insensitive indicators (2, 7–9). The excretion rate time plot, corresponding to the cumulative plot, should always be drawn also (7, 9). One would be unwise to infer constant formation rate of a metabolite, for example, unless the excretion rate time plot indicated an essentially maximum sustained rate *which commenced after some lag time* estimated by Eqs. 8 or 24.

Analogously, if all the drug administered in a dosage form is suspected of being absorbed at a constant rate, then the same consideration would apply as discussed here for constant-rate intravenous infusion. In such a case one would not expect the plasma concentration to plateau, nor the urinary excretion rate to become nearly constant, until the drug had been released and absorbed at a constant rate for a period of time which may be estimated by means of Eqs. 8 or 24. Since gastrointestinal transit time past the absorbing surface area is limited, and since this period of time is near or less than the lag time, t_a, for most drugs predicted by the equations in this report, it would be a rare event for constant-rate absorption to lead to a constant blood level or a constant urinary excretion rate after a single dose of drug in such a dosage form. For example, assume the one-compartment open model applies to the drug. If the half-life of elimination is 2.77 hr., corresponding to a rate constant of 0.25 hr.$^{-1}$, it would require about 12 hr. to reach 95% of the asymptotic plasma concentration and about 28 hr. to reach 99% of the asymptotic plasma concentration if the drug entered the blood at a constant rate. Gastrointestinal transit times *at the absorbing surface area* are usually of this order of magnitude or less.

If a metabolite is formed at a constant rate, due to saturation of an enzyme system metabolizing the drug, the above considerations indicate that it is theoretically impossible for the plot giving the cumulative urinary excretion of the metabolite as a function of time to be linear *and* the line extrapolate through the origin corresponding to zero excretion at zero time.

It should be noted that $t_a/t_{1/2}$ for the two-compartment open model is always greater than the corresponding value for the one-compartment open model for a given p value and when $0.125 \leq V_1/V_2 \leq 8$ and $0.1 \leq K_1/K_2 \leq 100$. Also, for given values of p and K_1/K_2, $t_a/t_{1/2}$ increases as V_1/V_2 decreases for the two-compartment open model. The examples shown in Figs. 3 and 4 are illustrative of the situation with very polar metabolites and drugs with short half-lives since a rate constant of 1 hr.$^{-1}$, corresponding to a half-life of 0.693 hr., was used in their construction. If the half-life of elimination is greater, the time scale on comparable plots would become greater. For example, Table I indicates that if the half-life of elimination is 6.93 hr. and the one-compartment model applies, then it would require 30 hr. to reach 95% of the maximum excretion rate if there was zero-order input to the blood; it would require greater than 30 hr. if the two-compartment open model applied.

CONCLUSIONS

The theoretical expectation is that if the cumulative urinary excretion curve is essentially linear, due to zero-order rate of formation of a metabolite or constant-rate intravenous infusion, then there must be an appreciable negative intercept when the linear portion is extrapolated back to zero time.[2] It is theoretically impossible for a cumulative urinary excretion curve to be linear *and* the line extrapolate through the origin corresponding to zero excretion at zero time.

[2] Proof of this for both the one- and two-compartment open models has been provided. Intuitively, one would expect the conclusions to hold for any compartment model.

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Keyphrases

Urinary excretion rates
Lag time—constant urinary excretion rate
Compartment models, open—one, two
Kinetic equations—zero-order plasma input, estimated maximum excretion
IV infusion—constant rate
Metabolite formation—constant rate

Validation of Methanol-Sulfuric Acid Colorimetry as a Stability Assay Method for Quinestrol by Comparison with a Gas Chromatographic Procedure

By LESTER CHAFETZ, MICHAEL G. BOUDJOUK, DIMITRI C. TSILIFONIS, and FOO SONG HOM

Excellent correlation was obtained between assay values by the methanol-sulfuric acid colorimetric method and a highly selective gas-chromatographic procedure, the specificity of which was demonstrated by separations on two different columns. Quinestrol, ethinyl estradiol 3-cyclopentyl ether, was assayed by both methods after intentional degradation by heating long periods in hydrogen peroxide, hydrochloric acid, or sodium hydroxide solutions, and the identifiable products were measured quantitatively by gas chromatography. The colorimetric method was found to be a valid stability assay for quinestrol.

COLORIMETRIC DETERMINATION of quinestrol, ethinyl estradiol 3-cyclopentyl ether, provides a facile and accurate means for measuring microgram amounts of the estrogen in dosage forms. Since colorimetry eliminates the need for specialized apparatus and technique and provides a considerable advantage in speed, it is preferred to the use of chromatographic techniques for routine and, especially, for occasional assays. The purpose of the study reported here was to determine the validity of the colorimetric assay used in these laboratories for determination of quinestrol in the presence of its degradation products.

The pink color formed by reaction of ethinyl estradiol or its 3-ethers, quinestrol and mestranol, with a methanol–sulfuric acid reagent was shown to be a highly selective means for determining these estrogenic steroids by Tsilifonis and Chafetz (1); several closely related estrogens give either no color or noninterfering yellow colors under the prescribed conditions. Khoury and Cali (2, 3) recently described a fluorometric assay for ethinyl estradiol and mestranol which makes use of 90% sulfuric acid as the fluorogenic reagent. The reaction product with either reagent appears to be identical. Excitation and fluorescence spectra obtained with methanol–sulfuric acid reagent and ethinyl estradiol and its ethers are identical with those reported by use of 90% sulfuric acid, and the methanol–sulfuric acid fluorophore has been used to measure nanogram amounts of quinestrol contamination in other drug dosage forms here.

Khoury and Cali (2, 3) inferred that sulfuric acid-induced fluorescence is specific for undegraded ethinyl estradiol from their finding that only the undegraded steroid gave a fluorescent spot on a thin-layer chromatogram of thermally or photochemically degraded material. This report describes validation of the previously reported colorimetric assay (1) as a stability method for quinestrol dosage forms. A linear correlation is demonstrated between assay results obtained by the colorimetric method and a specific gas chromatography procedure on quinestrol samples partly degraded by heating with acid, alkali, or hydrogen peroxide. A colorimetric procedure which obviates interference from ethinyl estradiol, the gas chromatographic method, and observations on the stability of quinestrol is presented here.

EXPERIMENTAL

Equipment and Supplies—Absolute methanol, Phillips "pure" grade isooctane, and common laboratory reagents were used. Ethinyl estradiol USP, estrone 3-cyclopentyl ether (Warner-Vister Institute), estrone NF, and quinestrol reference standard were used in this study. The separators used in the colorimetric assay were fitted with Teflon stopcocks. Absorbance measurements were made in 1-cm. silica cells in a Beckman DU instrument and a Cary model 14 recording spectrophotometer. The gas chromatographic instrument and operating parameters are described below.

The *chromogenic reagent* was made by cautiously

Received January 29, 1968, from the Pharmaceutical Research and Development Laboratories, Warner-Lambert Research Institute, Morris Plains, NJ 07950

Accepted for publication March 20, 1968.

Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Miami Beach Meeting, May 1968.

adding concentrated sulfuric acid to 30.0 ml. of chilled methanol in a 100-ml. volumetric flask, in small increments and with frequent agitation so that the mixture was made to the mark at room temperature. Too rapid addition of acid to alcohol results in a yellow reagent.

Degradation with Peroxide—Mixtures of 2.0 ml. of 1 mg./ml. methanolic quinestrol were heated at reflux in 250-ml. flasks with 50 ml. of methanol and 50 ml. of water containing 0.5, 1.0, 1.5, or 2.0 ml. of 30% hydrogen peroxide solution for about 22 hr. in one set of experiments and about 40 hr. for another. The mixtures were evaporated to dryness under reduced pressure in a rotary-film apparatus at about 40°. The residue was taken up in 15 ml. of chloroform and washed three times with 5-ml. volumes of water to remove residual peroxide. The washed chloroform was evaporated to dryness, and the residue was dissolved in 3.0 ml. of chloroform. The samples were apportioned for colorimetric and gas chromatographic assay.

Acid and Base Degradation—Mixtures of 2.0 ml. of 1 mg./ml. quinestrol in methanol were heated at reflux in 250-ml. flasks with 50 ml. of methanol and 50 ml. of 0.1 *N*, 1.0 *N*, 3.0 *N*, or 6 *N* hydrochloric acid or with 0.1 *N* or 1 *N* sodium hydroxide for periods of about 20 hr. The solutions were neutralized and evaporated to dryness at about 40°. The residues were extracted with 15 ml. of chloroform, the extracts were evaporated to dryness, and the residues were taken up in 3 ml. of chloroform. Portions of each sample were taken as assay samples in each method.

Colorimetric Method—Prepare the *standard preparation* by the following procedure: accurately weigh about 50 mg. of reference standard quinestrol, dissolve it in methanol, and dilute the solution to 100 ml. Further dilute 2.0 to 50 ml. with methanol to obtain a 20 mcg./ml. solution.

Dilute 1.0 ml. of the chloroform sample obtained by partial degradation to 25 ml. with methanol. Transfer 1 ml. containing the equivalent of about 20 mcg. of quinestrol to a 125-ml. separator. Add 10 ml. of water and 5 drops of concentrated hydrochloric acid. Extract the mixture with about 75 ml. of isooctane, shaking for 2 min. Allow the phases to separate, and discard the lower layer. Quantitatively transfer the isooctane to a second, *scrupulously dry* separator through a solvent-wetted cotton pledget filter, using about 10 ml. of fresh isooctane to effect the transfer. Equilibrate the isooctane solution with exactly 5.0 ml. of *chromogenic reagent* by shaking 2 min. Discard 2–3 drops of the pink lower phase through the stopcock bore, and collect about 4.5 ml. of the pink solution into a 15-ml. glass-stoppered centrifuge tube. Withdraw by pipet exactly 4.0 ml. and add exactly 0.4 ml. of absolute methanol. Mix thoroughly, and centrifuge the contents to dispel air bubbles. Determine the absorbance of the solution, A_u, *versus* a solvent blank at the maximum, about 540 mμ.

Concomitantly develop a color with 1.0 ml. of the *standard preparation*, beginning with, "Add 10 ml. of water and 5 drops of concentrated hydrochloric acid." Designate the concentration of quinestrol in the standard in mcg./ml. as C and the absorbance obtained as A_s.

$$\text{quinestrol, mcg./sample} = A_u/A_s \times C \times \text{dilution factors}$$

Gas Chromatography—Place a volume of the sample used for the colorimetric assay equivalent to 10–100 mcg. of quinestrol in a 7-ml. glass vial. Add 1.0 ml. of an 80 mcg./ml. chloroform solution of *n*-dotriacontane,[1] the *internal standard*. Evaporate the solvent on the steam bath with the aid of a gentle stream of nitrogen. Take up the cooled residue with 0.2 ml. of chloroform. Mix thoroughly and inject 5 μl. on the column of the gas chromatograph. The following conditions were used:

1. A 1.2-m. 6-mm. (4 ft. 1/4 in. o.d.) glass U-tube column packed with 4% OV-17[1] on 80/100-mesh Gas Chrom Q[1] was used in an F & M Scientific model 402 instrument, which provides on-column injection and dual columns and detectors. The temperature was maintained isothermally at 253° for the column and 263° for the flame-ionization-detector chamber. Helium carrier gas flow rate was maintained at 120 ml./min. and hydrogen gas flow to the detector was set at 60 ml./min.

2. A second system was used as a check on separations, where all of the operating parameters were identical with the first, but the column used was a 1.2-m. 6-mm. o.d. glass U-tube packed with 3% SE-30 on 80/100 mesh Diatoport S.[2]

The response factor for quinestrol was determined by injecting 10-, 50-, and 100-mcg. amounts of quinestrol with the internal standard. Where the response of quinestrol is R_Q, the internal standard response R_I, the weight of internal standard W_I, and the weight of quinestrol standard W_Q:

$$\text{Factor } (F) = R_Q/R_I \times W_I/W_Q$$

The amount of quinestrol in the sample is calculated:

$$\text{mcg. quinestrol} = R_Q/R_I \times W_I/F$$

Response factors were determined in a similar manner for ethinyl estradiol, estrone, and estrone-3-cyclopentyl ether.

RESULTS AND DISCUSSION

Specificity of the Gas Chromatographic Assay—Quantitative results were obtained using the OV-17 column described in system 1 above, however, the separations were checked on the alternate column given in system 2. Correspondence of sample peak retention times to those of the standard substances in two systems provides a high degree of specificity of identification. The retention times obtained for the standard substances on the two columns are given in Table I. The only quinestrol degradation products found in significant amounts were ethinyl estradiol and, in one case, estrone-3-cyclopentyl ether (*v.i.*). Quantitative measurements were made by the peak height method.

Comparison of Methods—Both methods provided recoveries of 98%. Relative standard deviations were 1.7% for the colorimetric assay and 1.6% for the gas chromatographic procedure. As noted earlier (1), the colorimetric method does not distinguish between quinestrol and ethinyl estradiol, and it has been used in these laboratories with a thin-layer limit test for ethinyl estradiol. (A procedure for selective colorimetric assay of quinestrol in the presence of ethinyl estradiol is described

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[2] F & M Scientific, Division of Hewlett-Packard, Avondale, Pa.

below.) Ethinyl estradiol was found in significant amounts, however, only in the experiments in which quinestrol was heated in at least 0.5 *M* hydrochloric acid for 20 hr. Figure 1 shows the correlation obtained by plotting the quinestrol assay value by the colorimetric method against the total of quinestrol and ethinyl estradiol (as quinestrol) determined by the gas chromatographic procedure. The close correspondence obtained between assay values on the same samples establishes the empirical colorimetric assay as a valid stability-indicating method for quinestrol, if provision is made to measure ethinyl estradiol by an independent method or eliminate it in the isolation scheme.

Degradation with Peroxide—Results obtained by both assay methods on the peroxide-degraded samples are presented in Table II. The pot temperature of the 50% methanol solutions was about 77°. Poor correlation was obtained between the amount of peroxide added and the percent of quinestrol which remained. This may reflect differences in handling the work-up after the reflux period, for residual peroxide was present in every case, and it would be concentrated during the evaporation steps. Failure to wash out peroxide residues led to low assays by the gas chromatographic procedure, indicating further reaction on the hot chromatographic column. The experimental design does not permit any estimates of the rate of quinestrol degradation by hydrogen peroxide, nor was this within the scope of the present study. Summation of the measurable peaks does not provide material balance; unidentified peaks were noted at retention times of 3.4 and 31.4 min. and on the reverse slope of the quinestrol peak (about 28 min.) using the OV-17 column.

Degradation with Acid—Heating quinestrol at reflux with 0.05 *M* hydrochloric acid in 50% methanol led to no detectable degradation. The amount of ethinyl estradiol formed by hydrolysis of the ether function increased with acid concentration as shown in Table III. The pot temperature was again about 77°. Unidentified peaks were noted in chromatograms run on the OV-17 column with the two higher concentrations of acid. A peak with a retention time of 34.6 min. was observed after heating with 1.5 *M* acid. Additional peaks were observed after heating with 3 *M* hydrochloric acid at 5.8, 14.6, 16.6, 41.8, and 50.4 min.

Table I—Retention Times of Standard Steroids on Two Columns

Compound	Retention Time, min. Column I	Retention Time, min. Column II
Estrone	8.0	3.2
Ethinyl estradiol	9.4	4.0
n-Dotriacontane	11.4	18.0
Estrone-3-cyclopentyl ether	22.4	9.0
Quinestrol	27.4	11.6

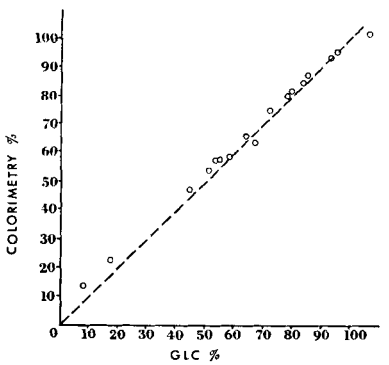

Fig. 1—Correlation of colorimetric and gas chromatographic assay values on degraded quinestrol samples. See text. Dashed line represents perfect correlation.

Degradation in Alkali—Heating quinestrol in 0.05 *M* alkali gave values of 95% quinestrol by the colorimetric method and 95.2% by gas chromatography. In this concentration of base, less than 1% of quinestrol was found converted to estrone-3-cyclopentyl ether, but unidentified peaks were noted with retention times of 1.6, 2.2, 3.2, and 5.2 min. In 0.5 *M* methanolic sodium hydroxide, 58% intact quinestrol was found by the colorimetric method and 54.7% by gas chromatography. Estrone-3-cyclopentyl ether was found in 6.5% concentration (expressed as quinestrol), and less than 1% estrone was seen. The unidentified peaks noted were the same as in the lower base concentration experiment. Talmage *et al.* (4) and Boughton and his co-workers (5) used estrone as the internal standard for gas chromatographic determination of ethinyl estradiol. It would appear that this is a poor choice, for the estrone is both a synthesis precursor and a possible degradation product. Langecker (6) has demonstrated the presence of estrone and acetylene after treating ethinyl estradiol with 1 *N* sodium hydroxide for 30 min.

Selective Colorimetric Assay for Quinestrol—The previously reported procedure (1) has been modified to provide fewer manipulations and to eliminate interference from ethinyl estradiol. The following procedure is recommended.

Transfer a weighed sample equivalent to about 20 mcg. of quinestrol to a 125-ml. separator containing 10 ml. of water and 1 ml. of methanol. Add 4–5 drops of concentrated hydrochloric acid, and extract the mixture with about 75 ml. of isooctane, shaking for 2 min. Allow the layers to separate, and discard the lower phase. Wash the isooctane layer with 5 ml. of 1 *N* sodium hydroxide, shaking the mixture about 1 min. Discard the alkali layer, and transfer the isooctane phase quantitatively to a second, *scrupulously dry* 125-ml. separator through a solvent-wetted cotton pledget filter, using about 10 ml. of fresh isooctane to effect the transfer. Continue the procedure as described above under *Colorimetric Method*, beginning with, "Equilibrate the isooctane solution with exactly 5.0 ml. of *chromogenic reagent*. . . ."

Use of 1 *N* sodium hydroxide to remove any ethinyl estradiol present was checked by adding 20- and 30-mcg. amounts of ethinyl estradiol to 20-mcg. amounts of quinestrol and going through the procedure described. There was no difference in absorbance values for quinestrol to which an equal or 1.5-fold excess of ethinyl estradiol was added and samples without added ethinyl estradiol. Recoveries of quinestrol added to placebo tablets were identical whether the alkali wash was used or water was employed.

SUMMARY AND CONCLUSIONS

Assay of quinestrol solutions partly degraded by heating with hydrogen peroxide, hydrochloric acid,

TABLE II—RESULTS OF PEROXIDE DEGRADATION OF QUINESTROL

30% Peroxide, ml.	Hr.	Percent Quinestrol Remaining				
		Colorimetric	GLC	EE[a]	ES[b]	EsCPE[c]
0.5	22	87.4	84.0	trace	trace	—
1.0	22	85.1	83.6	trace	trace	—
2.0	22	81.4	78.6	trace	trace	—
1.5	22	80.2	77.9	trace	trace	—
0.5	22	75.0	71.3	—	trace	—
1.0	22	65.4	63.9	—	<1%	—
1.5	22	58.6	58.4	—	<1%	—
1.0	40	58.0	53.0	trace	trace	<1%
2.0	40	54.0	51.3	trace	trace	trace
2.0	22	46.7	44.0	trace	<1%	—
0.5	40	21.9	18.2	trace	<1%	<1%
1.5	40	12.9	8.0	trace	trace	trace

[a] Ethinyl estradiol. [b] Estrone. [c] Estrone-3-cyclopentyl ether.

TABLE III—DEGRADATION OF QUINESTROL IN HYDROCHLORIC ACID

HCl	Percent Intact Quinestrol					
	Colorimetric	GLC	EE[a]	Q + EE[b]	ES[c]	EsCPE[d]
0.5 *M*	101	106	1.8	108	—	—
1.5 *M*	93	82.7	9.8	92.5	trace	1%
3 *M*	64	36.2	30.9	67.1	trace	1%

[a] Ethinyl estradiol calculated as quinestrol. [b] Total of quinestrol and ethinyl estradiol calculated as quinestrol. [c] Estrone. [d] Estrone-3-cyclopentyl ether.

or sodium hydroxide by a colorimetric method (1) and by a highly selective gas chromatographic method shows excellent correlation in values. The colorimetric method has thus been established as a valid stability-indicating assay for quinestrol, providing that ethinyl estradiol is measured by an independent limit test or eliminated in the isolation scheme. Ethinyl estradiol was found in significant amount only in those samples degraded by heating in 0.5 *M* or stronger hydrochloric acid. The specificity of the gas chromatographic procedure used was established by obtaining separations on two different columns. Both provided excellent separations of quinestrol from ethinyl estradiol, estrone, estrone-3-cyclopentyl ether, and unidentified peaks noted in several of the chromatograms.

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Keyphrases

Quinestrol—analysis
Stability assay—quinestrol dosage forms Colorimetric analysis
Methanol–sulfuric acid—color reagent
GLC—analysis

Gas-Liquid Chromatography of Niacin and Niacinamide

By A. R. PROSSER and A. J. SHEPPARD

A procedure has been developed for the qualitative and quantitative determination of niacin and niacinamide by gas-liquid chromatography. Niacin was determined as its ethyl ester (ethyl nicotinate) or as *N*-ethylnicotinamide. Niacinamide was determined as niacinamide or converted to either ethyl nicotinate or *N*-ethylnicotinamide. An immobile biphase of 2.5 percent NPGS–10 percent SE-30 was the most satisfactory immobile phase evaluated. The results revealed that, in general, this sensitive gas-liquid chromatographic method could detect 5, 8, and 10 ng. of ethyl nicotinate, *N*-ethylnicotinamide, and niacinamide, respectively, using the hydrogen flame-ionization detector. The β-argon ionization detector (Ra_{226}) detected 15, 20, and 25 ng. of each compound, respectively. It was found that increasing quantities of ethyl nicotinate, *N*-ethylniacinamide, and niacinamide gave linear responses.

NIACIN (nicotinic acid) and its analog niacinamide (nicotinic acid amide) are important members of the B-complex vitamins. Niacinamide is included as an ingredient in decavitamin capsule and tablet composition requirements of the USP (1).

Chemical and biological methods are now widely used. The 10th edition of "Official Methods of Analysis" of the A.O.A.C. (2) includes both a chemical and biological method; the USP method (1) is based on a chemical technique. The chemical methods generally utilize the König reaction (3, 4) in which pyridine and its derivatives react with cyanogen bromide and an aromatic amine. In this reaction polymethine dyes are formed and are measured spectrophotometrically at 450 mμ. However, the chemical method is undesirable because the color development is pH sensitive and has low stability, which makes it difficult to reproduce results.

Biological methods provide the most specific quantitative assay presently available. The dog and chicken have been used for biological assay for total niacin activity. However, microbiological methods have been more popular and are far more practical from the standpoint of sensitivity, time, and expense. The A.O.A.C. method (2) uses *Lactobacillus plantarum*, which measures total niacin activity and can be used to specifically assay nicotinic acid in the presence of nicotinamide if the latter is first converted to 3-aminopyridine (6). Johnson has used *Leuconostoc mesenteroides* to measure nicotinic acid (5).

A polarographic method (7) and a thin-layer chromatographic method (8, 9) are available but have not been widely accepted.

Although a number of satisfactory methods are available for their separation and identification, a more rapid and simple method specific for niacin and niacinamide is needed. Since previous work from this laboratory (10, 11) has shown that vitamin B_6 of the water-soluble vitamin series is amenable to gas-liquid chromatography (GLC), it was felt that niacin and its analogs could also be measured by GLC. The present report is a summary of the feasibility of utilizing the gas chromatograph for the analysis of niacin and niacinamide.

Received January 10, 1968, from the Division of Nutrition, Food and Drug Administration, Washington, DC 20204
Accepted for publication March 4, 1968.

EXPERIMENTAL

Instrumentation—A model 5000 series Barber-Colman gas chromatograph (Barber-Colman, Rockford, Illinois) fitted with a high temperature hydrogen flame-ionization detector (FID) and a β-argon ionization detector (AID) with a 56 μc Ra_{226} foil was used with a 5-mv., 2-sec., 28-cm.-(11-in.) strip-chart recorder.

Materials—Niacin, niacinamide, and ethyl nicotinate were purchased from Eastman Organic Chemicals, Distillation Products Industries, Rochester, New York. *N*-Ethylnicotinamide was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. Pure grade *n*-hexane was obtained from Phillips Petroleum Co., Special Products Division, Bartlesville, Oklahoma. All other reagents were A.R. grade and used without further purification. Whatman No. 4 filter paper was used for all filtrations.

Preparation of Derivatives—Ethyl nicotinate was prepared by two methods (12): (*a*) direct action of ethanol and sulfuric acid on either niacin or niacinamide; (*b*) interaction of niacin with thionyl chloride followed by the reaction of the nicotinyl chloride hydrochloride product with ethanol and subsequent neutralization. Two methods of preparing niacinamide were used: (*a*) treatment of ethyl nicotinate with concentrated ammonia (12); (*b*) action of thionyl chloride on niacin followed by treatment of the resulting acid chloride with ammonia (13). *N*-Ethylnicotinamide was prepared by the following reactions: (*a*) action of thionyl chloride on niacin and treatment of the resulting acid chloride with ethylamine hydrochloride; (*b*) direct action of ethylamine on niacin in the presence of phosphorus pentoxide; (*c*) action of an aqueous solution of ethylamine on ethyl nicotinate.

Standard Solutions—Niacinamide and *N*-ethylnicotinamide were dissolved in enough ethanol and ethyl nicotinate in enough *n*-hexane so that a constant injection volume of 2 μl. was maintained throughout the investigation. Calibration standards were prepared from commercially obtained materials.

Gas-Liquid Chromatography—Three different column packings were used in this study. Column

1: 183 cm. × 3 mm. i.d. packed with a biphase mixture of 2.5% neopentyl glycol succinate (NPGS) and 10% SE-30 (w/w) on 100/120 mesh Gas Chrom P (silanized). Operating conditions: ethyl nicotinate: column, 180°; detector, 230°; flash heater, 220°; carrier gas, 50 ml./min. Niacinamide and *N*-ethylnicotinamide: column, 230°; detector, 280°; flash heater, 270°; carrier gas, 50 ml./min. Column 2: 244 cm. × 3 mm. i.d. packed with 5% NPGS (w/w) on 100/120 mesh Gas Chrom P (silanized) for the analysis of ethyl nicotinate. Operating conditions: column, 180°; detector, 260°; flash heater, 250°; carrier gas, 80 ml./min. Column 3: 244 cm. × 4 mm. i.d. packed with 2% neopentyl glycol sebacate (NPGSeb) (w/w) on 100/120 mesh Gas Chrom P (silanized) for the analysis of niacinamide and *N*-ethylniacinamide mixtures. Operating conditions: column, 210°; detector, 250°; flash heater, 230°; carrier gas, 60 ml./min.

All columns were U-shaped Pyrex glass and were preconditioned 24 hr. at 235° with carrier-gas flow rate of 60 ml./min. Argon was used as the carrier gas throughout the study. Compressed air and hydrogen flow rates were 460 and 36 ml./min., respectively, with the FID. A d.c. cell voltage of 900 was used at all times with the AID. Electrometer output was 1×10^{-14} amp. when FID was used and 3×10^{-9} amp. when the AID was used. The electrometer output is stated as full-scale output into a 5-mv. recorder unless otherwise stated in the text. Peak areas were determined by triangulation (width at half-height × height = peak area).

RESULTS AND DISCUSSION

The applicability of gas chromatography to niacin is limited by the presence of an acid group in the niacin molecule, its insolubility in most organic solvents, and the fact that niacin sublimes. It was therefore necessary to convert niacin to a derivative volatile enough for GLC. Ethyl nicotinate and *N*-ethylnicotinamide were chosen because of their relatively low boiling points, ease of preparation, and stability. Although niacinamide has an amide group, it is soluble in some organic solvents, has a low melting point, and is suitable for GLC analysis.

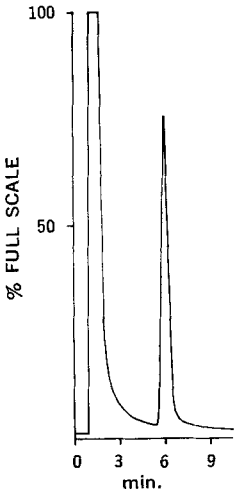

Fig. 1—Chromatograms of 1.0 mcg. ethyl nicotinate in n-hexane on a 2.5% NPGS–10% SE-30 biphase column at 180°.

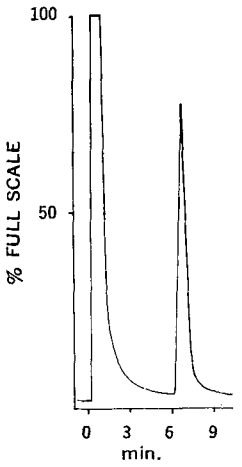

Fig. 2—Chromatogram of 2 mcg. niacinamide in ethanol on a 2.5% NPGS–10% SE-30 biphase column at 230°.

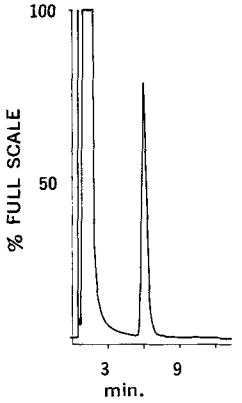

Fig. 3—Chromatogram of 1.25 mcg. ethyl nicotinate in n-hexane on a 5% NPGS column at 180°.

Derivative Preparation Efficiency—The yields of three derivatives were in good agreement with existing literature values (12, 13). GLC scans of the prepared derivatives indicated no contaminants present. The structures of the reaction products were confirmed by IR and UV spectroscopy.

Effect of Immobile Phase—The elution peaks of the three niacin analogs were typical adsorption isotherms on SE-30, QF-1, and biphase mixtures of NPGS–SE-30 when the ratio of NPGS to SE-30 was less than 1:4. The preceding immobile phases were rejected on the basis of their tailing adsorption isotherms. The 2.5% NPGS–10% SE-30 column at 180° gave a satisfacory elution peak for ethyl nicotinate (Fig. 1). When the temperature was increased to 230°, niacinamide produced a usable elution peak exhibiting slight tailing (Fig. 2). On this column, *N*-ethylniacinamide was found to precede niacinamide. The results suggest that by varying the column temperature and carrier solvents, GLC may be a potential means of screening for analogs of the vitamin.

Ethyl nicotinate exhibited a nearly symmetrical elution peak on the 5% NPGS immobile phase column (Fig. 3) whereas the other two analogs gave

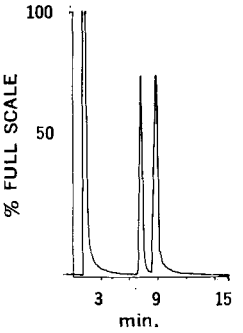

Fig. 4—Chromatogram of a mixture of 1.0 mcg. N-ethylniacinamide (7.6 min.) and 1.2 mcg. niacinamide (9.2 min.) on a 2% NPGSeb column at 210°.

broad tailing elution peaks at much longer retention times. The 2% NPGSeb column eluted *N*-ethylnicotinamide ahead of niacinamide (Fig. 4). At 210°, ethyl nicotinate was eluted along with the carrier solvent. This column will resolve all three niacin analogs at 180° but the retention times for *N*-ethylniacinamide and niacinamide are extended and the elutions peaks were quite broad and were, therefore, less desirable for quantitative analysis.

Effect of Quantity Injected—Response data for ethyl nicotinate, *N*-ethylnicotinamide, and niacinamide using the biphase column of 2.5% NPGS and 10% SE-30 are given in Table I. It can be seen from these data that as the quantity of compound increased the response was linear. The response lines for *N*-ethylnicotinamide and niacinamide are almost identical but the response line for ethyl nicotinate was not closely related to those of the other two analogs.

Sensitivity—The GLC method for niacin and niacinamide is very sensitive. By using the FID, an attenuator setting of 1, an electrometer output of 1×10^{-10} amp., and a 0–1-mv. recorder, 5 ng. of ethyl nicotinate and 10 ng. of niacinamide were detectable. The AID, with an attenuation of 1 and an electrometer output of 3×10^{-9} amp., detected 15, 20, and 25 ng. of ethyl nicotinate, *N*-ethylnicotinamide, and niacinamide, respectively. Quantitation at these low levels, however, is difficult because of the noise-to-signal ratio and because there is less control over the quantity injected.

CONCLUSIONS

It has been established that it is possible to detect and analyze three analogs of the vitamin niacin, *i.e.*, nicotinic acid as the ethyl ester, nicotinamide, and *N*-ethylnicotinamide. Studies are currently underway on applying the GLC analysis to pharmaceuticals containing niacin and niacinamide. Preliminary results indicate that the GLC analysis of the vitamin niacin is adaptable to pharmaceutical products.

TABLE I—RESPONSE OF ETHYL NICOTINATE, *N*-ETHYLNICOTINAMIDE, AND NIACINAMIDE[a]

	Peak Area,[b] mm.²		
mcg.	Ethyl Nicotinate	*N*-Ethyl-Nicotinamide	Niacinamide
0.5	25.0 ± 0.4	18.9 ± 0.5	9.8 ± 0.2
1.0	40.0 ± 1.4	31.3 ± 0.8	21.3 ± 0.2
2.0	70.0 ± 1.5	62.1 ± 1.3	45.0 ± 1.0
5.0	190.0 ± 2.5	124.2 ± 1.4	117.2 ± 1.3
10.0	384.0 ± 3.4	250.3 ± 2.9	245.4 ± 3.1
15.0	572.5 ± 4.4	362.5 ± 4.3	364.0 ± 2.7
20.0	757.5 ± 4.2	473.2 ± 4.7	477.8 ± 3.8

[a] 10 × attenuation, electrometer output of 1×10^{-10} amp. hydrogen flame detector, and a 2.5% NPGS–10% SE-30 column. [b] Mean of six analyses ± standard deviation.

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Keyphrases

Niacin—analysis
Niacinamide—analysis
Ethyl nicotinate, *N*-ethyl nicotinamide—derivatives
GLC—analysis

Influence of Certain Factors on the Coating of a Medicinal Agent on Core Tablets

By WERNER M. K. SEIDLER and E. J. ROWE

The influence of core tablet shape, mixing of coating powders, dilution ratio of diluent to medication, and composition of diluent on the efficiency in the coating of a medicinal onto core tablets by the conventional method was investigated. It was found that tablet shape and type of mixing did not influence the efficiency in this procedure. The overall percent recovery of medicament depending on the variations introduced was from 85 to 90.9 percent in the procedure. Significant factors were drug to diluent ratio and composition of the diluent. Two new methods of drug application were employed, namely, the air-atomized system and the airless-spray-coating system. The air-atomized system was found less efficient than the conventional coating procedure. The airless-spray-coating system, on the other hand, showed a nearly 100 percent efficiency in medication coating.

PROCESSES AND TECHNIQUES employed in the coating of tablets have been surrounded with secrecy. Only one specific report was found in the literature about the efficiency of coating a medicinal agent onto solid cores. Welti (1) investigated the efficiency of applying medication onto pellets on a small scale by the conventional coating method. He found that losses of medication are considerably smaller when the medicament is applied in a powder mixture to previously wetted pellets than when the medicament is applied in the form of a suspension. In general, Welti experienced a loss of medicament ranging from 18 to 44.8%. The loss was dependent on the size of the coating pan, the method of application, and the type of drying.

It was the purpose of this study (*a*) to investigate the efficiency of coating a medicament onto solid cores by the conventional coating procedure, (*b*) to present two new film-coating methods of medicament coating and to show their efficiency, and (*c*) to evaluate some factors influencing the efficiency of medicament coating with respect to the conventional coating procedure and the two new methods.

It was hoped that a comparison between these three methods of application would reduce the general loss of medication as experienced by Welti and give a better way for medicament coating.

Received May 8, 1967, from Eli Lilly and Co., Indianapolis, IN 46206

Accepted for publication February 14, 1968.

Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

Abstracted from a thesis presented to the Graduate Division of Butler University College of Pharmacy, Indianapolis, by Werner M. K. Seidler in partial fulfillment of the requirements for the degree of Master of Science.

The author wishes to thank Eli Lilly Research Laboratories, Indianapolis, Ind., for the supply of chemicals and use of equipment.

EXPERIMENTAL

Two types of tablet shape were used in all experiments: Type 1—round deep concave, and Type 2—elliptical. The tablet weight was in both cases 365 mg. The tablets were subcoated with four applications of a coating solution consisting of sucrose (35% w/v) and acacia (27% w/v) and a coating powder consisting of talc (87%) and acacia (13%) in the conventional manner before medication coating was applied. The medicament used in these experiments was quinine hydrochloride. Each tablet was coated with 15 mg. of quinine hydrochloride, theoretically. No excess was used in any of the experiments.

Conventional Coating Procedure—Twenty-four experiments were carried out to investigate the conventional coating procedure. In each experiment 5,000 subcoated tablets were placed into a 14-in. pear-shaped copper coating pan rotating at 24 r.p.m. The tablets were wetted with an aqueous sucrose-acacia solution after which a powdered diluent–medicament mixture was applied. Alternate wetting and powdered diluent–medicament application was continued until all of the powdered medicament mixture was applied. This was followed by two applications of syrup. Drying was accomplished after each addition with warm air and full exhaust. The tablets were then dried for 24 hr. at room temperature. Ten tablets were picked at random from each experiment and a single tablet assay performed for quinine hydrochloride.

The variations introduced in the conventional coating procedure involved (*a*) the ratios 50:50, 20:80, and 5:95 of powdered diluent to medicament, (*b*) the mixing of the components in a twin-shell blender and in a ball mill for 5 hr. in both cases, and (*c*) the use of two different powdered diluent–medicament mixtures. The composition of the powdered diluent formulas was as follows:

1.	Calcium sulfate	49% w/w
	Milk sugar	49% w/w
	Magnesium stearate	2% w/w
2.	Calcium carbonate	44.5% w/w
	Starch powder	6.0% w/w

Calcium sulfate	44.5% w/w
Silicon dioxide	5.0% w/w

The diluents were chosen as being representative of those commonly used in the pharmaceutical industry.

Film-Coating Procedure—In the film-coating procedure, utilizing the air-atomized system and the airless-spray system, quinine hydrochloride was incorporated in the film-forming solution. The two film-forming solutions used in the experiments consisted of:

1. Hydroxypropyl methylcellulose 10	3.0% w/v
Glycerin	0.3% v/v
Quinine hydrochloride	5.0% w/v
Methylene chloride and methanol 50:50 v/v, a sufficient quantity	
2. Polyvinylpyrrolidone	4.0% w/v
Acetylated monoglycerides type 9-40	1.0% v/v
Acetylated monoglycerides type 5-00	3.0% w/v
Quinone hydrochloride	7.5% w/v
Isopropanol and methylene chloride 40:60 v/v, a sufficient quantity	

For the application of quinine hydrochloride film-coating solution in the air-atomized system, a De Vilbiss spray gun with the spray nozzle No. 92 was used. A variation introduced in the air-atomized system involved a change in air pressure (10 p.s.i. and 25 p.s.i., respectively). The pan size, pan speed, and batch size were the same as in the conventional coating procedure. The coating solution was continuously sprayed onto a moving bed of tablets. The spray rate for Solution 1 was 17.2 ml./min.; for Solution 2 it was 11.4 ml./min., both at 10 p.s.i. At 25 p.s.i. the spray rate of Solution 1 was 23 ml./min.; for Solution 2 it was 19 ml./min. Sufficient exhaust was provided on the outside of the pan to carry off the solvent vapors during the spraying. After drying the tablets for 24 hr. at room temperature, 10 tablets were picked at random from each of eight experiments and assayed for quinine hydrochloride content.

In the airless-spray system the film-coating material to be applied was pumped under high pressure through a special nozzle. In four experiments a Nordson pump model No. 100 060 (16:1 pressure ratio) with a Nordson Versa gun was used. Nozzle 9C11 was used for coating Solution 1 with an air pressure of 500 p.s.i.; nozzle 6C7 for coating Solution 2 with an air pressure of 800 p.s.i. All experiments utilized a noncirculating system. To better accommodate the spray to the coating pan and to the tablets the lot size was increased to 7,500 subcoated tablets per experiment. The pan size and the pan speed were the same as in all previous experiments. The Nordson system did not allow a continuous spray, as was the case with the air-atomized system, since no air to facilitate drying was introduced during the actual spray cycle into the pan. A cycle of 3 sec. spraying time and 25 sec. drying time was established. No exhaust or hot air was used during the actual spray cycle. Again, after 24 hr. drying at room temperature, 10 tablets were selected at random from each experiment and assayed for quinine hydrochloride content.

Assay Procedure—The quinine hydrochloride content of each tablet was determined spectrophotometrically. Correction for the interference due to the water-soluble film-forming agent was determined from a placebo tablet subjected to the entire assay procedure.

RESULTS AND DISCUSSION

A total of 36 experiments was carried out (24 for the conventional coating procedure, 8 for the air-atomized system, and 4 for the airless-spray-coating system), and 358 single-tablet assays were performed. Table I gives the averages for the conventional coating procedure according to the variations introduced. The assay range differential is understood to be the difference in assay results between the lowest and the highest assay in this grouping. It can be seen that the type of mixing and tablet shape did not have any significant influence on the coating efficiency as shown by the percent recovery or on the assay range differential. The variations which were found to be significant are (*a*) the medicament ratio and (*b*) the type of powder diluent formula used. There was little difference between powdered medicament–diluent in the ratio of 50:50 and 20:80, but the 5:95 ratio showed the highest assay results and the least assay range differential. Powder diluent Formula 1 showed greater percent recovery than powder diluent Formula 2; however, the assay range differential was higher. The overall percent recovery in the conventional coating procedure, depending on the variations introduced, was from 85.0 to 90.9%.

Table II gives the averages for the film-coating procedures. In the air-atomized system the nature of the film-forming solution and the pressure applied have a significant influence on the quinine hydrochloride content of the finished tablets. The higher the pressure the greater the medicament loss. The overall recovery of quinine hydrochloride in this system ranges from 69.4 to 74.0%, depending on the air pressure and the film-forming solution used. The assay range differential is about the same regardless of the variations introduced.

In the airless-spray-coating system, the tablet shape seems to have an influence on the actual amount of quinine hydrochloride found on the tablet. Also, film-forming Solution 2 shows a greater percent recovery and a smaller assay range differential than film-forming Solution 1. However, it must be recalled that a larger nozzle and a lower pressure was required for film-forming Solution 1 in order to obtain a satisfactory spray. The range of the individual assay results was the greatest in the airless-spray-coating system compared to either the conventional coating procedure or the air-atomized system; however, the actual amount of quinine hydrochloride found on tablets was close to theory.

Statistical Evaluation—Analysis of variance showed (Table I) that in the conventional coating procedure the powder diluent–medicament ratios and the powder diluent formulas are factors which influence medicament coating. In the air-atomized system the pressure and, to a lesser degree, the film-forming solution are factors which influence medicament coating (Table II). In the airless-spray-coating system (Table II) the variations used appear to make no difference in the results.

TABLE I—AVERAGES FOR CONVENTIONAL COATING PROCEDURE AND STATISTICAL EVALUATION

Variation	Recovery, %	Assay Range Differential, mg.	Assay, mg.	Source of Variation	SS	df	MS	FP(F)
Shape				Shape	0.22	1	0.22	0.15
Round	87.2	2.7	13.1					
Ellipt.	87.5	3.1	13.1					
Mixing				Mixing	0.52	1	0.52	0.37
Twin-shell	86.6	2.8	13.1					
Ball mill	88.3	3.1	13.2					
Medicament ratio				Medicament ratio	33.11	2	16.56	$11.65 < 0.001^a$
50:50	85.8	3.3	12.9					
20:80	85.6	3.4	12.8					
5:95	90.9	2.0	13.7					
Powder diluent formula				Powder diluent formula	47.25	1	47.25	$33.23 < 0.001^a$
1	90.3	3.3	13.6					
2	85.0	2.5	12.7					
				Residue and error	329.84	232	1.422	
				Total	410.94	237		

[a] Statistically significant differences.

TABLE II—AVERAGES FOR FILM-COATING PROCEDURES AND STATISTICAL EVALUATION

Variation	Recovery, %	Assay Range Differential, mg.	Assay, mg.	Source of Variation	SS	df	MS	FP(F)
Air-Atomized System								
Shape				Shape	0.38	1	0.38	0.38
Round	71.6	2.9	10.8					
Ellipt.	72.5	2.1	10.9					
Film-form. solution				Film-form. solution	6.86	1	6.86	$6.88 < 0.01^a$
1	74.0	2.5	11.1					
2	70.1	2.5	10.5					
Air pressure, p.s.i.				Air pressure	12.81	1	12.91	$12.85 < 0.001^a$
10	74.8	2.4	11.2					
25	69.4	2.6	10.4					
				Residue and error	75.75	76	0.997	
				Total	95.80	79		
Airless-Spray System								
Shape				Shape	5.70	1	5.70	$2.52 > 0.05$
Round	103.2	3.9	15.6					
Ellipt.	98.6	5.6	14.8					
Film-form. solution				Film-form. solution	2.35	1	2.35	$1.04 > 0.05$
1	99.3	7.0	15.0					
2	102.5	3.1	15.4					
				Residue and error	83.54	37	2.26	
				Total	91.59	39		

[a] Statistically significant differences.

SUMMARY AND CONCLUSIONS

The following conclusions are drawn from this study:

1. In the conventional coating procedure the medicament loss was found to range from 0.7 to 24.3% based on individual assay.

2. The type of mixing of the powdered diluent–medicament mixture and the tablet shape did not influence the efficiency of the conventional coating procedure.

3. Best yields and smallest tablet-to-tablet variations in medicament content were obtained with the highest powder diluent–medication ratio.

4. Two new methods of application were employed, namely, the air-atomized coating system and the airless-spray-coating system.

5. With the two new methods it was found that the tablet shapes used in this study did not influence the drug content of the tablets.

6. The air-atomized system gave a more consistent range in tablet-to-tablet medicament content than either the conventional coating procedure or the airless-spray-coating system; however, the percent recovery of the applied medicament was the lowest, in the range of 65 to 75%, depending on the air pressure used.

7. The airless-spray-coating system was found

to be the most efficient method of medicament application to a tablet core. Nearly 100% quinine hydrochloride recovery was obtained with this method.

8. Of the three methods investigated, the air-atomized coating system showed the greatest loss of medicament. The airless-spray-coating system showed hardly any loss of medicament. The conventional coating procedure was intermediate in medicament content with respect to the atomized systems.

From this study there appears to be an advantage in applying medication by the airless-spray system. Such a system eliminates the excess medication which must be added in the conventional coating procedure and would thus result in a savings in labor and material.

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Keyphrases

Core tablets—drug coating
Physical parameters, effect—coating core tablets
Powder, sucrose–acacia solution—coating
Drug–diluent ratio, effect—core coating
Film coating—airless-spray, air-atomized systems

Notes

Enhanced Mortality of Selected Central Nervous System Depressants in Hypoexcretory Mice

By B. A. BECKER, K. L. HINDMAN*, and J. E. GIBSON

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CHOLESTATIC OR anuric mice may be more susceptible to some pharmacologic agents than mice without excretory impairment as shown by Gibson and Becker (1). In that work the acute lethality of ouabain was found to be enhanced in cholestatic mice; digoxin and digitoxin had higher mortality rates in anuric mice. Possible alteration in acute lethality of nine central nervous system depressant drugs were similarly studied in cholestatic and anuric mice: five phenothiazine tranquilizers (Table I) including two from the propyldimethylamine subgroup, promazine and chlorpromazine; three from the propylpiperazine subgroup, prochlorperazine, trifluoperazine, and perphenazine; the nonphenothiazine tranquilizer, meprobamate; and three sedative-hypnotics, pentobarbital, phenobarbital, and chloral hydrate.

METHODS

Male Swiss-Webster mice weighing 25–40 Gm. and allowed free access to food and water throughout the experiment were rendered anuric by penile ligation under light ether anesthesia by the method of Becker and Gibson (2). Cholestasis was induced by ligation of the common bile ducts of other male mice of the same strain under ether anesthesia. The surgical wound was closed by 9-mm. wound clips. The general experimental procedure followed that previously reported (1). Preliminary experiments were performed on intact mice to determine suitable dosages of the test agents. Since the anticipated responses were enhancement or no change in mortality rates, dosages were sought which would be in the low lethal range, *e.g.*, LD_{10}–LD_{50}, as enhancement of mortality rates by dosages in the high lethal range, LD_{50}–LD_{99}, would be difficult to demonstrate with any degree of statistical confidence. The selected drugs were administered intraperitoneally in aqueous solution or 0.5% sodium carboxymethylcellulose suspension. The desired dosage of the selected agent was administered 2 hr. after penile ligation (PL) or 24 hr. after bile duct ligation (BDL); sham-operated animals, similarly treated, served as standards. Vehicle-treated operated and sham-operated mice served as controls. Since none of the control animals died under the conditions used, control data were eliminated from

Received October 18, 1967, from the Department of Pharmacology, College of Medicine, University of Iowa, Iowa City, IA 52240

Accepted for publication December 11, 1967.

This research was supported in part by grant No. GM-12675 from the National Institutes of Health.

* Recipient of a College of Medicine Summer Fellowship.

to be the most efficient method of medicament application to a tablet core. Nearly 100% quinine hydrochloride recovery was obtained with this method.

8. Of the three methods investigated, the air-atomized coating system showed the greatest loss of medicament. The airless-spray-coating system showed hardly any loss of medicament. The conventional coating procedure was intermediate in medicament content with respect to the atomized systems.

From this study there appears to be an advantage in applying medication by the airless-spray system. Such a system eliminates the excess medication which must be added in the conventional coating procedure and would thus result in a savings in labor and material.

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Keyphrases

Core tablets—drug coating
Physical parameters, effect—coating core tablets
Powder, sucrose–acacia solution—coating
Drug–diluent ratio, effect—core coating
Film coating—airless-spray, air-atomized systems

Notes

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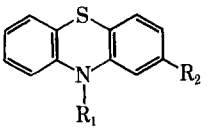
TABLE I—STRUCTURE OF PHENOTHIAZINE COMPOUNDS TESTED



Name	R_1	R_2
Subgroup A. Propyldimethylamine		
Promazine	$-CH_2-CH(CH_3)-CH_2-N(CH_3)_2$	H
Chlorpromazine	$-CH_2-CH_2-CH_2-N(CH_3)_2$	Cl
Subgroup B. Propylpiperazine		
Prochlorperazine	$-CH_2-CH_2-CH_2-N$ piperazine $-CH_3$	Cl
Trifluoperazine	$-CH_2-CH_2-CH_2-N$ piperazine $-CH_3$	CF_3
Perphenazine	$-CH_2-CH_2-CH_2-N$ piperazine $-CH_2-OH$	Cl

the results. The percent kill was recorded 12 hr. after drug administration to PL mice and 24 hr. after drug treatment of BDL mice. The 12-hr. observation period was selected in the case of PL mice as previous studies had uniformly shown PL mice to survive for more than 18 hr. before succumbing to the effects produced by anuria; a 24-hr. observation period was unsuitable because approximately 30% of PL mice died in that period (2). Standard errors of the percent kill and statistical significance ($p<0.05$) of differences in percent kill between hypoexcretory and standard groups were determined by the binomial expansion method (3).

RESULTS

In cholestatic mice, the 24-hr. acute mortality was significantly enhanced when the animals were treated with dosages of promazine, perphenazine, or meprobamate (Table II).

The agents which exhibited significantly elevated mortality rates in anuric mice were: prochlorperazine, trifluoperazine, perphenazine, meprobamate, and chloral hydrate (Table II).

Pentobarbital but not phenobarbital had significantly reduced mortality rates in either anuric or cholestatic mice (Table II).

DISCUSSION

Increase in the mortality rates of pharmacologic agents in hypoexcretory animals is presumably the consequence of the inability to excrete the agent or metabolic products. The metabolic products may be toxic, or on the other hand, accumulation of nontoxic metabolites may contribute to enhanced mortality rates by preventing the metabolic alteration of the parent substance according to the law of mass action.

Examination of the results presented in Table II reveals two agents, meprobamate and perphenazine, whose mortality rates are increased when either urinary or biliary excretion is impaired, suggesting that these compounds may be excreted by both major excretory routes in mice. The results also show an interesting structure-action correlation among the phenothiazines (Table I). Phenothiazines with propylpiperazine moieties are uniformly more toxic when the urinary route of excretion is blocked while the propyldimethylamine subgroup is not so affected. When the biliary route of excretion is impaired, the phenothiazines affected, promazine and perphenazine, are found to belong to different subgroups with respect to both N substitution and ring substitution (Table I).

Little is known of the metabolism and excretion of the phenothiazine compounds in the mouse. In man, rats, and dogs, however, the metabolism and excretion of the phenothiazines has been the subject of considerable investigation. The metabolism of the phenothiazines is extensive and the role of

TABLE II—PERCENT MORTALITY OF CNS DEPRESSANTS IN CHOLESTATIC AND ANURIC MICE

Drug	Dose[a] mg./Kg.	Cholestatic[b] Operated No.	Cholestatic[b] Operated % ± S.E.[d]	Cholestatic[b] Sham-operated No.	Cholestatic[b] Sham-operated % ± S.E.[d]	Anuric[c] Operated No.	Anuric[c] Operated % ± S.E.[f]	Anuric[c] Sham-operated No.	Anuric[c] Sham-operated % ± S.E.[f]
Promazine	40	29	21 ± 8	35	6 ± 4[e]	28	7 ± 5	33	6 ± 4
Chlorpromazine	100	34	41 ± 8	32	33 ± 8	34	56 ± 9	33	55 ± 9
Prochlorperazine	50	33	24 ± 7	31	10 ± 5	24	42 ± 10	23	9 ± 6[e]
Trifluoperazine	200	39	56 ± 8	41	54 ± 8	34	91 ± 5	32	66 ± 8[e]
Perphenazine	21	34	56 ± 9	34	15 ± 6[e]	24	21 ± 8	3	3 ± 3[e]
Meprobamate	390	22	32 ± 10	21	5 ± 5[e]	31	39 ± 9	33	8 ± 5[e]
Pentobarbital	88	33	3 ± 3	35	20 ± 7	11	0	11	46 ± 15
Phenobarbital	13	13	39 ± 14	8	25 ± 15	12	36 ± 14	11	36 ± 14
Chloral hydrate	690	12	25 ± 13	12	25 ± 13	33	64 ± 8	32	41 ± 9[e]

[a] By intraperitoneal administration. [b] Mice rendered cholestatic by common bile duct ligation 24 hr. before drug administration. [c] Mice rendered anuric by penile ligation 2 hr. before drug administration. [d] Percent dead ± standard error 24 hr. after drug administration. [e] Significantly enhanced lethality of operated *vs.* sham-operated mice ($p < 0.05$). [f] Percent dead ± standard error 12 hr. after drug administration.

metabolism in the excretion of these compounds is recognized (4). The excretion of chlorpromazine in rats is divided equally between the urinary and biliary routes, the major routes of drug excretion. Only about 12% of the original dose of chlorpromazine is recovered in the urine as the unchanged drug, whereas the remainder is present as metabolites (5). In the present study cholestasis or anuria did not increase the lethality of chlorpromazine in mice, indicating that either the urinary or biliary route of excretion may serve adequately to remove chlorpromazine and its metabolites in mice.

In contrast to chlorpromazine, promazine is excreted primarily in the urine (70–80%) by the rat with the remainder excreted *via* the feces (6). Under these conditions it might be expected that the mortality of animals treated with a toxic dose of promazine might be enhanced if the urinary route of excretion were blocked. In mice, however, it was observed that the lethality was enhanced if the biliary route of excretion was blocked. This finding suggests that the primary excretory route of promazine and its metabolites may be different in mice than in rats or that a toxic metabolite of promazine is excreted primarily *via* the biliary route.

Phenothiazines having propylpiperazine moieties may be mainly excreted in the bile. In rats, triethylperazine, a propylpiperazine derivative of phenothiazine, is excreted largely in the bile with less than 10% of the dose appearing unchanged in the urine when given either orally or parenterally (7). The enhanced lethality of these compounds in anuric mice (Table II), however, may indicate that the urinary route of excretion for these compounds is more important in mice than in rats. Perphenazine appears to be dependent upon the function of both the urinary and biliary excretory routes in mice for detoxication since blockade of either route significantly increased the acute lethality of the compound.

Meprobamate is metabolized *in vivo* to hydroxymeprobamate, a pharmacologically inactive and nontoxic product. Both meprobamate and hydroxymeprobamate are conjugated with glucuronic acid and excreted *via* the urinary route in dogs (8). Ten percent of administered meprobamate is excreted unchanged and the remainder is excreted as metabolite in the urine. In mice the lethality of meprobamate is significantly enhanced when either the urinary or biliary routes of excretion is blocked (Table II). Since meprobamate and its metabolites are almost exclusively excreted by the kidneys in other species, the enhanced lethality of meprobamate in cholestatic mice is somewhat surprising. One explanation is that the mouse metabolizes or excretes meprobamate very differently from other mammalian species. Another possible explanation may be inhibition of meprobamate metabolism in the liver due to bile duct ligation thus resulting in delayed detoxication of the drug.

Chloral hydrate is reduced *in vivo* primarily to trichloroethanol, an active metabolite, and partially to trichloroacetic acid, an inactive metabolite. Both metabolic products may be conjugated with glucuronic acid and excreted either in urine or, to a smaller extent, bile (9). In mice, the acute lethality of chloral hydrate is increased by the anuric condition which correlates well with metabolism and excretion of chloral hydrate in man.

The significant reduction of lethality rates seen in hypoexcretory mice treated with pentobarbital but not phenobarbital cannot be readily explained. One possibility is that metabolites of pentobarbital accumulate in the blood of hypoexcretory animals and affect the equilibrium of pentobarbital toward increased tissue storage. Increased metabolism due to induction of liver microsomal enzymes is not believed to be an important factor in view of the short time (12 or 24 hr.) available for enzyme induction by pentobarbital. If enzyme induction were a significant mechanism, phenobarbital would also be expected to show a protective effect.

The dearth of information concerning metabolism and excretion of drugs in the mouse is not limited to the phenothiazines. Very little data of this type can be found in the literature. The authors are aware that extrapolation from one species to another is hazardous and must be done with great care. The above comments on metabolism and excretion in other species are germane to the findings described only if the metabolism and excretion of the drugs discussed are qualitatively the same in the mouse as in the other species.

The procedures employed in this study are relatively simple and can be carried out in a reasonable number of animals in a short time. The information gained from such procedures may prove to be of value in predicting the route of excretion of the drugs and/or their biotransformation products, and possible toxic reactions in the presence of reduced liver or kidney function. Such data may be of value in the safety evaluation of new drugs.

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Keyphrases

Hypoexcretion—mortality effect, mice
CNS depressants—test drugs
Anuria—penile ligation
Cholestasis—bile duct ligation

Synthesis and Pharmacology of Some Pyrroles and Indan Amines: Hexahydro Indeno (1,2-*c*) Pyrroles and Indan Amines

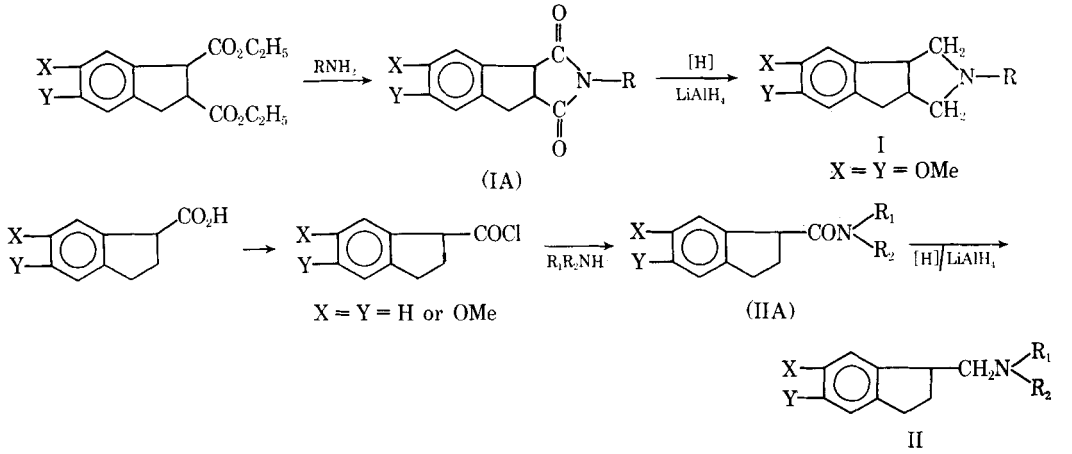
By SAMIR CHANDRA LAHIRI and BALAI PATHAK

Syntheses and pharmacology of a number of 1,2,3,3*a*,8,8*a*-hexahydro 5:6-dimethoxy indeno (1,2-*c*) pyrroles, 1-*N*-dialkyl amino methyl indans and 1-*N*-dialkyl amino methyl 5:6-dimethoxy indans were described. A few of these compounds showed oral hypoglycemic activity.

THIS PAPER, which is an extension of a previous one (1), is concerned with the syntheses of a few compounds of the types I and II and presents in summary form the salient features of their pharmacological activities.

RESULTS AND DISCUSSION

Chemistry—The reaction sequences leading to the formation of compounds I and II are shown n Scheme I:



Scheme I

Ethyl 5:6-dimethoxy indan 1,2-dicarboxylate (2) was heated with appropriate amine in a sealed tube to yield imides. The imides were reduced to indan pyrrolidines (I) by $LiAlH_4$ (3).

3-Carboxy indan-1-one (4) and its 5:6-dimethoxy derivative (5) were reduced to the corresponding carboxy acids by Clemmensen reduction (6, 7). The acids were converted to acid chlorides by thionyl chloride and were subsequently treated with appropriate primary or secondary amines to yield amides. The amides were converted to desired amines by $LiAlH_4$ reduction. In some cases, methylation of the secondary amines was carried out following a procedure of Bachmann (8). The hydrochloride salts were used for the pharmacological evaluation of hypoglycemic activity.

Structure Activity Relationship—Results of investigation of hypoglycemic activity of the compounds tested so far are summarized in Tables I and II. A close scrutiny of the above results revealed the following facts that among the compounds of the type I, substitution at N atom has some direct bearing on activity. Maximum activity was attained in the *n*-propyl or *n*-butyl substitution and activity disappeared in the *n*-amyl compound. The activity, however, reappeared in the *n*-hexyl compound of this series. Cyclohexyl or benzyl substitution also showed appreciable activity. Methoxy substitution at 5 and 5:6 positions of the aromatic ring also improved the activity in general (Table II). These compounds, however, were more effective against alloxan-induced diabetic animals than that of normal animals, and in a few cases they showed better response than the known standard drugs such as tolbutamide or chlorpropamide. One striking feature of these compounds is their slow onset of action and longer duration.

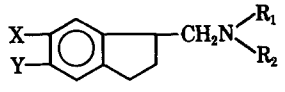
Among 1-*N*-dialkyl aminomethyl indans (Table I), activity was found to be very poor and methoxy substitution in the aromatic ring lowered the activitity in general. However, these compounds showed slight to moderate hypotensive, analgesic, and muscle-relaxant activities.

Complete pharmacology as well as mode of action of a few potent compounds along with their toxicology will be published soon. [1-Methyl-2-*N*-dialkyl aminomethyl indans and their 5:6 dimethoxy derivatives, however, showed appreciable hypoglycemic activity in both normal and alloxan-induced diabetic rabbits (9).]

Pharmacology Method—Normal, healthy, male rabbits, weighing between 1.5–1.8 Kg. were selected. Eight rabbits were used in each set. The animals were kept on fasting for 18 hr. prior to administration of the compound. Water was, however, allowed *ad libitum*. After taking the venous blood of the fasting rabbits, the animals were given the compounds (in the form of hydrochloride in aqueous solution) at the rate of 25 mg./Kg. of body weight by stomach tube and blood glucose concentration

Received August 4, 1967 from the Department of Applied Chemistry, University College of Science and Technology, Calcutta University, Calcutta 9, India.
Accepted for publication January 18, 1968.

TABLE I—1-*N*-DI-ALKYL AMINO METHYL INDANS (II)



(II)

(X = Y = H, or OMe)

X	Y	R_1	R_2	B.p., °C., mm.	Formula	% Carbon Calcd.	% Carbon Found	% Hydrogen Calcd.	% Hydrogen Found	% Nitrogen Calcd.	% Nitrogen Found	n_D	M.p., HCl	Activity 25 mg./Kg.
H	H	Me	Me	80–82/1	$C_{12}H_{17}N$	82.4	82.3	9.7	9.6	8.0	7.8	1.520	237–238[a]	±
H	H	Et	Et	98–100/1	$C_{14}H_{21}N$	82.7	82.6	10.3	10.3	6.9	6.7	1.509	124–126[b]	+++
H	H	*n*-Pr	*n*-Pr	110–114/.8	$C_{16}H_{25}N$	83.1	82.9	10.8	10.6	6.0	5.9	1.503	108–109[b]	++
H	H	*n*-Bu	*n*-Bu	105–110/.35	$C_{18}H_{29}N$	83.4	83.2	11.2	11.1	5.4	5.4	1.495	100–102[b]	++
H	H	H	*n*-Pr	80–85/.15	$C_{13}H_{19}N$	82.5	82.4	10.0	9.9	7.9	8.0	1.515	127–129[a]	++
H	H	H	*n*-Bu	98–102/.35	$C_{14}H_{21}N$	82.7	82.5	10.3	10.2	6.9	6.8	1.525	231–233[b]	++
H	H	Me	*n*-Pr	86–90/.25	$C_{14}H_{21}N$	82.7	82.6	10.3	10.1	6.9	6.8	1.509	131–132[b]	++
H	H	Me	*n*-Bu	128–132/2	$C_{15}H_{23}N$	82.9	82.7	10.6	10.6	6.4	6.3	1.503	138–139[a]	++
OMe	OMe	Me	Me	118–122/.6	$C_{14}H_{21}NO_2$	71.5	71.4	8.9	8.9	5.9	5.8	1.530	157–158[b]	−
OMe	OMe	Et	Et	120–122/.1	$C_{16}H_{25}NO_2$	73.0	72.9	9.5	9.4	5.3	5.4	1.526	140–142[b]	±
OMe	OMe	*n*-Pr	*n*-Pr	138–142/.1	$C_{18}H_{29}NO_2$	74.2	74.1	9.9	9.8	4.8	4.7	1.518	126–127[c]	−
OMe	OMe	*n*-Bu	*n*-Bu	144–148/.6	$C_{20}H_{33}NO_2$	75.2	75.0	10.3	10.2	4.8	4.7	1.509	230–232[a]	−
OMe	OMe	H	*n*-Pr	130–132/.4	$C_{15}H_{23}NO_2$	72.3	72.2	9.2	9.2	5.6	5.5	1.528	138–139[c]	±
OMe	OMe	H	*n*-Bu	152–156/.8	$C_{16}H_{25}NO_2$	73.0	72.9	9.5	9.4	5.3	5.3	1.523	143–145[a]	±
OMe	OMe	Me	*n*-Pr	149–151/.6	$C_{16}H_{25}NO_2$	73.0	72.8	9.5	9.3	5.3	5.3	1.516	131–132[a]	+
OMe	OMe	Me	*n*-Bu	163–166/.4	$C_{17}H_{27}NO_2$	73.3	73.2	9.7	9.8	5.0	4.9	1.511	134–135[a]	++

[a] Crystallized from ethyl acetate and ethanol. [b] Crystallized from ethyl acetate. [c] Crystallized from benzene. [d] − = inactive; ± = questionable; + = very slight; ++ = slight; +++ = moderate.

TABLE III—1-*N*-DI-ALKYL CARBOXY AMIDO INDANS (II-a)

(X = Y = H, or OMe)

Compd. Type	X	Y	R_1	R_2	B.p., °C., mm.	Formula	% Carbon Calcd.	% Carbon Found	% Hydrogen Calcd.	% Hydrogen Found	% Nitrogen Calcd.	% Nitrogen Found	n_D^{30}
IIA	H	H	Me	Me	140–145/0.8	$C_{12}H_{15}NO$	76.2	76.1	7.9	7.8	7.4	7.2	1.554
IIA	H	H	Et	Et	215–220/1.5	$C_{14}H_{19}NO$	77.4	77.2	8.7	8.7	7.4	7.2	1.529
IIA	H	H	*n*-Pr	*n*-Pr	234–240/1.5	$C_{16}H_{23}NO$	78.3	78.1	9.4	9.3	5.7	5.7	1.521
IIA	H	H	*n*-Bu	*n*-Bu	206–210/1	$C_{18}H_{27}NO$	79.1	79.0	9.9	9.8	5.1	5.0	1.511
IIA	H	H	H	*n*-Pr	204–206/1 m.p. 57–59[a]	$C_{13}H_{17}NO$	76.8	76.6	8.4	8.3	6.9	6.8	
IIA	H	H	H	*n*-Bu	207–210/1.5 m.p. 60–62[a]	$C_{14}H_{19}NO$	77.4	77.2	8.7	8.5	6.4	6.3	
IIA	OMe	OMe	Me	Me	163–165/.06	$C_{14}H_{19}O_3N$	67.4	67.2	7.6	7.5	5.6	5.5	1.546
IIA	OMe	OMe	Et	Et	160–166/.05	$C_{16}H_{23}O_3N$	69.3	69.1	8.3	8.2	5.0	5.0	1.538
IIA	OMe	OMe	*n*-Pr	*n*-Pr	212–215/.2	$C_{18}H_{27}O_3N$	70.8	70.6	8.8	8.6	4.6	4.5	1.533
IIA	OMe	OMe	*n*-Bu	*n*-Bu	196–202/.08	$C_{20}H_{31}O_3N$	72.0	71.9	9.3	9.1	4.2	4.1	1.513
IIA	OMe	OMe	H	*n*-Pr	m.p. 92–93[b]	$C_{15}H_{21}O_3N$	68.4	68.3	7.9	7.7	5.3	5.2	
IIA	OMe	OMe	H	*n*-Bu	m.p. 115–117[b]	$C_{16}H_{23}O_3N$	69.3	69.2	8.3	8.2	5.0	5.0	

[a] Crystallized from petroleum ether, 60–80°. [b] Crystallized from a mixture of benzene and petroleum ether, 60–80°.

TABLE II—ORAL HYPOGLYCEMIC ACTIVITY OF 1,2,3,3*a*,8,8*a*,-HEXAHYDRO INDENO (1,2-*c*) PYRROLES

(I)

(X = Y = H, OMe, X = H, Y = OMe)

X	Y	R	Hypoglycemic Effect at a Dose of 25 mg./Kg.[d]
H	H	Me[a]	+
H	H	Et[a]	±
H	H	*n*-Pr[a]	++
H	H	*n*-Bu[a]	+++
H	H	*n*-Am[a]	−
H	H	*n*-Hex[b]	++
H	H	Cyclo-hex[b]	+++
H	H	Benzyl[b]	++
H	OMe	Me[c]	−
H	OMe	Et[c]	+
H	OMe	*n*-Pr[c]	+++
H	OMe	*n*-Bu	+++
H	OMe	*n*-Am[c]	−
H	OMe	*n* Hex[c]	++
OMe	OMe	Me[a]	±
OMe	OMe	Et[a]	−
OMe	OMe	*n*-Pr[a]	+++
OMe	OMe	*n*-Bu[a]	++++
OMe	OMe	*n*-Hex	+++
OMe	OMe	Cyclo-hex	+++
OMe	OMe	Benzyl	+++
Tolbutamide			++++

[a] See Ref. 3. [b] See Ref. 1. [c] See Ref. 11. [d] − = no activity; ± = questionable; + = very slight; ++ = slight; +++ = moderate; ++++ = marked.

was observed at different hours up to 24 hr. The maximum fall of blood glucose concentration was observed in between the 9th–12th hour. The blood glucose estimation was carried out in a manner as described by Hagedorn and Jensen (10). The hypoglycemic activity of the compounds tested were detailed in Tables I and II.

EXPERIMENTAL[1]

***N*-Substituted 5:6-Dimethoxy Indan 1,2-Dicarboximides (Ia)**—A mixture of 5:6-dimethoxy indan 1,2-dicarboxylate (2) (0.05 mole) and an appropriate amine (0.1 mole) was heated in a sealed tube for 8–12 hr. at 150° and the imide isolated following a similar procedure as reported earlier (1,3).

N-*n*-Hexyl 5:6-dimethoxy indan 1,2-dicarboximide was boiled at 188–190°/0.8 mm.

Anal.—Calcd. for $C_{19}H_{25}NO_4$: C, 68.9; H, 7.5; N, 4.2. Found: C, 68.7; H, 7.4; N, 4.2.

N-Benzyl 5:6-dimethoxy indan 1:2-dicarboximide was boiled at 210–215°/0.15 mm.

Anal.—Calcd. for $C_{20}H_{19}NO_4$: C, 71.2; H. 5.6; N, 4.1. Found: C, 71.2; H, 5.5; N, 4.2.

N-Cyclohexyl 5:6-dimethoxy indan 1,2-dicarboximide was crystallized from petroleum ether (60–80°); m.p. 118–120°.

Anal.—Calcd. for $C_{19}H_{23}NO_4$: C, 69.3; H, 6.6; N, 4.2. Found: C, 69.2; H, 6.5; N, 4.2.

1,2,3,3a,8,8a-Hexahydro 5:6-Dimethoxy 2-Alkyl Indeno (1,2-*c*)Pyrroles (I)—The appropriate imides were reduced by refluxing with $LiAlH_4$ with absolute

[1] All melting points are corrected and determined in Gallenkamp apparatus. Boiling points are uncorrected.

ether and amines were separated in the usual manner.

1,2,3,3*a*,8,8*a*-Hexahydro 5:6-dimethoxy *N*-*n*-hexyl indeno (1,2-c) pyrrole was boiled at 186–188°/0.6 mm.

Anal.—Calcd. for $C_{19}H_{29}NO_2$: C, 75.2; H, 9.6; N, 4.6. Found: C, 75.1; H, 9.5; N, 4.5. Hydrochloride (hygroscopic) was crystallized from ethyl acetate, m.p. 118–120°.

1,2,3,3*a*,8,8*a*-Hexahydro 5:6-dimethoxy *N*-cyclohexyl indeno (1,2-*c*) pyrrole was boiled at 192–195°/0.6 mm.

Anal.—Calcd. for $C_{19}H_{27}NO_2$: C, 75.7; H, 8.9; N, 4.6. Found: C, 75.6; H, 8.7; N, 4.5. Hydrochloride was crystallized from a mixture of ethyl acetate and absolute ethanol, m.p. 200–202°.

1,2,3,3*a*,8,8*a*-Hexahydro 5:6-dimethoxy *N*-benzyl indeno (1,2-*c*) pyrrole was boiled at 192–196°/0.4 mm.

Anal.—Calcd. for $C_{20}H_{23}NO_2$: C, 77.6; H, 7.4; N, 4.5. Found: C, 77.4; H, 7.3; N, 4.4. Hydrochloride salt crystallized from ethyl acetate, m.p. 172–173°.

1-Chlorocarboxy Indans—Indan 1-carboxy acid (5, 6) was converted to acid chloride by reacting with thionyl chloride. It boiled at 140–145°/1.5 mm. (74%).

Anal.—Calcd. for $C_{10}H_9ClO$: C, 65.9; H, 4.6; Cl, 19-11. Found: C, 65.9; H, 4.88; Cl, 19.01.

1-Chlorocarboxy 5:6-Dimethoxy Indan—A mixture of 3,4-dimethoxy phenyl succinic acid (5) (16 Gm.) and polyphosphoric acid (740 Gm.) was heated at 100° for 3 hr. under stirring. The keto acid thus formed was extracted with chloroform. The yield was 80%, m.p. 190–191° dec. The keto acid was then subjected to Clemmensen reduction and the 1-carboxy 5:6-dimethoxy indan distilled at 176–180°/0.7 mm., crystallized from benzene, mp. 114–116°.

Anal.—Calcd. for $C_{12}H_{14}O_4$: C, 65.86; H, 6.30. Found: C, 64.82; H, 6.28. The acid chloride was prepared by reacting with thionyl chloride but it was not distilled as it decomposed on distillation.

1-N-Dialkyl Carboxy Amido Indans (IIa)—The 1-chlorocarboxy indan or 1-chlorocarboxy 5:6-dimethoxy indan (1 mole) was added dropwise to a mixture of an appropriate amine (1.5 mole) and sodium hydroxide solution (10%; 1 mole) cooled in an ice bath. The amide was extracted with a suitable solvent and either distilled under reduced pressure or crystallized from a suitable solvent. The physical properties and analyses of the amides are listed in Table I.

1-N-Dialkyl Aminomethyl Indans (II)—The amide (1 mole) so prepared was reduced with $LiAlH_4$ (2 mole) in dry ether under reflux for 8–12 hr. in the usual manner. In some cases, the secondary amines, thus prepared, were methylated by heating a mixture of amine, formic acid, and formaldehyde on an oil bath in the usual manner (8). The bases were characterized as their hydrochlorides and other physical properties and hypoglycemic activities were listed in Table I.

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Keyphrases

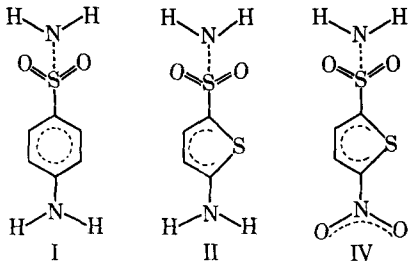
Pyrroles—synthesis
Indan amines—synthesis
Hypoglycemic activity—pyrroles, indan amines
Structure–activity relationship

Thiophene Analogs of Sulfanilamide: Bacterial Growth Inhibitors

By J. BULKACZ, M. A. APPLE*, J. C. CRAIG, and A. R. NAIK

Structure *versus* activity study of thiophene analogs of sulfanilamides indicated: (*a*) the parent aminothiophene-2-sulfonamide is a better bacterial growth inhibitor than is sulfanilamide (I); (*b*) 5-aminothiophene-2-sulfonamide (II) is a better inhibitor than the 4-amino isomer (III); (*c*) substitution of the sulfonamide N with groups which enhance I's antibacterial activity does not necessarily similarly enhance the activity of the thiophene analog (VI, VII); (*d*) 5-(IV) and 4-nitrothiophene-2-sulfonamides(V) are more inhibitory than the corresponding 5- and 4-amino compounds (II, III) with the 5-nitro compound (IV) being bactericidal at very low concentrations. Folic acid or PABA reversed the growth inhibition produced by the thiophene-2-sulfonamides, indicating both the nitro and amino thiophene-2-sulfonamides prevent intracellular folic acid biosynthesis.

THE MODERN era of drug treatment of many bacterial infections originated with the finding of a selective effect of sulfanilamide on organisms which synthesize their own folic acid from *p*-aminobenzoate (1–3). The essential nucleus, a *p*-aminophenyl-sulfonamide, has been modified and sometimes improved extensively, mostly by addition of groups to the sulfonamide nitrogen (4). In the increasing frequency of instances of bacterial drug resistance transmitted by the multiple-drug resistance RTF episome, most often resistance to streptomycin, chloramphenicol, and tetracycline are gained simultaneously by the invading pathogen; there is a smaller incidence of concomitant sulfanilamide (I) resistance (5). Improved forms of I might be effective in such cases of multiple-drug resistance, or in cases where current I derivatives are only marginally effective. The authors report here the superior antibacterial effectiveness of modified forms of I, in which the phenyl ring of the parent molecule of the sulfanilamide family of drugs has been replaced by a thiophene ring (II, IV).



I II IV

MATERIALS AND METHODS

Salmonella typhimurium and *Proteus vulgaris* were obtained from the University of California, San Francisco, collection.[1] These bacterial strains were maintained on sensitivity test (ST) agar (Colab, Chicago) or brain heart infusion (BHI) agar (Difco, Detroit) slants or plates and subcultured on the same media at 37° in a candle jar as needed for anaerobic-high CO_2 conditions (8). The bacterial cells used as inoculum were grown overnight in tubes containing 8 ml. of BHI broth for approximately 15 hr., centrifuged at about 1500 × g in an International Clinical centrifuge (I.E.C. Co., Boston) at 25° and suspended in a pH 7.0 sodium phosphate buffer. The growth flasks consist of 300-ml. conical flasks, fitted with a closed-tube side-arm about 2.5 cm. from the flask bottom. The drug, if any, was added to these flasks and diluted with the growth medium consisting of 15 ml. of BHI broth supplemented with 0.5% lactalbumin hydrolysate (Difco, Detroit) and 0.6 m*M* $NaHCO_3$. To this medium the bacterial cells were added to about 25 mcg../ml. of dry weight equivalent and flasks placed at 37° on a gyrotary shaker (New Brunswick Scientific, New Brunswick, N. J.) at two shakes per sec. The bacterial growth was followed spectrophotometrically at 30-min. intervals.

Details of the synthesis and properties of the thiophene analogs will be published (6, 7). The compounds used and their melting points were:

	Compounds	m.p.
II	5-aminothiophene-2-sulfonamide	137–138°
III	4-aminothiophene-2-sulfonamide	132–133°
IV	5-nitrothiophene-2-sulfonamide	135–136°
V	4-nitrothiophene-2-sulfonamide	165–166°
VI	2-(5-aminothiophene-2-sulfonamide) pyridine	162°
VII	2-(5-aminothiophene-2-sulfonamide) pyrimidine	204–205°

To estimate *in vivo* toxicity of a compound, 25-Gm. male albino mice, random bred Webster-Simonsen Swiss (Simonsen Laboratories, Gilroy, Calif.), were given a single intraperitoneal injection each morning for five consecutive mornings. The animals were weighed daily and survival followed for 30 days. A tolerated dose was considered that dose which produced a readily reversible weight loss not exceeding 12% at any time and which caused no mouse deaths during the 30-day period.

RESULTS AND DISCUSSION

Superior Antibacterial Activity Associated with Thiophene Ring Modification—As seen in Fig. 1, the thiophene ring modification (Structure II) produced an increase in the antibacterial activity against streptococcus AHT, known to be a human pathogen, above that seen under these conditions with the phenyl ring compound (Structure I). The 5-aminothiophene analog, II, is considerably more effective as a growth inhibitor than is the 4-amino isomer (Fig. 1). The medium of cultures containing II turned a violet-brown after 24 hr.

The growth inhibition by II is much greater than I when tested on *Salmonella*, *Streptococcus*, and *Proteus* (Table I). Growth rate was significantly

Received January 2, 1968, from the University of California Medical Center, San Francisco, CA 94122
Accepted for publication February 20, 1968.
Supported in part by grants USPHS CA-03175 (MAA), NIDR-Tr-DE-20 (JB).

[1] Streptococcus, human type, "AHT," was obtained from D. D. Zinner.

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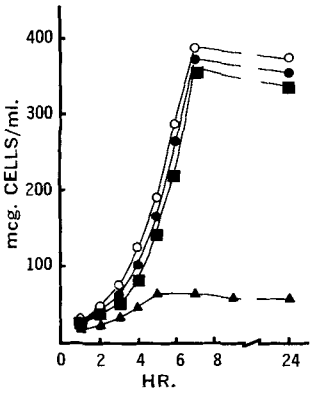

Fig. 1—Growth of Streptococcus *in the presence and absence of I and thiophene analogs of I. All drugs at 500 mcg./ml. Key: ○, control (no drugs); ●, I; ■, III; ▲, II.*

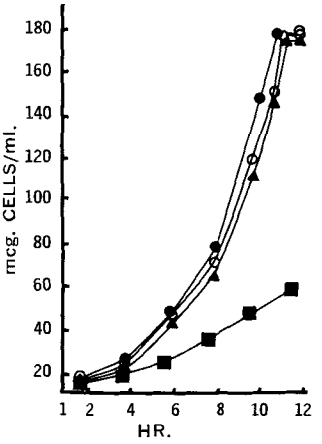

Fig. 2—Inhibition of Streptococcus *by IV and its reversal by PABA or folic acid. IV was added to 5 mcg./ml. Key: ○, control (no drugs); ■, IV only; ●, IV + 300 mcg./ml. PABA; ▲, IV + 100 mcg./ml. folic acid.*

slowed by II, which about doubled the time between cell divisions at concentrations at which I had no significant effect (Table I). Substitution of a pyrimidine on the thiophene sulfonamide nitrogen (Table I) produced a drug which was much more active than I.

Nitrothiophene Analogs As More Effective Growth Inhibitors Than Aminothiophene Analogs—The nitrothiophene compounds were both more inhibitory than the corresponding aminothiophene compounds (Table I). For example, I had no effect on *Salmonella* growth at 1 mg./ml., II inhibited *Salmonella* growth by 60% at 1 mg./ml., but both III and IV inhibited *Salmonella* growth by over 95% at concentrations of only 100 mcg./ml. (Table I). At 100 mcg./ml., IV was a more effective growth inhibitor than III against both *Salmonella* and *Streptococcus* (Table I).

Based on relative inhibition per mcg./ml. concentration, IV is about 1,000-fold more effective than I (*cf.* Fig. 2 and Table I).

Mechanism of Bacterial Growth Inhibition: Inhibition Reversal by Folate and p-Aminobenzoate—BHI growth medium contains folic acid and its metabolites. After limited growth at the slower rate by *Streptococcus* in the presence of II, the culture was found to be sterile: no viable cells could be found by the plating-out procedure using drug-free BHI or ST agar (9). The latter observation was

Table I—Effect of Sulfanilamide and its Thiophene Analogs on Growth of Three Diverse Bacteria

Drug Name Microorganism	Drug Concn., mcg./ml.	7-Hr. Growth, mg./L.	Inhibition, %	Division Time, min.
Streptococcus AHT				
None	—	400	—	75
Sulfanilamide (I)	1,000	300	25	85
	500	400	0	75
4-Aminothiophene-2-sulfonamide (III)	1,000	250	40	—
5-Aminothiophene-2-sulfonamide (II)	1,000	100	75	150
	500	150	60	—
4-Nitrothiophene-2-sulfonamide (V)	1,000	25	95	>600
	500	200	50	—
	100	335	15	—
5-Nitrothiophene-2-sulfonamide (IV)	1,000	20	95	—
	500	25	95	>600
	100	25	95	—
2-(5-Aminothiophene-2-sulfonamido) pyridine (VI)	500	300	25	100
2-(5-Aminothiophene-2-sulfonamido) pyrimidine (VII)	1,000	115	70	—
	500	250	40	—
Salmonella typhimurium				
None	—	4,700	—	26
Sulfanilamide (I)	1,000	4,700	0	27
5-Aminothiophene-2-sulfonamide (II)	1,000	1,850	60	50
4-Nitrothiophene-2-sulfonamide (V)	100	200	95	100
5-Nitrothiophene-2-sulfonamide (IV)	100	35	99	190
Proteus vulgaris				
None	—	3,000	—	28
Sulfanilamide (I)	1,000	2,250	25	30
5-Aminothiophene-2-sulfonamide (II)	1,000	900	70	65

also made for *Streptococcus* exposed to either IV or V at the second lowest concentrations used (Table I), suggesting the organisms incorporate the drugs as they grow and the drugs then diminish some essential activity to a lethal extent, in a manner similar to the less effective I.

Consistent with the possible mode of action of these thiophene analogs in antagonizing *p*-aminobenzoate metabolism, II is probably closer in its three-dimensional structure to *p*-aminobenzoate than is III, since the large S atom essentially replaces two carbon atoms.

Experiments with *Streptococcus* using 100 mcg./ml. of the nitro compounds or 500 mcg./ml. of the amino compounds showed no significant inhibition reversal by 25 mcg./ml. of PABA except in the case of III. The inhibition by V (150 mcg./ml.) was reversed by 300 mcg./ml. PABA. The latter concentration of PABA also served to release the inhibition induced by 2–5 mcg./ml. of IV (Fig. 2).

The reversal of the IV-induced inhibition by PABA or folate (Fig. 2) suggests the biosynthesis of folate is the site of action of the nitrothiophene-2-sulfonamides as well as the amino compounds. This could be accomplished by intracellular reduction of the nitro compounds to the corresponding amino compounds, making the amino compounds the active forms. The same concentration of PABA (300 mcg./ml.), completely reversing effective inhibition by IV, produced only a slight reversal of the same extent of inhibition by II.

However, nothing is yet known about the relative transport rates of these thiophenes into cells, so no conclusions can be drawn about the relative effective concentration; it appears the nitro compounds, if they are reduced to the amino form, may be transported to the site of intracellular activity at a greater rate than the corresponding amino compound.

Antibacterial Activity of II and IV Exceeds That of Similar Compounds—The carboxamide corresponding to II, 5-aminothiophene-2-carboxamide, was inactive against *Streptococcus*, *Escherichia*, or *Diplococcus*, even at concentrations many times that at which sulfanilamide was active (10). The 5-amino-3-thiophene sulfonamide isomer of II was not inhibitory to a wide variety of bacterial genera concentrations at least tenfold greater than those used for II (11). Thus, II is a new structure with much increased biological activity over similar structures.

Although the 5-nitrothiophene-3-sulfonamide isomer has antibacterial activity, it is 500–1,000-fold less active against *Streptococcus* per μM concentration than IV (6). More significantly, compared with the data of Woods on sulfanilamide reversal (1), IV is at least 10,000-fold less susceptible to reversibility by PABA than is sulfanilamide.

In Vivo Toxicity—The substitution of the thiophene ring for the phenyl ring of I increases the water solubility and thus decreases the partition coefficient. Such changes are less significant to this study *in vitro* than they would be to study of the drug in pathogen-infected animals. Although a few mcg./ml. of IV appeared to be bactericidal, a daily injection (5 days) of 100 mg./Kg. of IV to adult Swiss mice was tolerated with a reversible weight loss. This suggests a body fluid level of up to several hundred mcg./ml. could be attained.

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Keyphrases

Sulfanilamide—thiophene analogs
Structure–activity relationship—sulfanilamide analogs
Antibacterial activity—*in vitro*
Toxicity—*in vivo*, *in vitro*

Essential Oil of *Anemopsis californica*

By RAMESH N. ACHARYA* and MADHUKAR G. CHAUBAL

The constituents of the hydrodistilled essential oil from the roots and rhizomes of *Anemopsis californica* have been examined. Thymol was identified from the phenolic fraction of the oil. Gas chromatography on Hyprose SP-80 followed by collection of effluent fractions and comparison of their IR spectra with those of pure samples confirmed the presence of methyleugenol as the major constituent. A third constituent of the oil was found to be piperitone. Methyleugenol (approximately 55 percent v/v of the oil), thymol (13 percent), and piperitone (5 percent) together make up approximately 74 percent of the oil. A fourth constituent, tentatively identified as an aromatic unsaturated ether, and other constituents are being investigated.

ONLY TWO reports on the chemical constituents of the roots and rhizomes of *Anemopsis californica* (Nutt.) Hook and Arn. (Fam. *Saururaceae*, Yerba del Mansa) are available. Horton and Paul (1) first reported the presence of methyleugenol (4-allylveratrole) in the steam-distilled oil. Childs and Cole (2) later confirmed the occurrence of methyleugenol in the petrol ether extracts of the rhizomes and also reported the presence of leucoanthocyanidins and an unidentified crystalline compound in these extracts.

This paper reports the results to date of the investigation of the essential oil of *Anemopsis* (Mansa oil).

EXPERIMENTAL

Crude Drug—Hathaway Allied Products[1] supplied the whole crude drug (roots and rhizomes) in following lots: Lot RM-65-167, Lot RM-65-1029 and Lot RM-67-91.

Extraction of Essential Oil—Approximately 3 Kg. crude drug was crushed to 12–6 mm. size. The crushed drug was hydrodistilled for 30 hr. (batches of 300 Gm. drug + 1800 ml. water). The distillate was collected in a modified Clevinger's apparatus for oils lighter than water. After separation for 10 hr. the oil was removed and dried over anhydrous sodium sulfate for 30 hr. The batches were pooled for further analysis.

Thin-Layer Chromatography (TLC)—To monitor the separation steps TLC was used under the following conditions: *Equipment*—Desaga,[2] variable thickness spreader. *Carrier Plates*—glass, 8 × 8 or 2 × 8 in. *Adsorbent*—Silica Gel G (25 Gm. suspended in 50 ml. deionized water and spread over five 8 × 8 in. plates), 250 μ thick. *Activation*—dried at 110° for 2 hr. and stored over anhydrous calcium sulfate[3] before spotting. *Sample Size*—10 μl. *Solvent System*—benzene + ethylacetate, 95:5 v/v. *Development*—room temperature (22–27°), 15 cm., 35–40 min. *Spray Reagents*—(A) Anisaldehyde (anisaldehyde, 3 drops in a mixture of 10 ml. methanol and 1 ml. glacial acetic acid + 2 ml. concentrated sulfuric acid just before spraying. Sprayed plates heated at 95° for 7 min.). (B) 2:4 Dinitrophenylhydrazine (2:4 DNPH) (3). (C) Diazotized sulfanilic acid (3).

Functional Group Separation—Scheme I shows the procedure used to achieve gross separation of the constituents of Mansa oil.

A negative test with 2:4 DNPH on TLC indicated initially that the oil was devoid of carbonyls. However, Stahl (4) has pointed out that ketones such as menthone, piperitone, and fenchone should be present in larger amounts to yield a positive color reaction with the reagent. If such ketones were present in Mansa oil in low concentrations, the negative test could be expected. In order to further test for the presence of carbonyls a procedure reviewed by Parsons (5) was adopted as follows. Analytical grade diatomaceous earth,[4] 10 Gm. was triturated with a solution of 500 mg. of 2:4 DNPH in 6 ml. phosphoric acid (d = 1.75) and 4 ml. of deionized water. The impregnated diatomaceous earth was then packed in a glass column (2.1 cm. i. d.) filled with cyclohexane. The cyclohexane was run off the column and the diatomaceous earth washed with benzene to remove excess 2:4 DNPH. A solution of 0.5 ml. oil in 0.5 ml. cyclohexane was then applied to the column and the column eluted with cyclohexane until the eluate gave no color with vanillin-sulfuric acid reagent (3). The column was now eluted with benzene. The benzene effluent was deep orange-red; a characteristic color of 2:4-dinitrophenylhydrazones. It was therefore apparent that small amounts of carbonyl compounds were present in Mansa oil and that these could not be grossly separated by the usual 2:4 DNPH treatment of the oil. A method similar to that of Teitelbaum (6) was therefore used to obtain the carbonyl fraction from the phenol-free oil (Scheme I). A mixture of 5 Gm. phenol-free oil, 5 Gm. Girard "T"[5] reagent, and 0.1 Gm. of a cation exchanger[6] were refluxed in a 50-ml. round-bottom flask with 10 ml. absolute ethanol for 1.5 hr., during which period Girard "T" dissolved. The hot solution was then stirred into 40 ml. ice water and allowed to stand for 0.5 hr. The aqueous mixture was extracted with ether (15 ml. × 3) and the combined ether extracts were dried over Na_2SO_4 anhyd. Removal of ether *in vacuo* gave the noncarbonyl fraction (4.15 Gm.). The aqueous phase was acidified by 10 ml. concentrated HCl and kept overnight. The oily carbonyl fraction which separated was extracted with ether (20 ml. × 3), the combined ether extracts dried over Na_2SO_4 anhyd., and the ether removed *in vacuo*

Received October 9, 1967, from the Department of Pharmacognosy, School of Pharmacy, University of the Pacific, Stockton, CA 95204

Accepted for publication December 21, 1967.

Abstracted from a thesis submitted by R. N. Acharya to the Graduate School, University of the Pacific in partial fulfillment of the Master of Science degree requirements.

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[1] Hathaway Allied Products, Los Angeles, Calif.

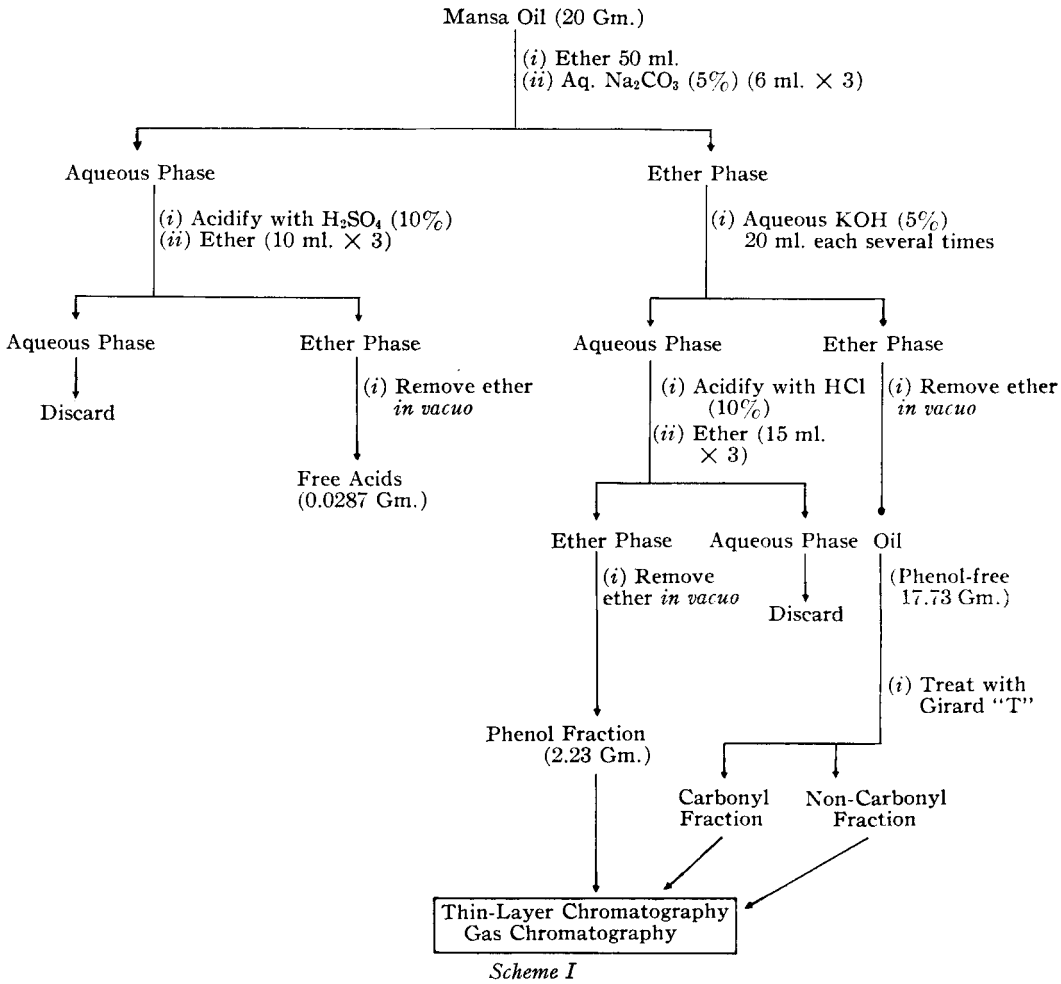
[2] Brinkmann Instruments Inc., Westbury, N. Y.

[3] Drierite, W. A. Hammond, Drierite Co., Xenia, Ohio.

[4] Celite 545, Johns-Manville Corp., New York, N. Y.

[5] Eastman Organic Chemicals.

[6] Dowex 50WX-8, Dow Chemical Co., Midland, Mich.



Scheme I

(0.85 Gm.). TLC of this carbonyl fraction now gave a 2:4 DNPH positive spot (Fig. 1B).

Gas Chromatography (GC) Apparatus—Autoprep[7] model A-700 with thermal conductivity detector. *Column*—Aluminum, 1/4 in. o.d., 20 ft. *Packing*—Hyprose SP-80,[7] 20% w/w on 60–80 mesh Chromosorb W (Hyprose SP-80, 20 Gm., in hot chloroform solution was deposited on 100 Gm. Chromosorb W with constant stirring. The solvent was then removed in a rotary evaporator under water-aspirator suction. The packed column was preconditioned overnight at 190° under a slow stream of nitrogen). *Carrier Gas*—Helium, 75 ml./min. at 50 p.s.i. inlet pressure. *Injection Port Temperature*—200°. *Column Temperature*—180°. *Detector Temperature*—250°. *Recorder Span*—1 mv. *Chart Speed*—1 in. 5 min. The effluent fractions were collected directly on KBr pellets placed in a KBr bottle[7] or by passing through a trap containing spectral grade carbon tetrachloride. The IR spectra were obtained with a Perkin-Elmer model 337 IR spectrophotometer.

RESULTS AND DISCUSSION

Approximately 250 Gm. of a dark green oil was collected. The average yield from several batches was 6.5% v/w of crude drug. Finer grinding of the crude drug increased the yield to approximately 7.5% however under these conditions charring of the fine powder occurred. The oil distilling in the initial stages was pale yellow gradually changing to dark green. The pooled oil had the following physical constants: d_{25}^{25} 0.9980; n_D^{25} 1.5195, and $[\alpha]_D^{25}$ less than −3.5° (dark color interfered).

The three spray reagents for TLC were selected so as to detect most of the common constituents of essential oils (3). Anisaldehyde gives colored spots with terpenes, phenols, steroids, and sugars and 10 well-marked spots were obtained with Mansa oil (Fig. 1A). The carbonyl compounds are detected, more or less specifically, with 2:4 DNPH. This reagent failed to give any colored spot with the oil. Diazotized sulfanilic acid gave only one yellow spot indicating the presence of one phenol (Fig. 1C).

Repeated extractions with aqueous KOH removed the phenol fraction from the oil. Addition of alkali to the oil changed the color of the oil to deep red as long as phenols were present. TLC of the dark brown oily phenol fraction (Scheme I) gave only one yellow spot with reagent (C) (Fig. 1D). The odor of the fraction was similar to that of thyme oil. Distillation of this fraction at 230–238° gave a clear heavy liquid which solidified on keeping at 4° for 4 hr. The oily film adhering to

[7] Wilkens Instrument and Research Inc., Walnut Creek, Calif.

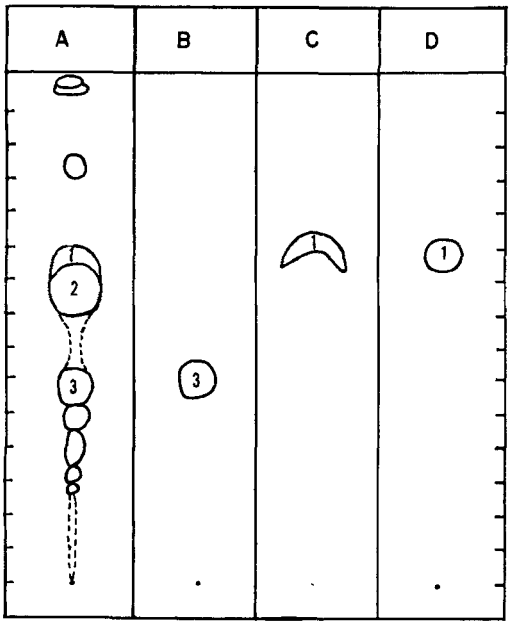


Fig. 1—Thin-layer chromatograms of hydrodistilled oil of Anemopsis californica. *Key: A, anisaldehyde; B, 2:4 DNPH, C, diazotized sulfanilic acid; carbonyl fraction of the oil; D, diazotized sulfanilic acid phenol fraction of the oil; 1, thymol; 2, methyleugenol; 3, piperitone.*

the solid was washed with chilled hexane and the solid recrystallized from boiling hexane (b.p. 68.7°). The purified solid was identified as thymol: m.p. 48–49° (49–50°) uncorrected; Positive thymolphthalein test; *N*-naphthylurethane—m.p. 106–107° (107–108°); α-naphthylurethane—m.p. 160° (161°); identical IR spectra, retention time (RT) on Hyprose SP-80 and R_f on TLC as that of pure thymol (reagent, Merck) (values in parentheses for thymol reagent, Merck).

The gas chromatogram of Mansa oil showed 20 peaks (Fig. 2). Four compounds (Peaks 1,2,3,12) were present in appreciable amounts and the authors concentrated on their identification. Tests with known samples[8] of common essential oil constituents had shown that, under these conditions, terpenes eluted out in the early region (0–5 min.), oxygenated compounds in the middle region (6–15 min.), and phenols and derivatives in the late region (16–40 min.) (Fig. 2). Injection of pure compounds showed that Peaks 1 and 2 (Fig. 2) were thymol and methyleugenol, respectively. The fractions from these peaks collected directly on KBr pellets gave IR spectra identical with those of the pure compounds. The GC of the carbonyl fraction (Scheme I) showed it to be enriched in the third major compound (Fig. 2, Peak 3). Successive injections of 5 μl. of the

[8] Supplied by Fritzsche Brothers Inc., New York, N. Y.

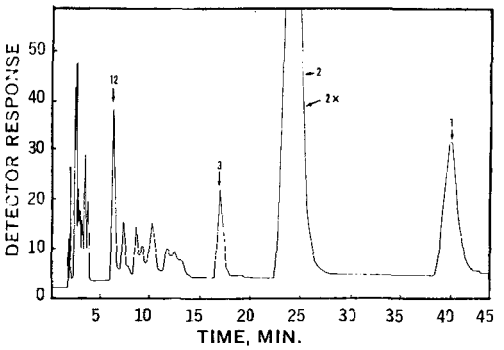


Fig. 2—Gas chromatogram of hydrodistilled oil o Anemopsis californica *(5 μl). Hyprose SP-80 stationary phase.*

carbonyl fraction were therefore used to collect sufficient quantity of Peak 3 in spectral grade carbon tetrachloride for IR analysis. The IR spectrum of the compound was identical with that of pure piperitone collected under identical conditions. The fourth compound (Fig. 2, Peak 12) collected in carbon tetrachloride gave an IR spectrum indicative of an aromatic ether (strong absorption at 1600–1500 $cm.^{-1}$ and at 1262 $cm.^{-1}$) with probably an isolated unsaturation (weak absorption at 1620 $cm.^{-1}$) and which is not monosubstituted (absence of absorption near 700 $cm.^{-1}$).

With pure methyleugenol and thymol as internal standards in GC the three identified compounds were found to be present in following amounts in the oil (% v/v); methyleugenol (55.30), thymol (13.57), and piperitone (5.19).

The aromatic ether and remaining 16 peaks constitute 25.94% of Mansa oil. In order to obtain sufficient quantities of these for identification, fractionation of the oil and subsequent GC on various stationary phases is under progress.

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Keyphrases

Anemopsis californica—essential oil
Mansa oil—*Anemopsis californica*
Methyleugenol, thymol, piperitone—isolated, identified
Column chromatography—separation
Cation-exchange resin—separation
TLC—separation monitoring
GLC—analysis
IR spectrophotometry—structure

Alkaloids of *Peumus boldus*. Isolation of (+) Reticuline and Isoboldine

By D. W. HUGHES, K. GENEST, and W. SKAKUM

Chromatographic investigation of alkaloid fractions from the leaves of *Peumus boldus* Molina revealed the presence of 17 alkaloidal components. Four of these were correlated with previously isolated aporphine constituents and two others were isolated and identified as (+) reticuline and isoboldine.

THE LEAVES of the Chilean evergreen shrub *Peumus boldus* Molina (boldo leaves) have long been used as a choleretic, diuretic, stomachic, sedative, and anthelmintic (1). Preparations from the leaf have not been official in the NF since 1936, but the drug is still used, especially in "over-the-counter" remedies. Some 40 preparations containing the drug are currently available in Canada. The activity of the leaves has been variously ascribed to the alkaloid, the glycoside, or the essential oil content (1).

Boldine (I, $R_1 = R_3 = H$, $R_2 = CH_3$) was isolated from *P. boldus* by Merck in 1922 (2) and its structure determined 10 years later (3). By paper chromatography Rüegger, in 1959 (4), detected the presence of 11 alkaloids and he isolated and characterized three of these: isocorydine (II, $R = CH_3$), norisocorydine (II, R = H), and *N*-methyllaurotetanine (I, $R_3 = H$, $R_1 = R_2 = CH_3$).

This paper describes preliminary separation schemes for the isolation of some of the unknown alkaloidal constituents of boldo leaves and the isolation and identification of (+)-reticuline (III, (R = H) and isoboldine (I, $R_2 = R_3 = H$, $R_1 = CH_3$).

EXPERIMENTAL AND RESULTS

TLC Procedures—TLC plates of Silica Gel G (Merck) (layer thickness 0.25 mm.) were prepared according to standard techniques[1] using either water (for basic solvent systems) or 0.1 *N* sodium hydroxide (for nonbasic solvent systems).

The most useful solvent systems were: *A*, chloroform–methanol (85:15); *B*, ethyl acetate–acetone–methanol (50:20:30); *C*, benzene–methanol (80:20); *D*, ethyl acetate–acetone–methanol–diethylamine (45:30:20:5). The most commonly used spray reagents were: A, potassium iodoplatinate (5); B, Gibb's (6).

Extraction—Boldo leaves[2] were ground to a coarse powder in a Wiley mill and defatted by portionwise extraction in a Soxhlet apparatus with petroleum ether (b.p. 30–60°) for 6 hr. No alkaloids could be detected in this extract by TLC after separation of any bases by acid extraction. One-kilogram portions of the defatted powder were moistened with 5% ammonium hydroxide (1 L. and exhaustively extracted with chloroform in a percolator. The combined extracts from 5 Kg. of boldo leaves were reduced to 1.5 L. in a flash evaporator and extracted four times with 1 *N* hydrochloric acid (500 ml.) and once with 4 *N* hydrochloric acid (500 ml.). The acid extracts were neutralized with ammonia (pH 8–9) and extracted with chloroform until alkaloids could no longer be detected in the extracts by TLC. The combined chloroform extracts were reduced to 200 ml. in a flash evaporator (extract *a*). TLC examination (system *A*, spray reagent A) revealed the presence of 5 main alkaloid-positive areas at R_f 0.7 (isocorydine),[3] R_f 0.6 (*N*-methyllaurotetanine and norisocorydine), R_f 0.5 (boldine), R_f 0.3, and R_f 0.2.

Countercurrent Distribution—Extract *a* formed the lower phase of the first tube of a seven-tube counter-current distribution (in separators) between chloroform and an aqueous solution of 1% tartaric acid and 10% sodium chloride (200 ml. of each phase in each tube). After distribution was completed, the pH of each aqueous phase was adjusted to 10 by addition of ammonia and the chloroform layers were separated. Each aqueous phase was then extracted three times with 100 ml. chloroform and the combined chloroform extracts from each tube washed (water) and reduced to 50 ml. in a flash evaporator. The contents of tubes 2–4 and 5–7 were combined and the two resulting solutions again subjected to the countercurrent distribution. The contents of tube 1 were distributed (7 tubes) between chloroform and an aqueous solution of pH 6 (McIlvaine buffer). All tubes from these distributions having similar groups of alkaloids were combined to give four solutions (*1*, *2*, *3*, *4*). TLC examination (solvent system *A*; spray reagents A and B) showed that boldine (R_f 0.50) was present mainly in solutions *2* and *3* and isocorydine (R_f 0.73), *N*-methyllaurotetanine (R_f 0.60), and norisocorydine (R_f 0.58) were mainly in solution *4*. Other alkaloid-positive spots were located at R_f 0.16, 0.17, and 0.30 in solution *1*, R_f 0.32 in solutions *2*, *3*, and *4*, R_f 0.55 and 0.65 in solution *3*, and R_f 0.18 in solution *4*. After further resolution of these four solutions by column chromatography and preparative TLC, 17 alkaloids could be detected by TLC to be present in the extract of boldo leaves. They were numbered 1–17 according to increasing R_f value in solvent system *A*.

Isolation of (+)-Reticuline—A slurry of silicic acid (100 mesh) and 0.1 *N* sodium hydroxide was filtered and the solid dried by suction and then at 100°. Solution *2* was evaporated on alkali-treated silicic acid (12 Gm.) and the dried and pulverized powder packed on top of a column of the same silicic acid (25 × 2.5 cm.). The column was developed first with chloroform and then with chloroform–methanol mixtures containing increasing concentrations of methanol. Forty-five fractions (15 ml.)

Received July 20, 1967, from the Research Laboratories, Food and Drug Directorate, Ottawa, Ontario, Canada.
Accepted for publication October 5, 1967.
Presented in part to the Pharmacognosy and Natural Products Section, APHA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
The authors thank Dr. G. Neville for the nuclear magnetic resonance data and Dr. Leo Levi for encouragement.

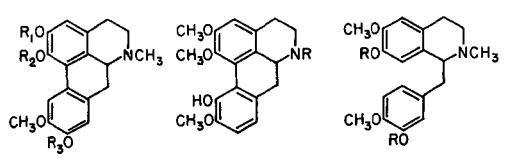
[1] Unoplan, Shandon Scientific Co. Ltd.

[2] Purchased from Dominion Herb Distributors, Montreal, Quebec, Canada.

[3] The authors are grateful to Dr. A. Rüegger, Sandoz Ltd. Basle, Switzerland, for authentic samples of isocorydine, norisocorydine, and *N*-methyllaurotetanine. Boldine was obtained from E. Merck (Darmstadt), Germany.

were collected and each fraction examined by TLC. Fractions 24–33 contained boldine (R_f 0.58; system *B*) in admixture with other alkaloids, mainly one (alkaloid 7) of R_f 0.50 (system *B*), characterized by giving a bright blue coloration with Gibb's reagent. Alkaloid 7 was concentrated by further column chromatography and a fraction was obtained which contained only boldine and alkaloid 7, in approximately equal amounts. After preparative TLC (system *A*) of this fraction, alkaloid 7 was obtained chromatographically pure. Further small amounts were obtained by preparative TLC of fractions obtained by chromatography of solutions *3* and *4*. The alkaloid was precipitated as an amorphous solid by *n*-hexane from ether solution, but attempts to crystallize it were unsuccessful. Yield 47 mg. (0.001%), m.p. 65–70°, $[\alpha]^{22}$ + 108°. The TLC (10 solvent systems, 11 spray reagents) and spectral (ultraviolet and infrared) properties of the alkaloid proved to be identical with those of an authentic sample of (±)-reticuline and the NMR spectrum ($CDCl_3$) was comparable with a published spectrum for (±)-reticuline taken in an unspecified solvent (7). It had a six-proton peak at 3.8 δ (two methoxyl groups), a three-proton peak at 2.44 δ (one *N*-methyl group), and a two-proton peak at 5.54 δ (two hydroxyl groups) which disappeared on addition of D_2O.

Alkaloid 7 was methylated with diazomethane in ethanol–ether solution for 20 hr., and the solution extracted with 1 *N* hydrochloric acid. This extract was made alkaline with ammonia, extracted in turn with chloroform, and the chloroform solution dried ($MgSO_4$), and evaporated. The product was amorphous and attempts to crystallize it were unsuccessful. It was shown to be homogeneous and identical with laudanosine[4] (III, R = CH_3) by TLC and infrared spectroscopy.



I II III

Isolation of Isoboldine—TLC comparison (system *A;* spray reagents A and B) of a sample of isoboldine[5] with solutions *1*, *2*, *3*, and *4* showed that alkaloid 11 (R_f 0.55) corresponded with isoboldine both in R_f and color reactions. The alkaloid was detected only in solution *3* and was concentrated by column chromatography and countercurrent distribution (chloroform *versus* 1% sodium carbonate, to separate it from boldine; chloroform *versus* 1% tartaric acid + 10% sodium chloride, to separate it from norisocorydine and *N*-methyllaurotetanine). Alkaloid 11 was obtained homogeneous only after preparative TLC and was shown to be identical with a sample of isoboldine by TLC (solvent systems *A–D*, 6 spray reagents) and by ultraviolet and infrared spectroscopy. Insufficient material was obtained for crystallization.

[4] Laudanosine was obtained from E. Merck (Darmstadt), Germany.

[5] The authors are grateful to Professor R. Tschesche, University of Bonn, West Germany, for an authentic sample of isoboldine and to Dr. R. H. F. Manske, Dominion Rubber Research Laboratory, Guelph, Ontario, Canada, for a sample of glaucine.

Alkaloid 11 was methylated with diazomethane in ethanol–ether solution for 72 hr. TLC examination of the product (3 solvent systems, 6 spray reagents) showed it to be identical with glaucine[5] (I, $R_1 = R_2 = R_3 = CH_3$).

DISCUSSION

Although the initial objective of this study was to characterize the alkaloids of boldo leaves by TLC alone, it soon became clear that, because of the large number of alkaloids present, preliminary resolution by countercurrent and chromatographic techniques was essential. After many purification steps it was possible to record unambiguously both color reactions and R_f values in various solvent systems for 17 alkaloids. Positive reactions with several alkaloidal spray reagents, in combination with the method of extraction, indicate that all 17 compounds are alkaloids. Furthermore, alkaloids 1–15 and 17 appear to be phenolic on the basis of positive reactions observed with other reagents. This was confirmed by repeated extraction of an ether solution of the alkaloids with 5 *N* sodium hydroxide, when only alkaloid 16 remained in the ether phase.

Considering the method of extraction used, the alkaloids detected in this study are probably nonquaternary. Preliminary work showed that acidification of the extracted boldo leaves, followed by further extraction with ethanol, yielded a fraction containing small amounts of quaternary bases. Alkaloids 8, 9, 14, 15, and 16 appear to be present in amounts too small to permit their isolation and identification, but work is in progress on the isolation and characterization of some of the other alkaloids, based on the purification processes described here.

Reticuline was originally isolated from *Cocculus laurifolius* and named coclanoline (8) but the name reticuline is now accepted by most workers and it has since been found in a number of plant species [*e.g.*, opium (*Papaver somniferum*) (7, 9), *Anona reticulata* (10), and *Romneyi coulteri* (11)]. Its isolation from *Peumus boldus* Molina is of interest since it is the first nonaporphine alkaloid to be isolated from this source and since it has been shown to be a key intermediate in the biogenesis of both the morphine (12) and berberine-protopine (13) series of alkaloids. It has also been postulated to be a biogenetic precursor of certain aporphines (14) and, although there is not yet direct *in vivo* evidence to support this hypothesis, reticuline has been converted *in vitro* in low yield into the aporphine isoboldine by oxidation with specific reagents (15). The two alkaloids have also been recently reported to occur together in opium (16). This prompted us, having isolated and characterized reticuline, to search for evidence for the presence of isoboldine in boldo leaves. The alkaloid was easily detected by TLC of solution *3* from the countercurrent distribution because of a characteristic green color given by it with potassium iodoplatinate reagent, all the other alkaloids giving the usual purple coloration. Separation of the pure alkaloid proved to be very difficult because of the presence of many interfering substances and because of the instability of the alkaloid. Enough was obtained, however, for comparison with an authentic sample of isoboldine by TLC, ultraviolet, and infrared spectroscopy. Furthermore, glaucine was shown by TLC identification to be the product of methylation with diazomethane.

The specific rotation of reticuline has been found to vary according to its origin between −55° and +132° and this is probably due to different rates of consumption of the two enantiomorphs in the biosynthetic pathways of the various species. Comparison with the value (+132°) found by Kusuda (8) for (+)-reticuline indicates that reticuline from *P. boldus* contains, at most, 10% (−)-reticuline.

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Alkaloids-*Peumus boldus*
(+)-Reticuline—isolation
Isoboldine—isolation
Countercurrent distribution
Column chromatography—separation
TLC—analysis
IR spectrophotometry—structure
UV spectrophotometry—structure
NMR spectrometry

Michaelis Constants for Isolated Cholinesterase Systems

By SAMUEL T. CHRISTIAN* and JAMES G. BEASLEY

The enzymatic hydrolysis of acetylcholine chloride by human plasma cholinesterase has been studied in detail. Results suggest that at substrate concentrations below 0.01 *M*, Michaelis-Menten kinetics are followed and enzyme hydrolysis rates are due to a single enzyme component. At higher substrate concentrations, a second enzyme component appears to contribute significantly to the total velocity of the reaction and a Lineweaver-Burk plot yields a hyperbolic-type curve. Apparent K_m values were calculated for the two components. The autohydrolysis rate for acetylcholine chloride at 26° was determined.

EVALUATION OF accurate Michaelis constants (K_m) has long been considered by the enzymologist to be practical only with highly purified isolated enzyme preparations. Nevertheless, enzyme kinetic constants from preparations of varying degrees of purity by investigators with divergent disciplinary backgrounds has led to the publication of a wide range of K_m values for most enzymes. This is particularly true in the case of human plasma (pseudo) cholinesterase (acylcholine acylhydrolase; E.C. 3.1.1.8) where many *apparent* K_m values (1–7) have been reported for the hydrolysis of butyrylcholine and benzoylcholine although comparatively little attention has been given to the hydrolysis of acetylcholine halides by the same enzyme. Acetylcholine is generally the substrate utilized in cholinesterase inhibitor evaluation.

In considering the variety of enzyme preparations (*e.g.*, commercial preparations,[1] Harvard fractions, IV-6-3 through IV-6-4 (8), Kabi fraction IV-6-3 (9), and completely unfractionated human serum (10), used for kinetic and inhibitor studies, it became of interest to ascertain the validity of expressing the Michaelis constants obtained with these preparations over wide ranges of substrate concentrations as valid, reproducible expressions of the true Michaelis constant.

METHOD

Acetylcholine chloride (Sigma Chemical Co.) was used as substrate in these studies; 17 different substrate concentrations covering a range from $3.00 \times 10^{-3}M$ to $1.50 \times 10^{-1}M$ were utilized. Enzyme initial velocity measurements were determined at 26.0° (±0.05°) with a continuous titration method (11) in a 0.15 *M* sodium chloride solution

Received October 19, 1967, from the Department of Medicinal Chemistry, University of Tennessee College of Pharmacy, Memphis, TN 38103
Accepted for publication February 7, 1968.
Support for this investigation was provided by grants from the National Science Foundation (GB-4453) and from Marion Laboratories, Kansas City, Missouri.
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The authors gratefully acknowledge helpful discussions with Dr. Charles Walter and the technical assistance of Mrs. Linda O. King and Mrs. Marjorie O. Clark.

[1] Sigma Chemical Co., Worthington Biochemical Corp., Cutter Laboratories, Inc., and A. B. Kabi (Stockholm, Sweden), among others.

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By SAMUEL T. CHRISTIAN* and JAMES G. BEASLEY

The enzymatic hydrolysis of acetylcholine chloride by human plasma cholinesterase has been studied in detail. Results suggest that at substrate concentrations below 0.01 *M*, Michaelis-Menten kinetics are followed and enzyme hydrolysis rates are due to a single enzyme component. At higher substrate concentrations, a second enzyme component appears to contribute significantly to the total velocity of the reaction and a Lineweaver-Burk plot yields a hyperbolic-type curve. Apparent K_m values were calculated for the two components. The autohydrolysis rate for acetylcholine chloride at 26° was determined.

EVALUATION OF accurate Michaelis constants (K_m) has long been considered by the enzymologist to be practical only with highly purified isolated enzyme preparations. Nevertheless, enzyme kinetic constants from preparations of varying degrees of purity by investigators with divergent disciplinary backgrounds has led to the publication of a wide range of K_m values for most enzymes. This is particularly true in the case of human plasma (pseudo) cholinesterase (acylcholine acylhydrolase; E.C. 3.1.1.8) where many *apparent* K_m values (1–7) have been reported for the hydrolysis of butyrylcholine and benzoylcholine although comparatively little attention has been given to the hydrolysis of acetylcholine halides by the same enzyme. Acetylcholine is generally the substrate utilized in cholinesterase inhibitor evaluation.

In considering the variety of enzyme preparations (*e.g.*, commercial preparations,[1] Harvard fractions, IV-6-3 through IV-6-4 (8), Kabi fraction IV-6-3 (9), and completely unfractionated human serum (10), used for kinetic and inhibitor studies, it became of interest to ascertain the validity of expressing the Michaelis constants obtained with these preparations over wide ranges of substrate concentrations as valid, reproducible expressions of the true Michaelis constant.

METHOD

Acetylcholine chloride (Sigma Chemical Co.) was used as substrate in these studies; 17 different substrate concentrations covering a range from $3.00 \times 10^{-3}M$ to $1.50 \times 10^{-1}M$ were utilized. Enzyme initial velocity measurements were determined at 26.0° (±0.05°) with a continuous titration method (11) in a 0.15 *M* sodium chloride solution

Received October 19, 1967, from the Department of Medicinal Chemistry, University of Tennessee College of Pharmacy, Memphis, TN 38103
Accepted for publication February 7, 1968.
Support for this investigation was provided by grants from the National Science Foundation (GB-4453) and from Marion Laboratories, Kansas City, Missouri.
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The authors gratefully acknowledge helpful discussions with Dr. Charles Walter and the technical assistance of Mrs. Linda O. King and Mrs. Marjorie O. Clark.

[1] Sigma Chemical Co., Worthington Biochemical Corp., Cutter Laboratories, Inc., and A. B. Kabi (Stockholm, Sweden), among others.

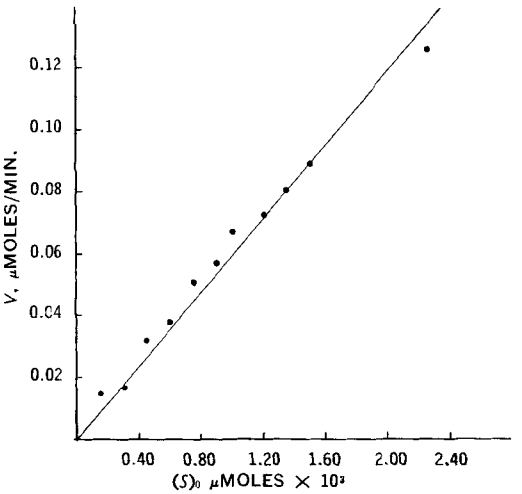

Fig. 1—Autohydrolysis of acetylcholine chloride at 26°. Key: S_0, abscissa, represents the initial micromolar substrate concentration; V, the ordinate, represents the reaction rate in μmoles/min. determined directly from titration curves for each substrate concentration; k = 5.59 × 10^{-5} min.$^{-1}$.

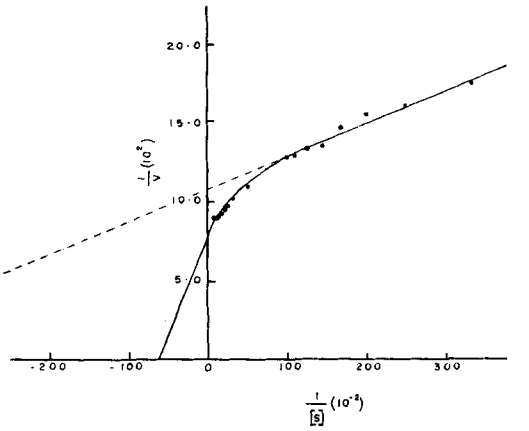

Fig. 2—Hydrolysis of acetylcholine chloride by human plasma cholinesterase at 26°, and pH 7.40 ± 0.05. Reactions were initiated by addition of 0.1 mg. of enzyme. Key: abscissa, reciprocal molar acetylcholine chloride concentration; ordinate, reciprocal enzyme initial velocity expressed in μmoles/min. per mg. of protein.

which was also 0.04 *M* in magnesium chloride. The total reaction volume was 15.0 ml. with each sample containing 0.1 mg. of enzyme (Type II Pseudo cholinesterase from human plasma; E.C. 3.1.1.8, Sigma Chemical Co.). The autohydrolysis of acetylcholine chloride was evaluated under the same conditions used for the enzyme reaction.

RESULTS AND DISCUSSION

The apparent first-order rate constant for the autohydrolysis of acetylcholine chloride as determined from Fig. 1 is 5.59 × 10^{-5} min.$^{-1}$. The effect of autohydrolysis on enzymatic initial velocities is negligible below substrate concentrations of 0.01 *M*; the data have been corrected for autohydrolysis effects at substrate concentrations above 0.01 *M*.

Walter (12), Dixon and Webb (13), and Reiner (14) have discussed the interesting case in which a reaction mixture containing two similar enzymes with different reaction rate constants is allowed to act on a single substrate; theoretically, if $K_{m1} \neq K_{m2}$, a Lineweaver-Burk (15) plot of such a reaction would show a hyperbolic relationship rather than the usual linear one. Reiner (14) has shown, by further theoretical treatment, that in order to distinguish between the two major enzyme components of such a system, the slopes of the two branches of the hyperbola must differ by 50% or more.

The authors have succeeded in demonstrating experimentally with human plasma cholinesterase[2] an actual example of a mixed enzyme system which adheres to this theoretical treatment.

A Lineweaver-Burk transformation constructed from our data for substrate concentrations ranging from 0.15 *M* to 0.003 *M* is presented in Fig. 2 and illustrates the hyperbolic-like relationship.

The results were not unexpected since Hess *et al.* (16) have previously demonstrated, by starch gel electrophoresis studies, the existence of six active butyrylcholinesterase fractions in human serum and Svensmark (17) has observed five fractions using DEAE-cellulose and DEAE-sephadex chromatography. However, from these studies it appears that in the enzyme preparations used, only two components were sufficiently active or present in sufficient concentration to contribute to the observed reaction rates.

A close look at the curve depicted in Fig. 2 shows that bending of the curve occurs at substrate concentrations greater than 0.01 *M*. Obviously, this bend is not caused by inhibition due to high ionic strength (18) but represents accelerated hydrolysis of substrate by a minor, kinetically distinct enzyme component.[3]

For substrate concentrations below 0.01 *M* it is apparent that this arm of the hyperbola is linear, as suggested by Walter (12) and is most probably due to a single, kinetically distinct major enzyme component. The K_m value determined for this component is 2.0 × 10^{-3} *M* (Fig. 2). This value is in good agreement with those previously reported using acetylcholine in low concentrations (<0.01 *M*) as a substrate (4, 20).

The wide range of K_m values previously reported may be due to the failure of some investigators to recognize the hyperbolic nature of this curve and to the inclusion of some high substrate concentration values in their K_m evaluation plots.

The relative importance of these findings should have special significance for investigators using plasma cholinesterase preparations to evaluate kinetically the effectiveness of various cholinesterase inhibitors.

From a practical standpoint, it appears safe to use acetylcholine in concentrations less than 0.01 *M* for evaluating enzyme-inhibitor kinetic constants[4] for

[2] Similar hyperbolic type curves were obtained from human plasma cholinesterase preparations supplied by Sigma Chemical Co. and by Cutter Laboratories, Inc. Berkeley, California.

[3] Humiston and Wright (19) have reported a similar break in the curve when acetylthiocholine iodide was used as a substrate for human plasma pseudocholinesterase.

[4] Dixon and Webb (13), in their treatment of a theoretical two enzyme system, suggest the practicability of this approach.

plasma cholinesterase systems. At these concentrations, the enzyme-substrate reaction follows classical Michaelis-Menten kinetics, suggesting a single enzyme–substrate interaction.[5] Although the apparent K_m value obtained from such plots appears reliable and reproducible, it does not necessarily represent a true K_m value but rather one intermediate between those for the two enzyme components present.

A complete kinetic analysis of pseudo (butyryl) cholinesterases must await separation and purification of each of the active enzyme species.

[5] The above suggestion does not apply to any rigorous kinetic treatment but is offered only as a guide for determining the relative potencies of cholinesterase inhibitors. Although unlikely, it is possible that specific inhibitors may have abnormally high affinities for minor enzyme components in impure cholinesterase preparations. Such a possibility cannot be ruled out completely on the basis of the available data.

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Keyphrases

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Hydrolysis—cholinesterase fractions

Correlation of Ratios of Drug Metabolism by Microsomal Subfractions with Partition Coefficients

By ERIC J. LIEN and CORWIN HANSCH

A strong correlation between ratios of drug metabolism by microsomal subfractions with 1-octanol/water partition coefficient was found. Explanation for the spread of ratios is presented.

A VARIETY OF drug-metabolizing enzymes appear to be distributed between rough and smooth particles derived from the endoplasmic reticulum of liver cells (1–6). Using density gradient centrifugation, Fouts' group has separated the smooth-surfaced particles from the denser, rough-surfaced particles and then studied the metabolism of various drugs by the two types of particles. The rates of metabolism for each type of particle were obtained in terms of micromoles of drug metabolized per hr. per mg. of microsomal nitrogen. The ratios of enzymic activity in the two types of particles differed by almost 10-fold (depending on the drug). Fouts interpreted the different ratios as indicating different concentrations (activities) of enzymes in the two types of tissues. The authors wish to show that an alternative explanation can be advanced from a consideration of the physical properties of the substrates.

Equation 1 was derived *via* the method of least squares from the data of Fouts (2) and Gram *et al.* (6) in Table I. These data were obtained using Rothschild's method of preparation of microsome fractions. In Eq. 1, $R_{(s/r)}$ stands for the ratio of enzyme activity in the two types of particles (smooth/rough) and P is the partition coefficient of the drug in 1-octanol/water (7, 8), n is the number of data points used in deriving the equation, r is the correlation coefficient, s is the standard deviation from regression, and the figures in parentheses are the 90% confidence intervals. Addition of a term in $(\log P)^2$ to Eq. 1 does not result in an improved correlation (9). Three of the $\log P$ values in Table I were calculated (7, 10, 11). The value for benzpyrene was based on $\log P = 3.37$ for naphthalene, codeine was based on 0.76 for morphine, and amphetamine was based on 1.41 for 2-phenylethylamine.

$$\log R_{(r/s)} = \underset{(\pm 0.035)}{-0.101} \log P + \underset{(\pm 0.108)}{0.859}$$

n	r	s	
10	0.885	0.116	(Eq. 1)

Received November 8, 1967, from Seaver Chemistry Laboratory, Pomona College, Claremont, CA 91711
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TABLE I—CORRELATION OF RATIOS OF ENZYMIC ACTIVITY (SMOOTH *Versus* ROUGH-SURFACED MICROSOMAL FRACTIONS OF RABBIT LIVER) WITH PARTITION COEFFICIENTS

	Substrate	Type of Reaction	Log P	Log $R_{(s/r)}$ Obs.[b]	Log $R_{(s/r)}$ Calcd.[d]
1	Benzpyrene	Aromatic hydroxylation	6.92	0.18[c]	0.16
2	Chlorpromazine	Ring-sulfur oxidation	5.35[a]	0.30	0.32
3	Zoxazolamine	Aromatic hydroxylation	2.46[a]	0.72[c]	0.61
4	*p*-Nitrobenzoic acid	Reduction of NO_2	1.83[a]	0.48	0.67
5	*l*-Amphetamine	Deamination	1.71	0.70	0.69
6	Hexobarbital	Aliphatic hydroxylation	1.47[a]	0.62	0.71
7	Codeine	*O*-Dealkylation	1.41	0.70	0.72
8	Acetanilide	Aromatic hydroxylation	1.16[a]	0.90	0.74
9	Aniline	Aromatic hydroxylation	0.90[a]	0.67[c]	0.77
10	Aminopyrene	*N*-Demethylation	0.76[a]	0.90	0.78

[a] Experimentally determined values. Other values calculated. [b] From *Reference 2*. [c] From *Reference 6*. [d] Calcd. using Eq. 1.

Using Dallner's method of preparing microsomal subfractions from rabbit liver, Gram *et al.* (6) studied the metabolism of eight drugs. Using log P values from Table I Eq. 2 has been derived, omitting data on azosulfamide[1] for which log P is not available.

$$\log R_{(s/r)} = \underset{(\pm 0.034)}{-0.091} \log P + \underset{(\pm 0.103)}{0.553}$$

$$n = 7 \quad r = 0.923 \quad s = 0.089 \quad \text{(Eq. 2)}$$

DISCUSSION

Equation 1 indicates that the more lipophilic the drug is (the higher log P is), the smaller the ratio of metabolism or, the more hydrophilic, the larger the ratio. The linear relation between log P and log $R_{(s/r)}$ does *not* imply the higher the ratio, the faster the metabolic change. In each case we are comparing the same drug in two different types of particles. In Table I there are 10 drugs of quite different chemical structure being metabolized by an unknown number of enzymes. Although the ratios in Table I can be explained by postulating different concentrations (or activities) of different enzymes in the two types of particles, this approach does not lend itself to rationalizing Eq. 1. An alternative postulate is that approximately the same activities of enzymes are in each type of particle, but the availability of the drug to the enzyme is rate limiting and depends on the milieu in which the enzyme is set. If one postulates that each type of particle contains enzymes in a very lipophilic setting, this would explain why hydrophobic drugs such as benzpyrene and chlorpromazine are metabolized at ratios near 1. The smooth particles would then appear to have a set of enzymes accessible to hydrophilic drugs which would explain why drugs with low log P values yield higher ratios. In this respect it is interesting to compare the four examples of aromatic hydroxylation in Table I. It seems likely that the same type of enzyme (12) would be involved in each case. If this is so, then the differences in R must be due to differences in availability of drug to enzyme. Most interesting is the fact that log $R_{(s/r)}$ is correlated with log P regardless of the type of reaction. This also points to access of drug to enzyme rather than a difference in enzyme activity in the two particles.

The correlation with Eq. 1 still leaves 22% of the variance in the data "unexplained" ($r^2 = 0.78$). This of course must be attributed to the second rate-limiting process, that of action of the enzyme on drug. For these processes no stereo or electronic terms have been included in Eq. 1. Although the slope of Eq. 2 is very close to that of Eq. 1, the intercept is different. Thus for *isolipophilic* drugs one would expect higher ratios using Rothschild's method of preparation. The dependence of log $R_{(s/r)}$ on log P appears to be the same.

Fouts' work is quite important for an ultimate understanding of the role of the liver in drug metabolism. Looked at from the point of view of Eq. 1, it appears to indicate two different kinds of setting for microsomal enzymes. It is further support for Brodie's (13) observation of the great dependence of drug metabolism on lipophilic character of the drugs (14).

[1] Neoprontosil, Winthrop Laboratories, New York, N. Y.

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Keyphrases

Drug metabolism—microsomal subfractions
Metabolism—partition coefficient ratios—correlated
Microsomal particles, smooth, rough—metabolism rates

Essential Oils and Their Constituents XXXVII. Isolation and Structure of Khusenol, a New Sesquiterpenic Primary Alcohol from Oil of Vetiver

By I. C. NIGAM, C. RADECKA, and H. KOMAE

A new sesquiterpenic tricyclic primary alcohol has been isolated from Angola vetiver oil. It was found to be identical with the product obtained by $LiAlH_4$ reduction of methyl khusenate, and on oxidation with CrO_3 and Ag_2O it gave khusenic acid. The alcohol has, therefore, been assigned structure I and named khusenol.

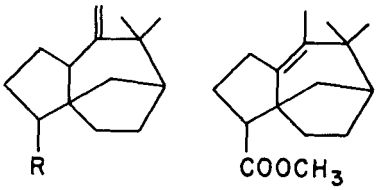
A NEW SESQUITERPENE alcohol has been determined to be the main constituent of the primary alcohol fraction of Angola vetiver oil. The present communication describes its isolation and properties, and records experimental evidence for its structure (I). The alcohol has been named "khusenol" in view of its structural relationship to another constituent of the oil, khusenic acid, which was shown to possess a novel carbon skeleton and assigned structure II (1).

A sample of angola vetiver oil, from which the free acids as well as the aldehydes and ketones had been removed previously, was treated with phthalic anhydride on a steam bath. The resulting hydrogen phthalate esters were saponified to give the primary alcohol fraction (35.5% of the oil sample), which was chiefly composed of khusenol (64% by GLC analysis). Fractional distillation of the primary alcohol fraction gave a 90% pure sample of khusenol. The alcohol may be characterized through its 3,5-dinitrobenzoate (m.p. 116°).

Dehydrogenation of khusenol with selenium at 300 ± 10° failed to yield any aromatic product. Catalytic hydrogenation showed the presence of one double bond, indicating the tricyclic nature of the compound. The infrared spectrum of the alcohol (Fig. 1) exhibited absorption bands at 1632 and 890 $cm.^{-1}$ characteristics of a $CH_2 = C <$ group. The NMR spectrum of the compound (Fig. 2) showed two triplets centered at 4.58 and 4.73δ, instead of the expected doublet for the olefinic protons, indicative of long range coupling. An additional NMR peak at 1.07δ with a shoulder ($\approx$1.06δ) was assigned to two methyl groups. A detailed study of the NMR spectra of khusenol, khusenic acid, and some related compounds will be reported shortly.

Treatment of khusenol with CrO_3–pyridine yielded a mixture of carbonyl compounds (mainly aldehydes, IR spectrum), which were oxidized with Ag_2O to the corresponding acids and converted into methyl esters by reaction with diazomethane. On gas chromatographic analysis, the mixture of esters was found to be chiefly composed of two products, one of which was methyl khusenate (III). Treatment of the ester mixture with HCl brought about isomerization of methyl khusenate to methyl isokhusenate (IV) (2).

The relationship between the alcohol and khusenic acid implied in these results was confirmed by comparing the properties of khusenol with those of the alcohol obtained by reduction of methyl khusenate with $LiAlH_4$. The IR and NMR spectra, and the m.p. of the 3,5-dinitrobenzoate of the two alcohols, as well as their gas and thin-layer chromatographic properties were identical. Khusenol is, therefore, assigned Structure I.



I, R = CH_2OH IV

II, R = COOH

III, R = $COOCH_3$

EXPERIMENTAL

Apparatus and other pertinent details for UV, IR, and NMR spectral measurements, microanalyses, and gas chromatographic determinations were described before (2).

Isolation of the Primary Alcohol Fraction of Angola Vetiver Oil—A sample of the oil (90.3 Gm.) from which the free acids as well as the aldehydes and ketones had been removed by reaction with Amberlite-IRA-400 ion-exchange resin (2, 3) and Girard T reagent (4), respectively, was treated with freshly sublimed phthalic anhydride (95 Gm.) on a steam bath for 2 hr. The resulting hydrogen phthalate esters of the primary alcohols were separated and saponified following the method of Elze as described by Sterrett (5). Yield of the primary alcohol fraction was 32.1 Gm. (35.5% of the oil). Gas-chromatographic examination of the isolate employing a 3% SE-30 column (2) showed the presence of one major constituent (64%; relative retention time 0.857, reference standard-phenanthrene).

Isolation of Khusenol—A portion (32.1 Gm.) of the fraction containing primary alcohols was submitted to fractional distillation (pressure: 3 mm.; reflux ratio: 10:1) using a Gallenkamp fractional distillation unit. When 11 fractions (total weight: 23.4 Gm.) had been collected, the distillation slowed down. At this stage the residue in the distillation pot was transferred to a small flask (25 ml.), and fractional distillation was resumed at 0.7 mm. pressure employing a Quickfit micro-distillation assembly. Three more fractions (total weight 5.3 Gm.) were collected. All 14 fractions were analyzed by gas chromatography on an SE-30 column (2) and found to be chiefly composed of khusenol. Fraction No. 13 (3.4 Gm.) was the purest (90%). An analytical specimen, prepared by gas chromatography on a 20% Reoplex 400 column (2), possessed the following properties. $b.p._{0.7}$ 140–142°; n_D^{27} 1.524;

Received August 31, 1967, from the Research Laboratories, Food and Drug Directorate, Ottawa, Ontario, Canada
Accepted for publication December 4, 1967.
Previous paper: Komae, H., and Nigam, I. C. *Perfumery Essent. Oil Record*, **58**, 710(1967).
The authors are grateful to Dr. Leo Levi for his interest in these investigations and to Dr. G. A. Neville for NMR measurements.

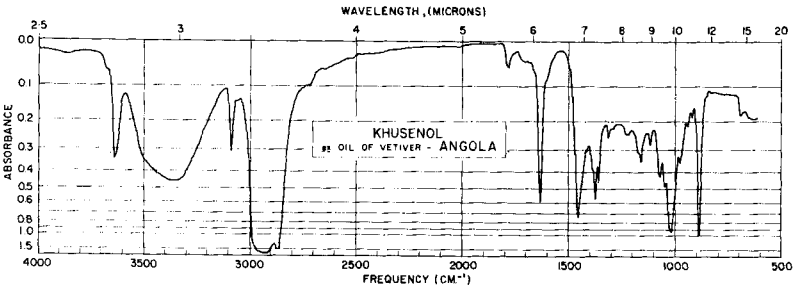


Fig. 1—Infrared spectrum of khusenol.

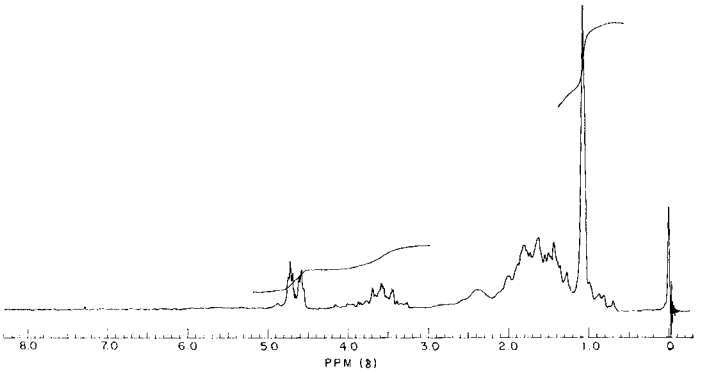


Fig. 2—NMR spectrum of khusenol. Solvent, $CDCl_3$; sweep width, 500. At sweep width of 100 a distinct shoulder near 1.06 δ was observed.

$[\alpha]_D^{29}$ + 14.5°; D_{26}^{26} 0.9740; 3,5-dinitrobenzoate, m.p. 116°.

Anal.—Calcd. for $C_{22}H_{26}O_6N_2$: C, 63.76; H, 6.32; N, 6.76. Found: C, 63.90; H 6.32; N, 6.75.

Dehydrogenation of Khusenol—A mixture of khusenol (381 mg.) and selenium (750 mg.) was heated at 300 ± 10° for 10 hr. under a stream of nitrogen. The reaction mixture was extracted with hexane (5 × 2 ml.), and the dark brown oil, which remained after evaporation of the solvent was chromatographed on alumina (grade I, 12 Gm.). Three fractions were collected: 1, (hexane, 30 ml.) 180 mg.; 2, (hexane, 30 ml.) 5 mg.; 3, (ether, 30 ml.) 58 mg. The fractions did not show any characteristic ultraviolet absorption and did not have any aromatic band in their infrared spectra.

Hydrogenation of Khusenol—Khusenol (135 mg. in glacial acetic acid (2 ml.) was hydrogenated in the presence of platinum oxide (5.2 mg.). The volume of absorbed hydrogen at 27°, 733 mm. was 16.6 ml. (1.2 moles).

Oxidation of Khusenol—Chromium trioxide (2.40 Gm.) in pyridine (36 ml.) was added to a solution of khusenol (2.98 Gm.) in pyridine (12 ml.) and the mixture was allowed to stand overnight at room temperature. The reaction mixture on conventional processing yielded a crude oxidation product (2.40 Gm.) which was purified by chromatography on grade II alumina (6) (Woelm; 100 Gm.). A mixture of hexane-benzene (3:1) eluted an oily product (468.2 mg., infrared absorption bands at 2712 and 1718 $cm.^{-1}$). Freshly prepared silver oxide, obtained from silver nitrate (169.2 mg.) (7) and a solution of sodium hydroxide (206.3 mg.) in water (2 ml.), was added to a portion of the product (107.1 mg.) dissolved in ethanol (0.2 ml.). The mixture was stirred at room temperature for 48 hr., filtered, acidified with 10% HCl, and extracted with ether. The yield of product was 100 mg.

Methylation of Acids—The above oxidation product (100 mg.) was dissolved in 3 ml. of a methanol–ether mixture (1:9) and methylated by introducing gaseous diazomethane (8). The product (99 mg.) on gas chromatographic analysis (SE 30 column) (2), showed 2 major peaks at 11.6 and 13.2 min. The former was identified as methyl khusenate.

Isomerization of Methyl Esters—The product of methylation (99 mg.) was refluxed with a mixture of ethanol (9 ml.) and concentrated HCl (1 ml.) for 1.5 hr. on a water bath. The usual processing of the reaction mixture yielded the isomerized esters (94 mg.). Gas chromatographic analysis of the product (SE-30 column) (2) showed the presence of two major constituents, appearing at 10.7 and 12.8 min. The earlier peak was identified as methyl isokhusenate. The peak corresponding to methyl khusenate was absent.

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Keyphrases

Khusenol—isolation, structure
Vetiver oil, Angola—khusenol isolation
UV spectrophotometry—structure
IR spectrophotometry—structure
NMR spectrometry—identity
GLC, TLC—identity

Uterine–Placental–Fetal Preparation *In Situ* on the Dog. Investigation of Metabolizing Activity and Tissue Distribution

By G. BENZI, F. BERTÉ, A. CREMA, and E. ARRIGONI

The extracorporeal perfusion technique *in situ* has been used to evaluate the metabolizing activity of the uterine–placental–fetal preparation on the dog. The pregnant uterus isolated from general circulation was supplied with a pump-oxygenator system. The method was tested studying the metabolic transformation of aminopyrine to 4-aminoantipyrine and *N*-acetyl-4-aminoantipyrine, and evaluating their distribution in fetal tissues.

TOXICOLOGICAL and pharmacological importance of drug metabolism in fetal and uterine tissues is well known. Nevertheless, the studies on pregnant animals *in toto* present complications related to maternal metabolism. The evaluation of the fetal physiological activities *in situ* can be performed while maintaining the fetus detached from its placental connection by an extracorporeal supporting system reproducing the placental circulation (1–7). Likewise, the uterine or placental influence on the transfer and utilization of the substances can be studied by the perfused placenta or pregnant uterus (8–14). Nevertheless, the lack of data prompted the study of drug metabolic activity of the fetus–uterus complex isolated *in situ*, so as to eliminate any interference of extrauterine tissues.

In a systematic investigation of the metabolism and tissue distribution during the pregnancy (15–17) the metabolic fate of aminopyrine was detected in the uterine–placental–fetal preparation *in situ* using a method of extracorporeal circulation. In the experimental arrangement, the pregnant uterus remained connected with the other parts of the body, except its blood circulation was supplied by a pump-oxygenator system.

EXPERIMENTAL

The experiments were carried out on 11 pregnant dogs (16.8 to 27.5 Kg.) preanesthetized with urethan (0.4 Gm./Kg. i.p.). Anesthesia was induced and maintained in closed circuit by nitrous oxide, cyclopropane, or ethyl ether. The animals were given artificial ventilation by a tracheal Warne tube after succinylcholine chloride (1 mg./Kg. i.v.) administration. Arterial blood pressure was measured from a cannula inserted into a femoral artery.

The abdomen was incised along the midline from xiphoid process to pubis; the uterine artery and vein on both sides of the uterus were isolated at the neck of the uterus. The vaginal, ovaric, and peritoneal vascular connections with the extrauterine tissues were occluded by ligature or compression. Both isolated uterine veins were ligated, cannulated, and connected with the venous reservoir of the pump-oxygenator system through the gravitational flow. Both isolated uterine arteries were cannulated and connected with the pump-oxygenator system; at this point the perfusion started.

Hematocrit, clotting time, pH, and arteriovenous blood oxygen were measured from samples of both extracorporeal and systemic circulation. The perfusion apparatus consists of: a venous reservoir, an oxygenator with gas meter, a roller-type pump with flow meter, a blood filter, an apparatus to eliminate the blood foam, a perfusion pressure regulator with manometer, and a blood exchanger with telethermometer (18).

Before the extracorporeal perfusion, the pump-oxygenator system was filled with 400–600 ml. of heparinized and defibrinated blood, previously obtained from the animal itself, filtered through cloth, preserved by ampicillin (1:10,000), and stored in a refrigerator. Before the perfusion the blood was filtered, diluted with Tyrode solution (to O_2 capacity = 13 ± 1 vol.%), and added to glucose (5%). The priming blood was circulated through the pump-oxygenator system, fully oxygenated, and warmed. A flow of O_2–CO_2 mixture (95:5) into the oxygenator was maintained at the rate of 5–10 L./min. During the extracorporeal circulation, the blood-flow rate was 10–12 ml./min./Kg.; the blood pressure was maintained at constant values of 80–110 mm. Hg; the time of perfusion was limited to 60 min.

The eventual leakage of perfusate into the systemic circulation was evaluated by taking samples from the general circulation of the blood and the lymph to verify the absence of the tested substance or its metabolites.

To investigate the metabolizing activity, the authors studied: (*a*) *demethylation*, by evaluating the transformation of aminopyrine to 4-aminoantipyrine using the method of Brodie and Axelrod (19) after addition of 100–200 mcg./ml. of aminopyrine into the extracorporeal circuit; (*b*) *acetylation*, by evaluating the transformation of 4-aminoantipyrine to *N*-acetyl-4-aminoantipyrine under the above-mentioned conditions and method.

At the end of the perfusion, the tissue distribution of aminopyrine and its two metabolites in some fetal organs (plasma, liver, kidney, lung, heart, brain, thymus, muscle, amniotic fluid, placenta) was determined, for every pregnant dog, pooling together the organs of its fetuses.

RESULTS

In the dog at eighth–ninth week of pregnancy, the uterine–placental–fetal preparation *in situ* shows a metabolizing activity. In fact, 29% (25 to 38%) of aminopyrine disappears in 60 min. and is partially replaced by the appearance of the 4-aminoantipyrine and *N*-acetyl-4-aminoantipyrine; an example is indicated in Fig. 1.

Figure 2 is a typical example of the fetal tissue concentration of aminopyrine and its two metabolites assayed after 60 min. of extracorporeal perfusion of an uterine–placental–fetal preparation. The following observations were made: (*a*) the diffusibility of aminopyrine across the placenta to the

Received August 11, 1967, from the Departments of Pharmacology of Pavia and Pisa Universities, Italy.
Accepted for publication October 5, 1967.
The authors are grateful to Mr. A. Grandini and Miss C. Provilli for technical assistance.

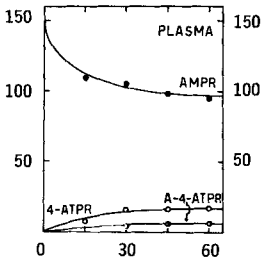

Fig. 1—Aminopyrine metabolism studied by the isolated uterine–placental–fetal preparation in situ *in a pregnant dog weighing 22.9 Kg. at ninth week of pregnancy; number of its fetuses = 6. Circulating blood in extracorporeal circuit = 680 ml.; blood flow rate of the extracorporeal circuit = 270 ml./min.; initial concentration of aminopyrine in the plasma of the extracorporeal circuit = 153 mcg./ml. On ordinate, the plasmatic concentrations (mcg./ml.) of aminopyrine* (AMPR), *4-aminoantipyrine* (4-ATPR), and N-*acetyl-4-aminoantipyrine* (A-4-ATPR). *On abscissa, time (in minutes) after aminopyrine addition.*

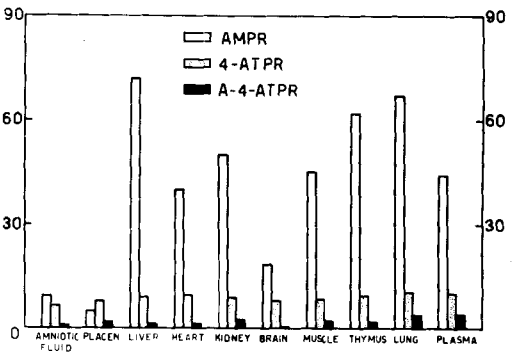

Fig. 2—Aminopyrine (AMPR), *4-aminoantipyrine* (4-ATPR), *and* N-*acetyl-4-aminoantipyrine* (A-4-ATPR) *concentrations (mcg./Gm. on ordinate) in the placental–fetal tissues 60 min. after adding aminopyrine into the extracorporeal circuit of the isolated uterine–placental–fetal preparation* in situ *of the pregnant dog as in Fig. 1. The concentrations are obtained by pooling together the organs from its six fetuses.*

fetuses; (*b*) the different concentrations of aminopyrine in the fetal organs studied; (*c*) the various proportions of aminopyrine to 4-aminoantipyrine and *N*-acetyl-4-aminoantipyrine in the fetal organs. Additional information will be necessary to state if these various proportions depend on a different ability in taking up the circulating metabolites.

In conclusion, the uterine–placental–fetal complex seems to be a useful method to investigate *in situ* both a detailed metabolizing activity and the drug-tissue distribution in the pregnant uterus normally connected with the body, except that its blood circulation is supplied by a pump-oxygenator system.

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Keyphrases

Uterine–placental–fetal preparation—*in situ*
Perfusion technique—drug administration
Metabolic activity
Fetal tissue—drug distribution

Radioprotective Effect of Magnesium Pemoline: A Comparison with Pemoline and Dextroamphetamine

By HOA LEVAN, and DELANO L. HEBRON

Magnesium pemoline, a central nervous system stimulant, is found to have a significant protective effect against ionizing radiations. Experimental results show that this drug is more potent than pemoline alone in exhibiting this radioprotective effect, and that dextroamphetamine does not possess this property. It is suggested that magnesium hydroxide, a component of magnesium pemoline, might enhance the absorption of pemoline to produce a more significant effect than when pemoline alone [without $Mg(OH)_2$] is used. The mechanism of this phenomenon is discussed, but no explanations are offered.

RECENTLY the authors reported the protective effect of magnesium pemoline against lethal doses of X-irradiations (1). Mice injected intraperitoneally with this drug showed a significant higher percentage of survival when exposed whole body to a total dose of 900 rads when compared with those injected with tragacanth. Further experiments were designed to compare the radioprotective effect of magnesium pemoline, of pemoline alone, and also that of another central nervous system stimulant, dextroamphetamine. Results of these studies are described and discussed in this paper.

METHOD

During the initial investigations, the experiments were designed to test the effect of magnesium pemoline on conditioned avoidance response in mice using X-rays as an unconditioned stimulus. Radioprotective effect of this drug was accidentally observed during these studies. Since then the experimental design has been changed to suit new objectives, although the same techniques were used in injecting and irradiating the animals.

Again CF_1 male mice (20–22 Gm.), 50 to 60 days old were used. The animals divided into five groups of 60 mice each were housed in standard plastic cages, 10 animals per cage. There was no restriction on food and water. On the first day of the experiment, 1 week after their arrival from the supplier, the mice in the first group were injected intraperitoneally with 0.6 ml. of magnesium pemoline (at a dose of 70 mg./Kg.), the second group with dextroamphetamine (0.7 mg./Kg.), the third group with pemoline alone (55 mg./Kg.), the fourth group with bacteriostatic water (0.3% tragacanth), and the fifth group with magnesium hydroxide, a component of magnesium pemoline (18 mg./Kg.). Magnesium pemoline, dextroamphetamine, pemoline, and magnesium hydroxide solutions were prepared with 0.3% tragacanth. All the mice were then exposed to 750 rads (at 80 rads/min.) of X-irradiations immediately after injection. A 400 Kv. Maxitron X-ray machine served as the source of radiation. The animals were then removed from the radiation room and returned to their original cages. Mortality in each group was observed and recorded daily. The experiments were repeated for three consecutive times. Data gathered from each group were then averaged each time and standard deviations obtained for all three experimental runs. Thus a total of 900 mice was used during this experiment.

Received December 8, 1967, from the University of Illinois, College of Medicine, Department of Radiology, Chicago, IL 60612

Accepted for publication January 19, 1968.

This work was supported in part by the Norval H. Pierce Research Fund, University of Illinois.

RESULTS AND DISCUSSION

As can be seen from Table I, the group injected with magnesium pemoline immediately before exposure to X-irradiation resisted against radiations longer than any of the other four groups. Comparing the magnesium pemoline group and the control group injected with tragacanth, one observes that this drug not only protects the animals against radiations (35% survival after 30 days) but also delays the damaging effect of radiation (100% survival on the seventh postirradiation day *versus* 79% in the control group). The rate of mortality was much less pronounced. On the thirteenth day after exposure while all the animals in the control group had died, 54% of the magnesium pemoline group still survived. Most of the mice in all groups died within the second postirradiation week. For the group treated with pemoline only, the delaying protective effect was not quite as significant when compared to the control (tragacanth) group. Nevertheless, 30-day mortality observation showed 15% of this group still survived, and at the end of the second week after exposure, 31% of the mice in this group were still alive *versus* 0% for the control group. Dextroamphetamine did not exhibit any protective effect against radiations. There was no significant difference between the mortality in this group and that in the control group. Magnesium pemoline is composed of 75 wt. % of pemoline and 25 wt. % of magnesium hydroxide. Table I also shows that the mice injected with $Mg(OH)_2$ died as fast as those in the control group and none survived 14 days after being exposed to lethal doses of ionizing radiations.

Comparing the radioprotective effect of magnesium pemoline and pemoline alone, one notes that the latter also offers protection but not as effectively as the former. On the eighth postirradiation day, the difference between the survival percentage of these two groups was quite significant ($p \ll 0.001$). Two weeks after exposure, this level of significance was still quite considerable ($0.05 < p < 0.02$). After 30 days while 15% of the animals in pemoline group survived, 35% were still alive in the magnesium pemoline group. Plotnikoff and Meekma (2) reported that magnesium pemoline was more potent in enhancing acquisition and retention of memory than pemoline alone, and that $Mg(OH)_2$ showed no significant effects. The results confirmed these findings as far as radioprotective effect of this drug was concerned. Comparing the doses used by these investigators and that used in these experi-

TABLE I—PERCENTAGE OF SURVIVAL OF 5 GROUPS OF MICE INJECTED WITH MAGNESIUM PEMOLINE, TRAGACANTH, $Mg(OH)_2$, DEXTROAMPHETAMINE, AND PEMOLINE, AND EXPOSED TO 750 RADS

Post-irradiation Days	Magnesium Pemoline	Tragacanth	$Mg(OH_2)$	Dextroamphetamine	Pemoline
1	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
2	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
3	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
4	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
5	100 ± 0	100 ± 0	98 ± 0.3	100 ± 0	98 ± 1.0
6	100 ± 0	94 ± 1.3	92 ± 0.7	87 ± 3.3	87 ± 1.5
7	100 ± 0	79 ± 2.9	79 ± 3.2	76 ± 3.5	75 ± 2.9
8	97 ± 1.2	57 ± 3.6	58 ± 2.6	52 ± 3.2	70 ± 0.2
9	94 ± 0.5	44 ± 2.5	48 ± 1.7	36 ± 1.6	64 ± 3.3
10	80 ± 2.6	24 ± 1.8	32 ± 1.7	29 ± 0.3	62 ± 1.1
11	69 ± 3.9	12 ± 2.4	25 ± 0.9	17 ± 2.5	54 ± 2.6
12	62 ± 0.2	7 ± 1.8	12 ± 1.3	8 ± 1.3	50 ± 1.7
13	54 ± 3.1	3 ± 0.5	9 ± 0.2	5 ± 0.2	40 ± 2.0
14	47 ± 1.5	0	6 ± 0.3	4 ± 0.1	31 ± 1.5
15	45 ± 0.2		0	0	29 ± 0.6
16	40 ± 1.1				25 ± 0.3
17	37 ± 0.6				20 ± 1.2
18	37 ± 0.6				15 ± 1.3
19	35 ± 0.3				15
20	35				15
21	35				15
22	35				15
23	35				15
24	35				15
25	35				15
26	35				15
27	35				15
28	35				15
29	35				15
30	35				15

ments, our dose was much higher (70 mg./Kg. *versus* 1.25–5.0 mg./Kg.). The authors are in the process of studying this radioprotective effect at lower doses of magnesium pemoline and higher doses of pemoline alone. It has been generally suggested that magnesium hydroxide serves as a carrier for pemoline, thus the magnesium hydroxide component enhances the absorption of this drug. If this is the case, perhaps one could verify this by increasing the amount of pemoline in order to compensate for the slower rate of absorption when only pemoline is used. In any case, pemoline alone was responsible for the protection against lethal doses of radiation. So far, no pharmacological findings have offered any clues that could be used to explain this phenomenon. Lange *et al.* (3) have observed significant anticonvulsant activity 15 to 30 min. after oral administration of magnesium pemoline (100 mg./Kg.) and 60 min. after the administration of pemoline alone of the same dose. This delayed effect of pemoline without magnesium hydroxide seems to agree with the less radioprotective effectiveness in these experiments; however whether one can associate these findings with Lange's observations is still a question for further investigations. Furthermore, if this suggestion is true it would be difficult to explain the long-term protective effect of magnesium pemoline against ionizing radiations that we have observed.

SUMMARY

The following observations resulted from this study: (*a*) Radioprotective effect of both magnesium pemoline and pemoline alone are quite significant. (*b*) Magnesium pemoline is more potent than pemoline alone in exhibiting protective effect against ionizing radiation. (*c*) Dextroamphetamine does not possess this radioprotective effect. (*d*) Magnesium hydroxide, a component of magnesium pemoline, seems to enhance the absorption rate of pemoline in this effect.

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Keyphrases

Radiation protective agent
Magnesium pemoline—radioprotective effect
X-Irradiation exposure—mice

Catharanthus Alkaloids XV. Isolation of Vindolinine from *C. lanceus* Leaves

By EDWARD M. MALONEY, HARRY H. S. FONG, NORMAN R. FARNSWORTH, RALPH N. BLOMSTER, and DONALD J. ABRAHAM

A continuing study of *Catharanthus lanceus* for antineoplastic alkaloids has resulted in the isolation of vindolinine (as HCl), which is reported for the first time from this plant. Perivine and yohimbine, reported previously, were also obtained in high yield.

In a previous communication (1) the isolation of perivine and yohimbine from the (B) fraction of *C. lanceus* leaves was reported. A reinvestigation of this fraction has resulted in the isolation of vindolinine, as the dihydrochloride salt. This is the first report on the isolation of vindolinine from *C. lanceus*, however it was initially isolated from the related *C. roseus* by two other groups (2, 3).

EXPERIMENTAL

Preparation of Crude Alkaloid Fraction—The alkaloid (B) fraction used in this investigation was derived from 267.5 Kg. of dried *Catharanthus lanceus* leaves, collected in Madagascar in July 1965.[1] Processing of the plant material as previously described (1) yielded 694 Gm. of crude alkaloid (B) fraction.

Isolation of Alkaloids—The crude fraction (694 Gm.) was dissolved in 6 L. of hot benzene, filtered, and cooled to room temperature. The residue on the filter pad was washed with cold benzene to remove brown coloring matter. This resulted in a yield of 43.92 Gm. of benzene-insoluble amorphous alkaloid, which was shown to be one-spot material following thin-layer chromatography (TLC), as previously described (4). The benzene filtrate, on standing at room temperature, formed crystals which were removed by filtration. The crystalline material when washed and dried, yielded 53.16 Gm. of alkaloid which was shown to be identical with the benzene-insoluble isolate. Concentration of the mother liquor to dryness *in vacuo*, and crystallization of the residue from acetone, yielded an additional 86.20 Gm. of alkaloid similar in all respects to the two previous crops. The three crystal crops were combined and an analytical sample was prepared by several crystallizations from hot acetone. After drying *in vacuo* at 50° for 24 hr. the crystals exhibited m.p. 232–233° dec. (cap., uncorr.). An ultraviolet absorption spectrum, in ethanol, showed maxima at 226 mμ (log $E^{1\%}_{1\,cm.}$ 3.20), 281 mμ (log $E^{1\%}_{1\,cm.}$ 2.52), and a shoulder at 290 mμ (log $E^{1\%}_{1\,cm.}$ 2.45). Thin-layer chromatography data in three solvent systems corresponded with that of yohimbine (4). An infrared spectrum (KBr) was identical with that of yohimbine, and the ultraviolet spectrum and melting point data were in agreement with published data for yohimbine (5).

After removal of the three crystal drops, the mother liquor was again taken to dryness and crystallized from hot acetone. After refrigeration, and standing overnight under refrigeration, crystals formed which were removed by filtration, washed with cold acetone, and dried to give a 79.9-Gm. yield. Thin-layer chromatography of this isolate indicated it to be similar, if not identical, with perivine (4). An analytical sample was prepared by several recrystallizations from acetone. The purified alkaloid was dried at 50° *in vacuo* for 24 hr., and was found to have m.p. 178–179° (cap., uncorr.). An ultraviolet absorption spectrum, in ethanol, exhibited a maximum at 316 mμ (log $E^{1\%}_{1\,cm.}$ 2.91) and a shoulder at 240 mμ, as well as an additional maximum being approached near 226 mμ. This spectrum is typical for the 2-acylindole moiety. An infrared spectrum of the isolate was identical with that of reference perivine, and the melting point and ultraviolet absorption data were consistent with literature values for perivine (5).

Following the removal of perivine, the mother liquor was again taken to dryness and 300 Gm. was dissolved in a minimum volume of benzene and the solution was applied to a chromatographic column (12 × 150 cm.) containing a benzene slurry of 14-Kg. Alcoa F-20 alumina, deactivated as previously described, by the addition of 120 ml. of 10% (w/v) acetic acid (1). Elution was initiated with benzene, followed by solvents and solvent mixtures of increasing polarity, and 1,000-ml. fractions were collected. The data for the operation of this column are presented in Table I. Fractions were combined on the basis of chromogenic responses of the resolved components to the ceric ammonium sulfate reagent (CAS), following TLC (4).

Fractions 28–101 (35.46 Gm.) from the column contained a major alkaloid which produced a red-orange color with the CAS reagent, and exhibited an R_f value consistent with that of vindolinine, in three different solvent systems (4). The alkaloid could not be induced to crystallize from the usual solvents, thus the fraction was taken to dryness, dissolved in ether, and HCl gas was passed through the ether solution. After 24 hr. the ether solution was filtered and the crude alkaloid hydrochloride was crystallized three times from methanol to yield 0.12 Gm. of pure vindolinine dihydrochloride. This exhibited m.p. 212–214° and an ultraviolet absorption spectrum having maxima at 245 mμ (log a_m 4.07) and 300 mμ (log a_m 3.63), with a minimum at 274 mμ (log a_m 3.35). An infrared spectrum (KBr) of the isolate was identical in all respects with that of a reference sample of vindolinine·2HCl[2]. The melting point and ultraviolet spectrum were in agreement with litera-

Received December 7, 1967, from the Department of Pharmacognosy, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15213

Accepted for publication January 17, 1968.

This investigation was supported, in part, by research grants CA-08228, CA-08509, and FR-5455 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and from the Thaw Fund, University of Pittsburgh, Pittsburgh, Pa.

[1] Supplied by the S. B. Penick and Company, New York, NY 10008. A reference herbarium specimen has been deposited at the above address.

[2] Supplied through the courtesy of G. H. Svoboda, Eli Lilly and Company, Indianapolis, Ind.

TABLE I—CHROMATOGRAPHIC SEPARATION OF *C. lanceus* LEAF ALKALOID (B) FRACTION[a]

Fraction No.[b]	Eluent	Fraction Wt., Gm.	Alkaloid Isolated	Yield, Gm.
1–3	Benzene	0.03		
4–7	Benzene	2.71		
8–18	Benzene	2.40		
19–27	Benzene	1.97		
28–101	Benzene	35.46	Vindolinine·2HCl	0.12
102–458	Benzene	29.50	Perivine	11.17
	Benzene–chloroform (99:1)			
	Benzene–chloroform (97:3)			
	Benzene–chloroform (95:5)			
459–580	Benzene–chloroform (3:1)	35.79	Perivine	13.56
			Yohimbine	9.42
581–608	Benzene–chloroform (3:1)	6.24	Yohimbine	3.46
609–623	Benzene–chloroform (3:1)	2.84	Yohimbine	0.91
624–640	Benzene–chloroform (3:1)	2.93	Yohimbine	1.09
641–735	Benzene–chloroform (3:1)	7.86		
736–788	Benzene–chloroform (2:1)	2.43		
789–806	Benzene–chloroform (1:1)	2.33		
807–842	Benzene–chloroform (1:1)	5.19		
843–901	Benzene–chloroform (1:1)	4.04		
902–1126	Chloroform	27.89		
1127–1151	Chloroform–methanol (99:1)	12.71		
1152–1518	Chloroform–methanol (99:1)	41.10		
	Chloroform–methanol (9:1)			
	Chloroform–methanol (3:1)			
	Chloroform–methanol (2:1)			
1519–1564	Methanol	14.65		

[a] 300 Gm. of alkaloid fraction was chromatographed on 14 Kg. of partially deactivated Alcoa F-20 alumina. [b] Each fraction was 1,000 ml. All fractions contained alkaloids.

ture reports for vindolinine·2HCl (3, 5), and the molecular weight for the alkaloid base was shown to be 336 by mass spectrometry (calcd. mol. wt., 336).

Fractions 102–458 (29.50 Gm.) from the column gave 11.17 Gm. of perivine on crystallization from acetone, benzene, and methanol. An additional 13.56 Gm. of perivine was obtained from fractions 459–580 (25.79 Gm.), from acetone, whereas the mother liquor of these fractions yielded 9.42 Gm. of yohimbine by crystallization from benzene and chloroform. An additional 5.46 Gm. of yohimbine was isolated from fractions 581–640 from the column, from benzene, chloroform, methanol, and acetone (see Table I for details).

This now brings to 19, the number of alkaloids that have been isolated from *C. lanceus* in this laboratory (1, 6–11).

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Keyphrases

Catharanthus lanceus leaves—alkaloids
Vindolinine, yohimbine, perivine—isolation
Column chromatography—separation
TLC—separation, identity
Mass spectrometry—mol. wt.
UV spectrophotometry—structure
IR spectrophotometry—structure

A New Flavone from *Salvia triloba L.f* (*Labiatae*)

By A. ULUBELEN, S. ÖZTÜRK, and S. IŞILDATICI

Salvigenin was isolated from the air-dried leaves and stems of *S. triloba*. Seventy percent aqueous ethanolic extract of the plant yields ursolic acid, oleonolic acid, β-sitosterol, carnosol, and the above-mentioned new flavone, when separated on a silicilic acid–diatomaceous earth (3:1) column. Methylation of pectolinarigenin, C, H, and methoxy group determination, alcoholic KOH degradation, and UV, IR, and NMR methods led to the establishment of the following structure for salvigenin: 5-OH-6,7,4′-OCH_3-flavone or pectolinarigenin-7-methyl ether.

Salvia triloba L.f (*Labiatae*) which is a shrub 0.5–1.5 M. tall, is distributed from the Marmara sea to the Mediterranean. The plant is also known as mountain apple in Turkey.

A survey in literature showed that various salvia species such as *S. officinalis* (1–12), *S. braclyodon* (13), *S. pratensis* and *S. sclerae* (14), *S. miltiorrhiza* (15), *S. spinoza* (16), *S. splendens* (17), *S. glutinoza* (18) have been studied and ursolic acid I and II, oleolonic acid, sclareol, carnosol, tanshinone I, II, III, β-sitosterol, thujone, carene, azulene, α and β pinene, camphor, borneol, bornyl acetate, pentagalactosido sucrose, and acetyl anthocyans have been isolated and identified.

However, there are only a few papers on *S. triloba*. In 1954 Leiner (19) studied the ether extracts of *S. triloba* in this school and obtained ursolic acid I and II, oleonolic acid, a paraffin, a polyterpene and an oily compound, a flavone aglicon, a flavone glycoside, and a bitter substance. Ursolic acid I and II and oleonolic acid were obtained from the *S. triloba* in Greece (20). Brieskorn (21) found the same acids in *S. triloba*.

EXPERIMENTAL

The plant was collected from Marmara island, Marmara sea, Turkey, in May 1967. Five-hundred grams of the air-dried leaves and stems were pulverized and extracted with 3 L. of 70% aqueous alcohol. Alcoholic extract was evaporated under a vacuum and 20 Gm. of a green precipitate (I) was separated. The brown aqueous part upon evaporation to dryness yielded 100 Gm. of a residue (II).

Fifteen grams of I was chromatographed on a 3 × 50 cm. column, using silicilic acid–diatomaceous earth (3:1). Fifty fractions, 100 ml. of each, were collected using the following solvents:

Solvents for Collecting Fractions	Number of the fractions
Petroleum ether	1–2
Petroleum ether–benzene (9:1)	3–4
Petroleum ether–benzene (1:1)	5–6
Benzene	7–11
Benzene–chloroform (9:1)	12–13
Benzene–chloroform (1:1)	14–15
Chloroform	16–17
Chloroform–ether (9:1)	18–25
Chloroform–ether (1:1)	26–27
Ether	27–40
Alcohol	40–50

Received October 26, 1967, from Faculty of Pharmacy, University of Istanbul, Istanbul, Turkey.

Accepted for publication January 11, 1968.

This investigation was supported by the Scientific and Technical Research Council of Turkey (TAG-96).

The authors wish to thank Dr. A. Baytop and Dr. N. Tanker, University of Istanbul, for the identification of this plant, and Dr. Brieskorn, University of Würzburg, for supplying carnosol; also Dr. H. Wagner, University of Münich, for supplying pectolinarigenin and for taking NMR curves and some of the IR curves.

Each fraction was checked on thin layer and those which gave the same spots were combined, thus 1–6, 7–8, 9–11, 12–18, 19–20, 21–23, 23–50 yielded seven fractions.

Fractions 1–8, being the essential oils of the plant, were not studied; fractions 23–50 were left aside together with the residue II for future studies.

Fractions 9–11 showed two distinct and five minor spots on thin layer; upon crystallization from boiling alcohol 70 mg. of a white crystalline compound was obtained. Thin layer, mixed m.p.'s, and IR curve comparison showed that this compound was β-sitosterol. The second compound was not obtained in the crystalline form.

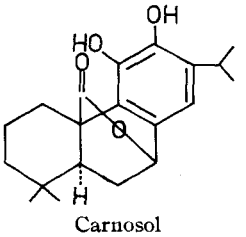
Fractions 12–18 when checked on thin layer showed one major and four minor spots; upon evaporation of the solvent these fractions yielded white needle-like crystals. Alcohol recrystallization yielded 1 Gm. of a compound; m.p. 286–290°, optical rotation $[\alpha]_D^{20}$ = +65 (in pyridin). Mixed m.p.'s and IR curve comparison as well as the optical rotation proved that this was ursolic acid.

Fractions 19 and 20 yielded two compounds, one of which was carnosol, the other was an unknown compound.

Fractions 21–23 yielded white crystals; alcohol recrystallization gave 250 mg. of a white crystalline material, m.p. 305°, optical rotation $[\alpha]_D^{20}$ = +83 (in methanol). Mixed m.p.'s and IR curve comparison as well as the optical rotation proved that this was oleonolic acid.

ISOLATION OF CARNOSOL

Fractions 19 and 20 when left aside yielded short, white, stick-like crystals (590 mg.) upon recrystallization from alcohol, the yield was 412 mg. Under the light, the crystals developed a blue-violet tinge. Melting point was 235–237°, UV spectra were taken using a Beckman DB recording instrument, $\lambda_{max.}$ 226 mμ (ε 12,100) and 288 mμ (ε 3,120) in ethanol. IR spectra (KBr) (Beckman IR-8) 3,570 $cm.^{-1}$ (intramolecular hydrogen bond), 3,330 $cm.^{-1}$ (OH groups), 2,980, 2,890 $cm.^{-1}$ (methyl groups), 1,720 $cm.^{-1}$ (lacton group), 1,580, 1,470, 1,450 $cm.^{-1}$ (aromatic ring), 1,380, 1,170 $cm.^{-1}$ (isopropyl group). NMR spectra (C_5D_5N) (TMS as internal



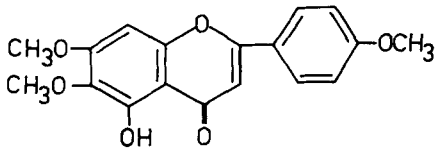
Carnosol

standard) (Varian Associates M-60) 0.8–1.05 p.p.m. (methyl groups in isopropyl chain), 1.3–1.5 p.p.m. (methyl groups in the nucleus), 6.93 p.p.m. (proton in the benzene ring), 5.62 p.p.m. (multiplet) (proton in the side chain), 1.65–4 p.p.m. (δ) (10 protons in the hydrogenated nucleus), and deuterium exchange show clearly the two OH groups.

Anal.—Calcd. for $C_{20}H_{26}O_4$: C, 72.72; H, 7.87. Found: C, 72.16; H, 7.87.

Infrared, NMR spectra, and analytical calculations suggested that this compound could be carnosol. Mixed m.p.'s and the IR curve comparison with the carnosol sample proved that these two compounds were identical.

Isolation and Identification of Salvigenin—The mother liquid of carnosol yielded green crystals; upon recrystallization from ethanol straw yellow-colored needles were obtained (70 mg.); m.p. 188°. UV maxima were 336 mμ (ϵ 31,000) and 280 mμ (ϵ 23,500). IR spectra suggested that this compound could be a flavone with a 5-OH group; 3,050 cm.$^{-1}$ (bonded OH), no free OH bands, 1,650 cm.$^{-1}$ (carbonyl group), 825 cm.$^{-1}$ (4′-OCH_3 group), 705 cm.$^{-1}$ (5-OH flavone). NMR spectra showed three methoxy groups at 4.0 p.p.m., 7.93, and 7.68 p.p.m. (aromatic H_2', H_6'); 7.1 and 6.93 (aromatic H_3', H_5'); 6.55 and 6.6 (aromatic H_8, H_3), and 11.6 p.p.m. (δ) (5-OH).



Salvigenin

Anal.—Calcd. for $C_{18}H_{16}O_6$: C, 65.85; H, 4.87; —OCH_3, 28.36. Found: C, 65.72; H, 5.1; —OCH_3, 26.96

Methylation of Pectolinarigenin—Ten milligrams of pectolinarigenin was dissolved in acetone and methylated with diazomethane in ether according to Merz (22). The reaction mixture was evaporated and the yellow residue was crystallized from ethyl acetate. A mixed m.p. of pectolinarigenin-7-methyl ether with salvigenin and the IR curve comparison showed that they were identical.

Hydrolysis of Salvigenin—Hydrolysis of salvigenin with 0.1 *N* H_2SO_4, then with more concentrated acids did not yield any sugar residue.

Degradation of Salvigenin—Twenty milligrams of salvigenin was refluxed with alcoholic KOH for about 5 hr. The acidic and the phenolic parts of the mixture were separated according to a previous paper (23). 4,5-Dimethoxy resorcinol and *p*-methoxy benzoic acid were found by thin-layer comparison.

SUMMARY

A new flavone was isolated from the leaves and the stems of *Salvia triloba L.f*, and its structure was found by using UV, IR, and NMR methods, as well as the alkaline degradation and the partial synthesis of the compound from pectolinarigenin. Carnosol which was found in *Salvia officinalis* was also found in this plant and identified as well as the following compounds: ursolic acid, oleonolic acid, and β-sitosterol utilizing thin-layer column chromatography, UV, IR, and when necessary, NMR techniques.

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Keyphrases

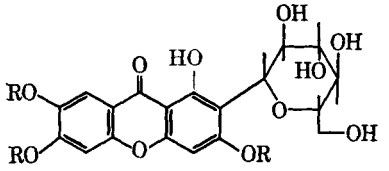
Salvia triloba—phytochemical investigation
Salvigenin—new flavone, isolated
Column chromatography—separation
TLC—identity
Optical rotation—identity
NMR spectrometry—identity
IR spectrophotometry—structure
UV spectrophotometry—structure

Occurrence of Mangiferin in *Hiptage madablota* Geartn.

By R. A. FINNEGAN, R. A. STEPHANI, G. GANGULI*, S. N. GANGULY*, and A. K. BHATTACHARYA*

A bright yellow, crystalline compound, m.p. 271–274°, isolated from the root-bark of *Hiptage madablota* Geartn. has been identified as mangiferin, 1,3,6,7-tetrahydroxy-2-C-β-D-glucosylxanthone, by direct comparison of its properties and those of its derived 3,6,7-trimethyl ether with those of authentic samples. Mangiferin was assayed in three biological test systems and the results are given.

In the course of studies on the structures of the endecaphyllins, a group of glucose polyesters of 3-nitropropanoic acid (1), the authors' attention was naturally directed to the report of Gorter (2) concerning hiptagin, a nitrogenous glucoside which was isolated from *Hiptage madablota* Geartn. (family *Malpighiacae*), since available evidence strongly suggested that hiptagin was very closely related to the endecaphyllins. Indeed, it was found that hiptagin (2) and endecaphyllin X (1) are identical and have the structure 1,2,4,6-tetra-*O*-(3-nitropropanoyl)-β-D-glucopyranoside (3). The authors deal here with the isolation of a new constituent of *H. madablota* and its identification as mangiferin (I).



I, R = H
II, R = CH_3

A granular solid which precipitated during acetone extraction of defatted root bark was collected and recrystallized several times from 50% aqueous alcohol to provide a bright yellow substance with melting point 271–274° dec. The spectroscopic properties of this substance pointed to the presence of a hydroxyxanthone moiety (4, 5) and quickly led to the supposition that the compound was mangiferin (I) on the basis of correspondence with published infrared (6), ultraviolet (4, 6, 7), and NMR (8) data in addition to agreement with the literature values for its melting point (9) and optical rotation (10). Furthermore, diazomethylation provided a derivative whose properties accorded with those of the known (7) trimethyl ether, II. Finally, the identification of the materials used was confirmed by direct comparison with authentic samples of I and II generously provided by Haynes (8, 11).

Although mangiferin has been long known as a constituent of the mango tree (*Mangifera indica* L., family *Anacardiaceae*), its structure has only recently been established (8, 12), thus permitting its inclusion in the rather unusual class of natural products, the *C*-glycosyl compounds. Reviews of earlier chemical studies have appeared (11). In addition to *M. indica*, and now *H. madablota*, the following species are reported to contain mangiferin: *Salacia prinoides* L. (*Hippocrateaceae*) (13), *Hedysarum obscurum* L. (*Papilionaceae*) (14), *Belamcanda chinensis* Adans (*Iridaceae*) (15), various *Iris* spp. belonging to sections Pogonoris, Apogon, and Pardanthopsis (*Iridacea* (15), *Athyrium mesosorum* Roth (*Polypodeaceae*) (16), *Apholoia madagascariensis* Clos (*Flacourtiaceae*) (17), *Cuscuta reflexa* Tourn. (*Convolvulaceae*) (17), *Smilax glycyphylla* Tourn. (*Liliacea* (10), *Madhuca utilis* Ridley (*Sapotaceae*) (7), and *Anemarrhenae rhizoma* B. (*Liliaceae*) (18).

The authors are aware of only two reports on biological properties of mangiferin. One of these describes increased diuresis in the adrenalectomized rat (19) while the other deals with the cardiotonic effects on the isolated frog heart (20). The cytotoxic effect of mangiferin on Sarcoma 180 cells grown in stationary cell culture has now been studied and the compound was found to be inactive. An ID_{50} value of 440 mcg./ml. was determined which is well outside the range (6 mcg./ml. or below) of potential interest. A number of more simply substituted xanthones have been subjected to this bioassay with a few showing significant activity. These data will be discussed in detail in a subsequent publication (21).

Results from two additional assays using cell-free systems from *Escherichia coli* have been kindly provided by Dr. C. Coutsogeorgopoulos at the Roswell Park Memorial Institute. Mangiferin (10^{-4} *M*) displayed low inhibitory activity (15–20%) on the polyuridylic acid-stimulated synthesis of polyphenylalanine (22) in the presence of ribosomes washed with ammonium chloride (23). In the second system, the reaction between puromycin and polylysyl-ribonucleic acid (23) was inhibited by mangiferin (10^{-4} *M*) to the extent of 35–40% whereas chloramphenicol showed 50–60% inhibition.

EXPERIMENTAL

Mangiferin—Finely ground, air-dried root bark (200 Gm.) of *H. madablota* was defatted with petroleum ether (b.p. 60–80°, 300 ml.) in a Soxhlet extractor for 30 hr. It was observed that after about 12 hr./extraction with acetone (300 ml.), a granular solid began to deposit. At the end of the extraction (48 hr.) the solid was collected on a filter and washed with acetone, yield 0.025%. Recrystallization from 50% aqueous ethanol afforded yellow crystals, m. p. 271–274° dec. Literature values, 271° (9), 268° (7), 269° (8); $\lambda_{max.}^{KBr}$ 3450–3100, 1650, 1625, 1600, 1570, 1500, 1260, 1100, 870 $cm.^{-1}$ $\lambda_{max}^{95\%\ ethanol}$ 244.5, 260.5, 321.0, 375.5 mμ, log ε 4.03, 4.13, 3.43, 3.41; $\lambda_{max.}^{95\%\ ethanol}$, NaOH 245, 261, 400 mμ, log ε 4.05, 4.10, 4.12. These spectra were identical with those of an authentic sample.

Received October 19, 1967, from the Department of Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication November 24, 1967.
This investigation was supported by grants GM 11412 and TG 050-0472E from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
* Department of Chemistry, University College of Science, Calcutta, 9, India.

Mangiferin Trimethyl Ether—A suspension of mangiferin (30 mg.) in 2 ml. methanol and 8 ml. ether was treated with an excess of ethereal diazomethane following the procedure of Hawthorne and co-workers (7). After twice being precipitated from aqueous acetone, the product had m.p. 298–299° and provided infrared and ultraviolet spectra identical with those of an authentic sample. Literature m.p. 298–299° (7).

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Keyphrases

Hiptage madablota
Mangiferin—isolation, identification
Biological activity—mangiferin
IR spectrophotometry—structure
UV spectrophotometry—structure

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By ROBERTSON PRATT and EVELYN JOHNSON*

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In an important review of vitamin B_{12} in the metabolism of microorganisms, Ford and Hutner (5) state "B_{12} . . . is not present in green plants and yeast; it is synthesized by bacteria and probably by blue-green, brown, and red algae, but not by green algae." Others have suggested that B_{12} found associated with seaweeds (whether red, brown, or green) can be attributed to bacteria living epiphytically on the algae (6, 7) or to bacterial contaminants in the distilled water used in preparing specimens for assay (8).

Referring specifically to *Chlorella* (species unspecified), Hutner (9) commented on the lack of B_{12} in the organism while, in contrast, Hashimoto reported significant levels of the vitamin (av. 61 μ mcg./mg. dry wt.) in "pure cultured" *Chlorella ellipsoidea* and he considered this a sufficiently high level to warrant further study (10).

METHODS

Chlorella vulgaris and *C. pyrenoidosa* were cultured aseptically by both the column and the round-bottom flask techniques described previously (11): light intensity in the former was 600 ftc. and in the latter 1250 ftc. Column cultures were harvested at different times from Day 5 to Day 21; flask cultures were harvested at intervals from 3 weeks to 10 weeks postinoculation.

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Received November 13, 1967, from the School of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122
Accepted for publication January 18, 1968.
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water were used for all assays. Standard curves were determined in each assay using USP cyanocobalamin reference standard.

Three basic extraction procedures were employed: (*a*) water extraction with heat, (*b*) refluxing with 70% isopropanol, and (*c*) extraction with perchloric acid. A number of variations of these were tried including (*a*) addition of sodium cyanide (10 mg./ml.), (*b*) buffering with 0.01 *N* sodium acetate or adjusting pH with HCl, (*c*) heating at 90° or autoclaving, (*d*) subsequent digestion with papain, and (*e*) preliminary mincing in a mortar with crystalon abrasive.

No single procedure seemed consistently or distinctly superior for releasing B_{12} (if present) from the algae. In some instances extracts prepared with sodium acetate seemed to be slightly inhibitory for the *Euglena* test organism; use of isopropanol resulted in an extract containing lipids and other cell material that interfered with turbidity readings during actual assay; the perchloric acid technique resulted in extracts significantly inhibitory to growth of *Euglena*, due to inability to completely remove the perchloric acid, and the papain technique gave a high blank reading that overshadowed any possible B_{12} in *Chlorella*.

Therefore, the method recommended by the Analytical Methods Committee (12) was used routinely. In this procedure a suspension of cells with 0.01% NaCN (pH 4.5 with HCl) was heated in a water bath at 90° for 30 min. Recovery of added B_{12} was approximately 110%.

RESULTS

In seven different experiments between October 1966 and August 1967, each entailing several assays of extracts prepared from one to five harvests made at different times during the culture period, no significant or consistent levels of B_{12} or B_{12}-like activity were detected. Most frequently no B_{12} was found. Low levels (0.1 to 5 μmcg./mg. dry wt.) were found sporadically, generally in cultures 14 days or more postinoculation.

Although aseptic techniques are employed in setting up the algal cultures, the danger of bacterial contamination having occurred during subsequent manipulation cannot be totally eliminated, especially for older cultures. Therefore, the possibility of low-level bacterial contamination as the cultures age must be considered as a possible source of B_{12}.

Moreover, the B_{12} levels found were generally so low that often they were within the range of error inherent in the extraction and assay procedures.

The possibility exists that extraction procedures were not adequate for releasing B_{12} from the *Chlorella* cells but, in view of the variety of procedures and modifications employed, it seems unlikely that all of the methods would have been totally inadequate. Nonetheless, the possibility that B_{12} is protein bound and not readily released or that the tough *Chlorella* cell wall prevents release should not be overlooked.

Other possibilities are (*a*) the synthetic medium employed for culturing the algae precluded or interfered with biosynthesis of the vitamin, and/or (*b*) B_{12} is synthesized but so quickly transferred in one or more various synthetic pathways that it never accumulates in assayable levels.

However, under the conditions employed for culture and for the variety of techniques for extracting the cells and assaying the extracts, the authors have been unable to demonstrate the presence of B_{12} or B_{12}-like activity in *Chlorella vulgaris* and *C. pyrenoidosa*.

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Keyphrases

Vitamin B_{12}
Chlorella species—vitamin B_{12} content
Cultures—column, round-bottom flask techniques

Studies on the Mechanism of Action of Salicylate II. Retardation of Wound Healing by Aspirin

By K. H. LEE

Aspirin, like cortisone, reduces the tensile strength of the healing skin wound in rats. The possible mechanism of action of these anti-inflammatory agents on wound healing has been discussed.

It is well known that cortisone reduces the rate of wound healing in experimental animals (1–3). It is also known that there is a time relationship between administration of cortisone and wound healing in rats (4). When cortisone is given during the entire healing period after infliction of the wounds, the healing processes are retarded. However, when cortisone is given 2 days or later after wound infliction, it exerts no effect on healing. Dunphy and Jackson (5) attribute the effect of cortisone on wound healing to its anti-inflammatory activity. Inflammation is a necessary process involved in the early stage of wound healing (6, 7). In the present study, it was found that aspirin, another anti-inflammatory agent, behaved exactly like cortisone in retardation of wound healing in rats.

METHODS

Wound Procedure—Sprague-Dawley rats of either sex weighing 450-500 Gm., were anesthetized with ethyl ether in an open mask. The hair on the back was depilatated with an electric clipper. One 6-cm. long incision was made, through the skin and cutaneous muscles, at a distance about 1.5 cm. from the midline on each side. No ligatures were used. Bleeding usually ceased spontaneously after a few min. The incisions were closed with continuous through-and-through sutures with stitches 0.5 cm. apart. Black silk surgical thread No. 000 or No. 30 and curved needles No. 19 were used. The continuous suture was pulled tight enough to secure good adaptation of the wound edges. The wounds were left undressed.

Administration of Drugs—Aspirin, neutralized with calculated amount of sodium carbonate solution before use, was fed to the rat daily through a short stomach tube (P.E. 160) attached to a blunted hypodermic needle (No. 17) connected to a 5-ml. syringe, starting 1 day before operation. Two dosage levels, 150 mg. and 75 mg./Kg. of body weight, were given to different groups of rats.

Measurement of Healing—Tensile strength, the force required to open a healing skin wound, was used to measure healing. The instrument for this measurement is called tensiometer which is usually designed on the same principle as the thread tester used in the textile industry (4). Since there was no commercially made tensiometer available, a simple instrument for the skin wound healing measurement was made. It consisted of a 6 × 12 in. board with one post of 4 in. long fixed on each side of the longer ends. The board was placed at the end of a table. A pulley (with bearing) was mounted on the top of one of the posts. An alligator clamp, with 1-cm. clamp width, was tied on the tip of the post without pulley by a piece of fishing line (20-lb. test monofilament) so that the clamp could reach the middle of the board. Another alligator clamp was tied on a longer piece of fishing line with a 1-L. polyethylene bottle tied on the other end. Before testing, the animal was anesthetized with ethyl ether in an open mask. The sutures of the wound were cut out with a pair of scissors. The animal was then placed on a stack of paper towels on the middle of the board. The amount of the towels could be adjusted so that the wound was on the same level of the tips of the posts. The clamps were then carefully clamped on the skin of the opposite sides of the wound at a distance of 0.5 cm. away from the wound. The longer piece of fishing line was placed on the pulley, and the position of the board was adjusted so that the polyethylene bottle was freely hanging in the air. Water was added to the polyethylene bottle at a rapid but constant rate by siphon from a large reservoir (20-L. bottle) until the wound began to open up. The amount of water in the polyethylene bottle was weighed and considered as the tensile strength of the wound. Two to three determinations were made on each wound. The mean of the determinations made on wounds on both sides of the animal was taken as the tensile strength of the wound. The tensile strengths reported in the paper were all measured on the seventh day after wounding.

RESULTS

Controls—The tensile strength of skin wound of untreated rats was determined after a healing period of 7 days. Usually three readings were taken on each 6-cm. long wound whenever possible. The tensile strength of wound on one side of the back did not differ greatly from that of the wound inflicted on the other side of the back. The averaged value for 21 animals tested was 438 ± 17.7 Gm. (Table I, Group I).

Treated Animals—Rats receiving an oral dose of 150 mg. of aspirin per kilogram of body weight daily, starting 1 day before wounding, have an averaged tensile strength of 262 ± 8.7 Gm. on the seventh day after wounding, which is about 40% inhibition (Table I, Group II). The inhibition is significant ($p < 0.001$).

When the dose of aspirin given was reduced to one half or 75 mg. (Group III), the averaged tensile strength of the wound was 343 ± 91 Gm. or 22% inhibition.

However, when the feeding of even the larger dose of aspirin (150 mg.) was started 2 days after the wounding, the averaged tensile strength was 350 ± 10.9 Gm. or 80% of the control. These results are in good agreement with those obtained by Sandberg (4) when cortisone was given under similar conditions.

Received November 13, 1967, from the School of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122

Accepted for publication January 2, 1968.

Presented to The American Society for Pharmacology and Experimental Therapeutics, August meeting, 1967, Washington, D. C.

* This work was supported in part by a grant-in-aid from Eli Lilly and Company, Indianapolis, Indiana.

TABLE I—TENSILE STRENGTH OF HEALING WOUNDS

Group[a]	No. of Animals	Aspirin Fed Daily: Amt., mg./Kg.	Aspirin Fed Daily: Time Started	Averaged Tensile Strength, Gm.	% of Control
I	21	—	—	438 ± 17.7	100
II	13	150	1 day before wounding	262 ± 8.7	60
III	12	75	1 day before wounding	343 ± 21.0	78
IV	7	150	2 days after wounding	350 ± 10.9	80

[a] Differences between I and II, I and III, I and IV, II and IV are significant ($p < 0.001$).

The body weight of every rat was recorded at the beginning and the end of the experiment. The averaged change in body weight did not, in most of the cases, vary more than 10 Gm.

DISCUSSION

The importance of inflammation in wound healing has long been recognized by surgeons (5). Experimentally, Selye (7) has shown that the connective tissue response to a granuloma pouch made by injecting air subcutaneously can be varied directly with the degree of inflammation created in the pouch. The injection of croton oil or other irritants into the pouch sets up an acute inflammatory reaction followed by a rapid growth in connective tissue. The injection of cortisone inhibits the inflammation and reduces the subsequent production of connective tissue. Other studies on the effect of cortisone on wound healing (4) have shown that if cortisone is given during the entire healing period, it inhibits the inflammatory response and subsequent healing processes. However, if cortisone is given some time after the wounding, so that the initial inflammatory response has already taken place, it has no effect upon the healing.

It has been suggested that lysosomal enzymes are responsible for inflammation (8). Lysosomes are cytoplasmic organells which contain the bulk of acid hydrolases (9). The proteolytic enzymes in lysosomes can break down plasma proteins into polypeptides. Some of these polypeptides may increase capillary permeability and thus cause inflammation. Anti-inflammatory steroids: cortisone, hydrocortisone, prednisone, prednisolone, betamethasone can protect lysosomal membrane (10, 11). The protective action on lysosomal membrane is one of the current theories on the mechanism of action of anti-inflammatory steroids (10).

Aspirin is another anti-inflammatory agent which also protects lysosomal membrane (12). In the present study, it has been shown that aspirin also retards wound healing in the same manner as cortisone. It is very likely that aspirin shares the same mechanism of action in retarding wound healing as cortisone. This belief is further strengthened by the fact that when aspirin was given 2 days after the wounding the inhibitory effect was greatly reduced. This followed the same pattern when cortisone was applied this way (4).

SUMMARY

It has been demonstrated that aspirin retards wound healing in the rat and the mechanism of this action has been discussed.

A simple laboratory-made tensiometer has been described.

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Keyphrases

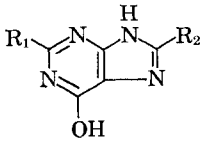
Salicylates—action mechanism
Aspirin—wound healing effect
Tensile strength—healing incision
Apparatus—tensile strength measurement

Pyrazoles I. Synthesis of 4-Hydroxypyrazolo[3,4-*d*]-*v*-triazine A New Analog of Hypoxanthine

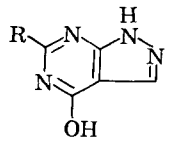
By C. C. CHENG, ROLAND K. ROBINS, KATHERINE C. CHENG, and DOLORES C. LIN

Treatment of 3-amino-4-pyrazolecarboxamide with nitrous acid yielded 3-diazo-4-pyrazolecarboxamide, which was cyclized to 4-hydroxypyrazolo[3,4-*d*]-*v*-triazine in aqueous solution. The latter is a structural analog of hypoxanthine as well as an aza analog of allopurinol.

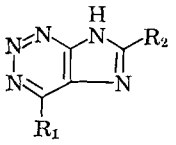
A STRUCTURAL ANALOG of hypoxanthine (Ia), 4-hydroxypyrazolo[3,4-*d*]pyrimidine (IIa, 4-HPP, allopurinol, Zyloprim), was synthesized and reported in 1956 (1, 2). Compound IIa has since been found to be effective against hyperuricemia associated with gout in man (3–11). It is remarkably well tolerated by patients, and serious side effects have not been encountered (12–16). Metabolic studies revealed that many compounds of this type are potent inhibitors of xanthine oxidase (4, 14, 17–23), the enzyme responsible for the oxidation of hypoxanthine (Ia) to xanthine (Ib) and to uric acid (Ic). This compound can, therefore, be used to reduce the hazard of uric acid nephropathy in patients with leukemias and lymphomas, in whom rapid lysis of cells is expected to occur (24). It has also been used to potentiate the therapeutic index of 6-mercaptopurine (17, 18).



I a, R_1, R_2 = H
b, R_1 = OH, R_2 = H
c, R_1, R_2 = OH



II a, R = H
b, R = OH



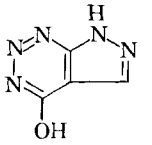
III a, R_1 = OH, R_2 = H
b, R_1 = NH_2, R_2 = H
c, R_1 = NH_2, R_2 = OH

Allopurinol itself is gradually metabolized *in vivo* by xanthine oxidase to 4,6-dihydroxypyrazolo[3,4-*d*]pyrimidine (25, 26) (IIb, isoxanthine, alloxanthine). Although IIb is still an inhibitor of xanthine oxidase, it is no longer as effective as allopurinol. Further metabolic oxidation of IIb to a uric acid-type analog is not feasible owing to the aza substituent in the original "purine-C_8" position.

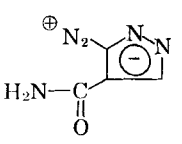
Some 2-azapurines (III, imidazo[4,5-*d*]-*v*-triazines) are also found to be inhibitors of xanthine oxidase (27). The most active one of this class, 2-azaadenine (IIIb), was reported to be rapidly attacked by this enzyme to form a somewhat less effective inhibitor, 8-hydroxy-2-azaadenine (27) (IIIc).

Since xanthine oxidase oxidizes purines at the 2, 6, or 8 position (28), synthesis of an aza analog of allopurinol (IIa), 4-hydroxypyrazolo[3,4-*d*]-*v*-triazine (IV) was proposed. Compound IV itself would not be subject to attack by xanthine oxidase, and thus could well be a better inhibitor of the enzyme. The fact that many 2-azapurines possess activity as inhibitors of neoplastic cells (29, 30) and of microorganisms (31) suggests that compounds such as IV might be useful as chemotherapeutic agents.

Treatment of 3-amino-4-pyrazolecarboxamide (1) with nitrous acid yielded a tan-colored compound which decomposed violently at *ca.* 165–170° on rapid heating. Although this product gave the correct elemental analysis for IV, a sharp, intense infrared absorption peak at the 4.5-μ region and a positive Bratton-Marshall test (32) indicated the presence of a diazo group. Hence the product was actually the uncyclized 3-diazo-4-pyrazolecarboxamide (V).



IV



V

Formation of a similar compound, 5-diazo-4-imidazolecarboxamide, from 5-amino-4-imidazolecarboxamide and nitrous acid has also been reported (33).

The desired cyclized compound, 4-hydroxypyrazolo[3,4-*d*]-*v*-triazine (IV, 4-HPT), can be readily obtained by treatment of V with dilute alkali or, less readily, with dilute acid. Compound IV did not give a positive Bratton-Marshall test (32), possessed no infrared absorption in the triple or cumulative double bond region. It can be recrystallized from water as a monohydrate.

Compound 4-HPT was tested as an inhibitor of xanthine oxidase from bovine milk. A 50% inhibition of the enzyme was observed by 4-HPT at a concentration of 7.4 μM in the presence of 8.1 μM of hypoxanthine as a substrate.

Received December 20, 1967, from the Midwest Research Institute, Kansas City, MO 64110
Accepted for publication January 31, 1968.
This investigation was supported in part by research grant No. C-2845 from the National Cancer Institute, and in part by contract No. DA-49-193-MD-2749 with U. S. Army Medical Research and Development Command. This paper is Contribution No. 257 from the Army Research Program on malaria.
The authors wish to thank Professor B. R. Baker for providing the information of xanthine oxidase inhibitory test, and to thank Mrs. Margaret L. Rounds and Mr. John R. Gravatt for their assistance in performing analytical and instrumental measurements.

EXPERIMENTAL[1]

3-Diazo-4-pyrazolecarboxamide (V)—To a finely powdered suspension of 35 Gm. (0.2 mole) of 3-amino-4-pyrazolecarboxamide hemisulfate (1) in 350 ml. of water cooled to 0° was added, with mechanical stirring, 16 Gm. (0.23 mole) of sodium nitrite dissolved in 60 ml. of water. The mixture was stirred for 3 hr. at 0° and the resulting tan solid was collected by filtration and washed successively with water, methanol, and ether. The yield after overnight air drying was 18 Gm. (66%), which, on rapid heating, decomposed violently with a sharp sound. The product, which is analytically pure, is surprisingly stable under ordinary storage conditions. It gave a positive Bratton-Marshall test (32) and exhibited a diazo absorption band at 4.5 μ. $\lambda_{max.}^{pH\ 1}$ 256 mμ (ϵ 6,000); $\lambda_{max.}^{pH\ 11}$ 311 mμ (ϵ 5,500).

Anal.—Calcd. for $C_4H_3N_5O$: C, 35.03; H, 2.20; N, 51.09. Found: C, 34.74; H, 2.36; N, 51.00.

4-Hydroxypyrazolo[3,4-*d*]-*v*-triazine Monohydrate (IV)—A white crystalline solid gradually separated on standing from the filtrate of the preceding experiment. The product was collected by filtration, washed with a small amount of cold water, and dried to yield 4 Gm. of IV. The compound, which is quite soluble in methanol, decomposed abruptly (but not as violently as V) at *ca.* 180° on rapid heating; slow heating did not cause it to decompose or melt up to 360°. The product is analytically pure but recrystallization from hot water changed the color of the solid from white to light yellow. The ultraviolet and infrared absorption spectra were, however, identical. Compound IV did not show a triple bond absorption peak and failed to yield a positive Bratton-Marshall test (32). $\lambda_{max.}^{pH\ 1}$ 270 mμ (ϵ 5,500); $\lambda_{max.}^{pH11}$ 312 mμ (ϵ 5,800).

Anal.—Calcd. for $C_4H_3N_5O \cdot H_2O$: C, 30.97; H, 3.25; N, 45.15; H_2O, 11.51. Found: C, 31.30; H, 3.53; N, 44.79; H_2O, 11.48.

The same product can also be obtained by the addition of 10 ml. of 10 *N* sodium hydroxide to the stirred suspension of 5 Gm. of V in 100 ml. of water. The resulting solution was allowed to stir for 40 min., filtered, and the filtrate acidified with dilute hydrochloric acid. Three and one-half grams of analytically pure light golden yellow plates were obtained.

[1] Melting points were taken on a Thomas-Hoover melting point apparatus. The infrared spectra were taken with a Perkin-Elmer infracord and the ultraviolet absorption spectra were determined with a Beckman DK-2 spectrophotometer.

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Keyphrases

Pyrazoles
4-Hydroxypyrazolo [3,4-*d*]-*v*-triazine—synthesis
Bratton-Marshall test—identity
IR spectrophotometry—structure
UV spectrophotometry—structure

Determination of 6-Demethyltetracycline HCl in 6-Demethylchlortetracycline HCl

By FORTUNATO S. CHICCARELLI

6-Demethyltetracycline, when heated with nitric acid, forms a stable colored derivative having an absorption maximum at 425 mμ. 6-Demethylchlortetracycline under the same condition is unstable. When 6-demethylchlortetracycline is heated with nitric acid, the color produced by the 6-demethyltetracycline fraction can be measured directly or extracted with *n*-butanol.

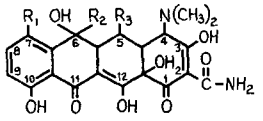
In the production of 6-demethylchlortetracycline (1)[1] small amounts of 6-demethyltetracycline are also formed. An automated method of assay for 6-demethylchlortetracycline based on the difference in the rates of conversion of these components to their respective anhydro forms has been presented (2). A manual procedure (3) based on the same principle has also been developed. To detect the presence of small concentrations of 6-demethyltetracycline in 6-demethylchlortetracycline, a direct analytical procedure was required.

Tetracycline, chlortetracycline, 6-demethyltetracycline, and 6-demethylchlortetracycline all undergo transformation to anhydro compounds when their respective solutions are heated with hydrochloric acid. The order of stability to HCl is 6-demethylchlortetracycline, 6-demethyltetracycline, chlortetracycline, and tetracycline with 6-demethylchlortetracycline being the most stable. Oxytetracycline forms colorless apoxytetracyclines (4).

With this in mind the above antibiotics were heated with other acids, one of which was nitric acid. A relatively stable intense yellow color remained in the 6-demethyltetracycline solution while the other solutions became relatively colorless. It was decided to investigate this difference in behavior.

EXPERIMENTAL

Scheme I shows the structural formula of the antibiotics tested. A diagrammatic scheme has been used to point up the differences and similarities.



	R_1	R_2	R_3
Tetracycline	H	CH_3	H
Chlortetracycline	Cl	CH_3	H
5-Hydroxytetracycline	H	CH_3	OH
6-demethyltetracycline	H	H	H
6-demethylchlortetracycline	Cl	H	H

Scheme I

Figure 1 shows the absorption spectra of tetracycline (TC), chlortetracycline (CTC), 5-hydroxytetracycline (OTC), 6-demethyltetracycline (DMTC), and 7-chloro-6-demethyltetracycline (DMCTC) from 300 mμ to 500 mμ at 25 mcg./ml. in 0.1 *N* hydrochloric acid.

Figure 2 shows the absorption spectra of each of the antibiotics after heating at 100° with nitric acid. Solutions of each were made with 200 mcg./ml. 0.07 *M* H_3PO_4. Forty ml. of the solution plus 20 ml. 4.0 *M* HNO_3 were heated in a water bath for 15 min. After cooling and diluting up to 100 ml., the absorption spectra were determined from 300 mμ–500 mμ. Of the five antibiotics studied, DMTC was unique in that a maximum appearing in the visible range (420–440 mμ) was retained. The product(s) of this reaction is now under study.

The effect of heating time at 100° with DMTC and DMCTC in the presence of nitric acid is shown in Table I. Solutions of each antibiotic were made with 200 mcg./ml. 0.07 *M* H_3PO_4. Forty ml. of this solution plus 20 ml. 4.0 *M* HNO_3 were heated in a boiling water bath for the time specified. At the end of each time interval the flasks were cooled and diluted up to 200 ml. with water. The absorbance of the solutions was determined at 425 mμ. The

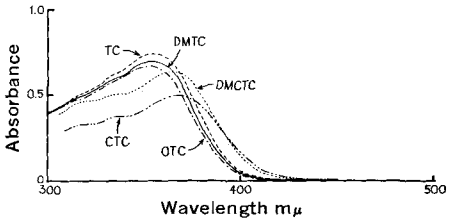


Fig. 1—Absorption spectra of tetracycline (TC), chlortetracycline (CTC), 5-hydroxytetracycline (OTC), 6-demethyltetracycline (DMTC), 6-demethylchlortetracycline (DMCTC) from 300 mμ to 500 mμ at 25 mcg./ml. in 0.1 N HCl.

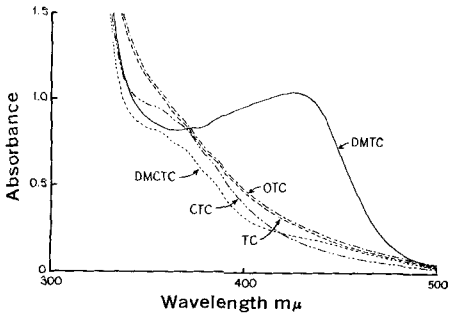


Fig. 2—Absorption spectra of tetracycline (TC), chlortetracycline (CTC), 5-hydroxytetracycline (OTC), 6-demethyltetracycline (DMTC), 6-demethylchlortetracycline (DMCTC), after heating at 100° with HNO_3 for 15 min.

Received September 28, 1967, from the Quality Control Section, Lederle Laboratories Division, American Cyanamid Co., Pearl River, NY 10965

Accepted for publication December 15, 1967.

The author thanks Mr. G. P. Cbrekian for his valuable assistance with this report.

[1] Declomycin is the registered trademark of American Cyanamid Co. for 6-demethylchlortetracycline HCl.

TABLE I—DIFFERENCE IN ABSORPTION WITH TIME OF HEATING AS A VARIABLE

Time, min.	Absorbance at 425 mμ DMCTC	DMTC
0	0.0	0.0
3	0.345	0.260
6	0.135	0.515
9	0.115	0.505
12	0.110	0.490
15	0.105	0.475
18	0.100	0.460
21	0.095	0.445

high absorbance value at 425 mμ after 3 min. heating suggests the formation of the DMCTC anhydro derivatives. Lower values at the 6-, 9-, 12-min. intervals may be attributed to oxidative cleavage of the anhydro molecule. The high absorbance at 425 mμ of the DMTC after heating in HNO_3 is probably due to a stable 7-nitro-anhydro DMTC.

Figure 3 shows the absorption spectra from 300 mμ to 500 mμ of butanol extracts of DMCTC standard solutions containing 0–8% DMTC. Determinations (*versus n*-butanol) were made with 1-cm. cells.

Figure 4 contains the spectra from 300 mμ to 500 mμ of standard solutions when measured directly with 10-cm. cells. The 0% treated standard solution is used in the reference cell.

Table II contains five separate results when a well-mixed synthetic sample of DMCTC containing 3% DMTC was assayed. The sample was made from standard DMCTC and DMTC.

Application of this procedure to refined DMCTC gave greater precision than the method previously used (5).

METHOD

Apparatus—Beckman spectrophotometer model DU 2400 equipped to handle 1-cm. or 10-cm. quartz cells. Cell correction was applied when necessary. Complete spectra were made with a Cary recording spectrophotometer.

Reagents—Nitric acid, 4.0 *M*, made from reagent grade which is free of yellow oxides of nitrogen; H_3PO_4, 0.07 *M*; *n*-butanol, reagent; NaCl, C.P. 6-demethylchlortetracycline HCl standard, 200 mcg./ml. (100 mg./500 ml.); and 6-demethyltetracycline HCl standard, 200 mcg./ml. (100 mg./500 ml.). Standard solutions are made in 0.07 *M* H_3PO_4.

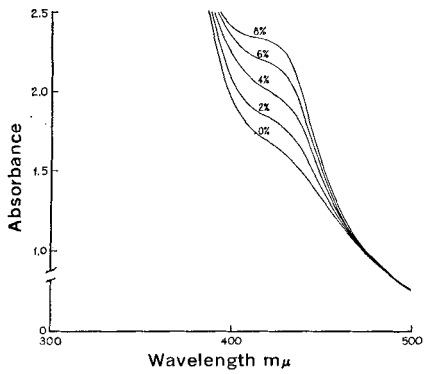


Fig. 3—Absorption spectra of demethyltetracycline (vs. n-butanol) standard solutions when measured after butanol extraction, with 1-cm. cells.

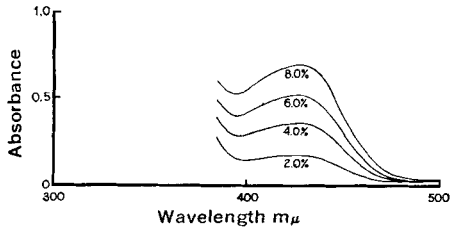


Fig. 4—Absorption spectra of demethyltetracycline standard solutions when measured directly, with 10-cm. cells.

TABLE II—RESULTS WITH SYNTHETIC SAMPLE

Determination	% DMTC
A	3.1
B	2.8
C	2.9
D	3.4
E	3.0

Procedure—Transfer an accurately weighed sample of about 100 mg. DMCTC to a 50-ml. volumetric flask. Add 0.07 *M* H_3PO_4 and mix until solution is complete. Make up to mark with 0.07 *M* H_3PO_4 and mix. Pipet a 40.0-ml. aliquot into a 100-ml. volumetric flask. Add 20 ml. 4.0 *M* HNO_3 and heat in a boiling water bath for exactly 15 min. with occasional swirling.

Standard solutions and samples should be treated at the same time. Pipet 2.0-, 4.0-, 6.0-, and 8.0-ml. aliquots of DMTC standard solution (200 mcg./ml., 0.07 *M* H_3PO_4) into separate 100-ml. volumetric flasks. Make each up to mark with DMCTC standard solution (200 mcg./ml., 0.07 *M* H_3PO_4) and mix. Transfer a 40.0-ml. aliquot of the DMCTC standard solution (0%) and 40.0-ml. aliquots from each of the 2.0, 4.0, 6.0, and 8.0% DMTC standards to separate 100-ml. volumetric flasks. To each add 20 ml. 4.0 *M* HNO_3 and heat in the same manner as the sample.

After heating is complete, remove all flasks, cool, and make to volume with water. Mix. The following alternatives can be used for the absorbance determination.

For 1-cm. cells transfer the solutions to individual separatory funnels containing 40 Gm. NaCl. Shake for 1 min. Add 10 ml. *n*-butanol and shake for 2 min. Collect the butanol extracts and centrifuge. Determine the absorbance (*versus n*-butanol) at 425 mμ using the 0.1 position of the scale. For 10-cm. cells fill the two 10-cm. cells with the 0% standard solution. Using one as the reference, measure the absorbance of the other at 425 mμ. The difference between this is the cell correction. Measure the standard and unknown solutions against the 0% reference solution at 425 mμ.

Plot a concentration curve using the 0, 2, 4, 6, and 8% absorbance values. Before the curve can be used, the sample absorbance must be corrected for the amount of antibiotic in the original weight taken.

Transfer a 5-ml. aliquot of the sample solution to a 100-ml. volumetric flask. Make up to mark with 0.07 *M* H_3PO_4 and mix. Determine its absorbance at 368 mμ. (Acid isobestic point of DMTC and DMCTC.) Repeat the determination with a 5-ml.

portion of the DMCTC standard solution treated in the same manner.

The corrected sample absorbance will give a direct measure of the DMTC when used with the standard curve.

Calculation—(*a*) mg. of antibiotic (DMTC and DMCTC) in sample weight:

$$\frac{\text{sample abs. at 368 m}\mu}{\text{standard abs. at 368 m}\mu} + 10.0 \times \frac{100}{1000} \times \frac{500}{5} = \text{mg.}$$

(*b*) corrected sample absorbance value to be used with concentration curve: $\frac{100}{\text{mg.}} \times$ abs. at 425 mμ (sample) = corrected value

SUMMARY

With the use of nitric acid, an assay for DMTC in DMCTC has been developed. DMCTC is unstable to nitric acid while DMTC is sufficiently stable for purpose of assay. The data presented suggest that DMTC could also be measured in the presence of CTC, TC, and OTC by this procedure.

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Keyphrases

6-Demethyltetracycline in 6-demethylchlortetracycline—analysis

6-Demethylchlortetracycline—nitric acid transformation

Spectrophotometry—analysis

Quantitative Thin-Layer Chromatography of Sympathomimetic Amines

By N. H. CHOULIS* and C. E. CAREY

Thin-layer chromatography for quantitative estimation of sympathomimetic amines has been used. Two methods were performed: the weight/area relationship and the elution of the spots and spectrophotometric determination in the ultraviolet. For the first method, solutions of the amines, in increasing concentration, were applied on cellulose thin layer, and a solution of unknown concentration of the same amines was run parallel to the standards. The spot was compared with the standards using a recording photoelectric densitometer. For the second method the standard spots were eluted from the cellulose plates and their absorption was measured. The absorptivity of a similarly eluted spot of unknown concentration, but parallel running, was measured and compared with the standards. The accuracy of the methods appeared to be between 95–98 percent.

THE APPLICATION of thin-layer chromatography to pharmaceutical analysis has been used increasingly during the past years. A number of methods for quantitative analysis have been employed since the technique was first introduced by Stahl (1) in 1956.

Seher (2) has suggested that in thin-layer chromatography the weight of the material and the spot area are proportional. Purdy and Truter (3) found that quantitation is based on a linear relationship existing between the square root of the area of a component after chromatographing, and the logarithm of the weight of the applied sample. According to them, Seher's data, as well as that of Stahl (4) and Breuner and Niederwieser (5), fit the relationship for loads of 1 to 80 mcg./spot. Pelka and Metcalf (6) examining long-chain tertiary amines, found the method applicable for loads of approximately 200 mcg.

Morrison and Orr (7), in an analysis of selected pharmaceutical mixtures in tablet and capsule forms and by using thin-layer chromatography, obtained quantitative results from the developed chromatograms. A linear relationship between the spot area, in sq. mm., and the weight of the spots was observed.

The method was applicable for concentrations up to 250 mcg. However, this method requires a satisfactory spray reagent, otherwise its use for quantitative work is doubtful.

Ganshirt *et al.* (8) used thin-layer chromatography for the quantitative assay of a number of bile acids, and Zollner *et al.* (9) found the method applicable for the quantitative estimation of cholesterol esters.

Millett *et al.* (10) described other techniques for

Received April 28, 1967, from the School of Pharmacy, Texas Southern University, Houston, TX 77004

Accepted for publication December 11, 1967.

Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

Aided by a Mead Johnson Research Grant for 1966–1967.

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portion of the DMCTC standard solution treated in the same manner.

The corrected sample absorbance will give a direct measure of the DMTC when used with the standard curve.

Calculation—(*a*) mg. of antibiotic (DMTC and DMCTC) in sample weight:

$$\frac{\text{sample abs. at 368 m}\mu}{\text{standard abs. at 368 m}\mu} + 10.0 \times \frac{100}{1000} \times \frac{500}{5} = \text{mg.}$$

(*b*) corrected sample absorbance value to be used with concentration curve: $\frac{100}{\text{mg.}} \times$ abs. at 425 mμ (sample) = corrected value

SUMMARY

With the use of nitric acid, an assay for DMTC in DMCTC has been developed. DMCTC is unstable to nitric acid while DMTC is sufficiently stable for purpose of assay. The data presented suggest that DMTC could also be measured in the presence of CTC, TC, and OTC by this procedure.

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Keyphrases

6-Demethyltetracycline in 6-demethylchlortetracycline—analysis

6-Demethylchlortetracycline—nitric acid transformation

Spectrophotometry—analysis

Quantitative Thin-Layer Chromatography of Sympathomimetic Amines

By N. H. CHOULIS* and C. E. CAREY

Thin-layer chromatography for quantitative estimation of sympathomimetic amines has been used. Two methods were performed: the weight/area relationship and the elution of the spots and spectrophotometric determination in the ultraviolet. For the first method, solutions of the amines, in increasing concentration, were applied on cellulose thin layer, and a solution of unknown concentration of the same amines was run parallel to the standards. The spot was compared with the standards using a recording photoelectric densitometer. For the second method the standard spots were eluted from the cellulose plates and their absorption was measured. The absorptivity of a similarly eluted spot of unknown concentration, but parallel running, was measured and compared with the standards. The accuracy of the methods appeared to be between 95–98 percent.

THE APPLICATION of thin-layer chromatography to pharmaceutical analysis has been used increasingly during the past years. A number of methods for quantitative analysis have been employed since the technique was first introduced by Stahl (1) in 1956.

Seher (2) has suggested that in thin-layer chromatography the weight of the material and the spot area are proportional. Purdy and Truter (3) found that quantitation is based on a linear relationship existing between the square root of the area of a component after chromatographing, and the logarithm of the weight of the applied sample. According to them, Seher's data, as well as that of Stahl (4) and Breuner and Niederwieser (5), fit the relationship for loads of 1 to 80 mcg./spot. Pelka and Metcalf (6) examining long-chain tertiary amines, found the method applicable for loads of approximately 200 mcg.

Morrison and Orr (7), in an analysis of selected pharmaceutical mixtures in tablet and capsule forms and by using thin-layer chromatography, obtained quantitative results from the developed chromatograms. A linear relationship between the spot area, in sq. mm., and the weight of the spots was observed.

The method was applicable for concentrations up to 250 mcg. However, this method requires a satisfactory spray reagent, otherwise its use for quantitative work is doubtful.

Ganshirt *et al.* (8) used thin-layer chromatography for the quantitative assay of a number of bile acids, and Zollner *et al.* (9) found the method applicable for the quantitative estimation of cholesterol esters.

Millett *et al.* (10) described other techniques for

Received April 28, 1967, from the School of Pharmacy, Texas Southern University, Houston, TX 77004

Accepted for publication December 11, 1967.

Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

Aided by a Mead Johnson Research Grant for 1966–1967.

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quantitative thin-layer chromatography using furoic acid and hydroxymethylfuroic acids as model compounds. Eble and Brooker (11) suggested an application of thin-layer chromatography for quantitative assay of tryptamine.

Choulis (12) recently used thin-layer chromatography for quantitative estimation of adrenaline.

The purpose of the present paper is to examine the applicability of the technique as a general method for quantitation for sympathomimetic amines in pharmaceutical formulations.

EXPERIMENTAL

Materials—Amphetamine hydrochloride, noradrenaline hydrochloride, normetanephrine hydrochloride, metanephrine hydrochloride, dopamine, ephedrine hydrochloride, isopropylarterenol hydrochloride, nordefrin hydrochloride, dopa, and phenylephrine hydrochloride were used.

Developing System—*n*-Butanol: acetic acid: water 4:1:5 v/v (the liquids were shaken together and set aside overnight; the organic layer was separated and used as the running solvent).

Detection—Amphetamine was detected by the *p*-nitroaniline diazo reagent described by Wickstrom and Salversen (13). The reagent was prepared by dissolving 0.25 Gm. *p*-nitroaniline in 25 ml. of 1 *N* hydrochloric acid and diluted to 50 ml. with ethanol. The solution was cooled in an ice bath and 0.1 Gm. sodium nitrate was added before use; the amphetamine was detected by the appearance of a pink spot.

Dopa, dopamine, and ephedrine hydrochloride were located by spraying with a solution of ninhydrin in *n*-butanol or acetone (0.2% w/v) and heating the plates at 100° for 4 min. (14).

All other amines were detected by using an aqueous solution containing 0.6% w/v potassium ferricyanide and 0.5% w/v sodium hydroxide (15).

An ultraviolet lamp was used also to locate the spots in cases where spraying reagents had to be avoided.

Preparation of the Plates—The plates (20 × 20 cm.) were coated with cellulose layer, 250 mμ thick, according to the method of Stahl (1), also (12).

General Procedure—Solutions of the amines were prepared by dissolving 200 mg. of the pure compounds in 10 ml. of a 5% solution of acetic acid in water, in 10-ml. volumetric flasks (master solutions).

Using accurately calibrated microliter pipets, samples of the solutions were applied to the plates, 1.5 cm. from the bottom edges, and ascending chromatograms were run at room temperature (about 23°). The solvent front was allowed to travel 10 cm.; the plates were removed, air dried, and sprayed with the appropriate reagent.

To all solvent systems used, small quantities of sodium metabisulfite were added as antioxidant. All chromatograms were carried out in chromatographic chambers enriched in nitrogen atmosphere.

Methods—(*a*) Spot area/concentration relationship—The relationship between spot area and concentration was established by spotting various concentrations of the amine solutions on thin layers. The chromatograms were developed and the spots were detected using various spray reagents. The detected chromatograms were positioned on the stage of a Photovolt Densitometer, model 52-C, and the spots were scanned perpendicularly to the direction of chromatographic development. Using a 1- × 10-mm. slit which covered the spots completely, the machine was adjusted to read zero absorbance at a point a few millimeters from the spot; scanning was begun and the peak area was traced on a Varicord recorder with a 6.5-in. paper. The area under the peak was integrated with the photovolt electronic integrator, and also calculated with the use of Gelman high-precision planimeter. Standard curves were constructed from plots of the area *versus* concentration of various amines.

(*b*) Ultraviolet absorption/concentration relationship—The relationship between ultraviolet absorption and concentration of the amines was established by spotting again various concentrations of the amine solutions on thin layers. The chromatograms were developed and the spots were detected using an ultraviolet lamp and marked. The marked areas, which corresponded to the spots, were carefully scraped off and the cellulose was quantitatively transferred to 20-ml. conical flasks. The amines were eluted from the coating material by adding about 7 ml. of the solvent; the flasks were shaken well for about 10 min., centrifuged, and filtered into 10-ml. volumetric flasks. Solvent was added to the volume. No amine residues were found on the filters testing with the appropriate amine reagent. A similar elution process was followed for a blank sample of the coating material alone.

Spectrophotometric measurements were carried out at λ maxima of each individual amine, using a Beckman DU spectrophotometer with 1-cm. cells, and standard curves of absorptivity *versus* concentration of the various amines (after reducing the absorbance due to the blank) were constructed.

RESULTS

Relationship Between Spot Area and Concentration—The quantitation of the amine spots, after spraying the chromatograms with the appropriate reagent, was calculated from plots of the peak areas *versus* concentrations with the standard deviation on the plate, and tabulated as indicated in Table I where the relationships for amphetamine hydrochloride and phenylephrine hydrochloride in concentration ranges between 1–15 mcg. were established. The average deviation from the theoretical value was calculated and expressed in percentages.

TABLE I—RECOVERY VALUES AND PERCENTAGE DEVIATION OF AMPHETAMINE HCl AND PHENYLEPHRINE HCl USING PHOTOELECTRIC DENSITOMETER

Compounds	Quantities Spotted on Plates, mcg.	Theoretical Values of Integrated Area Units, Pips	Received Mean-Values of Integrated Area Units, Pips	Av. Deviation from 100%
Amphetamine hydrochloride	1	8	8	
	3	16	16	
	5	32	31	−5
	7	48	46	
	9	54	58	
Phenylephrine hydrochloride	3	25	24	
	6	50	50	
	9	75	72	−3
	12	100	96	
	15	125	121	

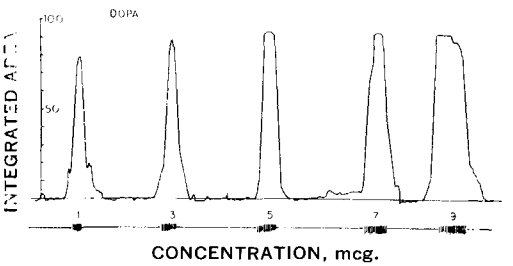

Fig. 1—Typical Varicord traced areas for dopa.

Figure 1 illustrates typical Varicord-traced areas for dopa, in concentration ranges between 1 and 9 mcg. Similar results were observed for all other examined amines using concentration areas between 2 and 40 mcg.

Relationship Between Ultraviolet Absorption and Concentration—The quantitation of the amine spots was determined from plots of absorbance *versus* concentration and tabulated, using the mean values from a number of measurements, as indicated in Table II, where the relationships of amphetamine hydrochloride and phenylephrine hydrochloride in concentration ranges between 0.1 and 5 mcg. were established.

Here also the average deviation from the theoretical value was calculated and expressed in percentages.

DISCUSSION

In the present investigation two methods for quantitative determination of sympathomimetic amines are employed, namely the spot area/concentration and the ultraviolet absorption/concentration.

All experiments were carried out in triplicate and the mean values were recorded.

For the first method, linear relationships between the spot areas and the weight of the compounds used were found using densitometric measurements. This relationship shows the dependence of the area of the spot upon the amount of the amine used. From the calculated mean values, a deviation of ±5% was observed.

Although the method is considered sufficiently accurate for routine pharmaceutical analysis, some limitations may be imposed. For example, a tendency to overload the capacity of the plates with an excess of spots, or even to overload the spots must be avoided. The best results were obtained with 25-mcg. or less quantities/spot, although higher concentrations can be determined accurately provided that the size of the initial spot application area should be as small as possible, due to the fact that the spot area is increased during the development of the chromatogram, and also that the photocell of the densitometer cannot detect areas outside its optical field.

Moreover, the temperature of the experiments (room temperature) must be constant, and the purity of the solvents used must be examined so that R_f values will be more reproducible.

The greater difficulty, however, is in the preparation of the appropriate plates, because variations in the thickness of the cellulose layer alter the light reflected by the background. Spray reagents which color the background will also lead to incorrect results, since the densitometer records the difference in intensity between the detected spot and the noncolored background.

Table II—Recovery Values and Percentage Deviation of Amphetamine HCl and Phenylephrine HCl Using Ultraviolet Spectrophotometer

Compounds	Quantities Spotted on Plates, mcg.	Theoretical Absorptivity Values	Absorptivity after Elution, Mean Value	Deviation from 100%
Amphetamine hydrochloride	0.3	0.06	0.06	
	0.6	0.12	0.11	
	0.9	0.18	0.19	−2
	1.2	0.24	0.24	
	1.5	0.30	0.28	
Phenylephrine hydrochloride	1	0.10	0.10	
	2	0.20	0.195	
	3	0.30	0.29	−4
	4	0.40	0.38	
	5	0.50	0.47	

Using the ultraviolet absorption/concentration method, linear relationships between the absorption density of the eluates and the weight of the compounds used were observed; the recovery of the compounds after elution of the spots was approximately 95% (±3%).

Previous observations (12) of a low recovery of the amines from cellulose layers had not recurred. Presumably the higher concentration of the acid solution used (5% instead of 2%) overpowered the influence of the absorption and partition forces, which otherwise operate exclusively on cellulose layers (16).

For quantitative estimation of sympathomimetic amines, the above-mentioned methods may be regarded as simple and accurate, and the use of these two techniques in routine microanalysis is suggested

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Keyphrases

Sympathomimetic amines—analysis
TLC—separation
Densitometry—spot area/concentration relationship
UV spectrophotometry—analysis

Nonaqueous Titration of Theophylline Combinations with Acetous Perchloric Acid

By M. B. DEVANI, C. J. SHISHOO, and D. J. BHUT

Synthetic mixtures of theophylline and sodium acetate, salicylate, and glycinate are titrated with acetous perchloric acid using a potentiometer for the detection of the end points. Differentiation of the equimolecular mixtures could be realized in the solvent mixture containing benzene–acetic anhydride–acetic acid (18:4:1). The method is applied to assay theophylline combinations and their tablets. The results are compared to those obtained by official methods.

IN PHARMACY, various combinations of purines with acetates, salicylates, and glycinates are employed to increase their solubility and effectiveness. Therapeutically, they are extensively used as myocardial stimulants, diuretics, and coronary dilators. Various assay procedures for theophylline in pharmaceuticals have been reviewed (1). In NF, USP, and BP methods, theophylline is precipitated with a silver nitrate solution from an ammoniacal aqueous extract of the preparation; the excess silver nitrate is titrated with a standard solution of ammonium thiocyanate. The method is reported to give erratic results (2). The present investigation was undertaken for devising a simpler method for routine analysis.

Theophylline, being amphoteric, has been titrated both as an acid (3) and as a base (4) in nonaqueous media. Recently, caffeine (5, 6) and theobromine (7) have been estimated as bases in mixtures in water-free media.

In the present report, combinations of theophylline with sodium salts of organic acids are analyzed by differentiating nonaqueous titration. The titration solvent is a mixture of benzene–acetic anhydride–acetic acid (18:4:1), and the titrant is 0.1 *N* acetous perchloric acid. Titration is effected potentiometrically, using a glass-calomel electrode system. The method is applied to assay these combinations in tablet forms. The results by this method compare favorably with those obtained by official methods.

EXPERIMENTAL

Apparatus—All titrations were performed potentiometrically with a pH meter (Polymetron) employing a glass electrode and a calomel electrode with a salt bridge. A saturated solution of lithium chloride in glacial acetic acid formed the bridge.

Reagents and Solutions—Theophylline USP was recrystallized from water and dried at 105°. Sodium acetate BP and sodium salicylate BP were recrystallized from alcohol and dried at 105°. Theophylline sodium acetate NF and theophylline sodium glycinate NF were used without further purification. Theophylline sodium was prepared by mixing theophylline and alcoholic sodium hydroxide (1 *N*) in equimolecular proportions and evaporated to dryness on a water bath. Other chemicals and all solvents used in this study were reagent grade and were employed without further purification. A solvent mixture consisting of 18 parts benzene, 4 parts acetic anhydride, and 1 part acetic acid (benzene–acetic anhydride–acetic acid) was prepared and stored in a reagent bottle. A 0.1 *N* solution of perchloric acid in glacial acetic acid was prepared and allowed to stand 24 hr. prior to standardization against potassium acid phthalate dissolved in glacial acetic acid.

Differentiating Titration of Theophylline and Sodium Salt of an Organic Acid—Approximately 2.0 meq. of each theophylline and sodium salt of an organic acid, accurately weighed, was transferred to a 100-ml. beaker and dissolved by stirring magnetically in 50 ml. of solvent mixture. The solution was titrated potentiometrically with 0.1 *N* acetous perchloric acid. The end points were determined from the inflections of the titration curve obtained by plotting millivolts (mv.) *versus* volume (ml.) of titrant added.

Analysis of Theophylline Sodium Acetate NF—About 50 mg. of the sample, accurately weighed, was dissolved in 50 ml. of the solvent mixture in a 100-ml. beaker. The solution was titrated potentiometrically with 0.1 *N* perchloric acid.

Analysis of Glycine or Sodium Glycinate—About 2.0 meq. of the sample, accurately weighed, was dissolved in a mixture of acetic acid (2.5 ml.) and

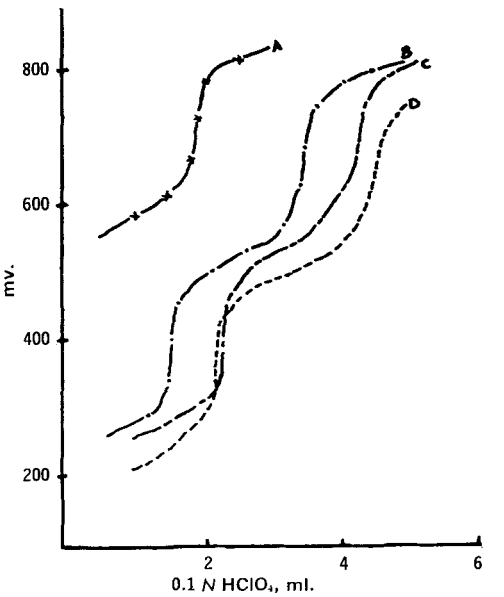

Fig. 1—Typical titration curves of theophylline mixtures against 0.1 N *acetous perchloric acid. Key:* A, *theophylline, 40.5 mg.;* B, *sodium salicylate, 26.7 mg. and theophylline, 41.2 mg.;* C, *sodium acetate, 21.0 mg. and theophylline, 41.2 mg.;* D, *theophylline sodium, 55.8 mg.*

Received December 1, 1967, from the Department of Pharmaceutical Chemistry, L.M. College of Pharmacy, Ahmedabad-9, India.

Accepted for publication March 18, 1968.

The authors are thankful to Dr. C. S. Shah for his interest in this work.

TABLE I—RECOVERIES OF THEOPHYLLINE AND SODIUM SALT OF ORGANIC ACIDS IN THEIR MIXTURES

Sample	Added, mg.		Found, mg.		Percent Recovery	
	Theophylline	Sodium Salt	Theophylline	Sodium Salt	Theophylline	Sodium Salt
Theophylline and Sodium Acetate (1:1)						
1	41.2	17.5	41.5	17.4	100.7	99.4
2	39.2	16.0	39.4	16.1	100.5	100.6
3	43.0	16.5	42.7	16.4	99.3	99.3
4	41.6	17.2	41.5	17.3	99.7	100.6
Mean					100.0	99.9
SD					0.66	0.73
Theophylline and Sodium Salicylate (1:1)						
1	41.5	30.0	41.7	30.3	100.4	101.0
2	38.0	33.3	37.8	33.1	99.5	99.4
3	40.9	31.5	41.0	31.4	100.2	99.6
4	41.2	32.5	41.0	32.7	99.5	100.6
Mean					99.9	100.1
SD					0.47	0.78
Theophylline and Sodium Glycinate (1:1)[a]						
1	41.7	20.2	41.5	20.1	99.5	99.5
2	41.5	19.5	41.7	19.3	100.4	98.9
3	40.4	18.7	40.4	18.7	100.0	100.0
4	38.7	19.0	38.6	19.1	99.7	100.5
Mean					99.9	99.7
SD					0.39	0.69

[a] Analyzed after treatment with salicylaldehyde.

acetic anhydride (10 ml.) in a 100-ml. beaker by warming on a water bath and allowed to stir for 5 min. Benzene (45 ml.) was added to the solution and titrated as described previously.

Analysis of Theophylline Sodium Glycinate NF—About 50 mg. of the sample, accurately weighed, was dissolved in a mixture of benzene (45 ml.) and acetic acid (2.5 ml.) by warming for 3 to 4 min. on a water bath. Salicylaldehyde (3 ml.) was mixed with it and stirred for 20 min. Acetic anhydride (10 ml.) was then added and the solution was stirred further for 5 min. The titration was carried out as described previously.

RESULTS AND DISCUSSION

The titration of purines in acetic acid against perchloric acid is reported to give unsatisfactory results. However, more distinct end points can be obtained by including acetic anhydride in the solvent mixture (5–8).

In preliminary studies, a series of solvent mixtures containing benzene, chloroform, acetic anhydride, and acetic acid in varying proportions were tested as titration media. Resolution of end points was most satisfactory in a mixture of benzene–acetic anhydride–acetic acid (18:4:1). Typical titration curves are illustrated in Fig. 1.

Curve A of Fig. 1 indicates only one potential break corresponding to theophylline content; whereas two distinct inflections are observed in the titration of theophylline sodium (Fig. 1, Curve D). The first end point corresponds to sodium content and the second to theophylline. Similar potential rises are noted in the titrations of equimolecular mixtures of theophylline-sodium salicylate and theophylline-sodium acetate (Fig. 1, Curves B and C). Both the components of each combination can be estimated with fair accuracy (Table I). The procedure was applied to determine the theophylline content of theophylline sodium acetate NF and its tablets. The difference between the first and second end points corresponds to the amount of theophylline. The results compare favorably with those obtained by NF method (Table II).

Figure 2, Curve E for theophylline sodium glycinate mixture indicates two inflections. The first corresponds to the sodium content and the second to the total basicity of theophylline and glycine. Since the theophylline–glycine mixture could not be differentiated in the solvent mixture used (Fig. 2, Curve B), the glycine was masked with salicylaldehyde. The resulting Schiff's base did not interfere with the titration of theophylline (Fig. 2, Curve D). Equimolecular mixtures of theophylline

TABLE II—ANALYSIS OF PHARMACEUTICAL PRODUCTS CONTAINING THEOPHYLLINE BY DIFFERENT METHODS

Preparations	Labeled Amount	Recovery of Theophylline by	
		NF Method	Proposed Method
Theophylline NF	—	99.8 ± 0.5[a]	100.0 ± 0.4[a]
Theophylline tablet	100 mg./tab.	102.2	101.5
Theophylline sodium acetate NF	—	63.0 ± 0.6[a]	63.1 ± 0.4[a]
Theophylline sodium acetate tablet	100 mg./tab.	61.0	62.2
	200 mg./tab.	120.9	119.7
Theophylline sodium glycinate NF	—	51.3 ± 0.5[a]	51.5 ± 0.6[a]
Theophylline sodium glycinate tablet	150 mg./tab.	76.4	77.5
	200 mg./tab.	102.0	100.6
	300 mg./tab.	150.5	151.6

Figures represent the percent of theophylline, and the standard deviation is calculated from at least five readings.

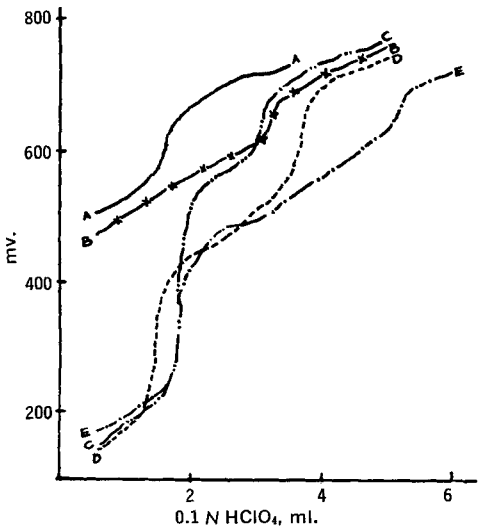

Fig. 2—Titration curves of glycine and its sodium salt individually as well as in combination with theophylline against 0.1 N acetous perchloric acid. Key: A, glycine, 15 mg.; B, glycine, 15 mg. and theophylline, 30 mg.; C, sodium glycinate, 17 mg.; D, sodium glycinate, 17 mg. and theophylline, 40 mg. (with salicylaldehyde treatment); E, sodium glycinate, 17 mg. and theophylline, 40 mg. (without salicylaldehyde treatment).

and sodium glycinate were analyzed by the modified method (Table I). The procedure was applied to determine the theophylline content of theophylline sodium glycinate NF and its tablets. The difference between the first and second end points corresponds to the amount of theophylline present. The results agree closely with those obtained by NF method (Table II).

The usual additives of the tablets except magnesium stearate do not seem to interfere with the determination of theophylline.

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Keyphrases

Theophylline combinations—nonaqueous titrations
Nonaqueous titration—acetous perchloric acid
Potentiometric analysis

Synthesis of Adamantyl Analogs of Analgesics

By A. NELSON VOLDENG, C. ALLEN BRADLEY, ROBERT D. KEE, EDDIE L. KING, and FRED L. MELDER

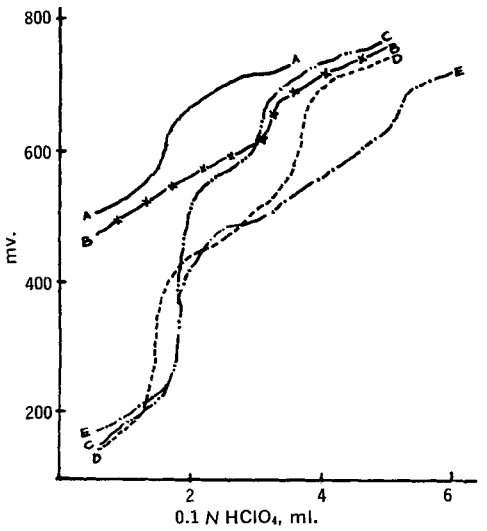
The synthesis of adamantyl esters related to heroin, meperidine, and aspirin is reported. Preliminary comparisons in mice indicate the adamantyl ester (V) is more potent and longer acting than meperidine hydrochloride.

THE INCORPORATION of the adamantyl moiety into drugs has been reported to increase the duration of action, and sometimes potency, of the parent compound. For example, 1-(1-adamantyl)-3-(*p*-tolylsulfonyl) urea, the adamantyl analog of tolbutamide, was found to be a very potent and long-acting oral hypoglycemic agent possessing activity two to fifteen times that of tolbutamide (1). The 17β-adamantoate ester of 19-nortestosterone is reported to be much longer acting with little androgenic activity as compared to other esters of the same steroid (2). This increase in duration of action was explained as being due to a decreased rate of enzymatic hydrolysis of the ester, thus making the 17β-hydroxyl group unavailable for biological oxidation to an inactive anabolic agent(2, 3).

In the penicillin series, resistance to penicillinase has been found by experiment to be associated with a side chain derived from a sterically hindered acid. Doyle and Nayler believe that restriction of rotation about the single bond linking the side chain to the amide carbonyl group constrains the molecule to adopt a conformation which is not readily accommodated at the active site of the enzyme (4). Substitution of the usual side chain with various adamantane carboxamide groups provides potent antibiotics that are penicillinase resistant and in some cases are active against Gram-negative bacteria (5–8).

Because of these profound pharmacological effects within different classes of drugs, the authors chose to prepare adamantyl analogs of known analgesics. The proposed compounds were expected to be much

Received January 24, 1968, from the School of Pharmacy, University of Arkansas Medical Center, Little Rock, AR 72205
Accepted for publication March 8, 1968.
This investigation was supported by grant NB 06759 from the National Institutes of Health, Bethesda, Md.



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Potentiometric analysis

Synthesis of Adamantyl Analogs of Analgesics

By A. NELSON VOLDENG, C. ALLEN BRADLEY, ROBERT D. KEE, EDDIE L. KING, and FRED L. MELDER

The synthesis of adamantyl esters related to heroin, meperidine, and aspirin is reported. Preliminary comparisons in mice indicate the adamantyl ester (V) is more potent and longer acting than meperidine hydrochloride.

THE INCORPORATION of the adamantyl moiety into drugs has been reported to increase the duration of action, and sometimes potency, of the parent compound. For example, 1-(1-adamantyl)-3-(*p*-tolylsulfonyl) urea, the adamantyl analog of tolbutamide, was found to be a very potent and long-acting oral hypoglycemic agent possessing activity two to fifteen times that of tolbutamide (1). The 17β-adamantoate ester of 19-nortestosterone is reported to be much longer acting with little androgenic activity as compared to other esters of the same steroid (2). This increase in duration of action was explained as being due to a decreased rate of enzymatic hydrolysis of the ester, thus making the 17β-hydroxyl group unavailable for biological oxidation to an inactive anabolic agent(2, 3).

In the penicillin series, resistance to penicillinase has been found by experiment to be associated with a side chain derived from a sterically hindered acid. Doyle and Nayler believe that restriction of rotation about the single bond linking the side chain to the amide carbonyl group constrains the molecule to adopt a conformation which is not readily accommodated at the active site of the enzyme (4). Substitution of the usual side chain with various adamantane carboxamide groups provides potent antibiotics that are penicillinase resistant and in some cases are active against Gram-negative bacteria (5–8).

Because of these profound pharmacological effects within different classes of drugs, the authors chose to prepare adamantyl analogs of known analgesics. The proposed compounds were expected to be much

Received January 24, 1968, from the School of Pharmacy, University of Arkansas Medical Center, Little Rock, AR 72205
Accepted for publication March 8, 1968.
This investigation was supported by grant NB 06759 from the National Institutes of Health, Bethesda, Md.

TABLE I—COMPARISON OF REACTION TIME (IN SEC.) OF MEPERIDINE AND ITS ADAMANTYL ANALOG (V) IN MICE[a]

Drug	Mean Reaction Time Recorded after Injection i.p., sec.[b] 0	30	60	90	120	150	180	210
Meperidine hydrochloride, 50 mg./Kg.	0.97 ± .11	1.21 ± .13	0.97 ± .11	0.87 ± .12	0.79 ± .18	0.83 ± .07	0.78 ± .05	0.78 ± .03
Adamantyl analog (V), 50 mg./Kg.	0.96 ± .14	1.2 ± .10	1.31 ± .16	1.17 ± .10	1.06 ± .10	1.00 ± .10	0.92 ± .06	0.85 ± .05
Adamantyl analog (V), 87 mg./Kg.[c]	0.91 ± .17	1.37 ± .22	1.18 ± .13	1.14 ± .13	1.10 ± .10	1.04 ± .07	0.94 ± .07	0.85 ± .05

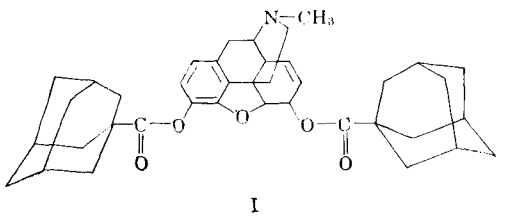
[a] Method: see *Reference 13*, 16 mice per dose, water bath temperature 56°. [b] Mean reaction time ±95% confidence limit. [c] Equimolar to 50 mg. meperidine hydrochloride.

longer acting since the ester function would not be hydrolyzed as rapidly *in vivo*. In addition, these analogs should still retain some activity since the moiety being replaced is not essential for the analgesic action.

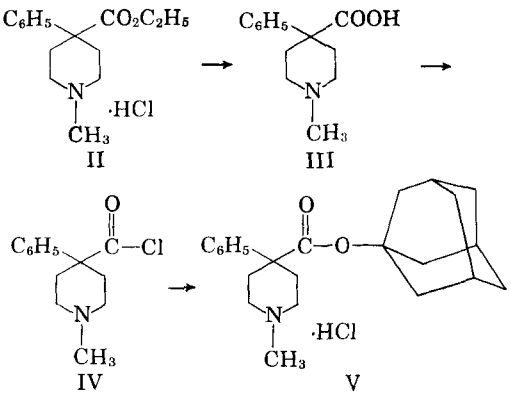
Certain esters related to dihydroheroin also possess analgesic activity (9). Both the 3,6-diglycyl and 3,6-di-(*o*-acetyl-L-tyrosyl) esters have the same toxicity as dihydromorphine but their analgesic actions are greater. Substitution of a propionyl or a carbomethoxy group for the 4-carboethoxy radical of meperidine does not materially alter activity (10). Other substitutions including larger ester functions such as phenyl or butyl at this point decrease the analgesic effect. Chlorthenoxazine, 2-(2-chloroethyl)-2,3-dihydro-1,3-benzoxazin-4-one, is an example of a cyclic derivative of aspirin (acetylsalicylic acid) and salicylamide. It is reported to be more potent and a longer-acting analgesic than either of the parent molecules (11).

The adamantyl analog of heroin, 3,6-diadamantoylmorphine (I), was prepared in 70% yield by reacting morphine with adamantoic anhydride (12). Attempts to simplify the procedure and improve the yield of I were not successful. Only starting materials could be obtained when morphine was heated with adamantoyl chloride (12) in the presence of pyridine, or when stirred with trifluoroacetic anhydride and adamantoic acid. The nitrate, sulfate, phosphate, and hydrochloride salts of the diester (I) were prepared but are not soluble in water. Since the compounds could not be pharmacologically evaluated by injection of a solution, the diester (I) was purified for analysis and testing as the free base.

Meperidine (II) was hydrolyzed to the acid (III) and esterified by reaction of the acid chloride (IV) with 1-adamantyl alcohol. Using the method of Ben-Bassat (13) comparison of the analgesic activity demonstrated the adamantyl analog to be more potent and longer acting than meperidine. The results are shown in Table I.

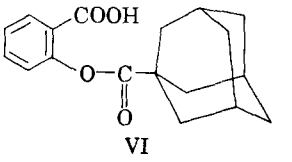


I



II III IV V

The adamantyl analog of aspirin, adamantoylsalicylic acid (VI), was prepared by reaction of salicylic acid with adamantoyl chloride (12) in the presence of pyridine.



VI

Attempts to prepare the adamantyl ester related to *d*-propoxyphene have been unsuccessful. The incorporation of the adamantyl moiety into appropriate positions of different classes of drugs and their pharmacological evaluation is presently being pursued in these laboratories.

EXPERIMENTAL[1]

3,6-Diadamantoylmorphine (I)—Anhydrous morphine was prepared by dissolving morphine sulfate in water and adding 10% sodium bicarbonate until precipitation was complete. The alkaloid was filtered, rinsed with water, and dried *in vacuo* for 24 hr. Anhydrous morphine (2.0 Gm., 0.007 mole) was added to a solution of 7.2 Gm., 0.020 mole, of adamantoic anhydride (12) in 15 ml. anhydrous toluene and the mixture heated for 24 hr. under reflux in an oil bath maintained at 130°. The solution was allowed to cool and the toluene removed *in*

[1] Melting points were determined on a Thomas-Hoover capillary melting point apparatus or a Fisher-Johns hot stage and are corrected. Infrared spectra were obtained using a Beckman IR10 spectrophotometer with KBr pellets. Elemental analyses were performed by Drs. G. Weiler and F. B. Strauss, Oxford, England. Adamantoic acid and 1-adamantanol were purchased from Aldrich Chemical Company.

vacuo. The oily residue was dissolved in anhydrous ether and a solution of hydrogen chloride in ether was added until precipitation was complete. The hydrochloride salt was filtered, added to a 10% sodium hydroxide solution (20 ml.), and extracted twice with 100-ml. portions of ether. The ether solutions were combined, washed with 20 ml. of water, dried over anhydrous magnesium sulfate, filtered, and the solvent removed *in vacuo*. A small amount of absolute methanol (5 ml.) was added to the oily residue, affording crystals of I (2.9 Gm., 70%) which were filtered, m.p. 239–241°. Recrystallization from ethyl acetate furnished an analytical sample, m.p. 240–241°. The infrared spectrum showed the characteristic ester C=O peaks at 1720 and 1750 cm.$^{-1}$ (14). The infrared spectrum of heroin, 3,6-diacetylmorphine (15) also showed two ester C=O peaks (1735 and 1755 cm.$^{-1}$).

Anal.—Calcd. for $C_{39}H_{47}NO_5$: C, 76.81; H, 7.76; N, 2.29. Found: C, 76.47; H, 7.65; N, 2.26.

1-Methyl-4-phenylisonipecotic Acid (III)—A solution of meperidine hydrochloride (II, 5.0 Gm., 0.018 mole) in 40 ml. of methanol was added to a solution of potassium hydroxide (5.0 Gm.) in 50 ml. of water and heated to reflux for 2 hr. The solution was allowed to cool and the methanol removed *in vacuo*. The alkaline solution was extracted with 30 ml. ether and adjusted to pH 6 with dilute hydrochloric acid. The white powder was filtered, rinsed with 50 ml. water, and dried for 24 hr. *in vacuo*, affording a quantitative yield of III, m.p. 310° dec. [lit. (16) m.p. 309–310°].

Adamantyl N-Methyl-4-phenylisonipecotate Hydrochloride (V)—A mixture of the acid III (2.7 Gm., 0.013 mole) and 5 ml. thionyl chloride was heated to reflux in an oil bath for 30 min., then 10 ml. of anhydrous benzene was added and heating continued for 30 min. After cooling, the acid chloride IV was filtered under suction and washed twice with 20 ml. anhydrous benzene as rapidly as possible. The white powder was quickly added to a solution of 1-adamantanol (3.0 Gm., 0.020 mole) in 20 ml. anhydrous benzene and the mixture was heated to reflux. A solution of anhydrous pyridine (1.2 ml., 0.015 mole) in 10 ml. of anhydrous benzene was slowly added and heating was continued for 4 hr. The flask was allowed to cool and the solvent removed *in vacuo*. To the white residue was added 80 ml. of water and the mixture was warmed to dissolve most of the material. After filtration under reduced pressure the insoluble material was stirred with 50 ml. of warm water. The mixture was filtered yielding 1.2 Gm. of unreacted 1-adamantanol. The aqueous solutions were combined, cooled in an ice bath, and 10% sodium carbonate was added until the extract was alkaline (pH 11). The mixture was placed in the refrigerator overnight and filtered affording 3.8 Gm. of the crude ester, V. Recrystallization from alcohol–water gave a white product which was dried *in vacuo* and dissolved in 30 ml. of anhydrous ether. A saturated solution of hydrogen chloride in anhydrous ether was slowly added with stirring and the white powder was filtered. Recrystallization from absolute alcohol gave an analytical sample of V (3.0 Gm., 60%), m.p. 267°. The infrared spectrum showed the characteristic ester C=O at 1725 cm.$^{-1}$ (14).

Anal.—Calcd. for $C_{23}H_{32}ClNO_2$: C, 70.84; H, 8.27; N, 3.59; Found: C, 70.74; H, 8.27; N, 4.31.

Adamantoyl Salicylic Acid (VI)—To a solution of 1.6 ml. of anhydrous pyridine and 2.7 Gm. of salicylic acid (0.020 mole) in 20 ml. of anhydrous benzene was added a solution of 3.9 Gm., 0.020 mole of adamantoyl chloride (12) in 20 ml. of anhydrous benzene. The mixture was heated to reflux for 3 hr. and allowed to stand at room temperature overnight. After filtration the solvent was removed *in vacuo*. The oily residue became crystalline upon standing at room temperature for 1 hr., affording 7 Gm. of crude VI, m.p. 145–147°. Recrystallization from alcohol–water gave an analytical sample of VI (4 Gm., 67%), m.p. 166–167°. The infrared spectrum showed the characteristic ester C=O at 1750 cm.$^{-1}$ and the broad acid C=O at 1690 cm.$^{-1}$ (14).

Anal.—Calcd. for $C_{18}H_{20}O_4$: C, 71.98; H, 6.71; Found: C, 71.91; H, 6.73.

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Keyphrases

Adamantyl analogs—synthesis
Analgesics—adamantyl analogs
IR spectrophotometry—structure
Meperidine adamantyl analog—analgesic activity

Polarographic Study of the Interaction Between the Antimonyl Ion and Pyrazolone Derivatives

By JOSEPH L. ROOSEMONT

The antimonyl ion was examined by polarography with the dropping Hg electrode. The half-wave potential was found to be a function of the pH of the buffer systems. In the presence of antipyrine and aminopyrine complex formation was noticed. The differential behavior of Pb(II) and As(III) under identical working conditions was studied.

A SCOPE OF polarographic research is the investigation of the behavior of electro-active substances in the appropriate supporting electrolytes. The objective may be in this connection either to determine the formation of selective complexes between the additive and the ion or to deduce the nature of these compounds.

In this paper the polarographic behavior of the antimonyl ion, compared to Pb(II) and As(III) is studied with regard to possible complex formation. This preliminary study deals particularly with the influence of the pH on complex formation. It is evident that one buffer possessing buffering capacity over a rather large pH range is certainly more suitable than a series of buffers of different composition and a limited pH range. A "ternary" buffer mixture is therefore proposed covering the slightly acid, the neutral, and the alkaline ranges. For the acid side an ascorbate buffer is utilized. Ascorbic acid has been proposed by Šušić (1) as a supporting electrolyte. This buffer has been included in the author's research in order to study the polarographic behavior of the ions in the presence of the pyrazolone derivatives in the acid range.

EXPERIMENTAL

Test Solutions—Solutions of 0.01 *M* potassium antimonyltartrate, lead acetate, and arsenic trioxide were prepared in bidistilled water. The arsenic trioxide solution was prepared by dissolving the required quantity in a minimal volume of 2 *M* NaOH, adjusting the solution to pH 7, and making up to volume.

Supporting Electrolytes—The used ternary buffer is 0.5 *M* in succinic acid, glycocoll, and tris-(hydroxymethyl)aminomethane. By mixing with appropriate volumes of 0.5 *M* NaOH a buffering action was observed between pH 4.4 and 12. Mold growth was avoided by addition of 50 mg./L. thymol. The corresponding supporting electrolyte was prepared by mixing an equal volume of buffer and 1 *M* KNO_3.

The ascorbate buffer was obtained by neutralizing a solution of ascorbic acid (0.25 *M*) with NaOH (0.25 *M*). Only the buffer range between pH 3.6 and 4.8 was studied. The buffer functioned as the supporting electrolyte.

The electrolytes were inert in those areas where the ions concerned were polarographically active. Unless indicated, no maximum suppressor was added.

Complexing Agents—The pyrazolone derivatives (antipyrine and aminopyrine) used for this purpose were also inert in the interesting voltage ranges.

Instrumentation—A polarograph of the Metrohm E 261 R type with Ag/AgCl reference electrode and automatic registration was used. All determinations were made under identical working conditions and with the same capillary (rate of flow = 2.75_0 mg./sec. for a Hg pressure = 50 cm. $T = 25° \pm 0.1°$). The $E_{1/2}$ values refer to the saturated calomel electrode.

Procedure—A fixed volume (0.2 ml.) of a stock solution was diluted with the supporting electrolyte at the required pH to a total volume of 20 ml. Oxygen was removed from the solution by passing through pure nitrogen during 10 min. and the polarograms were registered. The complexes with the pyrazolone derivatives were examined by making the solutions 0.01 *M* with respect to these compounds. When using the ascorbate buffer appearance of disturbing maxima was avoided by adding 0.2 ml. gelatin solution (0.5 Gm.%).

RESULTS AND DISCUSSION

For the antimonyl ion the apparent $E_{1/2}$ values as a function of the pH are indicated in Fig. 1. From this figure it appears that the $E_{1/2}$ values become more negative as the medium becomes more alkaline. In the presence of antipyrine a complex formation was observed. The half-wave potentials were found to shift towards more negative values. In the

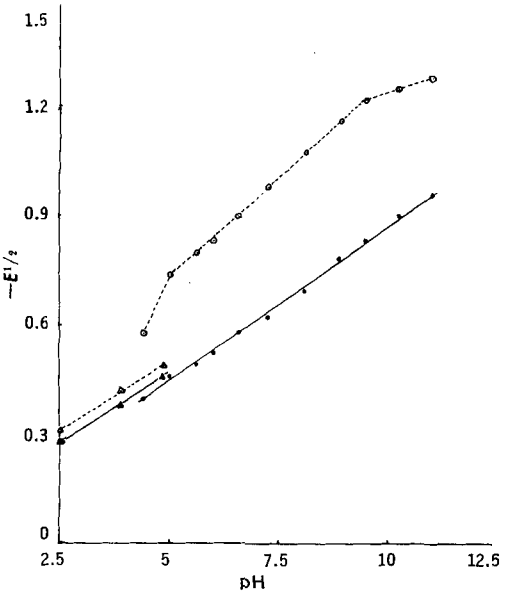

Fig. 1—Apparent half-wave potentials of the antimonyl ion as a function of the pH. Key: ●, in ternary buffer; ▲, in ascorbate buffer; ○, in ternary buffer with antipyrine; △, in ascorbate buffer with antipyrine.

Received January 5, 1968, from the Laboratory for Toxicology, University of Louvain, Belgium.
Accepted for publication March 13, 1968.

TABLE I—APPARENT $E_{1/2}$ VALUES OF Pb(II)

Buffer	pH	$-E_{1/2}$[a]
Ascorbate	2.5	0.39
	3.7	0.41
	4.2	0.42
	4.8	0.43
Ternary	4.5	0.47
	6.5	0.48
	8.0	0.49
	8.9	0.52
	11.0	0.58

[a] With reference to the saturated calomel electrode.

ascorbate buffer a nearly constant shift is observed and the slope of the straight lines is about 0.08_2 v./pH. In the ternary buffer a nearly identical slope (0.08_5 v./pH) was found for the antimonyl ion. In presence of antipyrine however three breaks were noticed, the slope of the middle part ($5 \leqslant$ pH $\leqslant 9.5$) being about 0.11_2 v./pH. Aminopyrine showed a similar effect on the polarographic behavior of the antimonyl ion.

With regard to Pb(II) the apparent $E_{1/2}$ values are indicated in Table I. Generally for lead a rather slight shift of the half-wave potentials is observed as a function of pH. The pyrazolones show a practically negligible shift of the $E_{1/2}$ values.

The As(III) behaves in an entirely different way: the reduction waves only originate sufficiently with a pH $\leqslant 2.5$. Beyond this limit value they are badly shaped or even absent. Adding antipyrine causes distortion of the waves. The same effect occurs with aminopyrine.

Polarograms registered in the alkaline range show beside the accustomed cathodical waves polarographic currents emerging from potential zero. It concerns apparently anodic waves. These anodic waves have been shown to be generated for Sb and As in a strongly alkaline medium (0.1 to 1 *M* in KOH) by Kolthoff and Probst (2). These waves begin to appear at about pH 9. A shift of the $E_{1/2}$ values seems also to appear when the pH values increase.

CONCLUSION

Apparently the antimonyl ion distinguishes itself from Pb(II) and As(III) by the values of the half-wave potentials in the described buffers and furthermore also by the interaction with pyrazolone derivatives. Only the antimonyl ion shows a definite tendency to complex formation.

For the As(III) the occurrence of waves is limited to a determined pH range; the waves are distorted in the presence of the pyrazolones. The described data will be further developed with regard to possible analytical applications.

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Keyphrases

Pyrazolone derivatives
Antimonyl ion—pyrazolone derivative interaction
Buffer system—"ternary"
Polarographic study—complex formation

Stability of Prednisolone in an Organic Vehicle

By W. H. BOWLES

Prednisolone (0.7042 Gm.) was dissolved in 35 ml. of a vehicle (CMP) composed of 50 percent camphor, 25 percent *m*-cresyl acetate, and 25 percent *p*-chlorophenol, a mixture used in dentistry as a pulp-capping agent to reduce sensitivity in dental restorations. This preparation was assayed by the blue tetrazolium reaction and compared with an identically treated sample of USP reference standard prednisolone. The prednisolone–CMP mixture was then incubated at 60° with aliquots collected for assay at intervals of 24 hr., 5 days, 1 week, 2 weeks, and 6 weeks. At the end of 2 weeks of incubation, 99.3 percent of the prednisolone still remained; after 6 weeks' incubation, the prednisolone concentration had dropped to 72.4 percent of the preincubation concentration. Compared to literature values on the thermal stability of aqueous preparations of prednisolone, these results indicate that prednisolone in CMP is quite stable.

THE WIDESPREAD use of natural and synthetic steroids as anti-inflammatory agents in recent years has led to some concern as to the stability of these compounds in various preparations. Most of these preparations are suspensions of the steroid in aqueous or buffered aqueous media. Granulation procedures used in the manufacture of such preparations often involve exposure of the steroid to moisture and elevated temperatures (1).

Studies of prednisolone in aqueous suspensions of solid buffering agents, such as magnesium oxide, magnesium trisilicate, aluminum hydroxide, and calcium carbonate, have shown that agents which

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Accepted for publication February 9, 1968.
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This study was supported by a grant from Wriner Laboratories, Inc., Dallas, Texas.

increase the pH of the suspension, *e.g.*, magnesium oxide, cause rapid deterioration of the prednisolone when incubated at 37.5 (1). Guttman and Meister (2) showed that the rate of degradation of prednisolone in aqueous alkaline suspensions was dependent upon the concentration of alkali. In this study, the rate of anaerobic degradation was only slightly below the rate of aerobic degradation.

In a study of the effect of the concentration of buffers on the rate of prednisolone breakdown, Oesterling and Guttman (3) found that the rate of degradation increased as the concentration of buffer increased. It was also shown that addition of a sequestering agent (EDTA-Na_2) could greatly decrease the rate of degradation of prednisolone. In this same series of studies, when the temperature was increased from 35° to 70° the rate of degradation was increased approximately sevenfold at pH 8.

The present study is concerned with the stability of prednisolone in an organic medium (CMP), composed of 50% camphor, 25% *m*-cresyl acetate, and 25% *p*-chlorophenol. This combination, with prednisolone in 1% concentration, has been used as a pulp-capping agent to reduce thermal sensitivity in dental restorations since about 1960 (4, 5). Mosteller (5, 6) has expressed doubt as to the stability of the combination beyond a period of 90 days. However, apparently no studies have been done on the stability of prednisolone in organic vehicles and in view of the relative instability of prednisolone in aqueous and buffered suspensions, it was deemed worthwhile to examine the stability of prednisolone in the camphor–*m*-cresyl acetate–*p*-chlorophenol (CMP) mixture.

METHODS AND MATERIALS

Prednisolone (0.7042 Gm.) was dissolved in 35.0 ml. of the CMP vehicle, giving a concentration of 20.12 mg./ml. of prednisolone in CMP. For analysis, 0.10-ml. samples of this solution were dissolved in 200 ml. of 95% ethanol, giving a calculated prednisolone concentration of 10.05 mcg./ml. of alcoholic solution.

A blank was prepared by dissolving 0.10 ml. CMP in 200 ml. of 95% alcohol. A reference standard was prepared by dissolving 60.0 mg. of USP reference standard prednisolone in 3.0 ml. CMP; 0.1 ml. of this mixture was added to 200 ml. 95% ethanol, giving a prednisolone concentration of 10.0 mcg./ml. of alcoholic solution. Blank, samples, and reference standard solutions all contained identical amounts of the CMP vehicle.

Twenty-milliliter aliquots of these solutions were assayed by the tetrazolium blue reaction (7) as follows: into each 20-ml. aliquot was pipeted 2.0 ml. of 0.5% tetrazolium blue in ethanol and 2.0 ml. of 10% tetramethylammonium hydroxide in ethanol. The solutions were mixed and the color allowed to develop for 90 min. in the dark.

The absorbances of reference standard and samples were read against the blank at a wavelength of 525 mμ in a Beckman DB-G spectrophotometer, with attached Sargent recorder, model SRL. After initial triplicate assays of the prednisolone–CMP preparation, the solution was incubated at 60° in a stoppered bottle. Samples (0.10 ml.) in triplicate were removed for assay at intervals of 24 hr., 5 days, 7 days, and 14 days. One additional sample was assayed after 6 weeks of incubation at 60°.

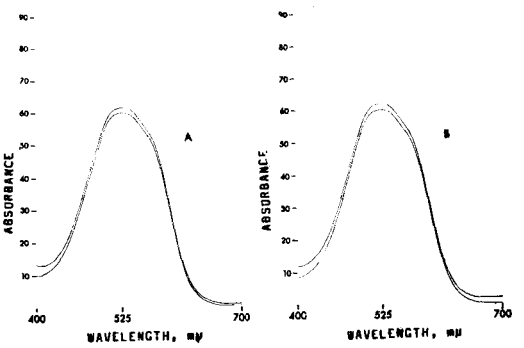

*Fig. 1—Stability of prednisolone after incubation at 60° in CMP (camphor—*m*-cresyl acetate—*p*-chlorophenol) compared to nonincubated USP reference standard prednisolone, as shown by tetrazolium blue assay. Key: upper curves, prednisolone; lower curves, nonincubated prednisolone; A, 24 hr.; B, 2 weeks.*

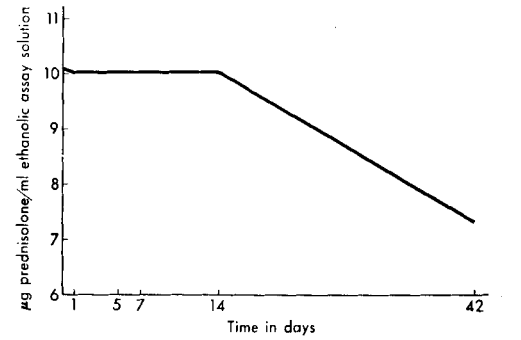

*Fig. 2—Stability of prednisolone in CMP (camphor—*m*-cresyl acetate—*p*-chlorophenol) incubated at 60°.*

RESULTS

Assay of the preincubation mixture gave a prednisolone concentration of 10.10 mcg./ml. of alcoholic solution. Assays after incubation at 60° for periods of 24 hr. to 2 weeks ranged from 10.03 to 10.05 mcg./ml. consistently (Fig. 1). After 6 weeks incubation of the prednisolone–CMP solution, the concentration of prednisolone was found to have dropped to 7.31 mcg./ml. of alcoholic assay solution (Fig. 2).

DISCUSSION

Prednisolone has been shown to be relatively unstable in aqueous suspensions at 35° (1), and even less stable when incubated at 35° in the presence of alkali (2) and trace metals (3). At pH 8, Oesterling and Guttman (3) obtained approximately a sevenfold increase in degradation rate when the temperature was increased from 35° to 70°. Thus the chief causes of prednisolone breakdown in pharmaceutical preparations have been shown to be alkalinity and trace metals, with degradation increasing as the temperature increases. In the organic vehicle, CMP, these catalysts of prednisolone degradation are absent and the preparation was found to be very stable at 60° for at least 2 weeks. Oesterling and Guttman (3) have shown prednisolone to be quite stable below pH 5. Thus good stability might be expected, since CMP is slightly acidic, due to the *p*-chlorophenol. It is suggested that the combina-

tion of 1% prednisolone in CMP, which is used in dentistry as a pulp-capping agent, would have a shelf-life of 2 to 5 years.

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Keyphrases

Prednisolone—stability in CMP
Degradation—prednisolone
Blue tetrazolium reaction—assay
IR spectrophotometry—analysis

Aster pilosus (*Compositae*) I. Isolation of Hyperoside (Quercetin-3-β-D-mono-galactoside) from the Leaves

By NORMAN R. FARNSWORTH, HILDEBERT WAGNER, LUDWIG HÖRHAMMER, and HANNS-PETER HÖRHAMMER

Aster pilosus **leaves were investigated and found to contain several flavonoids, as evidenced by thin-layer chromatography. Purification of a crude ethanol extract, followed by polyamide column chromatography, resulted in the isolation of one of the flavone constituents, which was found to be identical with hyperoside (quercetin-3-β-D-mono-galactoside).**

ALTHOUGH SEVERAL HUNDRED SPECIES of the genus *Aster* have been botanically described, relatively little is known of the phytochemical constituents of this group of plants. *Aster tataricus* roots have yielded the triterpenes friedelin, epifriedelanol, and shionone, in addition to astersaponin (hederagenin glucoside) (1–6) and the flavone quercetin (7). Quercetin, carotenoids, and galactose have also been detected in the pollen of this species (8). The latter three compounds have similarly been detected in the pollen grains of *Aster yomena* and *A. ageratoides* var. *ovatus* (8). *Aster tripolium* roots have yielded the polyacetylene compound 2-*trans*:8-*trans*-matricarianol (9) and *A. spinosus*, *cis*-lachnophyllum ester (10). Also, 2-*cis*: 8-*cis* matricaria ester has been isolated from the roots of *A. mongolicus*, *A. lautureanus*, and *A. novae angliae* (9), and angelic acid ester from *A. novae belgii* (11). Delphinidin diglycosides have been detected in the flowers of *A. amellus* var. *riverslea* and *A. sinensis* (12), cyanidin-3, 5-dimonoside in the leaves of *A. ericoides* (13), and callistephin (pelargonidin-3-glucoside), as well as chrysanthemin (cyanidin-3-glucoside), have been isolated from *A. chinensis* (14). A number of volatile oil constituents has also been identified in *Aster indicus* by means of gas chromatography (15).

Several *Aster* species have been used as folkloric remedies in the treatment of skin diseases (*A. novae angliae*) (16), rectal disorders, angina, eye inflammation, and stomach acidity (*A. amellus*, *A. tripolium*) (16), syphilis, bone caries, and coughs. (*A. bakerianus*) (17), intestinal parasites, and abdominal pain (*A. erigeroides*, *A. filifolius*) (17), and headache (*A. muricatus*) (17). In addition, various species have been recorded as being toxic to livestock, having strong emetic and purgative activity, producing depression, and having antiseptic properties (16, 17).

On the experimental side, *A. pilosus* var. *demotus* (18), *A. scaber* (19), *A. tataricus* (19), and *A. japonicus* (20) have been reported to elicit antitumor activity, whereas *A. divaricatus* extracts have shown strong inhibitory action against *Mycobacterium tuberculosis* (21). Defatted ethanol extracts of several native *Aster* species have been shown to elicit varying degrees of central system depression and/or autonomic activity in mice (22) and *A. divaricatus* is inhibitory for the pseudorabies virus in tissue culture tests (22).

No definitve phytochemical studies have been reported in the literature for *Aster pilosus* and biological activity reports on this species are limited (22–24).

On the basis of a lack of phytochemical information on *A. pilosus*, and because of interesting folkloric uses recorded for this genus of plants, a preliminary chemical study of the leaves was initiated.

EXPERIMENTAL

Plant Material—The plant material used in this investigation was the dried leaves of *Aster pilosus* Willd. (*Compositae*), collected during September 1966 at Pittsburgh, Pa. and authenticated by Dr. L. K. Henry, Carnegie Museum, Pittsburgh, Pa.

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Voucher specimens representative of the collection have been deposited in the herbaria of Carnegie Museum and the Department of Pharmacognosy, University of Pittsburgh. The plant material was air-dried and milled to a coarse powder prior to extraction.

Preparation of Crude Flavonoid Extract—The dried leaves (2.56 Kg.) were continuously extracted in a Lloyd extractor with skellysolve B (*n*-hexane) for 72 hr. and the resulting extract was concentrated to remove all traces of solvent by means of a flash evaporator *in vacuo* at 40°. The green, oily residue weighed 37.1 Gm. (Fraction *A*). Next, the skellysolve-B exhausted plant material was continuously extracted in the Lloyd extractor for 72 hr. with methanol, and the methanol extract was concentrated to dryness to yield 435 Gm. of a dark-green powder (Fraction *B*).

Thin-layer chromatography of Fraction *B* on Kieselgel G, and polyamide plates, as well as paper chromatographic examination, revealed the presence of several flavonoid components, as evidenced by color reactions of resolved components after treatment of the chromatograms with lead subacetate solution, and viewing under ultraviolet light (25). At least three major and two minor flavonoids could be detected in this manner.

A 210-Gm. batch of Fraction *B* was mixed with 3 L. of distilled water and heated on a steam bath for several hours until the extract was concentrated to about 1 L. The mixture was refrigerated overnight, during which time a considerable amount of nonflavonoid material precipitated from solution, which was removed by centrifugation. The clear supernatant was again concentrated on a steam bath to about 0.5 L., followed by refrigeration overnight. Very little precipitation occurred following this treatment, and the extract was again clarified by centrifugation to yield a clear brown solution. Thin-layer chromatography of this extract revealed the presence of chlorophyll, thus it was diluted with an equal volume of water and again concentrated to about 0.5 L. and refrigerated. This treatment resulted in an additional precipitation of nonflavonoid pigments, which were removed by centrifugation.

This clarified extract was next extracted six times in a separator with separate 200-ml. portions of ethyl acetate, the combined extracts being taken to dryness *in vacuo* to yield 14.37 Gm. of Fraction *B-1*. The aqueous phase was then extracted with six separate 200-ml. portions of ethyl acetate–methanol (95:5). Removal of solvent *in vacuo* yielded 3.01 Gm. of Fraction *B-2*.

Isolation of Hyperoside—A glass chromatographic column (5.5 × 55 cm.) was filled with a methanol slurry of fine particle size polyamide, prepared as previously described (26), to a packed height of 48 cm. in the column. Fraction *B-2* (3.1 Gm.) was dissolved in methanol, mixed with a small amount of polyamide, and allowed to air dry. The mixture was then placed on the top of the prepared column and methanol was continuously added, with 25-ml. fractions being collected and monitored by means of TLC on Kieselgel G plates, using methanol as the developing solvent. In this manner, a total of 95 fractions was collected and combined as follows on the basis of TLC data: 1–11, 12–21, 22–27, 28–39, 40–46, and 47–95. Combined fractions 47–95 were taken to dryness, and crystallized from methanol to yield 0.017 Gm. of pale yellow crystals. After drying the crystals *in vacuo* at 100° for 24 hr., they exhibited an m.p. of 226–229° dec. Thin-layer chromatography of the isolate on Kieselgel G plates, using a solvent system of ethyl acetate–methanol–water (100:16.5:13.5) revealed a single spot at R_f 0.38 after treatment of the chromatogram with lead subacetate solution, and viewing under ultraviolet light (366 mμ). Further, TLC of the isolate on polyamide, using methanol as the developing solvent, revealed a single spot at R_f 0.28 under the same conditions. An infrared absorption spectrum (KBr) was superimposable with those of reference samples of hyperoside (quercetin-3-β-D-mono-galactoside) derived from natural sources, and synthesized (Fig. 1). Thin-layer chromatography of the isolate in the two systems previously described, showed it to have an R_f value identical with the two samples of reference hyperoside.

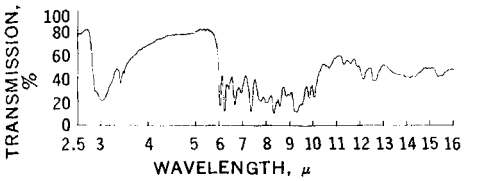

Fig. 1—Infrared absorption spectrum of hyperoside (KBr).

To further characterize the isolate, a small amount (2.0 mg.) in 5.0 ml. of methanol was hydrolyzed using 5.0 ml. of 10% hydrochloric acid under reflux for 2 hr. The hydrolysate was cooled and extracted several times with ether to remove the aglycone. Thin-layer chromatography of the concentrated ethereal extract on Kieselgel G plates, using a solvent system of benzene–pyridine–formic acid (36:9:5), showed the aglycone to have an R_f value of 0.25, and identical with that of reference quercetin, in addition to R_f values of 0.36 for the ethereal extract and reference quercetin after TLC on Kieselgel G, using a solvent system of toluene–ethyl formate–formic acid (5:4:1).

Identification of the sugar moiety was achieved by neutralizing the ether-extracted hydrolysate (*vide supra*) using silver oxide, concentrating the aqueous phase to about 0.5 ml., and spotting the extract on Schleicher and Schull No. 2043 b chromatography paper, followed by ascending development using solvent systems of isopropanol–pyridine–water–acetic acid (8:8:4:1), as well as *n*-butanol–pyridine–water (6:4:3), each for 18 hr. The aqueous hydrolysate and D-galactose both exhibited R_{GA} values of 1.0 following treatment of the dried chromatograms with benzidine spray reagent and heating at 105° for 15 min. These data are consistent with an identification of the isolate from *Aster pilosus* leaves as the flavone glycoside hyperoside (quercetin-3-β-D-mono-galactoside).

SUMMARY

An investigation of the leaves of *Aster pilosus* Willd. (*Compositae*) has resulted in the isolation of a flavone glycoside, which has been identified as hyperoside (quercetin-3-β-D-mono-galactoside). This flavone has been previously isolated from several higher plants (7), but this is the first report of its presence in any *Aster* species. The only flavonoid isolated from this genus previously is the flavone

aglycone quercetin, from *A. tataricus* roots and pollen (7, 8), and the pollen of *A. ageratoides* var. *ovatus* (8), as well as *A. yomena* (8).

The identity of additional flavonoids present in this plant will be reported at a later date.

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Keyphrases

Aster pilosus leaves
Quercetin-3-β-D-mono-galactoside—isolated
TLC—separation, identity
Column chromatography—separation
IR spectrophotometry—structure

Bacterial Contamination in Some Nonsterile Antibiotic Drugs

By MACK WHITE, FRANCES W. BOWMAN, and AMIEL KIRSHBAUM

Methods have been developed for the determination of microbial contamination in some nonsterile antibiotic preparations. Microorganism counts have been performed on both antibacterial and antifungal antibiotic powders. The highest counts were found in samples from the antifungal antibiotic.

In 1964, an extensive investigation into microbiological contamination of medical preparations was conducted in Sweden (1). A report of these findings was submitted to the Royal Swedish Medical Board, including a proposal for an upper limit of 100 bacteria per gram in nonsterile medicinals.

The *Antibiotic Regulations* of the Food and Drug Administration (FDA), the USP, and the NF require that antibiotics for injection be sterile, and they specify sterility tests for these preparations. None of these compendia, however, require sterility for oral or topical antibiotics, except for surgical powders and some ophthalmic and otic solutions. Tolerances are not specified in the official compendia for microbial contamination of nonsterile drugs except for one topical antibiotic preparation; the *Antibiotic Regulations* limits the contamination of this preparation to not more than 10 microorganisms per Gm. (2). No other oral or topical antibiotics undergo routine microbial examination as part of the antibiotic certification program.

Received January 10, 1968, from Division of Antibiotics and Insulin Certification, Food and Drug Administration, Washington, DC 20204
Accepted for publication February 9, 1968.

Recently, several batches of Agent F, an antifungal antibiotic, were found to be grossly contaminated with microorganisms, Although sterility is not required for the powder or dosage forms of this antifungal antibiotic, gross contamination would indicate a possible violation of the good manufacturing practices required by FDA (3). This possibility led to a study of the problem and to the development of suitable methods for the determination of microbial contamination in nonsterile antibiotic preparations.

EXPERIMENTAL

Materials—Prepare the following nutrient agar: peptone, 6.0 Gm.; yeast extract, 3.0 Gm.; beef extract, 1.5 Gm.; agar, 15.0 Gm.; and enough distilled water to make 1000.0 ml. The pH should be 6.5 to 6.6 after sterilization.

Prepare peptone water by dissolving 1 Gm. of peptic digest of animal tissue, USP or equivalent, in sufficient distilled water to make 1,000 ml. Dispense 99-ml. portions into flasks and sterilize in an autoclave at 121° for 20 min. The final pH should be 7.1 ± 0.1.

Method I—Powder—Place 1.0 Gm. of powder into 99 ml. of sterile peptone water. From this sus-

aglycone quercetin, from *A. tataricus* roots and pollen (7, 8), and the pollen of *A. ageratoides* var. *ovatus* (8), as well as *A. yomena* (8).

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Keyphrases

Aster pilosus leaves
Quercetin-3-β-D-mono-galactoside—isolated
TLC—separation, identity
Column chromatography—separation
IR spectrophotometry—structure

Bacterial Contamination in Some Nonsterile Antibiotic Drugs

By MACK WHITE, FRANCES W. BOWMAN, and AMIEL KIRSHBAUM

Methods have been developed for the determination of microbial contamination in some nonsterile antibiotic preparations. Microorganism counts have been performed on both antibacterial and antifungal antibiotic powders. The highest counts were found in samples from the antifungal antibiotic.

In 1964, an extensive investigation into microbiological contamination of medical preparations was conducted in Sweden (1). A report of these findings was submitted to the Royal Swedish Medical Board, including a proposal for an upper limit of 100 bacteria per gram in nonsterile medicinals.

The *Antibiotic Regulations* of the Food and Drug Administration (FDA), the USP, and the NF require that antibiotics for injection be sterile, and they specify sterility tests for these preparations. None of these compendia, however, require sterility for oral or topical antibiotics, except for surgical powders and some ophthalmic and otic solutions. Tolerances are not specified in the official compendia for microbial contamination of nonsterile drugs except for one topical antibiotic preparation; the *Antibiotic Regulations* limits the contamination of this preparation to not more than 10 microorganisms per Gm. (2). No other oral or topical antibiotics undergo routine microbial examination as part of the antibiotic certification program.

Received January 10, 1968, from Division of Antibiotics and Insulin Certification, Food and Drug Administration, Washington, DC 20204
Accepted for publication February 9, 1968.

Recently, several batches of Agent F, an antifungal antibiotic, were found to be grossly contaminated with microorganisms, Although sterility is not required for the powder or dosage forms of this antifungal antibiotic, gross contamination would indicate a possible violation of the good manufacturing practices required by FDA (3). This possibility led to a study of the problem and to the development of suitable methods for the determination of microbial contamination in nonsterile antibiotic preparations.

EXPERIMENTAL

Materials—Prepare the following nutrient agar: peptone, 6.0 Gm.; yeast extract, 3.0 Gm.; beef extract, 1.5 Gm.; agar, 15.0 Gm.; and enough distilled water to make 1000.0 ml. The pH should be 6.5 to 6.6 after sterilization.

Prepare peptone water by dissolving 1 Gm. of peptic digest of animal tissue, USP or equivalent, in sufficient distilled water to make 1,000 ml. Dispense 99-ml. portions into flasks and sterilize in an autoclave at 121° for 20 min. The final pH should be 7.1 ± 0.1.

Method I—Powder—Place 1.0 Gm. of powder into 99 ml. of sterile peptone water. From this sus-

pension, prepare a number of 10-fold serial dilutions by aseptically transferring 1 ml. to 9 ml. of sterile peptone water. From each dilution remove 1 ml. and add to separate sterile 20 × 100 mm. Petri dishes. Add 15 ml. of sterile liquid nutrient agar which has been cooled to 48–50°. Swirl the agar and allow to harden. Then add a 5-ml. overlay of nutrient agar and allow to harden. Invert the Petri dish and place in a 37° incubator for 48 hr. Remove and count colonies, using a Quebec colony counter.

Method II—Powder—Place a 0.45-μ solvent-resistant membrane filter, 47 mm. diameter, in a filter funnel fitted into a vacuum flask. Aseptically place 0.1 Gm. of powder into 50 ml. of sterile dimethylsulfoxide (DMSO) and allow to dissolve. Immediately filter the solution, using vacuum, through the membrane. Wash the membrane with 100 ml. of sterile peptone water, and allow to dry by continuing vacuum for 5 min. after filtration.

Place the membrane on the surface of 15 ml. of agar which has been previously allowed to harden in a Petri dish. Overlay with 5 ml. of agar and allow to harden. Incubate the plate in an inverted position for 48 hr. at 37°. Count the colonies that appear on the pad. (Ordinarily the agar overlay is not necessary, since the membrane pad is dry, and spreading growth will not occur. However, it is used here to prevent curling of the pad caused by the DMSO.)

Method III—Tablets—Use Method I, except use a sterile mortar and pestle to reduce the tablets to a powder for initial dilution. If 1 Gm. will not suspend properly, reduce initial concentration to 0.1 Gm.

Method IV—Ointment—Place 1 Gm. of ointment into 99 nl. of sterile *n*-hexane in a conical flask and shake until the ointment dissolves. Prepare a number of 10-fold serial dilutions by transferring 1-ml. aliquots to 9 ml. of sterile *n*-hexane. Filter each dilution and further proceed as described for filtration under II, omitting the 5-ml. agar overlay.

RESULTS AND DISCUSSION

Four different batches of contaminated powder were received for examination. Agar plate counts performed by conventional emulsion or surface swab methods gave unreliable results without any diminution in organisms per plate from lower to higher dilutions. The predominating contaminant was an aerobic, motile Gram-variable bacillus that resembled *Bacillus sphaericus* in its morphological and biochemical characteristics. According to Bergey's Manual (4), this organism usually grows in motile microcolonies of various shapes that move in large arcs from the point of inoculation and cover the plate in 1 day. The surface of poured agar plates should be dried by holding at room temperature for 2 or 3 days before use.

Therefore, unless extreme precautions are taken, the colonies do not remain as separate entities which can be related to a single parent; instead they will run together and counts will be erroneous. As an example, one count from another laboratory was reported to us as 2×10^{12} per Gm. Pelczar and Reid (5) report that it has been calculated that 1 Gm. of bacteria contains approximately 10^{12} bacteria. Therefore, a count of 2×10^{12} bacteria per Gm. of powder was unlikely even if the contaminating microorganisms were extremely minute. Such an inaccuracy results not from an arithmetic error, but from an illusion due to the spreading growth of the contaminant.

The use of the 5-ml. agar overlay described in Method I contains the individual colonies and prevents their spreading. Repeated tests showed good reproducibility with excellent arithmetic correlation of total counts obtained from different dilutions.

Because Agent F is relatively insoluble in water, the lowest dilution that was feasible was 10^{-2} as described in Method I. However, if the sample contains less than 100 organisms per Gm., the dilution in this technique precludes the recovery of microorganisms. Therefore, for samples contaminated with relatively few organisms, Method II should be used. In evaluating the results, the number of colonies are counted and multiplied by a factor of 10, since only 0.1 ml. is used, to obtain the number of organisms per Gm. Although the sample is dissolved in 50 ml. of DMSO, this dilution does not affect the final count because, during the filtration, the entire volume (50 ml.) passes through the membrane and the undiluted microorganisms are retained. Thus, if 8 colonies were observed on the membrane pad, the final count would be $8 \times 10 = 80$ organisms per Gm.

Using these techniques, counts for Agent F powder have ranged from 30 organisms per Gm to 4×10^8. The use of a grossly contaminated powder in the formulation of a dosage form such as a tablet or ointment resulted in a highly contaminated product. When the powder containing 4×10^8 microorganisms was used to make a tablet weighing 1 Gm. the tablet contained 9.4×10^7 microorganisms per Gm. The final dosage form was almost as heavily contaminated as the bulk although only a few mg. of the contaminated antibiotic was used in the formulation for each tablet. The excipient ingredients used in the tablet were also tested and less than 10 microorganisms per Gm. were found in each ingredient. Since the contamination of the tablet was greater than that originally introduced by the raw materials, the production procedures probably favored bacterial growth or additional contamination occurred during the manufacturing process. In any event, it was later shown that acceptable antibiotic preparations could not be made from heavily contaminated antibiotic powder.

After the manufacturing procedures were changed to yield a less contaminated powder, two batches of tablets were examined and found to be satisfactory. The antibiotic powder manufactured by the revised procedures contained 3×10^3 microorganisms per Gm. and the samples of the two batches of tablets made from this powder contained less than 50 microorganisms per Gm. An ointment was prepared from a powder having a count of 4×10^8 organisms per Gm. as an ingredient; the final product had a count of 4×10^5 per Gm. When a powder with a count of 1.2×10^2 was used in the formulation of an ointment, counts ranging from zero to a maximum of 100 organisms per Gm. were found in the ointment.

Because of the experiences with Agent F, other antibiotics were screened for bacterial counts. Among these were candicidin, kanamycin, bacitracin, neomycin, griseofulvin, nystatin, amphotericin, penicillin G, nafcillin, tyrothricin, and gramicidin.

TABLE I—VIABLE MICROORGANISMS PER GRAM FOUND IN NONSTERILE ANTIBIOTICS

Antibiotic Powders	Bacteria/Gm.	Molds/Gm.
Antifungal		
Antibiotic A	6,700	None detected
Antibiotic B	400	None detected
Antibiotic C	200	None detected
Antibiotic D	80	15
Antibiotic F (old process)	9,400,000	2
Antibiotic F (old process)	1,000,000	None detected
Antibiotic F (new process)	3,000	None detected
Antibiotic F (new process)	2,000	None detected
Antibacterial		
Nafcillin	None detected	None detected
Bacitracin	None detected	None detected
Neomycin	18	None detected
Zinc bacitracin	2	38
Penicillin	12	None detected
Tyrothricin	None detected	3

No other counts approached those of Agent F. As shown in Table I, the highest counts were found in samples of the antifungal antibiotics, indicating that they may present more of a problem than the antibacterial antibiotics.

The methods described here can be applied to bacterial counting of most antibiotics. However, the solvent systems should be modified according to the solubility characteristics of the antibiotic or antibiotic formulation under test. Any organic solvent used should, of course, be nontoxic to the contaminating microorganisms. Since the problem encountered with Agent F was gross bacterial contamination of a spore-forming organism, the tests were developed specifically for spore-forming bacteria. The techniques described may also be used for fungal contamination by using Sabouraud's agar in place of nutrient agar, incubating at 25° instead of 32°, and extending the incubation time from 2 to 5 days.

Antibiotic drugs, even those that are represented as being nonsterile, should be relatively free of microorganisms. Gross contamination is an indication of poor manufacturing practices. In addition, Curtis *et al.* (6) have stated that any organism, under certain conditions, can be considered pathogenic.

REFERENCES

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Keyphrases

Antibiotic preparations—bacterial contamination
Antifungal preparations—bacterial contamination
Agar overlay—test procedure

Synthesis of Imidazolthiones from 3,4-Diphenyl-4-oxazolin-2-thione

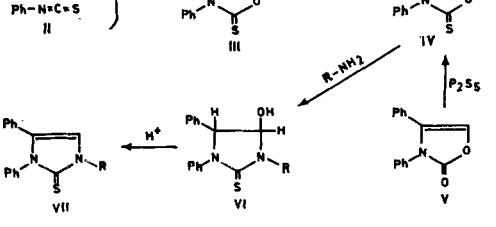
By S. CARBONI, E. GROTHE, and M. F. SAETTONE

The synthesis of some 4-hydroxyimidazolidin-2-thiones (VI) and 4-imidazolin-2-thiones (VII) by a method involving the reaction of 3,4-diphenyl-4-oxazolin-2-thione (IV) with primary amines is described.

A METHOD FOR THE preparation of imidazolones, based on the reaction of 5-unsubstituted 4-oxazolin-2-ones (such as V, Scheme I) with primary amines, was recently reported (1). In view of the interesting bacteriostatic (2, 3), fungistatic (3, 4), and CNS-depressant (5) activities reported for some 4-imidazolin- and imidazolidin-2-thiones, the authors have applied their method to the preparation of imidazolthiones from 4-oxazolin-2-thiones; some preliminary results are reported herein.

3,4-Diphenyl-4-oxazolin-2-thione (IV), prepared either from phenacyl alcohol (I) and phenyl isothiocyanate (II) through the intermediate 3,4-diphenyl-4-hydroxyoxazolidin-2-thione (III), or from V and P_2S_5, was chosen as a model compound for this study.

Treatment of IV with some primary amines gave good yields of 3,4-diphenyl-5-hydroxyimidazolidin-2-thiones (VI, see Table I). These could be easily dehydrated to afford the corresponding 4-imidazolin-2-thiones VII (Table II). In order to secure a structure proof for compounds of type VII, 1-



Scheme I

Received January 3, 1968, from the Institute of Pharmaceutical Chemistry, University of Pisa, 56100 Pisa, Italy.
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Treatment of IV with some primary amines gave good yields of 3,4-diphenyl-5-hydroxyimidazolidin-2-thiones (VI, see Table I). These could be easily dehydrated to afford the corresponding 4-imidazolin-2-thiones VII (Table II). In order to secure a structure proof for compounds of type VII, 1-

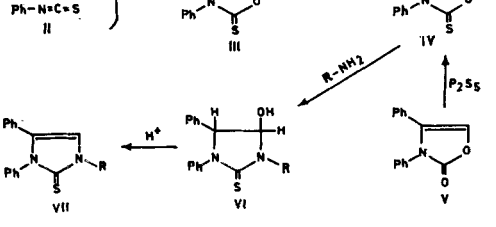

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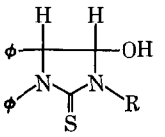
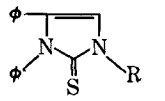
TABLE I—3,4-DIPHENYL-5-HYDROXYIMIDAZOLIDIN-2-THIONES



No.	R	M.p., °C.[a]	Yield, %	Formula	Anal., % Calcd. C	H	N	S	Found C	H	N	S
1	Benzyl	169–171	70	$C_{22}H_{20}N_2OS$	73.31	5.59	7.77	8.88	73.05	5.70	7.71	8.78
2	2-Diethylaminoethyl	119–121	68	$C_{21}H_{27}N_3OS$	68.27	7.37	11.37	8.66	68.15	7.30	11.44	8.87
3	2-Morpholinoethyl	183–185	87	$C_{21}H_{25}N_3O_2S$	65.78	6.57	10.96	8.34	65.57	6.73	11.02	8.45
4	2-Pyrrolidinoethyl	165–167	83	$C_{21}H_{25}N_3OS$	68.64	6.86	11.44	8.70	68.37	6.89	11.66	8.90

[a] Uncorrected.

TABLE II—3,4-DIPHENYL-4-IMIDAZOLIN-2-THIONES



No.	R	M.p., °C.[a]	Yield, %	Formula	Anal., % Calcd. C	H	N	S	Found C	H	N	S
5	Benzyl	177–178	92	$C_{22}H_{18}N_2S$	77.17	5.30	8.18	9.35	76.96	5.57	8.32	9.10
6	2-Diethylaminoethyl	108–109	95	$C_{21}H_{25}N_3S$	71.77	7.17	11.96	9.10	71.61	7.09	11.93	9.27
7	2-Diethylaminoethyl HCl	197–199	45	$C_{21}H_{26}ClN_3S$	65.01	6.75	10.83		64.98	6.98	10.96	
8	2-Morpholinoethyl	132–135	87	$C_{21}H_{23}N_3OS$	69.02	6.34	11.50	8.76	68.98	6.49	11.73	8.84
9	2-Morpholinoethyl HCl	206–209	64	$C_{21}H_{24}ClN_3OS$	62.76	6.01	10.45		62.93	6.24	10.38	
10	2-Pyrrolidinoethyl	129–131	94	$C_{21}H_{23}N_3S$	72.18	6.63	12.03	9.16	72.03	6.49	12.27	9.02
11	2-Pyrrolidinoethyl HCl	227–230	36	$C_{21}H_{24}ClN_3S$	65.35	6.26	10.89		65.62	6.57	11.00	

[a] Uncorrected.

benzyl-3,4-diphenyl-4-imidazolin-2-thione (Compound 5) was independently synthesized (Scheme II) from 2-benzylaminoacetophenone (VIII) and phenyl isothiocyanate (II), following a known synthetic method (6) for 4-oxazolin-2-thiones.

In the course of the work, compounds of type VII were observed to differ noticeably in reactivity from their oxygenated analogs, 4-imidazolin-2-ones. A chemical investigation on these compounds is now in progress and will be the object of a further communication.

All new compounds described herein were submitted to a primary biological screening[1] aimed at detecting CNS, autonomic, antifungal, antibacterial, and antiviral activity. Compound 2 exhibited weak antiviral activity *in vitro*, and compound 10 showed smooth muscle relaxant activity *in vitro* (minimal effective dose 10 mcg./ml.). Both levels of activity were considered too weak to consider further study.

EXPERIMENTAL[2]

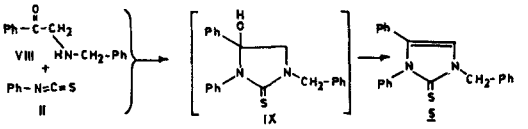
3,4-Diphenyl-4-oxazolin-2-thione (IV)—*From V*—A solution of 3,4-diphenyl-4-oxazolin-2-one (1) (V, 5.0 Gm., 0.021 mole) in xylene (150 ml.) was treated with P_2S_5 (10 Gm.) according to the procedure described by Gompper (7) for the preparation of 4,5-diphenyl-4-oxazolin-2-thione. The solution, after a 10-hr. reflux, afforded 2.85 Gm. (53%) of IV as colorless prisms, m.p. 159–161° after recrystallization from benzene, IR $\lambda_{max.}$ (nujol) 6.70, 6.91, 7.25, 7.42, 7.70, 8.81, 10.28, 10.61, 12.90, 13.31, 14.24 μ.

From I and II—A mixture of phenacyl alcohol (I, 13.6 Gm., 0.1 mole), phenyl isothiocyanate (II, 13.5 Gm., 0.1 mole), and pyridine (0.3 ml.) was heated at 120° for 4 hr., then allowed to cool. Trituration of the semisolid reaction mixture with benzene afforded 8.8 Gm. of an impure solid, which was fractionally crystallized from ethanol to afford 5.1 Gm. (18%) of a compound, m.p. 161–163° after recrystallization from benzene, IR $\lambda_{max.}$ (nujol) 3.17, 6.71, 6.94, 7.36, 7.68, 8.21, 9.41, 10.25, 13.02, 14.19 μ, which was identified as 3,4-diphenyl-4-hydroxyoxazolidin-2-thione[3] (III).

Anal.—Calcd. for $C_{15}H_{13}NO_2S$: C, 66.41; H, 4.83; N, 5.16. Found: C, 66.23; H, 4.63; N, 5.32.

Concentration of the mother liquor from III afforded unreacted phenacyl alcohol (2.0 Gm.), then a small amount (0.5 Gm.) of a compound with m.p. 173–175°, identified as 1,3,4-triphenyl-4-imidazolin-2-thione by comparison with an authentic sample[4].

A solution of III (8.13 Gm., 0.03 mole) in glacial acetic acid (25 ml.) was refluxed for 1 hr. After evaporation of the solvent under reduced pressure,



Scheme II

[1] Carried out by Bristol Laboratories, Syracuse, N. Y.

[2] Melting points were determined on a Kofler hot stage and are uncorrected.

[3] In no case phenacyl thiocarbanilate, open-chain tautomer of III, could be isolated. It should be pointed out that the oxygenated analog of III could never be obtained: phenacyl carbanilate, formed in the reaction between phenacyl alcohol and phenyl isocyanate, resisted all attempts at tautomerization to 3,4-diphenyl-4-hydroxyoxazolidin-2-one (*cf.* 1).

[4] Prepared according to McCombie and Scarborough (*cf.* 6). The compound originates probably in the reaction between *N,N'*-diphenylthiourea (formed as a secondary product in the reaction mixture) and phenacyl alcohol. Indeed, when equimolar amounts of these compounds were heated together at 160° for 4 hr., 1,3,4-triphenyl-4-imidazolin-2-thione was obtained in 67% yield.

the residue was crystallized from ethanol to afford 7.0 Gm. (92%) of IV, identical with the previously obtained material. The analytical sample was crystallized from ethanol and melted at 162–163°.

Anal.—Calcd. for $C_{15}H_{11}NOS$: C, 71.14; H, 4.37; N, 5.53. Found: C, 70.94; H, 4.24; N, 5.75.

Procedure for the Preparation of the 5-Hydroxyimidazolidin-2-thiones VI (Table I)—Solutions of IV (2.53 Gm., 0.01 mole) in the appropriate amine (benzylamine, 2-diethylaminoethylamine, 2-morpholinoethylamine or 2-pyrrolidinoethylamine, 15 Gm. *ca.*) were heated at 100° for 8–9 hr. The mixtures were then poured into ice cold water (1000 ml.); the product which separated was collected and crystallized from aqueous ethanol. A further crystallization from ethanol or from benzene–petroleum ether afforded the pure products in the yields reported in Table I.

Procedures for the Preparation of the 4-Imidazolin-2-thiones VII (Table II)—Compound 5 was prepared as follows: a solution of 1 (3.6 Gm., 0.01 mole) in ethanol (70 ml.) was treated with 1.0 ml. of concentrated H_2SO_4, then was heated to boiling. Crystalline 5 began to separate from the hot solution. After cooling, the product was collected and recrystallized from ethanol.

Compounds 6 and 8 were prepared by the same method, from 2 and 3, respectively. The acid ethanolic solution was treated with excess 10% Na_2CO_3 to precipitate the products; these were then crystallized from aqueous ethanol.

Compound 4 could best be dehydrated to 10 by the following procedure: a solution of 4 (1.1 Gm., 3.0 mmoles) in glacial acetic acid (2.0 ml.) was refluxed for 1 hr., then was poured into cold 10% Na_2CO_3 (50 ml.). The solid was collected and recrystallized from aqueous ethanol.

The hydrochlorides 7, 9, and 11 were prepared from the corresponding bases in the conventional fashion, and were purified by crystallization from absolute ethanol.

Structure Proof of 1-Benzyl-3,4-diphenyl-4-imidazolin-2-thione (Compound 5)—Treatment of 2-benzylaminoacetophenone hydrochloride (8) (VIII) with phenyl isothiocyanate, according to the procedure described by McCombie and Scarborough (*cf.* 6) for the preparation of 1,3,4-triphenyl-4-imidazolin-2-thione, gave a 15% yield of 5; IR $\lambda_{max.}$ (nujol) 6.70, 6.91, 7.15, 7.71, 7.95, 8.30, 9.35, 10.40, 13.40, 13.59, 14.24 μ.

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Keyphrases

Imidazolthiones—synthesis
3,4-Diphenyl-4-oxazolin-2-thione—derivatives
Pharmacological screening
IR spectrophotometry—structure

New Compounds: *N*-Hydroxy- and *N*-Acetoxy-Metabolites of *N,N'*-(2,7-Fluorenylene)bisacetamide

By NELLIE W. PITZER and FRANCIS E. RAY*

The partial catalytic reduction of 2,7-dinitrofluorene (I) in dimethylformamide, ethyl acetate, and acetic anhydride has yielded: *N*-hydroxy-*N*-(2-fluorenyl-7-nitro)acetamide (VII); *N*-acetoxy-*N*-(2-fluorenyl-7-nitro)acetamide (II); *N'*-hydroxy-*N,N'*-(2,7-fluorenylene)bisacetamide (XI); *N,N'*-dihydroxy-*N,N'*-(2,7-fluorenylene)bisacetamide (VIII); and *N,N'*-diacetoxy-*N,N'*-(2,7-fluorenylene)bisacetamide (IV).

THE ISOLATION and subsequent synthesis of *N*-hydroxy-*N*-(2-fluorenyl)acetamide as a proximate carcinogenic metabolite of *N*-(2-fluorenyl)-acetamide (1) led to the undertaking of the synthesis of a similar *N*-hydroxy compound as a possible metabolite for *N,N'*-(2,7-fluorenylene)bisacetamide (VI) which is known to produce carcinoma of the glandular stomach in rats (2).

Received August 28, 1967, from Pharmaceutical Chemistry Research Laboratory, University of Florida, Gainesville, FL 32601
Accepted for publication November 11, 1967.
This work was supported by Public Health Research grants No. 5 RO1 CA 08186 and No. 2 RO1 CA 07737 from the National Cancer Institute.
The authors wish to thank Dr. Danuta Malejka for her assistance in preparing this manuscript.
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The procedure for the catalytic partial reduction and the acetylation of 2-nitrofluorene in ethyl acetate and the subsequent hydrolysis in ammonium hydroxide (4) applied to 2,7-dinitrofluorene (I) gave a small yield of *N*-hydroxy-*N*-(2-fluorenyl-7-nitro)acetamide (VII).

After hydrolysis the ethyl acetate solution contained a mixture of reduction products. This prompted the use of *N*-(2-fluorenyl-7-nitro)acetamide (III) for catalytic reduction, as the only *N*-hy-

the residue was crystallized from ethanol to afford 7.0 Gm. (92%) of IV, identical with the previously obtained material. The analytical sample was crystallized from ethanol and melted at 162–163°.

Anal.—Calcd. for $C_{15}H_{11}NOS$: C, 71.14; H, 4.37; N, 5.53. Found: C, 70.94; H, 4.24; N, 5.75.

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Pharmacological screening
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New Compounds: *N*-Hydroxy- and *N*-Acetoxy-Metabolites of *N,N'*-(2,7-Fluorenylene)bisacetamide

By NELLIE W. PITZER and FRANCIS E. RAY*

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THE ISOLATION and subsequent synthesis of *N*-hydroxy-*N*-(2-fluorenyl)acetamide as a proximate carcinogenic metabolite of *N*-(2-fluorenyl)-acetamide (1) led to the undertaking of the synthesis of a similar *N*-hydroxy compound as a possible metabolite for *N,N'*-(2,7-fluorenylene)bisacetamide (VI) which is known to produce carcinoma of the glandular stomach in rats (2).

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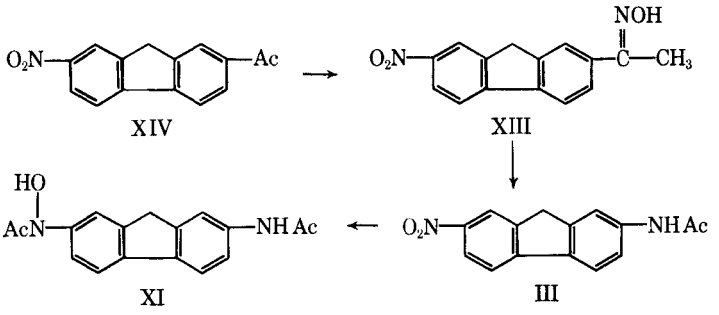
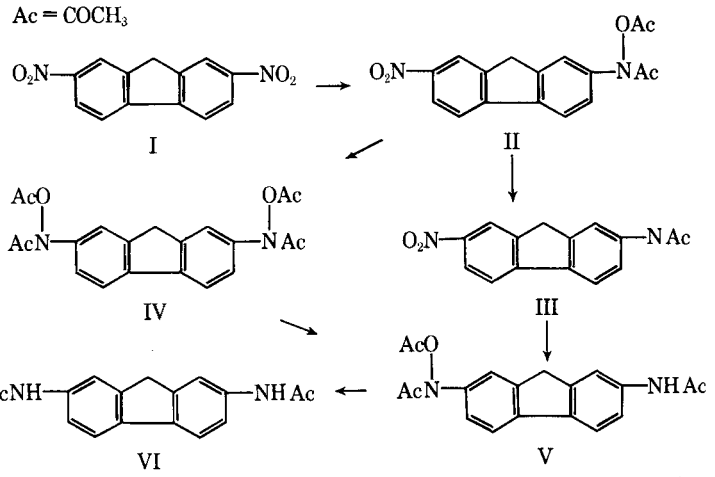


Scheme I



Scheme II (not isolated)

droxy derivative would then be *N'*-hydroxy-*N,N'*-(2,7-fluorenylene)bisacetamide (XI). The other probable product would be the known *N,N'*-(2,7-fluorenylene)bisacetamide (VI) (Scheme II).

A reduction of 2,7-dinitrofluorene in ethyl acetate and acetic anhydride yielded a compound, m.p. 248–249°, that was neither an *N*-hydroxy (negative to ferric chloride test) nor the known compounds, (III and VI). Microanalysis showed it to be *N,N'*-diacetoxy-*N,N'*-(2,7-fluorenylene)bisacetamide (IV) (Scheme II).

Since the one-step reduction from 2,7-dinitrofluorene (I) appeared to be unsatisfactory, due to the low solubility of I and the necessity for rapid reduction (25 min. or less), the two-step procedure with subsequent separation of products was devised.

EXPERIMENTAL

Ultraviolet spectra were obtained on a Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer in 95% ethanol. Microanalyses were performed by Schwartzkopf Microanalytical Laboratories.

N'-Hydroxy-N,N'-(2,7-fluorenylene)bisacetamide (XI) from N-(2-Fluorenyl-7-nitro)acetamide (III)—*2-Acetyl-7-nitrofluorene oxime (XIII)*[1]—To a well-stirred mixture of 2.5 Gm. (0.01 mole) 2-acetyl-7-nitrofluorene (XIV) (5) heated in 150 ml. of *n*-butanol was added 1.4 Gm. (0.02 mole) of hydroxylamine hydrochloride dissolved in 3 ml. of water. Sodium acetate (1.4 Gm.) was added and the suspension heated on a magnetic stir plate for 1 hr. A few milliliters of water added near the end of the heating was necessary for complete solution. The yellow oxime (XIII) precipitated upon cooling. Yield, 2.3 Gm., 88%, m.p. 229–230°. Several recrystallizations from glacial acetic acid raised the m.p. to 236°.

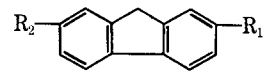
Anal.—Calcd. for $C_{15}H_{12}N_2O_3$: C, 67.15; H, 4.51; N, 10.44. Found: C, 66.77; H, 4.49; N, 10.38.

N-*(2-Fluorenyl-7-nitro)acetamide (III)*—The oxime (XIII) (5.4 Gm., 0.02 mole) was stirred into 200 Gm. of polyphosphoric acid and heated 3 hr. on the steam bath with occasional stirring. It was then poured into ice and water to decompose the acid, filtered, and washed well with water to give an orange solid (III). Yield, 5.6 Gm., 100%, m.p. 247–252°. Several recrystallizations from glacial acetic acid and from *n*-butanol yielded 4.5 Gm., m.p. 256–257° [Lit. (6) 250–253°]. Further proof of its identity was determined by preparation of two known derivatives: 2-amino-7-nitrofluorene [XII (7, 8)] and *N,N'*-(2,7-fluorenylene)bisacetamide [VI (9)].

N' - Hydroxy - N,N' - *(2,7 - fluorenylene)bisacetamide (XI)*—To a hot solution of 4 Gm. (0.015 mole) of *N*-(2-fluorenyl-7-nitro)acetamide (III) and 250 ml. of ethyl acetate in a pressure bottle was added as quickly as possible, 15 ml. of triethylamine, 20 ml. of acetic anhydride, and 0.15 Gm. of platinum oxide.

The hot mixture was placed on the Parr pressure reaction apparatus, and hydrogen was passed in

[1] This synthesis was begun by Ru-Jen L. Han in this laboratory.



VII, R_1 = N(OH)Ac, R_2 = NO_2
VIII, R_1 = R_2 = N(OH)Ac
IX, R_1 = NHAc, R_2 = NH_2
X, R_1 = R_2 = NH_2
XI, R_2 = N(OH)Ac, R_1 = NHAc
XII, R_1 = NH_2, R_2 = NO_2

(pressure dropped from 45 p.s.i. to 41.3 p.s.i.). The mixture was filtered, the filtrate was heated and stirred with 100 ml. of water and 50 ml. of ammonium hydroxide for 1 hr. The two layers were separated, the aqueous layer extracted twice with ethyl acetate. The combined ethyl acetate layers were washed with water, evaporated almost to dryness, ether added, and extracted with 0.5 *N* sodium hydroxide. The red alkaline solution was immediately acidified with concentrated hydrochloric acid. The precipitate was filtered, dissolved in boiling water, and the solution filtered hot through coarse filter paper and allowed to cool. Yield, 0.5 Gm., 11%, m.p. 177–179°. It gave a purple color with ferric chloride solution and formed a copper chelate, m.p. 235°. $\lambda_{max.}$, mμ 303 (ε 41,500), 311–317 sh (39,800).

Anal.—Calcd. for $C_{17}H_{16}N_2O_3$: C, 68.90; H, 5.44; N, 9.45. Found: C, 68.21; H, 5.64; N, 9.46.

N-Acetoxy and N-Hydroxy Derivatives of 2,7-Dinitrofluorene—N - *Acetoxy* - N - (2 - *fluorenyl* - 7-*nitro*)*acetamide* (*II*)—To a hot suspension of 3.8 Gm. of 2,7-dinitrofluorene (0.015 mole) and 100 ml. of dimethylformamide in a pressure bottle was added 20 ml. of acetic anhydride and 0.2 Gm. of platinum oxide. The hot mixture was placed on the Parr pressure apparatus and shaken with hydrogen (pressure dropped from 37 p.s.i. to 29 p.s.i. in less than 30 min.). The reaction mixture was filtered, the filtrate was stirred with 200 ml. of water for 1 hr., and filtered. Yield, 2.5 Gm., m.p. 160–166°. Combined runs (10 Gm.) of the crude material were extracted with benzene in a Soxhlet. Hexane, added carefully, precipitated out a red, gummy material. After most of this impurity was removed, a yellow, fluffy precipitate (II) formed spontaneously. Yield, 2.5 Gm., m.p. 174°. $\lambda_{max.}$, mμ 240–241 (ε 50,000), 336–341 (59,000).

Anal.—Calcd. for $C_{17}H_{14}N_2O_5$: C, 62.57; H, 4.37; N, 8.59. Found: C, 62.65; H, 4.34; N, 9.01.

N-*Hydroxy*-N-(*2-fluorenyl-7-nitro*)*acetamide* (*VII*)—Hydrolysis of II in ammonium hydroxide gave a yellow precipitate of the *N*-hydroxy, m.p. 204°. It gave a purple color with ferric chloride and formed a copper chelate, m.p. 235°. $\lambda_{max.}$, mμ 250–262 (ε 10,170), 355–361 (18,170).

Anal.—Calcd. for $C_{15}H_{12}N_2O_4$: C, 63.38; H, 4.26; N, 9.86. Found: C, 63.64; H, 4.60; N, 9.50.

N,N' - *Dihydroxy* - N,N' - (*2,7 - fluorenylene*)-*bisacetamide* (*VIII*) *and* N'-*Hydroxy*-N,N'-(*2,7-fluorenylene*)*bisacetamide* (*XI*)—To a hot suspension of 1.7 Gm. of *N*-hydroxy-*N*-(2-fluorenyl-7-nitro)-acetamide (VII) in 100 ml. of ethyl acetate in a pressure bottle was added 10 ml. of triethylamine, 15 ml. of acetic anhydride, 1 Gm. of 5% palladium on charcoal, and a few mg. of platinum oxide. The hot mixture was placed on the Parr pressure apparatus and shaken with hydrogen (pressure dropped from 37 p.s.i. to 35 p.s.i.). The reaction mixture was filtered and the filtrate stirred with 200 ml. of diluted ammonia for 1 hr. The products were then separated. A precipitate (VI) was filtered off. The aqueous and ethyl acetate layers of the filtrate were separated. The aqueous layer was extracted twice with ethyl acetate and discarded. The combined ethyl acetate solution was extracted: (*a*) with 5% sodium carbonate, which upon acidification with hydrochloric acid gave 0.39 Gm., m.p. 162–175°, of *N'*-hydroxy-*N,N'*-(2,7-fluorenylene)bisacetamide (XI). (*b*) With 0.5 *N* sodium hydroxide, which upon acidification yielded *N*-hydroxy-*N*-(2-fluorenyl-7-nitro)acetamide (VII). (*c*) With 5% sodium hydroxide, which upon acidification, yielded 0.18 Gm., m.p. 176–184°, of N,N'-dihydroxy - *N,N'* - (2,7 - fluorenylene)bisacetamide (VIII). A sample of VIII was prepared for analysis as follows: stirred with 0.5 *N* sodium hydroxide and filtered to remove any remaining VII and XI; dissolved in 5% sodium hydroxide, acidified, the precipitate filtered and dried; recrystallized from ethanol, m.p. 187–189°. It gave a purple color with ferric chloride and formed a copper chelate, m.p. 282°. $\lambda_{max.}$, mμ 305 (ε 34,700), 311–317 sh (30,400).

Anal.—Calcd. for $C_{17}H_{16}N_2O_4$: C, 65.37; H, 5.16; N, 8.97. Found: C, 65.23; H, 5.47; N, 8.56.

(*d*) With concentrated hydrochloric acid. The dark green solution was poured into water to give a precipitate of 2-acetylamino-7-aminofluorene (IX), m.p. 181–184° (6). $\lambda_{max.}$, mμ 301.5 (ε 45,000).

N,N' - *Diacetoxy* - N,N' - (*2,7 - fluorenylene*)*bisacetamide* (*IV*)—2,7-Dinitrofluorene (I) was reduced in ethyl acetate and acetic anhydride at 48 p.s.i. for 1 hr. and IV was isolated from the mixture of reaction products in trace amounts on a silica gel column eluted with ethyl acetate, m.p. 248–249°.

Anal.—Calcd. for $C_{21}H_{20}N_2O_6$: C, 63.63; H, 5.09; N, 7.07. Found: C, 62.89; H, 5.03; N, 6.57.

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Keyphrases

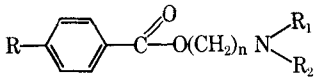
N,N'-(2,7-Fluorenylene)bisacetamide metabolites—synthesis
N-Hydroxy, *N*-acetoxy metabolites—*N,N'*-(2,7-fluorenylene)bisacetamide
UV spectrophotometry—structure

New Compounds: Certain Alkanolamine Esters Likely to Possess Anesthetic Activity

By L. R. ALPAD, M. KHALIFA, Y. M. ABOU-ZEID, and E. GABALI

The synthesis of a new series of alkanolamine esters is described. Esterification of 1,1′-iminodi-2-propanol with benzoyl chloride applying the Schotten-Baumann conditions afforded either the mono or diester according to the molecular ratio of the reactants but never the amide. With the more powerful acylating agent *p*-nitrobenzoyl chloride the diester-amide was always obtained even when equimolecular amounts of the reactants were used. Esterification of the *N*-methyl derivative of the amino alcohol with benzoyl chloride yielded in all cases the diester while esterification *via* the interaction of the dichloro analog of the amino alcohol and the appropriate silver salt invariably yielded the monoester even when excess of the silver salt was used.

THE VAST MAJORITY of the existing local anesthetics have been synthesized with the following fundamental structural requirements: an aromatic acid with or without a nuclear substituent in which the acid grouping has been esterified with an amino alcohol of simple or complex consititution.



Scheme I

In the present investigation, the mono- and diesters of 1,1′-iminodi-2-propanol (Table I) were prepared in the hope that the new products would show superior anesthetic activity[1] with less side effects.

The esterification of 1,1′-iminodi-2-propanol was successfully achieved *via* three different procedures: (*a*) the Schotten-Baumann conditions, (*b*) blocking the =NH prior to acylation to prevent amide formation, and (*c*) replacing the hydroxyl groups in the amino alcohol with halogen and reacting the halogeno derivative thus obtained with the appropriate silver salt.

Cope and Hancock (1), applying the Schotten-Baumann conditions to a number of alkyl amino alcohols, reported that the main product was the amide while Abou-Zeid and Gabali (2) disclosed that with diethanolamine only the diester was formed. With 1,1′-iminodi-2-propanol the authors have observed that the rate of the esterification depends solely on the molecular ratio of the reactants. When 1 mole of the amino alcohol interacted with 1 mole of benzoyl chloride, the monoester was produced, and if the amount of the acid chloride was doubled, the diester was obtained. The failure of amide formation with diethanolamine and 1,1′-iminodi-2-propanol may be attributed to the steric hindrance displayed by the bulky diethanol and di-isopropanol radicals flanking the =NH group. The fact that diethanolamine always affords the diester while 1,1′-iminodi-2-propanol affords either the mono- or diester according to conditions, may be easily accounted for by a consideration of the reactivity of the alcoholic groups in the two amino alcohols. It has long been known that in reactions in which the hydrogen atom of the alcoholic —OH is involved, a primary alcohol is far more reactive than a secondary (3). It follows, therefore, that the —OH in diethanolamine is more reactive than that in 1,1′-iminodi-2-propanol.

Using *p*-nitrobenzoyl chloride, which is a powerful acylating agent, resulted in attack of all available centers in the amino alcohol with the production of the diester amide, a result which is in accord with what has been reported earlier by other investigators (1, 2). As a consequence we resorted to Cope's method of blocking the =NH group by salt formation prior to acylation in order to prepare the diester amine which, however, was obtained but in poor yields. To improve the yield of the diester amine, its synthesis *via* the interaction of the dichloro analog of the amino alcohol and the appropriate silver salt was attempted. Application of this latter procedure, however, instead of affording the diester amine yielded a monoester with the second chlorine unreacted upon in spite of the use of excess of the silver salt. In the authors' opinion one of the contributing factors to the failure of the second chlorine atom to interact is steric hindrance.

EXPERIMENTAL

Schotten-Baumann Conditions—The dialkanolamine (the parent 1,1′-iminodi-2-propanol or its *N*-methyl derivative) (0.01 mole) mixed with 10% sodium hydroxide was treated with the acid chloride (0.01 or 0.02 mole) and cooling. In the case of benzoyl chloride, the insoluble viscous matter formed was washed and extracted with ether and the extract dried over anhydrous sodium sulfate. Then the ethereal extract was either concentrated until the separation of a crystalline solid mass (monoester) or completely evaporated and the oily material (diester) left converted to the corresponding picrate. The white solid forming with *p*-nitrobenzoyl chloride was washed with benzene and then crystallized again from benzene.

Cope's Conditions—1,1′-Iminodi-2-propanol (0.01 mole) dissolved in chloroform (25 ml.) was saturated with dry HCl gas till the separation of an oily top layer. Then finely powdered *p*-nitrobenzoyl chloride (0.01 mole) suspended in chloroform (25 ml.) was added and the whole was heated under reflux for 36 hr. The platelets separating were filtered, washed several times with ether, and crystallized from isopropyl alcohol.

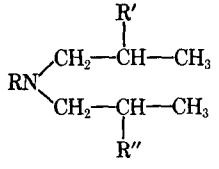
Received September 19, 1967, from the Faculty of Pharmacy, Cairo University, Cairo, U.A.R.

Accepted for publication November 29, 1967.

L. R. Alpad from the Philippines expresses her appreciation to the Ministry of El-Awkaf and the Supreme Council for Islamic Affairs of the United Arab Republic for the scholarship awarded to her.

[1] The products at present are under preliminary screening for possible anesthetic action or any useful pharmacological activity.

TABLE I—ESTERS OF 1,1′-IMINODI-2-PROPANOL AND OF THE *N*-METHYL DERIVATIVE



Name	R	R′	R″	Method of Prep.	Yield, %	Solvent of Crystallization[a]	M.p.°C.[c]	Formula	Anal., %[b] Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
β-Benzoyloxy-β′-hydroxy-di(*n*-propyl)amine picrate	H +	OH	$OCOC_6H_5$	1	90	A	186–187	$C_{19}H_{22}N_4O_{10}$[d]	48.92	4.72	12.01	49.14	4.89	12.11
β-Benzoyloxy-β′-hydroxy-di(*n*-propyl)amine	H	OH	$OCOC_6H_5$	1	31	B	87–88	$C_{13}H_{19}NO_3$	...	...	5.90	...	...	5.93
β,β′-Dibenzoyloxy-di-(*n*-propyl)amine picrate	H +	$OCOC_6H_5$	$OCOC_6H_5$	1	70	A	160–162	$C_{26}H_{26}N_4O_{11}$[d]	54.73	4.56	9.82	54.83	4.71	10.00
β,β′-Di-*p*-nitrobenzoyloxy-di(*n*-propyl)amine hydrochloride	H +	$OCOC_6H_4NO_2$-*p*	$OCOC_6H_4NO_2$-*p*	2	12	C	190–192 dec.	$C_{20}H_{22}ClN_3O_8$[e]	...	...	8.98	...	...	8.68
β-Chloro-β′-*p*-nitrobenzoyloxy-di(*n*-propyl)amine	H	Cl	$OCOC_6H_4NO_2$-*p*	3	60	B	158–159	$C_{13}H_{17}ClN_2O_4$	...	...	9.31	...	...	8.94
N-Methyl-β,β′-dibenzoyloxy-di(*n*-propyl)amine picrate	CH_3 +	$OCOC_6H_5$	$OCOC_6H_5$	1	21	A	142	$C_{27}H_{28}N_4O_{11}$	55.47	4.79	9.58	55.38	4.89	9.55
N-Methyl-β,β′-di-*p*-nitrobenzoyloxy-di(*n*-propyl) amine	CH_3	$OCOC_6H_4NO_2$-*p*	$OCOC_6H_4NO_2$-*p*	1	82	B	114–115	$C_{21}H_{23}N_3O_8$[d]	56.62	5.16	9.44	55.17	5.49	9.20

[a] A, ethanol; B, benzene; C, isopropyl alcohol. [b] Analyses performed by Alfred Bernhardt, Germany and El Nasr Laboratories, U.A.R. [c] Melting points were performed by the capillary tube method and are uncorrected. [d] Isolated as the picrate. [e] Isolated as the hydrochloride.

Halide–Silver Salt Method—*β,β'-Dichloro-di(n-propyl)amine Hydrochloride*—Thionyl chloride (12 ml.) was slowly added to 1,1'-iminodi-2-propanol (2 Gm.) with stirring and cooling until a homogeneous liquid was obtained. Then the excess thionyl chloride was distilled *in vacuo* and the residue was washed several times with chloroform and acetone. The salt that was obtained in 86% yield as white microcrystals melted at 197–198°.

Anal.—Calcd. for $C_6H_{14}Cl_3N$: C, 35.12; H, 6.83; Cl, 51.22; N, 6.83. Found: C, 34.77; H, 6.73; Cl, 51.52; N, 6.73.

The aqueous solution of the salt was rendered alkaline with cold saturated sodium hydrogen carbonate solution and the freed base rapidly extracted with ether. Then the extract was dried over anhydrous sodium sulfate for 24 hr. before the ether was evaporated *in vacuo*. The base thus obtained was immediately used in the condensation reaction.

Condensation with the Appropriate Silver Salt—The silver salt (3 Gm.) was intimately mixed with the halogeno base (0.9 Gm.) in a 100-ml. round-bottom flask. A condenser was fitted to the flask and the whole setup was dipped into a preheated oil bath at 130–140° for a few minutes. The mixture was then allowed to cool, then benzene (40 ml.) was added and the whole was refluxed for 48 hr. after which it was filtered while hot. Light feathery crystals cropped out on cooling and concentrating the benzene solution.

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Keyphrases

Alkanolamine esters
Monoester, 1,1'-iminodi-2-propanol—synthesis
Diester, 1,1-iminodi-2-propanol—synthesis
p-Nitrobenzoyl Cl—acylating agent

Communications

Enzyme Inhibitors XXII. Identity of Inhibitor Binding Site on Adenosine Deaminase

Sir:

Investigators concerned with active sites or receptor sites in macromolecules have interest in the number and types of binding sites that exist in the biological sample. In enzyme systems, whenever competitive inhibitors are studied, it is possible that the inhbitor is complexed at the active site of the enzyme. However, it is also possible that the inhibitor is complexed to an entirely different site. When two or more inhibitors of an enzyme have been prepared and evaluated as enzyme inhibitors, it is possible that they complex to the same site on the enzyme or to different sites. It occurred to us that it might be possible to obtain information concerning the identity or nonidentity of inhibitor binding sites by combining in one inhibitor the moieties which make a contribution to binding in two different inhibitors. For example, in a variety of 9-substituted adenines, it has been found that the adenine moiety of these inhibitors makes a contribution to binding to the enzyme, adenosine deaminase (1). Furthermore, it has been observed (2) that adenosine deaminase possesses a hydrophobic area which is important in the formation of a complex with the 9-substituent of some 9-*n*-alkyladenines (I). In addition, adenosine deaminase has a specific hydroxyl binding site (2, 3) which makes a contribution to complex formation of the hydroxyl group in a compound such as 9-(2-hydroxyethyl)adenine. Therefore, if the adenine moiety of the 9-*n*-alkyladenines and the 9-(2-hydroxyethyl)adenine (II) is complexed to the same site on the enzyme, it should be possible to prepare very potent inhibitors by combining in one molecule the alkyl chain and the 2-hydroxyethyl group at the 9 position of the adenine nucleus. Such a class of compounds would be the 9-(1-hydroxy-2-alkyl)adenines (III). These compounds were prepared by a modification of a general procedure (4) which involves the condensation of the appropriate amino alcohol with 5-amino-4,6-dichloropyrimidine. The resulting 5-amino-6-chloro-4-(1-hydroxy-2-alkylamino)pyrimidine was cyclized with triethyl

Halide–Silver Salt Method—*β,β'-Dichloro-di(n-propyl)amine Hydrochloride*—Thionyl chloride (12 ml.) was slowly added to 1,1'-iminodi-2-propanol (2 Gm.) with stirring and cooling until a homogeneous liquid was obtained. Then the excess thionyl chloride was distilled *in vacuo* and the residue was washed several times with chloroform and acetone. The salt that was obtained in 86% yield as white microcrystals melted at 197–198°.

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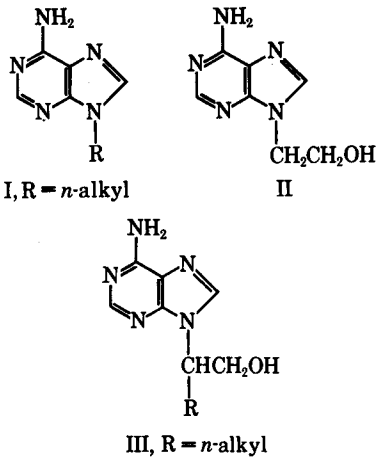
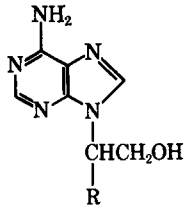


orthoformate to the 6-chloropurine intermediate, which on treatment with ammonia, gave the 9-(1-hydroxy-2-alkyl)adenines shown in Table I.[1]

When the series of compounds listed in Table I was evaluated as inhibitors of adenosine deaminase, it was found that as the alkyl group was lengthened, the compounds consistently became better inhibitors. Previously, on the basis of a study of the change in free energy of binding/methylene group with a series of 9-*n*-alkyladenines, it was found (2) that the $\Delta F/CH_2$ was greatest in those compounds bearing the 9-*n*-propyl through the 9-*n*-heptyl groups; *i.e.*, the main hydrophobic region of adenosine deaminase appears to extend from C_3 through C_7 in the conformation in which the 9-substituent is complexed to the enzyme. The average $\Delta F/CH_2$ group in proceeding from 9-*n*-propyl- through 9-*n*-heptyladenine was −350 cal. (2). An inspection of the $\Delta F/CH_2$ for the 9-(1-hydroxy-2-alkyl)adenines in Table I reveals that the main hydrophobic area also appears to terminate at approximately seven carbons in length[2] since the $\Delta F/CH_2$ for IX is −521 cal., whereas further increases in the carbon chain (X, XI, and XII) gave near minimum changes in free energy. However, note the unusually large change in free energy for the additional methylene unit when comparing V to VI. The magnitude of this change is clearly beyond simple hydrophobic transfer forces (5, 6) and might reflect a conformational change in the enzyme.

Finally, it should be noted that the 9-(1-hydroxy-2-alkyl)adenines are considerably more effective inhibitors than the corresponding parent

TABLE I—INHIBITION OF ADENOSINE DEAMINASE BY



Compd.[a]	R	$(I/S)_{0.5}$[b]	$\Delta F/CH_2$ (cal.)
II	H	1.1 ± 0.05[c]	—
IV	CH_3	1.2 ± 0.03	—
V	C_2H_5	0.49 ± 0.02	−521
VI	C_3H_7	0.071 ± 0.004	−1140
VII	C_4H_9	0.033 ± 0.006	−451
VIII	C_5H_{11}	0.015 ± 0.001	−467
IX	C_6H_{13}	0.0062 ± 0.0002	−521
X	C_7H_{15}	0.0047 ± 0.00003	−163
XI	C_8H_{17}	0.0039 ± 0.00002	−110
XII	C_9H_{19}	0.0030 ± 0.00006	−155

[a] None of these compounds served as substrates of adenosine deaminase. The enzyme (Type I, calf intestinal mucosa) was purchased from the Sigma Chemical Company. [b] The inhibition index $(I/S)_{0.5}$ is the ratio of the m*M* concentration of the inhibitor for 50% inhibition to the m*M* concentration of the substrate. The concentration of adenosine in all experiments was 0.066 m*M*. [c] Data taken from *Reference 2*.

inhibitors. For example, the $(I/S)_{0.5}$ for 9-*n*-heptyladenine is 0.32 (2) and for II is 1.1 (1), whereas the $(I/S)_{0.5}$ for IX is 0.0062. We interpret the enhanced inhibitory properties of the 9-(1-hydroxy-2-alkyl)adenines to be a result (*a*) of the identity of the adenine binding site utilized by I, II, and III and (*b*) of the close spatial relationship of the hydrophobic region and the hydroxyl binding region to the site to which adenine is bound on the enzyme.[3] Thus, the combination of a hydrophobic group and a properly positioned hydroxyl group at the 9 position of the adenine nucleus has resulted in the formation of compounds with greatly enhanced inhibitory properties.

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Received February 12, 1968.
Accepted for publication March 19, 1968.

[1] The details of the synthesis of these compounds as well as the intermediates will be published in the full paper. All compounds gave acceptable combustion analyses for carbon, hydrogen, and nitrogen.

[2] For the purpose of comparing the chain length of the 9-(1-hydroxy-2-alkyl)adenines to the 9-*n*-alkyladenines, we have ignored the hydroxymethyl group; *i.e.*, V has an alkyl chain length of three.

[3] The close spatial relationship of the hydrophobic region and the hydroxyl binding region may be native or may be the result of a conformational change in the enzyme induced by the formation of the E···I complex.

This investigation was supported by grant T-337B from the American Cancer Society, by a Public Health Service research grant 5-RO1-GM-09775-05, by a research career program award 5-K3-CA-18718-05 fom the National Cancer Institute, and a training grant 5-T1-GM-555-05 from the Division of Medical Sciences, Bethesda, Md.

Keyphrases

Enzyme inhibitors
9-(1-Hydroxy-2-alkyl)adenines—synthesis
Adenosine deaminase—inhibitor binding site identified

Apparent Nonsteady-State Dissolution Phenomenon in Solutions of Colloidal Solubilizers

Sir:

It has been demonstrated in recent reports (1–3) that the dissolution rate of solids in solutions of surfactant above the critical micelle concentration cannot be predicted by the Noyes-Whitney equation (4). Higuchi (1) has suggested that the dissolution process in solutions of colloidal solubilizers may be quantitated by the diffusion layer theory. In a previous report (3) we have found reasonable agreement of diffusion layer theory with initial dissolution rate data obtained under well-stirred conditions. We have recently observed, however, that the initial dissolution rates of salicylic acid in micellar solutions of a nonionic polyoxyethylene surfactant represent presteady-state values. Accordingly, the enhancement of apparent steady-state dissolution rate by the colloidal solubilizer is significantly greater than predicted by diffusion layer theory.

Details of the experimental procedure will be presented in a subsequent report. Briefly, in each dissolution experiment nondisintegrating disks of salicylic acid were used which provided a constant surface area over the entire experimental time period. Dissolution was followed at 37° at a 50 r.p.m. agitation rate (provided by an overhead blade) in either 0.1 *N* HCl or in various concentrations of polyoxyethylene (23) lauryl ether[1] dissolved in 0.1 *N* HCl. The amount of salicylic acid in solution at various intervals of time was determined spectrophotometrically at 304 mμ.

A kinetic method has been devised to ascertain steady-state dissolution rates which introduces the concept of an *excess dissolution rate, i.e.,* $(DR_c - DR_w)$ where DR_c is the dissolution rate per unit area in the colloid system and DR_w is the dissolution rate per unit area in an aqueous system under equivalent hydrodynamic conditions. Both the Dankwerts theory (1, 5) and the diffusion layer theory (1) predict a first-order dependence of excess dissolution rate on $(C_M - C')$, the micellar phase concentration gradient.

According to Gibaldi and Feldman (6), dissolution in a simple aqueous system under constant-surface and nonsink conditions follows first-order kinetics and a plot of log $(C_S - C)$ *versus* time is linear (where C_S is the solubility of the drug in the dissolution medium and C is the concentration of drug in solution at time t). Since the excess dissolution rate is proportional to a concentration gradient term, it follows that a plot of log $(DR_c - DR_w)$ *versus* time should be linear.

A representative plot of the log of excess dissolution rate as a function of time is shown in the figure. As noted in the plot a significant time lag occurs before apparent linearity (attainment of steady state) is observed. A similar plot of log dissolution rate in 0.1 *N* HCl *versus* time shows

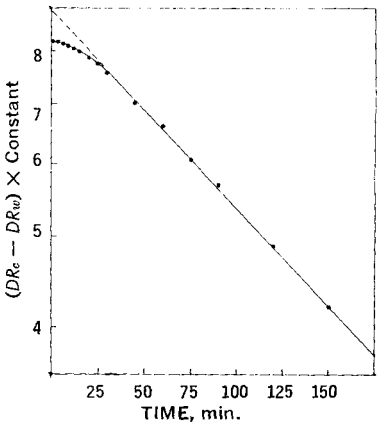

Fig. 1—Plot of the difference in dissolution rates of salicylic acid in 3% polyoxyethylene (23) lauryl ether solution, DR_c, *and 0.1 N HCl,* DR_w, *(on log scale)* versus *time under stirred conditions.*

[1] Brij 35 SP, Atlas Chemical, Wilmington, Del.

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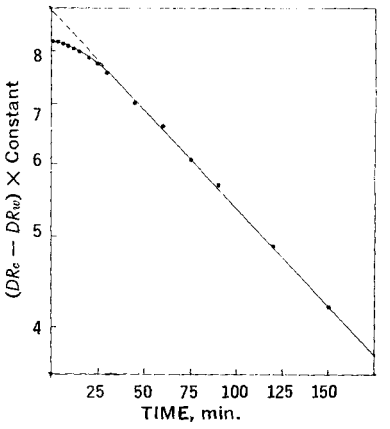

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excellent linearity with no evident time lag. Extrapolation of this plot to $t = 0$ yields the value of the solubility of the drug in 0.1 N HCl as predicted by theory.

The presteady-state phenomenon indicates that the apparent initial contribution of the micellar phase to the dissolution process is much less than its contribution at steady state. This disparity is evident when the ratios of dissolution rates in 3% polyoxyethylene (23) lauryl ether solution and in 0.1 N HCl are compared. The initial dissolution data suggest a 50% increase in dissolution rate while a comparison of steady-state data indicates a 90% increase in the apparent first-order rate constant for dissolution.

The present findings suggest the possibility of a more general phenomenon. Initial dissolution rate data (obtained from apparent zero-order rate plots) in systems of colloidal solubilizers may represent presteady-state conditions and must, therefore, be used cautiously in attempting to explain dissolution phenomena.

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Received February 12, 1968.
Accepted for publication April 1, 1968.
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Keyphrases

Colloidal solubilizer solutions—dissolution
Dissolution—apparent nonsteady state
Salicylic acid disks—dissolution rates

Observations on the Instability of Cyclamate in Hydroalcoholic Solution

Sir:

Cyclamic acid, widely used as such and as its sodium and calcium salts for sweetening pharmaceuticals and other products, is generally conceded to be extremely stable in solution (1, 2). Hydrolysis of cyclamic acid has been reported to occur only under very rigorous conditions (3–5). Studies in our laboratories have affirmed that aqueous solutions of cyclamate degrade to sulfuric acid and cyclohexylamine at very slow rates, which are proportional to hydrogen ion concentration. The half-life for a solution at 25° buffered at pH 2.0 is calculated to be 60 years!

Stability studies on experimental formulations led to the discovery that cyclamate sweeteners degrade at markedly faster rates in hydroalcoholic vehicles than would be predicted by their behavior in aqueous systems. In one instance, samples of an antibacterial amine formulation stored 24 months at room temperature were found to assay substantially higher than the initial value for total amine by nonaqueous titration. The formulation contained 0.1% amine in a hydroalcoholic vehicle sweetened with 0.6% sodium cyclamate and buffered at an apparent pH of 4.0. The increased titer was found to be due to cyclohexylamine formed from the cyclamate, and a material balance was obtained by gas-liquid chromatographic (GLC) assay of cyclohexylamine content. In a second case, formulations in 85% alcohol sweetened with calcium cyclamate and sodium saccharin were found unchanged after several weeks of storage, while similar formulations sweetened with calcium cyclamate and saccharin *acid* were found to have increased in apparent pH and deposited a precipitate, identified as calcium sulfate after isolation and the usual tests for the ions. Again, degradation of cyclamate was confirmed by GLC assay of the cyclohexylamine produced. The apparent pH of the stable formulations was about 6.5, while that of the labile ones was 3.7.

Unbuffered 1% (w/v) solutions of cyclamic acid[1] were made up in distilled water, absolute alcohol, and alcohol–water mixtures of graded composition. Portions of the solutions were heated in flint glass screw-capped bottles at 80°

[1] Hexamic acid, Abbott Laboratories, North Chicago, Ill.

excellent linearity with no evident time lag. Extrapolation of this plot to $t = 0$ yields the value of the solubility of the drug in 0.1 N HCl as predicted by theory.

The presteady-state phenomenon indicates that the apparent initial contribution of the micellar phase to the dissolution process is much less than its contribution at steady state. This disparity is evident when the ratios of dissolution rates in 3% polyoxyethylene (23) lauryl ether solution and in 0.1 N HCl are compared. The initial dissolution data suggest a 50% increase in dissolution rate while a comparison of steady-state data indicates a 90% increase in the apparent first-order rate constant for dissolution.

The present findings suggest the possibility of a more general phenomenon. Initial dissolution rate data (obtained from apparent zero-order rate plots) in systems of colloidal solubilizers may represent presteady-state conditions and must, therefore, be used cautiously in attempting to explain dissolution phenomena.

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Received February 12, 1968.
Accepted for publication April 1, 1968.
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Keyphrases

Colloidal solubilizer solutions—dissolution
Dissolution—apparent nonsteady state
Salicylic acid disks—dissolution rates

Observations on the Instability of Cyclamate in Hydroalcoholic Solution

Sir:

Cyclamic acid, widely used as such and as its sodium and calcium salts for sweetening pharmaceuticals and other products, is generally conceded to be extremely stable in solution (1, 2). Hydrolysis of cyclamic acid has been reported to occur only under very rigorous conditions (3–5). Studies in our laboratories have affirmed that aqueous solutions of cyclamate degrade to sulfuric acid and cyclohexylamine at very slow rates, which are proportional to hydrogen ion concentration. The half-life for a solution at 25° buffered at pH 2.0 is calculated to be 60 years!

Stability studies on experimental formulations led to the discovery that cyclamate sweeteners degrade at markedly faster rates in hydroalcoholic vehicles than would be predicted by their behavior in aqueous systems. In one instance, samples of an antibacterial amine formulation stored 24 months at room temperature were found to assay substantially higher than the initial value for total amine by nonaqueous titration. The formulation contained 0.1% amine in a hydroalcoholic vehicle sweetened with 0.6% sodium cyclamate and buffered at an apparent pH of 4.0. The increased titer was found to be due to cyclohexylamine formed from the cyclamate, and a material balance was obtained by gas-liquid chromatographic (GLC) assay of cyclohexylamine content. In a second case, formulations in 85% alcohol sweetened with calcium cyclamate and sodium saccharin were found unchanged after several weeks of storage, while similar formulations sweetened with calcium cyclamate and saccharin *acid* were found to have increased in apparent pH and deposited a precipitate, identified as calcium sulfate after isolation and the usual tests for the ions. Again, degradation of cyclamate was confirmed by GLC assay of the cyclohexylamine produced. The apparent pH of the stable formulations was about 6.5, while that of the labile ones was 3.7.

Unbuffered 1% (w/v) solutions of cyclamic acid[1] were made up in distilled water, absolute alcohol, and alcohol–water mixtures of graded composition. Portions of the solutions were heated in flint glass screw-capped bottles at 80°

[1] Hexamic acid, Abbott Laboratories, North Chicago, Ill.

TABLE I—RELATION OF ALCOHOL CONTENT, APPARENT pKa, AND EXTENT OF CYCLAMATE HYDROLYSIS

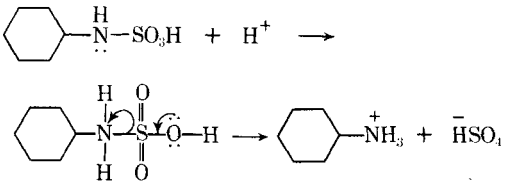
Alcohol			% Intact Cyclamate	
% (v/v)	mol. %	pKa	2 hr./80°	22 hr./80°
0	0	2.28	99.5	91.7
20	7.16	2.48	98.8	77.0
40	17.06	2.60	94.7	47.0
60	31.65	2.90	70.7	23.0
80	53.30	3.22	25.0	0
100	100.0	3.45	12.0	0

for periods of 2 and 22 hr., and the extent of degradation was determined by GLC assay of cyclohexylamine. Aliquots of the solutions were basified, and chloroform extracts were injected into a Perkin-Elmer model 801 gas chromatograph fitted with dual-flame ionization detectors. The GLC operating parameters were: (*a*) column—9.1 M. (6 ft.), 0.63 cm. (1/4 in.) o.d. glass helix packed with 5% potassium hydroxide, 10% polyethylene glycol 4000 on Anakrom ABS,[2] 80/90 mesh. (*b*) helium flow—40 ml./min. (*c*) temperature—column, 75°, injector block, 150°, detector, 150°.

Dimethylformamide was used as the internal standard. Under these conditions, cyclohexylamine has a retention time of 3 min. and the internal standard 7 min. Cyclohexylamine content was determined by the peak-height ratio method. Apparent pKa values were determined for each of the solutions at room temperature by potentiometric titration of a 50-ml. sample with 1 *N* sodium hydroxide added from a 1-ml. microburet.

The correlation of increasing alcohol content, and the concomitant increase in the apparent pKa of cyclamic acid, with the extent of hydrolysis is evident in Table I.

The degradation of unbuffered solutions of cyclamate would be expected to be autocatalytic, for the release of sulfuric acid increases hydrogen ion concentration. The results of these experiments are consistent with a mechanism involving attack by hydrogen ion on undissociated cyclamic acid, *viz.*;



More comprehensive studies on the degradation of cyclamate in low dielectric media are in progress and will be reported at a later date. This communication is intended to alert formulators to the necessity for avoiding an acidic pH range in cyclamate sweetened solutions of low dielectric constant. Such solutions should be buffered to at least 2 pH units above the apparent pKa of cyclamate in the vehicle.

[2] Analabs, Inc., Hamden, Conn.

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Received February 15, 1968.
Accepted for publication April 19, 1968.

Keyphrases

Cyclamate—instability
Instability—cyclamate–hydroalcoholic solution
pH effect—cyclamate instability

JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Council

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JANUARY TO DECEMBER, 1968

JOURNAL OF **Pharmaceutical Sciences**

July 1968 volume 57, number 7

Review Article

Pharmaceutical Sciences—1967

A Literature Review of Pharmaceutics

By LOUIS J. RAVIN and PETER D. BERNARDO

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Received from the Research and Development Division, Smith Kline & French Laboratories, Philadelphia, PA 19101
The authors thank Miss Mary E. Sivak for her assistance.

THIS REVIEW of the literature represents a comprehensive cross-section of the research and development efforts in various selected disciplines of the pharmaceutical sciences. It is the sixth survey in this series (1–5). Numerous periodicals and selected sections of *Chemical Abstracts* were abstracted. The *Pharmacognosy* section has been discontinued. In order to maintain continuity with the previous pharmaceutical sciences reviews of the *Journal of Pharmaceutical Sciences*, their general format was retained.

GENERAL PHARMACY

Solmssen discussed pharmaceutical research with respect to the synthesis and testing of new drugs (6). Another survey reviewed the federal, state, and foreign drug regulations regarding stability testing and formulation research (7). Comments on the empirical and the rational method for the development of new drugs were the subject of another review (8). The similarities and differences of the pharmacopeial

standards and specifications for bulk drugs and solid oral dosage forms were discussed (9). The location pattern of the pharmaceutical industry was outlined by Smith *et al.* (10). Another paper pointed out methods of stimulating creative output in small laboratory groups (11). The teaspoonful dosage was critically evaluated. The authors pointed out that there was a great deal of variation with this type of dosing (12). Schwartz elaborated on the serious problem of penicillin allergy (13).

Excellent review articles on gastrointestinal enzymes, over-the-counter (O-T-C) depilatories, O-T-C antiobesity drugs, O-T-C anthelminthics, O-T-C burn and sunburn remedies, O-T-C poison ivy and poison oak remedies, O-T-C external analgesics, O-T-C psoriasis and eczema remedies, O-T-C deodorants and antiperspirants, O-T-C internal analgesics, O-T-C hemorrhoidal preparations, and O-T-C preparations for disorders of the scalp appeared in the *Journal of the American Pharmaceutical Association* (14–27).

Preservatives—Bean discussed the use of preservatives to protect pharmaceuticals from contamination (28). Another study was concerned with the preservation of pharmaceutical and cosmetic preparations against microbial spoilage (29). Chemical preservation of ointments and creams in cosmetics and pharmaceuticals was the subject of another paper (30). In addition, the physical, chemical, microbiological, and toxicological properties of several new preservatives were reviewed and their application in dermatologic and cosmetic preparations was demonstrated (31). The interaction of nonionic surfactants with preservatives was shown to be responsible for partial inactivation of the preservative (32). Vitez utilized sodium hyposulfite to eliminate the bacteriostatic effect of various chemical preservatives during sterility testing (33). Several derivatives of β-naphthol were found to have antimicrobial properties in very low concentrations against both bacteria and fungi (34).

Flavor, Color, and Aroma—A concise history of the development regarding the use of color additives in foods, drugs, and cosmetics was presented (35). Another review was concerned with dyes commonly used in foods, pharmaceuticals, and cosmetics (36). Raff reviewed the concept of color as a psychological-physical phenomenon and gave examples illustrating this viewpoint (37). An account was also given of the fundamental mechanisms which occur when coloring matter absorbs light (38). Another paper considered the limitations of conventional color measurements from the point of view of applicability to routine storage testing (39). The suitability of some natural dyes for coloring sugar-coated tablets was investigated (40).

Bedoukian discussed the progress in the use of perfumery materials (41). Another review concerned itself with the updating of flavoring materials currently in use (42). The influences of the methods of perfuming and the requirements of the perfume for various types of preparations were considered (43).

Adjuvants—The effect of water, surfactants, and binders on the adhesion of solid particles was studied by a centrifugation method (44). Mendes evaluated a number of selected new materials as binders for tablets (45). The application of bentonite in the technology of tablet preparation was the subject of a paper (46). The role of various adjuvants in the mechanism of tablet disintegration was the subject of a review (47). Dextran was investigated as a binding agent (48). Kaplan studied the effect of varying the ratio of isopropanol–water granulating solution on various tablet parameters. He found that the ratio affected the hardness, thickness, and disintegration of tablets (49). The role of starch in the mechanism of tablet disintegration was elucidated (50). Another group studied holocellulose and technical cellulose as substances that support the disintegration of tablets (51). The change in flow properties of sodium bicarbonate in the presence of small amounts of magnesium stearate was observed (52). It was also pointed out that surface-active quaternary ammonium and pyridinium compounds are adsorbed on colloidal silicic acid to different degrees depending upon the concentration (53).

Polyvinyl alcohol was found to be a useful ingredient in vehicles for ophthalmic solutions (54). Parker investigated the effect of solution additives upon the stability of intravenous fluids (55). The concepts of ophthalmic compounding were also the subject of another paper (56). A review and discussion of the use of parenteral water-in-oil emulsions as adjuvants administered with allergens in the treatment of seasonal allergy appeared in the literature (57). The effect of various solvents on the stability of reserpine was described (58).

Stability—Tingstad discussed the various aspects that should be considered in the design of stability studies in product development (59). Another review article by Garrett elaborated on the stability of drug solutions (60). A

review of the chemical and physical properties of epinephrine as related to its use in glaucoma was published (61). Microdiffusional analysis was successfully applied to the stability of drugs in the solid state (62). Stability of reconstituted drugs and intravenous fluids received some attention in several articles (63, 64). Therapeutic incompatibilities caused by drug interactions were reviewed (65).

The chemical mechanism that explains the accelerated hydrolysis demonstrated by aspirin in combination with an alkaline stearate in a solid dosage form was elucidated by employing a suspension technique (66). Undissolved sulfadimidine removed benzoic acid from solution in sulfadimidine mixtures (67). The stability of sulfacetamide ophthalmic drops was discussed (68). The degradation of aspirin in commercial products containing acetaminophen was found to be accompanied by the formation of diacetyl-*p*-aminophenol or *p*-acetoxyacetanilid (69). In another article Pellerin discussed the value of analytical methods which indicate the degree of purity and the effect of the latter on the stability of pharmaceutical preparations (70). A colorimetric method was outlined for studying the stability of certified dyes (71). The comparative hydrolytic rates of *N*-substituted 6-aminothiouracils were obtained in acidic and alkaline media (72). Pearl-shaped granules were found to be the most stable form of vitamin A (73).

The relative stability of penicillin salts was similar at pH 1.5–4.5 (74). Another study gained some insight about the reactions possible in aqueous pharmaceutical systems of amines buffered with di- and polycarboxylic acids (75). The change in color of aqueous solutions of sodium sulfathiazole was related to decomposition (76). The autoxidation of sodium salicylate solutions was followed by polarographic methods (77). The stability of the amide group of glutamine derivatives in aqueous solution was determined (78). Another group studied the effect of pH, temperature, nature of the stabilizers, and conditions on the stability of pyridoxine (79). The effect of gamma radiation on the stability of several drugs packaged in glass and plastic containers was the subject of another investigation (80). The acid-catalyzed anaerobic degradation of isoniazid was elucidated (81). Acetylcysteine was susceptible to oxidation during nebulization (82). Rubber closures containing substances that are relatively inert should be used for insulin preparations (83). Racemization of drugs sometimes attributes to stability problems (84). Cod liver oil stability was examined using an accelerated aging test for evaluation (85). The stability of dextran during prolonged storage was investigated (86). The oxidation reactions involving white lotion, paraffin, and linoleic acid indicated that peroxides were the degradation products (87–89). Stability studies were also carried out with novobiocin, tartar emetic, alcoholic solutions of iodine, methoclopramide, belladonna alkaloids in antacid tablets, ethionamide, and sulfonamides (90–96). Barry and Shotton checked the influence of 1-hexadecanol on the acid-catalyzed hydrolysis of sodium dodecyl sulfate (97). Another group followed the reaction between boric acid and *p*-aminosalicylates by thermal and conductivity measurements (98). Benzoyl peroxide stability in pharmaceuticals was determined (99). An improved theoretical calculation of the stability ratio for colloidal systems was proposed (100). A simple method was also described for testing light absorption of various glass, plastic films, and other wrappings (101).

Stability Kinetics—Connors and Favilla described a method for kinetic studies of reactions in which the concentration of one reactant was held essentially constant by the addition of that reactant throughout the course of the reaction (102). The general concept of chemical kinetics in relation to the study of drug stability was reviewed (103). In another study on stability kinetics, Seydel demonstrated the application of reaction kinetics in predicting stability of drugs and derived rate equations related to drug degradation (104). Several papers appeared which discussed the stability of some cardenolide preparations (105–107). The hydrolysis of solubilized aspirin in solution buffered from pH 1.7 was studied at 37° (108). Another group presented data on the hydrolysis of aspirin in aqueous solutions of polysorbate 80 (109). In an excellent review article Garrett discussed the rate–pH profile in solvolytic degradations (110). An improved iodometric technique was used to investigate the kinetics of the reversible reaction between methionine and iodine (111). The ultraviolet light-catalyzed reaction of chlorpromazine under anaerobic conditions appeared to be different from the reaction under aerobic conditions (112). Several papers appeared on the stability of *N,N'*-di-[α(1-naphthyl)-propionyloxy-2-ethyl]piperazine (113, 114). A mechanism for the hydrolytic deamination of cytosine arabinoside in aqueous buffer was proposed (115). The degradation kinetics of 6-aminopenicillanic acid was the subject of another paper (116). Hurwitz established the mechanism for the

degradation reaction of *N*-chlorosuccinimide in aqueous solution (117). The mechanism of the base-catalyzed hydrolytic reactions of isoalloxazine was proposed (118) while Guttman and Platek were investigating the hydrolytic behavior of the same compounds. Kinetic data indicate both ionized and unionized species underwent specific base-catalyzed reactions but at different rates (119). Reactions involving cyclic acid anhydrides in aqueous solution were studied in an effort to understand biological mechanisms and in predicting pathways by which drug mixtures may undergo decomposition (120).

A method was described to determine the chemical stability of labeled fluocinolone acetonide incorporated into pharmaceutical preparations (121). The kinetics for the decomposition of chlorophenyl-benzylpiperazine derivatives was considered in another study (122). The hydrolysis of solubilized esters and amides was the subject of several papers (123, 124). The kinetics of solvolysis of various sydnones was investigated spectrophotometrically as a function of HCl and NaOH concentrations, pH, and temperature by Garrett and Mehta (125).

5-Methyl-3(2*H*)-furanone was formed from the acid-catalyzed solvolysis of 2-deoxy-D-ribose (126). The effect of certain additives on the hydrolysis of phenobarbital was shown (127). Methylparaben hydrolysis was also the subject of another paper (128). Fractional factorial experiments were used to evaluate related liquid formulations (129). A kinetic study of the reaction between dehydroacetic acid and primary amines in various solvents was investigated (130). The kinetics of degradation of chlorothiazide in acidic and alkaline solution was also reported (131, 132). The decomposition of morphine was followed as a function of temperature and pH (133).

Antibiotic Stability—Schwartz and Pflug described the mechanism of hydrolysis of penicillins catalyzed by catechol using benzylpenicillin and methicillin as model compounds (134). Potassium penicillin G was found to be incompatible with *p*-hydroxybenzoate. The authors isolated and identified the products of the reaction (135). Another group determined and eliminated the cause of the yellow discoloration in penicillin after sterilization (136). It was also shown that magnesium ions overcome the activity of novobiocin by forming a complex with the antibiotic (137). The stability of tetracycline hydrochloride in the presence of pyridine compounds, magnesium salts, and aliphatic and aromatic hydroxyacids was the subject of several papers (138, 139). Nystatin exhibited excellent stability on prolonged storage when kept under phosphorus pentoxide (140). Monciu *et al.* studied the interaction of chlortetracycline[1] with various components and related this effect to the duration of activity (141).

Vitamin Stability—The color stability of ascorbic acid tablets containing eight commonly used lubricants and glidants was studied by light reflectance (142). The stability of ascorbic acid tablets prepared by three different methods was evaluated (143). The discoloration of ascorbic acid tablets under prolonged storage was traced to ingredients commonly used in tablet formulations (144). Phytic acid derivatives have been used successfully to stabilize ascorbic acid (145). Sodium ascorbate solutions maintained satisfactory potency in the presence of sodium sulfite and carbon dioxide; however, on prolonged storage oxidation due to the presence of trace metals was observed (146). Another study dealt with the behavior of ascorbic acid solutions subjected to ultrasonic treatment (147). The rate of ascorbic acid decomposition under aerobic and anaerobic conditions was reported (148). Quantitative changes in L-ascorbic acid occurred in aqueous solution (149). The *in vitro* evaluation of the interaction of vitamins was discussed (150). Another group examined the photodegradation and stabilization of vitamin K_1 (151).

Ophthalmic solutions containing vitamins neutralized with bicarbonate were not irritating and were relatively stable (152). Multivitamin tablets containing vitamin A, D_2, and B_{12} coated with a gelatin–sugar coating showed better stability in artificial gastric juice (153). The factors affecting the stability of polyvitamin hydrosols were reviewed (154). Another group studied the stability of thiamine salts and calcium pantothenate in various vitamin B complex preparations (155). It was also pointed out that vitamin A preparations were more stable when vitamin A was converted to anhydrovitamin A (156).

PHARMACEUTICAL TECHNOLOGY

Knoechel *et al.* designed an instrumented rotary tablet machine and showed its applicability in pharmaceutical research and development and in production (157, 158). The spray pan method of coating tablets was described. It involved applying coating materials in solution or suspension as a spray from a centrifugal disk atomizer (159). Another group developed a small coating machine for enteric coating small

[1] Aureomycin, Lederle Laboratories, Pearl River, N. Y.

batches of tablets (160). An apparatus for studying the disintegration of tablet coatings was designed (161).

The pharmaceutical application of internal reflectance spectroscopy and UV spectroscopy was the subject of several papers (162, 163). An excellent review containing 60 references on spray drying and its application in pharmaceutical systems appeared in the literature (164). The use of differential scanning calorimetry and its application in pharmacy was commented on with reference to the detection of polymorphism and purity (165). The B.E.T. method for determining specific surface area was described in detail (166). Campiglio reviewed the apparatus and techniques for microfiltration and microcrystallization (167). Ultrasonic treatment of alumina gel did not alter its adsorption heat (168).

Parenterals—Several papers were published dealing with the danger and control of particulate matter in parenteral solutions. They pointed out methods of estimating the number of particles in injectable solutions and the danger involved when an excess of particles are present (169–171). Extemporaneous mixing of parenterals often presents problems (172, 173). They often show some type of incompatibility (174). A change in pH may be an important factor contributing to the problem (175).

The sorption, leaching, tensile, and other properties of a series of butadiene–acrylonitrile rubbers from the medium high acrylonitrile group were evaluated to determine the suitability of this type of closure for multiple-dose vials (176). Anschel discussed the requirements for suitable solvents and solubilizers for use in injectable preparations (177). The separation of microcrystals in injectables containing calcium gluconate was investigated (178). Parenteral solutions were found to develop precipitates after storage for 2 years indicating that hydrolysis can occur even in supposedly stabilized preparations (179).

Parenteral solutions of papaverine were stabilized by the addition of disodiumethylenediamine tetraacetate (180). Another investigation dealt with the stability of epinephrine[2] in parenteral form (181). Parenteral solutions of certain fat-soluble vitamins were also the subject of another paper (182). A comparison of the efficacy of several glass filters commonly used to filter parenterals appeared in the literature (183). The interaction of various types of rubber with medicament was reviewed (184).

[2] Adrenalin, Parke Davis & Co., Detroit, Mich.

Sterility—The dynamics of air sterilization was presented by Dorman (185). Another excellent review article was prepared describing sterilization with ethylene oxide in medicine and pharmacy (186). A new aseptic processing unit for sterile products was introduced (187). Filters for removing pyrogens and bacteria from pharmaceutical injection solutions were evaluated (188). Anderson studied the hydrolysis of sulfacetamide solutions at sterilization temperatures (189). The effect of preservatives on the heat sterilization of sesame oil was the subject of another paper (190). The causes and prevention of pH change in glucose solutions during sterilization were investigated (191).

Tablets and Capsules—The *in vitro* evaluation of physiological availability of compressed tablets was the subject of a review (192). The compression characteristics of some pharmaceutical materials were investigated (193). Hardness and disintegration time of tablets as a function of compression force were studied. The results indicated that there was no relationship between hardness and disintegration time in water (194). The effect of moisture on the behavior of sodium chloride under compression was investigated by measurement of compaction and ejection forces (195). Another investigation was concerned with the various changes of compression by pressure measurements (196). Abdel Khalek *et al.* elaborated on the effect of humidity on mechanical strength weights and disintegration rates of tablets (197). A quantitative evaluation of the dissolution rates of active ingredients from tablets was discussed. The authors checked filler binder, lubricant, disintegrating agent, and hardness on the dissolution rate of sodium salicylate from tablet bases (198).

A new technique was described for the observation of the stresses involved during a compaction operation (199). Hanus studied the thermodynamic and resistivity effects involved in the compression of solids (200). Graphic illustrations of the effects of air temperature, air velocity, and layer thickness on the drying process appeared in the literature (201). Meanwhile, the effect of particle size on the strength of sodium chloride tablets was measured. The results show that there was no simple relationship between crushing strength and particle size over the range of particle size studied (202). The disintegration of tablets containing triamterene was classified according to the type of dispersion obtained (203). High molecular weight film-forming compounds were used by Daragan for coating tablets (204). The determination of

certain parameters of free-flowing pharmaceutical products was carried out (205). It was proposed that the uniformity of weight test may sometimes replace individual chemical assay when the percent of active ingredient is high (206). Tablet coatings were the subject of another investigation (207).

Williams and Shields discussed the segregation of granules in a vibrated bed (208). Another group measured the drying rates of beds of magnesium carbonate powder wetted with various liquids (209). The shape of the core of a tablet was found to have some effect on its ease of sugar coating (210). A bowl granulation process was described for the granulation of lactose (211). Tablets were also prepared containing chemically incompatible materials (212). Various pill bases were evaluated to improve the technology of pill making (213). Dissolution studies were carried out for commercial brands of phenylbutazone and phenindione tablets (214, 215).

Suspensions—Various theories of dispersion were critically presented. Dispersions take place in two stages, *i.e.*, the wettability of the substance by the medium and the stability which is inversely related to the number of particles flocculating per unit time (216). A review containing 34 references classified disperse systems and described their general physical properties (217). A detailed study was made of the destabilization of dilute clay suspensions with a cationic polymer under controlled conditions of pH, ionic strength, initial clay concentration, and intensity and duration of agitation (218). The stability of white lotion was increased by using zinc oxide with a high sedimentation volume and by machine dispersal of the solids (219). In another paper the theoretical principles involved in the preparation of a physically stable suspension were discussed, and some examples of successful application of these principles in pharmaceuticals were given (221). Smith discussed the physical properties of suspensions (222). Crystal growth in aqueous suspensions was the subject of a detailed study (223). A reduced form of the particle-size distribution function, designated "self preserving," satisfied the Smoluchowski equation of coagulation by Brownian movement (224). The viscosities of dilute solutions of carboxymethylcellulose and methylcellulose influenced the sedimentation rate of barium sulfate particles (225, 226). The structural and filtration properties of kaolin and kaolin clay dispersions were shown to be affected by surfactants and electrolytes (227). A relationship describing the structural strength of disperse systems was derived (228).

Several articles discussing the preparation and stability of barium sulfate suspensions appeared in the literature (229, 230). A study of the translation and rotation of rigid spheres, rods, and disks, as well as the deformation of fluid drops in pulsatile and oscillatory flow was conducted (231). A graphical method was utilized to compute sedimentation coefficients (232). Zeta potential measurements can be obtained with an electrically driven pendulum with viscous damping (233). The properties and applications of several different gel systems were elaborated on in a number of papers (234–237). Lanolin derivatives were utilized to disperse various pigments in aqueous media (238). A polymeric preparation, K-4, stabilized suspensions of natural and monocationic forms of clays (239). Mueller and Schneider studied the wettability of granular material and the wetting action of surfactant solutions (240).

The Gibbs equation for the adsorption of surface-active monovalent and polyvalent ions in the presence and absence of neutral salt was extended (241). In another publication Samyn and Jung investigated the negative thixotropy in flocculated clay systems (242). Micelle formation, micelle size, and critical micelle concentration in the presence of dissolved macromolecules were thoroughly investigated (243–245). Another study was conducted to determine the formation and structure of sediments in pharmaceutical systems (246). The acceleration of sedimentation of fine solid suspensions was also examined (247). α-Dialkyl-β-aminopropionic acid was found most useful in the modification of flow properties of lipophilic vehicles (248). The technology of the production of microcrystalline suspensions was also investigated (249). The stability of dispersions of alumina and aluminum hydroxide was affected by various chain length alcohols and water (250). Light transmission measurements on suspensions were made by Hepplestone and Lewis (251).

Emulsions—A review containing 30 references dealt with emulsification and the selection of nonionic surfactants (252). Several papers also appeared which discussed the theory of emulsification, characteristics of the emulsifier, and finally the selection of the emulsifier (253, 254). The more important principles and some fundamental aspects of disperse systems were presented (255). Another review concentrated on the factors which determine the choice of emulsifying methods, the phase volume ratio,

and the emulsifiers (256). Two excellent papers appeared on emulsification. The authors stated that the HLB value and the rheological properties of the interfacial films were important factors affecting emulsion stability (257, 258). Jass stressed the importance of controlling the process variables to insure emulsion stability (259). Smoluchowski's equations were used to determine the kinetics of the rapid coagulation of emulsions (260). Negative interfacial tension in mixed films of soaps and long chain alcohols results from a large depression between the aqueous and oil phase with its adsorbed alcohol monolayers (261).

A thermodynamic study of emulsions showed that the mechanism of action of the emulsifier was twofold and involved surface phenomena and solution stability (262). Centrifugation was used to evaluate the stability of emulsions (263). The influence of pH on the stability of emulsions formulated with hydrophilic colloids was considered (264). Rimlinger elaborated on the influence of containers, chemical variation of emulsion constituents, and the power of the third solvent in preparing stable emulsion systems (265, 266). It was pointed out that oil-in-water emulsions can be obtained when the emulsifier–water ratio is such that the emulsifier is in micellar solution (267). A mechanism for the action of specific electrolytes on emulsion systems containing certain nonionic polyoxyethylene ethers as emulsifying agents was postulated (268). Electrolyte solutions did not affect the creaming of fat emulsions. The variation in creaming was attributed to the differences in composition or purity of the phosphatide emulsifiers and to the differences in properties of the respective oils (269). In another study Mizutani investigated the relation between phase inversion temperature, cloud point, and HLB values for emulsions prepared from nonionic emulsifiers (270). Pepsin was utilized as an emulsifier to prepare a stable system (271). The stability of an emulsion is increased by using a stabilizer with a high free energy of adsorption and as low a compressibility as possible in the monolayer (272). Particle-size changes were monitored in determining the stability of oil-in-water emulsions (273). A new method of studying the particle-size distribution of mechanically prepared hydrocarbon-in-water emulsions was presented (274). The effect of mixing of oils and of nonionic surfactants on the phase inversion temperatures of emulsions was considered (275). Another study involved spontaneous emulsification caused by the lowering of interfacial tension (276). The interfacial surface effects in a homogeneous multicomponent liquid mixture were measured (277). The possibility of the appearance of a synergism in the decrease of interphase tension was examined by mixtures of surfactants differing only in the structure of the polar groups (278). An effective method for measuring the emulsifying properties of surfactants was presented (279). Low-pressure capillary homogenization was used to prepare emulsions. The stability of the resultant emulsion was followed (280). The formation of microemulsions can be affected by different hydrocarbons (281). Cavitation mills produced more homogeneous creams than did colloid mills, however, after prolonged storage the creams produced by the cavitation mills showed poorer stability (282). Peroxides were found to affect the rate of emulsification of mercury in anhydrous lanolin (283). The charge on the disperse phase particles influenced the stability of some water-in-oil type emulsions (284). Admixing oils was shown to control the activity of phenols in oil-in-water dispersions during storage at different temperatures (285). The distribution of benzoic acid in some emulsified systems revealed that its fungistatic activity was dependent upon the amount of free acid in the aqueous phase (286). Conductivity measurements were used to follow the creaming rate of aggregate systems (287). The emulsifying properties of several surfactants were evaluated (288). A new polysaccharide gum was announced as a potential emulsifier for pharmaceutical systems (289). Gum arabic and tragacanth were determined to be the most satisfactory agents for maintaining the stability of paraffin oil emulsions (290). The method of preparation had an effect on the initial size distribution of oil-in-water emulsions (291).

Ointments and Creams—Schlossman discussed various hand creams and lotions and listed typical formulas (292). Several new water-washable bases and their properties were described (293). Oil-soluble lanolins were used in the preparation of typical creams (294, 295). Several papers were published describing typical ointment bases useful for various externally applied medicinals (296–299). Lukas described a new synthesis of ointments with fatty acid lead salts (300). Formulas for chloramphenicol ointment were also described (301).

The evaluation of the chemical stability of ointment bases was determined by the method of accelerated aging (302). Polymyxin B sulfate was found to be most stable in an ointment base containing methylcellulose and polyethylene glycol[3] (303). Antioxidants were effective in

[3] Carbowax, Union Carbide Corp., New York, N. Y.

preventing the breakdown of ointment bases hydrated with rose water (304). A synergistic behavior was observed when ointments were prepared by incorporating β-naphthol, iodochlorhydroxyquin[4] and undecylenic acid into water-soluble bases (305).

The rheological behavior of various ointments of the suspension type was investigated (306). Penetration measurements using a rotating viscometer slightly modified by the addition of penetrators permitted the determination of consistency of ointments (307). Whitworth and Becker studied the release of soluble sulfonamides from ointment bases through a membrane for a 6-hr. period (308). In a similar study the liberation of active substances from ointment bases was followed by dialysis (309).

Suppositories—The methods used in a physical study of suppositories were described in detail (310). Several papers were written on the compounding of colored suppositories (311, 312). Plaxco *et al.* studied the effect of some nonionic surfactants on the rate of release of drugs from suppositories. Their data indicate that the HLB value of the surfactants had an influence on the rate of release of the drug (313). Several substances were evaluated as possible viscosity increasing agents for the suppository base lasupol-G (314). The use of 1,3-butylene glycol as a glycerol substitute in suppositories was investigated (315). It was also pointed out that amidopyrine was unstable in the presence of certain agents in suppository bases (316). The formulation and evaluation of new rectal dosage forms including a study of microcrystalline cellulose–carboxymethylcellulose gels as suspension vehicles were reported by Wakling (317).

Aerosols—A discussion of the theory of foaming, foaming properties of nonionic surfactants, and of foam stabilizers and foam inhibitors was published (318). Nonionic aerosol foams were studied with respect to emulsion stability, bubble size, foam stability, stiffness, and drainage as various fatty alcohols or acids were added (319). Silicone antifoams are often used to decrease the tendency for foaming (320). Kuebler commented on the dosage accuracy in aerosol preparations. He stated that the filling of aerosol preparations entails some dosage variation which increases as the container size decreases, however, the variations are within limits (321). An outline of the available literature on aerosols was presented (322). The development and application of aerosols in pharmacy were reviewed in several articles (323, 324). The quality control requirements for aerosols were outlined (325). Several authors discussed the significance and determination of particle size for oral inhalation aerosols (326, 327). Sciarra wrote a review article concerning the particle size of topical aerosols (328).

Several additional papers were published which described the measurement of aerosol particles by light scattering and by acoustical measurements, respectively (329, 330). In another paper a series of electrolytes were dispersed from solutions with a modified Dautrebande-type aerosol generator (331). The modification of an aerosol generator resulted in the preparation of sodium chloride aerosols of narrow particle-size distribution (332). The particle-size distribution of an aerosol continually reinforced by the introduction of particles and undergoing Brownian coagulation was calculated by integrating the equation of Smoluchowski modified to include a feed term (333). The effect of a weak electric charge on the rate of coagulation was studied using dioctyl phthalate (334). Several additional papers discussed propellant blends and perfumes commonly used in aerosols (335–337). Richman studied pressurized foams with emphasis on rheological evaluation (338). An experimental method for measuring the drop size distribution in liquid sprays was presented (339).

Sustained Release—A review was presented describing the means of prolonging medicinal action of various drugs and the various factors involved (340). Recent achievements in the field of pharmaceutical preparations with prolonged action were the subject of a survey containing 300 references (341). Another survey appeared which emphasized the importance of timed-release preparations (342). Drug latency, durability, metabolic activation, and water solubility were reviewed with respect to sustained-release dosage forms (343). Stuart offered a table of variable-release dosage forms and of the principles of the timed-release dosage forms (344).

Several papers appeared elaborating on the use of microencapsulation of pharmaceuticals (345, 346). An attempt was made to develop a parenteral form of an antibiotic by coacervation (347). Luzzi and Gerraughty commented upon the effect of additives on the controlled release from microcapsules (348, 349).

A modified half-change method was suggested to maintain proper enzyme levels during a prolonged-action test (350). Coated granules have been investigated in the preparation of phar-

[4] Trademarked as Vioform, Ciba Pharmaceutical Co., Summit, N. J.

maceuticals with prolonged action (351). Sustained-action thiamine tablets were prepared. The author studied the *in vivo* and *in vitro* release patterns (352). Ion-exchange resin adsorbates of aspirin were prepared and evaluated for sustained action (353). A carboxyvinyl polymer[5] was found to affect the dissolution rate of a tablet. It was concluded that this agent could be used successfully to prepare sustained-action tablets (354). The effect of surfactant concentration on the release rate of sulfaethylthiadiazole from synthetic wax particles prepared by aqueous dispersion was studied (355). Long-action compressed tablet formulation was prepared from a plastic matrix. Both *in vitro* and *in vivo* tests were carried out on the finished forms (356). Newly developed coating laquers made from acrylic resins were also used to prepare depot medicinal dosage forms (357). Another paper appeared which described the preparation and effectiveness of long-acting diethylstilbestrol injections for veterinary use (358).

Cosmetics—Moxey published a survey dealing with perfumery and cosmetic material (359). Another review dealt with the structure of emulsions, emulsifiers, and their use in the cosmetic industry (360). The application of silicones in cosmetic preparations was the subject of another article (361). It contained formulas for a number of preparations. A paper dealing with the use of modern surfactants in the manufacture of cosmetics appeared in the literature (362). Bergwein reviewed hair creams, commenting on their history and development in the various forms (363). Several interesting articles appeared relating the role of glycerin in modern cosmetic products (364, 365). Another author discussed the use of glyceryl esters in cosmetic emulsions (366). Fatty acid esters of xylitol were experimented with as possible ingredients in the preparation of cosmetics (367). Schmolka elaborated on the application of pluronic polyols in the cosmetic industry. He pointed out that these agents may aid the cosmetic chemist in improving current formulations and developing new products (368).

Diisopropyl adipate was evaluated as an ingredient for cosmetic formulations (369). New types of pearl pigments have been developed for cosmetics which previously were not practical because of natural limitations (370). Cosmetic grade propoxylated ethers were discussed and formulations were suggested to illustrate their application (371). The use of polymer microspheres as raw materials for cosmetics and toiletries was suggested (372). Cotta *et al.* described some important points relative to the production and control of tissue protein and their use in cosmetics (373). The importance and the influence of grain size in pigments used in cosmetic preparations was the subject of another paper (374). The synthesis of two new groups of alcohol-soluble aluminum compounds was described. Their antiperspirant activity was also measured (375). Because of unique properties of silicones it was pointed out that a variety of performance characteristics can be built into cosmetic formulations (376). Other papers listed several new active substances that were useful for the care of the skin (377, 378). It was also found that the application of a lipid film containing dewaxed lanolin was of value both in maintaining hydration and in serving as a potential lip barrier (379). A synthetic copy of the natural moisturizing factor present in human skin was formulated based on amino acid analysis (380). Several papers were presented dealing with the evaluation of sun screen agents (381–383). The background and practical application of gel formation in nonionic systems in relation to cosmetic preparations were discussed (384). Fatty acid chain length had some effect on the physical properties of cosmetic emulsions (385).

Packaging—Beal *et al.* described a comprehensive study on pharmaceuticals stored in plastic containers (386). Another study pointed out that direct measurements of light transmission of plastics is affected by diffusion and is not a trustworthy test of suitability for packaging (387). An excellent review on the structure problems of silicates and glass as a construction material, on its resistance to heat change, and on the physical and chemical properties of pharmaceutical containers was published (388). The quality requirements of the pharmaceutical industry for flasks and ampuls were outlined (389). The research and development of safety closures was the subject of another paper (390). Rehm conducted the testing of water resistance of container glass (391). Autian presented a review of the interaction between medicaments and plastics (392). Several papers discussed the problem of permeability of various components through polyethylene and polypropylene containers (393–395). Corrosion problems in aerosol packaging were reviewed (396). High polymer plastics with their flexibility, shock resistance, and other helpful aspects lend themselves well for developing interesting packages for cosmetics (397).

EQUIPMENT

Several papers were published concerning the

[5] Carbopol 934, B. F. Goodrich Co., Cleveland, Ohio.

use of vibration mills (398–400). The performance of several industrial mixers was compared using segregating materials (401). An excellent review article on the application of ultrasonics in pharmacy was written by Skauen (402). Unit-dose packaging was the subject of another article (403). The use of new automated techniques for the pharmaceutical industry has been reported (404). Several typical spray driers were described and their application to pharmaceutical systems was discussed (405). Another paper described the mechanical bottling of oral solutions under protective gas (406). A review, containing 175 references, concerned with automatic adjustments of the meniscus, the dosing adapters for pipets, special devices, the sources of error, and calibration of the dosing adapters was published (407). The construction and use of laminar flow rooms were described by Soltis (408). Another article appeared which described equipment utilized in high speed film coating of tablets (409).

PHYSICAL PHARMACY

The various aspects concerned with dissolution and diffusion studies have produced a number of interesting papers. A new method of studying the diffusion of liquids through gels has been noted (410). Nixon *et al.* have determined the concentration effect on diffusion coefficient (411). In another study, based on the first principles of diffusion, the authors derived appropriate equations to predict the transport of solubilized systems (412). The effect of high surfactant concentration on the kinetics of dissolution, the importance of sink conditions, and the simultaneous determination of dissolution and partitioning rates *in vitro* have been demonstrated (413–415). In regard to the solid dosage form, Singh *et al.* described the release rates from the inert matrices in cases where there existed noninteracting drug mixtures, as well as mutually interacting drug mixtures (416, 417). Formulation effects on the tablet dissolution pattern were also investigated (418, 419). An apparatus for determining the drug release from tablets has been described (420). The dissolution phenomenon has also been reported in somewhat of a different but definitely pertinent interest, *i.e.*, the dissolution of phosphate kidney stones by citrate buffer (421). In addition to dissolution rate studies, the deaggregation behavior is likewise relevant. Aguiar *et al.* have developed equations and demonstrated such rates quantitatively (422). The association of dissolution to insoluble compounds immediately directs itself to the effect of particle size. In fact, it was suggested that the use of an appropriate particle-size terminology be used in pharmacy (423). A review of particle size and surface area and critical evaluation of measurement methods were discussed (424). The following methods for determining particle size were described: photosedimentometer, ultramicroscope, permeability, sedimentation balance, X-ray, turbidimetry, and light scattering (425–434). Other results indicate significant influence of a polar nature of the solution on cluster formation when hindered setting was used for determining particle size (435). A study on the dispersion of clay for particle size determination was made (436). Relative to this, studies on particle reduction to μ size, the effect of grinding on precipitated magnesium hydroxide, and a new technique for fractionating powders were performed (437–439). A comparative study of commercial griseofulvin with reference to official standards was also investigated (440).

Solubility—Solubility determinations of the following systems were reported: the solubility of antibiotics in 26 solvents, purines, pyrimidines, and their nucleosides in urea and sucrose solutions, vinbarbital in ethanol–water systems, sodium bicarbonate in glycerol, and some aminoalkylphenothiazine tranquilizers and related compounds in water (441–445).

The increase of aspirin–water solubility was accomplished through the interaction with urea (446). The effect of urea on the solubilities of benzoic acid and the effect of salicylic acid on the dissolution of some polymers was also determined (447, 448). The solubility relations and solubilization by nonionic surfactants were noted in a number of cases such as, the ascorbic acid–water–polysorbate 80 system, lecithin in antibiotic preparations, benzocaine and homatropine, testosterone, hydrocarbons, hormonal steroids, and chloramphenicol (449–455). Several papers described the increased yields of essential oils when nonionics were utilized in the extraction process (456–458). A review with 167 references outlined the solubilization of drug compounds by polysorbates[6] (460). A study was also conducted on the solubilization of water from its vapor pressure over nonionic and ionic surfactant solutions in various nonpolar solvents (461). Kuettel described saturated and supersaturated systems prepared by solubilization (462). The effect of the cation on solubilization by oil-soluble surfactants and solubilization of polymers in aqueous cationic surfactant solution has been noted (463, 464). The use of some synthetic

[6] Tweens, Atlas Chemical Co., Wilmington, Del.

surface-active substances as well as the solution of sulfonamides with polyglycols was reported (465, 466). Bjaastad and Hall have investigated the value measure of relative solvent polarity in micellar solubilization (467). The solubilization and rate of dissolution of drugs in the presence of physiologic concentrations of lysolecithin were determined (468).

Complexation—Patel presented data to show that antimicrobial activity of several preservatives is primarily a function of the free or unbound preservative (469). A series of papers elaborated on the interaction between drugs and macromolecules (470–472). The calculation and determination of stability and dissociation constants have been reported (473–475). The stability of metal complexes of salicylic acid derivatives and caffeine with various benzoic acids has been evaluated (476, 477). Seventeen drugs were tested against seven ion-exchange resins and activated charcoal for binding capacity *in vitro* (478). Diffuse reflectance studies of solid–solid interactions of drugs were made and in the case of dye–adjuvant chemisorption, the results indicate that a metallic or polyfunctional adsorbent molecule is necessary (479). Evidence of the following complex formations has also been noted: epinephrine with boric acid in aqueous solutions, benzoquinone solutions with indole derivatives, adrenochrome–adrenolutin, weak organic acids and phenols with nylon-6, anionic detergents with cellulose, and starch with organic substances (480–485). The effect of neutral salts in gelatin–gum arabic complexes was also determined (486). A report on the hydrogen acceptor properties of griseofulvin and related compounds, in order that its physical behavior would allow a more rational approach to the formulation of dosage forms, was also reported (487). Interactions of penicillin with deoxycholic acid polymer-like structures have been shown (488). Soluble copper chelates were suggested for addition to injectable solutions such as iron saccharate (489). Also appearing in the literature, was a paper which describes complex formation in aerosol emulsions and foams (490). The use of complexes as antacid preparations has been evaluated for a new aluminum hydroxide complex as well as metal complexes of tris(hydroxymethyl)aminomethane (491, 492). NMR spectroscopy has been used to study molecular interactions and mobility at liquid–liquid interfaces (493).

Surface Phenomena—Aspects of surface-active agents in pharmacy, in which their classification and properties are given, were described (494). A review contained historical, chemical, and biological aspects of surfactants (495). Discussions were also given for the modern theory of fluid surface tension, physical basis of wetting, and some aspects of classical surface thermodynamics (496–498). The properties and chemistry of silicones and clay material have been reported (499, 500). Descriptions of nonionic and anionic surfactants have been noted (501, 502). Another paper contained data on the bactericidal properties of straight-chained alkyltrimethylammonium bromides in a simple emulsion system (503). A paper by Beckett *et al.* contained data on surface-active betaines (504). A review on the use and analysis of sucrose fatty acid esters also appeared in the literature (505). The structure and rheology of sodium dodecyl sulfate–cetyl alcohol–water can be found in a series of papers (506–508). Several other papers have noted micellar properties of sodium dioctyl sulfate (509–511). A study using monooctyldecyl phosphate included data which showed the surface viscosity of the monolayer increased linearly with time and this rate increased as the pH was lowered (512). Studies of the surface viscosity of monomolecular films of *n*-chain alcohols were also conducted (513). Other aspects of surface-active agents which have been reported included monolayers formed by mixtures of anionic and cationic surfactants, surface excess in solutions of surface-active agents, and the effect of composition on the critical surface tension of polyvinyl butyral (514–516). Another report included the physiochemical properties of monoglyceride sulfonate–water systems near the critical micelle concentration (517). Definitions of kinetics and kinematic surface tension on the physiochemical surface characteristics of moving surfactant solutions were given (518). Electrocapillary curves for the interfaces between aqueous solutions of inorganic electrolytes and oils containing detergents were published (519). A number of papers have considered the following aspects of the micellar state: the relationship with cloud point, influence of temperature, the effect of tracer dyes on the size distribution and transport properties, density, hydration, shape and charge, and the ionic dependence of micelle number (520–524). The determination of critical micelle concentration of cationic surfactants has been made through the use of alizarine red S (525). The osmotic coefficient and equivalence conductance of aqueous solutions of medical amine salts were used to determine aggregating properties (526). Quantitative measurements of the interfacial free energy of black lipid membranes

separating two aqueous solutions were made (527). A number of papers have included various facets in the measurement of surface tensions of surfactant solutions including methods (528–533). Ho and Higuchi used the RIDL multichannel particle counter and size analyzer to develop a method for studying the kinetics of aggregation of denatured proteins (534). Another paper described the use of flow ultramicroscopy in particle-size analysis (535). The kinetics of monolayer desorption at constant surface pressure was analyzed (536). Fluorine magnetic resonance was used to investigate micelle structure while the sedimentation equilibrium method was used to determine micelle molecular weight (537–538).

A geometric model of floc formation using Smoluchowski's rate equation for perikinetic flocculation was developed (539). The colloidal–chemical properties of nonionic surfactants is included in another paper (540). A number of papers included discussions on colloid stability (541–546). Another author presented the effects of low concentrations of electrolytes upon electrophoretic mobility of aqueous colloidal suspensions (547). The description of the double-layer structure of colloidal electrolytes was also made (548). Temperature effect on micellar state of surface-active semicolloids in aqueous solutions has been noted (549). Also presented in the literature was a paper which contains the description of liquid–solids separation with particle growth (550). Papers specifically directed to pharmacy included data on the application of surface-active agents to pharmaceutical preparations, the effect of griseofulvin upon lipid films at the air–water interface, and the work on the formation of micelles in topical anesthetics (551-553).

A general equation for adsorption isotherm was derived and the factors involved in adsorption at the solid–liquid interface were described (554, 555). A method and determination of surface areas of solids has been described in separate papers (556, 557). Investigations of adsorption have included that of the effect of aprotic solvents on phenol alumina, the adsorption of alkaloid salts on silica gel, and the adsorption of mixtures of organic substances from aqueous solutions by activated charcoal (558–560).

The homogeneity of powder mixtures and the segregation kinetics of particulate solids were discussed (561, 562). The aggregation of particles during the grinding process and a report on the development of cohesion in granular materials during comminution were noted (563, 564). The effect of the particle size of granulated drugs on free-flowing properties can be found in another paper (565).

Crystallization—A study was conducted on the mechanism of crystallization of hydrocortisone by ultrasonic irradiation (566, 567). Several authors presented data on the development of optimal crystal shape of basic pharmaceutical materials. This included the influence of solvents and solvent mixtures on crystal shape (568). The conversion of six cholesterol esters from crystalline solid to the isotropic liquid during heating and cooling was studied (569). It was also found that the rate of crystallization depended upon the concentration of starting materials in studies of the crystalline aluminosilicates (570).

Separate studies on sulfathiazole and methylprednisolone described heats of transition and fusion and crystal growth and dissolution rate of the two polymorphic forms of both compounds (571–573). The polymorphic forms of chloramphenicol palmitate and stearate were examined by IR spectrophotometry (574). The polymorphic forms of cortisone acetate and sulfamethoxydiazine were also reported (575, 576). The phase transformation of theophylline hydrate to an anhydrous form was measured at a variety of temperatures using X-ray powder diffractometry (577).

Rheology—The viscosity of some suspensions was studied in which an equation was derived for the effective viscosity of low concentration emulsions containing surfactants (578). It was also stated that the measured viscosity of a suspension depends upon the diameter of the tube of the capillary viscometer used in determining it (579). A Ferrante-Shirley cone and plate viscometer was used to estimate the spreading characteristics of pharmaceutical semisolids (580). An evaluation of the rheological properties of monodisperse latex systems flow curves of thickened latexes was made (581). The structure and rheology of dispersed and flocculated colloidal systems was reviewed (582). Another study described the rheology of surfactant solutions by a novel viscometer based on the principle of harmonic damped oscillations (583). The mathematical criteria of the thixotropy of starch gels were reported (584). Other studies included the viscosity of phospholipid solutions and the development of a standard gel for interlab comparison (585, 586). The Stormer viscometer in the measurement of certain pharmaceutical systems was evaluated (587). Studies reported on clays include: thixotropy and rheopexy of

clay suspensions, negative thixotropy, colloidal and surface properties, sedimentary volumes, structure formation in hydrothermal conditions, and rheological properties (588–594). Another worker determined the sorption and exchange capacity of cations in clay materials (595). The rheology of some oil-in-water emulsions and the rheological changes in emulsions on aging have been reported (596–598).

The following agents have been evaluated as to rheological properties: methylcellulose hydrogels, ternary system of sodium carboxymethylcellulose–glycerol–water, surfactants, sugars in pectin solutions, and the effect of cations on γ-carrageenan (599–605). Another report contained a description of the five general stages in the formation of ionotropic gels (606). The rheological and stabilizing properties of some soaps of naphthenic acid were also investigated (607).

The application of rheology in pharmaceutical cosmetic practice with plastic gels was described in a survey on the main principles of rheology (608). A study on the rheology of a thixotropic lotion and the rheological characteristics of emulsion ointment bases appeared in the literature (609, 610). A paper describing the alteration in flow properties of light mineral oil with an organic derivative of a special montmorillonite[7] was also noted (611).

PHARMACOCHEMICAL ASPECTS

Antibiotics—A review included the new derivatives of semisynthetic penicillin and their clinical applications (612). The pharmaceutics of the semisynthetic penicillins, the compatibilities, and the incompatibilities with other compounds, were presented (613). Gibaldi and Schwartz have determined, from literature data, the kinetics of absorption and elimination in the dog of a new penicillin derivative, penamecillin (614). New antibiotic preparations and other biologically active agents of natural origin have been reported (615). The biochemical mechanism of action of antibacterial antibiotics has been reviewed and the physical-chemical properties of novobiocin were discussed (616, 617). Several authors investigated the hygroscopic properties, thermostability, and solubility of oleandomycin salts (618).

Radiopharmaceuticals—Radiopharmaceuticals were reviewed quite extensively (619). The development, preparation, and application of nuclear pharmaceuticals were described (620). The problems of radiochemical purity, analytical methods, label stability, isotope effects, tissue residues, and clinical studies were considered in a review article (621). Equations were derived without steady-state assumptions for inactivation of enzymes in dilute aqueous solution by ionizing radiation (622).

BIOPHARMACEUTICS

This area appears to be commanding progressively more interest and it probably is the single most investigated area in pharmacy. Interesting work but perhaps least pertinent is that dealing with molecular aspects of drug–receptor interactions as noted in these papers (623–626). An interesting review was presented on alicyclic amines showing relations between structure and pharmacologic activity (627). Others worth noting include the material basis of drug activity and the concepts of the mode of action of drugs in which specific absorption is suggested as a basis of biological activity (628–631).

Studies related to biological systems included the determination of acetylsalicylic acid and salicylic acid in plasma (632). The behavior of erythrocytes in various solvent systems has been investigated (633, 634). Also reported were the excretion levels in the saliva of dogs in which the excretion levels in saliva paralleled the blood levels for salicylate, pentobarbital, and sulfanilamide (635). It was also noted that saliva added to meals significantly retarded gastric emptying while a review pointed out that gastrin was the most potent stimulator of gastric digestive processes (636, 637). Included in the literature was a report that blood plasma was a Newtonian fluid over a wide range of shear rates to cover any likely situation in a living animal (638). Drug accumulation and the storage of drugs have been treated in separate papers (639, 640).

Numerous papers have dealt with a wide range of drug-binding phenomena. These include antibiotic–metal ion interactions, complex formation of tetracyclines with different components of blood serum, protein-binding of local anesthetics, binding of cortisol, binding of phenothiazine derivatives, and sulfonamide binding by serum albumin (641–646). Others included studies of norchlorcyclizine and norcyclizine and the thiazole moiety of thiamine (647, 648). The correlation of serum binding of penicillins with partition coefficients and studies of the binding of phenoxazone compounds were reported (649–651). The competition phenomena in binding of drugs to albumin were discussed (652). A review contained the agents which affect enzyme activity and another the enzymatic degradation

[7] Marketed as Bentone 38, National Lead Co., Brooklyn, N. Y.

of drugs in the organism (653, 654). In another paper, the effect of proteolytic enzymes on tetracycline absorption was studied (655). In separate studies, the *in vivo* degradation of penicilloic and penilloic acid and the hydrolysis of acetylsalicylic acid in human blood were discussed (656, 657).

Studies in which the routes of administration were compared included the comparison of oral and parenteral calcium administration, the difference between parenteral and rectal administration of thiamphenicol, and the rate of equipotent oral and intravenous doses of propranolol and H56/28 (658–660). Species comparisons were also made in which the binding of salicylate to plasma proteins in different species, the comparative metabolism of griseofulvin-^{14}C in the rat and rabbit, and the species difference in duration of action of cardiac glycosides were studied (661–663). The dose dependence of drug plasma level decline in dogs was investigated, and the statistical analysis of log-dose response bioassay experiments were described (664, 665).

Effects of Physiochemical Properties—Evidence of there being a definite pKa which a sulfonamide must possess in order to exhibit maximum activity was discounted (666). In another paper, it was noted that the carbonate derivative is the prodrug of trichlorethanol (667). In a study of pharmaceutical potentiation of drugs, it was reported that the long-chain alkyl derivatives of *para*-aminosalicylic acid were characterized by sustained release and maintenance of high levels in body fluids for long periods (668). In another study, the complex of salicylic acid with caffeine appeared to affect the absorption of this drug in the rat stomach (669). Other authors compared the sulfate and *para*-chlorophenoxyacetate of DL-amphetamine (670). The enteric absorption of formosulfathiazole and phthaloylsulfathiazole was also investigated (671).

Effects of Formulation—An interesting paper on the effect of adjuvants in the manufacture of pharmaceutical preparations was reported. It was noted that the release of antibacterial active drugs from aerosil, white clay, and talc was dependent on the structural relation between the drug and adjuvant (672). The review by Higuchi described the diffusional models useful in biopharmaceutics drug release rate processes (673). Using a new estimation method for release of drugs from dosage forms, an estimate was made of the actual sulfamethylthiadiazole release from the tablet in the gut (674).

Other authors compared the activity, fate, and absorption of chloramphenicol in different suspensions, while Aguiar *et al.* studied the effect of polymorphism on the availability of chloramphenicol from the chloramphenical palmitate, on the degree and velocity of hydrolysis, and the extent of resorption (675–677). The crystalline phases of fluprednisolone were reported in which a comparison of the physical properties and biological activities was made (678).

The action of tetracyline hydrochloride, oxytetracycline, and monomycin was prolonged through the use of polyvinylpyrrolidone aqueous solution (679). A description of the ability of certain additives to influence the absorption of salicylic acid from solutions was given (680). In another report, *in vivo* tests revealed a slight delay in absorption of a sulfa from a topical suppository base (681). A review of the biochemical and physiological mechanisms underlying fat-emulsion preparations also was written (682). A sensitive analytical method was described for the effect of formulation in phenmetrazine levels in man (683). Polymeric pharmaceutical coating materials as enteric coating *in vivo* were evaluated and the intestinal absorption of quinine from enteric-coated tablets was determined (684, 685). A report contained a method for studying the *in vivo* disintegration of orally administered pharmaceuticals (686). In studies of griseofulvin, in which the dissolution rate and absorption in man were compared, a good correlation was seen (687). Paul *et al.* related the effect of varying the crystal size of orally administered nitrofurantoin on absorption in the rat and man and emesis in dogs (688).

Absorption Control—A number of examples have been reported in which the absorption of a compound is altered by the presence of another. One study showed the effect of EDTA on intestinal absorption of sodium *p*-aminohippurate (689). Nikethamide[8] was found to produce a marked decrease in the blood levels of chloramphenicol, and methimazole was found to decrease the absorption of iron in rats (690, 691). Similar studies included the effect of certain nonsteroid antirheumatic drugs on the active amino acid transport across the small intestine and the effect of phenobarbital and glutethimide on the biological half-life of warfarin (692, 693). It was also reported that acetylsalicylic acid reduced the excretory rates of sodium, chloride, calcium, and magnesium ions by about 50% (694). Another author considered the retention and distribution of vitamin B_{12} activity and requirement for B_{12} following parenteral adminis-

[8] Coramine, Ciba Pharmaceutical Co., Summit, N. J.

tration of hydroxycobalamin (695). Uropepsin excretion and the effect of antacid materials, the effect of chelating agents on the excretion of metals, and the effect of probenecid on riboflavin absorption and excretion was also reported (696–698).

Serum levels of vitamin A and β-carotene in patients with and without dermal diseases were determined (699). In another paper, the excretion of trancyclomine sulfate was increased from 1 to 8% after acidification of the urine (700). Levy noted the effect of bed rest on the distribution and elimination of drugs (701).

Absorption Mechanism—A review by Weiner described the mechanisms of drug absorption and excretion (702). The theory of the molecular transport phenomena through thin membranes can be found in another paper (703). A diffusional model for transport rate studies across membranes was proposed by Stehle and Higuchi while another group related drug transport to its solubility parameters (704, 705). Studies utilizing the isolated rabbit mesentery and the urinary bladder of the rabbit have also been reported (706, 707). Electrochemical properties of membranes and anion-exchange resins with inorganic groups of different structure have been noted (708). Several papers included the transfer of compounds across the intestine. Among the compounds studied were L-selenomethionine, basic amino acids, quaternary ammonium ions, and leucomycin (709–712). An interesting paper describes the mechanisms of water transport through nonaqueous liquid membranes (713).

Kinetic Studies—A review on pharmacokinetics was prepared (714). Wagner and Metzler described the estimation of rate constants for absorption and elimination from blood concentration data (715). Equations for excretion rates and renal clearances of exogenous substances not actively reabsorbed have also been derived (716). It was also reported that blood level data and urinary excretion data may lead falsely to the conclusion of zero-order kinetics (717). Martin appraised some aspects concerning the interpretation of drug urinary excretion data (718). Another paper related kinetic considerations to the accrual and elimination of drug metabolites (719).

Pharmacokinetic studies were reported separately on the following compounds: salicylamide, paracetamol, pentobarbital, and ioglycamate (720–723). The digital computer was used to describe the kinetics of the distribution of estradiol-^{3}H (724). Kinetic constants for mixed inhibitors of cholinesterase were evaluated (725). Several authors reported on the effect of repeated oral dosage of nalidixic acid, while others derived a formula for calculating effective levels after repeated doses of hyoscyamine (726, 727). Subcutaneous absorption kinetics were also reported in which benzyl alcohol was studied (728).

Drug Absorption—Krueger-Thiemer and Hansen reported on the solution of pharmacological problems by computers, and a new computer program to determine biological constants was written by others (729–731). A report was also noted on the formal theory of drug-dosage regimens in which the desired plateau effect was not reached if the product of the rate constant for absorption and the dosage interval was low (732). Perrin described the mathematics of the three-phase *in vitro* model and the *in vitro* model for soluble drug absorption (733, 734). Another paper provided a method of estimating relative absorption of a drug in a series of clinical studies in which blood levels were measured after single and/or multiple doses (735). O'Reilly discussed the biological factors in dosage design in which drug absorption was related to membrane transport mechanisms (736, 737). The absorption profiles of drugs were reported (738). In another paper the buccal absorption of basic drugs was applied as an *in vivo* model of passive drug transfer through lipid membranes (739). Reports on the absorption, excretion, and metabolism of the following compounds were made: dextroamphetamine, barbiturate, riboflavin-5′-phosphate, methyl anthranilate, pentaerythritol tetranitrate, methyridine, haloperidol, propranolol, 5-methyl pyrazote-3-carboxylic acid, alloferin, fenfluramine, dimethylsulfoxide, pilocarpine, and lidocaine (740–752). A review of drug and chemical metabolism outlines the fate and distribution of drugs (753).

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Keyphrases

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Research Articles

Studies on the Mechanism of the Mannich Reaction

By W. LEWIS NOBLES* and N. D. POTTI

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FOR MORE THAN 60 years the Mannich reaction has been studied by several groups of workers, especially in the field of medicinal chemistry, primarily because of its synthetic utility and the favorable pharmacological properties of the Mannich bases and their derivatives. The mechanism of this reaction has been the subject of considerable discussion (1–13). Yet, thus far, no single mechanism which will account for all the experimental facts has been suggested.

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Accepted for publication October 17, 1967.
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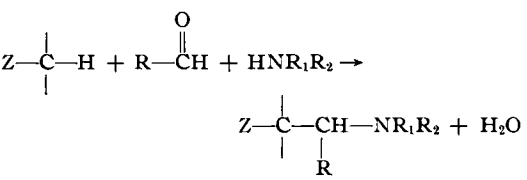
The reaction in its most generalized form may be represented as follows:



Several investigators (2, 5, 7, 14, 15) have indicated the possibility of considering a methylenebisamine as the most probable intermediate under normal reactions. A quasi-six-membered hydrogen-bonded transition state has been offered, in the case of the Mannich reaction with phenols as a plausible mechanism for the selective

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FOR MORE THAN 60 years the Mannich reaction has been studied by several groups of workers, especially in the field of medicinal chemistry, primarily because of its synthetic utility and the favorable pharmacological properties of the Mannich bases and their derivatives. The mechanism of this reaction has been the subject of considerable discussion (1–13). Yet, thus far, no single mechanism which will account for all the experimental facts has been suggested.

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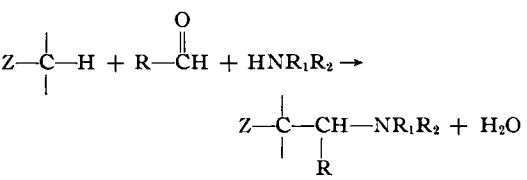


Several investigators (2, 5, 7, 14, 15) have indicated the possibility of considering a methylenebisamine as the most probable intermediate under normal reactions. A quasi-six-membered hydrogen-bonded transition state has been offered, in the case of the Mannich reaction with phenols as a plausible mechanism for the selective

formation of the *ortho*-substituted phenols (8). A similar complex transition state has been indicated (5) in the case of the Mannich reaction of nitroalkanes. Further strong intramolecular hydrogen bonding is indicated in Mannich bases in general (10,16).

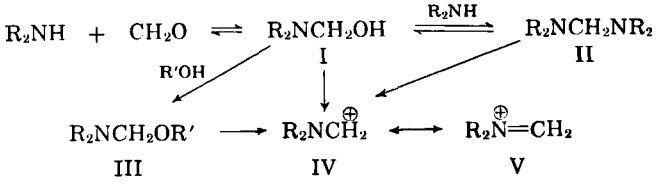
Methylenebispiperidine reacts faster than methylenebismorpholine in the Mannich reaction (5); since they are sterically quite similar, this has been attributed to their difference in basicity. One objective of the present investigation was to study the course of the Mannich reaction when an unsymmetrical methylenebisamine was used.

In previous mechanistic studies, there seems to have been little consideration of the steric effects of the reactants, even though there is a significant amount of published data available. The significance of this aspect is elaborated in this study to the point that it appears to account for several otherwise unexplained facts.

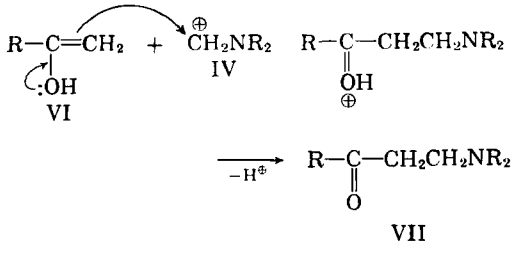
DISCUSSION AND RESULTS

The formation of a methylenebisamine (II) as the primary step of the Mannich reaction has been suggested by Lieberman and Wagner (2) even though later kinetic studies (3, 4) proved their mechanism to be incorrect. Alexander and Underhill (3) held the view that an aminomethylol (I) is the intermediate of the reaction. The possibility of the formation of an aminomethyl ether (III) in alcoholic medium also has been suggested (8, 17). (Scheme I.)

To date, one may accept most of the intermediates that have been proposed in the mechanistic considerations of this reaction. This is essentially true since, according to the current view (4, 18, 19), in slightly acidic medium the carbonium ion (IV) which is obtainable from all the above intermediates, attacks the enol form (VI) of the ketone in an electrophilic attack to give the Mannich base (VII).



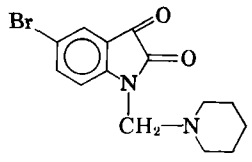
Scheme I



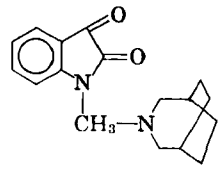
A more recent study (14) indicates that at 30° practically the entire quantity of the amine exists as the methylenebisamine when the secondary amine and formaldehyde are mixed. There is little evidence (17) to consider the aminomethyl ether as a possible intermediate in the normal Mannich reaction, since the rate of formation of this ether is very slow compared to the almost instantaneous formation of the methylenebisamine. Furthermore, Varma and Nobles (20), while attempting the Mannich reaction with indanedione, isolated a methylenebisamine dihydrochloride when the normal reaction failed. Fernandez and Fowler (5) have demonstrated the possibility of a six-membered hydrogen-bonded transition state in the Mannich reaction of nitroalkanes with a methylenebisamine. Burckhalter and Leib (8) offered a similar mechanism involving a chelated six-membered transition state in the Mannich reaction of phenol to account for the selective formation of the *ortho*-substituted derivative.

The present study extends the possibility of a similar six-membered (chelated) transition state to other types of Mannich reactions, especially those involving ketones and phenols as active hydrogen components. As a matter of fact, ketones, after enolization, appear to behave exactly as the phenols do from one mechanistic point of view.

Two unsymmetrical methylenebiasmines,[1] 5-bromo-1-piperidinomethylisatin (VIII) and *N*,*N*-3-azabicyclo[3.2.2.] nonylmethylisatin (IX) have been reacted with β-naphthol and acetophenone, respectively. The products obtained have been identified by independent synthesis as shown in Scheme II. The selective incorporation of the piperidine and AZBN moieties in the final product is in good agreement with the observation of Fernandez and Fowler (5).



VIII

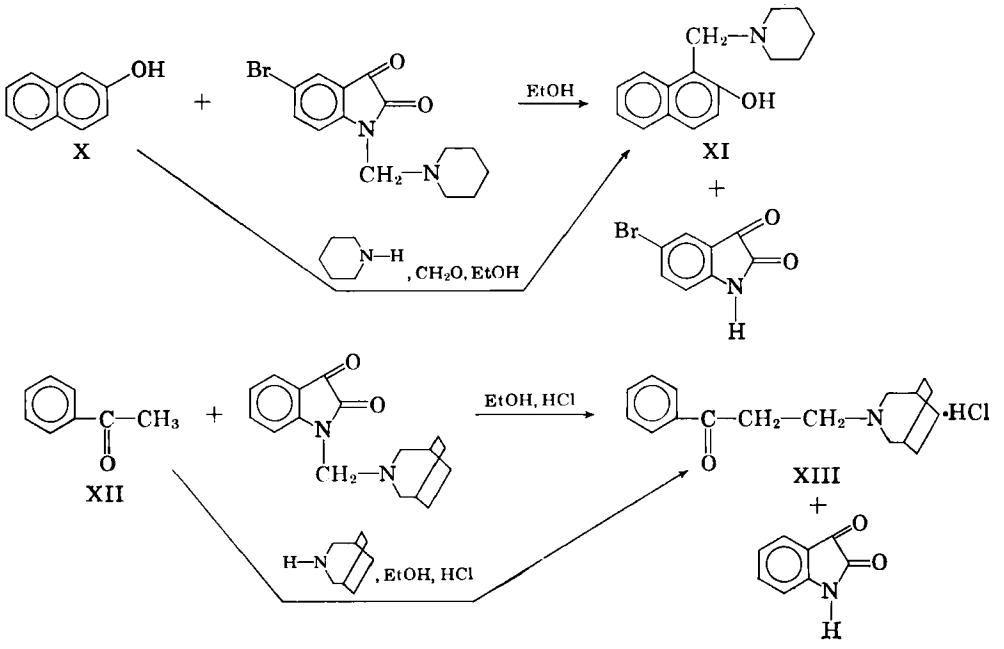


IX

The possible significance of this particular study is limited by the realization that isatin acts as a weak acid in aqueous solution, being soluble in cold alkali and yielding 2-aminophenylpyruvic acid on warming with aqueous alkali. Nonetheless, the leveling effect in comparing the relative strengths of acids and bases in various solvents should be noted; the range of comparisons is limited by the strength of the solvent as an acid or base.

Participation of isatin to date in the Mannich reaction has been limited to the observation that *N*-Mannich bases may be prepared by the action of

[1] Isatin is a very weak base (21) and forms a crystalline perchlorate, $C_8H_5NO_2 \cdot 2H_2O$.



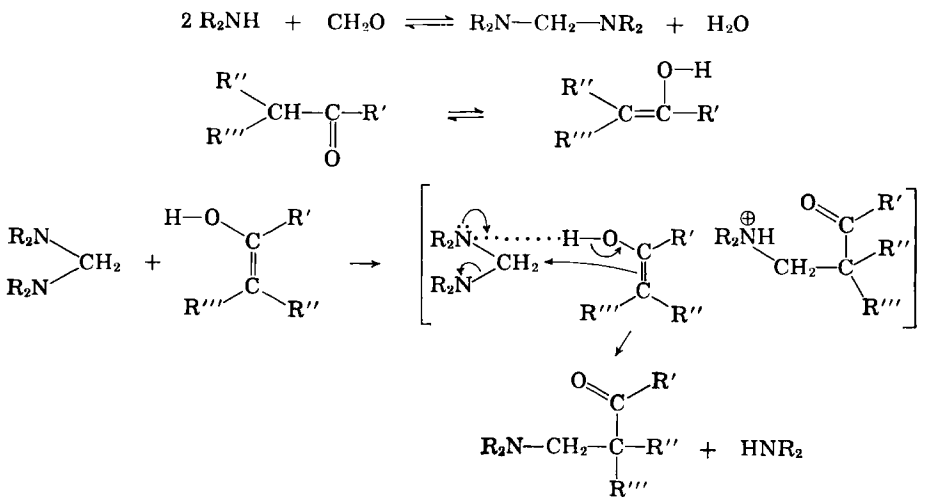
Scheme II

formaldehyde and a secondary amine with this lactam. Studies are currently underway in our laboratory to determine if it is possible to utilize the leveling effect of various solvents on amine strengths as bases in the utilization of isatin and related products as amine components in the Mannich reaction.

The stronger base should logically form a more effective hydrogen bond. The mechanism may be represented as in Scheme III; the primary step of the reaction is the formation of the methylenebisamine.

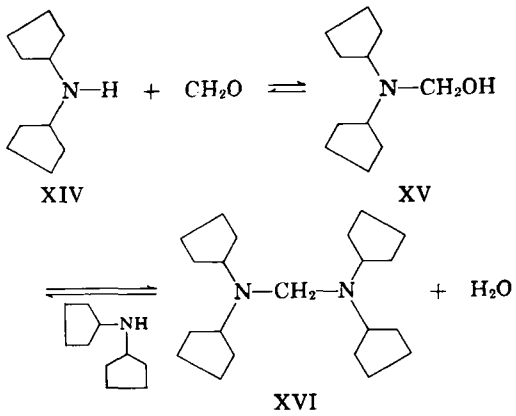
R′ and R″ may form a part of the ring in the case of cyclic ketones and phenols. In the case of phenols R‴ must necessarily be a hydrogen atom so that by enolization it can rearrange to the more stable phenol form.

Hitherto, it appears little attempt has been made to study the steric effects of the reactants. Diethylamine appears to behave quite differently in several situations (18, 22, 23) when compared with dimethylamine and piperidine. Dicyclohexylamine does not take part in the normal Mannich reaction (24). In the present study, an attempt has been made to condense dicyclopentylamine (XIV) both in acidic and basic media using acetophenone and β-naphthol, respectively, without success. Interestingly enough, heat is liberated when the amine is mixed with the formalin; this may indicate that the primary reaction, formation of the methylenebisamine, has taken place. Attempts to isolate the methylenebisamine dihydrochloride (XVI), however, have failed thus



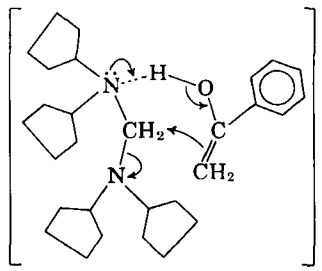
Scheme III

far. Instead, dicyclopentylamine (XIV) has been isolated; identity has been confirmed by independent synthesis. This is not surprising since the reaction is one of a reversible nature and a molecular model study indicates a considerable amount of steric hindrance would be involved in the formation of the methylenebisamine (XVI). But model studies would indicate no difficulty in forming the carbonium ion (XVII) from the aminomethylol (XV) and, according to the current view (4, 18, 19), XVII may result in the formation of the Mannich base (XIX) by an electrophilic attack on the enol form (XVIII) of the ketone.



The total failure of the reaction is contrary to the current view, but is in full agreement with the mechanism proposed; the attainment of the suggested transition state (XX) is difficult on steric grounds even if the methylenebisamine (XVI) were to be formed.

The present consideration satisfactorily explains the apparent existing ambiguity (18, 25) with respect to the predominant Mannich condensation at the most highly substituted position or the least substituted postion (12, 13, 26) in 2-substituted cyclic and branched ketones. Apparently, that which determines the nature of the products will depend on the steric environment of the ketone and the ability of the methylenebisamine to form an effective hydrogen-bonded six-membered (chelated) transition state. The situation can be explained as indicated in Scheme IV. The proportion of isomer A or B may vary from 0% to 100% depending upon the

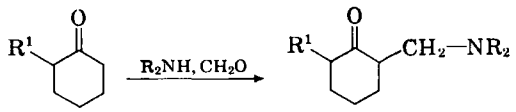


XX

steric contribution of the substituents R_1R_2 and R′; route I is preferrred but II is followed when these groups become more bulky in nature.

The reaction is controlled both thermodynamically and kinetically. House and Trost (18) found the ratio of isomer A to B (n = 3; R′, R_1, R_2 = CH_3) to be 70 to 30% and 65 to 35% in acidic and basic media, respectively, indicating that variation of pH does not affect the ratio of the isomers considerably. The proportion of B (n = 3, R′ = CH_3; R_1 = R_2 = C_2H_5) increases to about 42% when diethylamine is used (27) instead of dimethylamine as the former offers comparatively more resistance toward following route I.

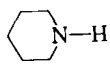
2-Methylcyclohexanone appears to be a borderline case. Reichert and Mayr (28) report the formation of several Mannich bases of the general formula XXII in very good yields from 2-alkylcyclohexanones (XXI) having very large substituents at C_2. The reaction follows route II as shown in Scheme IV predominantly.



XXI XXII

R^1 = —CH_2—CH(—CH_3)—$(CH_2)_n$—CH_3, n = 2, 3

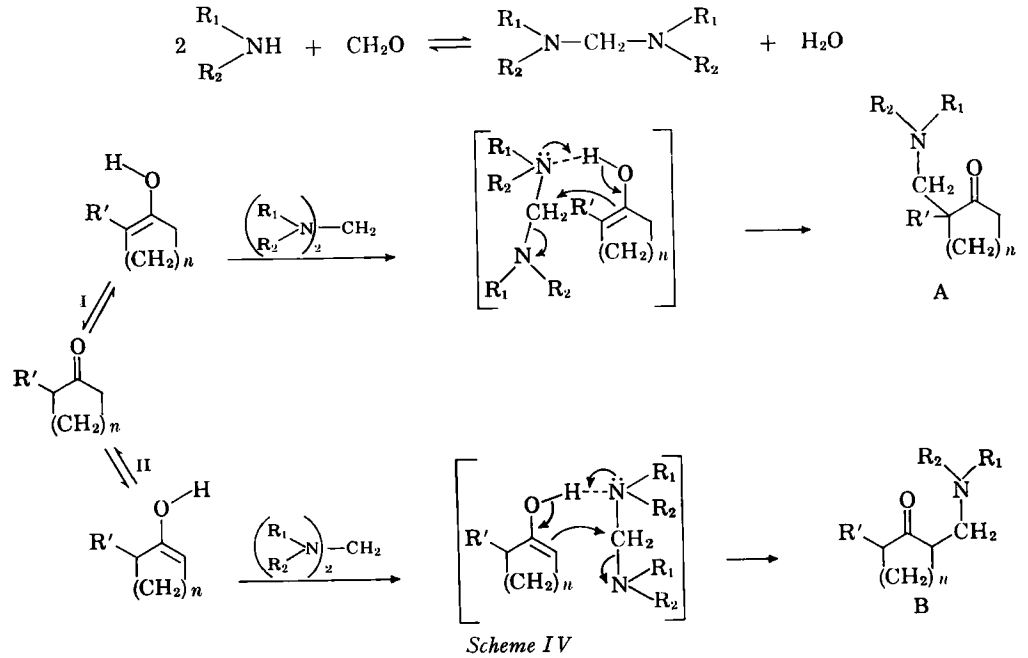
R_2NH =

$(CH_3)_2NH$, $(C_2H_5)_2NH$, 

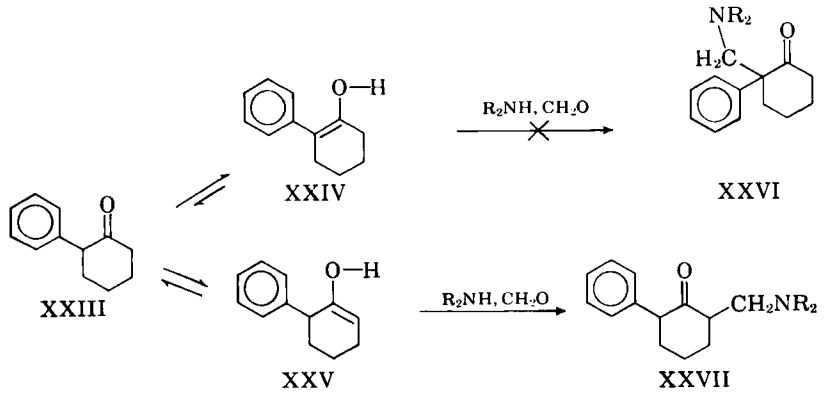
Bachmann and Wick (29) have demonstrated a similar course for 2-arylcyclohexanones; the less stable enol undergoes the Mannich condensation. For example, 2-phenylcyclohexanone (XXIII) gives the Mannich base XXVII in 62% yield. (Scheme V)

The same reasoning is applicable to the behavior of menthone (XXVIII) in the Mannich reaction (12). Mention may be made that the Mannich base XXX is less strained as compared to XXIX on the basis of nonbonded interactions.

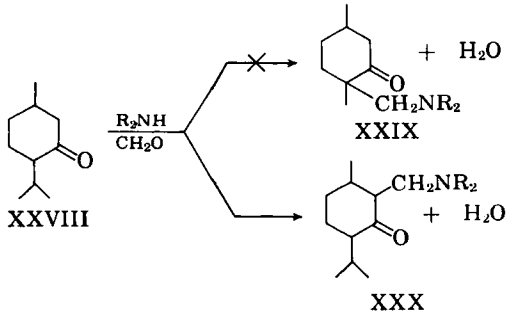
The proposed mechanism may be extended to explain the nature of the products obtained (22) from lawsone (XXXI). Contrary to expectation (12), the primary amine, instead of proceeding to the Mannich base XXXIII, gives the secondary amine, XXXII, which is the primary product of the reaction. When the complexity of the amine in-



Scheme IV



Scheme V

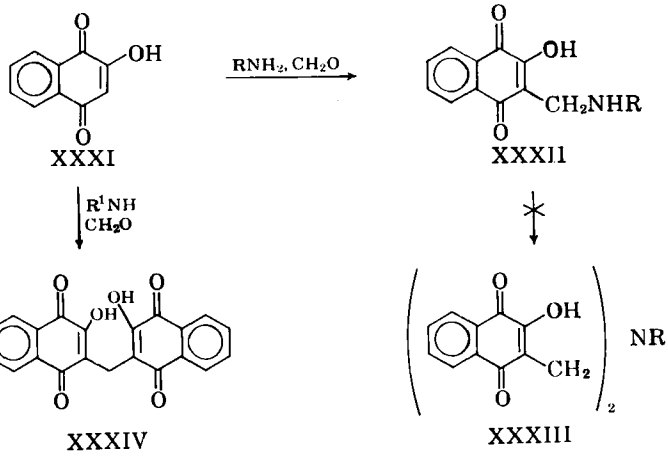


creases, the side reaction predominates so as to give the bis compound, XXXIV (Scheme VI).

Existing views (4, 18, 19) do not appear to explain all these facts; nor do they satisfactorily explain the selective formation of the *ortho* isomer in the case of the Mannich reaction with phenols. The *ortho* substitution of the phenol may be comparable to that of Claisen rearrangement (30) of allylic ethers, rather than a simple electrophilic substitution by the carbonium ion, IV, which is free to attack either the *ortho* or *para* position or both.

Para substitution in the *ortho* substituted phenols may take place through the participation of a second molecule of the methylenebisamine as is shown in Scheme VII; this makes it a termolecular reaction, the frequency of which is considerably less than that of a bimolecular reaction under identical conditions. Thus, it also explains why *ortho* substitution takes place in unsubstituted phenols in good yields.

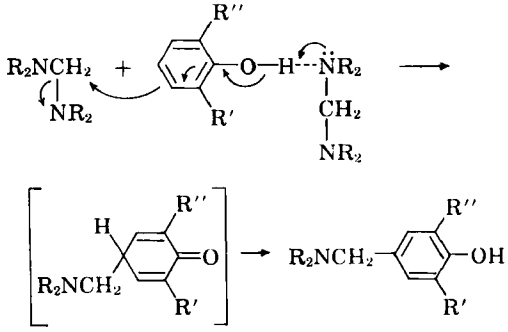
A considerable amount of confusion (4) exists as to the effect of pH on the Mannich reaction. Usually an acidic pH is not favorable for phenolic Mannich condensations. Too alkaline an environment also is not favorable since the sodium salt of naphthol (XXXV), under usual Mannich conditions, does not give the Mannich base XXXVI (Scheme VIII). Instead, it results in a resinous mass insoluble in both dilute alkali and dilute acid. The pH of the medium which affects unfavorably the formation and stability of the chelated transition state shown in Scheme III



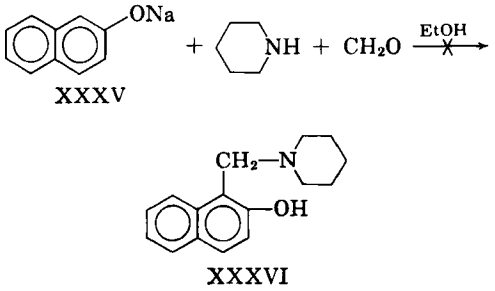
$RNH_2 =$ NH_2, $CH_3(CH_2)_3NH_2$

$R^1NH =$ $[CH_3(CH_2)_3]_2NH$, $(C_2H_5)_2NH$

Scheme VI



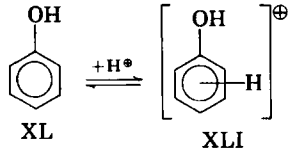
Scheme VII



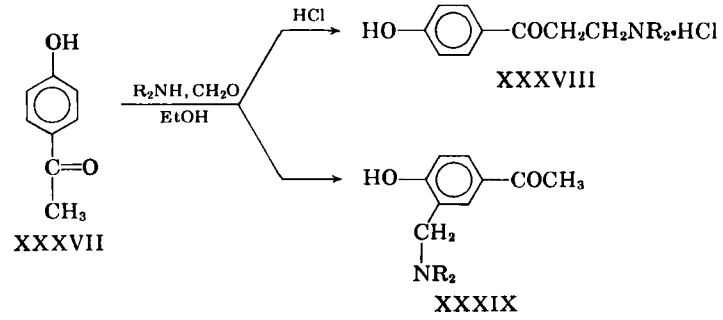
Scheme VIII

will delay the normal reaction and this in turn may result in the side reaction predominating.

Gautier and his associates (31) report the isolation of phenolic and ketonic Mannich bases from *p*-hydroxyacetophenone (XXXVII) with the reaction being carried out in basic and acidic media, respectively (Scheme IX). The products have been identified on the basis of the nuclear magnetic resonance spectra and also by chemical means. The acidic medium appears to deactivate the ring with respect to the Mannich condensation, whereas it will catalyze the enolization of the ketone, thus favoring the formation of the ketonic Mannich base, XXXVIII. This deactivation of the ring by acid may be attributed to the formation of a π-complex, XLI, between the phenol and the proton surrounding it. This results in the generation of a partial posi-



tive charge on the aromatic ring. It may be recalled that during the formation of the chelated transition state (Scheme III) the ring plays the role of a nucleophilic species and it is, therefore, evident that



Scheme IX

the aromatic ring carrying a partial positive charge will no longer effectively play the part of the nucleophilic species.

EXPERIMENTAL[2]

Essentially, the method followed by Varma and Nobles (32) was utilized for the preparation of VIII and IX.

5-Bromo-1-piperidinomethylisatin (VIII)—A mixture of 4.25 Gm. (0.05 mole) of piperidine, 11.3 Gm. (0.05 mole) of 5-bromoisatin, 5 ml. (0.06 mole) of formalin, and 50 ml. of ethanol (95%) was heated on a water bath for 1 hr. and then chilled in an ice bath. The crude product was filtered and crystallized from ethanol; yield 13 Gm. (81%), m.p. 135–136° [lit. (32) m.p. 135–136°].

N-N-3-Azabicyclo[3.2.2]nonylmethyl Isatin (IX)—A mixture of 7.3 Gm. (0.05 mole) of isatin, 6.25 Gm. (0.05 mole) of 3-azabicyclo[3.2.2]nonane, 5 ml. (0.06 mole) of formalin, and 50 ml. of ethanol (95%) was heated on a water bath for 1 hr. and chilled in an ice bath. The crude product was filtered and crystallized from ethanol; yield 9.2 Gm. (64.3%), m.p. 101–102°. [Lit. (32) m.p. 103–105°.]

2-Hydroxy-1-piperidinomethylnaphthalene (XI). Method A—A mixture of 1.44 Gm. (0.01 mole) of β-naphthol, 0.85 Gm. (0.01 mole) of piperidine, 1 ml. (0.01 mole) of formalin, and 3 ml. of ethanol was refluxed for 2 hr. on a water bath and chilled in an ice bath. The crude product was filtered and crystallized from ethanol; yield 1.6 Gm. (66%), m.p. 94–95°. [Lit. (33) m.p. 96°.]

Method B—From VIII—A solution of 3.23 Gm. (0.01 mole) of VIII in 15 ml. of ethanol was refluxed with 1.44 Gm. (0.01 mole) of β-naphthol, on a water bath for 15 min.; a yellow precipitate (2.1 Gm.) separated and was removed by filtration. This was found to be 5-bromoisatin, m.p. 255–256°. The filtrate was concentrated and chilled in an ice bath until crude XI weighing 1.8 Gm. (73%) separated. Crystallization from ethanol gave a colorless product, m.p. 94–95°. A mixed melting point determination with the product from *Method A* showed no depression. The infrared spectra of the products from both methods were found to be identical.

Phenyl-β-N-3-azabicyclo[3.2.2]nonylethyl Ketone Hydrochloride XIII. Method A—In a 100-ml. flask 2.4 Gm. (0.02 mole) acetophenone, 2.5 Gm. (0.02 mole) of 3-azabicyclo[3.2.2]nonane, 0.9 Gm. (0.03 mole) of paraformaldehyde, and 5 ml. of ethanol (95%) were mixed; the pH was adjusted to 5 with concentrated hydrochloric acid. The mixture was refluxed for 3 hr. The volume was reduced to one-half of its original volume and 30 ml. of acetone was added by stirring. The product separated on crystallization from ethanol; yield 3.4 Gm. (68%), m.p. 199–200°. [Lit. (34) m.p. 199–200°.]

Method B—From IX—A mixture of 1.2 Gm. (0.01 mole) of acetophenone, 2.84 Gm. (0.01 mole) of IX, and 5 ml. of ethanol was refluxed on a water bath for 1 hr. after adjusting the pH to 5 with concentrated hydrochloric acid; a yellow precipitate then appeared. It was filtered and identified as isatin, m.p. 197°. The filtrate was concentrated and 20 ml. of acetone was added; a light yellow precipitate, 1.9 Gm. (75%), separated; on crystallization from ethanol–acetone mixture this yielded colorless crystals, m.p. 198–199°. A mixture melting point determination with the sample from *Method A* showed no depression. The infrared spectra of both samples were found to be identical.

[2] All melting points were determined in a Thomas-Hoover capillary melting point apparatus and are uncorrected. The infrared spectra were determined on a Perkin-Elmer model 137 G Infracord spectrophotometer.

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Keyphrases

Mannich reaction—mechanism
Bisamines, unsymmetrical—reaction effect
Methylenebisamine—reaction intermediate
Steric factors—Mannich reaction mechanism
IR spectrophotometry—identity

Spray-Congealed Formulations of Sulfaethylthiadiazole (SETD) and Waxes for Prolonged-Release Medication

Effect of Wax

By ANTHONY G. CUSIMANO* and CHARLES H. BECKER

Various spray-congealed SETD-wax products were prepared by pneumatic atomization using six waxes (white wax USP, glyceryl tristearate, carnauba wax, hydrogenated castor oil, cetyl alcohol NF, and glyceryl monostearate), two nozzle sizes, 0.20 and 0.25 cm. (0.08 and 0.10 in.) i.d., and three concentrations of the surfactant sorbitan monostearate (0, 1, and 5 percent w/w). The geometric mean diameters and the volume-surface diameters were affected primarily by the wax and the nozzle size used. All of the main effects, *i.e.*, wax, nozzle size, and concentration of surfactant, had a significant effect on the percent of SETD released in acid pepsin and alkaline pancreatin media. In general, the dissolution rate increased as the concentration of surfactant was increased. Faster dissolution rates were noted with formulations atomized through the smaller of the two nozzles. The most important factor that had an effect on the dissolution behavior of these products was the wax used in a particular formulation. This effect depends upon the following factors: the physical properties of the wax and the drug–wax particles, the chemical composition of wax, and the composition of the dissolution medium.

THE USE of spray-congealing techniques for modifying the physical, chemical, or physiological behavior of drugs has gained increasing attention within recent years. This process has proved to be an effective means of controlling the particle size of drug–wax particles. Therefore, spray congealing may be quite useful in controlling the dissolution behavior of drugs intended for prolonged-release medication.

In 1958, the utilization of this process was reported for the first time in the pharmaceutical literature (1). A sustained-release powder of sulfamethylthiadiazole (SMTD) was prepared by mixing the drug with molten hydrogenated castor oil and atomizing the resulting suspension. The same workers prepared a sustained-release powder of sulfaethylthiadiazole (SETD) by spray congealing a suspension of the drug using a centrifugal wheel atomizer (2).

Spray congealing has also been employed by others for enhancing the stability and palatability of such compounds as iron salts, vitamin A, and the B vitamins (3–7).

Scott *et al.* (8), were probably the first pharmaceutical investigators to conduct an extensive study on the production factors influencing the size and distribution of spray-congealed particles. In their work, stearic acid–ethylcellulose mixtures were spray congealed using a specially instrumented spray dryer and a centrifugal wheel atomizer. The variables studied included atomizer wheel speed, feed rate, and feed viscosity.

Recently, Cox (9) studied the effect of various production variables, *i.e.*, siphon height and atomizing pressure, on the particle size and dissolution behavior of spray-congealed particles of SETD-beeswax and SETD-glyceryl tristearate.[1] John and Becker (10) studied the effect of surfactant, *i.e.*, sorbitan monooleate, on the particle size and dissolution behavior of similar spray-congealed particles.

The purpose of this investigation was to determine the effect of several production and formulation variables on the particle size and dissolution behavior of various spray-congealed particles. This work was intended primarily to evaluate the effect of various waxes on the parameters studied. The experimental work conformed to a factorial design such that all of the possible combinations of the treatment variables were formulated and investigated.

EXPERIMENTAL

Materials—All materials used in this study, except some of the waxes, conformed to USP or NF specifications, or were of analytical reagent quality. The drug, sulfaethylthiadiazole (SETD) was obtained from American Cyanamid Co.

The six waxes used were white wax USP, glyceryl

Received January 8, 1968, from the College of Pharmacy, University of Florida, Gainesville, FL 32601

Accepted for publication April 19, 1968.

Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

Abstracted in part from a dissertation presented by Anthony G. Cusimano to the Graduate School of the University of Florida, Gainesville, in partial fulfillment of Doctor of Philosophy degree requirements.

The authors express their appreciation to Glyco Chemicals, Inc., for supplying the Glycowax S-932 and the Aldo MS, Edible used in this study, and to Dr. John I. Thornby, J. Hillis Miller Health Center, University of Florida, Gainesville, for his assistance in programming the dissolution data. The assistance of the University of Florida Computing Center is also gratefully acknowledged.

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[1] Marketed as Glycowax S-932 by Glyco Chemicals, Inc., New York, N. Y.

tristearate, carnauba wax, hydrogenated castor oil,[2] cetyl alcohol NF, and glyceryl monostearate,[3] and these were designated as W_1, W_2, W_3, W_4, W_5, and W_6, respectively.

The surfactant, sorbitan monostearate,[4] was used in some of the formulations in concentrations of 0, 1, and 5% w/w, and these were designated as S_0, S_1, and S_5, respectively. Sorbitan monostearate was used rather than some of the more commonly employed surfactants because it is a solid at room temperature. This would eliminate or reduce the tendency of the spray-congealed products to become tacky.

Preparation of Spray-Congealed SETD-Wax Particles—Spray-congealed formulations were prepared using the six waxes as the carrier for the drug SETD. All formulations contained about 25% SETD by weight. These were atomized through one of two external-mixing pneumatic nozzles[5] having orifice diameters of 0.20 and 0.25 cm., and the latter were designated as N_8 and N_{10}, respectively. The apparatus used has been described previously (10).

The wax for a particular formulation was melted in a stainless steel beaker which was immersed in an oil bath maintained at 100 ± 2°. The sorbitan monostearate, where included, was dissolved in the melted wax after which the SETD was added. Suspension of the SETD was maintained by means of a variable speed mixer. A Teflon siphon tube, wrapped with a heating tape, maintained the melted wax–drug mixture at the desired temperature (100 ± 2°) while it passed up to the pneumatic nozzle. Dry air was passed through the nozzle at a pressure of 40 p.s.i. This was sufficient to deliver the melted wax–drug mixture to the nozzle, and then to bring about atomization. A higher pressure could not be used since this would have caused the molten mixture to congeal on the sides of the collection chamber rather than within it. Since the air was not heated prior to entering the nozzle, the latter was equipped with a heater and a thermocouple monitoring system. Thus, the spraying operation could be performed without interruption due to clogging of the nozzle by melted wax–drug congealing inside. The nozzle orifice was directed into the collection chamber such that the particles of melted wax–drug congealed in the air. This resulted in a very fine powder which was collected and then passed through a No. 30 U.S. Standard sieve before conducting any of the subsequent experiments.

Particle-Size Measurements—Particle-size measurements were conducted on each of the 36 products using a microscopic method. The microscope was equipped with a calibrated ocular micrometer. Calibration was performed with a stage micrometer, and each division of the ocular micrometer was equivalent to 1.175 μ at a magnification of 10×.

In order to measure the individual particles, deagglomeration of the samples was necessary. Adequate deagglomeration was attained by briefly shaking a small amount of the sample in a test tube with an aqueous solution of polysorbate 80[6] and washed sand.

Assay of Spray-Congealed SETD-Wax Particles—Each product was formulated to contain about 25% SETD by weight. Chemical analyses were then performed on each product to determine the exact SETD content.

Each sample was dissolved in hot chloroform, and the SETD was extracted with five portions of 0.10 *N* HCl. The acid extract was then assayed for SETD content by the Bratton-Marshall (11) procedure using a Klett-Summerson photoelectric colorimeter with a No. 54 filter.

A standard curve for SETD was also prepared and was found to be valid up to a concentration of 2.0 mg.% in SETD. Appropriate blank and standard solutions were run with all samples assayed.

In Vitro Dissolution Studies—The dissolution behavior of all spray-congealed products was determined using a rotating-bottle apparatus (12), and a procedure similar to that of Robinson and Swintosky (2).

About 0.5 g. of the product, equivalent to 0.1200 g. of SETD, as determined by prior assay, was accurately weighed into 90-ml. screw-capped bottles. To each bottle 60 ml. of acid pepsin medium (pH 1.1) was added. Duplicate samples were allowed to rotate end over end at a speed of 46 r.p.m. in a water bath maintained at 37 ± 1°. Samples were removed at specified time intervals, *i.e.*, at 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, and 2 hr.

The samples were filtered, and an appropriate aliquot of the filtrate was then assayed for SETD content. If all of the SETD were released during the dissolution run, then the solution assayed would be equivalent to 2.0 mg.% in SETD.

Samples were also run for extended periods of time, *i.e.*, 24 and 48 hr., although equilibrium was not attained in acid medium.

Dissolution studies were also conducted in an alkaline environment in a similar manner. Again the equivalent of 0.1200 g. of SETD was weighed into each bottle, and 60 ml. of alkaline pancreatin medium (pH 8.3) was added. Samples were removed for assay at 0.25, 0.5, 0.75, 1.0, 2.0, 3.0, 4.0, and 6.0 hr. Studies were also conducted for a period of 12 hr. at which time equilibrium was attained in most of the samples.

RESULTS AND DISCUSSION

Manufacture of Spray-Congealed SETD-Wax Products—Most of the spray-congealed SETD-wax products were fine powders that initially were free flowing. The SETD-carnauba wax products were the best from an esthetic viewpoint since they were very fine, free-flowing powders that were not tacky. In addition, they showed no tendency to become lumpy in storage. The SETD-glyceryl monostearate products showed the greatest tendency to become lumpy.

The ease with which the products were sprayed depended primarily upon the wax and the nozzle size used. The production rates were greatest when white wax, glyceryl tristearate, and cetyl alcohol were used as the drug carrier. Formulations con-

[2] Marketed as Castorwax MP-70 by the Baker Castor Oil Co., Bayonne, N. J.
[3] Marketed as Aldo MS, Edible by Glyco Chemicals, Inc., New York, N. Y.
[4] Marketed as Span 60 by Atlas Chemical Industries, Inc., Wilmington, Del.
[5] Available from Spraying Systems Co., Bellwood, Ill.
[6] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.

taining glyceryl monostearate were the most difficult to spray, and these became increasingly difficult to spray as the concentration of sorbitan monostearate in the formulation was increased. This was due primarily to an increase in the viscosity of the melt when sorbitan monostearate was added. Sorbitan monostearate had little effect on the ease of production of the other formulations.

As one would expect, the larger nozzle (0.25 cm.) gave higher production rates for a given wax formulation.

Particle-Size Analysis—The particle sizes of the various spray-congealed SETD-wax products were found to be log-normally distributed. The particle-size distributions of the SETD-white wax products are shown in Fig. 1. These linear log-probability plots are typical of those obtained with all of the waxes used in this study. Other investigators have observed the log-normal distribution of spray-congealed SETD-wax particles (9, 10).

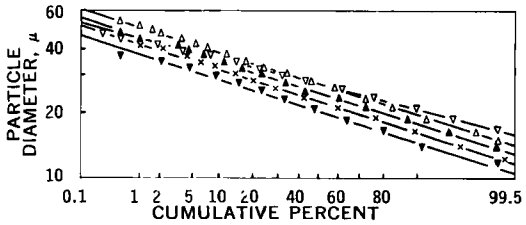

Fig. 1—Particle-size distributions by number of several spray-congealed SETD-white wax products. Key: ▼, $N_8S_0W_1$; ×, $N_8S_1W_1$; ▲, $N_8S_5W_1$; Δ, $N_{10}S_0W_1$; ▽, $N_{10}S_1W_1$.

TABLE I—ANALYSIS OF VARIANCE FOR GEOMETRIC MEAN DIAMETER ($\bar{d}$)

Source of Variation	*df*	*SS*	*MS*	*F* Value
Main effects				
W (Wax)	5	158.69	31.74	9.83[a]
N (Nozzle size)	1	190.85	190.85	59.09[a]
S (Sorbitan monostearate)	2	6.68	3.34	1.03
Interactions				
W × S	10	23.10	2.31	0.72
W × N	5	41.74	8.35	2.58[b]
N × S	2	4.76	2.38	0.74
Error	10	32.29	3.23	
Total	35	458.11		

[a] Significant at $p < 0.01$. [b] The wax–nozzle interaction was not significant at the 5% level.

The geometric mean diameters ($\bar{d}$) and the volume-surface diameters (d_{vs}) of these particles were affected primarily by the wax and the nozzle size used. Sorbitan monostearate had no significant effect on these parameters (Table I).

The smallest particles, in terms of the geometric mean diameter, were produced from the SETD-glyceryl monostearate formulations atomized through the 0.20-cm. nozzle. These averaged about 20 μ. The SETD-white wax and the SETD-glyceryl tristearate formulations gave the smallest particles from the 0.25-cm. nozzle. These averaged about 25–26 μ. The largest particles were produced from the SETD-cetyl alcohol and the SETD-hydrogenated castor oil formulations atomized through the 0.25-cm. nozzle (Table II).

The results obtained in this study are in qualitative agreement with those reported by Scott *et al.* (8). These workers found that the volume-surface diameter of spray-congealed stearic acid particles varied inversely with the feed viscosity. In the present study, the SETD-cetyl alcohol formulations gave the highest values for the volume-surface diameter, *i.e.*, about 32 μ for the 0.20-cm. nozzle, and about 42 μ for the 0.25-cm. nozzle. These formulations, prior to atomization, were observed to be the least viscous of all the waxes used. The SETD-glyceryl monostearate formulations were found to be the most viscous, and these gave relatively low values for the volume-surface diameter. This was particularly the case with the glyceryl monostearate formulations atomized through the 0.20-cm. nozzle where the volume-surface diameter was about 23 μ.

The wax and nozzle size used also had a significant effect on the other micromeritic parameters, *i.e.*, bulk density, percent porosity, and specific surface. Sorbitan monostearate had no significant effect on these parameters (Table III).

TABLE II—GEOMETRIC MEAN DIAMETERS ($\bar{d}$), GEOMETRIC STANDARD DEVIATIONS (σ), AND VOLUME-SURFACE DIAMETERS (d_{vs}) OF SEVERAL SPRAY-CONGEALED SETD-WAX PRODUCTS

Wax	Sample Symbol	Geometric Mean Diameter ($\bar{d}$), μ	Geometric Standard Deviation (σ)	Volume-Surface Diameter (d_{vs}), μ
Hydrogenated castor oil	$N_8S_0W_4$	25.4	1.2	28.1
	$N_8S_1W_4$	27.4	1.2	30.3
	$N_8S_5W_4$	23.4	1.2	25.6
	$N_{10}S_0W_4$	27.4	1.3	32.0
	$N_{10}S_1W_4$	33.7	1.3	38.2
	$N_{10}S_5W_4$	32.6	1.2	36.5
Cetyl alcohol	$N_8S_0W_5$	25.8	1.4	34.2
	$N_8S_1W_5$	25.2	1.4	32.6
	$N_8S_5W_5$	25.3	1.3	30.0
	$N_{10}S_0W_5$	32.7	1.4	43.8
	$N_{10}S_1W_5$	31.7	1.4	43.3
	$N_{10}S_5W_5$	28.5	1.4	39.1
Glyceryl monostearate, edible	$N_8S_0W_6$	19.3	1.2	21.8
	$N_8S_1W_6$	19.1	1.3	22.4
	$N_8S_5W_6$	20.7	1.3	23.9
	$N_{10}S_0W_6$	27.2	1.3	33.0
	$N_{10}S_1W_6$	27.4	1.3	32.3
	$N_{10}S_5W_6$	28.7	1.3	33.2

TABLE III—ANALYSIS OF VARIANCE FOR SPECIFIC SURFACE (S_w)

Source of Variation	*df*	*SS*	*MS*	*F* Value
Main effects				
W (Wax)	5	1,534,170	306,834	19.35[a]
N (Nozzle Size)	1	1,486,774	1,486,774	93.76[a]
S (Sorbitan monostearate)	2	39,947	19,974	1.26
Interactions				
W × S	10	137,458	13,746	0.87
W × N	5	359,716	71,943	4.54[a]
N × S	2	6,383	3,192	0.20
Error	10	158,567	15,857	
Total	35	3,723,015		

[a] Significant at $p < 0.01$.

The SETD-carnauba wax products were the least porous, and these gave relatively low values for the specific surface. In addition, the SETD-white wax products were the most porous, and these gave relatively high values for the specific surface. In general, products atomized through the smaller of the two nozzles gave higher percent porosities.

In Vitro Dissolution Behavior of Spray-Congealed SETD-Wax Products—The drug SETD is much more soluble in an alkaline medium than in an acid medium. Therefore, the dissolution rate was much less in acid pepsin medium than that in alkaline pancreatin medium. In all samples, the percent of SETD released in the alkaline medium was much greater than that in the acid medium during the same time interval (Table IV).

A greater amount of SETD was released during the initial 15-min. period than in any subsequent 15-min. period. The percent of SETD released, from samples containing no sorbitan monostearate, during the initial 15-min. period in acid pepsin medium may be taken as an estimate of the amount of drug partially exposed on the surface of the particles and any free drug present in the samples. However, in all samples, the percent of SETD released during the initial 15-min. period in alkaline pancreatin medium was greater than that in acid pepsin medium. This may be due to the nature of the medium itself, as well as to the solubility factor mentioned previously. The alkaline medium contains ox bile extract which is a powerful surfactant due to the presence of bile salts. Apparently, a significant amount of the spray-congealed particles are emulsified, disintegrated, or solubilized during the initial 15-min. exposure to the alkaline medium. A similar effect was noted, even in the acid medium, for those samples containing sorbitan monostearate.

All of the main effects, *i.e.*, wax, nozzle size, and concentration of sorbitan monostearate, had a significant effect on the percent of SETD released in both media (Table V).

Effect of Sorbitan Monostearate—In general, the dissolution rate increased significantly as the concentration of sorbitan monostearate was increased. This was noted with four of the six waxes used, *i.e.*, white wax, glyceryl tristearate, carnauba wax, and hydrogenated castor oil (Figs. 2 and 3). The surfactant lowers the interfacial tension between the drug–wax particles and the dissolution medium, particularly since some sorbitan monostearate may be extracted into the medium. Therefore, wetting of the drug–wax particles is promoted, and the amount of drug released increased with the concentration of surfactant. In addition, the dissolution medium can more readily enter the porous, waxy clusters and dissolve the drug within the matrix. The drug is then transported away from the matrix into the medium. Some of the drug–wax particles may actually be emulsified, disintegrated, or solubilized, allowing the drug to be more readily dissolved due to an increase in the total surface area available to the medium.

An unusual surfactant effect was noted in the case of the SETD-cetyl alcohol and the SETD-glyceryl

TABLE IV—*In Vitro* DISSOLUTION DATA FOR SETD-WHITE WAX PARTICLES CONTAINING 5% SORBITAN MONOSTEARATE AND ATOMIZED THROUGH a 0.20-cm. PNEUMATIC NOZZLE[a]

Time, Hr.	Colorimetric Readings of Two Determinations		Mg. SETD Released	% SETD Released	% Predicted Released
			Dissolution in Acid Pepsin Medium		
0.25	35	32	20.13	16.77	14.36
0.5	39	37	22.83	19.03	20.43
0.75	41	45	25.84	21.53	23.97
1.0	51	50	30.34	25.29	26.36
1.25	55	58	33.95	28.29	28.11
1.5	63	61	37.25	31.04	29.47
2	66	66	39.66	33.05	31.45
			Dissolution in Alkaline Pancreatin Medium		
0.25	86	85	51.37	42.81	40.96
0.5	109	112	66.39	55.33	56.61
0.75	128	125	76.01	63.34	65.14
1.0	142	143	85.62	71.35	70.61
2.0	159	163	96.74	80.61	81.34
3.0	171	175	103.95	86.62	86.03
4.0	180	181	108.45	90.38	88.73
6.0	187	184	111.46	92.88	91.78

[a] Sample number 19 ($N_8S_8W_1$); each sample weighed 0.4630 g.

TABLE V—ANALYSIS OF VARIANCE FOR PERCENT SETD RELEASED DURING INITIAL 15-MIN. PERIOD IN ALKALINE PANCREATIN MEDIUM

Source of Variation	*df*	*SS*	*MS*	*F* Value
Main effects				
W (Wax)	5	12,679.684	2,535.937	107.99[a]
N (Nozzle size)	1	391.314	391.314	16.66[a]
S (Sorbitan monostearate)	2	612.111	306.056	13.03[a]
Interactions				
W × S	10	833.060	83.306	3.55[b]
W × N	5	175.688	35.138	1.50
N × S	2	44.206	22.103	0.94
Error	10	234.832	23.483	
Total	35	14,970.895		

[a] Significant at $p < 0.01$. [b] The wax–sorbitan monostearate interaction was not significant at the 2.5% level.

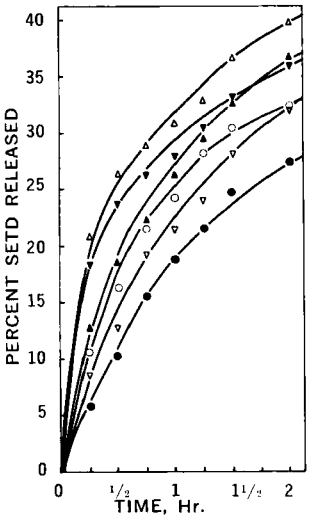

Fig. 2—In vitro *dissolution behavior of SETD-carnauba wax particles in acid pepsin medium. Key:* ▽, $N_8S_0W_3$; ▲, $N_8S_1W_3$; △, $N_8S_5W_3$; ●, $N_{10}S_0W_3$; ○, $N_{10}S_1W_3$; ▼, $N_{10}S_5W_3$.

monostearate particles, especially in acid pepsin medium. The amount of drug released from these particles decreased as the concentration of sorbitan monostearate increased (Fig. 4). This phenomenon may be explained in the following manner.

Although all of the waxes used in this study are insoluble in water, cetyl alcohol and glycerol monostearate differ from the others, with the exception of hydrogenated castor oil, in that they are somewhat hydrophilic. That is, cetyl alcohol and glyceryl monostearate are capable of being hydrated in an aqueous medium. The presence of sorbitan monostearate in the formulation reduces the tendency of these particles to become hydrated. This is to be expected since sorbitan monostearate has an HLB value of 4.7 and would make the drug–wax matrix more lipophilic than the one containing no sorbitan monostearate.

Effect of Nozzle Size—The nozzle used in atomizing the SETD-wax formulations also had a significant effect on the dissolution behavior of the spray-congealed particles. Faster dissolution rates were noted with formulations atomized through the 0.20-cm. nozzle than with the same formulations atomized through the 0.25-cm. nozzle.

This effect was apparently due to a difference in the total surface area of the particles prepared from

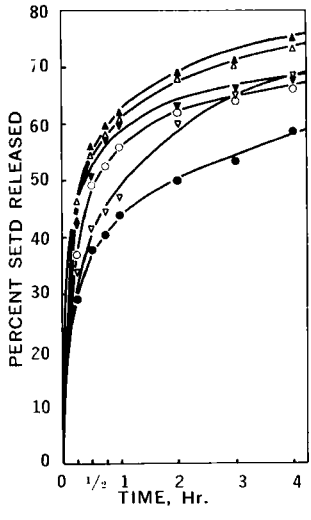

Fig. 3—In vitro *dissolution behavior of SETD-carnauba wax particles in alkaline pancreatin medium. Key:* ▽, $N_8S_0W_3$; ▲, $N_8S_1W_3$; △, $N_8S_5W_3$; ●, $N_{10}S_0W_3$; ○, $N_{10}S_1W_3$; ▼, $N_{10}S_5W_3$.

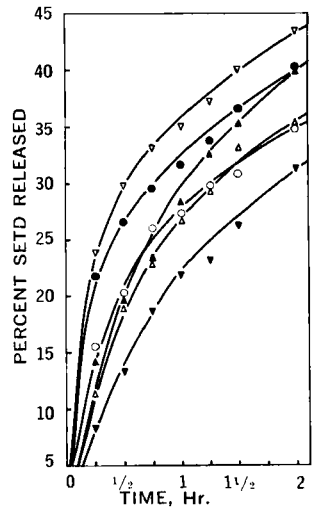

Fig. 4—In vitro *dissolution behavior of SETD-cetyl alcohol particles in acid pepsin medium. Key:* ▽, $N_8S_0W_5$; ▲, $N_8S_1W_5$; △, $N_8S_5W_5$; ●, $N_{10}S_0W_5$; ○, $N_{10}S_1W_5$; ▼, $N_{10}S_5W_5$.

the two nozzles. Particles having smaller volume-surface diameters, and therefore larger specific surfaces, were those prepared with the smaller of the two nozzles. For example, the SETD-white wax particles prepared with the 0.20-cm. nozzle had an average specific surface of 2,124 cm.²/g., whereas those prepared with the 0.25-cm. nozzle had an average specific surface of 1,858 cm.²/g. Similar results were obtained with the other waxes.

Effect of Wax—The most important factor that had an effect on the dissolution behavior of the spray-congealed SETD-wax particles was the wax used in a particular formulation. The effect of wax apparently depends upon the following factors: the physical properties of the wax and the drug–wax particles, the chemical composition of the wax, and the composition of the dissolution medium. For all waxes the amount of drug released after a certain time interval in alkaline pancreatin medium was greater than that in acid pepsin medium. This effect is due to three factors. The drug SETD is much more soluble in the alkaline medium than in the acid medium. Secondly, the alkaline medium can emulsify, disintegrate, or solubilize the particles much more readily. Finally, the constituents of these waxes, notably the fatty acids and fatty alcohols, would be expected to be more soluble in the alkaline medium. In addition, the fatty esters, present in some of the waxes, may be more readily degraded or hydrolyzed in the alkaline medium.

SETD-White Wax Particles—The SETD-white wax particles released between 10 to 33% of the drug after a 2-hr. exposure to acid pepsin medium. In alkaline pancreatin medium these particles released between 55 to 80% of the drug after 2 hr. and between 80 to 95% of the drug after 6 hr. Sorbitan monostearate had a significant effect in increasing the dissolution rate from these particles.

The primary constituents of white wax are cerotic acid and the fatty ester, myricyl palmitate. The fatty acid would be more soluble in the alkaline medium than in the acid medium. In addition, the ester may be more readily hydrolyzed in the alkaline medium. These factors would facilitate the disintegration of these particles and increase the dissolution rate in the alkaline medium.

SETD-Glyceryl Tristearate—The SETD-glyceryl tristearate particles released between 20 to 42% of the drug after 2 hr. in acid pepsin medium. In alkaline pancreatin medium these particles released between 25 to 60% of the drug after 2 hr. and between 38 to 80% of the drug after 6 hr. Again sorbitan monostearate significantly increased the amount of drug released after a certain time interval.

The dissolution rate was greater in the alkaline medium than in the acid medium. It is possible that the ester is more susceptible to alkaline-catalyzed hydrolysis than to acid-catalyzed hydrolysis.

In general, the SETD-glyceryl tristearate particles gave the slowest dissolution rates of the six waxes used. This was the case in both media, and it is not surprising since glyceryl tristearate consists essentially of the triglyceride ester of stearic acid. Triglyceride esters of fatty acids are less susceptible to either acid- or alkaline-catalyzed hydrolysis than are the corresponding di- or monoglyceride esters.

However, the SETD-glyceryl tristearate particles released more drug in the acid medium than the SETD-white wax particles, whereas the former released less drug in the alkaline medium. This effect is also believed to be due to the chemical composition of the two waxes. White wax contains a high percentage of free fatty acids, notably cerotic acid. These fatty acids are essentially insoluble in the acid medium, but are readily soluble in the alkaline medium. Glyceryl tristearate contains no free fatty acids, but does contain a high percentage of glyceryl tristearate. The latter is slowly hydrolyzed in acid and alkaline media, and therefore the glyceryl tristearate particles disintegrate quite slowly. In addition, these particles were not readily wetted by the dissolution media.

SETD-Carnauba Wax Particles—The SETD-carnauba wax particles released between 27 to 40% of the drug after 2 hr. in acid pepsin medium (Fig. 2). In alkaline pancreatin medium these particles released between 50 to 70% of the drug after 2 hr. (Fig. 3) and between 65 to 78% of the drug after 6 hr. Sorbitan monostearate increased the amount of drug released from these particles in both media.

Carnauba wax consists chiefly of myricyl cerotate with smaller amounts of myricyl alcohol, ceryl alcohol, and cerotic acid. Its fatty alcohol and fatty acid content probably accounts, in part, for the higher dissolution rates in the alkaline medium.

Again it was noted, just as with the SETD-glyceryl tristearate particles, that the SETD-canauba wax particles released more drug in acid pepsin medium than the SETD-white wax particles, whereas the former released less drug in alkaline pancreatin medium than the SETD-white wax particles. Carnauba wax contains a lower percentage of free fatty acids (acid value from 2 to 10) than white wax (acid value from 17 to 24), but the former contains a somewhat higher percentage of fatty esters (ester value from 75 to 85) than white wax (ester value from 72 to 79).

For the same reason, the SETD-carnauba wax particles released more drug in the alkaline medium than the SETD-glyceryl tristearate particles since glyceryl tristearate contains no free fatty acids.

SETD-Hydrogenated Castor Oil Particles—The SETD-hydrogenated castor oil particles released between 35 to 48% of the drug after 2 hr. in acid pepsin medium. In alkaline pancreatin medium these particles released between 75 to 92% of the drug after 2 hr. and between 92 to 100% of the drug after 6 hr. Sorbitan monostearate also increased the amount of drug released from these particles in both media.

Hydrogenated castor oil consists primarily of partially hydrogenated glyceryl triricinoleate. It contains very little free fatty acids (acid value of 2) but a significant amount of hydroxyl groups (hydroxyl value of 158). A relatively large proportion of its fatty ester content is in the form of triglycerides of unsaturated fatty acids (iodine value of 38). These factors may account, in part, for the observed dissolution behavior of these particles.

The hydroxyl groups in this wax make it somewhat hydrophilic such that the particles were easily wetted, particularly in the alkaline medium. This would also account for the relatively high dissolution rates in the acid medium. The latter effect is also due to the relatively low free fatty acid content. In addition, the unsaturated fatty esters present in this wax are apparently more susceptible to acid- and

alkaline-catalyzed hydrolysis than the saturated fatty esters present in some of the other waxes.

SETD-Cetyl Alcohol Particles—The SETD-cetyl alcohol particles released between 32 to 44% of the drug after 2 hr. in acid pepsin medium (Fig. 4). In alkaline pancreatin medium these particles released between 85 to 93% of the drug after 2 hr. and between 90 to 96% of the drug after 6 hr. (Fig. 5). In these products the amount of drug released decreased as the concentration of sorbitan monostearate increased.

The official alcohol is a mixture of solid, fatty alcohols consisting chiefly of cetyl alcohol. Although it is insoluble in water, it is capable of being hydrated. Thus, of the waxes used in this study, cetyl alcohol is one of the most hydrophilic. Glyceryl monostearate and hydrogenated castor oil are also somewhat hydrophilic. This property is evident from the fact that these particles were readily wetted by the dissolution media, particularly in the alkaline medium.

Nearly complete removal of the drug was attained from these particles after about 6 hr. in the alkaline medium. The dissolution rates in the acid medium were relatively high also, that is, compared with the SETD-white wax and the SETD-glyceryl tristearate particles. The latter effect is due to the hydrophilic character of cetyl alcohol and also to the fact that this material contains no free fatty acids or fatty esters.

SETD-Glyceryl Monostearate Particles—The SETD-glyceryl monostearate particles released between 45 to 51% of the drug after 2 hr. in acid pepsin medium. In alkaline pancreatin medium these particles released between 83 to 97% of the drug after 2 hr. and between 86 to 100% of the drug after 6 hr. In these products the amount of drug released decreased slightly as the concentration of sorbitan monostearate increased. This phenomenon was not as pronounced as in the case of the SETD-cetyl alcohol particles.

Glyceryl monostearate is a wax-like ester. It has an acid value of less than 5, and is somewhat hydrophilic since each molecule of the pure ester contains two hydroxyl groups. For this reason, this material was readily wetted in both media. The dissolution behavior of these particles was quite similar to that of the SETD-cetyl alcohol particles since both are somewhat hydrophilic. They differ in that the SETD-glyceryl monostearate particles released more drug in the acid medium.

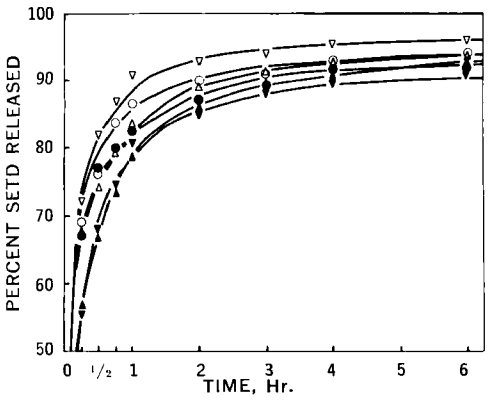

Fig. 5—In vitro *dissolution behavior of SETD-cetyl alcohol particles in alkaline pancreatin medium. Key:* ▽, $N_8S_0W_5$; ▲, $N_8S_1W_5$; △, $N_8S_5W_5$; ●, $N_{10}S_0W_5$; ○, $N_{10}S_1W_5$; ▼, $N_{10}S_5W_5$.

It is interesting to note the tremendous difference in the dissolution behavior between these particles and the SETD-glyceryl tristearate particles. In both media, the SETD-glyceryl monostearate particles released significantly greater amounts of the drug than the SETD-glyceryl tristearate particles. This may have been due to the combined effect of the following factors: the hydrophilic character of glyceryl monostearate, the fact that the SETD-glyceryl monostearate particles can be readily emulsified or disintegrated, and that glyceryl monostearate may be more readily hydrolyzed in acid and alkaline media than glyceryl tristearate.

In Vitro Dissolution Model for Spray-Congealed SETD-Wax Products—The dissolution data obtained in this investigation did not give a linear relationship when plotted in accordance with existing theories (13, 14). For example, a plot of the percent of SETD remaining in the particles ($C_e - C$) *versus* time was not linear. This would indicate that the dissolution kinetics were not zero order. In addition, a plot of the logarithm of the percent of SETD remaining in the particles *versus* time was not linear. Therefore, the dissolution process did not conform to first-order kinetics either. In accordance with the theory of Higuchi (14), the percent of SETD remaining in the particles was also plotted *versus* the square root of time. Again nonlinear relationships were obtained for all of the waxes used.

The nonlinear relationships obtained are not surprising in view of the assumptions involved in these theories. The most important assumption in both the Noyes-Whitney (13) dissolution rate law and the theory of Higuchi (14) is that the total surface area of the tablet or particles does not change significantly during the dissolution run. Therefore, it is presumed that the drug–wax matrix is completely inert or unaffected by the dissolution medium. However, this was not the case in the present study. For all of the waxes used, the particles were emulsified, disintegrated, or solubilized by the dissolution medium. This was particularly true in the alkaline medium.

A dissolution model previously proposed by Cox (9) was utilized in the present study. This model is represented by the equation

$$C = C_e[1 - (1 + t/d)^{-c}] \qquad \text{(Eq. 1)}$$

where C is the percent of drug in solution at a particular time (t), C_e is the percent of drug in solution at equilibrium, and c and d are constants for a particular formulation. This dissolution model is not a linear function of the unknown parameters c, d, and C_e. Therefore, a nonlinear least-squares curve fitting procedure was used to estimate the above parameters. Different values for c, d, and C_e were used in a computer program, and the estimated percent of drug in solution ($\hat{C}$) at a particular time (t) was calculated. The experimental values of the percent of drug in solution (C) at a particular time (t) were compared with the estimated values. The quantity $\Sigma\ (C - \hat{C})^2$ was minimized by adjusting the values of the three parameters, c, d, and C_e (Table VI).

TABLE VI—ESTIMATED[a] PARAMETERS FOR DISSOLUTION MODEL IN ACID PEPSIN MEDIUM

Wax	Sample Symbol	$C = C_e[1 - (1 + t/d)^{-c}]$ C_e	c	d	SS for Error	Instantaneous Dissolution Rate, $(c/d)C_e$
White wax	$N_8S_0W_1$	37.81	0.6630	1.7350	4.26	14.45
	$N_8S_1W_1$	46.68	0.3054	0.1894	16.09	75.27
	$N_8S_5W_1$	45.60	0.5160	0.2311	21.66	101.82
	$N_{10}S_0W_1$	45.00	0.3732	1.5000	14.65	11.20
	$N_{10}S_1W_1$	33.84	0.5669	0.5495	5.72	34.91
	$N_{10}S_5W_1$	69.85	0.1181	0.0537	2.20	153.60
Glyceryl tristearate	$N_8S_0W_2$	69.77	0.1971	0.1644	8.83	83.65
	$N_8S_1W_2$	66.27	0.2317	0.0809	9.76	189.80
	$N_8S_5W_2$	67.16	0.2389	0.0448	24.04	358.14
	$N_{10}S_0W_2$	76.00	0.1958	0.4845	7.59	30.71
	$N_{10}S_1W_2$	68.61	0.2039	0.1228	19.22	113.92
	$N_{10}S_5W_2$	75.53	0.1754	0.0366	14.75	361.97
Carnauba wax	$N_8S_0W_3$	45.30	2.8258	3.7450	4.93	34.18
	$N_8S_1W_3$	47.97	1.4304	1.2591	7.84	54.50
	$N_8S_5W_3$	54.24	0.4260	0.1341	13.64	172.31
	$N_{10}S_0W_3$	42.22	4.1761	6.6859	1.75	26.37
	$N_{10}S_1W_3$	46.91	1.0864	0.9886	3.13	51.55
	$N_{10}S_5W_3$	53.23	0.3834	0.1383	7.23	147.57
Hydrogenated castor oil	$N_8S_0W_4$	85.36	0.1664	0.0344	7.65	412.90
	$N_8S_1W_4$	81.04	0.1716	0.0172	3.35	808.52
	$N_8S_5W_4$	77.43	0.2012	0.0179	3.84	870.33
	$N_{10}S_0W_4$	86.80	0.1885	0.1338	3.95	122.29
	$N_{10}S_1W_4$	73.36	0.2726	0.0933	2.44	214.34
	$N_{10}S_5W_4$	75.06	0.2264	0.0284	1.91	598.37
Cetyl alcohol	$N_8S_0W_5$	64.39	0.3179	0.0808	13.37	253.34
	$N_8S_1W_5$	53.50	1.2642	1.1429	14.90	59.18
	$N_8S_5W_5$	58.40	0.5815	0.5311	13.08	63.94
	$N_{10}S_0W_5$	71.23	0.2144	0.0605	18.57	252.42
	$N_{10}S_1W_5$	74.59	0.2396	0.1678	48.77	106.51
	$N_{10}S_5W_5$	75.00	0.3151	0.5000	47.28	47.27
Glyceryl monostearate edible	$N_8S_0W_6$	78.23	0.1705	0.0056	14.75	2,381.82
	$N_8S_1W_6$	83.80	0.1420	0.0071	4.76	1,676.00
	$N_8S_5W_6$	60.06	0.3041	0.0216	5.40	845.57
	$N_{10}S_0W_6$	68.27	0.2518	0.0154	17.24	1,116.26
	$N_{10}S_1W_6$	68.83	0.2060	0.0086	6.02	1,648.72
	$N_{10}S_5W_6$	57.65	0.4379	0.0590	6.02	427.88

[a] Modified Gauss-Newton least-squares method (15).

Specifically, the procedure used in performing the nonlinear least-squares fit was a modified Gauss-Newton least-squares method (15). Setting up of the computer program and subsequent operations with the computer were performed in cooperation with the University of Florida Computing Center using an IBM 360 computer.

Equation 1 can be shown to be equivalent to

$$\log (C_e - C) = \log C_e + c \log d - c \log (t + d) \quad \text{(Eq. 2)}$$

Therefore, a plot of the logarithm of the percent of drug remaining in the particles $(C_e - C)$ *versus* the logarithm of $(t + d)$ should give a straight line.

In general, the agreement between the experimental and the predicted values for the dissolution data was good. The experimental data for the dissolution of SETD from the SETD-carnauba wax particles in acid pepsin medium are plotted on the lines predicted from the dissolution model in Fig. 6. The plot of the logarithm of the percent of SETD remaining in the particles *versus* the logarithm of $(t + d)$ was found to be reasonably linear. A similar plot for the dissolution of SETD from the SETD-cetyl alcohol particles in alkaline pancreatin medium is shown in Fig. 7. Again a reasonably good linear relationship was obtained.

Thus, the data obtained in this investigation, both in acid pepsin and in alkaline pancreatin media, agreed reasonably well with those predicted from a dissolution model in accordance with Eq. 1.

However, it should be pointed out that no correlation could be demonstrated between the instantaneous dissolution rates, *i.e.*, $(c/d)C_e$, and the parameters obtained from the particle-size studies, notably the specific surface. A possible explanation for this is as follows. In the particle-size studies, only individual particles were measured and not clusters of particles. In the samples used for the dissolution studies, many clusters of particles were also present in addition to the individual, spherical

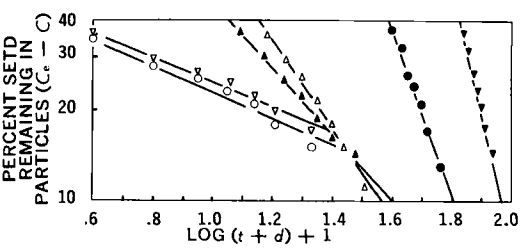

Fig. 6—Experimental data for percent SETD released from SETD-carnauba wax particles in acid pepsin medium, plotted on lines predicted from dissolution model. Key: ●, $N_8S_0W_3$; △, $N_8S_1W_3$; ○, $N_8S_5W_3$; ▼, $N_{10}S_0W_3$; ▲, $N_{10}S_1W_3$; ▽, $N_{10}S_5W_3$.

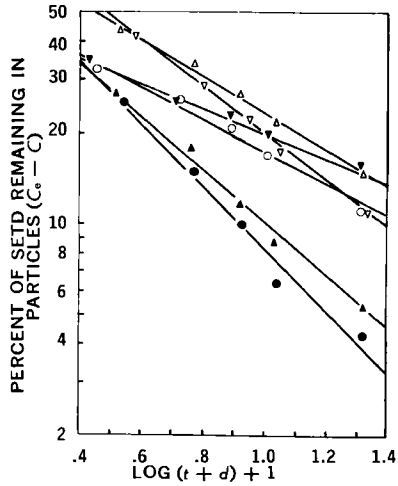

Fig. 7—Experimental data for percent SETD released from SETD-cetyl alcohol particles in alkaline pancreatin medium, plotted on lines predicted from dissolution model. Key: ●, $N_8S_0W_5$; Δ, $N_8S_1W_5$; ○, $N_8S_5W_5$; ▼, $N_{10}S_0W_5$; ▲, $N_{10}S_1W_5$; ▽, $N_{10}S_5W_5$.

particles. This would be the case particularly for those products that were somewhat tacky. Another factor is that many of the spray-congealed SETD-wax products were not easily wetted by the dissolution medium, at least initially. Some of the particles tended to agglomerate upon coming in contact with the dissolution medium, particularly in acid pepsin medium. As the dissolution process continued, these agglomerates were subsequently emulsified, disintegrated, or solubilized as were the individual particles.

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Keyphrases

Prolonged release dosage forms—spray congealed
Sulfaethylthiadiazole–wax formulations
Spray-congealed products—*in vitro* dissolution model
Particle size—wax, nozzle size effect
Dissolution rates—parameters affecting

Tumor-Inhibitory Activity of Pyrrolizidine Alkaloids

By C. C. J. CULVENOR

Eighteen pyrrolizidine alkaloids and several derivatives have been examined for tumor-inhibitory properties. Of these, 10 compounds show significant activity against one or more test tumors. Heliotrine, lasiocarpine, monocrotaline, spectabiline, and senecionine are highly active against the Walker 256 (intramuscular) system. The activity pattern is consistent with the tumor-inhibitory action being associated with the allylic ester function which imparts alkylating ability and is also responsible for hepatotoxicity.

PYRROLIZIDINE ALKALOIDS are found typically in species of *Senecio* (family *Compositae*, tribe *Senecioneae*), *Crotalaria* (family *Leguminosae*, tribe *Genistae*) and the subfamilies *Heliotropioideae* and *Boraginoideae* of the family *Boraginaceae* (1). They occur also in other genera of the tribes *Senecioneae* [*e.g.*, *Cacalia*, *Emilia* (2), *Erechtites*] and *Genistae* [*e.g.*, *Adenocarpus* (3), *Cytisus* (4)] as well as in the unrelated genera *Eupatorium* (*Compositae*) (5), *Thesium* (*Santalaceae*) (6), *Planchonella* (*Sapotaceae*) (7), *Lolium* (8), and *Festuca* (9) (*Gramineae*). The ability of some alkaloids of the group to cause an unusual chronic liver disease in animals (10) has promoted extensive study of their chemical and toxicological properties (*e.g.*, *References 1, 11, 12*). Platyphylline (I) (13) has no hepatotoxic action and has found use in the U.S.S.R. as an antispasmodic.

Received May 15, 1967, from the Division of Applied Chemistry, C.S.I.R.O. Chemical Research Laboratories, Melbourne, Australia.
Accepted for publication October 9, 1967.
This work has been carried out partly in collaboration with Cancer Chemotherapy National Service Center, Bethesda, Md., and partly in collaboration with Abbott Laboratories, North Chicago, Ill. Thanks are due to both organizations for permission to publish the screening results.

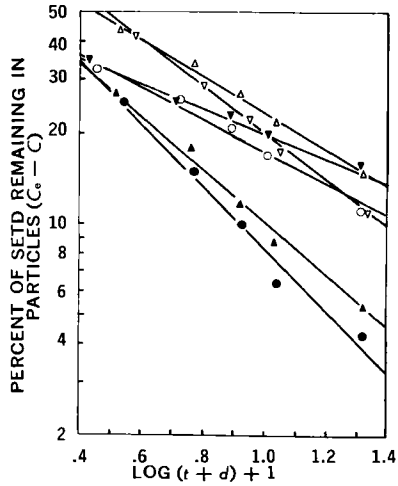

Fig. 7—Experimental data for percent SETD released from SETD-cetyl alcohol particles in alkaline pancreatin medium, plotted on lines predicted from dissolution model. Key: ●, $N_8S_0W_5$; Δ, $N_8S_1W_5$; ○, $N_8S_5W_5$; ▼, $N_{10}S_0W_5$; ▲, $N_{10}S_1W_5$; ▽, $N_{10}S_5W_5$.

particles. This would be the case particularly for those products that were somewhat tacky. Another factor is that many of the spray-congealed SETD-wax products were not easily wetted by the dissolution medium, at least initially. Some of the particles tended to agglomerate upon coming in contact with the dissolution medium, particularly in acid pepsin medium. As the dissolution process continued, these agglomerates were subsequently emulsified, disintegrated, or solubilized as were the individual particles.

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Keyphrases

Prolonged release dosage forms—spray congealed
Sulfaethylthiadiazole–wax formulations
Spray-congealed products—*in vitro* dissolution model
Particle size—wax, nozzle size effect
Dissolution rates—parameters affecting

Tumor-Inhibitory Activity of Pyrrolizidine Alkaloids

By C. C. J. CULVENOR

Eighteen pyrrolizidine alkaloids and several derivatives have been examined for tumor-inhibitory properties. Of these, 10 compounds show significant activity against one or more test tumors. Heliotrine, lasiocarpine, monocrotaline, spectabiline, and senecionine are highly active against the Walker 256 (intramuscular) system. The activity pattern is consistent with the tumor-inhibitory action being associated with the allylic ester function which imparts alkylating ability and is also responsible for hepatotoxicity.

PYRROLIZIDINE ALKALOIDS are found typically in species of *Senecio* (family *Compositae*, tribe *Senecioneae*), *Crotalaria* (family *Leguminosae*, tribe *Genistae*) and the subfamilies *Heliotropioideae* and *Boraginoideae* of the family *Boraginaceae* (1). They occur also in other genera of the tribes *Senecioneae* [*e.g.*, *Cacalia*, *Emilia* (2), *Erechtites*] and *Genistae* [*e.g.*, *Adenocarpus* (3), *Cytisus* (4)] as well as in the unrelated genera *Eupatorium* (*Compositae*) (5), *Thesium* (*Santalaceae*) (6), *Planchonella* (*Sapotaceae*) (7), *Lolium* (8), and *Festuca* (9) (*Gramineae*). The ability of some alkaloids of the group to cause an unusual chronic liver disease in animals (10) has promoted extensive study of their chemical and toxicological properties (*e.g.*, *References 1, 11, 12*). Platyphylline (I) (13) has no hepatotoxic action and has found use in the U.S.S.R. as an antispasmodic.

Received May 15, 1967, from the Division of Applied Chemistry, C.S.I.R.O. Chemical Research Laboratories, Melbourne, Australia.
Accepted for publication October 9, 1967.
This work has been carried out partly in collaboration with Cancer Chemotherapy National Service Center, Bethesda, Md., and partly in collaboration with Abbott Laboratories, North Chicago, Ill. Thanks are due to both organizations for permission to publish the screening results.

The hepatotoxic alkaloids have an irreversible, antimitotic effect on liver cells which leads to grossly enlarged cells and cell nuclei (megalocytosis). They cause chromosome breakage (14) and are mutagenic (15). These properties suggested possible antitumor activity, and in 1958, several alkaloids from this laboratory were screened by the Cancer Chemotherapy National Service Center against sarcoma 180, lymphoid leukemia L1210, and Ehrlich ascites cell culture. Weak activity against sarcoma 180 was observed with heliotrine, but heliotrine *N*-oxide, seneciphylline, sarracine *N*-oxide, jaconine hydrochloride, lasiocarpine, and retronecine hydrochloride were inactive. About the same time, Pukhal'skaya *et al.* (16) also investigated the action of heliotrine, viridiflorine, platyphylline, seneciphylline, lasiocarpine, and heliosupine on a transplanted hepatoma in mice, Ehrlich's adenocarcinoma, and sarcoma 45. The hepatoma was inhibited to the extent of 21% by lasiocarpine, 10% by seneciphylline, and 8% by heliotrine. The Ehrlich's tumor was inhibited 54% by heliosupine and 19% by seneciphylline. Sarcoma 45 was inhibited 28% by heliosupine and 17% by lasiocarpine.

More extensive testing has since been undertaken in response to two developments. First, it was recognized that the hepatotoxic alkaloids, which conform to the general formula (II), behave chemically as alkylating agents and that several of their biological properties are those of alkylating agents (17). This provided further rationale for possible antitumor action, and in particular, suggested testing against the Walker 256 tumor systems which are sensitive to alkylating agents (18). Second, a high level of activity was observed in monocrotaline (III), which was isolated as the tumor-inhibitory constituent of *Crotalaria spectabilis* (19). The main activity was against adenocarcinoma 755, the highest degree of inhibition achieved being 95% at a dose of 90 mg./Kg. Further testing by CCNSC disclosed activity against plasmacytoma, but in many other systems monocrotaline was inactive (20). This paper reports results of testing of 18 alkaloids, three alkaloid *N*-oxides, and two derivatives from this laboratory and discusses the structure-activity pattern within the series in relation to tumor-inhibitory activity.

EXPERIMENTAL

Alkaloids—The alkaloids examined were either available from other studies or were reisolated by the techniques previously employed. They are crispatine (21), echinatine (22), europine (23), fulvine (21), heleurine (23), heliotrine (24), jacobine (25), jaconine hydrochloride (25), lasiocarpine (24), 1-methylenepyrrolizidine (26), 1-methoxymethyl-1,2-epoxypyrrolizidine (27, 28), 1-methoxymethyl-7β-hydroxy-1,2-dehydropyrrolizidine (28), monocrotaline (29), senecionine (30), seneciphylline (25), spectabiline (31), and supinine (23).

Alkaloid N-Oxides—Sarracine *N*-oxide was isolated directly from *Senecio mikanioides* Otto (32). Heliotrine *N*-oxide and monocrotaline *N*-oxide were prepared by oxidation of the parent alkaloids as described previously (*References 24* and *29*, respectively).

Derivatives—Retronecine hydrochloride was obtained by hydrolysis of monocrotaline using the procedure described by Culvenor and Smith (31). 1 - Chloromethyl - 7α - hydroxy - 1,2 - dehydropyrrolizidine was prepared by reaction of heliotridine with thionyl chloride as described previously (33).

Testing Procedure—Testing was carried out in accordance with the Cancer Chemotherapy National Service Center protocols (34). The dosing regimens are as follows: adenocarcinoma 755, in mice, one dose daily for 11 days; lymphoid leukemia L1210, in mice, one dose daily until death; sarcoma 180, in mice, one dose daily for 7 days; Walker 256 (intramuscular), in rats, one dose daily for 4 days; Walker 256 (subcutaneous), in rats, one dose daily for 5 days, by dose response at 4 doses; and plasmacytoma 1, in hamsters, one dose daily for 15 days. The criterion for significant activity accepted by CCNSC is at least 58% decrease in tumor size compared with controls for solid tumors, and 25% increase in survival time for leukemia 1210 and 50% inhibition of KB cell culture at a concentration of 1 mcg./ml.

Results—The results obtained are summarized in Table I, in which plus and minus signs indicate whether the level of inhibition in the particular tumor system meets the criteria given in the preceding paragraph. Detailed results are given for the active compounds in Table II, replicates being omitted except where it seemed advisable to indicate a variability or reproducibility in the level of action.

DISCUSSION

It is apparent from Table I that tumor-inhibitory activity is widely exhibited among pyrrolizidine alkaloids, 10 of 23 compounds tested being active at a significant level against one or more tumor systems. The tumors most frequently inhibited were the Walker 256 (intramuscular) and adenocarcinoma 755, the level of activity against the former being in some cases very high. Thus complete or nearly complete destruction of this tumor was observed with heliotrine (IV) at a dose level of 125 mg./Kg., and T/C values[1] below 30 were attained at fairly low dose levels with fulvine (V), heliotrine *N*-oxide (VI), lasiocarpine (VII), monocrotaline (III), spectabiline (VIII), and senecionine (IX).

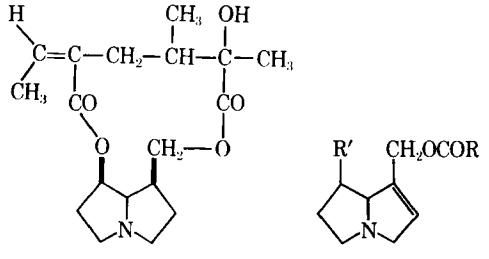
Against adenocarcinoma 755, the strongest activity is found in the bases of monocrotaline type, monocrotaline, spectabiline, and fulvine, and in the chloro-derivative (X); crispatine (XI), heliotrine, and supinine (XII) exhibit weak activity. Heliotrine, lasiocarpine, monocrotaline, and the chloro-

[1] T/C is the ratio of tumor weight or survival time of test animals to control animals, expressed as percent.

TABLE I—ANTITUMOR SCREENING RESULTS[a]

Alkaloid	NSC No.	CA	LE	SA	WM	WA	KB
Crispatine (XI)	89933	+	−	−	−	−	−
Echinatine (XVIII)	89937	−	−		−	−	−
Europine (XIII)	89939					−	−
Fulvine (V)	89932	+	−	−	+	+	−
Heleurine (XIV)	89940	−	−	−	−	−	−
Heliotrine (IV)	30620	+		+	+	−	−
Heliotrine *N*-oxide (VI)	30621	−	−	−	+		−
Jacobine (XVI)	89936		−		−		−
Jaconine (XVII) hydrochloride	30624		−	−	−		−
Lasiocarpine (VII)	30625		−	+	+	+	−
1-Methylenepyrrolizidine (XIX)	89941		−		−	−	
1-Methoxymethyl-1,2-epoxypyrrolizidine (XX)	89944						−
1-Methoxymethyl-7β-hydroxy-1,2-dehydropyrrolizidine (XXI)	89942						−
Monocrotaline (III)	28693	+	−	+	+	−	−
Monocrotaline *N*-oxide (XXV)	108378	−	−				
Sarracine *N*-oxide (XXIII)	30623	−	−	−	−		
Senecionine (IX)	89935	−	−		+	−	−
Seneciphylline (XV)	30622	−	−	−	−	−	−
Spectabiline (VIII)	89934	+	−		+	+	−
Supinine (XII)	89938	+	−	−	−	−	−
Retronecine (XXII) hydrochloride	30618		−	−			
1-Chloromethyl-7α-hydroxy-1,2-dehydropyrrolizidine (X)		+		+			−

[a] The tumor systems are CA, adenocarcinoma 755; LE, lymphoid leukemia L1210; SA, sarcoma 180; WM, Walker 256 intramuscular; WA, Walker 256 subcutaneous; KB, cell culture.



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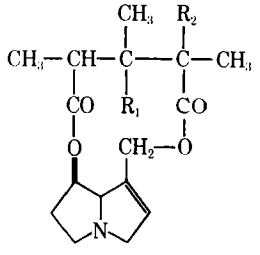
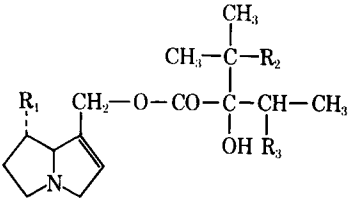
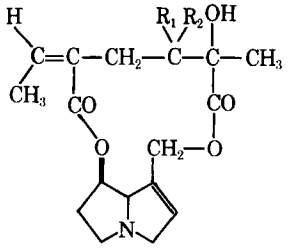
II



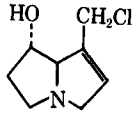
III, $R_1 = OH$, $R_2 = OH$
V, XI, $R_1 = OH$, $R_2 = H$
VIII, $R_1 = OCOCH_3$, $R_2 = OH$
XXV, III with N→O in place of N



IV, $R_1 = OH$, $R_2 = H$, $R_3 = OCH_3$
VI, IV with N→O in place of N
VII, $R_1 = CH_3-CH=C(CH_3)-CO-O$, $R_2 = OH$, $R_3 = OCH_3$
XII, $R_1 = H$, $R_2 = H$, $R_3 = OH$
XIII, $R_1 = OH$, $R_2 = OH$, $R_3 = OCH_3$
XIV, $R_1 = H$, $R_2 = H$, $R_3 = OCH_3$
XVIII, $R_1 = OH$, $R_2 = H$, $R_3 = OH$



IX, $R_1 = H$, $R_2 = CH_3$
XV, $R_1R_2 = CH_2$



X

derivative were active against sarcoma 180, and lasiocarpine, spectabiline, and fulvine were active (the last two only weakly) against the Walker 256 (subcutaneous) system. Activity was essentially confined to the solid tumors, all compounds being inactive against leukemia 1210 and KB cell culture, except that heliotrine, lasiocarpine, europine (XIII) and heleurine (XIV), evinced weak activity against the latter (ED_{50} 15–23 mcg./ml.) at a level lower than the CCNSC minimum standard (ED_{50} 1 mcg./ml.).

The active alkaloids are all of the allylic ester type which have a potential for alkylation (17). Of the bases of monocrotaline type (11-membered ring diesters), monocrotaline and spectabiline are highly active against WM and CA, fulvine of moderate activity, and crispatine of weak activity overshadowed by a high toxicity. The alkaloids of senecionine type (12-membered ring diesters) also appear to be generally of too high toxicity to the host to permit expression of activity against the

TABLE II—TUMOR-INHIBITORY ACTIVITY OF PYRROLIZIDINE ALKALOIDS[a]

Test System	Dose, mg./Kg.	Survivors	Animal Wt. Diff., T-C(g.)	T/C,[b] %	Tester[c]
Crispatine					
CA	90	0/6		Toxic	A
	45	0/6		Toxic	
	22.5	6/6	−1.3	50	
LE	20	6/6	−1.2	96	C
WM	20	5/6	−10	62	
	75	1/6	−5		
	50	0/6			
	25	2/6	−20		
	12.5	5/6	−10	104	
WA	3.5	6/6	−18	61	C
KB				(>100)[d]	C
Fulvine					
CA	90	0/6		Toxic	A
	45	5/6	−0.8	36	
	22.5	6/6	−1.6	56	
LE	400	4/4	−1.5	104	C
	200	4/4	0.8	98	
	100	4/4	0.7	98	
	50	4/4	0.4	98	
	50	6/6	0.0	112	
WM	200	4/6	−12	44	
	150	4/6	−9	49	
	100	5/6	−1	29	
	50	1/6	9		
	50	6/6	7	65	
	50	6/6	−2	26	
WA	3.8	6/6	−11	49	C
KB				(>100)[d]	
Heliotrine					
CA	90	4/5	−2.6	45	A
	45	5/5	−1.2	60	
	22.5	5/5	−0.7	104	
SA	125	5/6	−1.4	47	C
	125	6/6	−1.8	87	
WM	188	1/6	−5		
	125	6/6	−9	4	C
	125	5/6	−6	10	
	125	5/6	2	4	
	125	6/6	7	0	
	125	6/6	−7	5	
	125	5/6	−8	0	
	83	6/6	4	30	
	55	6/6	0	44	
WA	10	6/6	0	86	C
KB				(15)[d]	C
Heliotrine N-Oxide					
LE	88	6/6	−1.0	92	
SA	125	4/6	−0.1	65	
WM	100	6/6	6	45	C
	100	6/6	4	23	
	100	6/6	1	40	
EA	125	4/6	2.6	120	
Lasiocarpine					
CA	90	0/5			A
	45	1/5			
	22.5	2/5			
LE	27.9	6/6	−1.6	89	C
SA	66	0/6			C
	44	3/6	1.1		
	40	4/6	−2.4	40	
	26.6	6/6	−2.6	48	
	18	6/6	−2.1	73	
WM	40	5/6	−9	52	C
	40	4/6	−13	26	
	20	6/6	−11	18	
	20	6/6	−16	36	
	20	6/6	−17	34	
	20	6/6	−25	58	
	10	6/6	−12	64	
	5	6/6	−7	66	
WA	3.0	6/6	−10	32	C
KB				(25)[d]	C
Monocrotaline					
CA	240	5/5	−2.8	23	A
	180	5/5	−0.9	32	
	200	6/10	−4.8		C
	100	9/10	−5.4	9	
	200	10/10	−5.1	7	
	100	8/10	−5.1	7	
	50	9/10	−4.4	44	
	25	10/10	−2.6	65	
	180	7/10	−4.6	7	
	90	9/10	−3.1	15	
	45	10/10	−1.9	34	
	22.5	10/10	−1.3	45	
LE	135	7/7	−0.5	117	C
	90	7/7	−0.5	109	
	60	7/7	0.0	113	
	40	7/7	0.0	110	
	90	6/6	0.4	136	
SA	100	6/6	−1.7	33	C
	100	6/6	−1.0	47	
	100	2/6	−4.3		
	100	7/7	−2.5	43	
	100	6/6	−1.4	34	
	50	6/6	0.7	48	
WM	100	6/6	−1	0	C
	50	6/6	−2	4	
	25	6/6	−1	14	
	12.5	6/6	−5	17	
	6.25	6/6	−2	17	
	50	5/6	−16	22	
	25	5/6	−11	25	
	12.5	6/6	−4	43	
	6.25	6/6	−6	58	
WA	10	6/6	−17	74	C
Pl	84	10/10	−8	4	
	63	10/10	−7	3	
	42	9/10	−5	25	
	30	8/10	−3	25	C
	15	9/10	−9	38	
	7.5	8/10	−6	59	
KB				(>100)[d]	C
Spectabiline					
CA	60	5/6	...	18	A
	45	5/6	...	23	
LE	50	6/6	−1.2	112	C
WM	75	6/6	−3	7	C
	50	6/6	4	5	
	33	6/6	3	14	
	22	6/6	5	25	
	100	3/6	−23		C
	75	6/6	−26	4	
	50	6/6	−22	8	
	25	6/6	−11	14	
	12.5	6/6	−8	25	
	20	6/6	−7	14	
	10	6/6	−9	19	
	5	6/6	−7	46	
WA	4.5	6/6	−13	47	C
KB				(>100)[d]	
Supinine					
CA	90	6/6	−1.3	51	A
	45	6/6	0.3	88	
	22.5	6/6	1.2	122	
LE	200	6/6	0.6	95	C
WM	200	6/6	−0.7	99	C
WA	50	6/6	2	132	C
KB				(>100)[d]	C
1-Chloromethyl-7α-hydroxy-1,2-dehydropyrrolizidine					
CA	90	0/6		Toxic	A
	45	6/6	−3.4	82	
	22.5	6/6	−0.7	26	
Senecionine					
LE	50	6/6	−0.5	98	C
	30	4/4	−2.0	98	
	20	4/4	0.1	96	
WM	35	3/4	1	5	C
	30	2/4	2		
	25	4/4	−6	16	
	20	4/4	−10	21	
	15	4/4	3	57	
	40	0/4			
	30	2/4	−10		
	20	3/4	−2	22	
	40	6/6	−9	85	
	27	6/6	−6	45	
	18	6/6	−7	66	
	12	6/6	−5	93	
WA	5.5	6/6	−13	110	
KB				(>100)[d]	C

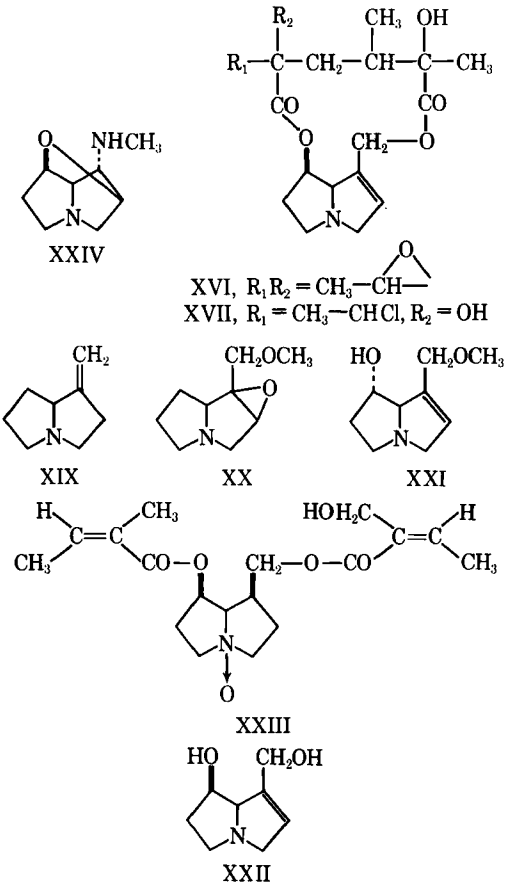
[a] The tumor systems are CA, adenocarcinoma 755; LE, lymphoid leukemia L1210; SA, sarcoma 180; WM, Walker 256 intramuscular; WA, Walker 256 subcutaneous; KB, cell culture; EA, Ehrlich ascites; Pl, plasmacytoma 1. [b] T/C is the ratio of tumor weight or survival time of test animals to control animals, expressed as percent. [c] A denotes testing by Dr. R. J. Stein, Abbott Laboratories, North Chicago. C denotes testing by Cancer Chemotherapy National Service Center, Bethesda, Md. [d] ED_{50} in mcg./ml.

tumors, although senecionine itself has shown high activity against WM at low dose levels. Senecionine (XV) almost meets the criterion for activity against WM at a dose of 50 mg./Kg., while jacobine (XVI) and jaconine (XVII) are inactive at nontoxic levels. Of the bases of heliotrine type (esters of monocarboxylic acids) heliotrine is strongly active against WM, lasiocarpine moderately active, and echinatine (XVIII), supinine, and heleurine essentially inactive.

On the whole, these results suggest that there is for tumor-inhibitory activity, an optimum level or set of levels for one or more of the interrelated properties, water solubility, lipid-water partition coefficient, and base strength. High hepatotoxicity is correlated with low water solubility and low base strength (2) as found for example in lasiocarpine and the macrocyclic diester alkaloids, excepting monocrotaline which has a moderate water solubility occasioned by two hydroxyl groups. Echinatine is very highly soluble in water and weakly hepatotoxic; it might be expected to have only poor access to the target cells. Supinine and heleurine are more highly basic than the other alkaloids [pK_a 9.6, compared with heliotrine, 8.5, and lasiocarpine, 7.8 (35)] and are so highly ionized at physiological pH that they partition only very slightly into a lipid phase (2). Thus monocrotaline and heliotrine appear to come closest to the optimum in properties although spectabiline and lasiocarpine, both more lipid-soluble, are almost as active. The restriction of even weak KB activity to the esters of monocarboxylic acids and the fact that europine (highly soluble in water) and heleurine (high base strength) are among the weakly inhibitory bases is probably a reflection of the different environment of the cell culture. High water solubility is apparently less damaging to activity in this system than high lipid solubility. Alternatively, there is the intriguing possibility that the macrocyclic alkaloids are too bulky to pass the cell membranes.

The structure-activity pattern and the high activity against the intramuscular Walker 256 system both support the idea that activity is connected with the alkylating ability of the allylic ester system (II). It is also significant that the chloro-derivative (X), which has the ester grouping replaced by a halogen atom and has strong *in vitro* alkylating reactivity, is active against two test tumors. Insofar as they have been tested, the other nonester alkaloids (XIX, XX, and XXI), as well as retronecine (XXII) and sarracine *N*-oxide (XXIII), the ester of a saturated pyrrolizidine aminoalcohol, have no inhibitory action. Thus antitumor activity seems quite definitely to be associated with the same functional region in the molecule as hepatotoxicity (17). A recent report by Aizenman *et al.* (36) that loline (XXIV) and *N*-methylloline have a weak inhibitory action against Ehrlich carcinoma suggests that it may become necessary to extend this concept of the structure-activity relationship, but this is scarcely warranted on the present evidence. It is possible that the shape of the pyrrolizidine ring, which is not unlike that of a purine, is another factor in promoting activity.

The *N*-oxides of monocrotaline and heliotrine were tested in the hope of achieving a more favorable ratio of desired activity to acute toxicity. Heliotrine *N*-oxide is active against the Walker 256



tumor but superiority over the tertiary base has not been demonstrated. Monocrotaline *N*-oxide is inactive against CA and LE but has not been tested against WM. From the same point of view, heleurine and supinine were also disappointing. Both are weakly hepatotoxic and thus presumably capable of biological alkylation, but do not appear to shorten the life span of rats (37). They were essentially inactive at the dose levels used.

The known acute and chronic effects of pyrrolizidine alkaloids militate against successful exploitation of the tumor-inhibitory activity which has been uncovered in this group of compounds. However, it may be possible to utilize the selective and apparently permanent and complete antimitotic action exerted by the alkaloids on parenchymal liver cells. If there is a similar effect on tumor cells derived from liver cells, it may be possible to achieve complete inhibition of hepatomas while leaving the liver capable of sustaining life for long periods.

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indicated that it is about 100 times as potent against *Herpes simplex* infections as IDU (10). Intrinsic potency is about 10 times greater but the solubility enhancement is also 10-fold. It also appears that its use is less conducive to the development of resistance (10).

Since the degradation products of nucleosides frequently interfere with the biological activity of its precursors (5, 11, 12), a full study of the kinetics of solvolysis of F_3TdR, I, is necessary to determine the optimum stability conditions for its pharmaceutical formulation and the products of its hydrolysis over the entire pH range. The comparative solvolytic rates of F_3TdR may also provide information on the contributory effects of strongly electronegative 5-substituents to the solvolysis of pyrimidine nucleosides which has been recently discussed in detail (13).

EXPERIMENTAL

Materials—5 - Trifluoromethyl - 2′ - deoxyuridine, F_3TdR, was obtained from P-L Biochemicals, Inc. and 5-trifluorothymine, F_3T, from Merck Sharp and Dohme. All solutions were made up in nitrogen-purged distilled water. Stock solutions of F_3TdR and F_3T were 0.7×10^{-2} *M* and 0.35×10^{-2} *M*, respectively, and were always prepared shortly before using. The pH values of buffer solutions were read at the temperature of the kinetic experiments. The pH values of NaOH and HCl solutions were obtained by calculation and extrapolation from the data of Harned and Owen (14). Ionic strength in all buffer solutions was adjusted to 0.1 with NaCl.

Instruments—A Cary model 15 spectrophotometer equipped with a repetitive scan accessory, automatic sample changer, and a thermostatically controlled cell compartment, and a Beckman DU spectrophotometer, DU-2 were used for ultraviolet absorbance readings. The pH was read on a Radiometer (Copenhagen, Denmark) pH meter equipped with a Beckman high temperature (pH range 1–14) glass electrode with a calomel reference electrode. For potentiometric titrations a Sargent automatic titrator model D was used.

Acid Solvolysis of F_3TdR—An aliquot (2.00 ml.) of F_3TdR stock solution was added to 98 ml. of thermally equilibrated HCl or acetate buffer to produce concentrations of 1.4×10^{-4} *M*. Samples were taken at appropriate time intervals, chilled to room temperature, and an equal volume of 1 *N* NaOH added. After 24 hr. at room temperature the increasing absorbance, *A*, to its asymptotic value A_∞ was read at 300 mμ (Fig. 1). From the slope of the plot $\log/A_\infty - A/$ *versus* time the apparent first-order rate constants were calculated for the temperatures listed in Table I.

Alkaline Solvolysis of F_3TdR—The initial transformation of F_3TdR to a presumed 5-hydroxydifluoromethyl-2′-deoxyuridine intermediate (VIII) was followed at 29.5° by adding 0.5 ml. of F_3TdR stock solution to 10 ml. of NaOH solutions to yield a 3.34×10^{-4} *M* solution. The spectral absorbance increased at 298.4 mμ to a readily obtainable asymptotic value (Fig. 2) prior to further reaction,

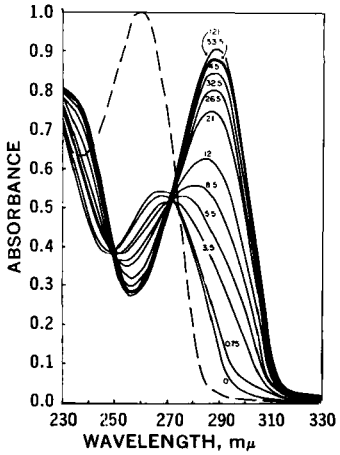


Fig. 1—Typical spectral changes of alkaline-treated (0.5 N NaOH, 24 hr. at room temperature) 5-trifluoromethyl-2′-deoxyuridine, F_3TdR, after the labeled hr. of degradation in 0.002 N HCl at 80.0°. The acid solutions of 1.4×10^{-4} M F_3TdR were diluted 1:1 with 1.0 N NaOH. The spectral end-product, $\lambda_{max.}$ 288 mμ, is 5-carboxyuracil anion. Key: ——, hr.; - - -, spectrum of 5-trifluoromethyluracil in 0.5 N NaOH.

and permitted the first-order rate constants at the cited NaOH concentrations of Table I to be determined. Similar studies were conducted in borate and phosphate buffers but valid asymptotic values for this first transformation were not easily obtained, probably because of the greater relative speed of the subsequent transformation.

At higher temperatures, *e.g.*, 47°, this first reaction was a fast process so that initial spectra (see 295 mμ in Fig. 3) already represented the complete transformation to the probable 5-hydroxydifluoromethyl-2′-deoxyuridine intermediate. The reaction was studied at a concentration of 1.05×10^{-4} *M* in various NaOH, borate, and phosphate buffer solutions listed in Table I with their pH values at 280 mμ and the rate constants determined. In buffer solutions below pH 8 the initial changes due to the first reaction were not as readily observable, and thus estimates of these rate constants were not as valid as the rate constants for the formation of 5-carboxy-2′-deoxyuridine from degrading F_3TdR.

Decarboxylation of 5-Carboxy-2′-deoxyuridine—The spectrum of 5-carboxy-2′-deoxyuridine was obtained very quickly at 80° in NaOH and borate buffers from F_3TdR. The spectrum then changed slowly to $\lambda_{max.}$ 261 mμ in base and 262 mμ in acid; these spectra are inidcative of 2′-deoxyuridine as the product. The reaction appeared to be pH independent in the alkaline region. 2′-Deoxyuridine was relatively stable in buffer below pH 9, but slowly degraded in strong NaOH with loss of absorbance.

Degradation of Trifluoromethyluracil, F_3T—An aliquot (2.00 ml.) of F_3T stock solution was added to 98 ml. of various NaOH and buffer solutions, preequilibrated at 34°. The reaction to 5-carboxyuracil was followed by monitoring the increasing absorbance at 290 mμ attributed to the 5-carboxyuracil anion. The derived first-order rate constants, solvent conditions, and pH values are given in Table II. This reaction is completed almost instantaneously at 80°. The subsequent

TABLE I—APPARENT FIRST-ORDER RATE CONSTANTS IN SEC.$^{-1}$ FOR THE HYDROLYSIS OF 5-TRIFLUOROMETHYL-2′-DEOXYURIDINE AND A PROBABLE INTERMEDIATE, 5-HYDROXYDIFLUOROMETHYL-2′-DEOXYURIDINE

80.0°				47.0°				29.5°			
Medium		pH[a]	10^5k[b]	Medium		pH[a]	10^5k[c]	Medium		pH[a]	10^5k[d]
[HCl]				[NaOH]	[H_3BO_3]			[NaOH]	[H_3BO_3]		
2.54		−0.34	7.97	0.962	—	13.40	32.2	0.126	—	12.80	205
1.407		−0.04	4.73	0.485	—	12.95	19.4	0.097	—	12.70	183
0.918		0.15	3.69	0.194	—	12.50	11.6	0.068	—	12.55	127
0.608		0.35	3.30	0.097	—	12.20	10.7	0.0485	—	12.40	118
0.217		0.80	2.27	0.0194	—	11.60	7.85	0.0194	—	12.10	46.0
0.100		1.10	2.14	0.0049	—	11.00	7.77	0.0097	—	11.75	22.5
0.010		2.05	2.16	0.050	0.050	9.57	8.29	0.0049	—	11.45	11.8
0.002		2.70	2.10	0.025	0.050	9.00	7.83	0.050	0.050	9.70	1.508
[$HC_2H_3O_2$]	[$C_2H_3O_2^-$]			[Na_2HPO_4]	[NaH_2PO_4]			0.025	0.050	9.00	0.973
1.00	0.100	3.75	1.95	0.033	0.002	8.00	6.10	0.012	0.050	8.30	0.945
0.100	0.100	4.75	2.57	0.025	0.025	6.70	1.18	[Na_2HPO_4]	[NaH_2PO_4]		
				0.068	0.010	5.90	0.255	0.033	0.002	8.00	0.936
				[$HC_2H_3O_2$]	[$C_2H_3O_2^-$]			0.006	0.031	7.40	0.540
				0.025	0.100	5.30	0.0753				
				0.100	0.100	4.60	0.0373				

[a] The pH values were calculated from pH = $-\log a_{HCl}$ [HCl] and pH = $pK_w - \log a_{NaOH}$ [NaOH], respectively, where the activity coefficients for the respective [HCl] and [NaOH] and the pK_w values at the several temperatures (12.63, 80.0°; 13.35, 47.0°; 13.83, 29.5°) were obtained from the literature (14). The pH values in the buffer solutions were measured at the stated temperatures and the ionic strengths of these buffer solutions were adjusted to 0.1 with NaCl. [b] These rate constants at 80.0° were obtained for the acid-catalyzed solvolysis of 1.40×10^{-4} *M* 5-trifluoromethyl-2′-deoxyuridine, I, to 5-trifluoromethyluracil, II, and deoxyribose by monitoring the appearance of 5-carboxyuracil, IV, derived from II after 24 hr. in 0.5 *N* NaOH at room temperature. Additional rate constants obtained for this transformation were: °C., [HCl], 10^5k; 70.0°; 2.54, 2.55; 60.0, 2.54, 0.570; 47.0, 2.54, 0.0994; 75.0°, 0.100, 1.1; 70.0°, 0.100, 0.617; 60.0°, 0.100, 0.253. [c] The rate constants at 47.0° in NaOH solutions were obtained for the hydroxyl ion catalyzed solvolysis of the anion of 5-hydroxydifluoromethyl-2′-deoxyuridine, VIII, to 5-carboxy-2′-deoxyuridine, IX ($\lambda_{max.}$ 272 mμ) after the rapid spectral changes at 295 mμ denoted completion of the formation of VIII from 1.05×10^{-4} *M* 5-trifluoromethyl-2′-deoxyuridine, I. Additional rate constants for the hydrolysis of VIII to IX were °C., [NaOH], pH, 10^5k; 38.0, 0.485, 13.1, 8.70; 26.2, 0.535, 13.6, 1.73. The buffer studies were conducted *in situ* and no significant amount of VIII could be observed indicative that these rate constants in the buffer solutions are for I → VIII with rapid degradation to IX. [d] Additional rate constants obtained at 60.0° for hydrolysis of I to IX through the intermediate VIII were: pH, 10^5k; 5.90 (phosphate), 1.24; 5.30 (acetate), 0.49. The rate constants at 29.5° in NaOH solution were obtained for the formation of the probable anion of 5-hydroxydifluoromethyl-2′-deoxyuridine, VIII, from 3.34×10^{-4} *M* trifluoromethyl-2′-deoxyuridine, I, a rapid reaction which is completed much before the hydrolysis of VIII to IX becomes significant as indicated by the monitoring of spectral absorbance at 295 mμ *in situ*. In the borate and phosphate buffer regions, there is no indication from the 290 and 295 mμ absorbances that VIII has more than a transitory existence and that its transformation to IX is very rapid. Thus, these rate constants in the buffer solutions are for I → VIII with rapid degradation to IX.

slower decarboxylation of 5-carboxyuracil to uracil ($\lambda_{max.}$ 283 mμ) was monitored spectrophotometrically.

Isolation of 5-Carboxyuracil—F_3T (200 mg.) was dissolved in 10 ml. of 0.5 *N* NaOH and heated for 30 min. at 50°. The cooled solution was adjusted to pH 3.0 with 2.5 *N* HCl. The refrigerated crystals were filtered after 3 days and dried under high vacuum at 60° for 3 hr. The yield was 180.8 mg., m.p. 284–285° dec. [lit. (15) m.p. 278° dec.]. The product was recrystallized from 50% aqueous ethanol; yield 62 mg., m.p. 285° dec. The com-

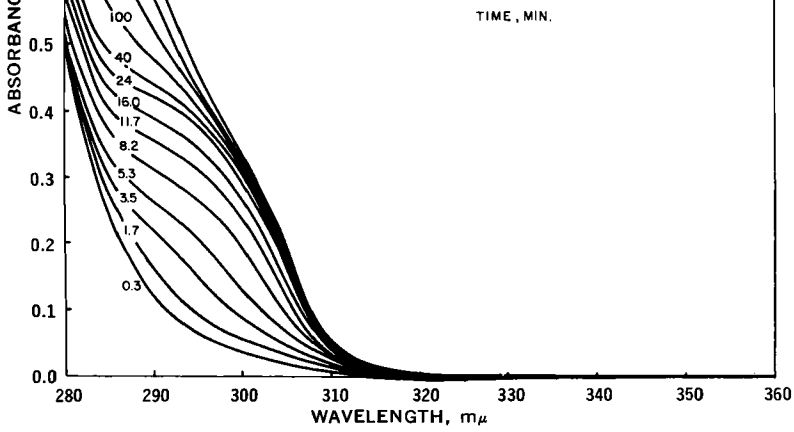


Fig. 2—Typical initial spectral changes for the degradation of 5-trifluoromethyl-2′-deoxyuridine, F_3TdR, in alkaline solution. The conditions were F_3TdR, 3.34×10^{-4} M in 0.126 N NaOH at 29.5°. Key: curves, min. after the start of the reaction; insert, typical plots of the absorbance at 295 mμ against time for the production of the probable 5-difluorohydroxymethyl-2′-deoxyuridine at several NaOH concentrations: ▲, 0.0485 N; ×, 0.097 N; Δ, 0.0049 N.

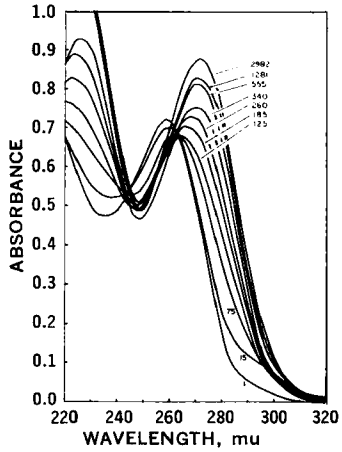


Fig. 3—Typical spectral changes of 5-trifluoromethyl-2′-deoxyuridine, F_3TdR, in alkaline solution. The conditions were F_3TdR, 1.05 × 10^{-4} M, in 0.020 N NaOH at 47.0°. Key: curves, min. after the start of the reaction. The rapid increase in absorbance in the range 290–310 mμ within the first 15 min. may be attributed to the production of a difluoro-monohydroxy intermediate.

pound is claimed to crystallize as the monohydrate (15), mol. wt. 174.1 + 18.0.

Anal.—Calcd. for $C_5H_4N_2O_4 \cdot H_2O$: C, 31.1; H, 3.12; N, 14.6. Calcd. for $C_5H_4N_2O_4$: C, 34.5; H, 3.45; N, 16.1. Found: C, 33.34; H, 3.22; N, 15.76.

Equivalent weight found by titration was 176.5, $pKa_1' = 4.25$, $pKa_2' = 8.90$.

Isolation of 5-Carboxy-2′-deoxyuridine—F_3TdR (200 mg.) was dissolved in 10 ml. of 1 *M* NaOH and heated for 7 hr. at 47°. The pH was adjusted to 6.8 with concentrated HCl. The solvent was removed *in vacuo*. The white residue was triturated with ethanol to which a few drops of concentrated HCl had been added. After filtering off the NaCl, the separated yellow filtrate was evaporated *in vacuo* at 60°. The yield was 115 mg. of yellow crystals, m.p. 165°. Repeated recrystallization from absolute ethanol yielded 31 mg. of a chloride-free product, m.p. 170° dec. The elementary analysis, mol. wt. 272.2, was as follows.

Anal.—Calcd. for $C_{10}H_{12}N_2O_7$: C, 44.12; H, 4.44; *N*, 10.29. Found: C, 44.64; H, 4.48; N, 10.20.

Paper Chromatography—Paper chromatography was used to monitor the basic degradation reactions from F_3TdR to 5-carboxy-2′-deoxyuridine, DU-5-COOH, and from F_3T to 5-carboxyuracil, U-5-COOH. Aliquots (25 μl.) of approximately 10^{-2} *M* aqueous solutions were spotted on Whatman No. 1 paper and developed with a mixture of ethyl acetate, formic acid, and water (21:3:1.5) (16). The substances were visualized by UV light (254 mμ). The acids were detected by spraying the dried chromatogram with alcoholic bromocresol green solution and gave yellow spots against a blue background. F_3TdR and F_3T move with the solvent front while the acids DU-5-COOH and U-5-COOH have an R_f value of ~0.5.

Table III lists the principal UV spectra and pKa′ values determined by potentiometric or spectrophotometric titration.

[1] The synthesis of this compound has been reported recently (Isenberg, N., and Heidelberger, C., *J. Med. Chem.* 10, 970(1967), colorless crystals, m. p. 158–159,° ε = 11,400 ($\lambda_{max.}$ 276 mμ, 0.1 *N* HCl), ε = 6,810 ($\lambda_{max.}$ 271 mμ, 0.1 *N* NaOH).

TABLE II—APPARENT FIRST-ORDER RATE CONSTANTS IN SEC.$^{-1}$ FOR THE HYDROLYSIS OF TRIFLUOROMETHYLURACIL[a] AT 34.0°

[NaOH]	pH[b]	10^4k
0.485	13.3	336
0.194	13.0	285
0.097	12.6	233
0.049	12.35	162
0.0194	11.95	78
0.0049	11.40	44.5

[a] Hydrolysis to 5-carboxyuracil. In borate buffer at pH 9.70 at 34.0°, $k = 5.17 \times 10^{-5}$. [b] Calculated from activity coefficients in the literature (14) where pH = pK_w − log a_{NaOH}[NaOH].

RESULTS AND DISCUSSION

Degradation in Mineral Acid—The glycoside bond of 5-trifluoromethyl-2′-deoxyuridine (F_3TdR), I, is readily cleaved in acid (Scheme I) in a manner common to pyrimidine deoxyribosides (13). The initial products are 5-trifluoromethyluracil (F_3T) (II) and deoxyribose, III. Deoxyribose is not stable under these acidic conditions; it is transformed in part to 5-methyl-3(2*H*)-furanone (17, 18), V, and levulinic acid (VI) (19). The absorbance of the former compound, V, $\lambda_{max.}$ 261 mμ which would interfere with the spectrophotometric monitoring of the F_3TdR, $\lambda_{max.}$ 261 mμ in 0.1 *N* HCl and $\lambda_{max.}$ 259 mμ in 0.1 *N* NaOH, can be removed (13, 17) by degradation of the reaction mixture in 0.5 *N* NaOH at room temperature for 24 hr.

The derived product of acidic solvolysis of I, which is F_3T, II, is completely hydrolyzed under these alkaline conditions to 5-carboxyuracil, IV ($\lambda_{max.}$ 289 mμ in 0.5 *N* NaOH) as shown in Scheme I. The acid-unreacted F_3TdR is also degraded under these alkaline conditions (Fig. 3). The 261 mμ $\lambda_{max.}$ shifts to 271 mμ $\lambda_{max.}$ in NaOH at room temperature in 24 hr., but contributes little to the change in the absorbance of IV at 295 mμ or 300 mμ in alkali. Thus the absorbance of IV at 295 mμ after 24 hr. at room temperature in 0.5 *N* NaOH may be taken as a measure of the extent of hydrolysis of I in acid solution. Typical spectra of the alkaline-treated acid degradations of I are given in Fig. 1. Typical first-order plots for the solvolysis of I in acid are given in Fig. 4 in accordance with the expression

$$\log |A_\infty - A| = -kt/2.303 + \log A_\infty \quad \text{(Eq. 1)}$$

where A is the absorbance with time, t, at 295 mμ after treatment for 24 hr. in 0.5 *N* NaOH at room temperature, A_∞ is the final absorbance, and k is the apparent first-order rate constant for the studies listed in Table I.

A plot of the apparent first-order rate constants (Table I) for the hydrolysis of F_3TdR, I, against [HCl] at 80.0° is linear with a finite intercept. This is indicative of a catalytic attack of hydrogen ion on the undissociated molecule, I, and a pH independent hydrolysis where

$$k = k_0 + k_{HCl}[HCl] \quad \text{(Eq. 2)}$$

where $k_0 = 2.0 \times 10^{-5}$ sec.$^{-1}$ and $k_{HCl} = 2.0 \times 10^{-5}$ L./mole/sec.

Degradation of F_3T—The 5-trifluoromethyluracil II, is known to produce 5-carboxyuracil, IV, in 0.1 *N* sodium bicarbonate and in 1.0 *N* NaOH solutions (2, 20). This can be followed readily by monitoring the absorbance at 290 mμ. Typical spectral changes in alkali with time are given in Fig. 5 and typical first-order rate constants are listed in Table II. Typical first-order plots are given in Fig. 6. At 34.0° the final absorbance was consistent with a stoichiometric yield of 5-carboxyuracil. Paper chromatographic monitoring of the reaction did not indicate any other processes but II → IV.

The rate constants can be well fitted by

$$k = k_{NaOH}'[NaOH]\, f_{u^-} \qquad \text{(Eq. 3)}$$

where $k_{NaOH}' = 4.5 \times 10^{-2}$ L./mole/sec. at 34.0°, where $f_{u^-} = [H^+]/([H^+] + K_a)$ is the fraction of 5-carboxyuracil that exists as the monoanion at the $[H^+]$ determined from the calculated pH (Table II). The K_{a_2}' is taken as 2.5×10^{-13}. This Eq. 3 indicates that the hydrolysis of F_3T, II, is effected by hydroxyl ion attack on the monoanion, or its kinetically equivalent water attack on the dianion at pH values more than one unit above the pK_{a_1}', 7.40 at 25.0°.

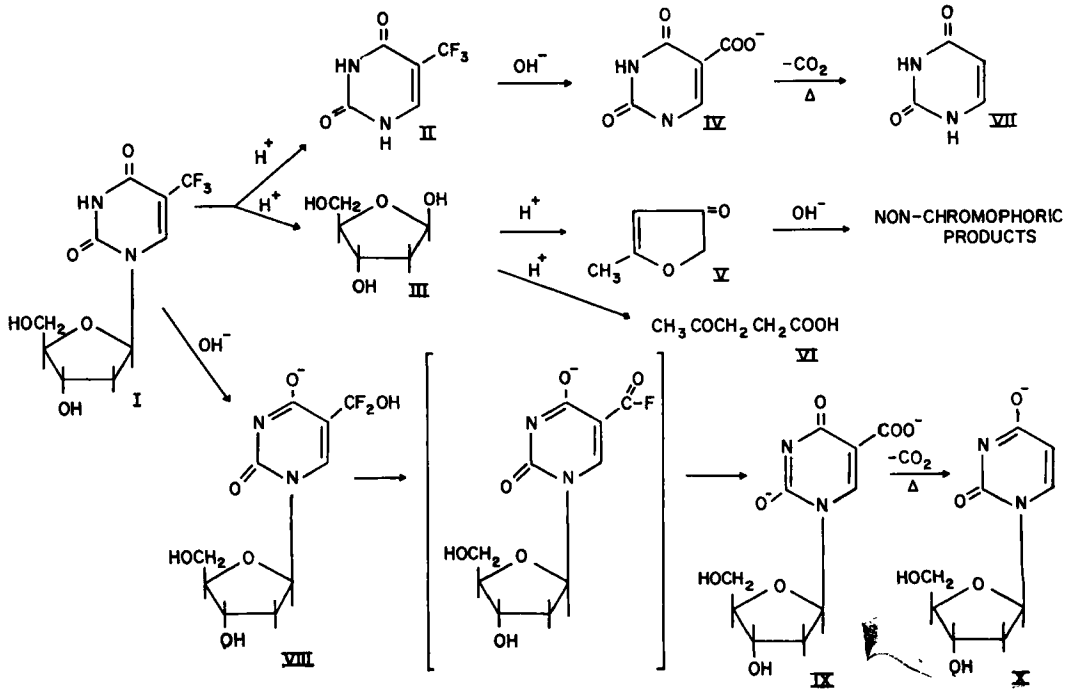
At elevated temperatures the anion of 5-carboxyuracil, IV, decarboxylates (19) to uracil, VII, but much more slowly than the production of IV from II. This is spectrally observable from the loss of absorbance at the 290 mμ $\lambda_{max.}$ in alkali of IV and the appearance of a 283 mμ $\lambda_{max.}$ with the lessened absorbance of the spectrum of VII.

At 80.0° in 0.02 *N* alkali the spectrum of 5-carboxyuracil, IV, is produced almost within 0.5 hr. from F_3T, II. The apparent first-order rate constants for the subsequent decarboxylation of IV were obtained from the slopes of typical first-order plots in accordance with Eq. 1. They were 8.17×10^{-7} and 2.07×10^{-7} sec.$^{-1}$ in 0.485 *N* (pH

TABLE III—SPECTRAL AND pKa′ VALUES

		M.p., °C.	pK_{a_1}'	pK_{a_2}'	0.1 *N* HCl $\lambda_{max.}$	0.1 *N* HCl ε	0.1 *N* NaOH $\lambda_{max.}$	0.1 *N* NaOH ε
5-Trifluoromethyl-2′-deoxyuridine, I (5-trifluorothymidine)	F_3TdR	186–189	7.85[a]	—	261	10,350	259	7,150
5-Hydroxydifluoromethyl-2′-deoxyuridine (5-hydroxydifluorothymidine)	HOF_2TdR	—	—	—	—	—	260[d]	7,000[d]
5-Carboxy-2′-deoxyuridine	DU-5-COOH	170	4.0[a]	9.8[a]	277	12,300	272	7,800
2′-Deoxyuridine	DU	161–163	9.3[a]	—	262	9,700	262	7,300
5-Trifluoromethyluracil (5-trifluorothymine)	F_3T	245–248	7.4[a]	12.6[c]	256	8,700	270	8,000[e]
5-Carboxyuracil	U-5-COOH	285	4.20[a] 4.25[b]	9.10[a] 8.90[b]	276	11,400	290	14,000
Uracil	U	335	9.0[a]	>13[a]	259	8,100	283	6,200

[a] Determined by spectrophotometric titration at 25.0°. [b] Determined by potentiometric titration at 25.0°. [c] Estimated from kinetic data at 34.0°. [d] Estimated. [e] Obtained by extrapolation to zero time.



Scheme I

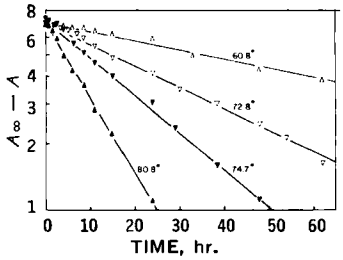


Fig. 4—Typical first-order plots for the degradation of 1.4 × 10⁻⁴ M 5-trifluoromethyl-2′-deoxyuridine in 0.1 N HCl at various temperatures. The absorbance values, A, were measured at 297.5 mμ for aliquots diluted 1:1 with 1.0 N NaOH and held for 24 hr. at room temperature where A_∞ is the asymptotic absorbance.

12.15) and 0.0218 *N* (pH 10.85) NaOH, respectively, at 80.0°. At 80.0° in pH 8.7 borate buffer, k = 2.0 × 10⁻⁷ sec.⁻¹ and in pH 6.7 phosphate buffer, k = 5.8 × 10⁻⁷ sec.⁻¹. At 80.0° in acetate buffers, k = 10.1 × 10⁻⁷ and 1.63 × 10⁻⁷ at pH values 4.75 and 3.75, respectively. It has been demonstrated that the major kinetic dependency for decarboxylation is by the attack of hydroxyl ion on the anion (21). Thus, it is probable from these rate constants that the specific catalytic rate constant for the attack of hydroxyl ion on the monoanion (or its kinetically equivalent water attack on the dianion) below the pKa_2' 9.0 (25.0°) but above the pKa_1' 4.2 (25.0°) of the carboxyl is of greater magnitude than the specific catalytic rate constant for the attack of hydroxyl ion on the dianion above the pKa_2'. The rate of decarboxylation most probably plateaus in the vicinity of this value and then decreases with solutions of decreasing pH values until values below the pKa_1' are reached. The values obtained in phosphate and acetate buffer solutions may be indicative of general base catalysis by phosphate and acetate ions where such catalysis has been reported for the decarboxylation of salicylic acids (21).

An expression consistent with the rate data excluding the values in acetate and phosphate buffers is

$$k = (k_0'' + k_{OH}''a_{NaOH}[NaOH])f_{u-} \quad \text{(Eq. 4)}$$

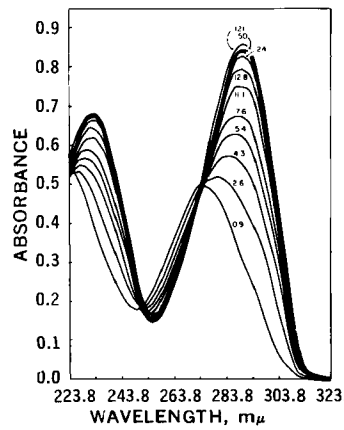


Fig. 5—Typical spectral changes for the degradation of 7.0 × 10⁻⁵ M 5-trifluoromethyluracil, in alkaline solution at low temperatures. The conditions were 0.200 N NaOH at 34.0°. Key: curves, min. after the start of the reaction.

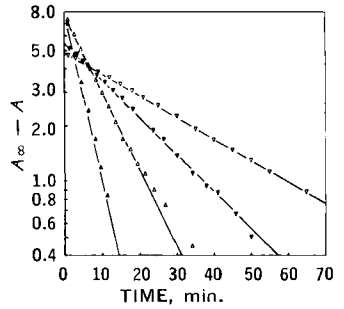


Fig. 6—Typical first-order plots for the degradation of 7.0 × 10⁻⁵ M 5-trifluoromethyluracil in various NaOH concentrations at 34.0°. Key: A, absorbance values were measured at 290 mμ where A_∞ is the asymptotic absorbance; curves and their respective N NaOH: ▽, 0.0049; ▼, 0.1094; Δ, 0.0485; ▲, 0.485.

where k_0'' = 2.0 × 10⁻⁷ sec.⁻¹, k_{OH}'' = 2.40 × 10⁻⁷ L./mole/sec., a_{NaOH} is the activity coefficient (14) for hydroxyl ion in [NaOH] solutions, and f_u = is the fraction of IV as the dianion at 80.0°.

The acid-catalyzed solvolysis of I in mineral acid at 80.0° produced the F_3T, II, or its hydrolytic product, IV, without any apparent decarboxylation. The alkaline-treated samples (room temperature, 24 hr.) from these studies produced a stoichiometric yield of the expected 5-carboxyuracil, IV. However, samples from the hydrolysis of I in acetate buffers at 80.0° at pH values of 3.7 and 4.7 when treated in alkali for 24 hr. did not produce a stoichiometric yield of desired 5-carboxyuracil. The presence of increasing amounts of uracil with time of acetate buffer degradation is consistent with the fact that 5-carboxyuracil is formed in these buffers at 80.0° and decarboxylated in the pH region of its pKa_1' 4.2 where it may exist in part as the carboxylate anion.

Since hydrolysis of II to IV and its subsequent decarboxylation to VII is a slow process with relation to the formation of II from I, an A_∞ corresponding to the theoretical yield of IV on alkaline treatment could be assumed. The initial points of the plot in accordance with Eq. 1 gave good estimates of the rate constants for hydrolysis of I in the acetate buffers at 80.0°.

Degradation of F_3TdR in Alkali—The facile degradation of F_3TdR, I, ($\lambda_{max.}$ 259 mμ) in alkaline solution is similar to that of F_3T in that the trifluoromethyl group is hydrolyzed to 5-carboxy-2′-deoxyuridine (DU-5-COOH), IX (272 mμ $\lambda_{max.}$ in 0.01 *N* NaOH), probably through alcohol, VIII, and acyl fluoride intermediates (Scheme I). The latter is most likely a highly reactive and transitory intermediate. This sequence is readily observable on changes in alkaline solution (Fig. 3). The spectra of alkaline-degrading I demonstrate a very fast initial increase in absorbance in the range 290–310 mμ (Fig. 3) which can be monitored with time at lower temperatures, *e.g.*, 30°. The apparent $\lambda_{max.}$ (260 mμ) of this intermediate is probably that of the curve for the 15-min. sample in 0.02 *M* NaOH at 47° and appears to be not very much different from the precursor, I (Fig. 3). This indicates that only minor changes in the electron patterns of the molecule are occurring, consistent with the structure of the 5-hydroxydifluoromethyl-2′-deoxyuridine, VIII, as the probable intermediate. The sub-

sequent production of a $\lambda_{max.}$, 272 mμ, is a slower process at these lower temperatures and the final absorbance and spectrum are consistent with the stoichiometric production of the anion of DU-5-COOH, IX, which was isolated from the alkaline-reacted solution and identified by elemental analysis and ultraviolet spectrum.

When the rapid increase of absorbance of higher concentrations of I in alkaline solutions is monitored at about 295 mμ at lower temperatures, *e.g.*, 30° (Fig. 2), plots of this absorbance against time permit the estimate of asymptotic values (Fig. 2 insert) before the further solvolysis to the carboxyl significantly affects the absorbance at this wavelength (Fig. 3). First-order plots of these data (Fig. 2 insert) in accordance with Eq. 1 permit estimates of the apparent first-order rate constants for the hydrolysis of I to VIII. These rate constants are listed in Table I and are consistent with the fact that the only significant process above pH 11 is the attack of hydroxyl ion on the monoanion where

$$k = k_{OH}' a_{NaOH}[NaOH] f_{u-} \quad \text{(Eq. 5)}$$

where k_{OH}' at 29.5° is 2.70 × 10^{-2} L./mole/sec., a_{NaOH} is the activity coefficient (14) for the specified [NaOH]. Above pH 11, the F_3TdR, I, exists as the monoanion since $pKa_1' = 7.85$ at 25°.

In the borate and phosphate buffer regions, first-order plots in accordance with Eq. 1 at both 280 and 290 mμ for data obtained at 47° were linear and of similar slopes for the complete duration of the reaction. No sequence of reactions could be observed at 30° and at these wavelengths in these buffer solutions, only the first-order process to IX. These facts are consistent with the premise that the intermediate, VIII, is rapidly hydrolyzed to IX in these lower pH regions by hydroxyl ion attack on the undissociated molecule (or its kinetically equivalent water attack on the monoanion) and that the rate-determining step is the hydrolysis to VIII.

At 80.0° in NaOH solutions, the hydrolysis of I to IX is too rapid to follow. However, it was spectrally observed that deoxyuridine (X) is rapidly produced by decarboxylation and slowly degrades subsequently.

Temperature Dependence—Rate constants for the several temperatures are given in Table I and its footnotes. The Arrhenius parameters were calculated from the plots of the logarithms of the rate constants in $sec.^{-1}$ against the reciprocal of the absolute temperature, T, in accordance with

$$\log k = \log P - (\Delta H_a/2.303R)(1/T) \quad \text{(Eq. 6)}$$

where R = 1.987 cal./degree/mole. The heats of activation, ΔH_a, in Kcal./mole and the log P values for the several conditions were: 2.54 N HCl, 29.7, 14.4; 0.100 N HCl, 26.9, 12.0; 0.485 N NaOH, 19.8, 9.78. The values in HCl are for hydrolysis of the nucleoside, I, to trifluoromethyluracil, II, and deoxyribose, III. The values in NaOH are for the hydrolysis of 5-hydroxydifluoromethyl-2′-deoxyuridine, VIII, to 5-carboxyl-2′-deoxyuridine, IX.

Log *k*-pH Profile—The log k-pH profiles for the degradation of F_3TdR, I, are given in Fig. 7 (solid lines) and are based on the data of Table I. They demonstrate maximum stability in the pH region 1–4 which may be assigned to solvent attack, k_0, on the undissociated molecule. The rising branch in

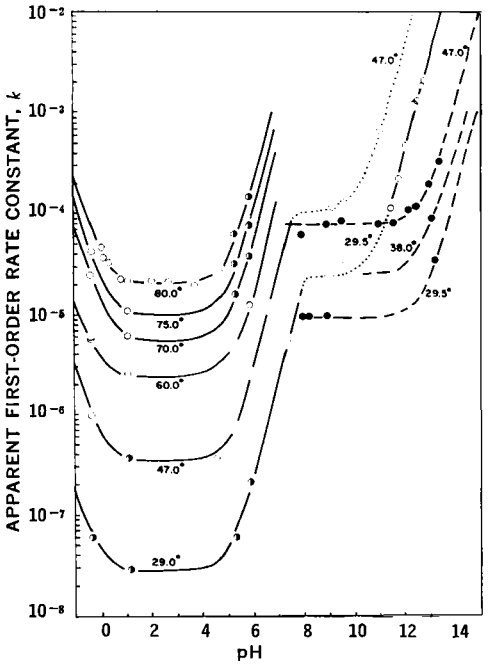


Fig. 7—Key: ——, *log k-pH profiles for the hydrolysis of 5-trifluoromethyl-2′-deoxyuridine and probable intermediate;* - - -, *5′-hydroxymethyl-2′-deoxyuridine where the lines are calculated;* ○, *represent data obtained from the loss of 5-trifluoromethyl-2′-deoxyuridine;* ◌, *estimated values with a degree of uncertainty;* ◑, *predicted from the Arrhenius relations; the dashed lines were calculated and fit the solid circles that represent data obtained for the formation of 5-carboxy-2′-deoxyuridine from the probable intermediate;* . . ., *estimated curves for the initial solvolysis of 5-trifluoromethyl-2′-deoxyuridine.*

the lower pH regions is consistent with hydrogen ion attack, $k_H[H^+]$, on the undissociated molecule. The products of these kinetic dependencies are F_3T, II, and deoxyribose, III (Scheme I).

The increasing rate constants with pH in the pH region 4–7.5 may be assigned to hydroxyl ion attack, $k_{OH}[OH^-]$ on the undissociated molecule and result in the 5-carboxyuracil-2′-deoxyribose, IX. Subsequent slow decarboxylation to X occurs at higher temperatures. No aberrations were observed in the spectra monitored in acetate or phosphate buffers (pH 4–7.5) as a function of time that could be assigned to a possible precursor, VIII, of the final product, IX. This can be explained on the basis of two possible premises. One explanation is that in the acetate and phosphate buffer regions, the HOF_2TdR, VIII, is more rapidly hydrolyzed than F_3TdR, I. Thus, the dashed lines (Fig. 7) for the presumed hydrolysis of HOF_2TdR, VIII to DU-5-COOH, IX by solvent attack on the dissociated molecule, k_0'' (or its kinetically equivalent hydroxyl ion attack on the undissociated molecule) may extend horizontally to lower pH values than the pKa′ of F_3TdR, I, *i.e.*, 7.9. This would imply that HOF_2TdR, VIII, has a significantly lower pKa_1' and that, even when it is completely undissociated, hydroxyl ion attack on this species is of much greater magnitude than hydroxyl ion attack, $k_{OH}[OH^-]$, on the undissociated F_3TdR, I.

TABLE IV—CATALYTIC RATE CONSTANTS[a] AND THERMODYNAMIC PARAMETERS[b] FOR THE CONSTRUCTION OF LOG k-pH PROFILES

	°C.							
	80.0	75.0	70.0	60.0	47.0	29.5	ΔH_a	log P
$10^6 k_0$	2.1	1.00	0.55	0.24	0.035	0.0028	27.1	16.2
$10^5 k_H$	2.2	1.45	0.80	0.13	0.029	0.00135	30.6	16.4
k_{OH}	430	460	250	130	48	14	17.0	13.3
k_{OH}'	—	—	—	—	0.13	0.026	18.2	11.6
$10^6 k_0''$	—	—	—	—	10.4[c]	2.5[c]	—	—
$10^5 k_0''$	—	—	—	—	7.85	0.97	22.3	11.1
$10^6 k_{OH}''$	—	—	—	—	25	10	10.3	3.5

[a] These catalytic rate constants may be used to predict the overall first-order rate constant for the hydrolysis of F_3TdR ($k = k_H[H^+] + k_0 + k_{OH}[OH^-]$ in the pH range -1 to 7.5 and $k = k_{OH}'[OH^-] + k_0'$ in the pH region >8) and of HOF_2TdR ($k = k_0'' + k_{OH}''[OH^-]$ in the pH region >8). [b] These values may predict the catalytic rate constant at other temperatures where $\log k = -(\Delta H_a/2.303R)(1/T) + \log P$. [c] These are estimated values based on approximations.

An alternative explanation is that in acetate and phosphate buffers, the spectrum of undissociated HOF_2TdR, VIII, is not significantly different than undissociated F_3TdR, I, and that the hydrolysis of the precursor I to VIII is much faster than the hydrolysis of VIII to IX so that the good first-order plots would still be obtained. This would lead to the conclusion that F_3TdR is more rapidly degraded than indicated in Fig. 7 for the pH range 5–7. However, in buffers about pH 10, there were no other spots observed on paper chromatographic monitoring that could be possibly assigned to a HOF_2TdR, VIII.

In the alkaline region the sequential steps could be readily analyzed kinetically. The data at 29.5° in the pH region > 11 (open circles, solid line, Fig. 7) is for the faster production of an asymptotic absorbance at 295–300 mμ (Fig. 2) than for the production of the spectrum assigned to DU-5-COOH, IX (Fig. 3) represented by the solid circles and dashed lines of Fig. 7. The intermediate is most probably HOF_2TdR, VIII, and is produced by hydroxyl ion attack, $k_{OH}'[OH^-]$, on the anion of F_3TdR, I. The dotted circles at 47.0° and 29.5° on the dotted lines are estimates of the rate constants in the borate buffer region, pH 8–11, and are not too accurate due to the small spectral difference at 295 mμ that had to be used. However, they do serve as a basis for estimating the stability of I in this intermediate pH region (dotted line, Fig. 7) where the rate constants for I and VIII are of the same order of magnitude.

The subsequent hydrolysis of the intermediate, the presumed VIII to IX, is represented by the dashed lines and the observed rate constants by the solid circles (Fig. 7). This obviously represents a solvent attack, k_0'', on the anion of VIII (or its kinetically equivalent hydroxyl ion attack on the undissociated molecule) and a hydroxyl ion attack, $k_{OH}''[OH^-]$, on the anion of VIII at higher pH values.

The basic expression used in the construction of the log k – pH profiles for the hydrolysis of F_3TdR (solid lines, Fig. 7) were

$$k = k_H[H^+] + k_0 + k_{OH}[OH^-] \qquad \text{(Eq. 7)}$$

in the pH region -1 to 7.5 (solid lines, Fig. 7), and

$$k = k_{OH}'[OH^-] + k_0' \qquad \text{(Eq. 8)}$$

in the pH region >8 (dotted and solid lines, Fig. 7). The expression used in the construction of the log k-pH profile for the appearance of DU-5-COOH, IX probably from the hydrolysis of HOF_2TdR, VIII was

$$k = k_0'' + k_{OH}''[OH^-] \qquad \text{(Eq. 9)}$$

in the pH region >8 (dashed lines, Fig. 7). The profiles of Fig. 7 were constructed from the $[H^+]$ and $[OH^-]$ in terms of their activities and the listed constants of Table IV. The drawn lines are calculated on the basis of the Eqs. 7–9 and are consistent with the experimental values of Fig. 7.

The half-life at 30° in the most stable pH range 1 to 4 is 280 days (580 days at 25°) and at pH 7.4 is 1.5 days (16 hr. at 37°). Thus solutions of F_3TR should be kept refrigerated. The products of degradation under the former conditions would be trifluorothymine and deoxyribose, and under the latter conditions would be fluoride ion, 5-carboxymethyl-2′-deoxyuridine and 5-hydroxydifluoromethyl-2′-deoxyuridine.

The obvious prediction is that F_3TdR incorporated into RNA or DNA will probably not remain as such. It should be readily transformed to the 5-carboxyl derivative at physiological pH values and temperatures. This would readily explain the fact that the use of F_3TdR is less conducive to the development of resistance than are the other nucleosidic antivirals (10).

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Keyphrases

Stability prediction—pharmaceuticals
5-Trifluoromethyl-2′-deoxyuridine—hydrolysis
pH effect—stability
Temperature effect—stability
Kinetic equations—degradation rates
Paper chromatography—degradation monitoring
UV spectrophotometry—degradation monitoring

Metabolism of β-Phenethylbiguanide

By PATRICK J. MURPHY and ARNE N. WICK

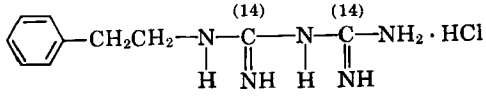
Carbon-14 labeled β-phenethylbiguanide (phenformin) was administered orally to male Sprague-Dawley rats. The radioactive material excreted in the urine was isolated and analyzed. The metabolic products were divided into "weak" and "strong" base components by their behavior on acidic ion-exchange resins. Chromatographic and spectroscopic evidence confirms the structure of the strong base fraction to be ρ-hydroxy-β-phenethylbiguanide. This hitherto unreported biguanide has been prepared synthetically for comparative purposes. The weak base metabolite has been shown to be the glucuronic acid conjugate of the phenolic biguanide. This fact was determined by acid hydrolysis to ρ-hydroxy-β-phenethylbiguanide and glucuronic acid. The more specific hydrolysis by β-glucuronidase confirmed the conjugate structure. The hydroxylated β-phenethylbiguanide along with its glucuronide conjugate were found to be present in the urine in approximately equal amounts. These two compounds seem to be the major metabolic products of β-phenethylbiguanide.

In diabetic humans, β-phenethylbiguanide hydrochloride has a hypoglycemic effect. Although this effect has been extensively studied, there is no unified concept which will explain the hypoglycemic action of this drug. Since the physiological activity of a chemotherapeutic agent is dependent in many cases (1) upon the mode of metabolism of the drug, an understanding of the metabolism of β-phenethylbiguanide may be helpful in elucidating the site and mode of action of the drug.

Preliminary studies in these laboratories have shown that the radioactivity of the labeled drug after administration is initially concentrated in the liver and gastric juice and is almost entirely eliminated in a 24-hr. period (2). Subsequent studies showed that the excreted metabolic products were chemically different from the ingested compound (3). The purpose of this study was the elucidation of the structure of the metabolic products excreted after the administration of β-phenethylbiguanide hydrochloride.

EXPERIMENTAL METHODS

In order to facilitate the metabolic studies, radioactive β-phenethylbiguanide hydrochloride synthesized by Walton (4), was used. The carbon-14 label was in the biguanide nucleus as depicted in the following:



Radioactivity was determined by the following means: (*a*) when the specific activity was to be determined the sample was oxidized to carbon dioxide by the "wet oxidation" method of Van Slyke and Folch (5) and precipitated as barium carbonate. The barium carbonate samples were mounted on planchets and counted for radioactivity in a Nuclear-Chicago gas-flow counter; (*b*) when relative radioactivity was desired, as in the case of column chromatography, aliquots were placed directly on the planchets, evaporated, and counted for radioactivity.

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Accepted for publication May 2, 1968.

Taken in part from the thesis of Patrick J. Murphy, submitted in partial fulfillment of the Master of Science degree requirements, San Diego State College, August 1964.

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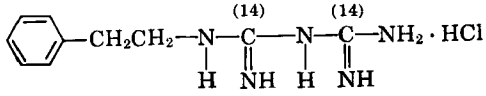

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used for the detection of biguanide metabolites was prepared 30 min. prior to use by mixing 10% solutions of sodium nitroprusside, potassium ferricyanide, and sodium hydroxide in a 1:1:1 ratio and diluting the solution with 9 vol. of water. The reagent gives a bright red color with guanidines and biguanides (6).

Ultraviolet spectra were run on a Cary model 14 recording spectrophotometer. Infrared spectra were run in potassium bromide pellets on a Perkin-Elmer Infracord spectrophotometer. Nuclear magnetic resonance spectra were obtained on a Varian A-60 spectrometer.

ISOLATION OF METABOLIC PRODUCTS

β-Phenethylbiguanide hydrochloride was administered in water solution by means of a stomach tube to 24 hr. fasted male Sprague-Dawley rats in doses of 100 mg./kg. body weight. The urine of the rats was collected at 8-hr. intervals for a 24-hr. period during which time the rats had access only to water. The urine, which was approximately 25 ml. per rat, was frozen upon collection and subsequently lyophilized.

Ion-Exchange Chromatography—The lyophilized urine was slurried with water, in approximately $^1/_6$ the original volume of urine, and the insoluble material removed by centrifugation. The clear solution was then placed on a 20 × 1.25-cm. column of Amberlite CG-50 (carboxylic acid—H^+ form) ion-exchange resin. This column retained all of the inorganic cations as well as strong organic bases. The column was rinsed with water and the eluate passed over a 20 × 1.25-cm. column of Amberlite CG-120 (sulfonic acid — H^+ form). The CG-120 resin retains all weak organic bases. The eluate from this column contains the neutral and acidic components of the urine. All of the radioactive components of the urine were retained by these columns and a summary of the distribution of this radioactivity is given in Table I.

The two columns were then separately eluted, the CG-50 resin with 0–0.1 *N* hydrochloric acid in a gradient elution manner, and the CG-120 resin with 0–0.5 *N* ammonium hydroxide by similar methods. The eluate from the CG-50 column thus contained the strong organic bases while the eluate from the CG-120 resin contained the weak bases.

The remainder of the administered radioactivity (45%) was found to be present in the stools of the animals. This radioactive material was soluble in water and upon separation on a CG-50 (H^+) column (as above) was found to consist of 77% strong base material. These results are also included in Table I.

Weak Base Metabolite—The isolated weak base fraction from 29 rats was extracted with 70 ml. of hot ethanol, the solution filtered, and the residue separated. Although a small amount of radioactivity was taken up in the extract, the majority of the material in this solution consisted of nonradioactive creatine and creatinine (as determined by chromatographic and spectroscopic means). The residue was then extracted with methanol, and isopropyl alcohol was added to the point of saturation. Concentration of the solution yielded three crops of material which gave a single biguanide-positive spot after paper chromatographic separation. These spots corresponded directly to the radioactive area.

Table I—Percentage Distribution of Excreted Radioactive Metabolites

	Total	Strong Base	Weak Base
Urine	56	28	28
Feces	45	35	Undetermined

In order to further purify the radioactive metabolite, 60 mg. obtained from the first three crops was placed on a column of pressure packed Whatman No. 1 powdered cellulose in *n*-butanol saturated with water. The column was eluted with 1.5 vol. of saturated butanol followed by a saturated butanol–acetic acid (3:1) solution. The fractions were collected in 10-ml. aliquots. The radioactivity came off the column in fractions 21–29. The main fraction was extracted with ether and the aqueous solution was then stripped to dryness to give 29 mg. of purified material.

Acid Hydrolysis of the Weak Base Metabolite—A solution of 3 mg. of the purified metabolite in 0.3 ml. of (1*N*) hydrochloric acid was heated for 2 hr. at 100°. At the end of the heating period 0.06 ml. was tested with barium chloride and the negative result indicated the lack of "ethereal sulfate" in the metabolite. A small portion (0.03 ml.) of the hydrolysate was tested for the presence of reducing sugar by the method described by Nelson (7). This test was strongly positive. A similar portion (0.03 ml.) was tested for the presence of phenol by the Folin-Ciocalteu method (8).

A comparison of the results obtained after hydrolysis with a blank run on the unhydrolyzed compound showed that approximately 1 mole of reducing sugar (using glucuronic acid for a standard curve) was released per mole of phenolic group obtained (using phenol for a standard curve).

Incubation with β-Glucuronidase—Incubation of the purified metabolite (5.2 mg.) with β-glucuronidase (5.7 mg., Sigma Chemical Co.) in 5 ml. of sodium acetate-acetic acid buffer (pH 5.8) was carried out for 18 hr. at 37° (9). After this time the protein was denatured with 1 ml. of 5% trichloroacetic acid, and the residue filtered off. The filtered material was washed with 0.2 ml. of water and the combined filtrates extracted with four 2-ml. portions of ether in order to remove excess trichloroacetic acid. The aqueous layer was aerated and then added to a volumetric flask and diluted to 10 ml. When the hydrolysate was assayed for phenol (8) and reducing sugar (7), the ratio was found to be approximately 1:1 (using the standard curves described above).

Strong Base Metabolite—The strong base fraction from 22 rats (335 mg.) was extracted with methanol. Almost the entire residue dissolved. The material was crystallized by means of concentration of the methanol solution and subsequent addition of isopropyl alcohol. These extracts contained the majority of the radioactivity present. The extracts were evaporated to dryness and the solid taken up in water to be used for chromatography.

Synthesis of p-Hydroxy-β-phenethylbiguanide Hydrochloride—Tyramine hydrochloride (2.5 g., 0.014 mole) was added to cyanoguanidine (1.8 g., 0.014 mole) in a 10-ml. conical flask. The flask

was heated in an oil bath to the fusion point (oil bath 175°) and then the bath temperature was raised to 190° for 2 hr. Upon cooling the mixture solidified. The solid glass-like material was dissolved in methanol and the solution concentrated until a precipitate was obtained upon cooling. The precipitate contained considerable unreacted starting material. One-half of the filtrate was added to a solution of cupric chloride dihydrate in 10 ml. of water and the pH adjusted to 5.6. The precipitated copper biguanide complex, along with any copper hydroxide formed, was centrifuged and washed with two 10-ml. portions of water. The solid material was slurried in 20 ml. of water and hydrogen sulfide gas was passed through the slurry for 1 hr. When the formation of copper sulfide was complete, the material was filtered and the solution lyophilized.

The grayish solid was extracted with methanol and the insoluble copper sulfide residue filtered off. The solution was evaporated to dryness and taken up in a minimum of hot ethanol. Upon cooling, 115 mg. of white crystals were obtained, m.p. 181–186°. Recrystallization from acetonitrile gave white crystals, m.p. 180–183°.[1] The NMR spectrum of this compound in trifluoroacetic acid confirms the structure as *p*-hydroxy-β-phenethylbiguanide. The symmetric aromatic quartet [$\delta = -7.21$ p.p.m.][2] is characteristic of *p*-substituted aromatic rings. The nitrogen protons of the protonated biguanide are observed slightly downfield of the aromatic protons. The methylene protons exhibit peaks at [$\delta = -3.13$ p.p.m.] and [$\delta = -3.92$ p.p.m.].[3]

The ultraviolet spectrum in water shows the absorption ($\lambda_{max.}$ 227 mμ, ε 15.8 × 10³) characteristic of the biguanide conjugated system (11). The infrared spectrum was consistent with the proposed structure [*e.g.*, NH peaks 3,320 cm.$^{-1}$, 1,653 cm.$^{-1}$ (12); aromatic 1,4-disubstitution peaks 1,620 cm.$^{-1}$, 832 cm.$^{-1}$] (13).

Chromatographic Identification of the Metabolite—The hydrolyzed weak base metabolite, the purified strong base metabolite, and synthetic *p*-hydroxy-β-phenethylbiguanide were chromatographed in acidic, neutral, and basic solvent systems. The results of these chromatograms are given in Table II. In all three systems the strong base metabolite, the weak base hydrolysate, and the synthetic *p*-hydroxy-β-phenethylbiguanide show the same R_f values, indicating that the three are identical.

Spectroscopic Identification of the Metabolites—The ultraviolet absorption spectra of synthetic *p*-hydroxy-β-phenethylbiguanide hydrochloride in neutral and basic solution were compared to the spectra obtained with the weak base hydrolysate, and the strong base metabolite in identical solutions. In all cases these spectra were identical, thus confirming that the metabolite was *p*-hydroxy-β-phenethylbiguanide hydrochloride.

The oxidation of aromatic rings in the process of metabolism is a well documented process (1). The observation that β-phenethylbiguanide is hydroxylated *in vivo* may have important implications as to its mode of action. Work on the physiological activity of this metabolite is currently under way.

[1] Analytically pure *p*-hydroxy-β-phenethylbiguanide hydrochloride subsequently supplied by U. S. Vitamin and Pharmaceutical Corp., New York, N. Y., was found to have identical physical characteristics as this material.

[2] Relative to external tetramethylsilane.

[3] The analysis of the structure and NMR spectra of protonated biguanides have been reported elsewhere (10). The readers are referred to the MS thesis of P. J. Murphy for the original spectra.

TABLE II—R_f VALUES OF URINARY METABOLITES AND CONTROLS[a]

Compound	R_f Value in Solvent System[b] A	B	C
Weak base (conj.)	0.25	0.20	—
Weak base (hydrol.)	0.17[c]	0.35[c]	0.65[c]
Strong base	0.17[d]	0.35[d]	0.65[d]
p-Hydroxy-β-phenethylbiguanide	0.17	0.35	0.65
β-Phenethylbiguanide	0.50	0.67	0.74

[a] Ascending technique at room temperature. [b] Solvent systems; A, *n*-butanol, saturated with water; B, *n*-butanol–formic acid–water (63:17:20); C, methanol–pyridine–water (80:2:20). [c] In each system the weak base hydrolysate gave a radioactive spot lower than the main spot. This spot is attributed to incomplete hydrolysis. [d] The reported spot is the major guanidine-positive spot, however a much weaker positive area was observed closer to the origin on all chromatograms. This spot is not presently explainable.

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Keyphrases

β-Phenethylbiguanide (C-14 labeled)—metabolism
Metabolites—isolated, identified
Radioactivity determination—relative, specific
Column chromatography—separation
Paper chromatography—identity
UV spectrophotometry—structure
IR spectrophotometry—structure
NMR spectrometry—identity

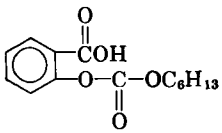
Pharmacology of the Hexylcarbonate of Salicylic Acid

By A. MISHER, H. J. ADAMS, J. J. FISHLER, and R. G. JONES

Gastric absorption of aspirin, salicylate, or acetic acid can result in increased mucosal permeability to H^+ ions leading to gastric bleeding. The pharmacology of the hexylcarbonate derivative of salicylic acid was investigated because its physical-chemical properties are such as could reduce the compound's availability to the gastric mucosa. It was found that the compound possesses antipyretic, analgesic, and anti-inflammatory activity qualitatively identical to those of aspirin. Its acute toxicity is less than that of aspirin in rats and mice and its gastric irritation potential is markedly lower in rats and dogs.

IT HAS BEEN SHOWN that the gastric absorption of aspirin, salicylate, or acetic acid can result in damage to the mucosa, making it more permeable to small ions including H^+ ions, and that the absorption of moderate quantities of H^+ ions through the insulted mucosa leads to gastric bleeding (1, 2).

The fact that aspirin administration is often associated with gastric irritation and hemorrhage is well documented in the literature (3). The authors' studies with carbonate derivatives of various drugs (4) suggested that salicylic acid might lend itself to prodrug formation, with retention of the antipyretic, analgesic, and anti-inflammatory activities, but with altered physical-chemical properties. Of the derivatives prepared, the *n*-hexyl-*O*-carboxyphenylcarbonate (SK&F 26070) (I) showed the most favorable profile being about only one-twentieth as soluble as aspirin in 0.1 *N* HCl at 37°, not producing acetic acid on hydrolysis, and having a much altered oil/water solubility coefficient (5). Changes such as these could reduce the compound's availability to the gastric mucosa.



I

These studies were conducted in order to determine the impact of these changes on the pharmacological profile of this compound and its potential to cause gastric irritation.

EXPERIMENTAL

Acute Oral Toxicity in Mice and Rats—Male Carworth Farms (CF_1-S) mice, weighing 18–24 Gm., and male Charles River rats, weighing 100–159 Gm., were randomly divided into groups of 10 and dosed orally with suspensions of aspirin or SK&F 26070. The maximum dose volume was 20 ml./Kg. Control animals received either 0.5% tragacanth or 1% methylcellulose (400 cps.) at corresponding dose volumes. Daily observations were made until no deaths had occurred for 2 consecutive days following the fifth day. Food and water were available *ad libitum*.

Gastric Irritation in Rats—Male Charles River rats, weighing 144–213 Gm., were fasted 18 hr. prior to use. They were then randomly divided into groups of 10 and dosed orally with aspirin or SK&F 26070 suspended in 1% methylcellulose (400 cps.). Drug concentrations were adjusted so that each animal received a dose volume of 10 ml./Kg.; 20 control animals received an equal dose volume of the vehicle. Two hours postdrug, the animals were sacrified with ether. The stomachs were excised, opened along the greater curvature, and gently rinsed with warm water. They were then examined under low magnification with a binocular microscope and rated positive or negative for hemorrhage and for ulceration, these evaluations were done on a "blind" basis.

Gastric Irritation in Dogs—Mongrel dogs of either sex, weighing 10–15 Kg., were randomly assigned identification numbers and divided into groups of four. Each group received one of the following treatments: aspirin, 25 mg./Kg.; aspirin, 75 mg./Kg.; SK&F 26070, 25 mg./Kg.; SK&F 26070, 75 mg./Kg.; empty gelatin capsules (control group). Each animal received six consecutive oral doses, spaced 12 hr. apart. The animals were sacrificed 4–5 hr. after the last dose. The stomachs were removed, opened, and the degree of irritation graded on a "blind" basis.

Antipyretic Activity in Rats—Eighty male Charles River rats were randomly divided into six drug groups of 10 and a control group of 20 animals. Immediately after a control rectal temperature had been taken, each animal received subcutaneously in the nape of the neck 1 ml./Kg. of a freshly prepared 20% suspension of brewer's yeast in isotonic saline. Rectal temperatures were taken 5 hr. later, and each animal then received a dose of drug or an equivalent dose volume of vehicle. Both aspirin and SK&F 26070 were administered orally at 50, 100, and 200 mg./Kg., suspended in 0.5% tragacanth, at a dose volume of 20 ml./Kg. Rectal temperatures were taken again 2 hr. postdrug. Food was withheld throughout the test period, but water was available *ad libitum*.

The results were evaluated quantitatively: if the final rectal temperature of an animal was at least 1° below the mean of the control group, the animal's

Received January 24, 1968, from the Research & Development Division, Smith Kline & French Laboratories, Philadelphia, PA 19101
Accepted for publication March 1, 1968.

response was negative. ED_{50}'s for the antipyretic activity of the two compounds were calculated by the Logit chi-square method of Berkson (6).

Analgesic Activity in Rats—The inflamed paw method of Randall and Selitto, which has been shown to be sensitive enough to detect the mild nonnarcotic analgesics (7), was used for this study. Male and female Dierolf Farms rats, weighing 80–110 Gm., were fasted 16 hr. before use. Inflammation was produced by the injection of 0.1 ml. of a freshly prepared 20% suspension of brewer's yeast into the plantar surface of the rat's left hind foot. Two and one-half hours after the yeast injection, pain threshold determinations (predrug control values) were made on the inflamed paw. Animals were then randomly divided into groups of five and dosed orally. Drugs were prepared as suspensions in 0.5% tragacanth, and concentrations were adjusted so that each animal received a dose volume of 20 ml./Kg.; 10 control animals received 20 ml./Kg. of vehicle. Threshold determinations were made at 30, 60, 120, 180, and 240 min. postdrug.

To determine pain thresholds, pressure was applied to the plantar surface of the inflamed paw at a constant rate of 20 mm. Hg/sec. by means of a Plexiglas cone attached to the plunger of a 10-ml. syringe; the syringe was connected to a pressure gauge, control valves, and a source of air pressure. The pain threshold is expressed as the pressure (in mm. Hg) required to induce struggle in the animal. Analgesic activity is expressed as the difference (Δ mm. Hg) between the pressure required to induce this behavior after drug administration as compared to the measurement before drug administration.

Anti-inflammatory Activity—Anti-inflammatory activity was tested in the rat in which polyarthritis was induced by the intradermal injection of adjuvant; the method used was similar to that described by Ward and Cloud (8) and by Glenn (9). Paw volumes were recorded by means of a modification of the method described by Winter and Nuss (10).

On the morning of Day 1, the plantar surface of the right hind paw of male Carworth Farms rats (initial weight 145–213 Gm.) was injected intradermally with adjuvant (0.1 ml. of a 2.5 mg./ml. suspension of *Mycobacterium butyricum*[1] in mineral oil). Immediately after the injection, animals were randomly divided into groups of 10, and the volume of each injected paw was measured by immersing the paw to the level of the lateral malleolus in a mercury bath which was connected through a closed, fluid-filled tube to the Microcord 44[2] recorder. Volumes could be measured accurately with reliability to the hundredth of a milliliter.

The drugs were administered immediately after this initial paw volume measurement, and again 3 hr. later. The doses of aspirin were 100 and 200 mg./Kg., b.i.d., the doses of SK&F 26070 were 148 and 293 mg./Kg., b.i.d. and were equimolar to those of aspirin. The drugs were suspended in 0.5% methylcellulose (4,000 cps.) and administered orally, b.i.d., at a dose volume of 10 ml./Kg. for 19 days.

From Day 2 through Day 19 the second dose was administered approximately 5 hr. after the first dose. One adjuvant control group received 0.5% methylcellulose (4,000 cps.) orally, b.i.d., at a dose volume of 10 ml./Kg. for 19 days. To provide "normal" values for the parameters measured, a group of 10 rats that received neither adjuvant nor treatment was used.

On Day 20, plasma inflammation units were determined according to the method outlined by Glenn and Kooyers (11). The animals were anesthetized with ether, and blood was obtained in heparinized syringes by cardiac puncture. The blood samples were placed into heparinized glass centrifuge tubes and centrifuged at about 1500 r.p.m. for 30 min. Plasma samples were withdrawn, and inflammation units were determined after diluting the plasma 1:200 with sterile physiological saline.

The initial turbidity readings of the diluted solutions were determined in a Coleman No. 9 nephelometer, set at full sensitivity, against the nephalos standard, $N = 19$. The diluted solutions were subsequently placed in a water bath at 56° for 30 min., cooled to room temperature, and turbidity again determined. The differences between initial and final nephelometric readings are expressed as "corrected inflammation units."

RESULTS

Acute Oral Toxicity in Mice and Rats—As shown in Tables I and II, milligram for milligram, aspirin is about twice as toxic orally in both mice and rats as SK&F 26070; on a molar basis, aspirin is significantly more toxic ($p = 0.05$) than SK&F 26070. With both drugs, most or all deaths occurred within 24 hr. postdrug.

TABLE I—ACUTE ORAL TOXICITY IN MICE

Drug	Dose, mg./Kg. (10 Animals/ Group)	Mortality, %	LD_{50} 95% Confidence Limits[a]
SK&F 26070	1000	0	2680 (2158–3777)
	1450	10	mg./Kg. 10.1 (8.1–
	2200	20	14.4) moles/Kg.
	3000	60	$\times 10^{-3}$
	4250	100	
Aspirin	750	0	1264 (1091–1478)
	1000	20	mg./Kg. 7.0(6.0–
	1250	50	8.2) moles/Kg. $\times$
	1500	70	10^{-3}
	2000	100	

[a] LD_{50}'s and 95% confidence limits were calculated by the Logit chi-square method of Berkson.

TABLE II—ACUTE ORAL TOXICITY IN RATS

Drug	Dose, mg./Kg. (10 Animals/ Group)	Mortality, %	LD_{50} 95% Confidence Limits[a]
SK&F 26070	2080	0	3447 (2680–4253)
	3000	40	mg./Kg. 13.0
	4300	70	(10.2–16.0) moles/
	6200	100	Kg. $\times 10^{-3}$
Aspirin	625	0	1366 (1003–1744)
	1250	40	mg./Kg. 7.6 (5.6–
	2000	80	9.7) moles/
	2500	100	Kg. $\times 10^{-3}$

[a] LD_{50}'s and 95% confidence limits were calculated by the Logit chi-square method of Berkson.

[1] Difco Laboratories, Detroit, Mich.
[2] Photovolt Corp., New York, N. Y.

TABLE III—GASTRIC IRRITATION IN RATS

Drug	Dose, mg./Kg., p.o. (10 Animals/Group)	Incidence of Gastric Hemorrhage, %	Incidence of Gastric Erosions %
SK&F 26070	84.4	30	0
	148	20	0
	259	30	0
Aspirin	57	70	20
	100	100	30
	175	100	90
1% Methylcellulose	10 ml./Kg.	0	0

Gastric Irritation in Rats—At equimolar dose levels, the incidence of hemorrhagic stomachs in rats that received SK&F 26070 was markedly lower than in rats that received aspirin (Table III). Even at the highest dose tested (259 mg./Kg.), SK&F 26070 produced no gastric erosions; the molar equivalent dose of aspirin, on the other hand, caused gastric erosions in 90% of the animals.

Gastric Irritation in Dogs—In dogs, the effects of the drugs were studied after repeated administration—six doses spaced 12 hr. apart—rather than after a single administration as in rats. The results are even more pronounced than in rats. The stomachs of all eight dogs that received SK&F 26070 were normal in appearance and indistinguishable from those of control animals. All four dogs that received the 25 mg./Kg. dose of aspirin and one of those that received the 75 mg./Kg. dose showed localized superficial mucosal irritation or inflammation; the other three dogs at the high dose showed mucosal irritation and isolated hemorrhage. On a molar basis, the high dose of SK&F 26070, which produced no gastric irritation, is about twice that of the low dose of aspirin, which caused gastric irritation in all four animals.

Antipyretic Activity in Yeast-Fevered Rats—At each dose level, the percent of animals tested that exhibited an antipyretic response to the drugs was identical for both SK&F 26070 and aspirin: 50 mg./Kg.—60%; 100 mg./Kg.—80%; 200 mg./Kg.—100%. Therefore, the two compounds appear equipotent, on a mg. for mg. basis, in antipyretic activity in this test procedure.

Analgesic Activity in Rats—At 200 mg./Kg., aspirin and SK&F 26070 caused a maximum increase in the pain threshold of the inflamed paw of 31 mm. Hg and 34 mm. Hg, respectively; at 400 mg./Kg., aspirin produced a maximum increase of 87 mm. Hg and SK&F 26070 effected a 72 mm. Hg increase. These data indicate that SK&F 26070 exhibits analgesic activity comparable to that of aspirin.

Anti-inflammatory Activity: Paw Volume Change—The onset of the polyarthritis was indicated by an increase in volume of the injected paw after an initial inflammatory phase of about 10 days in which the volume first increased and then decreased. In the vehicle control group, the onset of polyarthritis occurred between Days 9 and 11 (see Table IV). Statistical analysis of the data seems to indicate that the onset of drug activity occurred earlier with SK&F 26070 than with aspirin.

On Days 15–19, both dose levels of SK&F 26070 and of aspirin elicited similar degrees of inhibition of paw volume increase. On Days 17 and 19, the inhibition of paw volume increase by the high dose

TABLE IV—ANTI-INFLAMMATORY ACTIVITY: REDUCTION IN VOLUME OF INJECTED PAW

Compound	Dose, b.i.d. (10 Animals/Group) mg./Kg.	Mean Paw Volumes (ml.) on Days 1	3	5	7	9	11	13	15	17	19	ED_{50} with 95% Fieller Confidence Limits Moles/Kg. $\times 10^{-3}$	mg./Kg. b.i.d.
SK&F 26070	148	0.94	1.22	1.11	1.06[a]	1.03	1.11[a]	1.12[a]	1.20[a]	1.28[a]	1.30[a]	0.98	261.0
	293	0.90	1.16[a]	1.10	1.02[a]	0.94[a]	1.06[a]	1.01[a]	1.05[a]	1.11[a]	1.12[a]	(—[b])	(—[b])
Aspirin	100	0.95	1.18	1.17	1.13	1.09	1.23	1.17[a]	1.22[a]	1.31[a]	1.36[a]	0.99	178.3
	200	0.95	1.16	1.13	1.10	1.02[a]	1.12[a]	1.12[a]	1.04[a]	1.12[a]	1.12[a]	(0.67–2.6)	(120.7–468.4)
0.5% Methylcellulose, 4,000 cps.	10 ml./Kg.	0.95	1.26	1.09	1.15	1.14	1.34	1.36	1.48	1.59	1.62	—	—
Nonarthritic control	No treatment	0.68[a]	0.78[a]	0.71[a]	0.74[a]	0.67[a]	0.79[a]	0.73[a]	0.68[a]	0.66[a]	0.68[a]	—	—

[a] Significantly different from the methylcellulose group ($p = 0.05$). [b] Limits could not be calculated.

TABLE V—ANTI-INFLAMMATORY ACTIVITY: PLASMA INFLAMMATION UNITS IN RATS

Compound	Dose, (10 Rat/Group) mg./Kg.	Mean Corrected Inflammation Units Determined from Plasma Withdrawn on Day 20	ED_{50} with 95% Fieller Confidence Limits Moles/Kg. × 10^{-3} b.i.d.	mg./Kg. b.i.d.
SK&F 26070	148	49.2	0.92	245.0
	293	21.5	(0.62–3.86)	(165.1–1027.9)
Aspirin	100	41.8	0.84[a]	151.3[a]
	200	20.3		
0.5% Methocel, 4,000 cps.	10 ml./Kg.	57.2	—	—
Nonarthritic control	No treatment	5.1	—	—

[a] Limits could not be calculated.

of both drugs was significantly greater ($p = 0.05$) than elicited by the low dose of the two drugs. ED_{50}'s for inhibition of paw volume increase (doses that decreased by 50% the difference in paw volumes between the vehicle control and nonarthritic control groups) were calculated from the data of Day 19. The mean paw volumes for each day and the ED_{50}'s for Day 19 are listed in Table IV.

The mean paw volume of the nonarthritic control group remained relatively constant during the 19 days of this study. Apparently the extremeties do not increase in size in proportion with the weight gain of the rat during this interval of growth.

Plasma Inflammation Units—The mean "corrected plasma inflammation units," determined on Day 20, are shown in Table V. The "corrected plasma inflammation units" for the low dose of SK&F 26070 and aspirin are not significantly different from the vehicle control group. The mean "corrected inflammation units" for the high dose for both compounds were similar and statistically significantly different from that of the vehicle control group. ED_{50}'s were calculated and are shown in Table V.

DISCUSSION

Orally in the mouse and rat, SK&F 26070 is less toxic, both on a milligram for milligram and on a molar basis, than aspirin. It is also significantly less irritating to the gastric mucosa of the rat and dog, even when administered repeatedly as was done with dogs. Although one might conclude that the lower toxicity and reduced gastric irritation are due to an incomplete or a slower rate of absorption, a previous study showed that SK&F 26070 is absorbed as rapidly and completely as aspirin following oral administration to dogs (5).

SK&F 26070 exhibits antipyretic, analgesic, and anti-inflammatory activities, and its pharmacological profile is, therefore, qualitatively similar to that of aspirin. One can infer from this and from the *in vitro* studies of hydrolysis of the compound (5) that the prodrug is cleaved after oral administration (and possibly after absorption) and that the pharmacological activities are due to the resultant salicylate formation. SK&F 26070's reduced gastric liability may be due to a reduced availability of the compound to the gastric mucosa because of the physical properties previously discussed.

Although SK&F 26070's acute toxicity and gastric irritation potential are less than those of aspirin, it appears, on a molar basis, as potent as aspirin in reducing both paw volume and plasma inflammation units in the chronic study of adjuvant-induced arthritis in rats.

SUMMARY

SK&F 26070, the hexylcarbonate of salicylic acid, possesses antipyretic, analgesic, and anti-inflammatory activities qualitatively identical to those of aspirin.

Its acute toxicity is less than that of aspirin, and its gastric irritation potential is markedly lower. Upon chronic administration, SK&F 26070 is as potent as aspirin in reducing both paw volume and plasma inflammation units in rats with adjuvant-induced arthritis.

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Keyphrases

Salicylic acid hexylcarbonate
Pharmacological activity—salicylic acid hexylcarbonate
Nephelometry—plasma analysis
Gastric irritation—salicylic acid hexylcarbonate

Study of the Sympathomimetic Action of Cyclohexylamine, a Possible Metabolite of Cyclamate

By H. I. YAMAMURA, I. P. LEE, and R. L. DIXON

The pharmacological actions of cyclohexylamine on the autonomic nervous system were tested using the cat's blood pressure and nictitating membrane responses. Although cyclohexylamine had no effect upon the adrenergic, cholinergic, or histaminic responses as shown by epinephrine, norepinephrine, acetylcholine, or histamine, it was demonstrated to possess a pressor action, and this pressor activity could be blocked by phenoxybenzamine. The results indicated that cyclohexylamine may act by releasing the neural humoral transmitter substance norepinephrine because prior pretreatment with reserpine, which depletes the catecholamine stores, abolished the pressor effect. Upon subsequent administration of norepinephrine the pressor response returned. It was shown that there was no potentiation between cyclohexylamine and a monoamine oxidase inhibitor, but potentiation did occur between tyramine and the monoamine oxidase inhibitor.

CYCLAMATE AND saccharin are extensively used as noncaloric sweetening agents for various beverages and foods. Ingestion of cyclamate in excess of 5 Gm. per day is not an unreasonable amount considering its widespread use. Recently, Kojima and Ichibagase (1) and Leahy *et al.* (2) have indicated the possibility that a small portion of administered sodium cyclamate may be metabolized to cyclohexylamine. The possibility also exists that cyclohexylamine may be ingested either as impurity or due to spontaneous breakdown in various food products. These findings prompted this investigtion of cyclohexylamine in order to: (*a*) investigate the action of cyclohexylamine on the vascular system, (*b*) study the effect of cyclohexylamine on the action of various endogenous transmitter substances, and (*c*) consider the possible interaction between cyclohexylamine and monoamine oxidase inhibitors.

METHODS

Twenty-five cats of either sex, weighing between 2.5–3.5 Kg., were anesthetized with sodium pentobarbital (30 mg./Kg.) administered intraperitoneally (i.p.). Blood pressure was recorded from the cannulated left femoral artery using a physiograph "Four" recorder, E & M Pressure Transducer (model P-1000) and E & M Myograph (type B). The contractions of the nictitating membrane were determined after placing it under an initial tension of 5 Gm. A tracheal cannula was routinely inserted and artificial respiration was administered when necessary.

Drugs—The following drugs were used: *l*-epinephrine bitartrate (Sigma Chemical Co.); *l*-norepinephrine bitartrate (City Chemical Co.); tyramine HCl (Mann Research Laboratories); acetylcholine chloride (NBC); histamine phosphate (Burroughs Wellcome & Co.); cyclohexylamine HCl (K & K Laboratories, Inc.); reserpine (Ciba Pharmaceutical Co.); pargyline HCl (Abbott Laboratories); phenoxybenzamine HCl (Smith Kline and French Laboratories); and propranolol (Ayerst Laboratories). The concentrations of drugs used in all experiments were expressed on the basis of their salts, except for epinephrine and norepinephrine, which were expressed on the basis of their base forms.

RESULTS

General Action of Cyclohexylamine—Figure 1 shows a record taken from an experiment performed using an anesthetized cat. It can be seen that injections of 10 mcg./Kg. each of epinephrine and norepinephrine, 3 mcg./Kg. of acetylcholine, and 5 mcg./Kg. of histamine resulted in the characteristic responses of each drug. Cyclohexylamine (5 mg./Kg.) was then administered intravenously and a pressor response was noted. Subsequent administration of epinephrine, norepinephrine, acetylcholine, and histamine still produced their characteristic responses indicating that cyclohexylamine was not an adrenergic, cholinergic, or a histaminic blocker, but that it was capable of exerting a pressor action.

Reversal of Cyclohexylamine Response by Phenoxybenzamine—To determine if cyclohexylamine was a sympathomimetic amine, phenoxy-

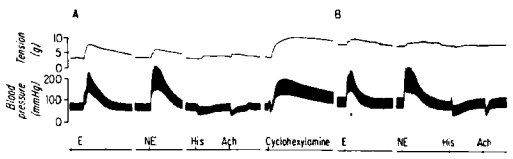

Fig. 1—The effects of epinephrine (E) (10 mcg./Kg.), norepinephrine (NE) (10 mcg./Kg.), histamine (His) (5 mcg./Kg.), and acetylcholine (ACh) (3 mcg./Kg.) before and after the administration of cyclohexylamine (5 mg./Kg.) on the cat's blood pressure and nictitating membrane.

Received November 2, 1967, from the Department of Pharmacology, School of Medicine, University of Washington, Seattle, WA 98105

Accepted for publication January 16, 1968.

This research was supported by the Sugar Research Foundation and by training grant No. GM0109 from the National Institutes of Health, U. S. Public Health Service.

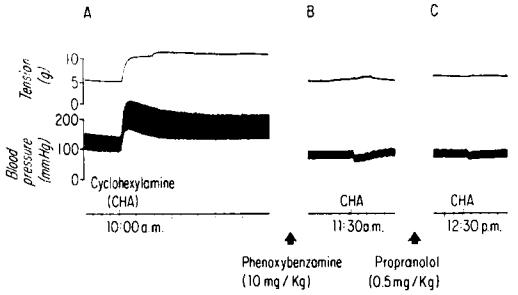

Fig. 2—The effect of cyclohexylamine (CHA) (5 mg./Kg.) on the cat's blood pressure and nictitating membrane following phenoxybenzamine (10 mg./Kg.) and propranolol (0.5 mg./Kg.).

benzamine (10 mg./Kg.), an α-adrenergic blocking agent, was administered intravenously. These results are presented in Fig. 2. After the adrenergic blockade was established, which required approximately 30 min., a second dose of cyclohexylamine (5 mg./Kg.) was given. The usual pressor response to cyclohexylamine was no longer noticed; rather a depressor response was now observed. Propranolol (0.5 mg./Kg.), a β-blocking agent, was then given to determine whether the depressor response could be blocked. Partial blockage of the depressor response was noted as seen in Fig. 2.

Effect of Cyclohexylamine After Pretreatment with Reserpine—Figure 3 demonstrates that an injection of 500 mcg./Kg. of tyramine, an indirect acting amine, caused a rise in blood pressure and produced a contraction of the nictitating membrane in a normal anesthetized cat. An injection of 5 mg./Kg. of cyclohexylamine also caused a large rise in blood pressure and a large and prolonged contraction of the nictitating membrane. In a subsequent study (Fig. 4), the successive injections of tyramine and cyclohexylamine were administered to a cat pretreated approximately 24 hr. with reserpine (1 mg./Kg. i.p.) in order to deplete the catecholamine stores. The effects of tyramine and cyclohexylamine, both on the blood pressure and nictitating membrane response, were diminished by the reserpine treatment. Norepinephrine (0.6 mg./Kg.) was then administered slowly into the femoral vein. This injection of norepinephrine caused a large rise in blood pressure and contraction of the nictitating membrane (Fig. 4). After the injection of norepinephrine, the blood pressure and contraction of the nictitating membrane returned to their initial level. An injection of tyramine (500 mcg./Kg.) followed by cyclohexylamine (5 mg./Kg.) was made again as shown in Fig. 4. The increase in the blood pressure was equal to that of control values and there was a noticeable contraction of the nictitating membrane which was absent after pretreatment with reserpine. The events shown in Fig. 4 were observed in each of three experiments. After the pressor action of tyramine and cyclohexylamine had been restored by administration of norepinephrine, the repeated injections of either tyramine or cyclohexylamine had a diminished effect. However, another injection of norepinephrine once again restored the pressor action.

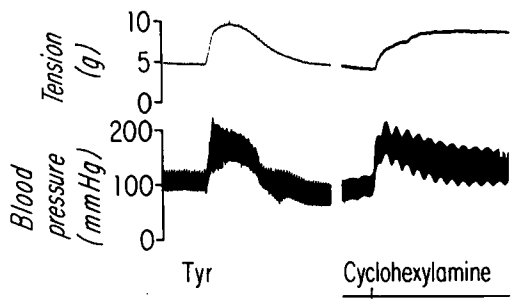

Fig. 3—The cat's pressor and nictitating membrane responses to tyramine (500 mcg./Kg.) and cyclohexylamine (5 mg./Kg.) in a normal anesthetized cat.

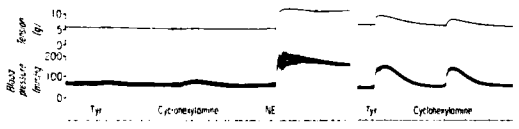

Fig. 4—The effect of slow administration of norepinephrine (0.6 mg./Kg.) on the cat's pressor and nictitating membrane responses to tyramine (500 mcg./Kg.) and cyclohexylamine (5 mg./Kg.) in a reserpinized (1 mg./Kg. i.p.) cat.

Effect of Cyclohexylamine After Pretreatment with Pargyline—These experiments were undertaken to determine if potentiation of the action of cyclohexylamine would be observed following animal pretreatment with a monoamine oxidase inhibitor. It should be recalled that pretreatment of patients with monoamine oxidase inhibitors resulted in a hypertensive crisis when these patients ingested the sympathomimetic agent, tyramine, contained in various foods (3, 4).

Therefore, pargyline (25 mg./Kg.), a monoamine oxidase inhibitor, was administered i.p. to cats 24 hr. prior to the start of the experiments. As can be seen in Fig. 5, pargyline pretreatment had no effect on the action of cyclohexylamine on either blood pressure or nictitating membrane response. However, in contrast, pretreatment with the monoamine oxidase inhibitor potentiated the responses seen after tyramine (500 mcg./Kg.). It can be seen that after tyramine there was a potentiation in both the pressor and in the nictitating membrane responses when compared to control values. Data obtained from all experimental animals are presented in Table I. Thus, there did not appear to be any significant interaction between this monoamine oxidase inhibitor and cyclohexylamine. This is most likely due to the fact that cyclohexylamine, in contrast to tyramine, is only a very weak substrate for MAO (5).

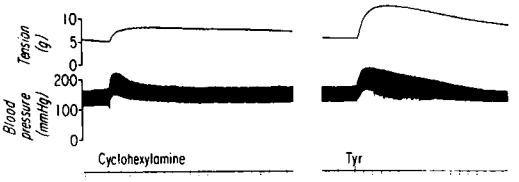

Fig. 5—The effect of cyclohexylamine (5 mg./Kg.) and tyramine (500 mcg./Kg.) on the pressor and nictitating membrane responses of a 24 hr. pargyline (25 mg./Kg. i.p.) pretreated cat.

TABLE I—CHANGE IN BLOOD PRESSURE[a] AND TENSION OF THE NICTITATING MEMBRANE IN CONTROL AND PARGYLINE-PRETREATED CATS

Experiment	Cyclohexylamine (5.0 mg./Kg.) Blood Pressure (mm. Hg)	Cyclohexylamine (5.0 mg./Kg.) Nictitating Membrane (Gm.)	Tyramine (500 mcg./Kg.) Blood Pressure (mm. Hg)	Tyramine (500 mcg./Kg.) Nictitating Membrane (Gm.)
Control	82 ± 8.2[b] (5)[c]	5.2 ± 0.62 (5)	64 ± 4.8 (7)	3.5 ± 0.71 (7)
Pargyline-pretreated (25 mg./Kg.)	73 ± 10.8 (6)	4.7 ± 0.98 (5)	78 ± 8.2[d] (6)	7.2 ± 0.52[d] (6)

[a] Blood pressure = diastolic pressure + 1/3 pulse pressure. [b] Mean value ± standard error of the mean. [c] Number of animals. [d] Significant difference from control ($p \leq 0.05$).

DISCUSSION

The data presented in this paper demonstrate that cyclohexylamine, a possible metabolite of cyclamate, possessed pressor activity and caused contraction of the nictitating membrane of the anesthetized cat. Cyclohexylamine had no effect upon the adrenergic, cholinergic, or histaminic responses elicited by epinephrine, norepinephrine, histamine, or acetylcholine.

The pressor activity of cyclohexylamine was shown to be due to the action of cyclohexylamine on the sympathetic nervous system. Prior administration of phenoxybenzamine, an irreversible competitive α-adrenergic receptor blocker, reversed the pressor response of cyclohexylamine and resulted in a depressor response which in turn was slightly blocked by the β-receptor blocking agent, propranolol.

Cyclohexylamine appeared to be an indirect acting sympathomimetic amine. After reserpine depletion of transmitter substance, cyclohexylamine was unable to produce its characteristic increase in blood pressure. The same result was seen with tyramine, which is a known indirect acting sympathomimetic amine. The responses to both tyramine and cyclohexylamine could be restored by slow administration of norepinephrine which has been shown to restore the depleted transmitter levels after reserpine pretreatment (6). Further evidence to indicate the indirect nature of cyclohexylamine was obtained by injecting cats repeatedly with the amine. After the first injection, the subsequent responses became progressively less until cyclohexylamine had only minimal effect. The same decrease in response has been demonstrated for tyramine.

Cyclohexylamine appeared to be like tyramine in many ways: (*a*) both produced hypertension in cats after i.v. injection, (*b*) both appeared to act indirectly by release of catecholamines, and (*c*) both were effective only after relatively high doses were administered. Recently, it was demonstrated that clinically useful monoamine oxidase inhibitors could potentiate or change the action of several other drugs and even certain foods (3, 4). For example, tyramine evoked, augmented, and prolonged hypertensive effects in animals and patients treated with inhibitors of monoamine oxidase. Usually innocuous amounts of tyramine present in certain food, including cheese caused hypertensive reactions in patients treated with the enzyme inhibitor.

Because cyclohexylamine produced a pressor response which closely resembled the pressor action of tyramine, and cyclohexylamine may possibly be ingested in many diets or produced from ingested substrate, its interaction with monoamine oxidase inhibitors was investigated. The action of cyclohexylamine was not potentiated in animals treated before with a monoamine oxidase inhibitor. This finding was in contrast to that demonstrated when tyramine was administered to animals pretreated with a monoamine oxidase inhibitor. After inhibition of this enzyme system the response to tyramine was potentiated and prolonged. The explanation for this dichotomy most likely is the fact that cyclohexylamine is not a substrate for monoamine oxidase; in fact, cyclohexylamine has been shown to be a weak inhibitor of the enzyme (5).

The possible clinical implications of these findings must wait until the metabolism and possible spontaneous breakdown of cyclamate are better understood. If an enzyme system is responsible for the hydrolysis of the artificial sweetening agent, then the possible substrate-induction of the enzyme must be considered. This, of course, could increase tremendously the amount of cyclohexylamine produced in the body and therefore also increase the possible toxicologic implications of the metabolite. Other pharmacological aspects concerning the interaction of the artificial sweetening agents and their metabolites are presently under investigation.

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Keyphrases

Cyclohexylamine—sympathomimetic activity
Pressor activity—cyclohexylamine
Phenoxybenzamine effect—cyclohexylamine activity
Reserpine effect—cyclohexylamine activity
Pargyline effect—cyclohexylamine activity

Absorption and Elimination Profile of Isoproterenol III

The Metabolic Fate of *dl*-Isoproterenol-7-^{3}H in the Dog

By W. D. CONWAY*, H. MINATOYA, A. M. LANDS, and J. M. SHEKOSKY

Intravenous injection of isoproterenol-7-^{3}H in dogs, in doses ranging from 0.4 to 1.6 mcg./kg. body weight, immediately induced tachycardia which declined with a half-life of about 1 min. The resulting levels of radioactivity in blood declined with a significantly longer half-life of 3–4 min. Unchanged isoproterenol accounted for less than 1 percent of the total radioactivity in plasma after intravenous medication. After oral administration of 15 mg. of isoproterenol-7-^{3}H hydrochloride to dogs, a biphasic time course was observed for both heart rate and radioactivity in blood with maxima occurring at 0.5 and 1–2 hr. after medication. The metabolic fate of isoproterenol was determined by the route of medication, the intravenous and intraportal route giving rise mainly to 3-*O*-methylisoproterenol, while oral medication was converted mainly to a sulfuric acid ester which was formed either by the intestinal flora or during passage through the intestinal wall. The rapid decrease in tachycardia after intravenous medication is consistent with the rapid conversion of the drug to inactive metabolites while the more prolonged tachycardia induced by oral medication results from slow absorption.

THE EFFICACY of the sympathomimetic amine, isoproterenol hydrochloride,[1] [1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol] in the treatment of bronchial asthma and other bronchopulmonary diseases as well as heart block, when administered by various routes of medication, prompted recent studies of the absorption profile of the drug in dogs (1, 2). The drug was found to be poorly absorbed from the stomach but well absorbed from the small intestine, proximal colon, rectum, and from the mucous membrane of the trachea as measured indirectly by the resulting tachycardia. The rapid disappearance of chronotropic activity observed after intravenous administration in contrast to the prolonged activity following oral medication and the relatively greater efficiency in producing tachycardia by infusion of the drug into the femoral vein in contrast to the gastroepiploic vein, prompted the biochemical studies of the metabolic fate of isoproterenol after various routes of administration. The tachycardia is believed to reflect only the concentration of free isoproterenol in plasma and it was hoped that this could be confirmed by direct measurement.

Received January 2, 1968, from Sterling-Winthrop Research Institute, Rensselaer, NY 12144

Accepted for publication March 4, 1968.

Presented to the American Society for Pharmacology and Experimental Therapeutics, Atlantic City, N. J., April 1966.

The authors thank Drs. G. A. Portmann and G. Levy for encouragement and many helpful suggestions. The radioactive material was synthesized by Dr. G. D. Diana. The authors also appreciate the competent technical assistance rendered by Mr. R. W. Piontek, Mr. A. Jensen, Mrs. S. Selberis, and Miss M. Gosztyla.

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[1] Trademarked as Isuprel, by Winthrop Labs., New York, N. Y.

EXPERIMENTAL

Materials—*dl*-Isoproterenol-7-^{3}H hydrochloride with a specific activity of 287 μc./mg. base was used for the intravenous injection studies. This was diluted with nonradioactive carrier to prepare medication with specific activities ranging from 4 to 66 μc./mg. base for the oral and intravenous infusion studies. The drug was dissolved in 5% dextrose solution containing sodium bisulfite (1:100,000) and ethylenediaminetetraacetic acid disodium salt (1:10,000). All doses are expressed in terms of isoproterenol base.

Animal Preparations—The heart rate was monitored by means of an electrocardiograph (Viso-Cardiette, Sanborn) using the Lead I attachments. Food was withheld 17–18 hr. prior to medication. Dogs of either sex, weighing between 8 and 17 kg., were anesthetized with sodium pentobarbital (30 mg./kg., i.v.). Both femoral veins were exposed, one for drug injection and the other for drawing blood *via* an implanted catheter. Urine samples were obtained periodically by rinsing the bladder with two 20-ml. portions of distilled water through an indwelling catheter. Ethylenediaminetetraacetic acid (100 p.p.m.) and α-thioglycerol (10 p.p.m.) were added to urine and blood samples as preservatives. All samples were kept frozen until analyzed. Where collection of bile was desired, the cystic duct was ligated and a polyethylene catheter was implanted in the common bile duct.

Conscious dogs of either sex, which weighed from 12 to 18 kg., were placed in sling-stand frames designed to keep the animal still but with a minimum of restraint. Blood was withdrawn periodically from the saphenous and radial veins. Urine was obtained periodically as described above.

Analytical Methods—Duplicate samples of blood (0.5 ml.) were dried and prepared for counting by a modification of the oxygen-flask combustion technique (3). Samples of urine were dissolved in a water-miscible scintillator for counting (4). Plasma samples (0.1 ml.) were digested with 1 *N* NaOH at 60° for 1 hr., neutralized with 0.5 ml. of oleic

acid, diluted with 1 ml. of water, and added to 15 ml. of the water-miscible scintillator. All samples were counted for 10 min. in a Packard Tricarb model 3324 liquid scintillation spectrometer operated at 2° and were corrected for quenching by an internal standard technique. The sensitivity of the assays was determined by the specific activity of the drug used in the particular experiment. Concentrations indicated as not significant in the text were based on net count rates less than 7 c.p.m.; corresponding to a standard error in the net count of ±20%.

Approximately 0.5 mg. each of authentic samples of isoproterenol (I), 3-*O*-methylisoproterenol (OMI), normetanephrine (NM), and norepinephrine (NE) were added to aliquots (1–2 ml.) of urine or plasma which contained 100,000–200,000 d.p.m. of tritium in the form of radiochemically labeled isoproterenol and its metabolites, after which the samples were adjusted to pH 8. Metabolites were then separated by ion-exchange chromatography on 1 × 3-cm. columns of Bio-Rex 70 resin (approx. 10 g., 200–325 mesh, Biorad Laboratories, Richmond, Calif.) developed with ammonium phosphate buffer (0.05 *M*, pH 8.0) containing 0.001 *M* thioglycerol as an antioxidant. The elution volumes of the compounds added to the sample were determined by continuous monitoring of the absorption of UV light (254 mμ) by the effluent. The effluent stream was also split to provide aliquots for monitoring the elution of radioactivity. The presence of conjugates was established by the shift in the elution pattern of metabolites brought about by enzymatic hydrolysis of samples prior to chromatography.

Paper electrophoresis was conducted using phosphate buffer (0.05 *M*, pH 8.0) and a voltage gradient of 900 v./56 cm. Authentic compounds, added as carriers, were located by spraying with iodoplatinate reagent. Mobilities, as cm., migrated toward the cathode in 1 hr., were: I, 11; OMI and NE, 12; NM, 13. Electrically neutral material migrated 3.5 cm. as a result of electroosmosis. The distribution of radioactivity was determined by cutting the electrophoregrams into 1-cm. strips which were soaked for 2 hr. in counting vials with 2 ml. of water, then mixed with 15 ml. of dioxane scintillator (4).

Conjugates of metabolites with glucuronic and sulfuric acids were hydrolyzed by adding 0.1 ml. of glucuronidase[2] mixture A to 2.0 ml. of plasma or urine (adjusted to pH 5.5 with acetate buffer) and incubating for 18 hr. at 37° in a closed vessel containing a nitrogen atmosphere and a drop of chloroform. This assay indicated that such an incubation mixture contained 77 arbitrary units of glucuronidase/ml. and 560 arbitrary units of sulfatase/ml. One arbitrary unit is defined as the quantity of enzyme required in 1 ml. of the reaction mixture to hydrolyze 1 μmole of substrate per hr. at 37°, pH 5.5, substrate concentration 0.0025 *M* in the presence of bovine serum albumin, 0.25 mg./ml. Substrates used in the assay were potassium *p*-nitrophenyl sulfate and *p*-nitrophenylglucosiduronic acid.

RESULTS

Chromatographic Analyses—The typical pattern for elution of metabolites from the weak cation exchanger is shown in Fig. 1. Neutral, acidic, and conjugated metabolites are not retained by the resin and the elution volume for these metabolites (approximately 8 ml.) represents the hold-up volume of the column. The authentic compounds, OMI, I, NM, and NE, were eluted in that order as indicated by the lower curve. The upper curves present the distribution of radioactivity in the column effluent from samples of a urine before and after hydrolysis with glucuronidase mixture A, a potent source of the enzymes β-glucuronidase and sulfatase. The radioactivity eluted in a particular peak before hydrolysis represents the free compound and that eluted after hydrolysis represents the total (free + conjugated) amount of the metabolite.

It is apparent from the shift of the pattern after glucuronidase treatment that the major metabolite in the particular urine sample illustrated is conjugated isoproterenol. From the difference in the radioactivity contained in each peak before and after enzymatic hydrolysis, the composition of this particular urine (as % of radioactivity in urine) can be calculated as I (free 0%; conjugate, 67.5%), OMI (free 6.7%; conjugate, 7.3%), NM (free 0.8%; conjugate, 0.0%), neutral and acidic materials (17.5%), NE (0%). NE was not detected as a metabolite in any urine or plasma sample. The presence of 0.8% NM in the sample illustrated is of borderline significance. Failure of the numbers to total 100% may result from rounding off errors or from elution of small amounts of radioactivity in areas not represented by discrete peaks. The results of many such analyses are presented later in the text and figures, usually calculated as a percentage of the administered dose. Where the concentrations of metabolites or conjugates are expressed as mcg./l., the figures refer to concentrations of molecularly equivalent solutions of isoproterenol.

Blood Levels After Intravenous Administration—Rapid intravenous injection of 0.4, 0.8, and 1.6 mcg./kg. of isoproterenol-^{3}H increased the heart rate of anesthetized dogs by 70, 105, and 120 b.p.m. within 30 sec. The tachycardia regressed with a

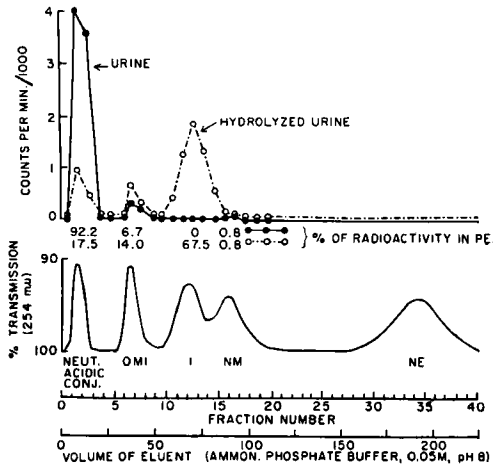

Fig. 1—Fractionation of metabolites on weak cation-exchange resin.

[2] Glusulase, Endo Products Inc., Richmond Hill, N. Y.

half-life ranging from 0.9 to 1.3 min. during the first 3 min. after medication (Fig. 2) and returned to the premedication rate within 5–7 min. On the other hand, the concentration of radioactivity in whole blood declined with a much longer half-life which ranged from 3.0 to 4.1 min. The heart rate is believed to reflect only the concentration of unchanged isoproterenol, whereas the tritium concentration represents the sum of all radioactive compounds in the blood.

In order to obtain a plasma level of radioactivity sufficiently high to enable estimation of the circulating metabolites, a dose of 131 mcg. isoproterenol-7-^{3}H/kg. (specific activity 287 μc./mg. base) in 1 ml. of aqueous solution was given by rapid injection into the femoral vein of one anesthetized dog. The tritium declined in two phases, a rapid phase with a half-life of about 5 min. and after 30 min. a second slower decline with a half-life of about 4 hr.

One minute after injection, ion-exchange chromatography indicated that the major metabolite in the plasma was OMI which accounted for 63% of the radioactivity. Isoproterenol amounted to less than 1% of the total radioactivity and, in fact, could not be detected. The NM content was also less than 1%. Neutral, acidic, and conjugated metabolites, which were not further resolved, made up 35% of the total. Electrophoretic analysis of the 3-min. plasma gave similar results, 65% OMI and 35% electrically neutral material; isoproterenol and acidic materials were not detectable.

Urinary Excretion—The rate of excretion of radioactivity in urine was similar for the four dogs given intravenous medication. After 6 hr. the amounts excreted were 84, 82, 70, and 83% following doses of 0.4, 0.8, 1.6, and 131 mcg./kg., respectively. The urine from the dog given the highest dose was examined in detail by ion-exchange chromatography and these results are summarized in Fig. 3, where the cumulative excretion of both total radioactivity and individual metabolites are shown. Free isoproterenol was found only in the early urine, 0.4% of the dose in the first 20 min., and another 0.2% in the next 10 min. Conjugated I was detected throughout the 6-hr. period although a major portion had been excreted within the first hour. NM and its conjugates accounted for only 3% of the dose. The metabolite excreted in largest amount at all times was OMI which, in the free form, accounted for 43% of the administered dose over the 6-hr. period. Another 10% was excreted in conjugated form. Neutral and acidic metabolites accounted for about 20% of the dose. The exact nature of this fraction has not yet been determined, but preliminary results involving fractionation on an anion-exchange resin indicate that less than 2% of the dose can be accounted for as 3-methoxy-4-hydroxymandelic acid.

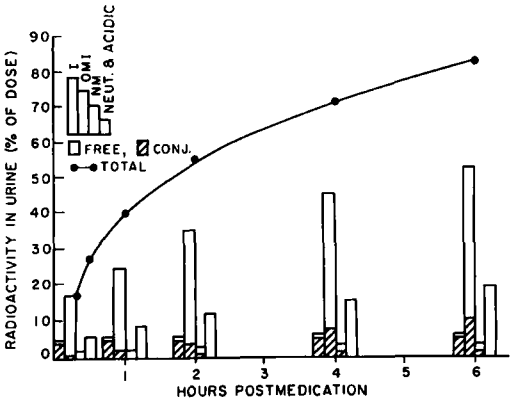

Fig. 3—Cumulative excretion of metabolites in the urine of dog No. 12 following rapid injection of 0.13 mg./kg. of isoproterenol-^{3}H into the femoral vein.

Blood Levels After Oral Administration—A dose of isoproterenol-7-^{3}H hydrochloride equivalent to about 0.8 mg./kg. was dissolved in 2 ml. of water and administered orally to four conscious dogs. The dose was delivered from a syringe at the back of the tongue and was followed immediately by a rinse with 5 ml. of water. The time course of the resulting tritium concentration in whole blood (Fig. 4) shows a distinct double peak in three of the four dogs. This double peak was also reflected in the heart rate.

The mean maximal increase in heart rate (173%) was obtained in 10 min. following medication, and the duration of action was approximately 3 hr. In comparison with the intravenous half-life of 1–2 min., this longer duration of heart-rate activity

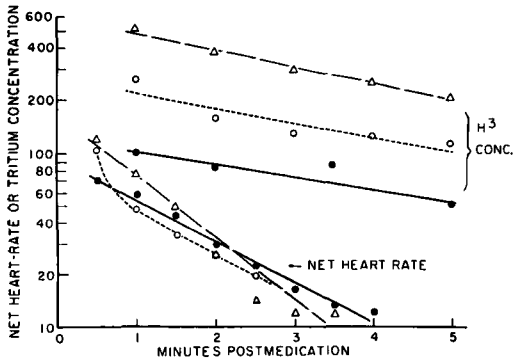

Fig. 2—Net heart rate (b.p.m. above premedication level) and tritium concentration (as 100 × the equivalent level of isoproterenol-^{3}H in mcg./l.) in whole blood following rapid intravenous injection of isoproterenol-^{3}H in dogs. Key (dose in mcg./kg.): ●, 0.4; ○ 0.8; △, 1.6.

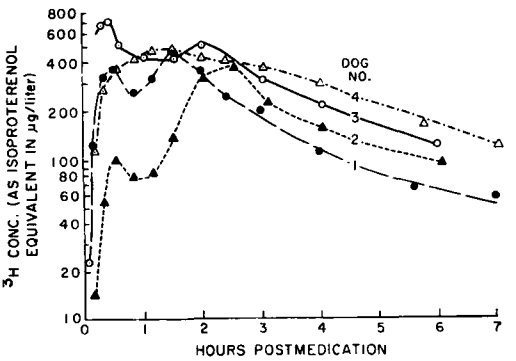

Fig. 4—Blood level of radioactivity following oral administration of isoproterenol-^{3}H to dogs as a solution of the hydrochloride salt. Key (dose, mg. base/kg.): ●, 0.78; ▲, 0.65; ○, 1.08; △, 0.82.

following oral medication indicated that the absorption from the intestinal tract was slow and prolonged. Slow absorption of oral medication was reflected also in the blood tritium level, which decreased at a considerably slower rate after oral than that after intravenous administration. At the third hour, when most of the cardiac effect had been terminated, the total blood tritium was still at a relatively high level of 270 mcg./l., reflecting mostly the total metabolites rather than active isoproterenol. In one experiment (dog No. 3) the plasma was analyzed by ion-exchange chromatography. Twenty minutes after medication, 5% of the radioactivity was in the form of OMI and 94% in the form of either neutral, acidic, or conjugated metabolites. Paper electrophoresis confirmed this and further indicated the presence of less than 5% in the form of acidic metabolites. Neither I nor NM was detected by either method. Estimation for tritiated water content of plasma indicated the conversion of 3% of the dose in 4 hr.

Urinary Excretion—The amount of radioactivity excreted in the urine of dogs within 6 hr. was 64, 45, 74, and 57%, respectively, of the administered dose. Chromatographic analysis of the urine from dog No. 3 (Fig. 5) indicated that less than 0.1% of the total dose was excreted as unchanged I. The major metabolite was conjugated I which accounted for 50% of the dose. Free OMI accounted for 5.8% of the dose and its conjugate accounted for another 6.2%. Less than 1% of either free or conjugated NM was found, and neutral and acidic metabolites, which have not been further identified, accounted for only 10% of the dose.

Plasma Levels After Intraduodenal Administration—The levels of radioactivity observed in three anesthetized dogs following direct injection of isoproterenol-^{3}H hydrochloride (in 2 ml. of water) into the duodenum are presented in Fig. 6. In contrast to the orally dosed dogs, only a single peak was observed. Chromatographic analysis of plasma samples indicated that the major metabolite at all times was conjugated I which, in one experiment, measured 70 mcg./l. at 10 min. and a maximum of 152 mcg./l. after 3 hr. Free drug levels ranging from 1 to 2.4 mcg./l. were measured in the

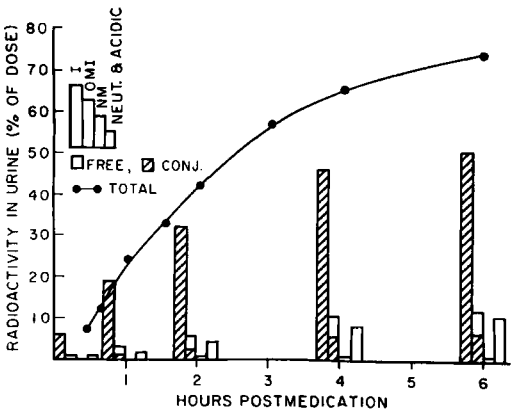

Fig. 5—Cumulative excretion of metabolites in the urine of dog No. 3 following oral administration of 1.08 mg./kg. of isoproterenol-^{3}H as the hydrochloride salt in solution.

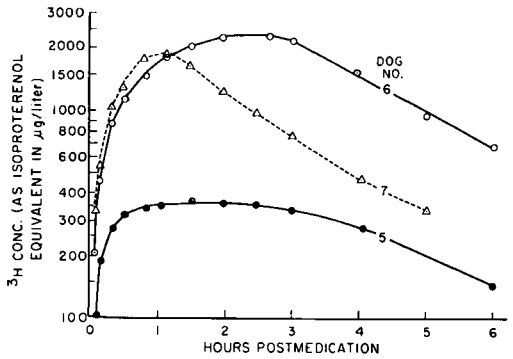

Fig. 6—Plasma level of radioactivity following intraduodenal administration of isoproterenol-^{3}H to anesthetized dogs. Key (mg. base/kg.): ●, 0.80; ○, 1.06; △, 1.07.

3-hr. period following medication, but these levels are so low as to be of doubtful significance. They do serve, however, to place an upper limit on the level of free drug to be expected under these conditions. From a level of 42 mcg./l. at 10 min., a maximum level of 66 mcg./l. of OMI was reached 30 min. after medication while its conjugates, which were labile to glucuronidase mixture A, attained a maximum of only 13 mcg./l. after 4 hr. NM was never present at a concentration above 2.4 mcg./l., but its conjugates were present at a concentration of 35 mcg./l. after 10 min. and a maximum concentration of 67 mcg./l. after 3 hr.

Urinary and Biliary Excretion—The excretion of radioactivity in the urine after intraduodenal administration ranged from 59 to 65% of the dose in 5–6 hr. after medication. However, biliary excretion was less than 2.6% of the dose. The drug was well absorbed under these conditions and after 5–6 hr. only 0.4–1.2% of the administered radioactivity remained unabsorbed from the small intestine.

Intraportal Infusion—In order to ascertain the fate of the drug after direct introduction into the portal system, isoproterenol-7-^{3}H in saline solution was infused into a branch of the mesenteric vein of dog No. 8 at a rate of 7.26 mcg. base/kg./min. for a period of 1 hr. (total dose 0.44 mg. base/kg.) in one experiment. This rate was felt to approximate that of absorption after oral medication.

The time course of radioactivity in plasma during and following the 1-hr. intraportal infusion of isoproterenol-7-^{3}H is given in Fig. 7. The effect on the heart rate is illustrated in Fig. 8. Although the level of radioactivity in the blood rose sixfold during the period of infusion, the heart rate, after the first 10 min., was found to be very constant at about 160 b.p.m. On cessation of the infusion, the heart rate returned to its preinfusion level within 10 min.

The cardiac effect during the steady state was relatively small considering the dose infused, indicating appreciable metabolic conversion of the drug on its passage through the liver. This was confirmed by estimation, using ion-exchange chromatography, of the plasma levels of free isoproterenol which were found to range from about 2–20 mcg./l. during the infusion. These levels were close to the limit of detection and should be regarded only as an order of magnitude. Only

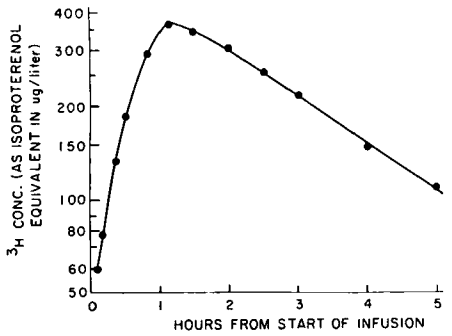

Fig. 7—Plasma level of radioactivity during and following infusion of isoproterenol-^{3}H into the mesenteric vein for 1 hr. at a rate of 0.44 mg. base/kg. body wt./hr. (dog No. 8).

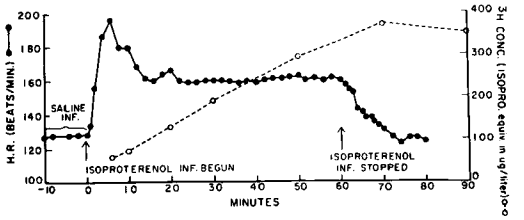

Fig. 8—Heart rate and plasma level of radioactivity during intraportal infusion of isoproterenol-^{3}H in anesthetized dog No. 8.

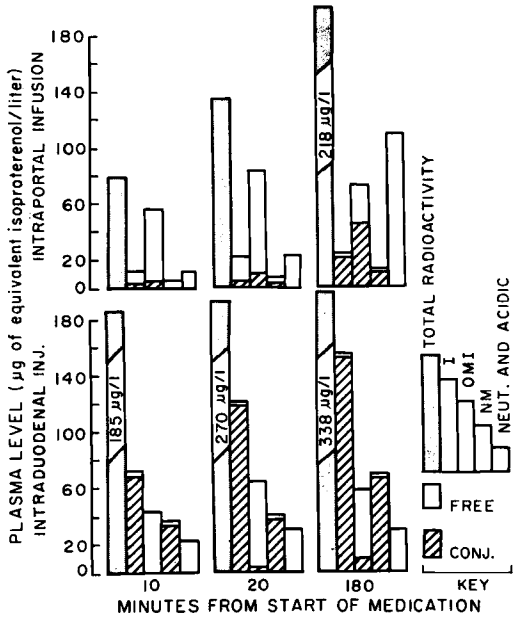

Fig. 9—Metabolites in plasma after intraduodenal administration (dog No. 5) and after the start of a 1-hr. intraportal infusion (dog No. 8).

slightly higher amounts of conjugated I were present ranging from 3 mcg./l. at 6 min. to 24 mcg./l. at 50 min. The levels of conjugated NM were also low each ranging from less than 3 mcg./l. at 6 min. to less than 11 mcg./l. at 50 min. The major metabolite present at all times was free OMI which rose from 36 mcg./l. at 6 min. to 108

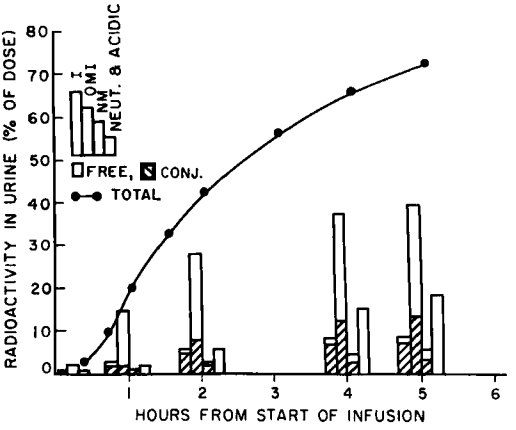

Fig. 10—Cumulative excretion of metabolites in the urine of dog No. 8 during and after infusion of isoproterenol-^{3}H into the mesenteric vein for 1 hr. at a rate of 0.073 mg. base/kg./min.

mcg./l. at 50 min. The level of conjugated OMI rose from 0 at 6 min. to 11 mcg./l. at 30 min. to 58 mcg./l. at 50 min. Unidentified neutral and acidic metabolites rose from 14 mcg./l. at 6 min. to 67 mcg./l. at 50 min.

A graphic comparison of the metabolites in plasma at 10, 20, and 180 min. after intraduodenal administration (dog No. 5) and after the start of the 1-hr. portal infusion (dog No. 8) of isoproterenol into the mesenteric vein is given in Fig. 9. It is readily apparent that the major metabolite in plasma at early times after intraduodenal administration was conjugated isoproterenol while after intraportal administration it was OMI.

Urinary and Biliary Excretion—Over a 5-hr. period from the start of portal infusion, 72% of the dose was excreted in the urine (Fig. 10). An additional 2.3% was excreted in bile. The major metabolites excreted in urine were OMI and its conjugates which together accounted for 40% of the dose. A comparison of the metabolites excreted in urine during the 0–4 hr. postmedication period with those excreted following oral administration and injection into the femoral vein is given in Fig. 11. It emphasizes the similarity of the excretion

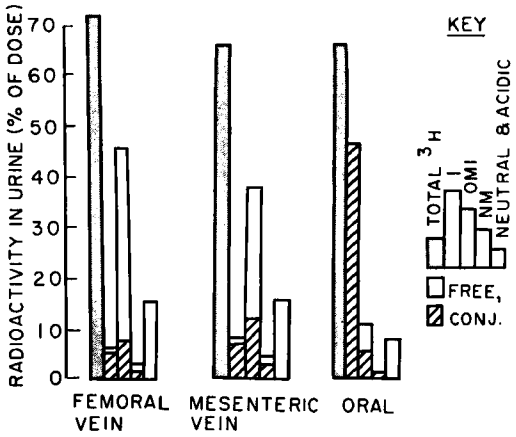

Fig. 11—Comparison of metabolites of isoproterenol-^{3}H excreted in the 0–4 hr. postmedication period after various routes of administration.

TABLE I—HYDROLYSIS WITH VARIOUS ENZYME PREPARATIONS
30–60 min. URINE, DOG NO. 3

Enzyme	Buffer	Enzyme Concn. (Effective Units/ml.) β-Glucuronidase	Sulfatase	Chromatographic Fractions, (% of ^3H in Sample) Neut. Acidic Conj.	OMI	I	NM
None	—	0	0	91.3	7.3	0.2	1.1
Glucuronidase mix. B[a]	Acetate	12	0	91.5	7.2	0.2	0.9
Liver β-gluc.	Acetate	29	0	90.5	8.1	0.2	0.6
Glucuronidase mix. A[b]	PO_4 (0.5 *M*)	35	11	78.7	9.4	8.0	3.7
Glucuronidase mix. A[b]	Acetate	77	560	5.3	9.5	82.3	2.2

[a] Ketodase. [b] Glusulase.

pattern after administration *via* either the femoral or mesenteric vein where OMI is the major metabolite and contrasts this with the pattern obtained after oral administration where mainly conjugated isoproterenol is excreted.

Nature of Conjugated Isoproterenol—As described under *Methods*, the various samples of urine and plasmas were hydrolyzed with glucuronidase mixture A, which contains both β-glucuronidase and sulfatase, in order to determine the relative amounts of free and conjugated material.

Hydrolysis of all urine samples from dog No. 3 with glucuronidase mixture B,[3] which contains glucuronidase, released no isoproterenol, indicating that the conjugate was a sulfuric acid ester. But since glucuronidase mixture B is a much less potent source of glucuronidase than is glucuronidase mixture A, the 30–60 min. urine sample from dog No. 3 was hydrolyzed with various sources of glucuronidase as summarized in Table I. In addition to glucuronidase mixture B, a liver glucuronidase preparation did not hydrolyze the conjugate, nor did glucuronidase mixture A in the presence of 0.5 *M* phosphate buffer, which inhibits 98% of the sulfatase activity while depressing glucuronidase by only half. Glucuronidase mixture A in the acetate buffer was the only effective agent, indicating that the conjugate is a sulfate.

Gastric Emptying Time—Enterohepatic recirculation was ruled out as the explanation of the double peaks observed in the blood level *versus* time curves after oral medication (Fig. 4), because it was found that only a small percent of the dose was excreted in bile (dogs No. 5–8). Preliminary studies have been carried out in three additional experiments to determine the location of the unabsorbed drug in the gastrointestinal tract at various times after oral medication. In two of the animals, which were sacrificed 1 hr. after medication, 55 and 29% of the administered radioactivity was still retained in the stomach. The stomach of the third dog sacrificed 2 hr. after medication contained only 1% of the administered radioactivity. In all cases less than 14% of the dose was found in the small intestine, indicating that the drug is readily absorbed from this area when it is released from the stomach.

DISCUSSION

The absorption and elimination profile of isoproterenol in dogs has been studied previously using the increase in heart rate as an indirect measure of the plasma level (1, 2). Only two other studies (5, 6) on the metabolic fate of the drug have been reported. However, pertinent metabolic studies have been carried out on the related drugs *dl*-nordefrin[4] and 4-(2-methyl-aminoethyl)pyrocatechol,[5] and several studies on epinephrine have been reported and will be reviewed in the following discussion.

Sjoerdsma (5) has reported that, when intravenously administered, the *d*-isomer of isoproterenol is converted to its 3-*O*-methyl derivative and has utilized this reaction to measure catechol *O*-methylating activity in man. No other metabolic studies in humans have been reported.

After intravenous administration of approximately 23 mcg. of isoproterenol-^3H/kg. to rats, Hertting (6) found that one-half of the administered radioactivity was excreted in urine over an 8-hr. period and 77% within 24 hr. The remainder was accounted for in the bile in a form which was not readily absorbed from the intestine. In addition to 10% of the dose in unchanged form, he found the urine to contain 18% as isoproterenol glucuronide, 14% as the *O*-methyl derivative, and 35% as the glucuronide conjugate of OMI. He was unable to detect any deaminated metabolites and concluded that the drug was metabolized exclusively by *O*-methylation and conjugation.

These results show that while isoproterenol given intravenously to dogs is metabolized largely by *O*-methylation, the orally administered drug is converted mainly to a sulfuric acid ester. Only traces of unchanged drug were excreted after i.v. administration and none after oral medication. The excretion of 10–20% of the dose as unidentified neutral and acidic products and up to 3% as normetanephrine or its conjugates indicates that monoamine oxidase plays a secondary role in the metabolism of the drug by the dog.

Haggendal (16) found that conjugation did not play a significant role in the metabolism of epinephrine and norepinephrine by humans when the plasma levels of these hormones were increased either by insulin administration or performance of muscular work. His conclusion was based on the fact that there was no concomitant increase in the levels of their conjugates. In contrast, after oral administration of epinephrine he found conjugation to be the predominant metabolic pathway. Since previous workers had proposed that circulating catecholamines are metabolized by *O*-methylation, primarily in liver tissue (17, 18), Haggendal suggested that the oral medication may be con-

[3] Ketodase, Warner-Chilcott, Morris Plains, N. J.

[4] Corbasil.

[5] Epinine.

jugated by the intestinal flora or in the intestinal mucosa.

Oral doses of epinephrine administered to humans have been reported to be inactivated by formation of the sulfuric acid conjugate (7–9) and after oral administration to rabbits by formation of the glucuronic acid conjugate (10, 11). Likewise more than half of the 50-mg. oral doses of the related drugs *dl*-nordefrin, $3,4\text{-}(OH)_2C_6H_3CHOHCH(NH_2)CH_3$, and 4-(2-methyl-aminoethyl)pyrocatechol, $3,4\text{-}(OH)_2C_6H_3CH_2CH_2HNCH_3$, were eliminated in conjugated form in the urine of humans and dogs within 9 hr. (7, 9).

In contrast, after intravenous infusion of epinephrine in humans (approximately 0.18–0.3 mcg./kg./min. for 0.5 to 1 hr.), urinary excretion of metanephrine or its conjugates was reported to account for almost half the dose while most of the remainder was excreted in the form of deaminated products (mainly 3-methoxy-4-hydroxymandelic acid). Little free or conjugated epinephrine was excreted (12, 13). In a particularly detailed study of the excretion products after intravenous injection (approximately 0.07 mcg./kg.) of epinephrine, only a small percent of the dose was excreted within 24 hr. as epinephrine or its conjugates whereas 23% was excreted as methoxyhydroxymandelic acid and 26% as the sulfuric acid conjugate of metanephrine (14).

The conclusion by Resnick (15) that *O*-methylation represented the predominant pathway in the metabolism of orally ingested epinephrine was unwarranted since he examined the metabolites only after subjecting the urine to acid hydrolysis and did not measure the amount of unconjugated metabolites prior to hydrolysis. In fact his identification of the hydrolysis products, epinephrine and metanephrine, were only qualitative and he did not determine their relative amounts.

The inactivation of orally administered isoproterenol by conjugation with sulfate is therefore consistent with the reported findings for related catecholamines. The fact that virtually identical patterns of urinary metabolites were found following either intraportal infusion of isoproterenol or injection into the femoral vein in dogs indicates that the oral medication is metabolized by conjugation to sulfate either within the intestinal tract or during its passage through the intestinal wall.

The observation of a biphasic absorption of the oral medication as indicated by the double peak in the plasma level and heart rate *versus* time curves at first suggested enterohepatic circulation of the drug. But this was ruled out by the demonstration that only a small amount was excreted in the bile. A biphasic curve was not obtained after intravenous or intraduodenal administration. Preliminary studies of the distribution of radioactivity in the gastrointestinal tract of dogs after oral medication showed a significant proportion of the dose remaining in the stomach 1 hr. after medication. This suggested that a gastric emptying cycle may be responsible for the biphasic absorption. However, the exact cause of the phenomenon is uncertain at present.

Attempts to extend these studies to the direct measurement of isoproterenol and its metabolites in plasma were only partially successful. It was possible to demonstrate that the major metabolite in plasma after intraduodenal administration was a sulfuric acid ester of isoproterenol, while after intraportal infusion the major metabolite was 3-*O*-methylisoproterenol. These results are analogous to the authors' analysis of urinary metabolites after oral and intravenous medication, respectively. Metabolism of the drug was demonstrated to be quite rapid as evidenced by the finding that the unchanged drug constituted less than 1% of the radioactivity in plasma 1 min. after an intravenous injection.

Isoproterenol disappears from the circulation with a half-life of a few minutes as indicated by the decrease in the induced tachycardia following intravenous administration of the drug. Initial disappearance of radioactivity from the blood occurs at a significantly slower rate. The initial regression of tachycardia results mainly from metabolic conversion of isoproterenol to inactive products while the slower elimination of total radioactivity reflects the slow excretion of these metabolites.

Orally administered isoproterenol is slowly absorbed and is less efficient than intravenous medication in inducing tachycardia in dogs. This lower efficiency results from inactivation of a large portion of the medication by conversion to a sulfuric acid ester either before or during absorption from the small intestine. Even that fraction of the drug which escapes this conjugation is then subjected to deactivation by conversion to the 3-*O*-methyl derivative on passage through the liver. Thus, only a very small fraction of an oral dose reaches the general circulation in an active form even on the first circuit of the blood through the body.

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Keyphrases

Isoproterenol—absorption, elimination
dl-Isoproterenol-7-^{3}H—metabolic fate
Metabolites—administration route effect
Column chromatography—separation
Radioactivity distribution—blood, urine
Electrophoresis, paper—identity, metabolites
UV spectrophotometry—analysis

Blood Plasma Levels and Elimination of Salts of 2-PAM in Man After Oral Administration

By ALBERT A. KONDRITZER, PETER ZVIRBLIS, A. GOODMAN, and S. H. PAPLANUS

The absorption and elimination of pralidoxime salts after oral administration to human volunteers have been studied at a dosage of 1.5–10 g. of drug for a 70-kg. man. The biological half-life, calculated from blood plasma values and urinary excretion rates, was approximately 1.7 hr. A 10-fold increase in dosage of the pralidoxime salts increased the peak plasma level of oxime 3.5-fold. The average total amount of pralidoxime recovered in the urine was 27 percent. In subjects given the iodide salt, symptoms of iodism were observed; all other clinical tests were negative. Some observations were made on the behavior of a bis-quaternary oxime.

A NUMBER of oximes have been found to reactivate the cholinesterases that have been inactivated by organic esters of phosphoric acid derivatives (1). One quaternary oxime in particular has proved effective in combination with atropine in the treatment of poisoning by some of these toxic chemicals and in reducing their toxicity in various animal species (2). Salts of the oxime, chemically known as 2-hydroxyiminomethyl-1-methylpyridinium chloride, iodide, and methane sulfonate, are commonly referred to as 2-PAMCl, 2-PAMI, and P-2-S, respectively. Pralidoxime (3) is the generic name given to the oxime moiety.

Few studies have been carried out in man of plasma levels after oral administration of salts of 2-PAM. Sundwall (4) reported that the maximum concentration in plasma of man after oral administration of 45 mg./kg. of P-2-S, packed in gelatin capsules, was about 4 mcg./ml. and that a concentration over 3.5 mcg./ml. was maintained from 1 to 4 hr. These results may be compared with peak plasma concentrations, averaging 15 mcg./ml., which were reached 20 min. after an intramuscular injection of 30 mg./kg.

The half-life for 2-PAM iodide in the blood serum of man after its intravenous administration in a dose of 15 mg./kg. has been reported (5) to be 0.9 hr. Most of the injected oxime quickly passed into the urine and no oxime was detected in the spinal fluid 70 min. after its injection.

The present investigation was carried out to measure the concentration of oxime in plasma and urine of man after ingestion of various salts of 2-PAM in order to determine the relation of the amounts ingested to the attainable concentrations and to acquire information concerning rates of excretion.

Received February 29, 1968, from the Physiological Chemistry Branch, Medical Research Laboratory, Edgewood Arsenal, MD 21010

Accepted for publication April 11, 1968.

MATERIALS AND METHODS

Organic and inorganic salts of 2-PAM, differing in water solubility, were used in this study (6). Adult male volunteers were given aqueous solutions of the 2-PAM salts by mouth; 400 ml. of water was drunk during the hour before and 100 ml. of water each hour for 5 hr. after the ingestion of the drug to insure good hydration and urine collections.

The following information was sought; (*a*) plasma levels of oxime at various times after ingestion, (*b*) rates of disappearance of oxime from blood, (*c*) urinary excretion of oxime, and (*d*) biological half-life of the oxime, calculated from plasma and urine data. The concentrations of the oxime in plasma, urine, and feces were measured by ultraviolet spectrophotometry, based on procedures previously described (7). The oxime salts, dosage, and number of subjects are listed in Table I.

In addition to the 2-PAM salts listed in the table, the bis-quaternary oxime *N,N'*-trimethylenebis-(pyridinium-4-aldoxime) dibromide, commonly referred to as TMB-4 (8), was given to three subjects at a dose of 1 g./70 kg.

Clinical laboratory tests were carried out on the volunteers to uncover indications of any pathologic damage associated with the ingestion of the 2-PAM salts as follows. (*a*) Urinalysis, including microscopic examination, before and the day after the test. (*b*) Blood examination, including hemoglobin, total and differential white blood cells, before and the day after the test. (*c*) Liver function tests, including alkaline phosphatase and serum glutamic-pyruvic transaminase, before and on the morning after the test. (*d*) In addition to the oxime measurements red

TABLE I—SALTS OF 2-PAM AND DOSE GIVEN ORALLY

2-PAM Salt	Mol. Wt.	g. Oxime g. Salt	Dose[a] g. Salt/70-kg. Man		
Chloride	172.6	0.79	1.5 (4)		3.7 (3)
Lactate	226.2	0.61	2 (6)		5 (9)
Dihydrogen phosphate	234.2	0.59	2 (3)		5 (3)
Methane sulfonate	237.3	0.58	2 (6)		5 (4)
Iodide	264.1	0.52	5 (3)	8 (3)	10 (3)

[a] Number of subjects is given in parentheses; the average weight was 72 kg. with a range of 63–84 kg.

cell and plasma cholinesterase activity measurements were made two or three times on separate days before the test, on the morning of the test, 3 and 6 hr. after ingestion of the oxime, and the morning after the test.

During the tests, the volunteers were observed closely for changes in blood pressure, pulse rate, and respiratory rate, and for development of abnormalities in their neurologic status; they were questioned repeatedly concerning their subjective reactions. At regular time intervals after ingestion of the oximes, muscular function was checked by means of a grip dynamometer and the standard step tests.

RESULTS AND DISCUSSION

Blood Plasma—Oxime was found in the blood plasma within 15 min. after oral administration. The concentration rose rapidly, approached a peak at 2–3 hr., and then declined at an approximately first-order rate. The first part of the curve results predominantly from absorption, and at the peak of the curve the rate of absorption is about equal to the rate of elimination; the latter portion of the curve results predominantly from elimination of the oxime. Average oxime absorption-elimination curves for plasma are shown in Figs. 1, 2, and 3. Absorption-elimination curves as a function of dosage of one salt, namely the iodide, are given in Fig. 1. The peak concentration of oxime in plasma increased with the increasing dosages. The time of reaching peak oxime concentration was slightly delayed with increasing dosages, from 2 hr. for the 5-g. dose to 2.5–3 hr. for the 10-g. dose. Evidently absorption of the oxime from the gut is prolonged to some degree with increasing dosage.

Figure 2 compares the rates of absorption and elimination of the lactate and dihydrogen phosphate salts after oral administration. At the dose of 2 g./70-kg. man, the peak plasma concentration obtained was higher with the lactate (6 mcg./ml. after 2 hr.). With the lactate salt a plasma concentration of mcg./ml. was reached in approximately 1.25 hr.

P-2-S and 2-PAMCl are compared in Fig. 3, which shows the plasma molar levels obtained when equimolar amounts of each of the two salts were given at two dosage levels. The results here indicate

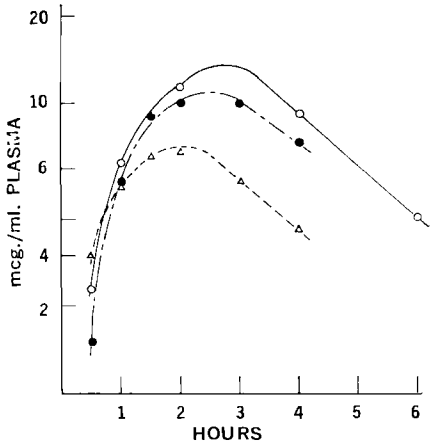

Fig. 1—Plasma concentrations of 2-PAM iodide in man after oral administration of 5, 8, and 10 g./70 kg. of body weight. Key: ●, 8 g.; ○, 10 g.; Δ, 5 g.

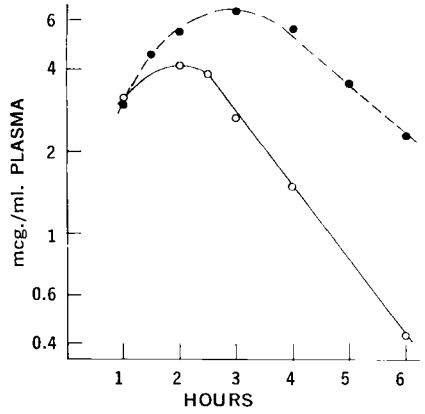

Fig. 2—Plasma concentrations of the lactate and dihydrogen phosphate salts of 2-PAM in man after oral administration. Key: ●, 2 g. lactate; ○, 2 g. phosphate.

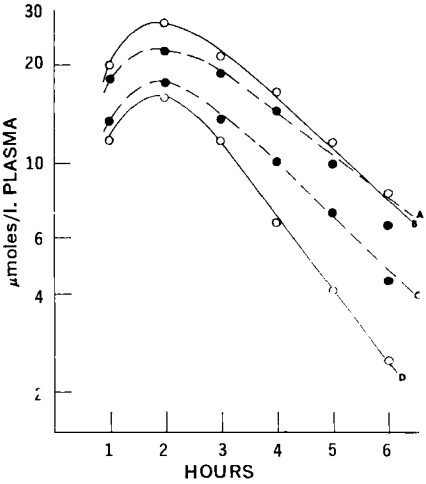

Fig. 3—Plasma concentrations of 2-PAM chloride and 2-PAM methane sulfonate (P-2-S). Key: A, 21.6 mmoles/70 kg. PAM-Cl; B, 21.6 mmoles/70 kg. P-2-S; C, 8.6 mmoles/70 kg. PAM-Cl; D, 8.6 mmoles/70 kg. P-2-S.

clearly that these salts are very similar with respect to absorption into and elimination from blood plasma.

The relationship between peak plasma levels of oxime and oral doses of the various salts may be approximated by an equation of the type

$$Y = kX^n$$

where Y is the oxime concentration in μmoles/l. of plasma, X is the dose in mmoles/70-kg. man, k is a constant, equal to Y when X is 1, n is a constant that describes the change in Y with change in X. The log form of this equation is plotted in Fig. 4. The best values of the constants were calculated by the method of least squares; the calculated value of log k is 0.79 and that of n (slope) is 0.54. Variability among individual results precludes any statement concerning statistically significant differences in peak oxime plasma levels resulting from the various 2-PAM salts. However, within the limits of this variability the estimation can be made that a 10-fold increase in dosage of any of the 2-PAM salts would increase the peak plasma level of oxime 3.5-fold.

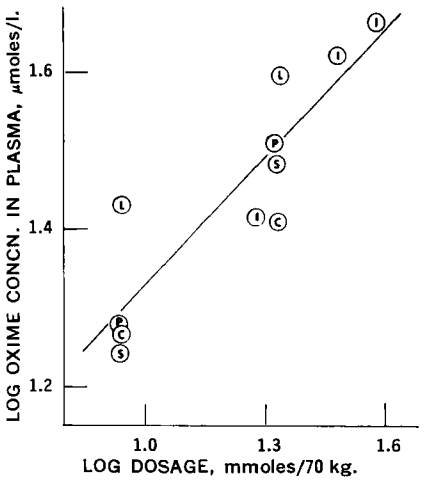

Fig. 4—Relationship between maximum oxime concentration reached in plasma and oral dosage of PAM salts. Key: C, chloride; I, iodide; L, lactate; P, dihydrogen phosphate; and S, methane sulfonate. The equation of the line is log Y = 0.79 + 0.54 log X.

Biological Half-Life—A considerable portion of the plasma concentration curve approaches or resembles the curve for a first-order process or reaction where the decrease in concentration of a reacting substance at a given time, t, is proportional to the concentration present at that time (9). Stated in logarithmic form,

$$\log C = \log C_0 - kt/2.303 \qquad \text{(Eq. 1)}$$

where C is concentration at time t, C_0 is concentration at time zero, t_0, k is the specific rate constant that characterizes the process.

A plot of log C *versus* time, t, gives a straight line; the slope of the line is equal to $-k/2.303$, from which the rate constant, k, is calculated. The time for the plasma concentration to be reduced by one-half may be defined as the biological half-life (10), and is equal to $0.693/k$. This value has been calculated from the plasma data for the 2-PAM salts; the best estimates are given in Table II.

Urinary Excretion—A valuable addition to the literature of urinary excretion equations was that which enabled a specific velocity constant, k, to be derived from the rate of urinary excretion by means of the equation

$$\log dC/dt = kt/2.303 + \log(-kC_0) \qquad \text{(Eq. 2)}$$

Table II—Biological Half-Life of 2-PAM Salts Given Orally in Man

2-PAM Salt	Mean Half-Life (hr.)[a] By Plasma	Mean Half-Life (hr.)[a] By Urine
Chloride	1.7 (7)	1.7 (7)
Lactate	1.7 (9)	1.7 (9)
Phosphate	1.3 (5)	1.5 (5)
Methane sulfonate	1.7 (10)	1.6 (10)
Iodide	2.0 (9)	2.2 (5)

[a] Figures in parentheses indicate number of subjects used.

where, after the attainment of pseudo-steady-state diffusion conditions in the body, C may be defined as the amount of unexcreted substance after a single dose, and C_0 is the apparent maximal amount of excretable substance (11). A plot of the change in C for each equal increment of t is a straight line having the same slope as a plot of Eq. 1. Application of Eq. 2 to urinary excretion data requires a measurement of the drug or substance excreted in a series of reasonably equal time intervals.

Plots illustrating the urinary excretion rates of 2-PAM salts are given in Fig. 5. In Fig. 6 a plot is shown of urinary excretion in which 2-PAMCl and P-2-S are compared; four subjects were used for each salt and all subjects were studied during the same day. The hourly points, in millimoles excreted per hour, were obtained from an average Cartesian coordinate plot for each salt. The excretion rate is approximately the same for both salts. The plateau between 2 and 3 hr. indicates the point where absorption is about equal to excretion of the oxime and agrees with the data on peak oxime levels found in plasma.

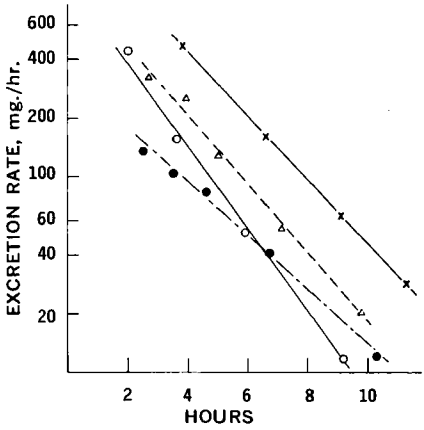

Fig. 5—Urinary excretion rate of salts of 2-PAM in man after oral administration. Key: ×, 10 g. iodide; Δ, 5 g. lactate; ○, 5 g. phosphate; ●, 2 g. P-2-S.

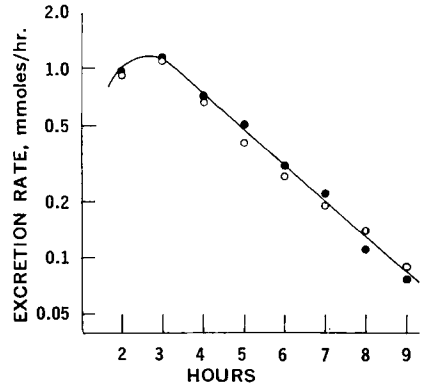

Fig. 6—Comparison of urinary excretion rates of 2-PAMCl and P-2-S following oral administration of 21.6 mmole/70 kg. Points were interpolated from Cartesian coordinate plots of the experimental data and are averages of four subjects for each salt. Key: ○, P-2-S; ●, PAM-Cl.

The concentrations of oxime in urine at various times of excretion were used to calculate the rate constant, k, and biological half-life, $t_{1/2}$ (Table II). Most of the urine samples were reasonably spaced; where the sample or urine collection spacing was too variable, values were interpolated from a smooth Cartesian coordinate plot. The procedure used, of necessity, is not a rigorously correct mathematical application of the equation. However, its use in this manner constitutes, under the conditions of the experiment, a reasonably good approximate evaluation of the total disappearance and excretion rates of the oximes.

An analysis of variance of the data revealed no significant difference at the 95% confidence limit in half-life among the various salts. The mean biological half-life of the five oxime salts was 1.7 hr. with a coefficient of variation of 24%. Statistical comparison of 35 paired replicates showed no significant difference between half-life calculated from plasma data and that calculated from urinary excretion data.

The average total amount of oxime recovered in the urine for the various 2-PAM salts was 27%; the standard deviation of individual recoveries was 9%.

Clinical Findings—With regard to the clinical laboratory tests, the following findings were made: (*a*) no changes were observed in the hematologic status of any of the subjects; liver and kidney functions were also unaffected. (*b*) A 20% drop in both RBC and plasma cholinesterase activity occurred in the men given TMB-4. No change in cholinesterase values occurred after ingestion of the other oxime salts. (*c*) No consistent changes in blood pressure, pulse, or respiratory rate occurred; no effect on either neurologic status or muscular performance was observed. (*d*) No subjective complaints were made by the volunteers, with the exception of those receiving 2-PAM iodide. These subjects had signs and symptoms of iodism: coryza, pharyngeal burning, and painful parotid glands. Two of the 10 subjects receiving 2-PAM iodide had detectable parotid enlargements, and in one subject the swelling was pronounced. These findings appeared to be unrelated to the dosage, inasmuch as the most severe case was a subject who received the lowest dose of 2-PAM iodide (5 g./70 kg.).

General Observations—The amount of oxime found in the feces among 18 subjects, taken at random, was variable, ranging from 5 to 35% of the doses administered. No correlation was observed between the amount found in the feces and either the urinary excretion or the plasma levels. No oxime could be found in the feces of one subject who had taken 2 g./70 kg. of the lactate salt. When this subject was given P-2-S and at another time the dihydrogen phosphate salt, no oxime could be found in the feces. It was later established that this individual harbored intestinal parasites. No studies were carried out to establish a correlation between these observations.

The peak plasma level achieved with the bis-quaternary oxime TMB-4 was approximately 1 mcg./ml., which caused no apparent clinical discomfort; however, all three volunteers suffered a 20–25% reduction in RBC and plasma cholinesterase. This latter result cannot be considered a conclusive characteristic since only three subjects were used.

Recovery of the bis-oxime in the urine over 24 hr. averaged less than 3% for each of three subjects, and the amount found in the feces was about 2% in two subjects. In one *in vitro* experiment 0.1 mg./ml. of 2-PAMI and of TMB-4 each were mixed with aliquots of a 1:3 suspension of a control fecal sample and allowed to remain at room temperature overnight. Recoveries from the samples were 80% for 2-PAMI and 8% for TMB-4, indicating that considerable destruction of TMB-4 may occur in the lower gut.

During the course of these trials the effect of withholding water (dehydration) from the subjects before and during the period of absorption of the oxime salt was studied in three volunteers who were given 5 g. of 2-PAM lactate. The subjects drank no fluids the night before and no water for 8 hr. after ingestion of the material. A significantly lesser amount of oxime was excreted in the urine of these volunteers compared with those subjects who were "hydrated"; no significant differences in blood plasma levels were observed.

Probenecid, which inhibits tubular secretion of substances such as penicillin and phenosulfonphthalein dye and inhibits tubular reabsorption of uric acid, was given in divided doses of 0.5 g. to three volunteers before ingestion of 2 g. of 2-PAM lactate, at the time of ingestion, and 1 hr. after ingestion. The probenecid did not increase or prolong plasma levels of the oxime and did not significantly alter its excretion in urine.

SUMMARY

1. Adult male volunteers were given, orally, aqueous solutions of each of the chloride, iodide, dihydrogen phosphate, methane sulfonate, and lactate salts of pralidoxime in varying amounts. A measurable amount of oxime was found in blood plasma within 15 min.; the concentration rose rapidly, reached a peak at 2–3 hr., and then declined at a rate that approximated a first-order process.

2. The rates of absorption into and elimination from plasma of 2-PAMCl and P-2-S were practically identical.

3. The equation, $\log Y = 0.79 + 0.54 \log X$, gives an approximate relationship between peak plasma levels of oxime (Y) and oral doses (X) of the various salts. Accordingly, a 10-fold increase in dosage of the 2-PAM salts increased the peak plasma level of oxime 3.5-fold.

4. The biological half-life in man of the 2-PAM salts given orally, calculated from blood plasma values and urinary excretion rates, was 1.7 hr., with a coefficient of variation of 24%.

5. The average total amount of 2-PAM recovered in the urine was 27%; the standard derivation of individual recoveries was 9%. Considerably less TMB-4 was excreted *via* the urine; an average of 3% was recovered during 24 hr. among three subjects.

6. Clinical changes observed were (*a*) iodism symptoms in subjects given 2-PAMI and (*b*) a 20% decrease in both RBC and plasma cholinesterase in subjects given 1 g./70 kg. of TMB-4. All other clinical tests were negative.

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Keyphrases

Pralidoxime salts—absorption, elimination
Absorption, elimination rates—pralidoxime salts
Biologic half-life—pralidoxime salts
Pharmacological activity—pralidoxime salts

4-Acetamidophenyl 2,2,2-Trichloroethyl Carbonate

Particle Size Studies in Animals and Man

By LEWIS W. DITTERT*, H. JACK ADAMS†, FRED ALEXANDER, CLIFFORD W. CHONG, THEODORE ELLISON‡, and JOSEPH V. SWINTOSKY*

4-Acetamidophenyl 2,2,2-trichloroethyl carbonate (ATC) was prepared in coarse, regular, and fine particle sizes by sieving and grinding. The powders were found to dissolve in water at significantly different rates in a mechanically stirred system. When administered orally in suspensions to mice, the LD_{50}'s of the ATC powders were as follows: coarse—3340 mg./Kg.; regular—2461 mg./Kg.; and fine—1796 mg./Kg. The three ATC powders produced significantly different blood plasma concentrations of total acetaminophen in mice during the 0- to 2-hr. period after oral administration. In humans, the blood plasma concentration curves produced by oral administration of coarse and regular ATC were nearly identical. However, both had lower peaks and slower rates of decline than those produced by fine particle ATC or acetaminophen. Forty-eight-hour recovery of acetaminophen from human urine indicated that all the ATC powders were as efficiently absorbed as acetaminophen itself. It was concluded that ATC is a true prodrug of acetaminophen and that the acetaminophen blood plasma concentrations produced by orally administered ATC can be controlled to some degree by controlling its particle size.

ONE OF THE MAIN objectives of preparing prodrugs is to influence the dose–time–action profiles of drugs with known pharmacologic activities. The authors' studies with the prodrug carbonate esters of acetaminophen (1, 2) have been directed primarily toward prolonging its duration of action. This is done conveniently when the prodrug is much less soluble in water and has a slower dissolution rate in aqueous fluids than the parent drug. Under these circumstances, the appearance of the parent drug in the body is slowed by the slow dissolution of the prodrug in the gastrointestinal tract. Previous studies have indicated that the dissolution rates of the acetaminophen prodrugs, rather than their rates of hydrolysis, determine the rates at which acetaminophen is released to the tissues following their oral administration (2).

4-Acetamidophenyl 2,2,2-trichloroethyl carbonate (ATC) is much less soluble in water than acetaminophen (1). If the duration of action of ATC is indeed controlled by its dissolution rate in the gastrointestinal tract, then it would be expected that the particle size of ATC powder administered orally might have a pronounced influence on its dose–time–action profile. This paper describes the influence of particle size on oral toxicity of ATC in mice, on the blood plasma concentrations of total acetaminophen it produces in mice and in humans, and on the urinary excretion of acetaminophen after its oral administration to humans.

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EXPERIMENTAL

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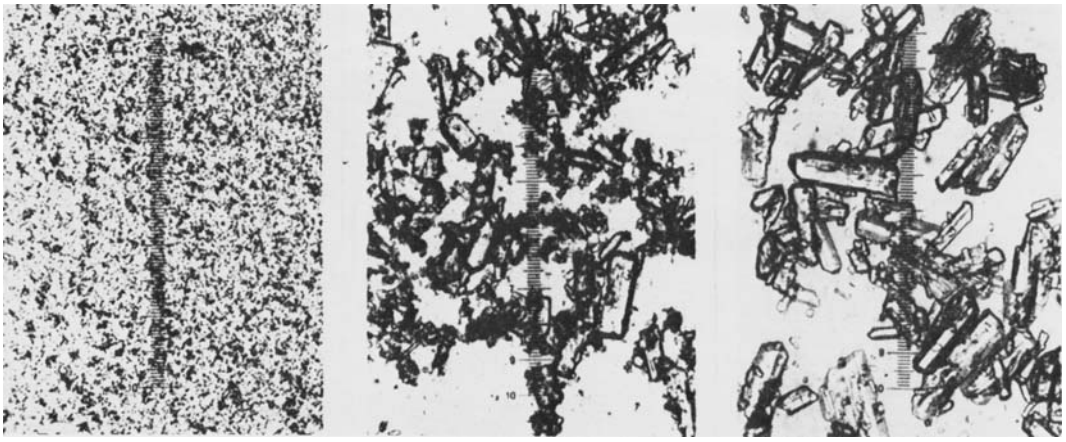

Fig. 1—Photomicrographs of the fine, regular, and coarse-particle ATC used in this study (scale = 6.03 μ/small division).

fine particle sizes by the following techniques: coarse—ATC particles which passed through a U. S. No. 140 mesh screen (hole size 150 μ) and remained on a U. S. No. 170 mesh screen (hole size 88 μ). Regular—ATC passed once through a U. S. No. 80 mesh screen in a Fitzpatrick model M comminuting mill (The W. J. Fitzpatrick Co., Chicago, Ill.) with hammers forward. Fine—ATC passed once through a fluid energy mill (Trost Jet Mill, Helme Products, Inc., Helmetta, N. J.).

Photomicrographs (125× magnification) of the powders are shown in Fig. 1. The volume surface mean diameters (d_{vs}) of the regular and fine powders were measured by air permeation using a Fisher subsieve sizer (Fisher Scientific Co.). After preparing and measuring the particle sizes of the powders, colloidal silicon dioxide[1] was added to comprise 2% by weight of the final powder to aid in dispersion.

Dissolution Rates of ATC Powders—The rate of dissolution of each of the ATC powders was determined by a method similar to that used by Parrott *et al.* (3). The apparatus consisted of a 2-L. three-neck round-bottom flask stirred by a glass impeller driven by a constant-speed electric motor (720 r.p.m.). The flask contained 1,500 ml. of 0.01% dioctyl sodium sulfosuccinate[2] solution which immediately wetted and dispersed the powders. One-hundred and fifty milligrams of the powders (about twice the amount necessary to make a saturated solution) was added through a powder funnel, and the appearance of ATC in solution was followed spectrophotometrically.

Oral Toxicity in Mice—Male Carworth Farms mice, weighing between 12 and 18 Gm., were randomly divided into groups of 10 and dosed orally with drug suspended in a 0.5% dispersion of gum tragacanth in water. Concentrations were calculated so that each animal received a dose volume of 20 ml./Kg. Control animals received 20 ml./Kg. of 0.5% tragacanth. After dosing, the animals were observed daily for 7 days; if no deaths occurred on the sixth and seventh days, the study was terminated. If deaths occurred during this 2-day period, the study was continued until no deaths occurred for two consecutive days. LD_{50}'s and potency ratios with 95% confidence limits were calculated by the Logit chi-square method of Berkson (4).

Blood Plasma Concentrations of Acetaminophen in Mice—Male Carworth Farms mice, weighing between 12 and 18 Gm., were randomly divided into groups of 10 and dosed orally with 1,000 mg./Kg. of drug suspended in a 0.5% dispersion of gum tragacanth in water. Concentrations were calculated so that each animal received a dose volume of 20 ml./Kg. At selected time intervals after dosing (0, 15, and 30 min., 1, 2, 4, and 6 hr.), heparinized blood was collected and pooled for each treatment group. The blood was centrifuged and the plasma analyzed for total acetaminophen (free + conjugated) by the method described below.

Protocols for Blood and Urine Studies in Humans—Twelve adult males were employed in a 6 × 6 crossover study with 6 subjects receiving 1 Gm. of coarse-particle ATC and 6 subjects receiving 1 Gm. of fine-particle ATC in hard gelatin capsules. Specimens of oxalated blood were collected at 0, 0.5, 1, 2, 3, 4, 7, 10, 12, and 14 hr. postdrug administration. Total urines were collected in polyethylene bottles at the following 8-hr. periods: 0, 0–8, 8–16, 16–24, 24–32, 32–40, and 40–48 hr. postdrug administration. (In this study, no changes in protocol were necessary.)

In a second study, 12 adult males (6 × 6 crossover) were given 1 Gm. of regular-particle ATC and 463 mg. of acetaminophen (molar equivalent of 1 Gm. of ATC) in hard gelatin capsules. Specimens of oxalated blood were collected at 0, 2, 4, 6, 8, 10, and 12 hr. postdrug, and total urine was collected in polyethylene bottles at 0, 2, 4, 6, 8, 10, 12, 14, 16, 24, 30, 36, and 48 hr. postdrug. (In this study, one subject was eliminated; and another, scheduled to receive acetaminophen during the second part of the crossover, was dropped.)

Analytical Method for Acetaminophen in Blood Plasma and Urine—The method employed for the determination of total acetaminophen (free + conjugated) in blood plasma and urine is similar to that used by Brodie and Axelrod (5). All samples were stored at −15° until assayed.

[1] Cab-O-Sil, Cabot Corp., Boston, Mass.
[2] Aerosol OT, American Cyanamid Co., Pearl River, N. Y.

One milliliter of the plasma or urine was transferred to a 12-ml. Pyrex graduated centrifuge tube containing 1 ml. of concentrated hydrochloric acid and diluted to 5.5 ml. with distilled water. The unstoppered tube was then placed in an autoclave at 15 lb. pressure for 1.5 hr. The tube was cooled to room temperature and the volume adjusted to 6 ml. A 5-ml. aliquot of the reaction mixture was transferred to a 40-ml. centrifuge tube containing 5 Gm. of dibasic potassium phosphate. The tube was shaken on a mechanical shaker for 5 min. (The pH of the final solution is between 7 and 8.) Twenty-five milliliters of isoamyl alcohol–ether solvent (prewashed with base, acid, and distilled water) was added, and the tube was shaken mechanically for 10 min. and then centrifuged for 10 min. to separate the phases. Twenty milliliters of the ether phase was transferred to a 40-ml. Pyrex glass-stoppered bottle containing 7 ml. of 0.01 *N* HCl, and the mixture was shaken mechanically for 5 min. and centrifuged for 5 min. Four milliliters of the aqueous phase was transferred to a colorimeter tube, and 1 ml. of a freshly prepared phenol solution (1% in water) was added and mixed. One milliliter of a freshly prepared sodium hypobromite solution (sufficient bromine water added to 1 *N* sodium carbonate solution to produce a slightly yellow color) was added, and the mixture was allowed to stand at room temperature for 20 min. The percent transmittance was read on a Coleman Junior spectrophotometer at 620 mμ against a blank consisting of 1 ml. of pretest plasma or urine treated in the same manner as the test specimens. The concentrations of acetaminophen in the specimens were determined by interpolation of a standard curve of transmittance *versus* concentration of *p*-aminophenol hydrochloride. Freshly prepared standard solutions of *p*-aminophenol hydrochloride were run through the analytical procedure along with the test and blank plasma and urine specimens.

RESULTS AND DISCUSSION

Photomicrographs of the coarse, regular, and fine ATC powders (Fig. 1) show that the coarse powder was of a relatively uniform particle size with nearly all particles falling in the 90- to 150-μ size range. The size range of this powder can be estimated from the photomicrograph in which each small division on the scale has a value of 6.03 μ. The fine powder also appears to be relatively uniform in particle size and was found by air permeation measurements to have a volume surface mean diameter (d_{vs}) of about 3.3 μ. The regular powder was not uniform in size and appears to be a mixture of relatively small particles (2–10 μ) and relatively large particles (80–100 μ). It was found to have a d_{vs} of about 22 μ. Dissolution rate experiments with the three powders showed that the solutions approached saturation at significantly different rates in the first 60 min. (see Fig. 2). Thus, significant differences in the onset and peak magnitude of the biological effects of ATC in the three particle sizes were anticipated.

Table I shows the LD_{50}'s of the three ATC powders when administered orally to mice. The LD_{50}'s and relative potencies were calculated by the Logit chi-square method of Berkson (4). The results show that both regular- and coarse-particle ATC were significantly less toxic than fine-particle ATC at the 95% confidence level. The toxicities of the regular and coarse ATC powders were not significantly different from each other at the 95% confidence level, but the actual LD_{50}'s strongly suggest that there were differences in the availabilities of these two powders for absorption in mice.

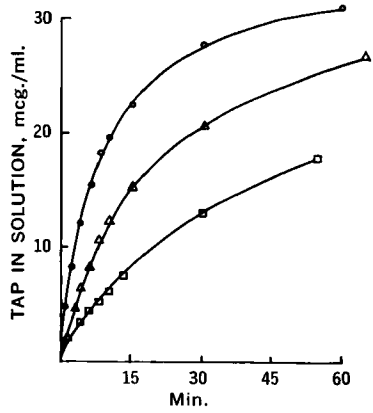

Fig. 2—Plots showing the relative rates of dissolution of fine (O-O), regular (Δ-Δ), and coarse (□-□) particle ATC powders in 0.01% dioctyl sodium sulfosuccinate solution in an apparatus similar to that used by Parrott et al. *(3). The plots illustrate different rates of* in vitro *dissolution and suggest that the powders would release ATC for absorption from the gastrointestinal tract at considerably different rates following oral administration.*

It was felt that a more sensitive test of availability, such as determination of drug plasma concentrations, might demonstrate even more unequivocally the differences in the availabilities of the three powder forms of ATC. Therefore, groups of mice were dosed orally with 1,000 mg./Kg. of the powders, and plasma concentrations of total (free + conjugated) acetaminophen were determined at various time intervals postdrug administration. The results in Fig. 3 show that, regardless of particle size, all three ATC powders produced peak plasma acetaminophen concentrations at about 1-hr. postdrug, suggesting that in each case absorption of ATC in the mouse was essentially ended after about

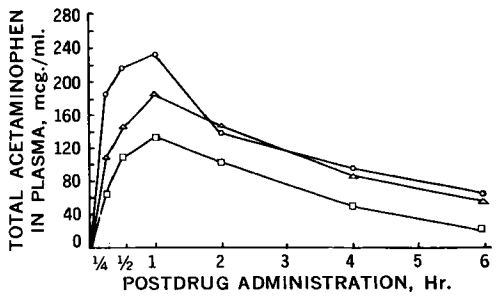

Fig. 3—Plots of plasma concentrations of total acetaminophen (free + conjugated) in mice following oral administration of 1,000 mg./Kg. of fine (O-O), regular (Δ-Δ), and coarse (□-□) particle ATC powders suspended in a 0.5% dispersion of gum tragacanth in water. The plots suggest that, in each case, the absorption of ATC was essentially ended after about 1 hr. The plasma concentrations in the 0- to 2-hr. period are dependent upon the particle sizes of the powders and, presumably, are controlled by their rates of dissolution

1 hr. However, Fig. 3 also shows that there were substantial differences in the plasma concentrations of acetaminophen produced by the powders during the first 2 hr. Thus, the fine-particle material gave rise to a substantially higher maximum plasma concentration during the first hour than the regular material; and the regular material gave rise to a substantially higher maximum plasma concentration than the coarse material.

Between 2 and 6 hr., the disappearance rates of acetaminophen from the plasma appeared to be identical for each of the three particle sizes of ATC. This supports the view that absorption of the administered doses was ended before 2 hr. The coarse-particle material gave the lowest peak and the lowest overall plasma concentrations of acetaminophen at all collection times, suggesting that the fraction of the dose absorbed was lowest from this form of ATC. The regular and fine-particle materials gave virtually superimposable plasma concentration curves between 2 and 6 hr. suggesting that the fractions of the doses absorbed were virtually identical from these two forms of ATC.

The higher plasma concentrations observed for the fine-particle ATC during the first hour suggests that absorption was fastest from this material. Also, the sudden drop in plasma concentration from Hour 1 to Hour 2 suggests that absorption-distribution equilibrium was not maintained during the first hour of absorption of fine-particle ATC. However, after absorption was ended, at about Hour 1, distribution equilibrium between the blood and other tissues was established by Hour 2; and the plasma concentrations observed thereafter were virtually the same as for the regular particle size ATC powder.

Figure 4 shows a plot of the LD_{50}'s of the three ATC powders (Table I) *versus* the peak plasma concentrations of total acetaminophen (log scale) at 1 hr. postdrug administration shown in Fig. 3. This plot shows that there was good correlation between the toxicities of the ATC powders and the peak plasma concentrations. The latter, in turn, appear to be related to the rate of absorption. In the mouse, the rate of absorption appeared to be a function of the particle size; therefore, there was excellent correlation between: (*a*) particle size and acute oral toxicity, (*b*) particle size and peak plasma concentration, and (*c*) peak plasma concentration and acute oral toxicity.

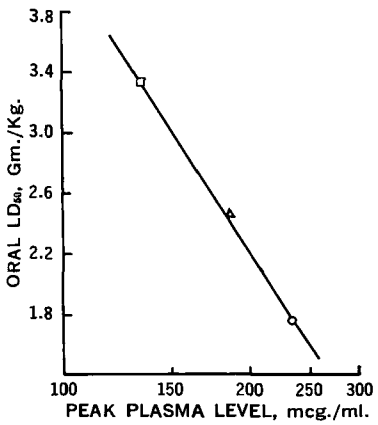

Fig. 4—Plot showing the correlation between the LD_{50}'s (Table I) and the peak plasma concentrations of total acetaminophen (log scale) at 1-hr. postdrug (Fig. 2) produced by oral administration of coarse (□), regular (Δ), and fine (○) particle ATC to mice.

TABLE I—ORAL LD_{50}'S AND POTENCY RATIOS WITH 95% CONFIDENCE LIMITS FOR COARSE, REGULAR, AND FINE-PARTICLE ATC POWDERS IN MICE[a]

LD_{50}	mg./Kg.
Coarse	3340 (1800–3710)
Regular	2461 (1820–2905)
Fine	1796 (1341–2079)
Relative Toxicities	
Coarse *vs.* regular	1.24 (0.93–1.59)[b]
Regular *vs.* fine	1.38 (1.12–1.73)[c]
Coarse *vs.* fine	1.71 (1.23–2.10)[c]

[a] Data calculated by the Logit chi-square method of Berkson (4). [b] Not significant at the 95% level. [c] Significant at the 95% level.

The results obtained with the three particle sizes of ATC in mice stirred interest to determine how particle size would affect the performance of ATC in humans. Two 6 × 6 crossover studies were carried out to compare (*a*) coarse-particle ATC with fine-particle ATC, and (*b*) regular-particle ATC with acetaminophen with respect to the plasma concentrations of acetaminophen produced in humans. Plots of the time profiles of plasma total acetaminophen in subjects administered 1 Gm. of coarse, regular, or fine-particle ATC or 436 mg. of acetaminophen (molar equivalent of 1 Gm. of ATC) orally in hard gelatin capsules are shown in Fig. 5.

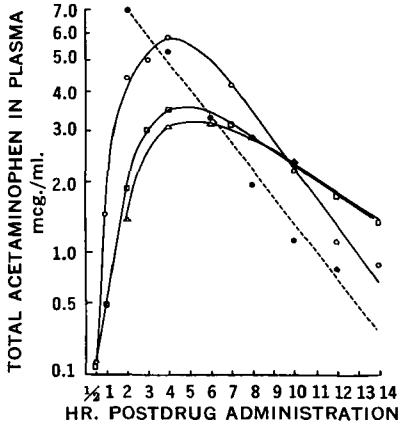

Fig. 5—Semilog plots of plasma concentrations of total acetaminophen (free + conjugated) in humans following oral administration of 1 Gm. of fine (○-○), regular (Δ-Δ), or coarse (□-□) ATC powders or 463 mg. of acetaminophen (●-●) (molar equivalent of 1 Gm. of ATC) in hard gelatin capsules. The study was carried out in two parts on 6 × 6 crossover plans. The acetaminophen and fine-particle ATC curves descend at rates corresponding to half-lives of about 3.4 and 3.2 hr., respectively, suggesting that no further absorption or conversion of ATC to acetaminophen took place after about 6 hr. following oral administration of fine-particle ATC. The regular and coarse-particle ATC curves descend at a rate corresponding to a half-life of about 6 hr. suggesting that absorption of ATC and conversion to acetaminophen was continuing well beyond 6 hr., perhaps as long as 14 hr., with these powders.

TABLE II—AVERAGE URINARY RECOVERY OF TOTAL (FREE + CONJUGATED) ACETAMINOPHEN FOLLOWING ORAL ADMINISTRATION OF COARSE, REGULAR, OR FINE-PARTICLE ATC OR ACETAMINOPHEN TO HUMANS (6 × 6 CROSSOVER)

Material Administered	Dose	Total Acetaminophen (mg./ml.) Collected per Postdrug Period (hr.) 0–8	8–16	16–24	24–32	32–40	40–48	Total	(Limits)
Acetaminophen	463 mg.	189.4 (40.9%)	70.7 (15.3%)	20.1 (4.3%)	←——7.8——→	←——(1.7%)——→		288.0 (62.2%)	(84.4–413.1) (18.2–89.3%)
Coarse ATC	1 Gm.	100.1 (21.6%)	142.3 (30.7%)	35.8 (7.7%)	23.9 (5.2%)	6.6 (1.4%)	2.2 (0.5%)	310.9 (67.1%)	(179.5–413.2) (38.8–89.2%)
Regular ATC	1 Gm.	112.3 (24.3%)	127.5 (27.6%)	41.7 (9.0%)	←——25.0——→	←——(5.4%)——→		306.5 (66.3%)	(207.4–432.1) (44.8–93.4%)
Fine ATC	1 Gm.	111.5 (24.1%)	134.6 (29.1%)	77.4 (16.7%)	10.1 (2.2%)	4.0 (0.9%)	1.9 (0.4%)	339.5 (73.3%)	(236.4–413.1) (51.1–89.2%)

The plasma concentration curves of regular and coarse-particle ATC were very similar, confirming the wide degree of overlap in the range of particle sizes in these two powders. In humans, where the gastrointestinal tract is much longer than in the mouse, proportionately smaller doses were given; and both the large and small particles in the regular-particle ATC powder were probably completely dissolved and absorbed. Since there was a relatively high proportion, on a weight basis, of coarse particles in regular-particle ATC powder (see Fig. 1), it was reasonable to expect that this powder would behave more like coarse-particle ATC in humans in which a large fraction of the administered dose is dissolved and absorbed.

Figure 5 shows that after peak drug concentrations were reached, the rates of plasma clearance with regular and coarse particle ATC were slower (half-life about 6 hr.) than that observed with acetaminophen (half-life about 3.4 hr.). Gibaldi and Schwartz (6) have shown that prolonged absorption of a prodrug of penicillin G gave a blood concentration plot of penicillin G with the appearance of prolonged excretion; that is, the half-life of the descending curve was much longer than that observed following administration of penicillin G itself. They pointed out that the apparent half-life from the "tail" of the plot was an artifact caused by absorption of the prodrug during the period in which the blood concentration curve was descending. Similarly, the slower apparent rate of clearance of acetaminophen from the blood of humans administered regular and coarse-particle ATC is probably due to prolonged or delayed absorption of the larger particles in these powders.

On the other hand, the plasma concentrations of acetaminophen produced by fine-particle ATC reached a higher, slightly earlier peak (4 hr.) than those produced by coarse or regular-particle ATC, and the rate of plasma clearance (half-life about 3.2 hr.) in the fine-particle case was the same as that observed for acetaminophen (half-life about 3.4 hr). This effect is probably due to "complete" absorption of fine-particle ATC within about 5 hr. postdrug and is indicative that ATC was completely converted to acetaminophen (and trichloroethanol) by about 5 hr. postdrug. Thus it appears that the low aqueous solubility of ATC would enable the formulator to lower the peak and extend the duration of acetaminophen (and presumably also trichloroethanol) blood levels by merely controlling the particle size of administered ATC powder.

The plasma concentration data obtained with mice suggested that coarse-particle ATC was not as completely absorbed as regular or fine-particle ATC, and it was of interest to know if this was also true in humans. Therefore, the urines of the subjects administered coarse, regular, or fine-particle ATC powder or a molar equivalent dose of acetaminophen were collected for 48 hr. following administration of the drug and analyzed for total acetaminophen content. The results shown in Table II suggest great variability in absorption efficiency from subject to subject with a given form of the drug. Also, the absorption efficiencies vary to the degree that the ranges overlap for each form of ATC and for acetaminophen. However, there is a trend toward greatest absorption efficiency for the fine form of ATC. Perhaps more importantly, these results confirm that ATC is a true prodrug of acetaminophen which releases molar equivalent amounts of acetaminophen to the tissues at a slower rate following oral administration than does acetaminophen itself. As a result, when a coarse powder form of ATC is administered to humans, acetaminophen blood levels show a profile which is suggestive of a slower, more prolonged release and absorption of this prodrug.

CONCLUSIONS

1. In mice, only a fraction of the dose of ATC appeared to be absorbed orally from the coarse powder form. The dissolution rate of the ATC powder controlled the blood plasma concentrations of acetaminophen (and presumably also trichloroethanol) during the first 2 hr. after oral dosing. The peak plasma concentration attained during this period was directly related to the LD_{50} of the powder.

2. In humans, coarse, regular, and fine-particle ATC powders appeared to be completely absorbed orally. The slow dissolution of the larger particles in the coarse and regular ATC powders caused the plasma concentrations of acetaminophen to reach lower peaks and descend at slower rates than those following oral dosing with acetaminophen or fine-particle ATC.

3. The low aqueous solubility of ATC would allow the formulator to control, to some extent, the blood levels and, presumably, the pharmacologic activity of ATC given orally. Thus, a prodrug might be employed to produce a sustained action of the parent drug in cases where the parent drug may not lend itself readily to formulation in common sustained-release dosage forms.

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Keyphrases

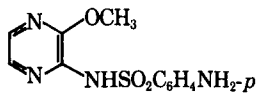
Prodrugs—acetaminophen carbonate esters
4-Acetamidophenyl 2,2,2-trichloroethyl carbonate
Particle size, effect—absorption, excretion, toxicity
LD_{50} values—acetaminophen carbonate esters
Plasma levels—acetaminophen
Urinary excretion—acetaminophen

Selective Acid-Catalyzed Hydrolyses of Methoxysulfanilamidodiazines

By VINCENT S. VENTURELLA*

The dilute acid hydrolysis of 3-methoxy-6-sulfanilamidopyridazine and several methoxysulfanilamidopyrimidines has been studied. Experiments show that in cases where an intermediate 2-pyrimidone is a possible postulation, further hydrolysis usually leads to the formation of sulfanilamide and the corresponding hydroxypyrimidine. A multistage route for the acidic degradation of 3-methoxy-6-sulfanilamidopyridazine, 2,4-dimethoxy-6-sulfanilamidopyrimidine, and 2-methylthio-4-methoxy-6-sulfanilamidopyrimidine is proposed.

RECENTLY, during a routine evaluation of the acidic degradation of the sulfanilamidopyrazine (I), it was found that the normally expected dilute acid cleavage to sulfanilic acid and 2-amino-3-methoxypyrazine did not occur (1), but that the products formed were sulfanilamide and 2,3-dihydroxypyrazine (II). Such a result



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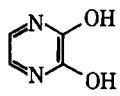

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is in part unexpected, due to the fact that a 60% HBr solution is routinely used to effect the cleavage of difunctional methoxypyrazines (2), although there is a report (3) that 2,3-disulfanilamidopyrazine forms II under similar conditions. Since the result obtained with (I) may be due to the activation of the CH_3O position by the p-$H_2NC_6H_4SO_2NH$ group, it was desirable to test the mutual activation of these groups on the other substituent in similar sulfanilamidodiazines, such as those with the pyridazine and pyrimidine rings. The hydrolyses (except where noted) were carried out in refluxing 2 *N* HCl.

Hydrolysis of 3-methoxy-6-sulfanilamidopyridazine (III) (sulfamethoxypyridazine)[1] gave the expected sulfanilic acid, but activation of the ether position occurred forming 3-hydroxy-6-aminopyridazine. This result is surprising, even though Jacobs (4) states that the pyridazine ring is susceptible to substitution by nucleophilic reagents, because the present study has shown that a CH_3O group in the 3 position is not susceptible to nucleophilic displacement under the reaction conditions employed. In 2 *N* HCl solutions, neither 3-methoxy nor 3-methoxy-6-aminopyridazine (IV) formed more than a nominal amount of hydrolyzed material (3-hydroxypyridazine; 3-hydroxy-6-aminopyridazine, respectively) after several hours reflux followed by standing in solution for 24–72 hr. This seemingly anomalous result demands further study although a partial explanation can be made on the basis of an induced increase in the basicity of N^2 in the substituted pyridazine. The basicity increase is probably lacking or very weak in 3-methoxy- and 3-methoxy-6-aminopyridazine; a *point which*

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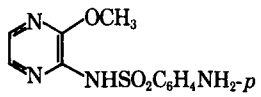

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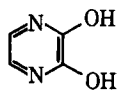

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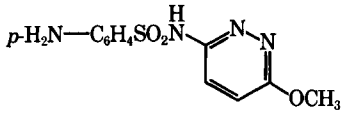
would account for the lack of acid-catalyzed aqueous nucleophilic displacement.



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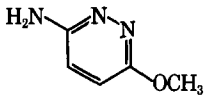
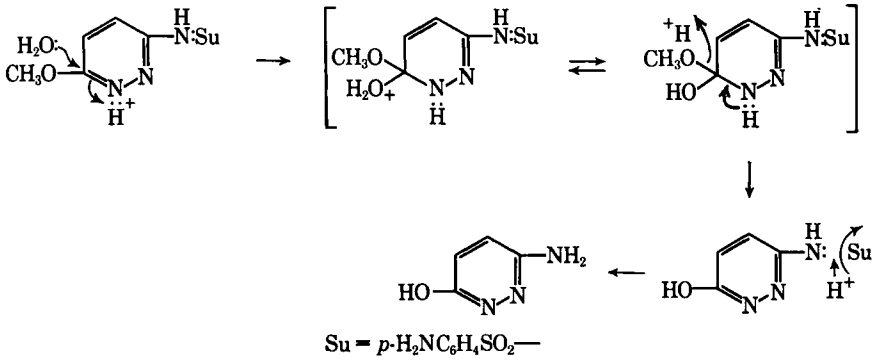


(IV)

A partial explanation which is then possible for the acid hydrolysis of III may be represented by Scheme I. In this scheme, an S_NAr attack is presumed to occur at the methoxyl position. This attack is aided by the induced electrophilic center produced by an electron migration promoted by the protonation of N^2.

hr. with 3.6 *N* HCl and 64.4% after 5.5 hr. with 3.6 *N* H_2SO_4.

Similar dilute acid hydrolysis of 4-methoxy-6-sulfanilamidopyrimidine, 4,6-dimethoxy-2-sulfanilamidopyrimidine, and 2-methylthio-4-methoxy-6-sulfanilamidopyrimidine (V) formed sulfanilic acid and the corresponding hydroxyaminopyrimidine (2-methylthio-4-hydroxy-6-aminopyrimidine with V). The 6 sulfanilamido compounds presumably hydrolyze by a nucleophilic alkoxy displacement *via* the S_NAr mechanism. This can then be followed by either normal SO_2NH bond cleavage or a second S_NAr nucleophilic displacement. In order for hydrolytic displacement of the sulfanilamido group to occur, the results of this study suggest that a 2-pyrimidone intermediate is needed, although there is no definitive proof of requirement. An incomplete hydrolysate of 4-methoxy-6-sulfanilamidopyrimidine was subjected to thin-layer



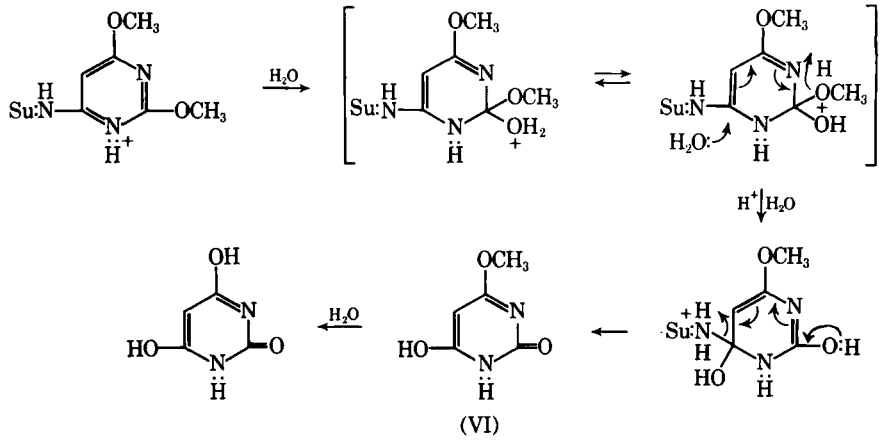
Su = *p*-$H_2NC_6H_4SO_2$—

Scheme I

Of the pyrimidines tested, 2,4-dimethoxy-6-sulfanilamidopyrimidine (sulfadimethoxine)[2] gave a novel result, yielding sulfanilamide and barbituric acid. The novelty of this result rests in the nucleophilic displacement of the sulfanilamido group, whereas one normally would expect nucleophilic displacement of the OCH_3 groups on the basis of the known S_NAr reaction which occurs at the 2,4, or 6 pyrimidyl alkoxy centers in acid solution (5, 6). By comparison with the experimental work of Daniels *et al.*, who used 2-methoxypyrimidine (7), there was a greater amount of reaction completed in a comparable period of time in the current study thereby indicating that CH_3O displacement in the hydrolysis of sulfadimethoxine is indeed accelerated by the presence of the sulfanilamido group. In the present study, essentially quantitative results were obtained after 4 to 6 hr. reflux, whereas the Daniels report (7) showed 40% completion in 1.5 chromatography (Silica Gel G, 0.5 mm., 70 Me_2CO; 30 *n*-BuOH; 30 NH_4OH) and showed an extra spot at R_f 0.52. This substance was isolated from an identical macrobatch and was shown to be 4-methoxy-5-aminopyrimidine [m.p. 148.5–150°; lit. (8), 150–151°]. The appearance of this compound thus points to the fact that when normal sulfa cleavage occurs, CH_3O^- displacement is more resistant.

However, when V was subjected to incomplete hydrolysis in 6 *N* HCl, 2-hydroxy-4-methoxy-6-sulfanilamidopyrimidine was isolated along with 2,6-dihydroxy-4-methoxypyrimidine as the intermediate hydrolytic products. This result was expected in view of the lability of 2-SR substituents of pyrimidines toward 6 *N* HCl (9). Either of the two intermediates then upon further reaction proceed along the sulfadimethoxine pathway since 2,6-dihydroxy-4-methoxypyrimidine (or its 2-pyrimidone tautomer) can be shown to be an intermediate during the sulfadimethoxine hydrolysis (Scheme II). This route is logical since the

[2] Madribon, kindly supplied through the courtesy of Dr. W. E. Scott, Hoffmann-La Roche Inc., Nutley, N. J.



(VI)

Su = p-$H_2NC_6H_4SO_2$—

Scheme II

2 position is more reactive in acid solution than either the 4 or 6 position in the absence of a retarding group such as the 2-methylthio group which is known to be unreactive in compounds similar to V in 2 *N* HCl (9).

Considering the results of the hydrolyses obtained with sulfadimethoxine and with V (2 *N* HCl and 6 *N* HCl) together with the established fact that a structure such as VI is favored in aqueous solution, a possible stepwise reaction for the hydrolysis of sulfadimethoxine can be represented by Scheme II. Alternately one might propose protonation of the other ring nitrogen first in the structure in Scheme II to give an incipient cross-conjugated cation. This alternative however is unlikely since neither 4-methoxy-6-sulfanilamidopyrimidine, 4,6-dimethoxy-2-sulfanilamidopyrimidine, nor V showed nucleophilic displacement of the sulfonamido group in 2 *N* HCl.

It is noteworthy that in neither of the other pyrimidines tested can an intermediate such as VI be postulated, the absence of which promotes normal SO_2NH bond cleavage and S_NAr attack at the CH_3O center (6).

In an effort to determine what normally might be expected to occur in the nonetheric sulfanilamidopyrimidines, 4-sulfanilamidopyrimidine, sulfadiazine, sulfamerazine, and sulfamethazine were subjected to similar hydrolytic experiments. In each of these four compounds the absence of a CH_3O function or the absence of a group that would yield a 2-pyrimidone (or 2-hydroxypyrimidine) function, based upon the present experimental results, would suggest that normal p-NH_2-$C_6H_4SO_2NH$ cleavage should occur. The lack of formation of sulfanilamide during the 2 *N* hydrolysis of 4-sulfanilamidopyrimidine, sulfadiazine, sulfamerazine, and sulfamethazine lends further support to the argument that the net effect of the CH_3O group is to promote the nucleophilic displacement of the p-NH_2-C_6H_4-SO_2NH group. In all the cases concerning the nonetheric sulfanilamidopyrimidines tested in this study, sulfanilic acid and the corresponding aminopyrimidine was isolated indicating the need for the CH_3O substituent in order to effect S_NAr displacement of the sulfanilamido group. It is also worthy of note that even CH_3 substitution, as in sulfamerazine and sulfamethazine, will not promote an S_NAr effect, and a CH_3O group in either the 4 or 6 position alone does not exhibit the unusual S_NAr sulfonamido displacement effect noted with sulfadimethoxine.

EXPERIMENTAL[3]

Isolated compounds were authenticated by comparison with physical constants appearing in the literature or with authentic samples (m.p., UV, IR spectra), mixture melting points, TLC comparison and/or by elemental analysis.

The procedure used for the hydrolysis of III, and the isolation of products is typical of that followed for all of the pyridazines and pyrimidines tested except V in 6 *N* HCl. The results of the hydrolytic experiments are collected in Tables I and II along with pertinent analytical data.

Hydrolysis of 3-Methoxy-6-sulfanilamidopyridazine (III)—Twenty grams (0.071 mole) of III was dissolved in 125 ml. of 2 *N* hydrochloric acid and was heated to reflux for 4 hr. The mixture was cooled and filtered, the solid washed with 50 ml. of cold 2 *N* hydrochloric acid, and suction dried to give a light yellow solid which crystallized from boiling water as white plates, m.p. 280–285° (decomposition); m.p. undepressed on admixture with sulfanilic

[3] All melting points were taken on a Thomas-Hoover apparatus and are corrected. UV spectra were obtained by use of the B. and L. Spectronic 505 or Cary model 15 (courtesy of George Irwin, SKF Laboratories). IR spectra were recorded on Perkin-Elmer model 137 spectrometer.

TABLE I—HYDROLYTIC PRODUCTS OF TESTED PYRIDAZINES

Substituents (wt. used; mole)	Vol. 2 *N* HCl; Reaction Time, hr.	Product(s) Isolated	Yield	M.p.	Supporting Anal. Data
3-Methoxy-6-sulfanilamido (20 Gm.; 0.071)	125 ml.; 4	Sulfanilic acid	11.4 Gm., 93%	280–285° (dec.)[a]	λ_{max}. pH 10 = 258 mμ[b]; IR identical to commercial sample[c]; analysis for $C_6H_8N_2O_2S$: C, 41.61; H, 4.07; N; 8.09. Found: C, 41.43; H, 4.04; N, 8.32.
		3-Hydroxy-6-amino-pyridazine	7.2 Gm., 91.5%	243–244.5° (dec.)[d]	ν(cm.$^{-1}$) 3440, 3315, 1685 (KBr); *p*-AcNHC$_6$H$_4$SO$_2$-NH-deriv. m.p. 198–199.2°[e]
3-Methoxy- (5 Gm.; 0.045)	90 ml.; 2	3-Methoxy-pyridazine	4.1 Gm., 84.4%	—	TLC R_f 0.67 (0.25 mm., Silica Gel G, AcOH, *n*-BuOH, Me_2CO; 40; 40; 20)[f]
	90 ml.; 6	3-Hydroxy-pyridazine hydrate	0.17 Gm., 3.4%	72–73.5°[g]	R_f 0.55 (3-methoxypyridazine TLC parameters)
3-Methoxy-6-amino-[h] (10 Gm., 0.08)	70 ml.; 5	3-Hydroxy-6-amino-pyridazine	0.13 Gm., 1.5%	235–237° (dec.)[d]	m.p. undepressed with hydrolysis product of III; 9.6 Gm. (0.077 mole) of starting material isolated

[a] Mixed m.p. showed no depression. [b] Repressed at pH 2 (9). [c] Distillation Products Ind., Rochester, N. Y. [d] Lit. (10) reports m.p. 245–246°. [e] Lit. (10) reports m.p. 200–202°. [f] R_f for starting material. [g] Lit. (11) reports 70–71°. [h] Aldrich Chem. Corp., Milwaukee, Wis.

TABLE II—HYDROLYTIC PRODUCTS OF TESTED PYRIMIDINES

Substituents (wt. used; moles)	Vol. of HCl; Reaction Time, hr.	Product(s) Isolated	Yield	M.p.	Supporting Anal. Data
2,4-Dimethoxy-6-sulfanilamido- (20 Gm., 0.065)	150 ml. (2 *N*); 5	Barbituric acid	10.8 Gm., 98.6%[a]	242–243.5°	λ_{max}. 256 mμ, log ϵ 4.34 (pH 3.2), pH − $\Delta\epsilon$ analysis positive.[b] Calcd. for $C_4H_4N_2O_3$: C, 37.50; H, 3.12; N, 21.92. Found: C, 38.05; H, 3.36; N, 22.26
		Sulfanilamide	7.9 Gm., 65%[c]	162–163.5°[d]	λ_{max}. 255, 312 mμ (H_2O). Calcd. for $C_6H_8N_2O_2S$: C, 41.84; H, 4.68; N, 16.27. Found: C, 41.91; H, 4.96; N, 15.38
4-Methoxy-6-sulfanilamido- (10 Gm., 0.036)	120 ml. (2 *N*); 6	Sulfanilic acid[e]	3.43 Gm., 56%	286–288° (dec.)	Calcd. for $C_6H_8N_2O_2S$: C, 41.61; H, 4.07; N, 8.09. Found: C, 41.94; H, 4.22; N, 8.00
		4-Hydroxy-6-amino-pyrimidine	2.05 Gm., 52%	261–262°[f]	*g*
4-Methoxy-6-sulfanilamido- (5.6 Gm., 0.02)	75 ml. (2 *N*); 1	4-Methoxy-6-amino-pyrimidine	0.9 Gm., 36%	148.5–150°[h]	R_f 0.52 (Silica Gel G, 0.5 mm., Me_2CO, *n*-BuOH; NH_4OH: (70:30:30)
		Sulfanilic acid[e]	1.1 Gm., 32.1%	284–287° (dec.)	3.3 Gm. of starting material isolated
4,6-Dimethoxy-2-sulfanilamido- (15 Gm., 0.054)	125 ml. (2 *N*); 4	Sulfanilic acid[e]	6.7 Gm., 70%	283–287° (dec.)	
		2-Amino-4,6-dihydroxy-pyrimidine	5.0 Gm., 72.4%	300°	Mixed m.p., IR, UV, qualitatively identical to authentic sample. R_f 0.33; Silica Gel G, 0.5 mm[i], Me_2CO; *n*-BuOH; NH_4OH (70:30:30): Authentic sample[i] gave the same R_f
2-Methylthio-4-methoxy-6-sulfanilamido- (10 Gm., 0.031)	120 ml. (2 *N*); 5	Sulfanilic acid[e]	4.0 Gm., 75%	280–284° (dec.)	
		2-Methylthio-4-hydroxy-6-amino-pyrimidine	3.5 Gm., 72%	261–263° (dec.)[j]	1.1 Gm. (11%) of starting material isolated. 4-Chloro-analog, m.p. 129–130°[k]
(12.6 Gm., 0.038)	150 ml. (6 *N*); 6	Barbituric acid	3.7 Gm., 59%[a]	241–243°	Spectroscopically similar to that isolated from 2,4-dimethoxy-6-sulfanilamido-pyrimidine
		Sulfanilamide	4.3 Gm., 65%	163–164°[d]	2.2 Gm. (21.5%) starting material isolated

(*Continued on next page.*)

TABLE II—(*Continued.*)

Substituents (wt. used; moles)	Vol. of HCl; Reaction Time, hr.	Product(s) Isolated	Yield	M.p.	Supporting Anal. Data
(4.9 Gm., 0.015)	65 ml. (6 *N*); 0.75	Barbituric acid	0.10 Gm., 40%[a]	243–244.5°	
		2-Hydroxy-4-methoxy-6-sulfanilamidopyrimidine	0.82 Gm., 18.5%	166–167.5°	Analysis for $C_{11}H_{12}N_4O_4S$: C, 46.16; H, 4.23; N, 19.57. Found: C, 45.80; H, 4.10; N, 20.18
		2,6-Dihydroxy-4-methoxypyrimidine[l]	0.21 Gm., 9.9%	204–206°	Analysis for $C_5H_6N_2O_3$: C, 42.26; H, 4.25; N, 19.70. Found: C, 42.00; H, 4.19; N, 19.41; 2.4 Gm. (49%) of starting material isolated
4-Sulfanilamido- (5.0 Gm., 0.02)	45 ml. (2 *N*); 5	Sulfanilic acid[e]	1.1 Gm., 32.1%	282–285° (dec.)	
		4-Aminopyrimidine	0.84 Gm., 46.5%	147–148°[m]	$\lambda_{max.}$ H_2O 233 mμ (ε 10,950), 264 mμ (ε 3802).[n] 2.6 Gm. of starting material isolated.
2-Sulfanilamido- (20 Gm., 0.08)	190 ml. (2 *N*); 4	Sulfanilic acid[e]	5.0 Gm., 36.1%	284–287° (dec.)	8.6 Gm. of sulfadiazine remaining in hydrolysis mixture
		2-Aminopyrimidine hydrochloride[o]	4.2 Gm., crude, 40.1%	221–222° (dec.)	ν (cm.$^{-1}$) (KBr) 2710–2720 (salt), 1580, 1610 (aromatic-atypical of 2-hydroxypyrimidine); no carbonyl absorption (as shown by 2-hydroxypyrimidine) (see *Ref. 17*); free base from aq. K_2CO_3 extracted with EtOAc, m.p. 121–122.5° (commercial[i] material melted at 124–126°); 2-acetylamino-deriv., m.p. 149–151° [Lit. (18) reports 145–146.5°]
Sulfamerazine (10 Gm., 0.038)	150 ml. (2 *N*); 7	Sulfanilic acid[e]	6.2 Gm., 94.5%	282–285° (dec.)	
		2-Amino-4-methylpyrimidine	3.5 Gm., 85%	155–157.1°	Lit. (18) reports m.p. 156.5–157.5°; analysis for $C_5H_7N_3$: C, 55.11; H, 6.41; N, 38.48. Found: C, 55.39; H, 6.19; N, 38.02
Sulfamethazine (10 Gm., 0.036)	135 ml. (2 *N*); 6	Sulfanilic acid[e]	5.9 Gm., 94.7%	283–285° (dec.)	
		2-Amino-4,6-dimethylpyrimidine	3.8 Gm., 87%	149–151°	Lit. (19) reports m.p. 152–154°; analysis for $C_6H_9N_3$: C, 58.40; H, 7.31; N, 34.18. Found: C, 58.10; H, 7.22; N, 33.97

[a] As the dihydrate. [b] Reported $\lambda_{max.}$ 257 mμ (pH 3.0); log ε 4.04 (*Ref. 12*). [c] An additional 2.1 Gm. (19%) of impure product was obtained from the recrystallization liquor. [d] Undepressed when admixed with authentic sulfanilamide (Distillation Products Industries, Rochester, N. Y.). [e] Identical to that obtained from the hydrolysis of 3-methoxy-6-sulfanilamidopyridazine (Table I). [f] Lit. (14) reports m.p. 263–264°. [g] 3.1 Gm. of 4-methoxy-6-sulfanilamidopyrimidine recovered. [h] Lit. (8) reports m.p. 150–151°. [i] Aldrich Chem. Co., Milwaukee, Wisc. [j] Lit. (15) reports m.p. 260–263° (dec.). [k] Prepared according to Baker *et al.* (15); reported m.p. 125–128°. [l] 2,6-Dichloro analog, m.p. 51–53° prepared according to Baddiley and Topham (16) (see experimental part). [m] Lit. (17) reports m.p. 150–151°. [n] Lit. (14) reports $\lambda_{max.}$ 232 mμ (ε 11,300) and 265 mμ (ε 3780). [o] The rust colored solid was extensively hydrated as isolated giving a higher melting product than reported. Confirmation of the product was obtained by drying for 10 hr. at 85° *in vacuo* over P_2O_5 yielding a product which melted at 205–208° (dec.); m.p. 173–175° (dec.) when admixed with authentic 2-hydroxypyrimidine.HCl.

acid.[4] The weight of crude material was 11.4 Gm. (93%), calculated as sulfanilic acid.

The filtrate after isolation was carefully neutralized with solid potassium carbonate, cooled in an ice bath, and filtered to give a 91.5% yield of the bland colored 3-hydroxy-6-aminopyridazine (7.2 Gm.), m.p. 243–244.5° (decomposition) after recrystallization from ethanol–water [lit. (11), m.p. 245–246°].

3-Methoxypyridazine—Twenty-five grams (0.17 mole) of 3,6-dichloropyridazine was treated with 1.05 equivalents of sodium methoxide in methanol according to the procedure described by Druey (21) and gave 11.6 Gm. (0.08 mole; 47%) of 3-methoxy-6-chloropyridazine (from boiling hexane, m.p. 87.9–89°; reported 90°). The product was dissolved in 120 ml. of anhydrous methanol and hydrogenated over 0.8 Gm. of Pd/C (22) for 4.5 hr., the dark reaction solution filtered, the filtrate evaporated under reduced pressure to a moist residue, the mixture neutralized with a saturated solution of potassium carbonate, and extracted with 380 ml. of methylene chloride. The extract was dried over

[4] Distillation Products Industries, Rochester, N. Y.

anhydrous magnesium sulfate, the solvent was removed *in vacuo*, and the product distilled to give 4.1 Gm. (0.037 mole) (46%) of a light yellow oil, b.p.$_8$ 76–79° (Druey reports 86–87°, 13 mm.).

4-Chloro-6-sulfanilamidopyrimidine—Twenty-five grams (0.168 mole) of 4,6-dichloropyrimidine,[5] 68.5 Gm. of sodium sulfanilamide, and 68.5 Gm. of acetamide were heated in the absence of solvent and the mixture worked up to yield 14.3 Gm. (0.05 mole; 29.5%) of product as the hydrochloride, m.p. 330–334° (decomposition), in the manner described by Shepherd (23), by acidifying the final solution to pH 3 rather than pH 4, followed by overnight refrigeration.

4-Sulfanilamidopyrimidine—Fourteen grams (0.047 mole) of 4-chloro-6-sulfanilamidopyrimidine was hydrogenated at 3 atm. in 82 ml. of 1.7 *N* sodium hydroxide for 75 min. (H_2 uptake complete) over 2.45 Gm. of 10% Pd/C catalyst (23). The filtered solution was neutralized with 1 *N* hydrochloric acid, refrigerated overnight, and filtered. The yellow solid was crystallized from hot acetonitrile to give 6.8 Gm. (0.031 mole; 63.5%) of 4-sulfanilamidopyrimidine as white microcrystals, vacuum dried at 55°, m.p. 225–227° [lit. (23), m.p. 232–232.5°].

4,6-Dimethoxy-2-sulfanilamidopyrimidine—The general methods described by Braker *et al.* (24) were used with some modifications in the preparation of this compound in 21% overall yield.

Fifty grams (0.392 mole) of 4,6-dihydroxy-2-aminopyrimidine was treated with 200 ml. of phosphorus oxychloride, added all at once, the mixture diluted with 20 ml. of dimethylaniline, stirred mechanically, and refluxed for 3 hr. The mixture was cooled in an ice bath and poured slowly onto 800 Gm. of cracked ice moistened with 50 ml. of ether. After all the ice had melted, the mixture was brought to pH 6 with concentrated ammonia and extracted continuously with ether for 36 hr. The ether extract was washed with saturated sodium chloride solution, dried over anhydrous magnesium sulfate, and evaporated to dryness, *in vacuo*. The solid was crystallized from a 1:1 ether–hexane mixture and recrystallized from the same solvent to give 34.2 Gm. (0.21 mole; 53%) of 4,6-dichloro-2-aminopyrimidine, m.p. 220–223° (reported by Braker as 223–225°).

The purified dichloro compound (0.20 mole; 32.8 Gm.) was suspended and stirred in 250 ml. of absolute methanol and treated with 3.5 molar equivalents (37.1 Gm.) of solid sodium methoxide and the mixture refluxed for 10 hr. The suspension was cooled to 5°, filtered, the filtrate evaporated to dryness, and the residue triturated with 50 ml. of water. The solid was filtered, washed with three 10-ml. portions of cold water, and vacuum dried at 45° over calcium chloride to yield 30.1 Gm. (0.19 mole; 97%) of 4,6-dimethoxy-2-aminopyrimidine, m.p. 95–97° [lit. (24) m.p. 94–95°] after recrystallization from hot hexane.

The purified dimethoxy compound (29.5 Gm., 0.18 mole) was mixed with 65 ml. of pyridine and 20 ml. of cyclohexane. The mixture was cooled in an ice salt bath, stirred, and treated portionwise with 52.4 Gm. (0.22 mole) of acetylsulfanilylchloride. The mixture was stirred for 3 hr. at room temperature after an initial 30-min. reaction time at 0°. The solvent was evaporated *in vacuo*, the residue treated with 150 ml. of water, the water evaporated under vacuum to give a solid which was crystallized from hot acetone. The yield of 2-(N^1-acetylsulfanilamido)-4,6-dimethoxypyrimidine was 58.2 Gm. (0.16 mole; 91%); m.p. 242–244° (decomposition) [lit. (24) m.p. 240–241° (decomposition)]. The *N*-acetyl sulfonamide was boiled with 180 ml. of 10% aqueous sodium hydroxide solution for 2 hr., the mixture cooled, and acidified with acetic acid to pH 4. The resulting suspension was refrigerated at 5° for 48 hr., filtered, the precipitate washed with 150 ml. of ice water, and suction dried. The white solid obtained was recrystallized from hot methanol–water and vacuum dried at 45° for 12 hr. to give 23.6 Gm. (0.076 mole; 47%) of 4,6-dimethoxy-2-sulfanilamidopyrimidine, m.p. 177–178° (*Reference 24* reports the same m.p.) as white microcrystals.

2-Methylthio-4-methoxy-6-sulfanilamidopyrimidine (V)—This compound was prepared in a manner substantially similar to that reported by Budesinsky *et al.* (25). Twenty-five grams (0.143 mole) of 2-methylthio-4-chloro-6-aminopyrimidine was suspended in 200 ml. of methanol containing 8.65 Gm. (0.16 mole) of sodium methoxide, and the mixture refluxed for 12 hr. The hazy solution was cooled to −10°, the mixture was filtered, the filtrate evaporated to dryness under reduced pressure, the solid suspended, and macerated with 250 ml. of water. The solid was removed by suction filtration and dried in air overnight to give 18.3 Gm. (0.107 mole) (74.8%) of the white microcrystalline 2-methylthio-4-methoxy-6-aminopyrimidine which melted at 106.2–108° after recrystallization from 50% aqueous methanol [lit. (16), m.p. 110°].

Anal.—Calcd. for $C_6H_9N_3OS$: C, 42.08; H, 5.29; N, 24.54. Found: C, 41.77; H, 5.06; N, 24.81.

Reaction of the isolated methoxy compound with acetylsulfanilylchloride in pyridine (24) followed by alkaline hydrolysis gave 23.1 Gm. (66.2%) of V, m.p. 171.5–173°, after recrystallization from ethanol–water [lit. (23), m.p. 171°].

Anal.—Calcd. for $C_{12}H_{14}N_4O_3S_2$: C, 44.15; H, 4.32; N, 17.16. Found: C, 44.70; H, 4.12; N, 17.45.

2,6-Dichloro-4-methoxypyrimidine—One-hundred sixty mg. (0.0011 mole) of 2,6-dihydroxy-4-methoxypyrimidine (isolated from the incomplete 6 *N* hydrochloric acid hydrolysis of 2-methylthio-4-methoxy-6-sulfanilamidopyrimidine) and 1.5 ml. of phosphorus oxychloride were diluted with 2 ml. of dimethylaniline and the mixture refluxed for 1.5 hr. The cooled mixture was poured onto 25 Gm. of cracked ice and extracted with seven 10-ml. portions of ether. The ether extracts were washed with saturated sodium bicarbonate solution, dried over anhydrous sodium sulfate, and distilled under reduced pressure to give 110 mg. (0.00062 mole) (56%) of the 2,6-dichloro derivative, b.p.$_5$ 132–135°. The oil solidified on standing and was crystallized from hot methanol, m.p. 51–53° [reported (26) to melt at 51°].

[5] K & K Laboratories, Plainview, N. Y.

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Crystal and Molecular Structure of 6-Thiopurine Riboside

By ELI SHEFTER

The molecular structure of 6-thiopurine riboside (commonly known as 6-mercaptopurine riboside) has been determined by X-ray crystallographic analysis. The material crystallizes from water as orthorhombic needles (space group $P2_12_12_1$) with unit cell dimensions of $a = 8.622$ Å., $b = 13.624$ Å., and $c = 20.262$ Å. There are two unique molecules in the asymmetric unit of the cell. The structure was solved by the heavy atom technique and refined by block diagonal least squares. The final R index is 0.067. In general, the bond lengths and angles agree with those found in other nucleoside structures. The C6-S distances of the two molecules (average 1.669 ± 0.002 Å.) together with the presence of a hydrogen on each of the N1 atoms indicates that the molecules are in the thiolactam configuration rather than the mercapto form. The glycosidic torsion angles (ϕ_{CN}) are +135° and +144° for the two molecules. The *syn* conformation about each of the CN bonds is stabilized by O5′-H...N3 intramolecular hydrogen bonds. The furanose rings are puckered; C2′ being displaced *endo* in both molecules. Each of the unique sulfurs is involved in a C-H...S interaction, while one is also participating in an O-H...S hydrogen bond.

6-Thiopurine (commonly known as 6-mercaptopurine) has been well established as an effective antineoplastic agent. It has been postulated that the compound exerts its effect at an enzymatic level., *i.e.*, through the purine metabolic pathway (1). In order to act as an inhibitor, it must first be converted to its active form, the ribonucleotide. In the hope of providing information on the electronic and steric configuration of this active species, the crystal and molecular structure of the riboside has been investigated.

Received January 18, 1968, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication March 5, 1968.
Presented to Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, April 1968.
This research was supported in part by grant No. CA 10104 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.
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The compound was obtained from Sigma Chemical Co. (St. Louis, Missouri) and crystallized from water. Orthorhombic needles so derived have the following data:

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$b = 13.624 \pm 0.002$ Å. $D_{calcd.} = 1.586$ for $Z = 8$

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TABLE I—FINAL POSITIONAL AND THERMAL PARAMETERS FOR NONHYDROGEN ATOMS AND THEIR ESTIMATED *SD*'S IN PARENTHESES, × 10^4[a]

Name	x/a	y/b	z/c	b_{11}	b_{22}	b_{33}	b_{12}	b_{13}	b_{23}
1S	9356(2)	−6936(1)	2913(1)	133(2)	47(1)	22(1)	−25(3)	−20(2)	−14(1)
1N1	7708(7)	−7216(4)	1808(3)	127(8)	35(3)	19(1)	4(8)	11(6)	−5(3)
1C2	6872(9)	−6943(4)	1267(3)	153(10)	30(3)	14(1)	26(10)	−16(6)	8(3)
1N3	6710(7)	−6059(4)	1056(2)	131(8)	34(3)	13(1)	8(8)	−4(5)	−3(3)
1C4	7490(7)	−5406(4)	1430(3)	90(7)	27(3)	14(1)	9(8)	10(5)	5(3)
1C5	8348(7)	−5586(4)	1993(3)	91(8)	35(3)	13(1)	1(9)	18(5)	2(3)
1C6	8472(7)	−6562(4)	2224(3)	88(7)	30(3)	17(1)	−11(8)	15(6)	−1(3)
1N7	8948(7)	−4745(4)	2259(3)	107(7)	40(3)	18(1)	−7(8)	−18(5)	12(3)
1C8	8455(8)	−4052(4)	1847(3)	117(9)	26(3)	20(2)	−3(9)	−17(6)	14(3)
1N9	7576(6)	−4397(4)	1337(2)	107(7)	26(2)	14(1)	−2(7)	5(5)	6(3)
1C1′	7096(8)	−3822(4)	779(3)	102(8)	24(3)	16(1)	−6(8)	−6(6)	3(3)
1O1′	5517(5)	−3992(3)	643(2)	98(6)	43(2)	16(1)	−14(7)	1(4)	1(3)
1C2′	7961(7)	−4063(4)	141(3)	94(7)	25(3)	14(1)	23(8)	0(6)	−7(3)
1O2′	9487(5)	−3706(3)	111(2)	93(6)	35(2)	25(1)	12(6)	0(5)	−17(3)
1C3′	6857(8)	−3609(5)	−354(3)	110(9)	31(3)	19(2)	−12(9)	−1(6)	−13(4)
1O3′	7065(6)	−2574(4)	−364(3)	143(8)	37(3)	34(2)	−17(8)	1(6)	−28(3)
1C4′	5254(8)	−3899(5)	−64(3)	97(8)	42(3)	17(1)	−17(9)	15(6)	7(4)
1C5′	4593(9)	−4831(5)	−329(3)	130(10)	44(4)	19(2)	−25(11)	−7(7)	7(4)
1O5	5604(7)	−5664(3)	−259(2)	175(8)	35(2)	20(1)	13(8)	−14(6)	9(3)
2S	−403(2)	−6728(1)	−19(1)	127(2)	46(1)	15(1)	−6(2)	−9(2)	−1(1)
2N1	1198(7)	−7638(4)	920(3)	142(9)	28(2)	18(1)	−6(8)	−2(6)	10(3)
2C2	2132(9)	−7753(5)	1448(3)	170(12)	30(3)	17(1)	−7(10)	1(8)	−2(4)
2N3	2771(8)	−7019(4)	1773(3)	160(9)	31(3)	16(1)	3(9)	−13(6)	−14(3)
2C4	2442(7)	−6131(4)	1502(3)	94(8)	34(3)	13(1)	−25(9)	2(5)	0(3)
2C5	1520(8)	−5959(4)	957(3)	110(8)	30(3)	12(1)	−12(9)	2(6)	3(3)
2C6	820(7)	−6774(5)	617(3)	104(8)	36(3)	12(1)	−1(9)	22(5)	5(3)
2N7	1427(6)	−4963(4)	821(2)	106(7)	37(3)	13(1)	−14(8)	0(5)	−5(3)
2C8	2283(9)	−4558(5)	1286(3)	132(10)	33(3)	17(1)	5(10)	10(6)	−1(3)
2N9	2919(6)	−5225(4)	1719(2)	101(7)	35(3)	13(1)	−10(7)	0(5)	−2(3)
2C1′	3681(7)	−4957(4)	2340(3)	84(8)	29(3)	20(2)	17(8)	−9(6)	5(3)
2O1′	4938(6)	−5601(4)	2442(2)	91(6)	54(3)	21(1)	−11(7)	5(4)	−11(3)
2C2′	2648(8)	−5063(5)	2937(3)	102(8)	35(3)	17(1)	15(9)	9(6)	8(4)
2O2′	1600(6)	−4285(3)	3019(2)	127(7)	38(2)	25(1)	−36(7)	−11(5)	24(3)
2C3′	3861(8)	−5147(5)	3476(3)	113(9)	50(4)	16(2)	−2(11)	−24(6)	4(4)
2O3′	4413(7)	−4179(4)	3625(3)	196(10)	50(3)	24(1)	−1(10)	−50(6)	18(3)
2C4′	5127(8)	−5739(5)	3144(3)	84(8)	39(3)	23(2)	13(9)	−10(6)	10(4)
2C5′	5103(9)	−6846(6)	3292(4)	152(11)	44(4)	23(2)	−2(12)	−19(8)	−9(5)
2O5′	3682(7)	−7280(4)	3125(2)	169(9)	49(3)	22(1)	38(9)	−12(6)	−20(3)

[a] Temperature factors in the form; exp = $(b_{11}h^2 + b_{22}k^2 + b_{33}l^2 + b_{12}hk + b_{13}hl + b_{23}kl)$.

The cell dimensions and the subsequent intensity measurements were carried out at 22 ± 4°, with Ni filtered Cu Kα radiation.

The intensities were measured by the stationary crystal-stationary counter technique using balanced Ni-Co filters. A General Electric XRD-6 diffractometer was utilized for this purpose. In the range of measurement (0 to 150° in 2 Θ), 2,729 unique reflections out of a possible number of 2,799 had intensities significantly greater than their background counts. These data were corrected for Lorentz and polarization factors and $\alpha_1 - \alpha_2$ splitting. An adjustment for the anisotropy of transmission of X-rays as a function of the angle ϕ was made as a means of correcting for absorption. The crystal used to collect the data was approximately 0.5 mm. along a (which in turn was parallel to the ϕ axis) and approximately 0.1 mm. in diameter. The structure factors were put on an absolute scale by a Wilson plot (2).

A three-dimensional Patterson synthesis calculated with only the $|F_0^2|$ terms having a value of $\sin \Theta/\lambda$ greater than 0.5 enabled the positions of the two unique sulfurs to be found. The coordinates of the 36 other nonhydrogen atoms were located in subsequent electron density Fourier syntheses. The positional and isotropic thermal parameters of these atoms were refined by least squares using a block diagonal approximation to the normal equations. A modified version of ACA program No. 317 of Gantzel, Sparks, and Trueblood (unpublished) was used for this purpose. Anisotropic temperature factors were introduced after the R value ($\Sigma||F_0| - |F_0||/\Sigma|F_0|$) was less than 0.2. After two cycles of least squares, a difference electron density map was calculated and 18 of the 24 hydrogens were easily located. The least squares was continued, including these atoms with isotropic temperature factors, until the shifts were less than $^1/_3$ of their estimated standard deviations (*SD*'s). A final difference map was then calculated and the positions of the four other hydrogens were obtained. The weighting scheme utilized in the final cycles of refinement was $W^{-1} = [(|F_0| - 15)/10]^2 + 1$, such that $\langle W\Delta^2 \rangle$ was constant throughout the range of $|F_0|$ (16). The unobserved data were given zero weight. The final R value for the observed data is 0.067.[1]

Throughout the above calculations, the atomic form factors utilized were those of Cromer and Waber (3) with the exception of hydrogen for which those in the International Tables of Crystallography (4) were used.

A listing of the fractional coordinates and temperature factors for the various atoms may be found in Tables I and II. Since the absolute configuration

[1] A list of the observed and calculated structure factors has been deposited in the Health Sciences Library, State University of New York at Buffalo, under the title of this publication.

TABLE II—POSITIONAL ($\times 10^4$) AND THERMAL PARAMETERS OF HYDROGENS AND THEIR RESPECTIVE ESTIMATED *SD*'S IN PARENTHESES

Name	Atom to Which it is Attached	x/a	y/b	z/c	B_{iso} (A^2)[b]
H1	1N1	780(7)	−786(7)	201(4)	2.3(2.9)
H2	1C2	616(8)	−745(5)	99(3)	0.1(1.3)
H3	1C8	881(7)	−330(4)	192(3)	−0.7(1.0)
H4	1C1′	740(6)	−309(4)	92(2)	−1.5(0.9)
H5	1C2′	795(9)	−478(6)	15(4)	0.9(1.6)
H6	1O2′	1054	−404	18	[a]
H7	1C3′	702(7)	−379(4)	−80(3)	−0.5(1.1)
H8	1O3′	619	−210	−21	[a]
H9	1C4′	459(9)	−328(6)	−32(4)	1.2(1.5)
H10	1C5′	351(11)	−499(6)	−6(4)	2.7(1.8)
H11	1C5′	436(8)	−486(5)	−84(3)	0.3(1.3)
H12	1O5′	578(11)	−564(7)	15(4)	1.9(1.8)
H13	2N1	88(11)	−816(9)	68(4)	2.4(2.0)
H14	2C2	222(8)	−841(5)	160(3)	−0.1(1.2)
H15	2C8	236(9)	−388(5)	124(3)	0.7(1.4)
H16	2C1′	397(8)	−412(5)	221(3)	−0.6(1.1)
H17	2C2′	202(8)	−565(5)	285(3)	0.4(1.3)
H18	2O2′	66	−424	273	[a]
H19	2C3′	353(8)	−543(5)	391(3)	−0.2(1.2)
H20	2O3′	466(11)	−422(7)	412(4)	3.1(1.9)
H21	2C4′	613(10)	−540(6)	325(4)	1.6(1.7)
H22	2C5′	588	−730	293	[a]
H23	2C5′	541(12)	−696(7)	376(5)	4.1(2.2)
H24	2O5′	338(12)	−717(7)	259(5)	2.8(2.3)

[a] The use of the scattering curve for the free hydrogen atom usually results in anomalously low values for *B* [Jensen, L. H., and Sundaralingham, M., *Science*, **145**, 1185(1964)]. [b] These were found in the final difference electron density synthesis.

was not determined, the positional parameters were set so that they adhere to the usual convention for D-ribosides. The prefix number on each atom denotes to which of the two unique molecules the atom belongs. The estimated *SD*'s in these tables were calculated from the inverses of the full normal equation blocks. The estimated *SD*'s in the positional parameters of the nonhydrogen atoms, except for the sulfurs, are approximately 0.006 Å. and for the sulfurs 0.002 Å. These *SD*'s correspond to an uncertainty of about 0.009 Å. and 0.4° in bond lengths and angles involving the C, N, and O atoms and in the case of the C—S bonds, 0.006 Å. The errors in the hydrogen positions are at least ten times as great as those for the atoms to which they are attached, *i.e.*, average bond length error of 0.09 Å.

The intramolecular bond distances and angles for the compound are shown in Figs. 1 and 2. The angles involving the hydrogens are not presented in these figures, and only those relevant to the discussion will be presented because of their estimated *SD*'s. They are, however, in good agreement with their expected values. In general, the bond distances and angles in the two unique purine moieties are considered to be similar, *i.e.*, using the levels of significance suggested by Cruickshank and Robertson (6), the comparative differences are not highly significant.

DISCUSSION

The bond distances of both purine residues together with the placement of a hydrogen on each of

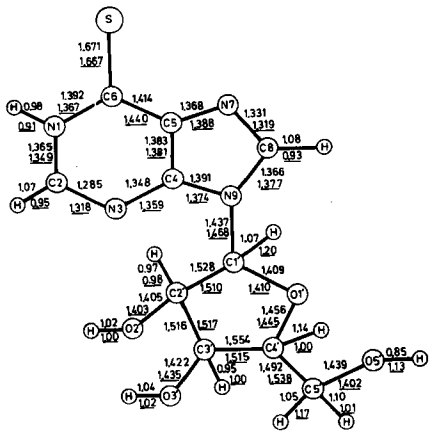

Fig. 1—Schematic drawing of molecule showing bond lengths. The distances for molecule 2 are underlined.

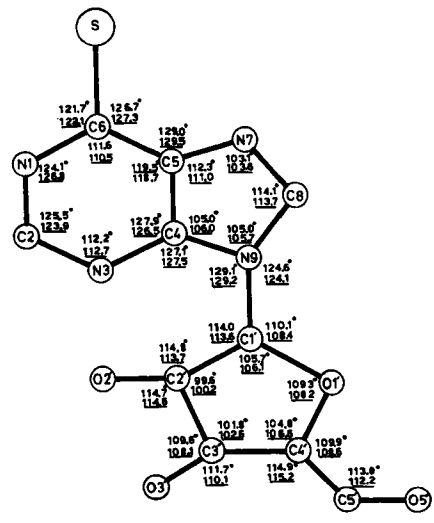

Fig. 2—Bond angles. Those obtained for molecule 2 are underlined.

TABLE III—LEAST-SQUARES PLANES[a]

Atoms Comprising L.S. Plane	Displacement (Å.) Molecule 1	Displacement (Å.) Molecule 2	Other Atoms	Displacement (Å.) Molecule 1	Displacement (Å.) Molecule 2
N1	−0.021	0.014	S	0.109	0.091
C2	0.002	−0.013	C1′	−0.211	0.250
N3	0.019	0.003	H(N1)	0.04	−0.10
C4	−0.006	−0.007	H(C2)	0.11	0.04
C5	−0.003	−0.002	H(C8)	−0.05	−0.04
C6	0.014	−0.002		—	—
N7	0.007	−0.003		—	—
C8	0.000	−0.001		—	—
N9	−0.011	0.010		—	—
C1′	—	—	C2′	0.612	0.632
O1′	—	—	C3′	−0.055	0.032
C4′	—	—	C5′	1.191	1.179

[a] Calculated by the method of Schomaker, V., Waser, J., Marsh, R. E., and Bergman, G., *Acta Cryst.*, **12**, 600(1959).

the N(1) atoms, indicate that at least in the solid state the molecule exists in the thiolactam form rather than the mercapto form. This configuration has also been observed in the crystal structures of 6-mercaptopurine (5) and 2,4-dithiouracil (17). The C—S bond length in each of the two unique nucleoside molecules agrees well with the corresponding distance in the former structure; 1.673 ± 0.003 Å. Based on the compilation of structural data of sulfur compounds by Abrahams (13), the 1.67-Å. length corresponds to approximately 20% single bond character. It thus appears that though the thione configuration, (—NH—C(=S)—), is the predominant valence state, the zwitterionic amidic form, (—+NH = C(—S⁻)—), makes a substantial contribution to the overall resonance.

With the exception of the imidazole portion of the purine residue, the bond lengths and angles are in quite good agreement with those found for 6-mercaptopurine. The disparity observed between the imidazole rings of the two structures is expected as the free base is protonated at N7 rather than at N9.

The least-squares planes calculated through the purine residues (see Table III) show that the atoms comprising the two rings are essentially coplanar. Both C1′ and S are significantly displaced from these least-squares planes. In molecule 1, they are displaced on opposite sides while in molecule 2, they are on the same side of the plane. It is not uncommon in nucleoside structures to find exocyclic atoms significantly out of the base plane. The equations of the least-squares planes are as follows:

$$\text{purine 1}(-0.8208X + 0.1171Y + 0.5591Z = 4.5373 \text{ Å.})$$

$$\text{purine 2}(-0.8044X + 0.0714Y + 0.5898Z = 0.4884 \text{ Å.})$$

X, Y, and Z are the coordinates measured in Å. along a, b, and c, respectively.

The torsion angles (ϕ_{CN}) about the glycosidic bonds (7, 8) in molecules 1 and 2 are +135° and +144°, respectively. The *syn* conformations in the two molecules are stabilized by 05′-H...N3 intramolecular hydrogen bonds. Though the majority of X-ray structural analyses on purine nucleosides show a preference for the *anti* conformation in the solid state, there have been three other nucleosidic compounds reported in the *syn* configuration; formycin, an antibiotic (18), adenosine 3′-5′-cyclic phosphate (19), and deoxyguanosine in the hydrogen bonded complex with 5-bromodeoxycytidine (14).

TABLE IV—CONFORMATIONAL ANGLES

Angle	Molecule 1	Molecule 2	Deoxyquanosine
$\phi C_1' \rightarrow C_2'$	39.3	41.2	30.61
$\phi C_2' \rightarrow C_3'$	−40.6	−36.9	−30.9
$\phi C_3' \rightarrow C_4'$	27.8	23.3	20.2
$\phi C_4' \rightarrow O_1'$	−2.1	1.3	−2.1
$\phi O_1' \rightarrow C_1'$	−24.6	−25.8	−17.6
ϕ_{0c}	55.4	56.7	42.8
ϕ_{00}	62.5	62.7	70.7

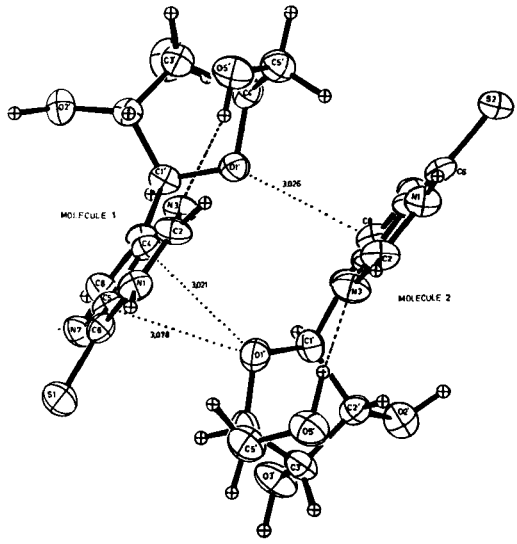

Fig. 3—View of the two unique molecules as seen down the b *axis. The thermal ellipsoids enclose a probability density of 0.50 for the nonhydrogen atoms (15).*

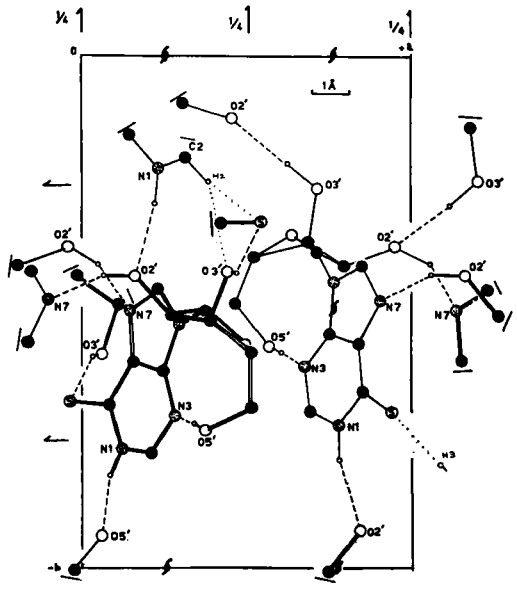

Fig. 4—Hydrogen bonding scheme about the two unique molecules as viewed down the c *axis. Dashed lines represent proposed hydrogen bonding scheme, while dotted lines are short contacts of the C-H variety. Key:* ●, *carbon;* ⊛, *nitrogen;* ○, *oxygen;* —, *molecule 1;* =, *molecule 2.*

The former two have chemical features not typical of the natural nucleosides and -tides and, therefore, a comparison with these will not be offered. The latter compound exhibits a similar intramolecular hydrogen bond. It has been shown (7, 9) using molecular models that purine nucleosides have very little restriction to rotation about the glycosidic bond, in contrast to pyrimidine nucleosides.

Although the glycosidic bond lengths fall within the usual range for nucleosidic compounds, they do appear to be significantly different (at 95% confidence level) from each other. This may be the result of the variation in the rotation angles about the glycosidic bonds and the adjacent furanose bonds. It has been shown that rotation about single bonds involving hetero-atoms having unshared pairs of electrons can influence neighboring bond lengths and angles (10).

The puckering of the furanose rings is such that C2′ is displaced on the same side of the five-member rings as C5′. *Endo* puckering of C2′ was also observed in the *syn* structure of deoxyguanosine (14). Since the usual sugar distortion in nucleotides and -sides involves C2′ and/or C3′, it can be described as a twist about the C2′—C3′ bond relative to the plane of the other three ring atoms (8). In these terms, molecule 1 shows a C2′ (*endo*)—C3′ (*exo*) conformation, while the other has a C2′ (*endo*)—C3′ (*endo*) conformation (*cf.* Table III). The overall configuration of the sugar residues can best be described in terms of torsional angles about each of the ring bonds. These twist angles, as defined by Brown and Levy (11) and Sundaralingam (10) are given in Table IV, for the two unique riboses with those for the furanose moiety in deoxyguanosine. The agreement of these angles among the three sugars is excellent. These angles probably reflect the most stable sugar configuration for the *syn* conformer.

The orientation of the exocyclic C5′-05′ bond with reference to furanose ring is usually described by its projected angle down C4′-C5′ with respect to 01′ and C3′ (12). These angles are referred to as ϕ_{00} and ϕ_{0c}, respectively. The intramolecular 05′-H... N3 interactions constrain the values of these angles to be in the vicinity of 60° (*i.e.*, gauche).

Distortions from tetrahedral symmetry of the ribose carbons are commonly found in the crystal structures of nucleosides and -tides. These deformations are possibly the result of intermolecular associations and the nonbonding interactions within the ribose structure. These reasons may also be re-

TABLE V—SHORT INTERMOLECULAR CONTACTS

Hydrogen Bonds		Pertinent Distances and Angles	
Acceptor	Donor[a]		
1O2′	1O3′-H8 (1)	1O3′...1O2′	2.871 Å.
		H8...1O2′	1.85 Å.
		1O3′-H8...1O2′	168°
1O5′	2N1-H13 (2)	2N1...1O5′	2.722 Å.
		H13...1O5′	1.83 Å.
		2N1-H13...1O5′	168°
1N3	1O5′-H12	1O5′...1N3	2.882 Å.
		H12...1N3	2.08 Å.
		1O5′-H12...1N3	158°
1N7	2O2′-H18 (3)	2O2 ...1N7	2.828 Å.
		H18...1N7	1.89 Å.
		2O2′-H18...1N7	155°
2S	2O3′-H20 (4)	2O3′...2S	3.133 Å.
		H20...2S	2.27 Å.
		2O3′-H20...2S	142°
		2C3′-2O3′-H	103°
2O2′	1N1-H1 (5)	1N1...2O2′	2.902 Å.
		H1...2O2′	2.00 Å.
		1N1-H....2O2′	151°
2N3	2O5′-H24	2O5′...2N3	2.871 Å.
		H24...2N3	1.74 Å.
		2O5′-H24...2N3	175°
2N7	1O2′-H6 (6)	1O2′...2N7	2.793 Å.
		H6...2N7	2.02 Å.
		1O2′-H6...2N7	131°
Other Short Contacts			
		1S...H3 (10)	2.47 Å.
		1O1′...2C8	3.026
		1O3′...H6 (7)	2.60
		2S...H(2) (8)	2.64
		2O5′...1C8 (9)	3.030
		2O5′...H3 (9)	2.56
		2O5′...H4 (9)	2.41
		2O1′...1C4	3.021
		2O1′...1C5	3.078
		2O3′...H(2) (5)	2.53
		2S...H(2)-1C2 (8)	162°
		1S...H3-1C8 (10)	155°

[a] The value in parentheses following the donor atom name denotes its equivalent position in fractional coordinates, as follows: (1) $^1/_2 + x, ^1/_2 - y, -z$, (2) $^1/_2 + x, ^3/_2 - y, -z$, (3) $1 + x, y, z$, (4) $^1/_2 - x, 1 - y, z - ^1/_2$, (5) $1 - x, y - ^1/_2, ^1/_2 - z$, (6) $x - 1, y, z$, (7) $x - ^1/_2, ^1/_2 - y, -z$, (8) $x - ^1/_2, ^3/_2 - y, -z$, (9) $1 - x, ^1/_2 + y, ^1/_2 - z$, (10) $2 - y - ^1/_2, ^1/_2 - z$.

sponsible for the differences in the bond lengths and angles between the two unique sugars. The two ribose moieties have bond lengths and angles which closely resemble those found in other nucleosidic structures.

The two unique molecules in the asymmetric unit (as shown in Fig. 3) appear to be approximately related by a noncrystallographic twofold axis nearly parallel to the *b* axis. The only short contacts between the two molecules shown in Fig. 3 are those involving 1O1′...2C8, 2O1′...1C4 and 2O1′...1C5, which have distances of 3.026 Å., 3.021 Å., and 3.078 Å., respectively. These are slightly less than the sum of the van der Waals radii of the respective atoms (3.1 Å.).

The arrangement of the hydrogen bonds around the two unique nucleosides are shown in Fig. 4. The pertinent distances and angles associated with the various hydrogen bridges are given in Table V. An interesting facet of the proposed intermolecular bonding scheme is the presence of a 2O3′-H...2S hydrogen interaction. The 2O3′ proton is considerably displaced from the plane of the purine nucleus and the C6-S...H angle also deviates considerably from linearity (116°). These distortions may possibly result from the valence state of the sulfur. The O3′-H...S distance of 3.133 Å. is much shorter than the O-H...S length reported in the structure of 6-mercaptopurine hydrate (3.374 Å.). There is also a very short contact between the 1C2 hydrogen (H2) and 2S, which is suggestive of a C-H...S interaction (20). The other sulfur (1S) appears to be only participating in a C-H type interaction and by virtue of its length (H3...1S 2.47 Å.) is a relatively strong hydrogen bond.

There are two N1-H...O hydrogen bonds. The 2N1-H...1O5′ length is 2.722 Å., while the 1N1-H...2O2′ interaction is 2.902 Å. The difference in the strengths of these two hydrogen bonds appears to be attributable to differences about N1 in the two unique residues. The N1-C6 bond length of molecule 2 is 0.025 Å. shorter than the same bond in molecule 1, and the C6-N1-C2 angle 2.7° greater than in molecule 1. Though these differences are only significant at the 95% confidence level, they are consistent with the marked inequality of the hydrogen bond lengths, *i.e.*, the proton on 2N1 is more acidic in character than the one on 1N1.

Each of the O2′ atoms is participating in the hydrogen bonding scheme as both a donor and acceptor, while the O3′ atoms only appear to be acting as donors. The other short intermolecular contacts are listed in Table V. Although some of these H contacts are slightly shorter than normal van der Waals contacts, only two are felt to be of a significant nature (mentioned above).

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Keyphrases

6-Thiopurine riboside
Crystal, molecular structure—6-thiopurine-riboside
Heavy atom technique—structure
X-Ray crystallography—analysis

Structural Studies on Complexes II

Crystal and Molecular Structure of a 1:1 Caffeine and 5-Chlorosalicyclic Acid Complex

By ELI SHEFTER

Crystals of a 1:1 complex of caffeine and 5-chlorosalicylic acid are orthorhombic with space group P *bcn*. The unit cell parameters are a = 7.952 Å., b = 16.303 Å., and c = 25.491 Å. The structure was solved by a symbolic addition procedure. The positional and thermal parameters were refined by least squares to an R value of 0.077. The caffeine molecule shows significant deviations from planarity. The molecules are held together in the lattice by O—H...N and C—H...O intermolecular hydrogen bonds. Polarization bonding also seems to play a dominant role in the stacking of the molecules in the lattice.

THE ABILITY of xanthine and purine derivatives to form association complexes with a wide variety of biologically important molecules is well documented in the literature. It has been shown that caffeine, through its complexing behavior, can influence the rates of hydrolysis of various molecules (1, 2) and also inhibit salivary amylase (3). To date there appears to be very little information reported as to the exact mode of the interaction in these "complexes." Recent structural studies on complexes of tetramethyluric acid with a variety of aromatic hydrocarbons (4–6) have indicated that polarization bonding is of primary importance. The structure analysis of the 1:1 complex of caffeine and 5-chlorosalicylic acid was undertaken as part of a continuing study to gain further insight into the nature of the forces that are responsible for intermolecular complexes between xanthines and purines with substituted aromatic compounds of biological and pharmaceutical importance.

EXPERIMENTAL

Orthorhombic prisms of the complex were obtained from an aqueous solution saturated with the two respective molecules. The following crystallographic data were measured for these crystals:

a = 7.952 ± 0.003 Å. $\alpha = \beta = \gamma = 90.0°$
b = 16.303 ± 0.003 Space group $P\ bcn$ (uniquely defined)
c = 25.491 ± 0.004 ρ measured = 1.47 ± 0.01 Gm./cm.^{3}
ρ calcd. (assuming 8 molecules of a 1:1 complex) = 1.462 Gm./cm.^{3}

The density was determined in a mixture of chloroform and methylene chloride. The unit-cell parameters were established by means of least-squares refinement of 30 general *hkl* reflections measured on a GE XRD-6 diffractometer. The temperature during these measurements and the subsequent intensity collection was approximately 22 ± 4°.

The intensity data were measured by the stationary crystal-stationary counter technique with balanced nickel and cobalt filters. The size of the crystal utilized was approximately 0.9 mm. × 0.3 mm. × 0.2 mm. These data were converted to structure factor amplitudes, $|F|$s, by applying α_1-α_2 splitting corrections, Lorentz-polarization factors, and an absorption factor based on the anisotropy of transmission of the X-rays as a function of the angle ϕ. The $|F|$'s were put on an absolute scale by use of Wilson's statistics (7), and then converted to their respective normalized structure factors, $|E|$'s. In the sphere of the data collection (0°–125° in 2 θ) 2519 reflections out of a possible number of 2,646 had values greater than twice the standard deviation of their measurement.

A trial structure was obtained using the symbolic addition procedure. The phases of 180 normalized structure factors, all having values of $|E|$ greater than 1.75, were determined using the Sayre equation (8), which is the same as the Σ_2 formula of Hauptman and Karle (9). A program written by Long was utilized for this purpose (10). Three linearly independent reflections (2.1.4, 2.2.1, 1.1.14) each having a large number of interactions were assigned positive signs in order to specify the origin. Aside from the origin-determining planes four other structure factors having large $|E|$ values (4.1.8, 1.2.10, 3.1.6, and 3.1.10) were allowed to take up each of the 16 possible phase combinations. The program in an interactive manner generated the phases for the other 173 reflections for the 16 sets of phases. A consistency index, C,

$$C = \frac{\langle |E_A \Sigma_{A=B+C} E_B E_C| \rangle}{\langle |E_A| \Sigma_{A=B+C} |E_B|\,|E_C| \rangle}$$

Received January 12, 1968, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14121

Accepted for publication February 19, 1968.

Presented to Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

This investigation was supported by grants CA-10104 and A-3909 from the Public Health Service, and also in part by the National Science Foundation (G202-07 and GB-2029).

The author is indebted to the SUNY/B Computing Center for a generous gift of computing time, to Dr. K. N. Trueblood of the Chemistry Department at U.C.L.A. for his interest and programs, and to Mrs. Phyllis Sackman for her technical assistance.

A list of the calculated and observed structure factors has been deposited in the Health Sciences Laboratory at the State University of New York at Buffalo.

where the sums are overall pairs of reflections B and C for which $B + C = A$. $\langle\rangle$ means the average overall values of A. A high value for this index usually indicates a consistent set of phases. A E-Fourier map was calculated from the solution with the highest C value (0.70). The whole molecule was not recognizable from this map, but the xanthine moiety clearly stood out. The positions of these atoms were then utilized to calculate an electron-density Fourier synthesis from which the positions of all the nonhydrogen atoms were located. The reason for the ambiguous nature of the E-map was due to the fact that a number of the phases were incorrectly assigned by the program.

The positional and thermal parameters were refined by least squares using a block-diagonal approximation to the normal equations. A modified version of the ACA program 317 (Gantzel, Sparks, and Trueblood, unpublished) was utilized. After anisotropic temperature factors were introduced and the usual reliability index (R value) was 0.10, the positions of the hydrogen atoms were found from a three-dimensional Fourier difference synthesis. These atoms were included in the final cycles of least squares with fixed isotropic B's of 5.0 Å.^{2}. The refinement was concluded when the parameter shifts were less than a third of their calculated e.s.d.'s (estimated standard deviations). In the last stages of least squares the weighing scheme utilized was $1/w = [(|F_0| - 15)/30]^2 + 1$, such that $\langle w\Delta^2\rangle$ was constant over the whole range of $|F_0|$s. The unobserved data were given zero weight in the last cycles. The final R index for the observed reflections is 0.077 and the goodness of fit, $(\Sigma w|F_0 - F_0^2|/m - n)$, is 1.23.

The nonhydrogen atomic parameters and their e.s.d.'s are given in Table I, and those for the hydrogen atoms in Table II. The e.s.d.'s were calculated from the inverses of the full normal equation blocks.

Throughout the above calculations the atomic scattering factors used were those of Cromer and Waber (11), except for hydrogen which was taken from the *International Tables for X-ray Crystallography* (12).

TABLE II—REFINED POSITIONAL PARAMETERS AND THEIR E.S.D.'S OF THE HYDROGENS × 10^3[a]

Name	x/a	y/b	z/c
H1	533(6)	240(3)	186(2)
H2	521(6)	288(3)	157(2)
H3	681(6)	235(3)	165(2)
H4	278(6)	210(3)	−30(2)
H5	156(6)	173(3)	−17(2)
H6	216(6)	253(3)	− 1(2)
H7	448(6)	−137(3)	112(2)
H8	633(6)	− 87(3)	112(2)
H9	495(6)	− 73(3)	154(2)
H10	302(6)	− 94(3)	33(2)
H11	163(6)	225(3)	218(2)
H12	272(6)	76(3)	257(2)
H13	59(6)	− 65(3)	149(2)
H14	− 38(6)	191(3)	103(2)
H15	−160(6)	− 25(3)	36(2)

[a] E.s.d.'s of the hydrogens × 10^3 in parentheses. Isotropic temperature factors (B's) were fixed at 5.0 Å.^{2}.

The results of a rigid body analysis of the temperature factors for the two residues of the complex are shown in Table III. The method of Schomaker and Trueblood (13, 14) was utilized to calculate the librational and translational tensors. The root mean-square difference between the observed and calculated Uij's were 0.0033 and 0.0041 for the caffeine and 5-chlorosalicylic acid molecules, respectively. The caffeine has a substantially smaller thermal motion than the salicylic acid moiety, which is consistent with the intermolecular bonding in the lattice (see below). Neither the librational nor the translational motions of the two molecules are significantly anisotropic.

DISCUSSION OF RESULTS

The intramolecular bond distances and angles of the two residues composing the complex are shown

TABLE I—LEAST-SQUARES PARAMETERS AND THEIR ESTIMATED STANDARD DEVIATIONS × 10^4[a]

Name	x/a	y/b	z/c	B_{11}	B_{22}	B_{33}	B_{12}	B_{13}	B_{23}
CN1	4939(4)	1911(2)	1177(1)	159(5)	34(1)	15(1)	− 3(5)	−11(3)	− 7(1)
CC2	3955(4)	2261(2)	786(1)	148(6)	35(1)	15(1)	−18(5)	15(3)	1(2)
CN3	3278(4)	1723(2)	422(1)	153(5)	31(1)	15(1)	5(4)	−18(3)	5(1)
CC4	3480(4)	896(2)	486(1)	136(6)	30(1)	12(1)	− 8(5)	− 1(3)	− 1(1)
CC5	4403(4)	579(2)	883(1)	154(6)	28(1)	12(1)	7(5)	− 1(3)	1(1)
CC6	5258(4)	1073(2)	1264(1)	132(6)	38(1)	12(1)	− 3(5)	− 5(3)	2(1)
CN7	4282(4)	−264(2)	835(1)	164(5)	28(1)	16(1)	2(4)	− 2(3)	6(1)
CC8	3305(5)	−393(2)	417(2)	172(7)	29(1)	19(1)	−17(5)	1(4)	− 4(2)
CN9	2782(4)	298(2)	186(1)	159(6)	34(1)	15(1)	−25(4)	−12(3)	0(1)
CO10	6124(4)	840(2)	1621(1)	218(6)	53(1)	16(1)	16(5)	−45(3)	4(1)
CC11	5728(6)	2493(3)	1546(2)	273(10)	53(2)	25(1)	− 4(8)	−46(5)	−27(2)
CO12	3708(3)	2991(1)	761(1)	224(6)	25(1)	26(1)	1(4)	− 4(3)	3(1)
CC13	2164(6)	2055(3)	12(2)	262(10)	45(2)	21(1)	7(7)	−40(5)	19(2)
CC14	5005(6)	−891(2)	1179(2)	231(8)	39(2)	25(1)	12(7)	− 6(5)	22(2)
C1	2391(2)	−936(1)	2388(1)	314(3)	108(1)	21(0)	76(3)	−41(2)	23(1)
SC1	208(5)	565(2)	1345(1)	151(6)	38(1)	14(1)	−29(6)	7(3)	− 4(2)
SC2	602(5)	1343(3)	1544(2)	177(8)	47(2)	19(1)	−41(6)	31(4)	− 9(2)
SC3	1560(6)	1417(3)	1999(2)	224(9)	75(3)	19(1)	−71(9)	11(5)	−18(2)
SC4	2115(6)	731(4)	2250(2)	185(9)	109(3)	15(1)	−72(9)	−10(4)	−24(3)
SC5	1718(5)	− 57(3)	2054(2)	170(7)	76(3)	15(1)	25(8)	− 5(4)	4(2)
SC6	795(5)	−133(2)	1606(1)	158(7)	54(2)	14(1)	−12(6)	0(4)	− 2(4)
SC7	− 773(4)	501(2)	859(1)	129(6)	41(1)	16(1)	−10(5)	7(3)	4(2)
SO8	−1084(3)	−249(1)	704(1)	222(5)	37(1)	18(1)	−22(4)	−36(3)	− 3(1)
SO9	−1289(3)	1106(2)	619(1)	236(6)	39(1)	20(5)	8(4)	−18(3)	9(1)
SO10	94(5)	2038(2)	1312(1)	326(8)	38(1)	30(1)	−32(5)	15(4)	− 9(2)

[a] Thermal parameters B_{ij} are defined by the temperature factor = exp − $(h^2B_{11} + k^2B_{22} + l^2B_{33} + hkB_{12} + hlB_{13} + klB_{23})$.
[b] E.s.d.'s in parentheses.

TABLE III—RESULTS OF RIGID-BODY ANALYSIS OF THERMAL PARAMETERS[a]

	Eigenvalues Cl-S.A.	Eigenvalues Caff.	Eigenvectors (10^4)[b] Cl-S.A.			Eigenvectors (10^4)[b] Caff.		
Librational tensor, **L**	31.4 (°)²	24.9	3465	−0696	9355	−1112	9910	0747
	17.6	15.8	9371	−0185	−3484	−9887	−1027	−1096
	13.8	9.6	0413	9974	0590	−1008	−0861	9912
Translational tensor, **T**	Å.²							
	0.068	0.041	−3254	8965	−3005	−2974	8921	3402
	0.046	0.039	−4206	−4219	−8032	−8518	−0869	−5167
	0.036	0.033	−8469	−1349	5144	−4314	−4434	7857
			Cl-S.A.			Caff.		
Symmetrized screw tensor, **S**		206	−277	−183	139	64	− 4	× 10^5 rad. Å.
			−131	361		−111	8	
				− 75			−28	

[a] Calculated by the method of Schomaker and Trueblood (13, 14). [b] These are the direction cosines of the eigenvectors relative to *a*, *b*, and *c*, respectively. **L** is independent of the origin, **T** and **S** depend upon it, and here they are given relative to the unique origin which symmetrizes **S**.

in Figs. 1 and 2. These distances and angles were corrected for the effects of molecular libration by the method of Cruickshank (15). The distances and angles for the nonhydrogen atoms have average e.s.d.'s of 0.007 Å. and 0.3°. The distances and angles for the hydrogens have an average uncertainty of approximately 0.07 Å. and 2°. These e.s.d.'s include an assumed e.s.d. of 0.002 Å. for the uncertainty in intramolecular bond distances and angles corrected for librational motion.

In general the bond distances and angles of the caffeine moiety of the complex agree fairly well with

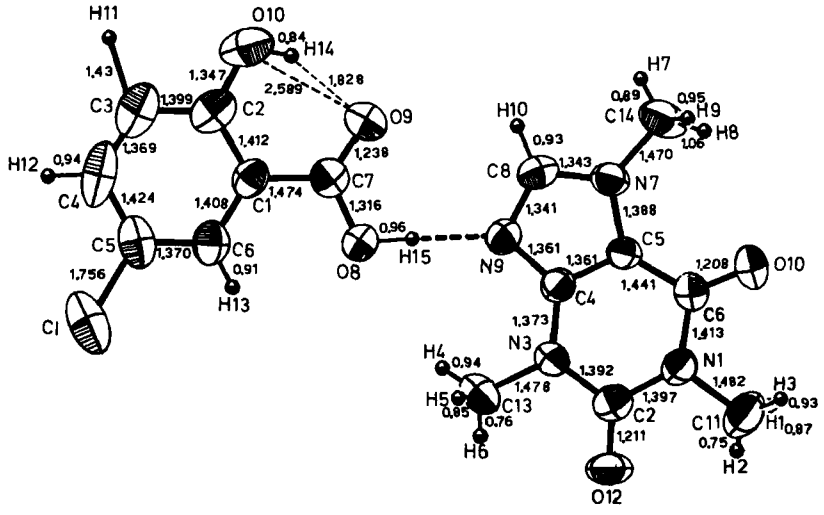

Fig. 1—Thermally corrected intramolecular distances. The thermal ellipsoids in this figure, and the others, enclose a probability density of 0.50 (30), for the nonhydrogen atoms.

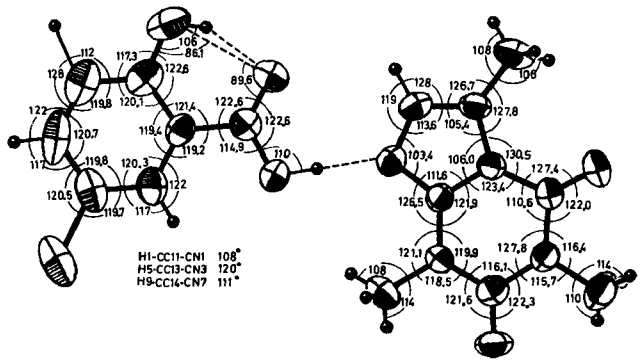

Fig. 2—Thermally corrected intramolecular angles.

those found in the structure of caffeine hydrate (16). There are however some differences worth noting; these are between the C6—O10, C6—N1, N3—C4, C4—N9,and C4—C5 bonds, whose values were found to be 1.26 Å., 1.36 Å., 1.42 Å., 1.31 Å., and 1.32 Å., respectively, in the caffeine structure. These discrepancies may reflect the higher uncertainty in the X-ray study of Sutor (av. e.s.d. ~ 0.02 Å. in bond lengths), or possibly the effect of intermolecular polarization bonding. The former of these reasons appears to be the more likely as the size of the differences in the intramolecular xanthine bonds are much smaller when these results are compared to the theophylline structure (17); *i.e.*, they all fall within 2.5 times the e.s.d.'s of that structure.

The atoms comprising the purine ring show a significant degree of puckering (see Table IV). These deviations most likely reflect the contribution of some sp^3 character to valence state of the nitrogens. This finding is not unusual as a variety of pyrimidine and purine structures have been observed to be significantly nonplanar (19).

There is a remarkably good similarity between the precise structure determination of salicylic acid (20) and the 5-chloro derivative in this complex. Only two bond lengths, the C5—C4 and C1—C7, differ by more than 2 e.s.d.'s The values for these bonds are 1.384 Å. and 1.457 Å., respectively, in the salicylic acid structure. The substitution of the electronegative chlorine atom in the five position of the ring, undoubtedly is a major factor for these observed differences. As already pointed out by Cochran (21) and Sundaralingam and Jensen (20), there appears to be a substantial contribution of the quinoid structure to the possible valence states of the molecule.

TABLE IV—LEAST-SQUARES PLANES[a]

Atoms Comprising L.S. plane	Deviation Å.	Other Atoms	Deviation Å.
SC1	−0.002	SC7	0.024
SC2	−0.001	SO8	0.017
SC3	0.001	SO9	0.031
SC4	0.003	SO10	0.002
SC5	−0.006	C1	−0.045
SC6	0.006	H11	−0.19
		H12	−0.04
		H13	0.03
		H14	0.07
		H15	0.15
CN1	0.013	CO10	−0.047
CC2	0.052	CC11	0.030
CN3	−0.030	CO12	0.144
CC4	−0.031	CC13	0.065
CC5	−0.023	CC14	0.091
CC6	−0.028	H10	0.10
CN7	0.019		
CC8	0.033		
CN9	−0.005		

[a] Calculated by the method of Schomaker *et al.* (18).

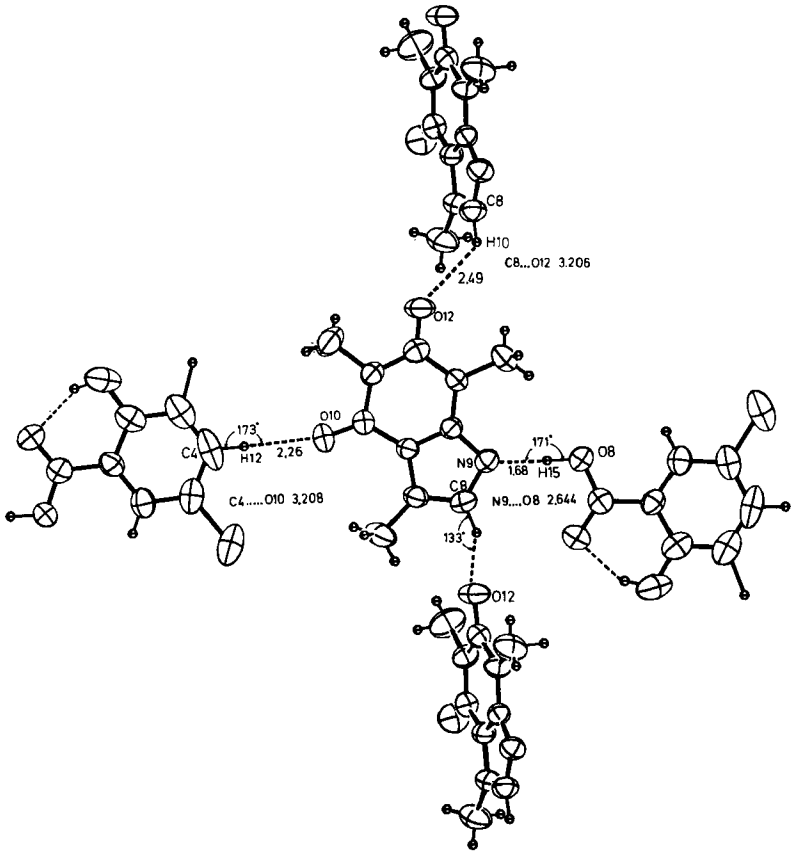

Fig. 3—A general view of the packing, showing the short intermolecular contacts.

The phenyl ring atoms are coplanar (see Table IV), within experimental error. The equation for this plane is $-0.8358\ X - 0.0142\ Y + 0.5488\ Z = 1.7274$ Å., where X, Y, and Z are in Å. measured along a, b, and c. The chlorine atom and the carboxyl group displacements from this plane are significant. In regard to the chlorine atom, it is most interesting to note that the displacement of the hydrogen in the five position of the salicylic acid structure is also significantly displaced from the ring. In the present instance the chlorine does not appear to have any short intermolecular contacts to it which might cause this distortion. The displacement of the carboxyl group from the least-squares plane is not unreasonable, as in both the structures of salicylic acid and 2-amino-3-methyl benzoic acid (22) a similar effect is observed.

The most dominant intermolecular bond observed in the complex is the N9...H—O8 interaction (see Fig. 3). The 2.644 Å. N...O length together with the 1.68 Å. H...N9 distance indicate this to be a relatively strong interaction. In general O—H...N bonds found in structures where the dominating proton comes from an acidic group, the hydrogen bond lengths are usually low. In the structures of nicotinic acid (23), hydroxy-L-proline (24), and 1,4-piperazine-α,α^1-dibutyric acid (25) the N...H—O lengths are 2.66, 2.67, and 2.60 Å., respectively. In most other types of O—H...N bridges the lengths are considerably longer as evidenced by the average value of 2.80 ± 0.09 Å. tabulated by Pimental and McClellan (26).

There are two other short intermolecular interactions that appear to be joining the molecules in chains throughout the crystal lattice (see Fig. 3). These are between SC4—H...O10 and CC8—H...O12. The hydrogen to oxygen distance in these two bridges are 2.26 and 2.49 Å., respectively, which are much less than the sum of the van der Waals radii of the atoms involved (2.6 Å.). There are numerous examples of this type of bonding in the recent literature. In the structures of both caffeine hydrate and theophylline hydrate the C8 hydrogen was also found (27) to be participating in a hydrogen bond with a carbonyl oxygen. The resonance in the purine system is probably responsible for the acidic nature of this proton. The acidic character of the SC4 hydrogen possibly results from the inductive effect of the chlorine atom. It is of interest to point out while on the subject of C—H hydrogen bonds, that spectroscopic evidence shows that C—H...O=C bonding plays a significant role in the stability of polypeptides (28).

The general features of the stacking of the molecules are shown in Fig. 4. Some of the distances between the stacked planes are listed in Table V. Though none of these lengths are shorter than the sum of the van der Waals radii of the atoms concerned, the general arrangement of the stacked molecules tends to indicate polarization bonding between layers. This idea has been discussed in a previous communication on this structure (29). The greatest degree of stacking overlap occurs between the salicylic acid moiety at $-x, -y, -z$ (drawn with the thickest bonds in Fig. 4) and the caffeine at x, y, z. Only the carboxyl group overlaps the caffeine on the underside, *i.e.*, at $1 - x, -y, -z$ (thinnest lines in Fig. 4). Similar observations have been reported by Damiani *et al.* (4–6) in the complexes of tetramethyluric acid and some aromatic hydrocarbons. The perpendicular separations between planes in the latter structures were 3.45 Å.

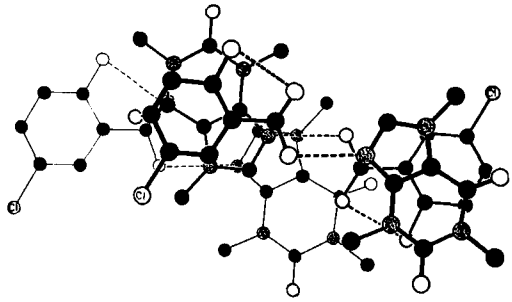

Fig. 4—The stacking arrangement viewed in the direction perpendicular to the molecular plane of caffeine. Broadest bonds indicate molecules at −x, −y, −z, *next broadest bonds indicate molecules at* x, y, z, *and the thinnest represents the molecules at* 1 − x, −y, −z. *Key:* ●, *C;* ⊛, *N;* ○, *O.*

TABLE V—INTERMOLECULAR DISTANCES LESS THAN 3.45 Å.[a]

Atom	to Atom	Equivalent Position	Distance, Å.
CC6	SC7	$1 - x, -y, -z$	3.350
CC6	SO9	$1 - x, -y, -z$	3.202
CC2	SO10	$-x, -y, -z$	3.370
CN3	SO10	$-x, -y, -z$	3.438
CC5	SC1	$-x, -y, -z$	3.443
CN7	SC6	$-x, -y, -z$	3.405
CO10	SC1	$1 - x, -y, -z$	3.354
CO10	SC7	$1 - x, -y, -z$	3.189
CO10	SO9	$1 - x, -y, -z$	3.309

[a] Excluding those shown in Fig. 3.

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Keyphrases

Complexes—structural studies
Caffeine-5-chlorosalicylic acid complex
Crystal structure
Molecular structure

Comparative Study of a New Fluorometric Assay Mestranol Ether in Tablets with GLC and Colorimetric Assays

By ROBERT J. TEMPLETON, WILLIAM A. ARNETT, and IVAN M. JAKOVLJEVIC

Mestranol (17α-ethinylestradiol-3-methyl ether) has been found to react with methanol–sulfuric acid mixture of a critical composition to give a stable fluorophor of high intensity. This fluorescence permits the analysis of mestranol in the submicrogram range. The use of varying proportions of methanol and sulfuric acid enables the analyst to measure the fluorescence at several different wavelengths. The analyst can choose the reagent which gives him the selectivity necessary for his analytical problems. Colorimetric, GLC, and TLC methods for the determination of mestranol are also discussed and compared with the fluorometric method. The described method is satisfactory for both control and stability measurements of mestranol.

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The fluorometric procedure described by the authors is quite simple. The reagent develops a fluorophor at room temperature, which is highly intense and stable for at least 30 min. This

Received December 7, 1967, from the Analytical Development Department, Eli Lilly and Company, Indianapolis, IN 46206
Accepted for publication March 13, 1968.
The authors wish to acknowledge the assistance of Dr. H. Latham Breunig in the statistical analysis of the data.

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Keyphrases

Complexes—structural studies
Caffeine-5-chlorosalicylic acid complex
Crystal structure
Molecular structure

Comparative Study of a New Fluorometric Assay Mestranol Ether in Tablets with GLC and Colorimetric Assays

By ROBERT J. TEMPLETON, WILLIAM A. ARNETT, and IVAN M. JAKOVLJEVIC

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paper will compare the newly developed fluorometric procedure with the authors' versions of the colorimetric and GLC assays. The ability of these methods to measure stability will also be discussed. TLC has been used to detect degradation products as well as to demonstrate and confirm the quantitative results of the other procedures, and a brief discussion is included. Because of the simplicity and sensitivity of this method, it will find application in biological analysis, trace analysis, and contamination control.

EXPERIMENTAL

General Procedure for Tablet Extraction

The tablets used for assay evaluation in this paper were labeled to contain 80 mcg. mestranol per tablet. The bulk of the tablet excipient material is sodium bicarbonate.

One tablet was weighed accurately and placed in a 50-ml. volumetric flask. Three drops of a 20% aqueous solution of sodium hydroxide and 2 ml. of water were added to the flask. The tablet was then dissolved by placing the flask in an ultrasonic cleaner for about 5 min. Approximately 25 ml. of chloroform was added to the flask, which was then shaken for 30 min. on a wrist-action shaker. Sufficient chloroform was added to raise all of the aqueous layer into the neck of the flask. The aqueous layer was aspirated and discarded, and the chloroform was transferred to a 100-ml. volumetric flask through anhydrous sodium sulfate. The content of the flask was diluted to volume with chloroform. This is the sample solution containing approximately 0.8 mcg. mestranol per ml. which is used in each of the described assays.

Fluorescence Assay Procedure

Apparatus—An Aminco-Bowman spectrophotofluorometer equipped with a xenon lamp, a IP21 photomultiplier tube, and silica cells was used to check all activation and emission wavelengths. A Turner model 110 filter fluorometer equipped with a G.E. F4T4 lamp and matched borosilicate cells was employed for all quantitative fluorometric measurements. Kodak Wratten filter 47B which has peak transmission at 430 mμ was used as the primary filter, and Corning filter 3385 which cuts off below 480 mμ as the secondary filter.

Reagent—To develop fluorescence, a 50% v/v mixture of absolute methanol and concentrated sulfuric acid (50% reagent) was used. To prevent heat build-up, the 50% reagent was prepared in an ice bath by adding sulfuric acid dropwise to a chilled flask of methanol.

Tablet Assay Procedure—Two milliliters of the sample solution containing about 1.6 mcg. of mestranol was pipeted into a test tube (18 × 120 mm.) and carefully evaporated to dryness using air and mild heat. The sample was redissolved with 0.1 ml. of methanol and then 5 ml. of the 50% reagent was added to the test tube. After shaking to mix thoroughly, the tube was allowed to stand at room temperature for 1 hr. to obtain maximum fluorescence development. The solution was then transferred to a Turner cell and the fluorescence measured against a reagent blank. To obtain on-scale readings with solutions in the concentration range of 0.1–0.4 mcg. per ml., a 1× aperture setting was used and a 10% transmittance density filter was combined with the secondary filter.

Colorimetric Assay Procedure

Apparatus—A Beckman model DU spectrophotometer with 1-cm. cells was used.

Reagent—The reagent used to develop the chromophor was 30% v/v mixture of methanol in concentrated sulfuric acid (30% reagent). The reagent was prepared in the same manner as the 50% reagent.

Tablet Assay Procedure—A 20-ml. aliquot of the sample solution, corresponding to 16 mcg. of mestranol, was pipeted into a 25-ml. volumetric flask. This solution was evaporated to dryness under a current of air and with gentle warming. Four drops of methanol, from a 1-ml. pipet, was added to redissolve the residue, and then 10 ml. of 30% reagent was pipeted into the flask. The contents were mixed well and the absorbance was read after 10 min. at 545 mμ against a reagent blank. A mestranol standard of about the same concentration was treated in the same way.

Gas-Liquid Chromatographic Procedure

Apparatus—F and M model 402 equipped with dual hydrogen flame detectors was used.

Column—A U-shaped glass column, 61 cm. (2 ft.) × 4 mm. i.d. was used. The column was cleaned with chromic acid cleaning solution and silanized before packing.

Liquid Phase—A silicone gum rubber, W-98 (F and M Scientific Corp., cat. No. LP-166).

Inert Support—Diatoport S, 80/100 mesh (a diatomaceous earth specially treated and silanized by F and M Scientific Corp.). The glass wool used to plug the ends of the column was also silanized.

Preparation of Column Packing—A 1% solution of W-98 in toluene was prepared by dissolving 3.0 Gm. of W-98 in 300 ml. of toluene with constant stirring on a steam bath. Fifty grams of 80/100 mesh Diatoport S was added to the W-98 solution in a liter filter flask. Vacuum was applied to degas the solution and it was allowed to stand for 5 min. The support was then separated from the solution by vacuum filtration. The support was spread and air dried for 24 hr. To remove the last traces of toluene, the support was heated at 100° for 1–2 hr.

Column Conditioning—The column was "no-flow" conditioned for 1 hr. at 325°. After cooling to room temperature, the carrier gas flow was started and the column was conditioned at 275° for 24 hr.

Instrumental Parameters—The instrument was operated isothermally at a column temperature of 205°. The injection port temperature was set at 230° and the detector temperature at 260°. Helium was used as carrier gas with the flow rate at 60–70 ml./min. Oxygen was used for operating the hydrogen flame detector. The range setting was at 10, and the attenuation at 16×.

Preparation of Mestranol Standard Solution—An accurately weighed and diluted solution of pure mestranol in chloroform containing 10 mcg./ml. was used in most cases.

Internal Standard—Cholestane (5α-cholestane

TLC homogeneous, Mann Research Laboratories, Inc.) was selected as the internal standard because of the stability, solubility, and retention time relative to mestranol. A cholestane solution at a concentration of 25 mcg./ml. of chloroform was used.

Preparation of Standard Curve—Internal standard solution in the amount of 2.0 ml., corresponding to 50 mcg. of cholestane, was pipeted into each of five 100-ml. beakers. Then 1.0, 2.0, 3.0, 4.0, and 5.0-ml. aliquots of 10 mcg./ml. of mestranol standard solution were pipeted into each beaker, respectively. Each solution was mixed and evaporated to dryness under a stream of air and mild temperature. The residue was redissolved with 1.0 ml. of chloroform, and approximately 5 μl. of the solution was injected into the gas chromatograph. The peak heights of mestranol and cholestane were measured to the nearest 0.5 mm. from the top of the peak to the corrected base line. The standard ratio of the mestranol peak height to the cholestane peak height was calculated.

Tablet Assay Procedure—Two milliliters of the 25 mcg./ml. cholestane solution was pipeted into a 100-ml. beaker. Then 50 ml. of the sample solution was pipeted into the same beaker. This mixture was then carefully evaporated to dryness under a stream of air and with gentle warming. One milliliter of chloroform was added to the dried residue to redissolve the sample, and 5 μl. of this solution was injected into the gas chromatograph. The sample ratio was calculated in the same way that the standard ratios were calculated and was used to determine the tablet strength.

Thin-Layer Chromatography

Up to 100 mcg. of material was applied to a 250-μ Silica Gel F_{254} plate supplied by Brinkmann Instruments Inc. The plate was developed in a saturated chamber containing chloroform–ethanol, 29:1. The solvent front was allowed to travel 15 cm. To visualize the spots the plate was sprayed with 30% reagent, heated at 110° for 10 min., and then viewed under long-wavelength UV light.

RESULTS AND DISCUSSION

Reagent Composition and Fluorescence Formation—Table I summarizes the characteristics of fluorescence formed by reacting mestranol with reagents of varying methanol–sulfuric acid concentration. It was found that two and possibly three distinct fluorophors are formed by mestranol. Using other reagent proportions than those described here, no other fluorophor was found. When the fluorophor formed with 40% reagent was examined, the spectra indicated that a mixture of the fluorophors of 30 and 50% reagents had been formed. The fluorophor of 60% reagent was the same as that of 50% reagent, but was less intense.

Table I—Color and Fluorescence Characteristics of Mestranol

	Color	Fluorescence, mμ Max. Activ.	Max. Emiss.
Sulfuric acid, concentrated	Orange-red	525	555
30% Reagent	Pink	545	565
50% Reagent	None	468	498

The activation and emission curves of these fluorophors were plotted using an Aminco-Bowman spectrophotofluorometer. In order to compare the intensity of fluorophors, instrument settings, and sample concentrations were the same for all of the spectra (0.2 mcg./ml.). The spectra of mestranol in concentrated sulfuric acid, in 30 and 50% reagents are shown in Fig. 1. These spectra are not energy corrected. The formation of two different and highly intense fluorophors, may allow the analyst to selectively eliminate fluorescence interferences due to other steroids by making a choice of either 30 or 50% reagent. Work is in progress on application of these reagents to the analysis of other steroids.

Detection of Mestranol by Fluorescence—To determine the lowest detection limits of mestranol by this procedure, aliquots of a 0.01 mcg./ml. solution of mestranol were evaporated and treated with 50% reagent. With the aperture set at 10 and no density filter, a solution of 0.001 mcg. (1 nanogram) mestranol per ml. of 50% reagent gave a reading of 22 galvanometer units. The linearity of the measurements at different concentrations in the nanogram range is shown in Fig. 2. Since the tablet assay method described here is in the microgram range, the instrument sensitivity had to be decreased. This was done by using an aperture of 1× and a 10% transmittance density filter in conjunction with the secondary filter. It was found to be more convenient to do this, than to make additional sample dilutions. The linearity of the measurements up to 2 mcg. is shown in Fig. 2. To improve

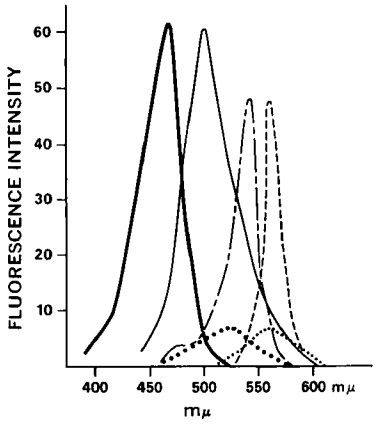

Fig. 1—Comparative activation and emission spectra of mestranol. Key: ——, activation in 50% reagent; ——, emission in 50% reagent; — —, activation in 30% reagent; ---, emission in 30% reagent; ●●●, activation in sulfuric acid; · · ·, emission in sulfuric acid

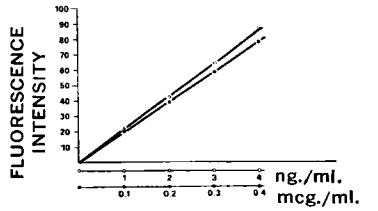

Fig. 2—Linearity of measurements of mestranol by fluorescence.

the reproducibility of the assay it was necessary to dissolve the residue of mestranol in a small volume (0.1 ml.) of methanol before adding the 50% reagent. Insufficient heat was evolved to require cooling, and the reagent properties were not adversely affected by additional methanol. The calculation of precision on a series of five standards assayed by this technique gave a relative standard deviation of ±0.63%.

Fluorescence Development—The reaction of the 50% reagent and mestranol to form the fluorophor does not go to completion at once. At room temperature, the intensity of the fluorescence increased until it reached maximum intensity about 50 min. after addition of the reagent to the sample. The maximum fluorescence was maintained from 50 to 90 min., when it began to decrease slowly as seen in Fig. 3. Based on these findings, it was decided to read all samples about 1 hr. after the addition of the reagent.

Colorimetric Determination—Colorimetric analysis with the 30% reagent has been the standard method in these laboratories for several years. It has been used along with various extraction techniques for both control and stability assays. The assay requires a sample concentration of about 2.0 mcg./ml. of 30% reagent. The calculation of precision on a series of five standards assayed by this technique gave a relative standard deviation of ±0.30%.

Gas Chromatographic Determination—Gas chromatography is a more specific technique for measuring mestranol, because it is a separation technique as well as a quantitation technique. Using the conditions described here, mestranol was chromatographed with no evidence of decomposition on the column. This was done without resorting to derivative formation, which would complicate the procedure. No problems were experienced due to interference from tablet excipients. They were apparently eliminated in the extraction procedure or during the chromatography. A typical chromatogram of mestranol and cholestane is shown in Fig. 4. Since only 0.25 mcg. of mestranol was injected on column in this procedure, the sensitivity of detection should make the procedure applicable in cases where much less sample is available to the analyst. An aliquot of the sample solution containing 40–50 mcg. of mestranol was usually taken for the assay. The procedure proved to be linear in response from 10 to 50 mcg. The precision of measurement by GLC was determined by calculating the standard ratios of 10 sample injections from the same solution, and gave a relative standard deviation of ±0.63%.

Measurement of Stability—In order to evaluate the application of these methods for stability measurement, tablets were artificially degraded with heat and humidity. Several tablets were placed in an area of high humidity at a temperature of about 65° for 14 days (Group I tablets). Another group of tablets was placed in an oven at 105° for 3 days (Group II tablets). These tablets were then

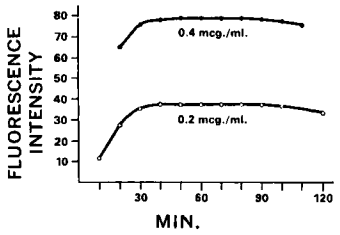

Fig. 3—Development and stability of fluorophor.

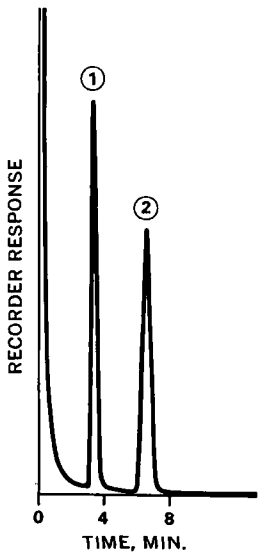

Fig. 4—Chromatogram of mestranol–cholestane mixture. Key: 1, mestranol; 2, cholestane.

TABLE II—COMPARISON OF METHODS FOR STABILITY MEASUREMENTS

	mcg. per Tablet		
	Color	Fluorescence	GLC
Tablets before aging	84.7	85.9	85.6
Group I	47.8	48.4	46.7
Group II	9.0	4.5	3.3

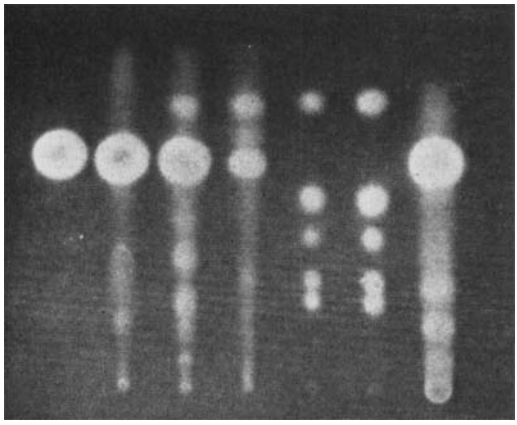

Fig. 5—TLC of mestranol and related steroids. Key: lane 1, (left to right) mestranol standard; lane 2, extract of fresh mestranol tablet; lane 3, extract of Group I tablets; lane 4, extract of Group II tablets; lane 5, mixture of steroids containing estrone methyl ether (highest R_f), estradiol methyl ether, estrone, ethinyl estradiol, and estradiol and appear in that order —1 mcg./steroid; lane 6, mixture of steroids—2 mcg./steroid; lane 7, methanol solution of mestranol exposed to Hanovia irradiation.

TABLE III—STATISTICAL COMPARISON OF METHODS[a]

Analyst	Color	Fluorescence	GLC	Analyst Means
A	706.5	716.5	714.1	712.4
B	690.7	701.7	706.3	699.6
C	699.8	695.6	—	697.7
				Grand mean
Method means	699.1	704.6	710.1	704.0
Estimates of Variation				
Variance[b] s_x^2	45.09	66.78	61.55	
Standard deviation s_x	6.72	8.17	7.85	
Rel. standard deviation c_x	0.96%	1.16%	1.10%	

[a] Results in mcg./Gm. [b] Individual tablets "within" analysts.

assayed by the three procedures described here and the results were compared as shown in Table II. Group I tablets retained only about 55% of their original strength, while in Group II tablets only trace amounts of mestranol remain. These observations were confirmed by the use of TLC.

By the GLC technique all known breakdown products of mestranol are separated from the parent compound. Because of this specificity the GLC assay is a positive measure of stability. Since the fluorometric and colorimetric results correlate very closely to the GLC results, all three methods are considered to be satisfactory stability assays.

Thin-Layer Chromatography—This technique was used as a qualitative assay to confirm the results by the other methods. The tablets were extracted in the same way as for the GLC measurements, then the desired portion of the tablet extract was applied to the plate. As is shown in Fig. 5, degradation of mestranol is observed in the tablets of Groups I and II. The figure also shows the result of exposing a methanol solution of mestranol raw material to irradiation by UV lamp. By this artificial means of accelerating decomposition, it was possible to form at least five decomposition products of mestranol, none of which corresponded to the related steroids chromatographed in lanes 5 and 6. However, the extract from the heated tablets, as seen in lane 4, contains components identifiable by this procedure as estrone methyl ether and 17α-ethinylestradiol. These results are not in agreement with the conclusions of Shroff and Grodsky (5) who state that "mestranol does not undergo any decomposition when stored under various conditions."

Comparison of Assay Methods—In order to evaluate the tablet assay procedures described in this paper, a study was run to compare the three different methods of measurement. The assays were performed by three different analysts. All samples were taken from a single lot of tablets. Each analyst extracted six single-tablet samples and then used aliquots of the extract for the different measurement procedures. The results were reported in mcg. of mestranol per Gm. to e'iminate the effect of tablet weight differences. The results are summarized in Table III. The means of six determinations by each analyst are shown.

An analysis of variance suggested that the variation of individual determinations within each of the three methods appeared to be consistent and that no significant difference among assay methods was shown. The grand mean of the experiment (n = 48) was not significantly different from the theory of 705.3 mcg. mestranol per Gm.

Assay of Ethinyl Estradiol—Ethinyl estradiol is the other estrogen besides mestranol which is widely used in oral contraceptive tablets. All three of the procedures applied to mestranol in this paper are applicable to the assay of ethinyl estradiol. The fluorescence spectrum of ethinyl estradiol is almost identical with that of mestranol in 50% reagent. Ethinyl estradiol forms a pink color with 30% reagent that has an absorbance maximum at 538 mμ instead of the 545 mμ of mestranol. It may also be assayed by GLC with the same operating parameters described for mestranol. It can be chromatographed without derivative formation and is eluted approximately 30 sec. after mestranol under the stated instrumental conditions.

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Keyphrases

Mestranol tablets—analysis
Fluorometry—analysis
Sulfuric acid-methanol—reagent
GLC, colorimetry, fluorometry—comparison
TLC—analysis confirmation
Degradation, mestranol—detected

Fenfluramine Metabolism

By ROBERT B. BRUCE and WILLIAM R. MAYNARD, JR.

The metabolism of fenfluramine in man results in the excretion of unchanged fenfluramine, deethylated fenfluramine, and *m*-trifluoromethylhippuric acid. Methods are described for the determinations of these three compounds and the total metabolites in urine by GLC. The *m*-trifluoromethylhippuric acid accounts for 66 to 93 percent of the dose and 79 to 100 percent of the dose may be accounted for as these three metabolites.

FENFLURAMINE [*N*-ethyl-α-methyl-*m*-(trifluoromethyl)-phenethylamine hydrochloride] is an effective anorexigenic agent showing little or no CNS stimulant properties (1). Structurally, this compound is similar to the amphetamines.

Duhault and Fenard (2) have reported a study of the excretion, distribution, and metabolism of fenfluramine using methyl orange complex for its determination. They found that fenfluramine persists in the animal body for considerably longer than amphetamine. The total excretion following oral doses in man and in dogs was 27 to 38% of the dose over a 5- to 6-day period. Relatively large amounts were found in fatty tissues of the dog. These authors found that the drug is deethylated but could find little or no evidence for hydroxylation, as occurs with amphetamine. Beckett and Brookes (3), using gas chromatographic methods, have studied the dependence of the rate of excretion on urinary pH. They found that some 50% of fenfluramine was excreted unchanged or deethylated under acidic urinary conditions, but could account for only 6 to 23% with uncontrolled acidity of the urine.

The relatively low recoveries of the amines, which has been confirmed in these laboratories, led the authors to investigate the fate of the remainder of the drug in the animal body. Extensive use of gas chromatography in these studies was made to determine the amounts of unchanged drug and its metabolities in biological materials. Blood levels in man and the dog have been determined by GLC. The results given below indicate that the major portion of an oral dose of fenfluramine in man is excreted as *m*-trifluoromethylhippuric acid (Scheme I).

EXPERIMENTAL

Human Study—Two normal, healthy males were given a 60-mg. tablet of fenfluramine as a single dose, and urine was collected at intervals for 72 hr. Ammonium chloride was administered the previous day and throughout the period of urine collection, in an attempt to maintain an acidic urine. All urine samples were kept in the refrigerator until analysis. The pH of each sample was determined with a pH meter immediately upon receipt. Blood samples of 20 ml. were collected at intervals during the first 8 hr. for analysis.

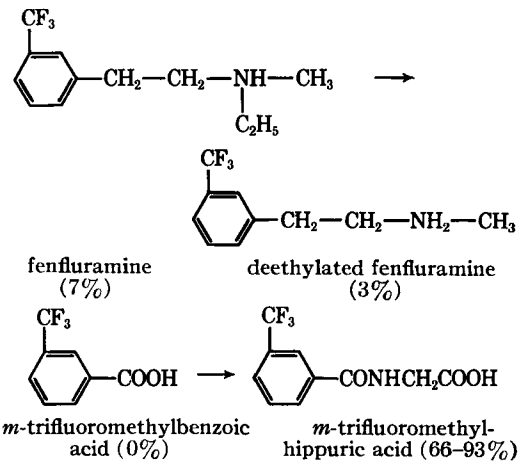
Dog Study—A mongrel male dog weighing 11.7 kg. was given a divided oral dose of 117 mg. of fenfluramine, and urine was collected for 5 days and stored under refrigeration.

Materials—*m*-Trifluoromethylbenzoic acid was purchased from J. T. Baker Chemical Co.

m-Trifluoromethylhippuric acid was prepared by reaction of *m*-trifluoromethylbenzoic acid with thionyl chloride to give *m*-trifluoromethylbenzoyl chloride. The acid chloride was then reacted with glycine to give the hippuric acid.

METHODS OF ANALYSIS

Fenfluramine and Norfenfluramine—These compounds were determined by gas-liquid chromatography according to a modification of a method described by Beckett and Rowland (4). An aliquot of 20.0 ml. of urine or 15.0 ml. of blood was made alkaline with 2 ml. of 2 *N* NaOH and extracted three times with 10-ml. portions of ethyl ether, centrifuging to separate the phases. The combined ether extracts were dried over anhydrous sodium sulfate and evaporated to 0.10 ml. under a gentle stream of nitrogen. Aliquots of 1 to 3 μl. were injected into the gas chromatograph under the follow-



Scheme I—Excretion of fenfluramine and metabolites by man

Received February 27, 1968, from the Research Laboratories, A. H. Robins Company, Inc., Richmond, VA 23220
Accepted for publication April 8, 1968.

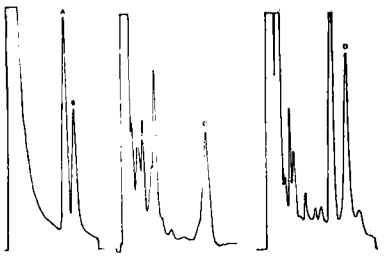


Fig. 1—Gas chromatograms obtained from the analysis of urine samples (Subject A) showing (a) fenfluramine, (b) deethylated fenfluramine, (c) the methyl ester of m-trifluoromethylhippuric acid, and (d) the methyl ester of m-trifluoromethylbenzoic acid. The large peak immediately preceding (d) is the ester of benzoic acid. See text for conditions.

ing conditions, and the peak heights compared with those of a standard.

Detector–flame ionization; column—10% polyethylene glycol[1] 20 M and 5% KOH on HMDS treated Chromosorb W, 2.1 m. × 0.31 cm. (7 ft. × 1/8 in.); column temperature—120°.

The retention times of fenfluramine and norfenfluramine under these conditions were 4 and 4.5 min., respectively. The peaks were sharp and well separated (Fig. 1). No interference has been found from control urine samples carried through the procedures. Recoveries varied from 98 to 102% and peak height was proportional to concentration over the range of 10 to 200 ng. (Standard deviation = ±2.2 at the 100-ng. level.)

Total Fenfluramine Metabolities—The following procedure was designed to determine fenfluramine and all metabolities that might be formed. However, any metabolite in which the benzene ring was altered would not be determined.

An aliquot of 2.0 ml. of urine was oxidized with 10 ml. of saturated potassium permanganate and 0.5 ml. of 2 *N* sodium hydroxide by heating for 1 hr. in the steam bath. The cooled solution was then extracted with a total of 20 ml. of ether in three portions and the acids repartitioned into 10 ml. of sodium bicarbonate. The sodium bicarbonate was acidified and the acids returned to ether by extracting three times with a total of 15 ml. The ether solution was treated with diazomethane, washed with 3 × 5-ml. portions of water, dried over anhydrous sodium sulfate, and concentrated to 0.10 ml. An aliquot of 2 μl. was injected into the gas chromatograph.

The major interference that was expected in this determination was benzoic acid, which was formed during the oxidation in relatively large amounts. The methyl esters of benzoic acid and trifluoromethylbenzoic acid are easily separated by GLC under the following conditions:

Column—10% 20 polyethylene glycol M on Gas Chrom Q, 1.2 m. × 0.31 cm. (4 ft. × 1/8 in.) at 75°; flow rate—25 ml./min.; injection port temperature —250°; detector–flame ionization.

The retention time for methyl trifluoromethylbenzoate is 5 min. and that of methyl benzoate is 4 min. (Fig. 1).

m-Trifluoromethylbenzoic Acid—Acidified urine was extracted with ether and carried through the procedure used for total metabolites, omitting the oxidation steps.

m-Trifluoromethylhippuric Acid—The hippuric acid derivative could not easily be extracted from urine without extracting interfering materials. It could be separated from these materials by the use of an ion-exchange column. A column,[2] (200-400 mesh) 12 × 500 mm., was prepared in 95% ethanol. A sample of 4.0 ml. of urine was placed on the column and the column was washed with 330 ml. of 95% ethanol. The trifluoromethylhippuric acid was then eluted with 70 ml. of 0.1 *N* hydrochloric acid in 95% ethanol. The ethanol solution was evaporated to dryness, the residue taken up in 10 ml. of ether, and methylated with diazomethane. After washing with water, the ether solution was evaporated to 0.10 ml. and 2 μl. were injected into the gas chromatograph. Conditions: Column—10% polyethylene glycol 20 M on Gas Chrom Q, 1.2 m. × 1.2 cm. at 190°; flow rate—30 ml./min.; injection port temperature—300°. Both hippuric and trifluoromethylhippuric acids are eluted from the ion-exchange column under these conditions but they are easily separated by GLC. The retention time of methyl trifluoromethylhippurate is 10 min. and that of the methyl hippurate is 14 min. (Fig. 1).

IDENTIFICATION OF METABOLITES

The correspondence of retention times found by GLC was an indication that the metabolites from urine were the same as those of authentic compounds. Beckett and Brookes established the presence of fenfluramine and norfenfluramine.

In addition, the following information as to identity of the other metabolites was found.

m-Trifluoromethylbenzoic Acid—A sample of acidified dog urine was continuously extracted with ethyl ether and the acids were separated from phenols by sodium bicarbonate partitioning. The resulting bicarbonate solution was acidified and the acids reextracted into ether. An aliquot of the ether was concentrated and used for thin-layer chromatography on silica gel. Using a solvent system consisting of benzene, methanol, and acetic acid (90:10:4) two spots were found that corresponded to *m*-trifluoromethylbenzoic acid and its glycine conjugate.

In order to separate a larger quantity of the *m*-trifluoromethylbenzoic acid, a partition column of 0.1 *N* sulfuric acid on silicic acid was prepared. A portion of the ether extract was added to the column and it was eluted with skellysolve B. Fractions were collected and monitored by UV and TLC. Those fractions corresponding to the *m*-trifluoromethylbenzoic acid were combined and evaporated to dryness. The infrared spectrum of the isolated material was identical with that of an authentic sample of *m*-trifluoromethylbenzoic acid. A melting point following sublimation was 101–102°, and a mixed melting point 100–101°.

Anal.—Calcd: C, 50.33; H, 2.64. Found: C, 50.56; H, 2.71.

m-Trifluoromethylhippuric Acid—Although this acid could not be separated in sufficient quantity and purity from urine for infrared identification and elemental analysis, the GLC and TLC results are good evidence for its identification.

[1] Carbowax, Union Carbide Corp., New York, N. Y.

[2] Dowex-IX-8(⁻OH), Dow Chemical Corp., Midland, Mich.

TABLE I—BLOOD LEVELS OF FENFLURAMINE IN TWO HUMAN SUBJECTS FOLLOWING AN ORAL DOSE OF 60 mg. OF DRUG

Time After Dose, hr.	Fenfluramine, mcg./100 ml. of Blood Subject A	Subject B
Control	0	0
1	2.3	0.9
2	2.6	2.3
3	2.9	2.9
4	1.2	1.5
6	1.2	1.2
8	0.6	0.9

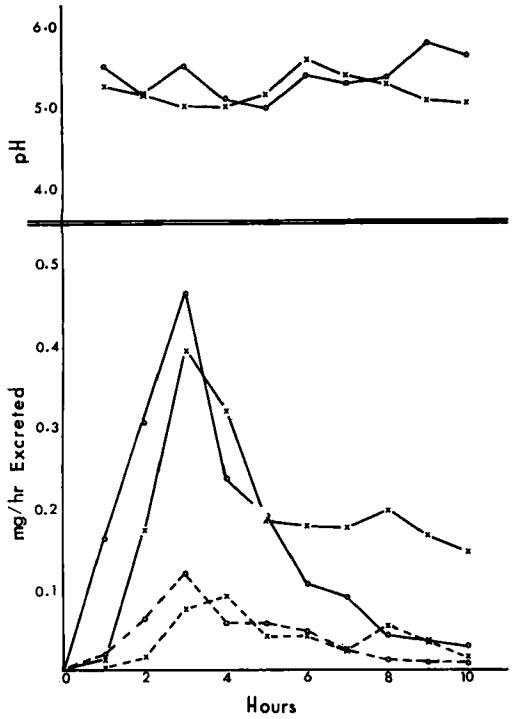


Fig. 2—The urine pH and rates of excretion of fenfluramine (solid line) and deethylated fenfluramine (dashed line) in two normal males.

Hydroxylation—The procedure as described by Axelrod (5) was used to determine whether a hydroxylated product might occur in the urine. No reaction was obtained with 1-nitroso-2-naphthol using either hydrolyzed or unhydrolyzed urine.

RESULTS AND DISCUSSION

The results of the blood level study in man are shown in Table I. These results indicate that fenfluramine is readily absorbed and persists for some time in the blood stream. Even in the final sample 8 hr. after the dose, measurable concentrations are still present. The maximum concentration appears at about the third hour. The persistence of fenfluramine in the body is in agreement with previous studies by Duhault and Fenard (2).

Determination of blood levels in dogs, that received 2 mg./kg. of fenfluramine, showed that both fenfluramine and norfenfluramine were present. However, no norfenfluramine has been found in blood during any of the studies on humans.

Typical results of the urinary excretion of fenfluramine and norfenfluramine are shown in Fig. 2. Although it was attempted to maintain an acidic urine, the pH did not reach levels below 5, as did those reported by Beckett and Brookes. The maximum excretion rate occurred at around 3 hr. which was at the same time as the maximum blood levels. Urine was continued to be collected from these subjects for 72 hr. Determinations of the acidic metabolic products were carried out on samples of urine pooled for 1–10 hr., 10–24 hr., 24–48 hr., and 48–72 hr. These results are shown in Table II. The excellent agreement found between the sum of individual metabolites and the total by oxidation indicates the accuracy of the methods and also that the oxidation procedure results give the desired results.

Although free trifluoromethylbenzoic acid was found in dog urine, none could be detected in human urine. It can be seen from these results that the main excretory product of fenfluramine in man is the trifluoromethylhippuric acid. These two subjects excreted approximately 10% of the dose as fenfluramine and its deethylated metabolite, which corresponds closely with that amount found by others with uncontrolled pH (5). In one subject (A), 93% of the dose was excreted as *m*-trifluoromethylhippuric acid and in the other, 66% was excreted in the 72-hr. period.

Amphetamine and methamphetamine also un-

TABLE II—EXCRETION OF FENFLURAMINE, NORFENFLURAMINE, *m*-TRIFLUOROMETHYLHIPPURIC ACID, AND TOTAL METABOLITES FROM TWO SUBJECTS WHO RECEIVED A SINGLE 60-mg. DOSE OF THE DRUG

Subject	Sample Time, hr.	Fenfluramine, mg.[a]	Norfenfluramine, mg.[a]	*m*-Trifluoromethylhippuric Acid, mg.[a]	Sum of Individual Metabolites, mg.	Total by Oxidation, mg.[a]
A	1–10	2.17	0.69	12.86	15.72	16.65
	10–24	0.95	0.47	10.33	11.75	12.05
	24–48	0.49	0.40	24.84	25.73	25.03
	48–72	0.32	0.37	7.81	8.50	8.90
Total		3.93	1.93	55.84	61.70	62.63
% of dose		6.55	3.22	93.07	102.9	104.4
B	1–10	1.92	0.51	10.89	13.32	13.11
	10–24	1.68	0.80	8.51	10.99	10.07
	24–48	0.72	0.58	10.11	11.41	11.10
	48–72	0.15	0.17	10.23	10.55	13.02
Total		4.47	2.06	39.74	46.27	47.30
% of Dose		7.45	3.43	66.23	77.12	78.83

[a] All results are calculated in terms of the dose-fenfluramine hydrochloride.

dergo side-chain oxidations. Oxidation occurs through the stages of benzyl methyl ketone and 1-phenyl-propane-2-ol to the acid. A corresponding mechanism would be expected to occur with fenfluramine. The amphetamines also undergo ring oxidation to give the phenol. The presence of the trifluoromethyl group on the benzene ring of fenfluramine appears to prevent such oxidation. The phenolic derivative of fenfluramine was not available to determine whether the reaction given by Axelrod would take place, but the recovery of 77 to 100% of the administered drug as fenfluramine and its two metabolites suggests that if such oxidation does occur, it can be only to a very small extent.

Previous observations (2, 5) have indicated that fenfluramine is rapidly absorbed into the tissues and is then slowly released into the extracellular fluid. This is also indicated by the present studies. The two amines continue to be excreted over at least a 72-hr. period and during the first 48 hr. *m*-trifluoromethylhippuric acid is excreted at an almost continuous rate.

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(2) Duhault, J., and Fenard, S., *Arch. Intern. Pharmacodyn.*, **158**, 251(1965).
(3) Beckett, A. H., and Brookes, L. G., *J. Pharm. Pharmacol.*, **19**, 42S(1967).
(4) Beckett, A. H., and Rowland, M., *ibid.*, **16**, 27T (1954).
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Keyphrases

Fenfluramine—metabolism
m-Trifluoromethylhippuric acid, de-ethylated fenfluramine—metabolites
Blood levels—fenfluramine
Urinary excretion—fenfluramine and metabolites
GLC—analysis

Structure-Activity Relations in an Imidazoline Series Prepared for Their Analgesic Properties

By W. BROCK NEELY*, H. C. WHITE†, and A. RUDZIK‡

A specific pharmacological property of a series of substituted imidazolines was examined in an effort to determine the structure-activity relationships that might be present. The best measure of the chemical reactivity was based on a charge-transfer complex between the drug and the receptor site. Using the molecular orbital technique for measuring this reactivity index, and combining with the partition coefficient in the manner described by Hansch 94.4 percent correlation was found for the biological activity.

STRUCTURE-ACTIVITY relationships have been an intriguing subject for organic and biological chemists for many years. At the same time, it is an area that has seen many frustrations and disappointments. The reasons for the failures are readily appreciated when the complexity of the biological system is taken into consideration. In attempting any type of rigid structure-activity study all of the the biological parameters such as active and passive transport, metabolism to a more or less active agent, nonspecific binding to proteins, and finally the reaction at the receptor site, must be taken into account. Obviously, the system is much too complicated and too little understood for a detailed analysis. As a starting point, a simple model is required. In such a case, if the model works, *i.e.*, can predict the data, it may give new insights into the mechanism of action. Conversely, if the model fails, then one must look for the reasons; this in itself could provide new concepts in the area under investigation.

The model used in this study is the one that has been developed by Hansch and his co-workers (1) and used extensively in a number of different biological applications (1–5). In effect, the model assumes that the amount of drug found at the receptor site is related to a single substituent constant which is derived from octanol–water partition-coefficient measurements. The reaction of the chemical at the receptor site is related to the rate constant. The model is illustrated by Eq. 1.

$$\log \text{B.R.} = -\alpha\pi^2 + b\pi + c \log k_x + d \qquad \text{(Eq. 1)}$$

where B.R. = biological response; π = log

Received March 13, 1968, from the Dow Chemical Company, Midland, MI 48640
Accepted for publication April 11, 1968.
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(P_x/P_h); P_x, P_h = partition coefficient of substituted compound P_x and parent compound P_h; k_x = rate constant; a, b, c, d = equation constants.

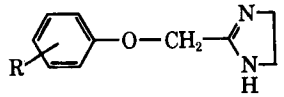
In attempting to arrive at some measure of the reactivity of a drug with a receptor site it is desirable to have some idea of what the mechanism might be. For example, in interpreting the insecticidal data of organophosphates, it is assumed that those phosphates which are most susceptible to nucleophilic attack will be the insecticidal agents. Consequently, a measure of the reactivity is the rate of hydrolysis of the phosphate by hydroxide ion. There are many papers that deal with this subject, but only a few are cited (6–8).

In the present case, the authors do not have a mechanism for the pharmacological activity (which in this case is assumed to be analgesic action); consequently, there is a problem of deciding on an appropriate reactivity index for the imidazoline compounds, I. What is required is a technique for estimating different electronic parameters and attempting to deduce which index correlates with the biological response. This requirement is met by the use of the simple Hückel molecular orbital (MO) technique. The theory and applications of MO to problems in biochemistry have been described by many authors (9–12). In using these electronic parameters as a measure of the rate constant in Eq. 1 the following assumption must be remembered. The rate constant k is related to the energy of activation E by means of the Arrhenius Eq. 2. The assumption

$$k = Ae^{-\Delta E/RT} \quad \text{(Eq. 2)}$$
$$\text{or } \log k = A - B\Delta E$$

where A and B are constants, is that the various indexes calculated from MO theory are linearly related to ΔE and hence may be substituted for $\log k$ in the regression equation, Eq. 1.

This paper reports the results in using the Hansch approach to correlate the pharmacologic activity, derived from an analgesic test, with the structure of a series of imidazoline derivatives, I.

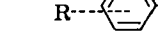


I

MATERIALS AND METHODS

Synthesis of Aryloxyacetonitrile—A mixture of 0.10 mole of the appropriate phenol, 0.10 mole of anhydrous K_2CO_3, 0.11 mole of chloroacetonitrile, and 100 ml. of dimethyl sulfoxide was stirred at 75° for 3 hr. The reaction mixture was poured into 100 g. of ice and the product extracted with methylene chloride. The product was then isolated by vacuum distillation through a 40-cm. Vigreux column. The identification was made with a Varian A-60 nuclear magnetic resonance spectrometer with a 10% $CDCl_3$ solution. The physical properties and yields are listed in Table I.

TABLE I—ARYLOXYACETONITRILE



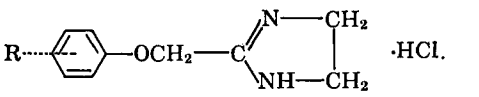
R	Yield, %	B.p., 2 mm., °C.	M.p., °C.[a]
2,6-di Cl	66	114–118	39–40[b]
2,6-di Br	85	135–138	63–64
2-Br, 6-Cl	88	126–128	
2,6-di CH_3	60	125–127[c]	
2,6-di OCH_3	69	133–137[d]	
2,6-di Cl, 3-CH_3	72	138–141	48–49

[a] Thomas-Hoover capillary m.p. apparatus, uncorr. [b] Campbell (15) reports m.p. 40–43°. [c] Barron *et al.* (16) reports b.p. 88–92° (*p*, 5–1.0 mm.). [d] Clark *et al.* (17) report m.p. 94–95° by a different method of synthesis. The authors' material showed no sign of crystallization even at 0°. The NMR spectrum of this material confirmed unambiguously the structure assigned.

Synthesis of the Imidazolines—A mixture of 0.10 mole of the aryloxyacetonitrile, 0.10 mole of 1,2-ethylenediamine monotosylate, and 100 ml. of *o*-dichlorobenzene was heated with stirring under a nitrogen blanket until ammonia evolution ceased. This usually required 1–2 hr. at 170–180°. The reaction mixture was poured into water, one equivalent of NaOH added, and the imidazoline extracted into methylene chloride. After the solvent was removed under vacuum, the residue was dissolved in isopropyl alcohol and a slight excess of anhydrous HCl in isopropyl alcohol added. The resulting imidazoline hydrochloride was then collected by filtration and recrystallized from isopropyl alcohol. Identification was by chloride equivalent and NMR. The spectra in D_2O in all cases were consistent with the expected substitutions in the aromatic regions, as well as the methylene bridge and the ethylene portion of the imidazoline ring. The properties of the imidazolines studied in this investigation are shown in Table II.

Pharmacology—Adult male mice (Cox) weighing

TABLE II—2-(ARYLOXYMETHYL)-2-IMIDAZOLINES



R	Yield, %	M.p. °C. (dec.)[a]	Chloride, Calcd.	Equivalent, Found
2,6-di Cl	57	221–222	281.58	283
2,6-di Br	57	230–231	370.50	365
2-Br, 6-Cl	40	214–216	326.05	329
2,6-di CH_3	76	224–226[b]	240.72	242
2,6-di OCH_3	54	188–190	272.74	272
2,6-di Cl, 3-CH_3	46	229–230	296.61	295

[a] Thomas-Hoover capillary m.p. apparatus, uncorr. [b] Laboratories Dausse, S. A., French pat. 1,312,410 (1962), report m.p. 219°.

18–22 g. were used in these tests. Relative potency was measured by the ability of these compounds to antagonize the HCl-induced writhing response. Writhing was induced by the intraperitoneal injection of 10 ml./kg. of a 0.1 *N* HCl solution. The compounds to be tested were administered orally 30 min. prior to the HCl. The results are expressed as the dose that will effectively prevent 50% of the animals from exhibiting the characteristic writhing. This test has been used as a measure of a drug's ability to cause analgesia (13). The ED_{50} values are given in Table II.

Reactivity Index—Calculated Value—The simple MO calculations were carried out as described by the Pullmans (9). There are many indexes that may be deduced from this type of procedure. The one that appeared to have the most significance for measuring the biological activity of the imidazolines was the energy of the highest occupied molecular orbital (HOMO). This index is a relative measure of the ability of an electron to be transferred to an acceptor molecule.

The calculations were performed on the substituted phenol present in the imidazoline structure. This simplification was made since it could be assumed that any perturbation caused by the imidazole would be insulated from the rest of the molecule by the methylene group. The parameters used for the heteroatoms in this study are listed in previous publications (6, 9).

Experimental Value—The oxidation potentials of the phenols, corresponding to the imidazoline derivatives, were determined with a Leeds and Northrup Electron chromograph type E instrument. This was equipped with 0.6 cm. (1/4 in.) graphite (ultrapure spectroscopic electrodes from Ultra Carbon Corp.) and a calomel reference electrode. The electrolyte was a 1 *M* acetic acid and 1 *M* sodium acetate buffer. The procedure was as follows: the phenol was made 0.001 *M* in methanol, 5 ml. was diluted to 10 ml. with the electrolyte, the solution was placed in the cell, and the potential was scanned from 0 to 1 v. The oxidation potential was recorded as the midpoint of the resulting curve.

Partition Coefficient—The substituent constant π was calculated from the values given by Hansch (5).

Calculations—The MO calculations and the regression analyses were performed by the Computation Laboratory, The Dow Chemical Company, using a Burroughs B5500 computer.

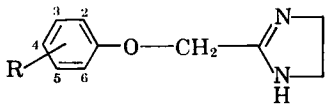
RESULTS

The data collected for this series are shown in Table III. In order to ensure that the calculated value for the energy of the highest occupied molecular orbital was relevant, the oxidation potential was determined for the various phenols. The correlation between the experimental and the calculated values are shown in Fig. 1.

Using the Hansch procedure (1) the biological data in Table III were regressed against the two parameters, π and HOMO. The analysis generated Eqs. 3–5 where

$$\log ED_{50} = -0.445\pi + 1.015 \qquad r = 0.640 \quad r^2 = 0.406 \quad \text{(Eq. 3)}$$

TABLE III—BIOLOGICAL AND CHEMICAL PARAMETERS FOR A SERIES OF IMIDAZOLINE DERIVATIVES



Substituent (R)	ED_{50}[a]	π[b]	HOMO[c]	Oxidation[d] Potential, v.
2,6-diCH_3	0.6	1.36	0.716	0.469
2-Br, 6-Cl	2.0	1.34	0.802	0.642
2,6-di Br	3.2	1.50	0.797	0.634
2,6-di Cl	3.3	1.18	0.808	0.659
2,6-diCl, 3-CH_3	5.4[e]	1.69	0.789	0.625
2,6-di OCH_3	25	−0.66	0.618	0.363

[a] Oral dose in mg./kg. (HCl induced rat writhing test for analgesics). [b] Determined from Hansch's values (5). [c] Energy of the highest occupied molecular orbital of the substituted phenol. The smaller the value, the greater the ease of electron donation. [d] Oxidation potential of substituted phenol determined by means of a Leeds and Northrup Electro chromograph type E. [e] Estimated value derived from the value for a subcutaneous dose of this derivative compared to a similar type of injection of the other derivatives.

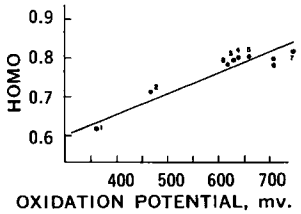


Fig. 1—Plot of HOMO against the oxidation potential (mv.) of a series of phenols. Key: 1, 2,6-di OCH_3; 2, 2,6-di CH_3; 3, 2,6-di Br; 4, 2,-Br, 6-Cl; 5, 2,6-di Cl; 6, phenol; 7, 3,4-di Cl; 8, 2,6-di Cl, 3-CH_3. The values of HOMO and the oxidation potential in mv. for the two phenols not listed in Table III are: phenol (0.711 and 705 mv.), 3,4-diCl (0-811 and 765 mv.).

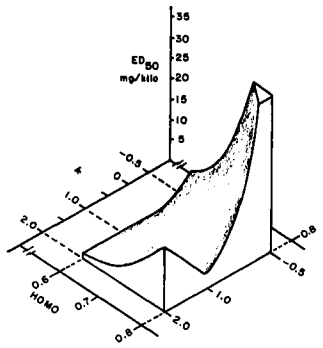


Fig. 2—Structure-activity surface generated by Eq. 5. The most potent analgesic has the smallest ED_{50}. The data for this figure are listed in Table III.

$$\log ED_{50} = +0.655\pi^2 - 1.00\pi + 0.455 \qquad 0.728 \quad 0.531 \quad \text{(Eq. 4)}$$

$$\log ED_{50} = 0.945\pi^2 - 1.850\pi + 7.90\,(\text{HOMO}) - 5.117 \qquad 0.975 \quad 0.944 \quad \text{(Eq. 5)}$$

r is the correlation coefficient. The good correlation

shown by r is indicated by r^2 (*i.e.*, 94.4% of the variance is "explained" by Eq. 5). An F test indicated that the inclusion of the HOMO term in Eq. 5 is significant at the 5% level, whereas Eqs. 3 and 4 were considerably less than 95% significant. There was no correlation when the biological response was regressed using the HOMO parameter by itself (Fig. 2).

DISCUSSION

One of the important items to emerge from this study is the role played by the substituent constant π. As Eq. 4 indicates 50% of the activity may be correlated with π. Consequently, the balance between the hydrophobicity and hydrophilicity of a chemical (as expressed by π) would seem to be critical. One interpretation of these data is that the partition coefficient has a strong influence on the actual amount of drug found on the receptor site. This occurs through a "random walk" process in the organism and finally association with the site itself. The recent work of Hansch (4) dealing with the influence of π on the binding of small molecules to bovine serum albumin helps to substantiate this hypothesis. As the drug builds up a concentration on the receptor site the intrinsic reactivity takes over and through the formation of a charge-transfer complex elicits the observed response. Green and Malrieu (11) made use of the concept of charge-transfer complex in study of a series of indole derivatives. In essence this type of complex may be formed when a compound capable of donating electrons is mixed with a receptor possessing the capacity to accept electrons.

In a recent review article on the molecular basis of drug action, Mautner (14) raised the question as to the molecular significance of the Hansch-type of correlation. The authors agree that the correlation does not predict a specific mechanism where the reactivity of the actual functional groups is implicated. The phosphorylation of the esteratic site in cholinesterase, as a mechanism for inhibition, is an example of a molecular description (6, 7). On the other hand, these correlations do provide a working model for speculating on the mode of action. In the present case, the interpretation of the results suggests a model which can be tested by further experimentation. The model that emerges consists of a receptor site which has the ability to accept electrons from an electron-donating drug. The association is probably similar to a Michaelis-Menten-type situation, hence the biological activity would have a short half-life relative to a drug that forms an actual C—C covalent linkage. The ability of the two parameters, π and HOMO, to correlate with the biological response would also indicate a type of drug that was not very selective in its action. While this model does not give a molecular mechanism, it may provide insight as to how this particular series of chemicals elicit their analgesic-like activity.

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Keyphrases

Imidazolines—synthesis
NMR spectrometry—identity
Pharmacological activity—imidazolines
Structure-activity relations—analgesic
Reactivity index—molecular orbital technique

Identification of Sugars from Rates of Oxime Formation

By THOMAS J. MIKKELSON and JOSEPH R. ROBINSON

The concept of using precise kinetic measurements to aid in the characterization of organic compounds has been applied to sugars. Hydroxylamine reacts with sugars to form oximes; the rate of the reaction can be conveniently followed spectrophotometrically. At pH 6.5 and with an excess of hydroxylamine the rate of oxime formation follows apparent first-order kinetics and is sufficiently discriminating to allow identification of many sugars. Kinetic evidence obtained in this study indicates that the reaction of ketoses and aldoses with hydroxylamine proceeds through a carbinolamine addition compound intermediate, analogous to that proposed for other aldehydes and ketones.

IDENTIFICATION OF an unknown organic compound is usually carried out by a combination of qualitative elemental analysis, determination of the functional groups present in the molecule by chemical or spectroscopic means, determination of constants such as melting point, density, refractive index, *etc.*, and ultimately preparation of a derivative. In many cases a clear distinction between possible choices cannot be made on the basis of the classical approach (and the full range of possible spectroscopic methods is not always available), and therefore a further criterion for identification is desirable. An earlier publication (1) has introduced the concept of using precise kinetic measurements as an additional aid in the characterization of organic compounds. It is the purpose of the present report to extend the concept to another class of organic compounds, namely, carbohydrates.

Sugars represent a class of organic compounds that are difficult to characterize, due partly to their great water solubility, and also to the large number of functional groups in the molecules. Numerous methods have been proposed (2–6) for the identification of sugars, the most common being chromatographic techniques. These procedures usually involve the preparation and purification of a derivative; this is at best difficult, and in addition it may not be very discriminating and may be limited to a small number of compounds.

Phenylhydrazine, semicarbazide, and similar nitrogenous bases react with sugars to form compounds that, because of their low water solubility, can be used as derivatives for identification purposes. The rate of this reaction has been used for many years as a qualitative test for identifying sugars, *e.g.*, the rate of osazone formation using phenylhydrazine as the base. Since qualitative kinetic differences exist with this reaction, it was expected that reactions with other more water-soluble bases, such as hydroxylamine, would also show kinetic differences. Appreciable water solubility of the reactants and products is essential for a study such as this in order to have homogeneous reaction conditions and also to encompass as many members of the carbohydrate class as possible.

Gladding and Purves (7) utilized the reaction of hydroxylamine and sugars to determine quantitatively the purity of various starch and cellulose derivatives. The study indicated that the rate of oxime formation of sugars did follow apparent first-order kinetics, but was nondiscriminating in that sugars could be grouped into only two categories, fast and slow reactants. This last observation is to be expected in view of the reaction conditions used and the currently accepted mechanism for oxime formation (8).

Reactions between hydroxylamine and various aldehydes and ketones have been studied extensively (8), although to the authors' knowledge the reaction between hydroxylamine and sugars has not yet been reported. It is the aim of this study to investigate the feasibility of using the reaction between hydroxylamine and sugars as a method to distinguish between various sugars, and, in addition, to compare the mechanism of this reaction with the proposed mechanism for other aldehydes and ketones.

EXPERIMENTAL

Reagents and Apparatus—Hydroxylamine hydrochloride (Baker reagent grade) was used without further purification. Sugars were obtained from customary commercial sources, and their decomposition points were the sole criteria of purity (9). Representative sugars were repeatedly recrystallized to establish that impurities normally present in sugars had no effect on the rate of oxime formation. Water was glass-distilled from alkaline permanganate. All other chemicals employed were either analytical or reagent grade purity.

Received February 26, 1968, from the School of Pharmacy, University of Wisconsin, Madison, WI 53706

Accepted for publication April 22, 1968.

This research was supported by a grant from the Research Committee of the University of Wisconsin.

This paper is Part II in the series "Precise Kinetic Measurements Applied to the Identification of Organic Compounds," for the first paper see *Reference 1.*

pH measurements and adjustments were made on a Corning model 12 pH meter with an expanded scale using a Beckman-type E-3 wide range glass electrode. The pH meter-electrode system was standardized against a phosphate buffer as described by Bates (10).

Water-bath temperatures were maintained to ±0.1° with Sargent Thermonitor electronic relays.

Kinetic Procedure for Spectrophotometric Determinations—The standard buffer solution employed in all runs was prepared by weighing 0.1 mole (6.950 g.) of hydroxylamine hydrochloride and dissolving to a volume of approximately 95 ml. with water. This solution was titrated to a pH of 6.50 with a saturated sodium hydroxide solution, and brought to a precise volume of 100 ml. with water. The solution was equilibrated to 25 ± 0.1° and brought to a final pH of 6.50 with the addition of one or two drops of concentrated NaOH or HCl solution.

Six milliliters of buffer solution were introduced into a 2-cm. absorption cell (or 3 ml. of buffer into a 1-cm. cell) together with 50 μl. of a 1 *M* aqueous solution of sugar. Mixing of the solutions was accomplished by shaking the absorbance cell prior to placement into the cell compartment. Absorbance changes were measured on a Cary model 15 recording spectrophotometer with thermostated cell compartments. The appearance of all the sugar oximes was followed at 245 mμ with pure buffer as a blank.

By following the experimental conditions as specified a large excess of the free base, hydroxylamine, is present and pseudo first-order kinetics ensue. From semilogarithmic plots of $(A_\infty - A_t)$ (absorbance at time infinity minus absorbance at time t) against time or by the Guggenheim method (11), pseudo first-order rate constants were obtained.

The convenience of the relatively rapid rates of aldose-oxime formation enabled these reactions to be followed by a direct recording method on the instrument. Because of the slower rates of oxime formation for the ketoses (L-sorbose and D-fructose), a nonrecording technique was employed by measuring absorbance at 1,800-sec. intervals with the absorbance cells kept at 25 ± 0.1° in a constant-temperature bath between measurements.

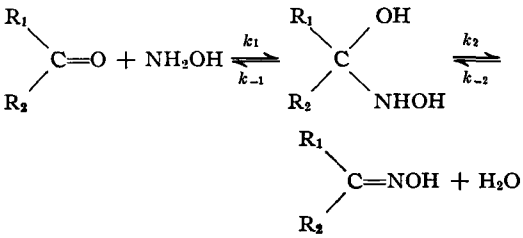
The procedure was altered slightly for some of the polysaccharides. The buffer solution was used as described above but 100 μl. of the sugar solution (cellobiose 0.5 *M*, lactose 0.5 *M*, and raffinose 0.25 *M*) was employed. This change in procedure was required by the decreased molar solubility of these di- and trisaccharides.

Formation and Characterization of Sugar Oximes—Mannose oxime was prepared by the method of Wolfrom and Thompson (12). The melting point of the oxime was found to be 174–176° [lit. m.p. 175–177° (12)]. Elemental analysis yielded N = 7.15%, calculated N = 7.175%. The molar absorptivity of mannose oxime was ϵ_{245} = 38.8.

Attempts to prepare fructose oxime by the method of Wohl (13) were considered unsuccessful. Repeated recrystallization of the product yielded a crystalline material, m.p. 120–122° [lit. m.p. 118° (13)]. However, elemental analysis for nitrogen content was more than two and a half times the calculated amount, and, in addition, the molar absorptivity was more than two and a half times the expected value. The authors assume that degradation of the fructose occurred during the reaction due to the rather harsh reaction conditions, and a species other than fructose oxime is formed.

KINETIC RESULTS AND DISCUSSION

Jencks (8), as well as others (14–16), has suggested that hydroxylamine, semicarbazide, and other similar nitrogeneous bases react with carbonyl functional groups to form additional compound intermediates which subsequently break down to product. The general reaction scheme for oxime formation can be written as:



It was also suggested that the characteristic bell-shaped pH profile obtained for reactions such as this was due to a change in rate-limiting step, which is attributed to two opposing effects. At pH values below the rate maximum there is a decrease in the concentration of the attacking free nitrogen base, and the formation of the carbinolamine addition compound is rate-determining. At pH values above the rate maximum general acid-catalyzed dehydration of the carbinolamine intermediate leading to the oxime is rate-determining (8). The pH-rate maximum is the result of a transition between these two opposing effects.

The partial pH profiles for formation of the oxime from an aldose and ketose exhibit the characteristic pH-rate maximum as shown in Fig. 1. No attempt was made to determine rates beyond the buffer capacity of hydroxylamine (below pH 4 and above pH 8), and the pH dependence is assumed to follow the profile reported for other carbonyl compounds.

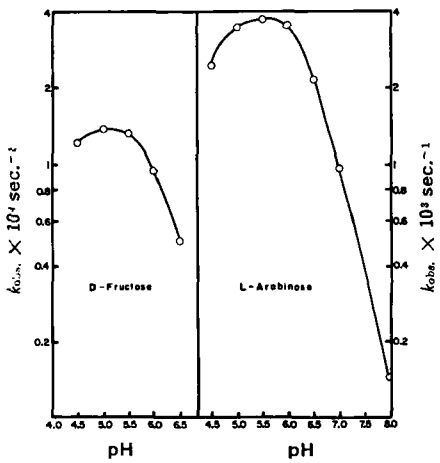


Fig. 1—Partial pH profiles for the formation of a keto- and an aldo-sugar oxime from the reaction of hydroxylamine and the respective sugar at 25° in aqueous solution.

Jencks (8) has found that the first-order rate constants for the reaction of hydroxylamine with ketones or aldehydes increase with the concentrations of free base and hydrogen ions so that the overall reaction is third order. However, at higher concentration of base the rate at a given pH becomes independent of the concentration of free base and approaches first-order kinetics; this was determined to be the case for the reaction between sugars and hydroxylamine (the terminal portions above pH 6, of the pH profiles shown in Fig. 1, have slopes that approach one). At acid pH (low concentration of nitrogen base) the first-order rate constants were found to increase with increasing free hydroxylamine, indicating that the reaction is second order with respect to hydroxylamine in this region, which is in agreement with published work.

Elemental analysis of mannose oxime indicates that only 1 mole of hydroxylamine is consumed per mole of oxime formed, unlike the reactions between sugars and other nitrogenous bases such as phenylhydrazine, which results in the addition of 2 moles of the base to 1 mole of sugar. The oxime reaction is second order with respect to hydroxylamine, but this is because the reaction is general acid-catalyzed and the second mole of hydroxylamine is serving as a general acid. From the above data the authors conclude that keto and aldo sugars react with hydroxylamine to form oximes *via* the same mechanism that is proposed for other aldehydes and ketones.

There is the possibility with aldo and keto sugars that tautomeric conversion from the cyclic ring structure to the open chain analog prior to reaction with hydroxylamine could be rate-limiting in this sequence (17). However, the rate of mutarotation for many of the sugars does not parallel the rate of oxime formation; *e.g.*, fructose shows the slowest rate of oxime formation, but is reported (7) to have a relatively fast rate of mutarotation. Although sugars in the solid state as well as in solution exist mainly in the cyclic ring structure, it is assumed, based on the above, that conversion to the open chain is not rate-determining in the reaction sequence.

Quantitative conversion of the aldo sugar to oxime occurred in the reaction, as calculated *via* the molar absorptivity of mannose oxime. The keto sugar oximes (fructose and sorbose) have molar absorptivities that are three times greater than the aldo sugars, as observed from the spectrophotometric data. It is assumed that the keto sugars are also quantitatively converted to oximes.

ANALYTICAL RESULTS

Figure 2 shows typical ultraviolet spectra of the formation of a sugar oxime at various time intervals. This time course absorbance profile demonstrates that the reaction can conveniently be followed at 245 mμ. Because of the absence of any appreciable absorbance by sugars in the concentrations employed and at a wavelength of measurement, the observed absorbance change was due solely to formation of the sugar oxime.

Choice of reaction conditions is always important in a study of this type. In this study reaction conditions are even more critical than usual in that the variables pH and hydroxylamine concentration affect not only the rate but also the order of the reaction. At pH values below the maximum, pseudo first-order conditions are approached but not rigorously adhered to because of the decreased concentration of the attacking free nitrogen base. At the pH maximum, spectral interference due to overlap of the reaction sequence is observed. At pH values above the maximum, pseudo first-order conditions rigorously hold. The choice of pH 6.50 for the kinetic determinations was based on two factors. First, pseudo first-order conditions exist. Second, the rates of oxime formation are sufficiently rapid at this pH so that the procedure is not self-prohibitive with respect to time.

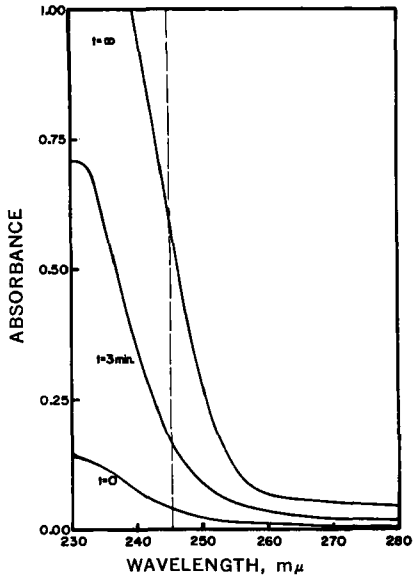


Fig. 2—Absorbance spectra of D-*arabinose-oxime ormation in 1* M *hydroxylamine buffer at pH = 6.50, 25°. Dashed line (245 mμ) indicates the wavelength at which the rates of oxime formation were followed.*

Table I lists the observed first-order rate con-

TABLE I—FIRST-ORDER RATE CONSTANTS FOR THE FORMATION OF OXIMES FROM THE REACTIONS OF VARIOUS SUGARS AND HYDROXYLAMINE IN AQUEOUS SOLUTION

Sugar	Decomposition Point	$k_{obs.} \times 10^3$ (sec.$^{-1}$)[a]	SD[b]	No. of Determinations
Raffinose	80 (hyd.)	0.673	0.023	4
D-Glucose	90 (hyd.)	0.208	0.009	4
D-Ribose	95	2.450	0.080	4
Maltose	100	0.180	0.010	4
D-Fructose	104	0.050	0.003	2
L-Rhamnose	105	0.841	0.037	7
D-Mannose	132	1.330	0.063	4
D-Xylose	145	1.360	0.030	6
D-Arabinose	158	1.890	0.105	4
L-Arabinose	160	1.920	0.089	9
L-Sorbose	160	0.023	0.002	2
D-Galactose	170	1.200	0.065	5
Gentiobiose	186	0.222	0.002	3
Lactose	203	0.322	0.004	4
Cellobiose	225	0.260	0.014	6

[a] At pH = 6.50 in 1.0 *M* hydroxylamine solution at 25°. [b] $SD = [\Sigma(x_i - \bar{x})^2/n - 1]^{1/2}$.

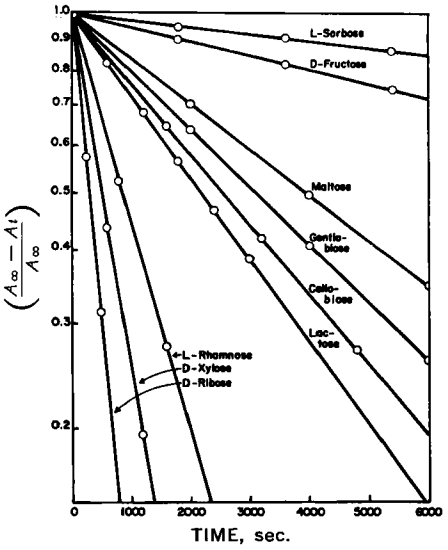


Fig. 3—Normalized representation of some sugar-oxime formations. The reactions were carried out in 1 M hydroxylamine, pH = 6.5 at 25°.

stants for sugar-oxime formations as well as sugar decomposition points. It should be noted that no rate constant for sucrose appears; sucrose does not form an oxime. Glyceraldehyde and ascorbic acid were also investigated, but the reactions did not appear to follow apparent first-order kinetics.

Figure 3 illustrates a normalized graphical representation of some sugar-oxime formations. This displays the approximate 125-fold difference in rate observed between the fastest and slowest oxime formations.

Variation of the ionic strength from 1.0 to 3.0 had a negligible effect on the rate. For this type of reaction only a secondary salt effect would be expected.

DISCUSSION

Utilizing kinetic measurements as an aid in the identification of the sugars as described above gives one the important advantage of a rapid and precise method without the necessity of a common derivative of the sugar. It is apparent from Table I that, with a combination of decomposition point and rate constant of oxime formation, it is possible to differentiate any sugar listed in the table.

The more than 10-fold difference in rate between the keto and aldo sugars is unexpected on the basis of steric and inductive effects. The structural difference between the ketoses and aldoses reported here is the presence of a hydroxymethyl group alpha to the carbonyl group in the ketoses and a hydrogen in the aldoses. Although the authors would predict a small steric effect for dehydration of the tetrahedral intermediate, this effect together with inductive effect cannot explain the difference in rate.

The procedure suggested here for identifying sugars is a relatively sensitive method. For example, a typical experiment with any of the aldoses included in this study would require approximately 5×10^{-5} mole of sugar, while the ketoses would require approximately 1.7×10^{-5} mole of sugar. Naturally this sensitivity could be increased 10-fold by using the expanded scale on the spectrophotometer. Many times only a limited amount of unknown sugar is available for identification, and therefore a sensitive characterization procedure is desirable.

Spectroscopic techniques (NMR, IR, *etc.*) are limited in differentiating among members of this class of compounds. The procedure suggested here for identifying sugars is rapid, sensitive, and quite discriminating. As with most solution kinetic measurements the limiting factor in reproducibility lies in the pH measurements.

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Keyphrases

Sugars—identification
Oxime formation rate—sugar identification
Hydroxylamine HCl—reactant
UV spectrophotometry—oxime formation monitoring

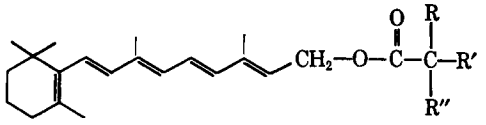
Preparation and Stability of a New Series of Sterically Hindered Esters of Vitamin A. I

By A. J. FORLANO*, C. I. JAROWSKI, and H. F. HAMMER

A series of α,α-dialkyl substituted aliphatic esters of vitamin A was prepared. The stability of these compounds was compared with vitamin A palmitate in hydroalcoholic acidic media, alkaline media, and under oxidative conditions. In all cases steric hindrance on the α-carbon of the fatty acid group increased the stability of the esters in solution. Under oxidative conditions it appeared that a long aliphatic chain was necessary for maximal stability. These results indicate that an ester such as vitamin A α,α-dimethylpalmitate could demonstrate optimal stability under all the conditions examined.

FORLANO AND HARRIS (1) demonstrated that the introduction of an electronegative group in the α-position of the acid portion of vitamin A esters increased stability toward acid-catalyzed elimination in anhydrous ethanol. The rate of anhydrovitamin A formation in anhydrous acidic media was slower for the α-chloropropionate than it was for either the palmitate or acetate. However, the resultant polarization of the ester linkage in the α-chloropropionate also rendered the ester more susceptible to solvent-catalyzed elimination in the hydroalcoholic media. These results suggested that vitamin A esters should be prepared from acids containing electropositive groups in the α-position to provide greater stability toward solvent-catalyzed degradation.

Vitamin A trimethylacetate (I) (R,R′, R″ = CH_3) was the first ester prepared using electropositive groups in the acid portion of the ester.



I, R, R′ and R″ = CH_3 or larger alkyl groups

This ester was more resistant toward solvolysis in an alcoholic medium but it also proved to be more stable than the acetate or palmitate toward acid-catalyzed elimination. Since this ester possessed greater stability in both media, it became obvious that steric and electromeric effects were both important considerations in the stability of vitamin A esters. The steric hindrance exerts an "umbrella-type" blocking effect which mechanically prevents reactive reagents from attacking the carbonyl group of the ester. The data obtained in this study showed that there was a direct relation between acidic and basic solution stability and the size of the groups in the α position of the ester. α,α-Disubstituted fatty acid esters were considerably more stable than the palmitate in anhydrous ethanolic 0.01 *N* HCl, 95% and 70% aqueous ethanolic 0.1 *N* HCl, 70% aqueous ethanol, and in alcoholic base. This suggested that reactions which involve either hydrolysis of the ester linkage or lysis of the alkyl-oxygen bond as the initiating step of the degradation, can be diminished appreciably by introducing steric hindrance on the carbon atom adjacent to the carbonyl group.

Oxidation studies of the sterically hindered esters were not very promising when compared with the palmitate in a vegetable oil (Wesson oil). These tests were run at a level of 1,000,000 units of vitamin A ester per gram of solution with and without antioxidants. Only the α,α-methylethylcaproate of the sterically hindered series of esters exhibited any appreciable resistance to oxidation, but it was not as resistant as the palmitate. When the pure compounds used in this study were subjected to oxidation tests, the crystalline pivalate (trimethylacetate) appeared to be the most stable ester. Since the pivalate is a solid and the others are liquid, it was not unreasonable to assume that its apparent stability was due to reduced surface area or to a coating of oxidized material on the surface which prevented the uniform penetration of oxygen through the crystal. This produced results that were misleading as far as the rate of oxidation under use conditions were concerned; consequently, all the oxidation tests were performed in Wesson oil at a level of 10^6 units of vitamin A per gram of solution. This procedure eliminated the variables of physical form and molecular concentration, since these solutions were all of the same molarity. The results of this test indicated that good oxidative stability in vitamin A esters required a high degree of liposolubility and a long aliphatic chain. Molecular models of the palmitate showed that it is possible for the aliphatic portion of the molecule to orient itself alongside the vitamin A

Received February 2, 1968, from Chas. Pfizer & Co., Inc., Laboratories, Brooklyn, NY 10017
Accepted for publication March 5, 1968.
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chain and thus afford a mechanical barrier against attack by oxygen. The relationship between acid chain length and resistance to oxidation clearly showed that chain length was a critical factor. On the basis of resistance to acid degradation, alkaline hydrolysis, solvent-catalyzed degradation, and oxidation, the α,α-methylethylcaproate was found to be most stable of the sterically hindered esters. The major disadvantage of this compound was its inability to resist oxidation to the same extent as the palmitate. Based on the observed results it was theorized that a vitamin A ester of maximal stability should have (*a*) steric hindrance in the α-position of the acid group, and (*b*) a long aliphatic chain. An ester that could fulfill these requirements would be vitamin A α,α-dimethylpalmitate. The study of the preparation and properties of this ester will be discussed in a future publication.

The balance of this paper deals with the methods of preparation, determination of degradation rates, and comparisons of the stability of these esters. Vitamin A β-cyclopentylpropionate and tetrahydrophthalate were also included in this study. While they are not classical aliphatic-type fatty acid esters, it was desirable to observe the effect of other types of steric hindrance.

EXPERIMENTAL

Preparation of the Esters

The esters were prepared and purified using the method described by Forlano and Harris (1). Essentially, vitamin A alcohol dissolved in a methylene chloride–pyridine mixture was treated with the respective acid chloride or anhydride. The acids or anhydrides were obtained from K & K Laboratories, Jamaica, N. Y. The reaction mixtures were purified by alumina column chromatography, and the esters were isolated as viscous yellow oils after evaporating the solvent. The pivalate crystallized from ethanol upon refrigeration as a yellow solid (m.p. 52–55°), and the monotetrahydrophthalate was crystallized from warm petroleum ether (30–60°) and had an m.p. 94–97°. The esters were identified by their IR and UV spectra which are typical for vitamin A esters along with the absorptivity (*a*) 327 mμ, which is a specific identification factor for each ester. The *a* and the wavelength of maximum absorption for each of the esters are presented in Table I. Vitamin A alcohol and acetate are also included in this table for comparison purposes. They were not prepared in this study.

TABLE I—OBSERVED *a* AND $\lambda_{max.}$ IN ISOPROPANOL FOR THE VITAMIN A COMPOUNDS USED IN THIS STUDY

Compd.	m$\mu_{max.}$	*a*
Trimethylacetate	327	130.8
Triethylacetate	327	115.0
α,α-Dimethylvalerate	328	120.9
α,α-Methylethylcaproate	327	118.1
β-Cyclopentylpropionate	326	122.0
Monotetrahydrophthalate	327	118.9
Palmitate	326	93.6
Alcohol	325	183.5[a]
Acetate	326	155.0[a]

[a] These values were obtained from "Merck Index," 7th ed., (1960).

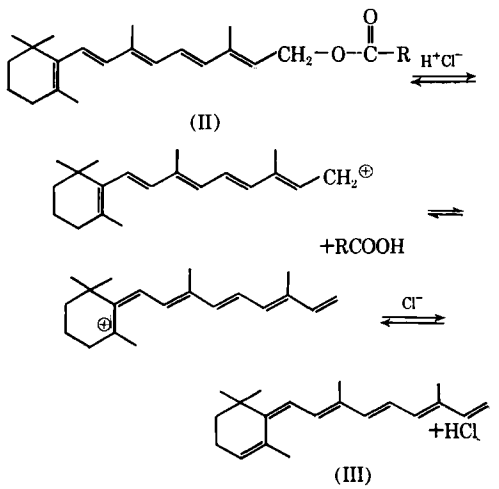
Stability Testing

In order to compare the stability of the hindered esters to that of the palmitate, they were subjected to the tests described below. Since vitamin A palmitate is the most stable ester commercially available, it was chosen as the standard with which to evaluate the new esters. The commercial material, however, was not suitable for these comparative tests, since it contained small quantities of antioxidants. Consequently, the standard used in these experiments was made by the same method used for the other esters.

Stability of Vitamin A Esters in Anhydrous Ethanolic 0.01 *N* HCl—*Part I*—A sufficient quantity of vitamin A ester was added to anhydrous ethanolic 0.01 *N* HCl, such that the initial UV absorbance at the maximum for vitamin A esters (327 mμ) was 0.700. The solutions were maintained at a constant temperature of 22 ± 1°, and changes in UV absorbance at 327 and 391 mμ were measured every 30 min. Anhydrovitamin A, one of the major degradation products under the conditions of this experiment, has a maximum absorbance at 391 mμ and an absorbance at 327 mμ, the maximum for vitamin A, equal to 0.2 the absorbance at 391 mμ. Since vitamin A has a negligible absorption at 391 mμ, the following correction factor for the absorbance for vitamin A at 327 mμ was determined:

$$A_{327\ m\mu\ (corr.)} = \left[A_{327\ m\mu\ (obs.)} - (0.2\ A_{391\ m\mu\ (obs.)})\right]$$

A is the UV absorbance at the specific wavelength. A plot of log $A_{327\ m\mu(corr.)}$, a measure of the concentration of vitamin A in solution, *versus* time was linear; consequently, the first-order rate constants were determined from the slope of this line. Under these conditions, the principal degradation pathway proceeds from the ester (II) to anhydrovitamin A (III), as shown below.



In the following tables, the "stability factor" represents the ratio of the rate constant of the specified ester compared to the rate constant of vitamin A palmitate. The first-order degradation rates are given in Table II.

Stability of Vitamin A Esters in 95% Ethanolic 0.1*N* HCl—*Part II*—The testing procedure used in this case was similar to that used in Part I, except that the solvent here was 0.1 *N* HCl in 95% ethanol. The results are presented in Table III.

Stability of Vitamin A Esters in 70% Ethanolic 0.1 *N* HCl—*Part III*—The testing procedure used in this section is outlined under Part I, except that the solvent here was 0.1 *N* HCl in 70% aqueous ethanol, and the results are presented in Table IV.

Stability of Vitamin A Esters in 70% Ethanol—*Part IV*—Since the degradation rate at 22° was too slow to be measured satisfactorily, the procedure used in this section was similar to that used under Part I, except that the reaction was run at 42 ± 1°, and the solvent here is 70% aqueous ethanol. The results are given in Table V.

Alkaline Hydrolysis of Vitamin A Esters—*Part V*—The method used to determine the resistance to alkaline hydrolysis was a modification of the procedure of Isler *et al.* (2) where 2.0 mmoles of ester was dissolved in 50 ml. of *n*-butanol and 50 ml. of 90% aqueous ethanol containing 2.0 mmoles of NaOH. The solutions were maintained in a constant temperature bath at 42 ± 1° and 10-ml. samples were removed for assay at 45, 80, 170, 235, 285, 330, and 375 min. The quantity of base consumed, a measure of the rate of hydrolysis, was determined by titrating the samples with 0.008 *N* HCl using thymol blue indicator. Since a plot of log concentration *versus* time was linear, it appeared that the reaction followed first-order kinetics. The rates of hydrolysis were determined from the slope of the line and the results are presented in Table VI.

Oxidation Studies—*Part VI*—The first test involved direct exposure of 0.2 ± 0.05 Gm. of accurately weighed pure compound to air at 37°, evenly spread over the surface of a 20-ml. opened Wheaton vial. The rate of oxidation, however, was too rapid to measure accurately; consequently, the experiment was performed at 22°. The amount of vitamin A remaining at any time was determined by dissolving the entire sample in isopropyl alcohol and measuring UV absorbance at 310, 325, and 334 mμ. Since the degradation products of these esters interfered with the direct measurement of vitamin A from the absorbance at 325 mμ, a correction factor (3) was applied to determine the portion of the absorbance at 325 mμ due to vitamin A.

The correction factor used in this experiment to determine the *a* 325 mμ for a 1:25,000 dilution of the sample, is shown below:

$$a_{(\text{corr.})\ (325\ \text{m}\mu)} = \frac{7A_{325(\text{obs.})} - 2.625A_{310(\text{obs.})} - 4.375A_{334(\text{obs.})}}{\text{weight of sample in Gm.}} \times 25$$

A plot of log *a* (corr.) *versus* time was a straight line; consequently, the first-order rate constants were calculated from the slope of the line. The results are shown in Table VII. There did not

Table II—Vitamin A Ester Degradation Rates in Anhydrous Ethanolic 0.01 *N* HCl

Vitamin A Ester	First-Order Rate Constant $(k^1/2.303) \times 10^4$ hr.$^{-1}$	Stability Factor
Palmitate	21.2	1.00
β-Cyclopentylpropionate	21.2	1.00
Monotetrahydrophthalate	11.4	1.85
Pivalate	3.4	6.25
α-Methyl-α-ethylcaproate (MEC)	3.4	6.25
α,α-Dimethylvalerate (DMV)	3.2	6.70
Triethylacetate (TEA)	2.7	7.87

Table III—Vitamin A Ester Degradation Rates in 95% Ethanolic 0.1 *N* HCl

Vitamin A Ester	First-Order Rate Constant $(k^1/2.303) \times 10^4$ hr.$^{-1}$	Stability Factor
β-Cyclopentylpropionate	19.6	0.99
Palmitate	19.4	1.00
Monotetrahydrophthalate	10.0	1.94
Pivalate	6.1	3.15
α-Methyl-α-ethylcaproate	5.0	3.88
α,α-Dimethylvalerate	5.0	3.88
Triethylacetate	4.5	4.30

Table IV—Vitamin A Ester Degradation Rates in 70% Ethanolic 0.1 *N* HCl

Vitamin A Ester	First-Order Rate Constant $(k^1/2.303) \times 10^4$ hr.$^{-1}$	Stability Factor
β-Cyclopentylpropionate	18.4	0.96
Palmitate	17.7	1.00
Monotetrahydrophthalate	11.8	1.50
Pivalate	6.0	2.96
α-Methyl-α-ethylcaproate	5.9	3.00
α,α-Dimethylvalerate	5.4	3.28
Triethylacetate	3.2	5.52

Table V—Vitamin A Degradation Rates in 70% Ethanol at 42°

Vitamin A Ester	First-Order Rate Constant $(k^1/2.303) \times 10^4$ hr.$^{-1}$	Stability Factor
Monotetrahydrophthalate	20.0	0.63
Palmitate	12.5	1.00
β-Cyclopentylpropionate	12.2	1.02
Pivalate	6.5	1.93
α,α-Dimethylvalerate	4.8	2.60
α-Methyl-α-ethylcaproate	2.7	4.63
Triethylacetate	2.2	5.70

Table VI—Rates of Vitamin A Ester Hydrolysis by NaOH in *n*-Butanol–90% Ethanol Mixture

Vitamin A Ester	First-Order Rate Constant $(k^1/2.303) \times 10^5$ min.$^{-1}$	Stability Factor
Palmitate	161.0	1.00
β-Cyclopentylpropionate	58.8	2.73
Pivalate	30.1	4.72
Monotetrahydrophthalate	34.9	5.24
α,α-Dimethylvalerate	13.4	12.05
Triethylacetate	8.1	20.00
α-Methyl-α-ethylcaproate	6.2	26.31

TABLE VII—OXIDATION RATES OF VITAMIN A ESTERS (PURE COMPOUNDS) EXPOSED TO AIR AT 22°[a]

Vitamin A Ester	First-Order Rate Constant ($k^1/2.303$) × 10^4 hr.$^{-1}$	Stability Factor
Palmitate	433.0	1.00
α-Methyl-α-ethylcaproate	228.0	1.90
β-Cyclopentylpropionate	216.0	2.00
Monotetrahydrophthalate[b]	175.0	2.47
Triethylacetate	142.2	3.05
α,α-Dimethylvalerate	71.4	6.06
Pivalate[b]	9.5	43.30

[a] It is noteworthy that the oxidation pattern of the pure compounds is quite different from oxidation in solution. This may be related to differences in physical form which is eliminated by solution studies. [b] Crystalline esters.

appear to be any induction period in this study, however, this is not surprising since no antioxidant was present.

Subsequently, the oxidative stability of these esters was determined under use conditions in Wesson oil solutions with butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) as antioxidants. The esters were dissolved in commercial Wesson oil at a level of 1,000,000 units per gram of solution and the antioxidants at a level normally used in commercial preparations were added. Accurately weighed samples of 0.5 Gm. were placed into 20-ml. Wheaton vials and transferred to a 37° oven with free access to air. Since plots of log concentration of vitamin A *versus.* time in this case were not linear, the length of the "induction period" was considered the criterion of stability. This period represents the time lag before oxidation begins after the sample is exposed to air. Following the induction period, oxidation and destruction of the vitamin A molecule proceeds rapidly. The cyclopentylpropionate and monotetrahydrophthalate were not included in the solution experiments because of their insolubility in Wesson oil at the level of 10^6 units per gram. The lengths of these periods are given in Table VIII.

The oxidation tests in Wesson oil solutions were repeated without antioxidants and at a lower temperature (22°). The same conditions were used as in the previous section, except that the antioxidants were not included. Under these conditions it appeared that esters having the longer chain lengths in the acid portion have a measurable induction period which is not true of the short chain esters. The results are presented in Table IX.

TABLE VIII—INDUCTION PERIODS FOR VITAMIN A ESTER SOLUTIONS IN VEGETABLE OIL WITH ANTIOXIDANTS AT 37° (10^6 UNITS OF VITAMIN A PER GRAM)

Vitamin A Ester	Induction Period, hr.
Palmitate	161
α-Methyl-α-ethylcaproate	150
Triethylacetate	32
α,α-Dimethylvalerate	32
Pivalate	30

TABLE IX—INDUCTION PERIODS FOR VITAMIN A ESTER SOLUTIONS IN VEGETABLE OIL WITHOUT ANTIOXIDANTS AT 22° (10^6 UNITS OF VITAMIN A PER GRAM)

Vitamin A Ester	Induction Period, hr.
Palmitate	53
α-Methyl-α-ethylcaproate	41
α,α-Dimethylvalerate	0
Pivalate	0
Triethylacetate	0

SUMMARY AND CONCLUSIONS

The results of the stability testing of the new esters when compared to vitamin A palmitate indicate the following:

1. Steric hindrance in the α-position of the acid portion of vitamin A esters confers stability with respect to acidic or alkaline degradation.

2. Vitamin A α,α-dimethylvalerate (DMV), α-methyl-α-ethylcaproate (MEC), and triethylacetate (TEA), were four to eight times more stable than the palmitate in anhydrous and hydroalcoholic acidic solutions.

3. The DMV, MEC, and TEA esters were five times more stable than the palmitate in 70% aqueous ethanol.

4. The DMV, MEC, and TEA esters were approximately 20 times more stable than the palmitate in alkaline hydrolysis studies.

5. The α,α-methylethylcaproate was found to be the most stable sterically hindered ester of the series because of its superior stability in solution relative to the palmitate, and its ability to compare somewhat favorably with the latter ester when subjected to air oxidation studies.

6. Since the palmitate and α,α-methylethylcaproate both showed stability, it can be considered that a long aliphatic chain (C_6—C_{16}) in the fatty acid portion is necessary for resistance toward oxidation. A future publication will deal with a sterically hindered ester, having a long aliphatic chain such as vitamin A α,α-dimethylpalmitate.

7. The β-cyclopentylpropionate and monotetrahydrophthalate were not as stable as the sterically hindered aliphatic esters under any of the conditions examined.

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Keyphrases

Vitamin A esters, α,α-dialkyl substituted—synthesis

Degradation rates—sterically hindered vitamin A esters

Column chromatography—separation

IR spectrophotometry—identity

UV spectrophotometry—identity

Preferred Conformation of Serotonin and a Postulate on the Nature of its Receptor from Molecular Orbital Calculations

By LEMONT B. KIER

Using extended Hückel molecular orbital theory, the preferred conformation of serotonin (5-hydroxytryptamine) I has been calculated. The internitrogen distance was calculated to be 5.84 Å. and the quaternary nitrogen to oxygen distance was calculated to be 6.96 Å. The complementary features of the serotonin receptor are predicted and the relationship of serotonin in its preferred conformation to the serotonin antagonist, LSD, is noted as an explanation of LSD's antagonism.

OVER THE past decade, an enormous amount of information has accumulated on serotonin (5-hydroxytryptamine) (I). A recent book on this subject by Garattini and Valzelli brings together much of this material (1). Of considerable interest to many scientists is the nature of the biological receptor interacting with the molecule. Gaddum and his group concluded, after a series of investigations, that the effect of serotonin on smooth muscle is specific and is not related to the effect of acetylcholine or histamine (2–5). The term "tryptamine receptors" was proposed to classify the receptors of the smooth muscle sensitive to serotonin. In reporting a study of antagonists, Barlow suggests that tryptamine and serotonin receptors may not be identical (6). This concept of the specific effect of serotonin is supported by results from numerous studies of known chemical mediators on serotonin-induced smooth-muscle stimulation (1).

A further elaboration of the serotonin-receptor concept was proposed by Gaddum, who offered evidence for two types of serotonin receptors, M and D (4). The M receptors were those blocked by morphine and the D receptors those blocked by dibenzyline. He found that the D receptors, located in the plain muscle fibers, were also blocked by LSD, gramine, or dihydroergotamine, while the M receptors, not accurately localized, were not easily blocked by these drugs (7–8).

Other authors have explained the dual-receptor-type effects by suggesting that serotonin stimulates organs containing smooth muscle by a direct action on the smooth muscle fiber, and by an indirect action on the nervous tissue (9–12).

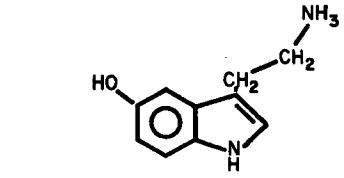
The question of whether serotonin interacts with two different types of receptors or functions both as a direct and indirect acting agent has not been resolved. The two-receptor concept is reminiscent of that proposed for the action of acetylcholine while the direct-indirect action is typified by ephedrine. In connection with the acetylcholine dual-receptor concept, Martin-Smith, Smail, and Stenlake have recently proposed that acetylcholine acts at both receptors (muscarinic and nicotinic) by being able to assume two different conformations (13).

Recently, this problem of the muscarinic and nicotinic actions has been considered by the author in studies on the preferred conformations of acetylcholine, muscarine, muscarone, and nicotine (14, 15). By performing quantum mechanical calculations using extended Hückel theory, the total energy of each molecule was minimized as a function of geometry and calculated preferred conformations were obtained. In the case of acetylcholine, the author reported that the molecule exhibited some degree of conformational flexibility and could exhibit a preferred conformation over a range of one bond-angle variation. At one point in this variable energy minimum, the acetylcholine conformation corresponded to the preferred conformations of muscarine and muscarone. The heteroatoms in each molecule bore a similar relationship to each other and were similarly related to the other muscarinics. From this it was possible to deduce the nature of the muscarinic receptor and to deduce the muscarinic conformation of acetylcholine (14).

It was also found that acetylcholine in a preferred conformation different from its muscarinic conformation placed key atoms in a relationship nearly identical to that of comparable key atoms found for nicotine in its calculated preferred conformation (15).

These studies supported the idea of dual conformers functioning at two different receptors. Further support for this theory was found in the most recent study on histamine (16). In this case, it was found from these calculations that histamine exists in two different preferred conformations, and it was possible to relate one

Received February 26, 1968, from Batelle Memorial Institute, Columbus, OH 43201
Accepted for publication March 21, 1968.



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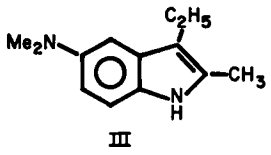

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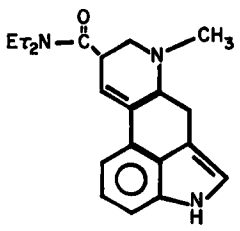

II

preferred conformation to a postulated receptor associated with one histamine action. The second preferred conformation was inferred to be complementary to a second histamine receptor.

From these studies it is possible to propose a theory concerning dual-receptor involvement that provides a rationale for considering the serotonin molecule by quantum mechanical methods. Essentially, it was postulated that *an observed dual action of a molecule may be explained by the presence of two preferred conformers of the molecule in equilibrium.* As a corollary, it can be stated that *if a molecule can exist in two nearly equally preferred conformations, an observed dual action of that molecule can be explained by the interaction of these different conformers with different receptors.*

If these postulates are assumed to be valid, it is readily apparent that quantum-mechanical calculations of the preferred conformation of the serotonin molecule may well lead to some understanding of its activity.

CALCULATIONS

The calculations used in this work have been described in detail elsewhere (14, 17). Briefly, the expansion of a molecular orbital as a linear combination of atomic orbitals yields

$$\Psi_i = \sum C_{ij}\phi_j$$

upon minimizing the total energy, a set of Hückel equations,

$$\sum_{i=j}^{n}[H_{ij} - ES_{ij}]C_{ij} = 0 \qquad j = 1,2 \ldots . n$$

where Ψ is the molecular orbital wave function, H the Hamiltonian operator, E the energy, S the overlap integral, and C the orbital coefficient. The calculations are performed for the valence electrons in s and p orbitals, using Slater orbitals as a basis set. The input parameters consist of the Slater exponent and the Coulomb integral, approximated by the ionization potential. All overlap integrals are considered. The resonance integrals are approximated by the equation

$$H_{ij} = 0.5\,K\,(H_{ii} + H_{jj})S_{ij}$$

with K set at 1.75. The total electronic energy is computed to be the sum of doubly occupied molecular orbitals,

$$\epsilon = 2\sum E_i$$

The total molecular energies can be written as

$$E = 2\sum E_i + \sum_{n,n'} Enn' - \sum_{e,e'} Eee'$$

where Enn' and Eee' are nuclear-nuclear and electron-electron repulsion energies. The success of the calculations in predicting preferred conformations from minimum energies lies in the fact that the method of selecting the H_{ij} values must simulate, within the calculated electronic energies, the contribution of nuclear repulsions to the total energy (17, 18). Thus, the nuclear-nuclear and electron-electron repulsion energies cancel approximately, and the simple sum of one-electron energies behaves similarly to the true molecular energy (19). A characteristic of the extended Hückel theory is the frequently correct prediction of equilibrium conformation with an exaggeration of barrier heights due to overestimation of nonbonded interactions.

For the calculations in this study, it was necessary to input precise three-dimensional coordinates for each atom. This otherwise very difficult task was expedited by a computer program that was obtained from the Indiana University Quantum Chemistry Program Exchange. The program performs a series of vector summations with previously determined planes of atoms as references. The program can be keyed to output the atom coordinates on punched cards in the fields required for the extended Hückel program. The bond angles and lengths used in the coordinates program were derived from standard values proposed by Pople and Gordon (20). Bond lengths were assumed not to vary in the different conformational models. To simulate the molecule in an *in vivo* environment, the author considered the amino-alkyl group as being protonated.

RESULTS

Calculations made every 45° for rotation of the bond joining the side chain to the ring through 180° revealed an energy minimum at $\theta_{ring-C} = 90°$ (Fig. 1). At this angle, the C—C bond of the side chain projects directly away from the plane of the ring system. Throughout this rotational pattern, the side chain is held in an all-*trans* conformation. The energy drop is not severe as the bond is rotated away from the all-planar conformation depicted in 1. An energy difference of 0.1 ev. exists between $\theta_{ring-C} = 45°$ and $\theta_{ring-C} = 90°$ (see Fig. 1). As the side chain rotates past the 90° minimum toward the benzene side of the ring system, the energy rises steeply, with a 1.5-ev. difference between $\theta_{ring-C} = 90°$ and $\theta_{ring-C} = 180°$. As has been pointed out, the energy barriers are exaggerated in these calculations; nevertheless, the values calculated must still be regarded as being considerable, based upon com-

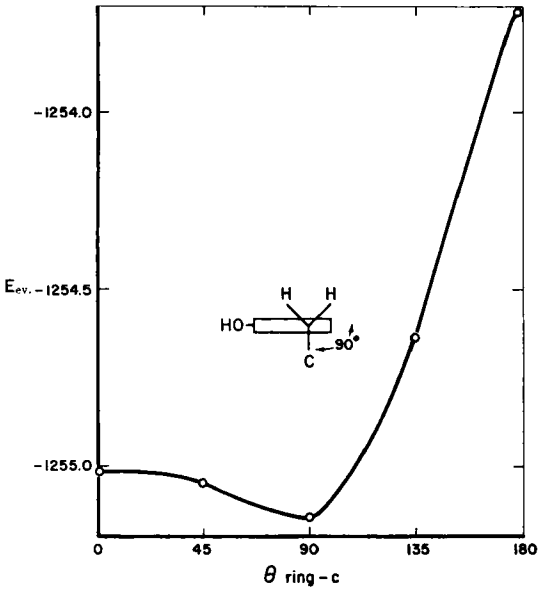

Fig. 1—Angle of rotation of ring to side chain bond vs. *energy.*

parisons of calculated with experimental values (17).

A further search for the energy minimum was conducted by varying the C—C bond of the side chain through 360°. This was done for the $\theta_{ring-C} = 90°$ and the $\theta_{ring-C} = 45°$ conformations. It was felt prudent to consider the latter conformation since the energy difference between the two was not so great as to exclude the 45° conformation as being part of a preferred conformation. The C—C rotation *versus* energy is depicted in Fig. 2. The minimum energy for this rotational model occurred for $\theta_{C-C} = 180°$, or on all-*trans* side chain. This minimum was found for both the $\theta_{ring-C} = 90°$ and the $\theta_{ring-C} = 45°$ conformations, although the former conformation had a substantially lower energy.

At the energy minimum, $\theta_{ring-C} = 90°$, $\theta_{C-C} = 180°$, the internitrogen distance was calculated to be 5.84 Å. The (*tetetete*)N-to-0 distance was calculated to be 6.96 Å. while the ($tr^2trtr\pi$)N-to-0 distance was 5.61 Å. The charge densities are displayed in Fig. 3, in which the values are for ($\sigma + \pi$) electrons. Note that the nitrogen atom of the quaternary group is negatively charged. The author has previously commented on this finding (16). The net charge of the quaternary group (including the α and β atoms) is +1.018.

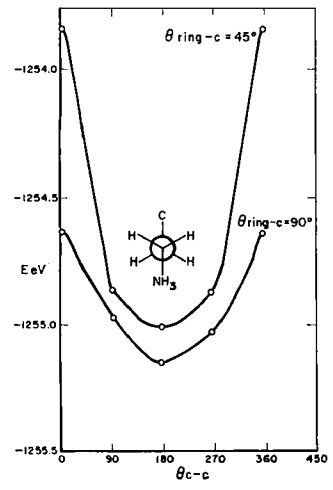

Fig. 2—Angle of rotation of side chain carbon to carbon bond vs. *energy.*

DISCUSSION

From these calculations it was found that serotonin exists in the single-energy preferred conformation shown in Fig. 4. From the energy *versus* angle profiles, it is evident that the molecule has a fair degree of rigidity in this conformation. Thus, a pattern of heteroatoms may be presented to a receptor, as shown in Fig. 5. The choice of these three atoms as being the essential ones for activity is speculative but reasonable. The inclusion of the oxygen in this category is based on the knowledge that, although tryptamine will produce some serotonin effects in the gut, its activity is feeble (2). In addition, as previously noted, antagonist actions suggest that serotonin and tryptamine receptors are different (6).

Because serotonin has only one preferred conformation, as indicated by these calculations, its

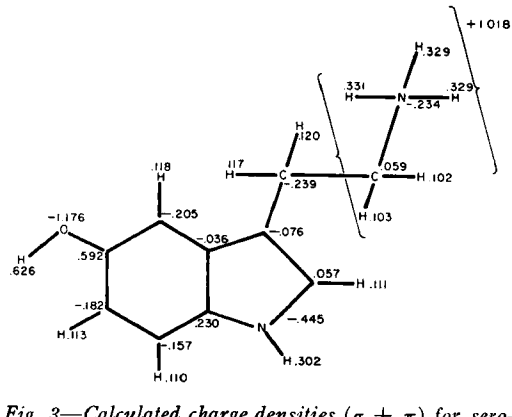

Fig. 3—Calculated charge densities ($\sigma + \pi$) for serotonin.

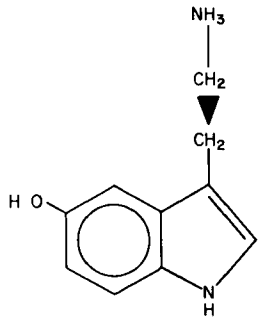

Fig. 4—Calculated preferred conformation of serotonin.

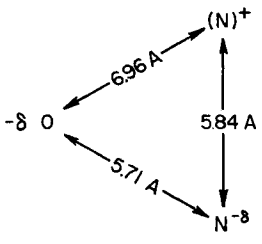

Fig. 5—Structural features proposed as being complementary to the serotonin receptor.

activity cannot be explained by the dual-conformation concept. However, the calculated inability of serotonin to exist in more than one preferred conformation does not completely exclude the possibility that the molecule could interact with two different receptors. There is the remote possibility that this could occur if the molecule presented alternate faces to each of two approximately mirror-image receptor patterns. Thus, each receptor would have its own set of stereospecific requirements for an agonist and each face of the serotonin molecule would satisfy these requirements. It remains to be conclusively demonstrated whether there are two receptors for serotonin. An explanation for the accommodation of serotonin to each is possible from this work. This possibility is considered remote since the quaternary N projects well above the plane of the rings, hence only one side of the molecule presents a planar aspect to a receptor. The alternate side of the molecule does not permit a close approach of the quaternary N if the other nitrogen and the oxygen atoms are anchored to a receptor. The alternative hypothesis of both direct and indirect effects is easier to explain since, as in the case of indirect-acting adrenergic agents, lower stereospecific requirements are present. Thus, only two of the three serotonin heteroatoms may be involved in an indirect action.

A comparison of the calculations reported here with previous calculations on the histamine molecule reveals that the two molecules in their preferred conformations do not have their heteroatoms separated by a similar distance (16). This suggests that the two molecules are not involved with the same receptor.

It is instructive to compare the results of these calculations with the dimensions of the more rigid, potent serotonin antagonists. The most prominent of these compounds is lysergic acid diethylamide (LSD) (II). It is known to be an antagonist for virtually all actions of serotonin (1). Because it is such an effective antagonist, it is reasonable to assume that the molecule successfully binds with enough of the serotonin receptor to exclude serotonin although it does not have the full complement of structural features necessary to produce an efficacious complex. Measurements on accurate scale models of LSD indicate a distance between the amino and the pyrrole nitrogen atoms to be 6.0 ± 0.2 Å. At physiological pH, the *N*-methyl group would be protonated. Therefore, it is evident that these two nitrogen atoms could interact with portions of the serotonin receptor complementary to the quaternary and pyrrole nitrogens of serotonin, based upon their positions in the calculated preferred conformation. Derivatives of lysergic acid would also present these same atoms to the serotonin receptor, which accounts for the prominence of antagonism in this class of compounds.

A second antagonist molecule from which one can readily derive values of interatomic distances is 2-methyl-3-ethyl-5-dimethylaminoindole (medmain) (III) (21). The internitrogen distance in this molecule, estimated from a scale model, is about 5.75 Å. This closely approximates the 5.61 Å. distance calculated to separate the oxygen from the pyrrole nitrogen of serotonin.

From these calculations on the serotonin molecule, it can be concluded that there is a single preferred conformation and that the relative preference is fairly high. In this preferred conformation, the molecule presents three heteroatoms to a receptor in a predictable manner, hence a complementary pattern of forces on the receptor can be deduced. The antagonism of LSD and other inhibitor molecules can now be explained in terms of this postulated receptor, since comparable interatomic distances are found in these antagonist molecules. This finding also tends to verify speculation on the serotonin receptor (Fig. 5).

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Keyphrases

Serotonin—preferred conformation
Single-energy preferred conformation—serotonin
Dual-receptor theory
Receptors proposed—serotonin
LSD antagonism—serotonin

Acceleration of Peritoneal Dialysis by Surface-Active Agents

By S. C. PENZOTTI and A. M. MATTOCKS

Peritoneal dialysis is an effective method of removing endogenous poisons, but it is a slow process. In a search for agents to accelerate dialysis across the peritoneum, a number of wetting agents were tested and used in the peritoneal fluid. Dialysis tests were run on rabbits with radioactive urea and rate constants were calculated from dialysate and blood curves. The anionic and cationic wetting agents had significant effects, while the nonionic wetting agents were much weaker. On the basis of effectiveness and gross toxicity dioctyl sodium sulfosuccinate 0.01 percent was chosen as the most promising accelerator. It was evaluated in more detail and found to accelerate the dialysis of urea to about four times the rate of controls. When tested with radioactive creatinine the acceleration was two to threefold. It was concluded that dioctyl sodium sulfosuccinate is worthy of the further work required to bring it to the stage of clinical trial.

PERITONEAL DIALYSIS is an effective means for removal of endogenous poisons (1). It has the advantage over the artificial kidney of requiring no special equipment or facilities and being a relatively simple technique which might be performed in any physician's office. It has the serious disadvantage of being slow to clear the body of toxic agents, requiring up to 72 hr. in treatment of kidney failure and being so slow in removal of drugs as to have questionable value in suicidal or accidental drug poisonings (2, 3). If the process could be accelerated this would be of utmost importance, making possible the treatment of a larger number of uremic patients in a given institution and offering an effective treatment for drug poisoning in any locality.

In a search for substances which might accelerate diffusion across the peritoneal membrane, wetting agents were among the first type of compound to be considered. It is conceivable that incomplete wetting of the membrane by the dialysis fluid might occur with the common solutions, possibly due to fatty material on the membrane surface or on other tissues where penetration of the fluid to the membrane might be blocked. Also, diffusion acceleration of unknown nature has been observed in laboratory experiments with cellophane membranes when certain wetting agents were added (4). In this laboratory it was found that tris-hydroxymethyl aminomethane (THAM), a substance with weak surface-active properties, accelerated peritoneal dialysis by an unknown mechanism (5).

A group of common wetting agents was selected for test in peritoneal dialysis, including some anionic, cationic, and nonionic compounds. Experiments were planned to observe dialysis rates of urea and gross toxic effects with one to three animals as a screening test, then to select optimum concentrations of any found to be effective, finally to run a series of tests with the selected concentration of the best agents found. Since creatinine as well as urea is used as an indicator of the progress of peritoneal dialysis, it was planned to test the most promising of the agents for its effect on the dialysis of creatinine also.

As was pointed out in a previous paper (5), the effectiveness of peritoneal dialysis is best evaluated by means of a rate constant, since the amount dialyzed in a given time is dependent on the blood-dialysate gradient at each instant of time throughout the period, while the blood level is affected by urinary excretion and metabolism as well as diffusion into dialysate. Thus, experiments were set up to measure both blood level and dialysate level over the period of experiment, allowing the rate constant to be calculated from suitable equations.

MATERIALS AND METHODS

Healthy, mature male albino rabbits were selected as the experimental animal. No general anesthesia was used, but 50 mg. of chlorpromazine hydrochloride was administered intramuscularly 20 min. prior to the beginning of the experiment. A urinary catheter was inserted and 1 Gm. of ^{14}C-tagged urea (200 mg./ml.) was injected intravenously. Twenty to 30 min. was allowed for distribution of the drug throughout the body fluids before beginning dialysis, then 150 ml. of dialysis fluid was introduced into the peritoneum by means of a pediatric-size peritoneal catheter. Blood samples were taken from the marginal ear vein. Dialysate samples were withdrawn through the peritoneal catheter, taking care to return contents of the outlet tubing and catheter after removal of the sample. Samples of blood and dialysate were removed at 10–15 min. intervals over periods up to 200 min. Urine

Received September 19, 1967, from the School of Pharmacy, University of North Carolina, Chapel Hill, NC 27515
Accepted for publication November 29, 1967.
Supported in part by grant 1-RO1-AC-00312 from the National Center for Urban and Industrial Health, U. S. Public Health Service, Bethesda, Md.
The authors wish to acknowledge with gratitude the valuable assistance of Jane Turner Queen and Harry F. McArver in the laboratory procedures.

samples were taken but are not used in this analysis and are not presented here. With creatinine dialysis the same procedure was used except that 0.5 Gm. of ^{14}C-tagged creatinine (50 mg./ml.) was injected and the beginning of dialysis was delayed until about 1 hr. after injection in most cases.

The urea injection was prepared by dissolving an amount of radioactive urea equivalent to about 70 microcuries and sufficient stable urea to make 10 Gm. total urea in bacteriostatic water for injection (0.9% benzyl alcohol) to make 50 ml. This was sterilized by filtration and stored in the refrigerator.

Creatinine injection was prepared by dissolving an amount of creatinine-1-^{14}C equivalent to about 70 microcuries and sufficient stable creatinine to make a total of 5 Gm. in water for injection, then adding 0.2 ml. of 0.2 *N* hydrochloric acid and sufficient water for injection to make 100 ml. The solution was filtered, sealed in multidose injection vials, and autoclaved. The acid and heat were used to insure that any traces of creatine would be converted to creatinine.

The dialysate solution used for control was a commercial formula, Inpersol No. 46244—Abbott containing dextrose 1.5%, sodium lactate 0.5%, sodium chloride 0.56%, calcium chloride USP 0.026%, and magnesium chloride hexahydrate 0.015%. The surface-active agents were merely dissolved in this fluid to prepare the test solutions, except where concentrations of wetting agents were high enough to alter significantly the osmolality. In such cases the same formula was used except that sufficient dextrose or dextrose and sodum chloride was deleted from the formula to balance the number of moles of added substance. The only fluids of this type of the data tables of this paper are those containing THAM.

The wetting agents used, their grade and source were: sodium desoxychlorate, purified, (Fisher); polysorbate 60 and polysorbate 85,[1] pharmaceutical grade; trishydroxymethyl aminomethane (THAM), primary standard (Fisher); sodium lauryl sulfate USP (Amend); benzalkonium chloride[2] 17% solution; dioctyl sodium sulfosuccinate (DSS), solid, (Fisher); cetyl trimethyl ammonium bromide (CTAB), technical, (Matheson); and cetyl trimethyl ammonium chloride (CTAC), practical, (Fisher).

Measurements of concentrations in blood and dialysate were made by standard radioactive counting methods. For blood, a 0.2-ml. sample of whole blood or serum was added to 1 ml. of 6% trichloroacetic acid in a centrifuge tube and immediately shaken vigorously, then centrifuged. A 0.5-ml. aliquot of the clear supernatant was added to 10 ml. of dioxane phosphor solution and counted. For dialysate, 0.5 ml. of the solution was transferred to the counting vial containing 10 ml. of phosphor solution for counting. An accurate dilution of the injection used served as a standard which was counted with each set of samples. The dioxane phosphor solution contained 0.7% PPO, 0.005% POPOP, and 5% reagent naphthalene in spectro quality dioxane. All samples were counted with 1% error or better in a Packard 314-X Tri-Carb liquid scintillation counter and corrected for quenching by channels ratio.

[1] Marketed as Tweens 60 and 85 by Atlas Chemical Corp., Wilmington, Del.
[2] Marketed as Zephiran by Winthrop, New York, N. Y.

RESULTS AND TREATMENT OF THE DATA

The dialysis rate constant describing the diffusion from blood to dialysis fluid is depicted as k_1 in the following equation:

$$\begin{array}{c} \overset{k_1}{\rightleftarrows} \\ \mathrm{B} \rightleftarrows \mathrm{D} \\ k_3 \downarrow \quad k_2 \\ \mathrm{U} + (\mathrm{M}) \end{array} \qquad \text{(Eq. 1)}$$

where B represents the compound, urea or creatinine, in blood, D, in dialysate, and U and M represent urinary excretion and metabolism, respectively. Values of k_1 were calculated from the equations of Rescigno and Segre (6) as previously described (5) using an analog computer. These rate constants are tabulated in Table I. Figures 1 and 2 illustrate curves obtained with controls and effective accelerating agents, respectively.

DISCUSSION AND CONCLUSIONS

In the design of these experiments, it was planned to run each test solution on a minimum number of animals as a screening test, then to run repeated tests with more animals where comparisons of rate constants with controls were desired. Thus, only in the cases where the agents showed promise as accelerators were more than two tests run. Substances were judged to be promising if no gross toxicity was evident and the rate constants were two to three times those of controls.

From the data it is seen that polysorbate 60, a nonionic wetting agent, appeared to have a weak effect at relatively high concentration. The low concentration of polysorbate, 0.01%, was run to determine whether this solution, with surface tension almost as low as the 1.8% solution, might give an equal effect. Since it did not, it appears, with this limited experience, that the polysorbate effect was not due simply to lowered surface tension. Polysorbate plus THAM was run to obtain an indication as to whether the two compounds affect dialysis by the same mechanism. Since the results showed a higher rate than either had alone, the indication, based on limited data, is that the two mechanisms are not the same.

In general, results show that both anionic and cationic wetting agents increase the rate of dialysis of urea across the peritoneal membrane and that the effect reaches a maximum, with higher concentrations yielding no appreciable increase in dialysis rate. The cationic agents, CTAB, CTAC, and benzalkonium chloride were all effective with the maximum in each case being at about 0.02%. With these agents, however, toxic effects and deaths were detected at concentrations 2–3 times as high as the most effective level. With benzalkonium chloride, deaths resulted with 0.02%. All deaths observed were similar, respiratory in nature, accompanied by fall in blood pressure, convulsions, appearance of a deep orange to red color in urine and dialysis fluid, and occasionally appreciable hemolysis as seen in the hematocrit. Because of the toxic manifestations observed at levels close to the effective concentrations, attention was focused on the less toxic anionic wetting agents.

TABLE I—DIALYSIS RATE CONSTANTS, k_1, FOR UREA AND CREATININE DIALYSIS WITH VARIOUS PERITONEAL FLUIDS

Fluid	Animal No.	Weight, Kg.	k_1, min.$^{-1}$ × 10^2
Urea Dialysis			
Polysorbate 60 1.8%	8	3.51	1.3
	9	3.08	1.7
Polysorbate 60 1.8% plus THAM 1.8%	10	3.32	3.9
Polysorbate 60 0.01%	11	2.55	0.5
Polysorbate 85 1.8%	29	2.79	1.1
Dimethylsulfoxide 1.2%	12	3.60	1.9
Benzalkonium chloride 0.02%	20	4.27	5.0[a]
	21	3.19	4.9
	22	3.13	3.9[a]
	23	3.51	4.4[a]
	24	3.61	4.8
Average		3.54	4.6
Benzalkonium chloride 0.1%	19	3.56	5.9[a]
CTAB 0.02%	33	2.79	5.3
	34	4.15	4.2
	35	4.43	4.9
	36	2.84	3.3
	37	2.77	4.9
Average		3.40	4.5
CTAC 0.02%	42	2.49	3.7
	45	3.07	2.8
CTAC 0.01%	43	3.05	2.6
	46	3.33	3.4
CTAC 0.05%	47	2.90	4.9
	48	3.58	4.6
Sodium lauryl sulfate 0.2%	27	2.87	2.8
	28	3.27	7.7
	30	3.33	3.1
	31	2.45	2.7
	32	4.02	5.3
Average		3.19	4.3
DSS 0.01%	16	2.63	4.4
	17	3.19	4.2
	18	3.61	3.4
	25	3.99	4.6
	26	4.08	5.3
	50	3.13	3.5
	51	2.98	4.5
	53	3.34	4.9
	55	4.26	3.0
	57	3.49	2.9
Average		3.47	4.1
DSS 0.18%	13	3.42	3.7
DSS 0.033%	14	3.80	5.0
DSS 0.02%	15	2.87	5.0
DSS 0.04%	38	2.79	4.3
	44	3.04	4.6
DSS 0.001%	39	4.02	0.8
DSS 0.005%	40	2.86	2.5
	41	3.96	3.1
Control	1	3.61	0.5[b]
	2	3.87	0.6[b]
	3	4.46	0.9[b]
	4	3.96	1.2[b]
	5	4.26	1.0[b]
	6	2.09	1.3[b]
	7	1.90	1.3[b]
	49	2.51	1.3
	52	2.71	0.8
	54	3.44	1.1
	56	3.78	1.0
	58	3.55	1.2
Average		3.34	1.0
Creatinine Dialysis			
DSS 0.01%	61	3.22	2.0
	63	2.79	1.5
	66	3.53	1.6
	67	3.97	1.3
Average		3.38	1.6
Control	59	2.45	0.48
	60	3.21	0.63
	62	3.27	0.84
	64	3.00	0.77
	65	3.10	0.53
Average		3.01	0.62

[a] Animal died shortly after last sample taken. [b] Controls taken from earlier data (5).

The difference in effect of CTAB and CTAC is unexpected, but since both are mixtures rather than pure compounds, it is presumed that certain effective components are present in greater proportions in one mixture than the other. The bromide ion would be undesirable in dialysis fluid since bromism might result. It was for this reason that the chloride was tested.

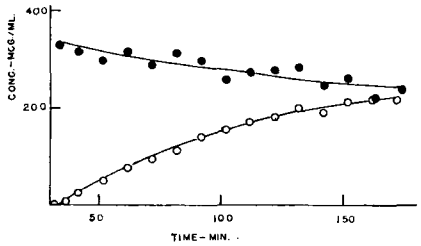

Fig. 1—Dialysis with control fluid, animal no. 54. Key: ●, blood; ○, dialysate.

Among the anionic agents tested, sodium lauryl sulfate, DSS, and sodium desoxycholate, DSS was the most effective. No gross toxicity was observed

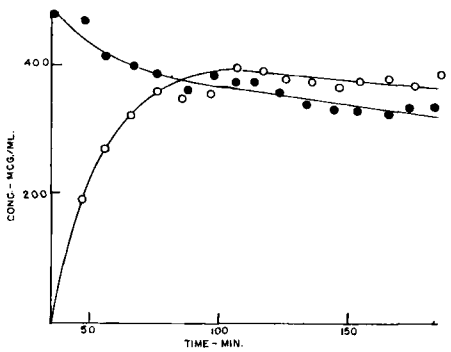

Fig. 2—Dialysis with an effective wetting agent, 0.01% DSS, animal no. 16. Key: ●, blood; ○, dialysate.

at levels five times the concentration of maximum activity, 0.01%. Sodium lauryl sulfate required a concentration of 0.2% to yield dialysis rate roughly equivalent to those with 0.01% DDS. Higher concentrations of sodium lauryl sulfate were not tested, since the 0.2% caused irritation in the abdomen evidenced by squirming and discomfort of the animal when the solution was introduced. It did not show any evidence of permanent damage, however. The rates obtained with sodium lauryl sulfate varied more than with any other agents used.

From these experiments it was concluded that DSS was the most promising of the wetting agents, accelerating the dialysis rate for urea to about four times that of the controls. This would constitute a significant improvement in the dialysis process.

Since DSS appeared to be the most promising agent it was also tested for its effects on the dialysis of creatinine. As seen in Table I, it also accelerates the removal of the compound, rates being about three times the controls.

The experiments with sodium desoxycholate were quite surprising. Death resulted with concentrations as low as 0.01%, and when lower concentrations were tested the effect on dialysis rate decreased as toxicity appeared to decrease. At nontoxic levels no acceleration of dialysis was observed.

The results of this work indicate that DSS may be a useful accelerator of peritoneal dialysis and is deserving of further work. The compound accelerates the dialysis of urea and creatinine, but whether it also accelerates the removal of the multitude of other substances, some known, some unknown, which accumulate in uremia must be determined by experiments which assess the effect of general uremic symptoms and long-term treatment of the diseased condition. Before DSS can be evaluated in man, of course, a measure of its absorption and accumulation in the body must be made and correlated with possible toxic effects which might result. It seems possible that once a given level of DSS is established in the system that the concentration in the dialysis fluid might be reduced without loss of effect. These are problems which must be faced in further work leading toward clinical trials.

This work also suggests the possibility of using DSS or other wetting agents in the removal of drugs such as salicylates and barbiturates which are commonly encountered in poisonings. This study is being conducted in this laboratory.

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Keyphrases

Peritoneal dialysis
Surfactants in dialysis solutions
Dialysis acceleration—surfactants
^{14}C-labeled urea—test compound
^{14}C-labeled creatinine—test compound
Liquid scintillation counting—analysis

Studies with Ion-Exchange Resins on Cinchona Alkaloids II

Effects of the Ionic Form and of the Solvent Medium

By C. V. BHAT, B. R. KAMATH, R. S. SHAH, S. S. KANHERE, and S. L. BAFNA

The effect of the ionic form of the sulfonic acid cation-exchange resins of different degree of crosslinking and of the added sulfuric acid on the equilibrium exchange of four cinchona alkaloid sulfates as well as the equilibrium uptake of four cinchona alkaloid bases by the hydrogen form of the same resins from six aliphatic alcohols has been studied and the results are discussed.

EARLIER (1) the equilibrium exchange of four cinchona alkaloid (quinine, quinidine, cinchonine, and cinchonidine) sulfates on styrene divinylbenzene copolymer based sulfonic acid cation-exchange resins of different degree of crosslinking and particle size in hydrogen form had been studied. This includes the study on the effect of the ionic form of such resins and of added sulfuric acid on the equilibrium exchange of the four cinchona alkaloid sulfates as well as the

Received October 16, 1967, from the Department of Chemistry, M.S. University of Barada, Barada, India.
Accepted for publication April 10, 1968.

at levels five times the concentration of maximum activity, 0.01%. Sodium lauryl sulfate required a concentration of 0.2% to yield dialysis rate roughly equivalent to those with 0.01% DDS. Higher concentrations of sodium lauryl sulfate were not tested, since the 0.2% caused irritation in the abdomen evidenced by squirming and discomfort of the animal when the solution was introduced. It did not show any evidence of permanent damage, however. The rates obtained with sodium lauryl sulfate varied more than with any other agents used.

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Since DSS appeared to be the most promising agent it was also tested for its effects on the dialysis of creatinine. As seen in Table I, it also accelerates the removal of the compound, rates being about three times the controls.

The experiments with sodium desoxycholate were quite surprising. Death resulted with concentrations as low as 0.01%, and when lower concentrations were tested the effect on dialysis rate decreased as toxicity appeared to decrease. At nontoxic levels no acceleration of dialysis was observed.

The results of this work indicate that DSS may be a useful accelerator of peritoneal dialysis and is deserving of further work. The compound accelerates the dialysis of urea and creatinine, but whether it also accelerates the removal of the multitude of other substances, some known, some unknown, which accumulate in uremia must be determined by experiments which assess the effect of general uremic symptoms and long-term treatment of the diseased condition. Before DSS can be evaluated in man, of course, a measure of its absorption and accumulation in the body must be made and correlated with possible toxic effects which might result. It seems possible that once a given level of DSS is established in the system that the concentration in the dialysis fluid might be reduced without loss of effect. These are problems which must be faced in further work leading toward clinical trials.

This work also suggests the possibility of using DSS or other wetting agents in the removal of drugs such as salicylates and barbiturates which are commonly encountered in poisonings. This study is being conducted in this laboratory.

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Keyphrases

Peritoneal dialysis
Surfactants in dialysis solutions
Dialysis acceleration—surfactants
^{14}C-labeled urea—test compound
^{14}C-labeled creatinine—test compound
Liquid scintillation counting—analysis

Studies with Ion-Exchange Resins on Cinchona Alkaloids II

Effects of the Ionic Form and of the Solvent Medium

By C. V. BHAT, B. R. KAMATH, R. S. SHAH, S. S. KANHERE, and S. L. BAFNA

The effect of the ionic form of the sulfonic acid cation-exchange resins of different degree of crosslinking and of the added sulfuric acid on the equilibrium exchange of four cinchona alkaloid sulfates as well as the equilibrium uptake of four cinchona alkaloid bases by the hydrogen form of the same resins from six aliphatic alcohols has been studied and the results are discussed.

EARLIER (1) the equilibrium exchange of four cinchona alkaloid (quinine, quinidine, cinchonine, and cinchonidine) sulfates on styrene divinylbenzene copolymer based sulfonic acid cation-exchange resins of different degree of crosslinking and particle size in hydrogen form had been studied. This includes the study on the effect of the ionic form of such resins and of added sulfuric acid on the equilibrium exchange of the four cinchona alkaloid sulfates as well as the

Received October 16, 1967, from the Department of Chemistry, M.S. University of Barada, Barada, India.
Accepted for publication April 10, 1968.

equilibrium uptake of the four cinchona alkaloid bases by the same resins in hydrogen form from six aliphatic alcohols at room temperature (~30°).

EXPERIMENTAL

Resins (1, 2)—Styrene divinylbenzene copolymer based sulfonic acid cation-exchange resins of degree of crosslinking (% nominal divinylbenzene content) as 2, 4, and 8 (further referred to as resins X2, X4, and X8)[1] of −20, +50; −50, +100; and −50, +100 mesh size, respectively. The resins were conditioned, regenerated, air dried, and moisture content and capacity were estimated (3, 4). The percent moisture and the capacity in meq. per oven-dry Gm. were: X2, 20.4, 5.20; X4, 24.0, 5.12; and X8, 27.0, 5.09. The different ionic forms of the resins were obtained by passing excess of the salt or hydroxide solution through the resin bed. The resins were then washed, filtered, air dried, moisture content determined, and capacity of the air-dry resin calculated from the oven-dry capacity in the hydrogen form.

Resin IR-200[2] of −10, +60 mesh size, which is presumably styrene divinylbenzene copolymer based sulfonic acid cation-exchange resin of relative degree of crosslinking of about 20 but has an expanded structure. The resin was conditioned, regenerated, air dried, and percent moisture content and capacity in meq. per oven-dry Gm. were 25.5 and 4.78.

Chemicals and Solvents (5)—Quinine sulfate (Government quinine factory, Madras), quinidine sulfate (P. B. Howard, London), cinchonine sulfate (B. D. H. London), and cinchonidine sulfate (Fluka, A.G., Switzerland) were crystallized several times from hot water and stored in well-stoppered colored bottles. The composition was $Q_2H_2SO_4 \cdot nH_2O$ where Q denotes the alkaloid base. This was established from estimation of water content by drying in an oven (100 ± 3°), sulfate estimation as barium sulfate gravimetrically and estimation of the precipitated alkaloid base by weighing and by ultraviolet absorption in alcoholic solution.

For the preparation of the cinchona alkaloid bases about 0.5 *N* sodium hydroxide solution was added to alkaloid sulfate solution in slight excess, heated to about 80°, filtered hot, washed free of alkali, recrystallized (quinine from 40% v/v aqueous ethyl alcohol, quinidine from 80% aqueous ethyl alcohol, and cinchonine and cinchonidine from distilled ethyl alcohol), dried at 100 ± 2°, and stored.

The alcohols were of C.P. grade. Absolute ethyl alcohol was prepared by refluxing distilled ethyl alcohol in the presence of anhydrous calcium oxide for 6 hr. and then distilling it.

Procedure—The alkaloid sulfate (1 mole equal to two equivalents) solutions were prepared by dissolving weighed amounts of the respective alkaloid sulfates (1) in distilled water in volumetric flasks. The concentration of the solutions was rechecked gravimetrically by sulfate estimation as barium sulfate and by ultraviolet absorption (5). The alkaloid base (1 mole equal to one equivalent) solutions were prepared by dissolving the weighed amount of the alkaloid bases in the respective alcohols in the volumetric flasks. The concentration of the solutions was rechecked by ultraviolet absorption. To study the equilibrium of alkaloid sulfates and bases with the resins, weighed amounts of air-dried resins were contacted with the aqueous sulfate solutions or the solutions of the bases in the alcohols of known concentration in well-stoppered flasks with frequent shaking at room temperature (~30°). Preliminary work was carried out to find the approximate time within which equilibrium was attained. After sufficiently more time than this (about 4–40 days depending on X), the solutions were analyzed for alkaloid concentration by ultraviolet absorption. The ultraviolet absorption measurements were made with a Beckman model DU spectrophotometer using 10-mm. matched quartz cells. Preliminary work also indicated that for small changes in temperature the value of P_R did not change significantly.

[1] Dowex 50W, Dow Chemical Co., Midland, Mich.
[2] Amberlite 1R-200, Rohm and Haas Co., Philadelphia, Pa.

RESULTS AND DISCUSSION

Effect of the Ionic Form of the Resin—The synthetic organic ion-exchange resins (6, 7) consist of an irregular, three-dimensional network of hydrocarbon chains, to which ionogenic groups are attached and the surplus electric charge is balanced by mobile counter ions. The hydrocarbon network is hydrophobic but the ionogenic groups are hydrophilic, and hence when the resin particle is placed in water, it sorbs water and swells to a limited extent. The amount of water sorbed and the extent of swelling would depend on the degree of crosslinking and the counter ions.

The equilibrium exchange of the four cinchona alkaloid sulfates in aqueous solution with resins X2, X4, and X8 in lithium, sodium, potassium, magnesium, and aluminum forms was studied and the calculated values of P_R were practically constant when P_A was varied. Table I gives an illustration of the type of results obtained, and the values of P_R obtained from such data are given in Table II.

It is suggested that the value of the equilibrium

Table I—Equilibrium Exchange of Quinine Sulfate with Resin X4 in K Form

$[\bar{M}]_i$	$[A]_i$	$[\bar{A}]_e$	P_A	P_R
1.32	2.16	0.57	26.4	43.2
2.21	2.16	0.97	44.9	43.9
3.08	2.16	1.33	61.6	43.2
3.34	2.08	1.44	69.2	43.1
4.46	2.08	1.92	92.3	43.1

Table II—Values of P_R for Equilibrium Exchange of Cinchona Alkaloid Sulfates with Resins in Different Ionic Forms

Ionic Form →		Li	Na	K	Mg	Al
Alkaloid Sulfate	Resin			P_R		
Quinine	X2	65	65	61	50	31
	X4	50	45	43	36	25
	X8	37	30	28	23	22
Quinidine	X2	66	62	56	44	28
	X4	51	43	39	32	20
	X8	38	29	27	21	18
Cincho-	X2	—	82	68	—	—
nine	X4	—	65	60	—	
	X8	—	42	44	—	—
Cinchoni-	X2	95	85	70	—	—
dine	X4	75	62	62	—	—
	X8	51	47	42	—	

constant for the exchange of the cation QH^+ is large and the equilibrium

$$\overline{M^{+y}} + y\overline{QH^+} \rightleftharpoons M^{+y} + y\overline{QH^+}$$

($\overline{M^{+y}}$ and $\overline{QH^+}$ denote the cations in the resin phase and M^{+y} and QH^+ denote the cations in the solution phase; y denotes the valence of M) is shifted very much to the right; however, all the replaceable ions in the resin phase are not accessible for exchange or the available capacity is only a fraction of the total capacity, probably due to the size of the organic counter ions. It is likely that the marked shift of the equilibrium to the right is aided by nonexchange interactions (1, 3).

For resins X2, X4, and X8 in lithium, sodium, and potassium forms, the values of P_R for the four cinchona alkaloid sulfates are in the order Li > Na > K which is also the order of the values of water content of the swollen resins (6, 7). For resins in the ionic forms of ions of different valence (sodium, magnesium, and aluminum), the order of the P_R values is monovalent > divalent > trivalent. The value of P_R decreases as the degree of crosslinking of the resin increases. This may be attributed to the decrease in the swollen volume of the resin as the degree of crosslinking increases and hence the number of accessible exchange sites for the organic cations in the interior of the resin decreases.

In general, the results (Table II) indicate that the P_R values for the optical isomers (quinine, quinidine cinchonine, and cinchonidine) are not substantially different. But the values of P_R for quinine and quinidine sulfates are lower than those for cinchonine and cinchonidine sulfates for the resin in the same ionic form. A possible reason for this should be the difference in the size of quinine or quinidine as compared to that of cinchonine or cinchonidine.

Effect of Added Sulfuric Acid—The equilibrium exchange of the four cinchona alkaloid sulfates with the hydrogen form of the resins X9, X8, and IR-200 with added sulfuric acid (1 N and 0.1N) was studied at room temperature. Table III gives the effect of added sulfuric acid on the equilibrium exchange of quinidine sulfate and cinchonine sulfate. The results for quinidine sulfate and cinchonine sulfate were practically the same as those for quinine sulfate and cinchonidine sulfate.

The values of P_R either decrease to a small extent or are constant with increase in P_A. The nonexchange interactions would tend to increase the extent of exchange with increase in $[A]_e$. This may be feasible to some extent by further expansion of and/or further separation between the segments in the swollen or expanded resin network and should depend on the degree of crosslinking and the extent to which the exchange has already occurred. If the further expansion and/or segment separation is sufficient to accommodate some more organic cations by exchange, P_R should increase with increase in $[A]_e$ or decrease in P_R. On the other hand, if the further expansion and/or segment separation is too small to accommodate some more organic cations by exchange, P_R should remain practically constant with increase in $[A]_e$ or decrease in P_A. The difference in the values of P_R of quinine sulfate and quinidine sulfate and those of cinchonine sulfate and cinchonidine sulfate is either relatively small or practically nil when the concentration of the added sulfuric acid is relatively large (0.1 N to 1 N). The difference in the values of P_R in the presence and absence of added sulfuric acid may possibly be due to the regenerative effect of H^+ ions and the deswelling of the resin particles for the gel-type resins (X4 and X8) and mainly due to the former for expanded structure resin IR-200.

Table IV—Equilibrium Uptake of Quinine from *n*-Propyl Alcohol by Resin IR-200 in Hydrogen Form

$[\bar{H}]_i$	$[A]_i$	$[\bar{A}]_e$	P_A	P_R
11.9	14.4	2.73	19.0	23.0
20.2	14.4	4.68	32.5	23.2
25.2	14.4	5.95	41.3	23.6
27.4	14.4	6.40	44.4	23.4
31.6	14.4	7.42	51.5	23.5
37.7	14.4	8.97	62.3	23.8

Effect of Solvent Medium (Aliphatic Alcohols)—The equilibrium uptake of the four alkaloid bases, quinine, quinidine, cinchonine, and cinchonidine, from dilute solution in six aliphatic alcohols (methyl alcohol, absolute ethyl alcohol, *n*-propyl alcohol, *n*-butyl alcohol, *t*-amyl alcohol, and *n*-hexyl alcohol) by resins X2, X4, X8, and IR-200 in hydrogen form was studied at room temperature. The value of P_R was practically independent of P_A. Table IV gives an illustration of the type of results obtained. The values of P_R obtained from such data are summarized in Table V; it also includes the values of P_R obtained for *n*-amyl alcohol by plotting P_R against the number of straight chain carbon atoms in the *n*-alcohol molecule for each resin.

The values of P_R for the optical isomers were not significantly different. The values of P_R for quinine or quinidine are different from those for cinchonine or cinchonidine in methyl alcohol. In ethyl alcohol (absolute) and *n*-propyl alcohol the values of P_R for the four bases are fairly close for each resin.

Table III—Effect of Added Sulfuric Acid on the Equilibrium Exchange of Cinchona Alkaloid Sulfates with Sulfonic Acid Resins in Hydrogen Form

Alkaloid →	Quinidine Sulfate						Cinchonine Sulfate					
H_2SO_4 →	0.1 N			1 N			0.1 N			1 N		
Resin	$[\bar{A}]_e$	P_A	P_R	$[\bar{A}]_e$	P_A	P_R	$[\bar{A}]_e$	P_A	P_R	$[\bar{A}]_e$	P_A	P_R
X4	0.53	51.2	45.3	0.23	22.5	29.7	0.37	38.3	46.6	0.29	30.5	25.2
	0.69	66.3	44.3	0.34	32.5	28.8	0.66	68.3	42.1	0.36	37.9	23.7
	0.84	81.2	43.4	0.52	50.2	26.9	0.80	83.2	40.8	0.42	43.9	21.7
X8	0.30	29.0	39.8	0.11	10.4	29.3	0.31	32.3	42.8	0.22	23.1	29.9
	0.45	42.6	39.5	0.41	39.3	27.6	0.46	48.4	41.3	0.40	41.4	26.8
	0.59	55.9	39.1	0.59	56.5	26.4	0.60	62.1	39.8	0.47	49.4	25.5
	0.72	68.6	38.4	—	—	—	0.71	73.8	38.0	—	—	—
IR-200	0.31	33.3	25.3	0.26	27.3	16.9	0.34	34.7	27.4	0.36	36.5	17.4
	0.44	46.6	25.2	0.34	36.4	16.5	0.46	47.1	26.8	0.45	46.3	16.7
	0.63	67.5	24.4	0.59	63.4	15.3	0.57	58.5	26.1	0.55	55.7	15.9

TABLE V—VALUES OF P_R FOR THE UPTAKE OF CINCHONA ALKALOID BASES FROM ALIPHATIC ALCOHOLS BY SULFONIC ACID CATION-EXCHANGE RESINS IN HYDROGEN FORM

Alkaloid → / Resin → / Alcohol	Quinine or Quinidine				Cinchonine or Cinchonidine			
	X2	X4	X8	IR-200	X2	X4	X8	IR-200
Methyl	42	33	23	30	67	38	26	34
Ethyl (abs.)	34	26	16	30	—	25	17	33
n-Propyl	23	16	09	27	24	18	11	31
n-Butyl	24	15	07	28	32	20	09	33
n-Amyl	29	14	07	30	44	25	10	33
tert-Amyl	18	11	08	28	25	23	13	37
n-Hexyl	37	14	08	32	63	29	11	39

With further increase in the chain length of the alcohol molecule (*n*-butyl and *n*-hexyl alcohols), the difference in the P_R values of quinine and quinidine and those of cinchonine or cinchonidine again becomes significant. A possible reason for this type of behavior should be the size of the organic counter ions relative to the swollen volume of the resin particle in the different alcohols. The value of P_R decreases with increase in the value of X for the gel-type resins X2, X4, and X8 due to the decrease in the swollen volume of the resin with increase in X. With increase in the chain length of the alcohol molecule, for resin of lower X, X2, the value of P_R for the four bases first decreases and then increases. With increase in the value of X, that is for resins X4 and X8, with increase in the chain length of the alcohol molecule, the value of P_R first decreases and then remains almost unchanged. With resin IR-200 which has expanded structure, the value of P_R first decreases and then increases with increase in the chain length of the alcohol molecule. The comparison of the values of P_R for *t*-amyl alcohol and the calculated values of P_R for *n*-amyl alcohol indicates that for resin of lower degree of crosslinking, X2, the value for *n*-amyl alcohol is higher than that for *t*-amyl alcohol. For resins of higher degree of crosslinking, X4 and X8 and for resin IR-200, the values of P_R for *n*- and *t*-amyl alcohols are fairly close. The variation in the values of P_R for the series of alcohols should be attributed to the variation in the swollen volume of the resin in the alcohols.

Nomenclature—

$[A]_i$ = initial concentration of the alkaloid sulfate or base solution in meq./l.

$[\bar{A}]_e$ = the meq. of alkaloid in the resin phase per liter of solution, at equilibrium.

$[A]_e$ = the meq. of alkaloid sulfate or base in the solution outside the resin phase, per liter of solution, at equilibrium.

$[\bar{M}]_i$ = the meq. of resin per liter of the solution in the M form, initially.

P_A = $100[\bar{A}]_e/[A]_i$ = the % exchange of alkaloid sulfate or base at equilibrium.

P_R = $100[\bar{A}]_e/[\bar{M}]_i$ = the % resin capacity exchanged at equilibrium.

X = the relative degree of crosslinking (% nominal divinylbenzene content).

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Keyphrases

Cinchona alkaloids
Ion-exchange resins, cinchona alkaloids—equilibrium exchange
UV spectrophotometry—analysis

Effect of pH and Dye Concentration on the Extraction of a Thiamine Dye Salt by an Organic Solvent

By V. DAS GUPTA*, DONALD E. CADWALLADER, HARVEY B. HERMAN†, and IRWIN L. HONIGBERG

The effect of pH and dye concentration on the extraction of thiamine dye salt by an organic solvent has been studied. An equation which expresses the dependence of the thiamine dye salt extracted with changes in pH was derived. The theoretical and experimental data were found to be in good agreement.

In a previous report (1) concerning an acid dye assay method for thiamine using bromothymol blue, it was observed that the amount of thiamine dye salt extracted by chloroform varied with pH and dye concentration. The variation in the amount of salt extracted from an aqueous solution by an organic solvent with changes in pH and dye concentration is well documented (2). Schill (3, 4) has recently reported the effect of various experimental conditions on the extraction of bromothymol blue amine salts. The purpose of this study was to derive an equation that expresses the dependence of the thiamine dye salt extracted with changes in pH and dye concentration, and to prove the validity of this equation by comparing theoretical and experimental data.

THEORETICAL

With the use of a thermodynamic argument Divatia and Biles (5) showed that the alkyl amine salts of tropaeolin 00 in the organic phase were in equilibrium with the ionic forms of amine and dye in the aqueous phase. In their studies they called the equilibrium constant for such a phenomenon the apparent partition coefficient and expressed it as

$$K = \frac{S}{A^+ \times D^-}$$

where S = concentration of salt in the organic phase; A^+ = concentration of dissociated amine in the aqueous phase; and D^- = concentration of dissociated dye in the aqueous phase.

In the authors' studies an equivalent equation was expressed as follows:

$$K = \frac{S}{[HI^-]_a[Th^+]_a} \qquad \text{(Eq. 1)}$$

where S = concentration of thiamine dye salt in the organic phase; $[Th^+]_a$ = concentration of thiamine in the aqueous phase (thiamine has one positive charge); $[HI^-]_a$ = concentration of dissociated dye in water.

The term equilibrium constant was preferred over the apparent partition coefficient because the latter gave the impression that the equilibrium was between the concentrations of the thiamine dye salt in two phases.

An equation relating the amount of thiamine dye salt extracted in a single portion of chloroform (when the volumes of immiscible solvents are equal) to changes in pH was derived using the following symbols:

S = Concentration of thiamine dye salt in the organic phase in moles/L. This also represents the concentration of dye or thiamine in the thiamine dye salt (Eq. 1).

$[C_{H_2I}]$ = Total concentration of the dye in both phases in moles/l.

$[C_{H_2I}]_a$ = Total concentration of the dye in aqueous phase in moles/l.

$[H_2I]_{ua}$ = Concentration of the undissociated dye in aqueous phase in moles/l.

$[H_2I]_c$ = Concentration of free dye in chloroform in moles/l.

$[HI^-]_a$ = Concentration of dissociated dye in water in moles/l.

A_0 = $[H^+]^2/([H^+]^2 + K_1[H^+] + K_1K_2)$ = fraction of $[H_2I]_{ua}$ at a given pH (6).

A_1 = $K_1[H^+]/([H^+]^2 + K_1[H^+] + K_1K_2)$ = fraction of $[HI^-]_a$ at a given pH (6).

K_1 = First acid dissociation constant of bromothymol blue.

K_2 = Second acid dissociation constant of bromothymol blue.

D = Distribution coefficient of the dye.

$[C_{Th}]$ = Total concentration of thiamine in both phases in moles/l.

$[C_{Th}]_a$ = Total concentration of thiamine in aqueous phase in moles/l.

$[TH^+]_a$ = Concentration of undissociated thiamine in aqueous phase in moles/l.

$[TH]_c$ = Concentration of free thiamine in chloroform phase in moles/l.

K_3 = Acid dissociation constant of thiamine.

K = Equilibrium constant (Eq. 1).

The equilibrium expression for the thiamine salt of bromothymol blue in chloroform and water is given by Eq. 1 which may be rearranged to give:

$$K[HI^-]_a[TH^+]_a - S = 0 \qquad \text{(Eq. 2)}$$

Received September 29, 1967, from the School of Pharmacy, University of Georgia, Athens, GA 30601

Accepted for publication March 4, 1968.

Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

Abstracted in part from a thesis submitted by V. Das Gupta to the Graduate School of the University of Georgia in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Adapted from the manuscript by V. Das Gupta which received the 1967 Lunsford Richardson Pharmacy Award.

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To solve for $[HI^-]_a$ in terms of $[H^+]$, K_1, K_2, and $[C_{H_2I}]$ let

$$[C_{H_2I}] = [C_{H_2I}]_a + S + [H_2I]_c \quad \text{(Eq. 3)}$$

and

$$[H_2I]_c = D[H_2I]_{ua} \quad \text{(Eq. 4)}$$

The expression for determining $[H_2I]_{ua}$ in an aqueous phase is

$$[H_2I]_{ua} = [C_{H_2I}]_a A_0 \quad \text{(Eq. 5)}$$

therefore, Eq. 4 can be expressed as

$$[H_2I]_c = D[C_{H_2I}]_a A_0 \quad \text{(Eq. 6)}$$

The expression for determining $[HI^-]_a$ in an aqueous phase is

$$[HI^-]_a = [C_{H_2I}]_a A_1 \quad \text{or} \quad [C_{H_2I}]_a = \frac{[HI^-]_a}{A_1} \quad \text{(Eq. 7)}$$

Substituting Eq. 6 and 7 in Eq. 3 and rearranging gives

$$[C_{H_2I}] - S = \frac{[HI^-]_a}{A_1} + D[HI^-]_a \frac{A_0}{A_1}$$

or

$$[C_{H_2I}] - S = [HI^-]_a \left[\frac{1}{A_1} + D\frac{A_0}{A_1}\right]$$

or

$$[C_{H_2I}] - S = [HI^-]_a \left[\frac{1}{A_1} + D\frac{[H^+]}{K_1}\right]$$

therefore,

$$[HI^-]_a = [[C_{H_2I}] - S] \frac{A_1 K_1}{K_1 + A_1 D[H^+]} \quad \text{(Eq. 8)}$$

To solve for $[Th^+]_a$ in terms of $[H^+]$, K_3 and $[C_{Th}]$ let $[C_{Th}] = [C_{Th}]_a + [Th]_c + S$. Since $[Th]_c$ is very small (Table IV), the equation becomes

$$[C_{Th}] = [C_{Th}]_a + S \quad \text{(Eq. 9)}$$

The expression for determining $[Th^+]_a$ in an aqueous solution is

$$[Th^+]_a = [C_{Th}]_a \frac{K_3}{[H^+] + K_3}$$

or

$$[C_{Th}]_a = [Th^+]_a \frac{[H^+] + K_3}{K_3} \quad \text{(Eq. 10)}$$

Substituting Eq. 10 in 9 we obtain $[C_{Th}] - S = [Th^+]_a [[H^+] + K_3/K_3]$ or

$$[[C_{Th}] - S] \frac{K_3}{[H^+] + K_3} = [Th^+]_a \quad \text{(Eq. 11)}$$

Substituting the values of $[HI^-]_a$ and $[Th^+]_a$ from Eqs. 8 and 11 into Eq. 2 we obtain the following final equation:

$$K \left\{[(C_{H_2I}) - S] \frac{A_1 K_1}{K_1 + A_1 D[H^+]} \times [(C_{Th}) - S] \frac{K_3}{[H^+] + K_3}\right\} - S = 0 \quad \text{(Eq. 12)}$$

To prove the validity of Eq. 12 known values were substituted for the various terms and the equation solved for S with the aid of an IBM 7094 computer.[1]

[1] Computer Center, University of Georgia, Athens, Ga.

The values used were as follows: $K = 3.0 \times 10^5$ l./mole (Table II). $[C_{H_2I}] = 2 \times 10^{-4}$, 1×10^{-4}, and 0.5×10^{-4} moles/l. (same as the experimental concentrations). $[C_{Th}] = 1.186 \times 10^{-5}$ moles/l. (usual concentration used for the assay). D = Distribution coefficient of the dye (Table III). $K_1 = 1$. Bromothymol blue is a strong acid dye and should have more than one pKa value. It was found that bromothymol blue undergoes a color change from deep red to deep yellow between theoretical pH values of -1 and 1, therefore, the first pKa value was estimated to be approximately zero.

$K_2 = 1.0 \times 10^{-7}$ (*Reference 7*)

$K_3 = 1.58 \times 10^{-5}$ (*Reference 8*)

$[H^+]$-different values ranging from 1×10^{-1} to 1×10^{-11}. Results are presented in Table I and Fig. 1.

DISCUSSION

Several factors strongly influenced the extractability of thiamine dye salt in the organic phase. A change in dye concentration affects the amount of

TABLE I—EFFECT OF pH AND DYE CONCENTRATION ON THE CONCENTRATION OF THIAMINE DYE SALT EXTRACTED IN CHLOROFORM (ACCORDING TO EQUATION 12)[a] WHEN THIAMINE CONCENTRATION IS 1.186×10^{-5} MOLE/l.

pH	Thiamine Dye Salt Concn. (mole/l.) × 10^6 when Dye Concn. (mole/l.) × 10^4 is 0.5	1.0	2.0
1.0	1.87×10^{-6}	3.75×10^{-6}	7.50×10^{-6}
2.0	1.87×10^{-4}	3.75×10^{-4}	7.49×10^{-4}
3.0	1.83×10^{-2}	3.66×10^{-2}	7.30×10^{-2}
4.3	3.35	5.27	7.34
5.2	9.66	10.70	11.30
5.6	10.40	11.10	11.50
6.0	10.60	11.20	11.50
6.2	10.60	11.30	11.50
6.6	10.50	11.20	11.50
7.0	10.10	11.00	11.40
7.4	9.20	10.40	11.10
8.0	6.43	8.46	9.93
10.0	1.74×10^{-1}	3.43×10^{-1}	6.67×10^{-1}
11.0	1.77×10^{-2}	3.54×10^{-2}	7.06×10^{-2}

[a] Similar results were obtained when solved quadratically.

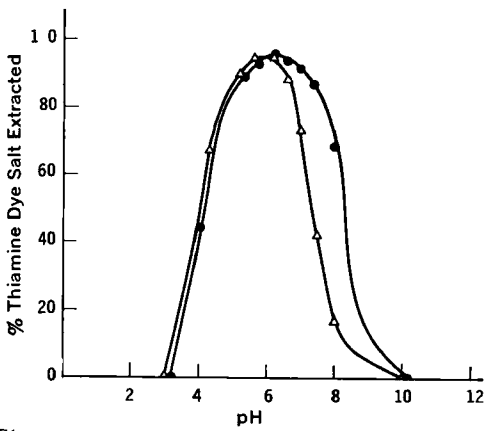

Fig. 1—Effect of pH on the extraction of thiamine dye salt when dye concentration is 1×10^{-4} mole/l. Key: ●, theoretical; Δ, experimental. Data taken from Table II, Reference 1.

salt extracted by the first 10-ml. portion of chloroform (1). This can be explained by Eq. 1.

$$K = \frac{S}{[HI^-]_a\,[Th^+]_a}$$

From this equation it can be seen that if $[HI^-]_a$ decreases S will decrease to keep K constant.

The change in the amount of thiamine dye salt extracted by the first 10-ml. portion of chloroform with a change in pH (1) can also be explained by Eq. 1. Since K is constant it is apparent that a change in the concentration of either $[HI^-]_a$ or $[Th^+]_a$ will change the value of S.

Using Eq. 1, a theoretical equation (Eq. 12) was derived (*viz. Theoretical*) to determine the changes in the amount of thiamine dye salt extracted with changes in pH. This equation was programmed for an IBM 7094 computer, and when solved within the pH range of 1–11, the amount of thiamine dye salt predicted was in excellent agreement with the experimental data (Fig. 1). Between the pH range of 7.0 to 8.0, the theoretical values were higher than the experimental values probably due to an error introduced by using the reported value for K_2 (10^{-7}). A K_2 value of 10^{-6} for bromothymol blue gave better theoretical results. The K_1 value of bromothymol blue was not in good agreement with the value reported by Schill (9), however, changes in K_1 values on either side of the approximated value did not affect the results.

The experimental and theoretical data indicate that the extraction of thiamine dye salt with a single 10-ml. portion of chloroform, between the pH range of 5.2–6.6 is greater than 88.4% (Fig. 1). This pH range appears to be the best since three 10-ml. portions of chloroform will extract more than 99.8% of thiamine dye salt.

Although the above equation was derived specifically for a thiamine-bromothymol blue system, no such limitations are placed on this mathematical expression. Therefore, it is suggested that [Th] can be any base and $[H_2I]$ can be any acidic dye. This would provide a theoretical foundation for the well-documented empirical observations of the dependence of the concentration of salt extracted on pH.

The site of reaction in thiamine may be either the amino group of the pyrimidine moiety or the quaternary nitrogen of the thiazole moiety. The amino group may be reacting with the sulfonic acid group of the dye since it is possible to form the salt by directly adding thiamine mononitrate and bromothymol blue in chloroform. On the other hand Eq. 1 predicts the possibility of reaction between the quaternary nitrogen and the sulfonic acid group as follows:

$$HI^- + TH^+ = [HI^-\,TH^+]$$

EXPERIMENTAL

Reagents—All chemicals and reagents used were either USP, NF, or ACS grade. Bromothymol blue was purchased from Fisher Scientific Company and was used without further purification.

Solutions—Various solutions were prepared using procedures as reported earlier (1). The ionic strength of the solutions was not adjusted but solutions were prepared in 0.05 M phosphate buffer.

Analytical Procedures—Procedures for determination of thiamine dye salt in organic phase, determination of dye in aqueous and organic phases, and determination of thiamine in aqueous and organic phases have been described (1).

Determination of Equilibrium Constant—The equilibrium constant was determined at three different pH values (5.6, 6.0, and 6.5). To run these experiments solutions containing 10 mcg./ml. of thiamine mononitrate were prepared in various buffer solutions and the thiamine dye salt determined according to *Procedure B* of a previous report (1). Thiamine in the aqueous phase was determined by the USP method (10) and the dye in the aqueous phase was determined by the method described earlier (1).

To determine the equilibrium constant the thiamine mononitrate present in the aqueous phase was assumed to be completely undissociated since the pKa of thiamine mononitrate is 4.8. The concentration of the dissociated dye $(HI^-)_a$ in the aqueous phase was calculated using Eq. 7. Results of the experiments are presented in Table II.

Determination of Distribution Coefficients—To determine the distribution coefficient (D) for bromothymol blue, 10.0 ml. of buffer solution of appropriate pH was vigorously shaken with 10.0 ml. of dye in chloroform solution ($6.7 \times 10^{-5} M$) in a 125-ml. separator for 1 min. The phases were allowed to separate and the dye content in both phases was determined using previously described methods (1). To determine the distribution coefficient the concentration of the undissociated dye in the aqueous phase was calculated using Eq. 5. Results are presented in Table III.

TABLE II—DISTRIBUTION OF THIAMINE AND DYE BETWEEN CHLOROFORM AND WATER (AT VARIOUS pH VALUES) FOR THE DETERMINATION OF EQUILIBRIUM CONSTANT[a]

	Concn. in mole/l. × 10^6				
	Thiamine Dye Salt in Chloroform	Thiamine Mononitrate in Water	Dye in Water: Total	Dye in Water: Diss.[b]	Equilibrium Constant in (l./mole × 10^{-6})
pH					
5.6	11.3	0.6	53.0	50.9	4.3
6.0	11.3	0.6	82.0	74.6	2.7
6.5	11.0	0.86	85.0	64.7	2.0
					Av. 3.0

[a] Each value is an average of three experiments. [b] Calculated using Eq. 7.

TABLE III—DISTRIBUTION OF BROMOTHYMOL BLUE BETWEEN CHLOROFORM AND WATER (AT VARIOUS pH VALUES) FOR THE DETERMINATION OF DISTRIBUTION COEFFICIENT[a]

	Concn. of Dye (Absorbance × 100) in			
pH	Chloroform	Water (Total)	Water (Undiss.[b] × 10^{-5})	$D \times 10^{-5}$[c]
6.0	6.40	38.70	3.52	1.80
5.5	15.35	29.50	9.05	1.69
5.0	25.00	19.90	19.90	1.24
4.0	41.20	4.00	40.00	1.03

[a] Each absorbance value is an average of three experiments. [b] Calculated using Eq. 5. [c] In the theoretical equation an average value of 1.5 × 10 (pH 5.0 to 6.0) was used.

TABLE IV—DISTRIBUTION OF THIAMINE HYDROCHLORIDE AND MONONITRATE BETWEEN WATER AND CHLOROFORM (AT VARIOUS pH VALUES) FOR THE DETERMINATION OF DISTRIBUTION COEFFICIENT[a]

pH	% Thiamine Salt in Chloroform[b]	% Thiamine Salt in Water (Total)	% Thiamine Salt in Water (Undiss.[c])	D
Thiamine HCl				
8	0.8	99.2	99.2	0.008
6	0.6	99.4	93.8	0.006
2	0.0	100.0	0.0	0.00
Thiamine Mononitrate				
8	1.2	98.8	98.8	0.012
6	0.8	99.2	93.6	0.008
2	0.0	100.0	0.0	0.00

[a] Each value is an average of three experiments. [b] Calculated by difference. [c] Calculated using Henderson-Hasselbach equation.

To determine the D for thiamine, 15 mg. of thiamine hydrochloride or thiamine mononitrate was dissolved in 25.0 ml. of the buffer solution of appropriate pH and shaken vigorously with 25.0 ml. of chloroform in a 125-ml. separator for 1 min. The phases were allowed to separate and the thiamine content in the aqueous phase was determined using the method described earlier (1). To determine the distribution coefficient, the concentration of undissociated thiamine in the aqueous phase was calculated using the Henderson-Hasselbach equation, and the concentration of thiamine in the chloroform phase was calculated by difference. Results are presented in Table IV.

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Keyphrases

Thiamine—bromothymol blue salt
Extraction—thiamine dye salt
Bromothymol blue concentration—salt extraction
pH effect—salt extraction
Equation—pH dependence of salt extraction

Structure and Activity Relationships in Molluscicides

By I. NABIH* and M. T. ELWASIMI

Noninfected snails of the *Biomphalari* and *Bulinus* types (intermediate hosts for *Schistosoma mansoni* and *Schistosoma hematobium*, respectively) can oxidize instantly and intensively *p*-phenylenediamine dihydrochloride and its derivatives to the corresponding colored trivalent nitrogen-free radicals. This reaction fails to occur with snails infected with miracedia of *Schistosomes*. The reaction is inhibited through inhibitors specific for enzymes of the protoporphyrin type, also through the potent molluscicide (bayluscide). Studies revealed the enzyme involved in the reaction is the peroxidase, along with the catalase. Both enzymes were found to be present in both types of snails.

BILHARZIASIS is a world health problem; it infects millions of people all over the world and causes a tremendous loss in economy and manpower.

To bring the disease under control different aspects would be encountered, mainly the irradication of snails, the intermediate vector of the parasite, through the application of molluscicides. The chemotherapy of the disease is still inadequate particularly in the areas where infection is widely distributed, due to the serious side effects the available agents exhibit when given either orally or by injection.

In the development and search for new molluscicides, extensive work that included investigations of thousands of compounds (mostly of phenolic type) was carried out (1–3). These investigations showed that activity of these phenolic compounds depends upon the nature of substituents on the aromatic ring. The most active were pentachlorophenol and 2,4-dinitro-6-

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Accepted for publication February 16, 1968.
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cyclohexylphenol, whereas those bearing bulky groups on the ring were inactive, *e.g.*, 2,6-di-*t*-butylphenol. Also compounds of the quinoid structure such as benzoquinone and naphthoquinone showed significant activity. Of the aminophenols, 4-aminophenol was active while 2- and 3-aminophenols were inactive. The sample of 4-aminophenol used by the investigators was described to be colored, thus suggesting the transformation to quinoimine or benzoquinone. It had also been suggested that it may be worthwhile to consider the possibility of the conversion of those active phenols within the snails' body to quinoid-type substances with the toxic effect due to that type (3).

Chemically, many phenols can be oxidized to form a phenoxy radical, which may lead to a quinoid-type structure through dimerization or disproportionation. The transformation depends upon the nature of the substituents on the aromatic ring, *e.g.*, 4-substituted 2,6-di-*t*-butylphenoxy radical is a reasonably stable species in solution (4), also the dimerization of 2,4,6-tri-*t*-butylphenoxy radical is inhibited through the bulky *t*-butyl groups (5). This may explain the failure of those phenols bearing bulky groups to show any molluscicidal activity as the apparent biologically formed phenoxy radical, that may undergo the subsequent transformation to the quinoid structure, is prohibited by these bulky substituents on the ring.

This correlation between the molluscicidal activity of those types of phenols bearing nonbulky groups and the chemical oxidation of related types to quinones *via* free radical suggested the present work, to study the possibility of the biological formation of free radicals by snails and to detect the biological system involved in this process.

p-Phenylenediamine and its derivatives were chosen as substrates as they can be chemically oxidized to the colored trivalent nitrogen-free radicals that include the so-called "Wurster's salts." The color formation renders a possible spectrophotometric means to detect and follow the different effects on the reaction. These radicals are formed, chemically, through the oxidation of *p*-phenylenediamine derivatives, *e.g.*, with bromine (6).

Two representatives of this group, namely *p*-phenylenediamine dihydrochloride (I) and *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride (II) were chosen. Both are readily soluble in water and can be prepared in a high state of purity by methods described by Nabih (7) and Michaelis (6), respectively.

Both compounds were submitted independently in appropriate dilutions to the biological action of two types of freshly collected water snails acting as the intermediate hosts for *Schistosoma mansoni* and *Schistosoma hematobium*, namely *Biomphalaria alexandrina* and *Bulinus truncatus*, respectively.

Both types can oxidize I instantly in the typical transformation pathway that usually follows when it is chemically oxidized with bromine, a transient yellow color forms, turning to green. Spectroscopic measurements showed the product to be identical with that obtained through the chemical oxidation, having absorption maxima at 462 mμ and 479 mμ (8).

Also, both types of snails were able to oxidize II to an intensive red color. Spectroscopic measurements showed the product to be identical with the corresponding free radical obtained when II was submitted to the chemical oxidation with one equivalent of bromine. It showed absorption maxima at wavelengths of 515 and 550 mμ (8).

When an excess of snails was used in the reaction, then the color of the solution faded and gradually disappeared. The same disintegration of color occurred when its solution was treated with a second equivalent of bromine to change it to the corresponding *p*-quinonediimine form (9) (Fig. 1).

When these experiments were applied on snails of the *Biomphalaria* type infected with *miracidia* of *Schistosome* using II as the substrate, the characteristic red color of the free radical either developed in a slow and faint pattern or did not at all (Fig. 2). This technique offered an easy and rapid way to differentiate between infected and noninfected snails when collected in patches from the field.

These findings suggested the presence of the enzyme peroxidase in the snail's body that may be involved in these transformation processes.

Peroxidase is an enzyme that contains a ferriprotoporphyrin hematin prosthetic group. It acts on hydrogen peroxide to cause catalytic oxi-

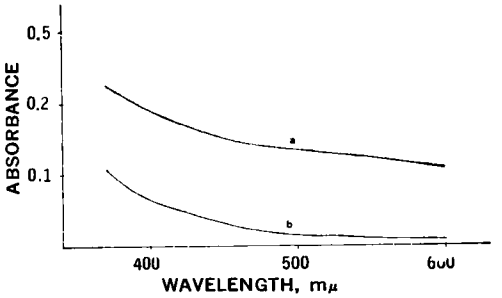

Fig. 1—Further oxidative effect on a solution of II. Key: a, Biomphalaria *snail;* b, *a 2nd equivalent of bromine.*

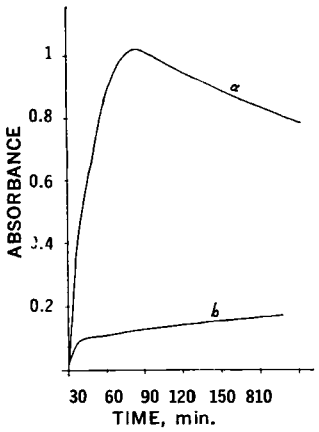

Fig. 2—Rate of color development in a solution of II. Key: a, *noninfected* Biomphalaria *snail;* b, *infected* Biomphalaria *snail.*

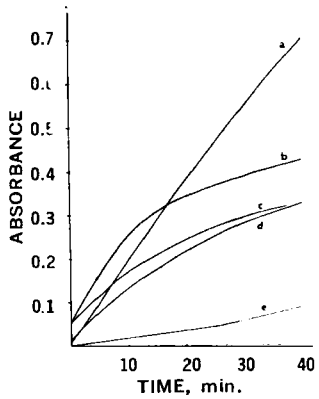

Fig. 3—Rate of color development in a solution of II by Biomphalaria *snails in presence of different inhibitors. Key:* a, *without;* b, *azide;* c, *bayluscide;* d, *cyanide;* e, *sulfide.*

dation of many organic compounds of the amine type, *e.g.*, *p*-phenylenediamine (10), phenolic type (11), and hydroquinones (12, 13).

It catalyzes generally the reaction:

$$AH_2 + H_2O_2 \rightarrow 2H_2O + A$$

The proposed mechanism of action goes through a free radical intermediate (14).

The authors' studies showed that cyanide, sulfide, and azide, inhibitors specific for enzymes of the protoporphyrin type, could inhibit the foregoing reactions when applied under the same experimental conditions on solutions of II (Fig. 3).

They also tried the inhibitory effect of the potent molluscicide, 5,2′-dichloro-4-nitrosalicylanilide (bayluscide) which showed an inhibitory effect on the reaction.

Further, when freshly collected snails of both types were placed in a hydrogen peroxide solution of an appropriate dilution ($1 \times 10^{-3}M$) evolution of gaseous oxygen took place. This suggested the presence of the enzyme catalase, beside the peroxidase. Both enzymes are closely related and are referred to as hydroperoxidases as both use hydrogen peroxide as a common substrate. Snails infected with *Schistosoma mansoni* did not show the same decomposing effect on hydrogen peroxide as the noninfected snails (Fig. 4).

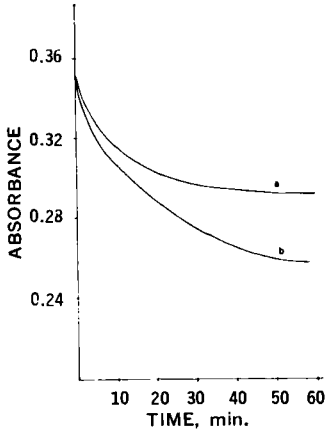

Fig. 4—Rate of decomposition of hydrogen peroxide. Key: a, *infected* Biomphalaria *snail;* b, *noninfected* Biomphalaria *snail.*

The inhibitory effect of some agents that can block the catalytic decomposition of hydrogen peroxide to its components (oxygen and water) have been investigated in connection with the snails' reaction, including cyanide, azide, sulfide, fluoride, hydroxylamine, and bayluscide.

Estimations of the rate of decomposition were made from titrimetric and spectrophotometric measurements for the residual peroxide.

These studies suggest the presence of both enzymes, peroxidase and catalase, in the snail's body, which are the specific catalysts for the foregoing reactions. The former is apparently involved in the biological oxidation of compounds of phenolic and amine types to quinones through the initial formation of free radicals within the snail's body. The quantitative assays for each in some different species of snails are now under determination and will be reported later.

MATERIALS AND METHODS

Preparation of Materials

p-Phenylenediamine Dihydrochloride (I)—Prepared in a high state of purity either by the method described in (7) or through the direct passage of dry HCl gas in absolute ethanol solution of pure *p*-phenylenediamine, then purified and recrystallized (6).

N,N-Dimethyl p-Phenylenediamine Dihydrochloride (II)—It could be obtained in a pure form by the passage of dry HCl gas in dry ether solution of

the free base, followed by purification and recrystallization (6).

The solutions of I and II used in all experiments were freshly prepared in bidistilled water solution.

All spectrophotometric measurements were run on a Unicam Sp. 500 spectrophotometer and cells with a light path of 1 cm.

The snails used were freshly collected from the field.

Oxidative Effect of Biomphalaria Snails on I

One snail of the *Biomphalaria* type (diameter 9 mm.) was immersed in 20 ml. (1×10^{-3} *M*) solution of I. The color developed instantly; after 2 min. the snail was removed and the spectroscopic measurements were run.

These measurements showed peaks of maximum absorption at 462 and 479 mμ (6).

The same peaks appeared when the measurements were run on the formed colored solution when a *Bulinus* snail (7 mm. diameter) was treated as above, with *Biomphalaria*, using I ($1 \times 10^{-3}M$) solution, as substrate.

Oxidative Effect of Biomphalaria Snails on II

One snail of the *Biomphalaria* type (diameter 7 mm.) was immersed in 20 ml. ($1 \times 10^{-3}M$) solution of II; after 2 min., the snail was removed and the absorption spectrum for the developed color was determined. Two peaks showed at 515 and 550 mμ (6).

The same peaks appeared when a snail of the *Bulinus* type (7 mm. diameter) was similarly treated as above using II in solution as substrate.

Further Oxidative Effect of Biomphalaria Snails on II—Fifty snails of nearly equal diameters (mean diameter 1 cm.) immersed in 20 ml. ($1 \times 10^{-3}M$ solution) of II. After 10 hr. the solution became colorless and spectrophotometric measurements were run (Fig. 1-*a*). Parallel, a bromine water solution was added to 10 ml. (1×10^{-3} *M* solution) of II till the red color disappeared, then spectrophotometric measurements were run (Fig. 1-*b*).

Oxidative Effect of Noninfected and Infected Biomphalaria Snails on II—A noninfected snail (8 mm. diameter) was immersed in 10 ml. ($1 \times 10^{-3}M$ solution) of II; the variation in absorbance was measured against time at wavelength 515 mμ (Fig. 2-*a*). The same was run using an infected snail (8 mm. diameter) (Fig. 2-*b*). Similar results were obtained with noninfected and infected snails of the *Bulinus* type.

The Inhibitory Effect of Some Agents on Color Development with II Using Biomphalaria Snails—The color developed by II using *Biomphalaria* snails is compared in five experiments using one snail (1 mm. diameter each) for each experiment:

a, 5 ml. of II ($1 \times 10^{-3}M$) + 5 ml. bidistilled water
b, 5 ml. of II ($1 \times 10^{-3}M$) + 5 ml. sodium azide ($1 \times 10^{-2}M$)
c, 5 ml. of II ($1 \times 10^{-3}M$) + 5 ml. *bayluscide* ($1 \times 10^{-3}M$)
d, 5 ml. of II ($1 \times 10^{-3}M$) + 5 ml. KCN ($1 \times 10^{-4}M$)
e, 5 ml. of II ($1 \times 10^{-3}M$) + 5 ml. Na_2S ($2 \times 10^{-4}M$)

Spectrophotometric measurements were run every 10 min. (Fig. 3) at a wavelength of 515 mμ.

Decomposing Effect of Noninfected and Infected Snails of Biomphalaria Type on H_2O_2 Solution—A noninfected snail (8 mm. diameter) was immersed in 10 ml. ($1 \times 10^{-3}M$ solution) of H_2O_2. An infected snail (8 mm. diameter) was immersed in 10 ml. ($1 \times 10^{-3}M$ solution) of H_2O_2. Spectrophotometric measurements, at a wavelength of 240 mμ, were carried out every 5 min. for both solutions. The results show that the rate of decomposition with the infected snail is slower than with the noninfected one (Fig. 4).

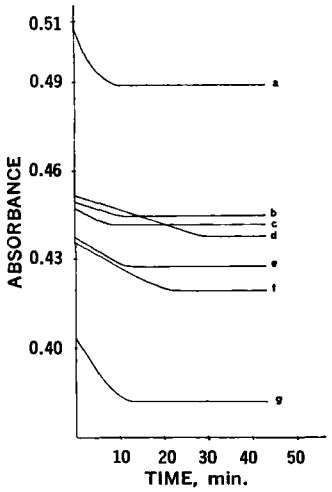

Fig. 5—Rate of decomposition of hydrogen peroxide by Biomphalaria *snails in presence of different inhibitors. Key:* a, *sulfide;* b, *azide;* c, *bayluscide;* d, *fluoride;* e, *hydroxylamine;* f, *cyanide;* g, *without.*

Rate of Decomposition of Hydrogen Peroxide by Fresh Snails and its Inhibition by Different Inhibitors—Decomposition was spectrophotometrically measured through changes in the absorbance at 240 mμ, using for each measurement one snail of *Biomphalaria* type (8 mm. diameter) + 5 ml. H_2O_2 ($1 \times 10^{-2}M$) + 5 ml. of the inhibitor of the following concentrations (Fig. 5):

a, Na_2S ($2 \times 10^{-5}M$)
b, Sodium azide ($2 \times 10^{-3}M$)
c, Bayluscide ($2 \times 10^{-4}M$)
d, Sodium fluoride ($2 \times 10^{-3}M$)
e, Hydroxylamine hydrochloride ($2 \times 10^{-3}M$)
f, Potassium cyanide ($2 \times 10^{-5}M$)
g, Bidistilled water as control.

The results show that the rate of decomposition of the peroxide is depressed through the presence of the inhibitors.

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Keyphrases

Molluscicides—structure-activity relationship
Snails—species of *Biomphalaria, Bulinus*
Schistosoma—infecting organism
P-Phenylenediamine—color reagent
Colorimetric analysis—spectrophotometry

Salt Effects in the Spectrophotometric Examination of a Complex of *N'*-Methylnicotinamide Cation

By DANA BROOKE* and DAVID E. GUTTMAN†

The extent of complex formation, as determined spectrally, between the *N'*-methylnicotinamide cation and the electron donor, 8-chlorotheophyllinate anion, was found to vary greatly when different salts were used to adjust ionic strength. The degree of complex formation when sodium perchlorate was used was found to be much smaller than when sodium acetate was used. An examination of these salt effects indicated that the observed behavior was explicable on the basis that ion pair formation occurred between the pyridinium cation and perchlorate anion.

THE NECESSITY of maintaining a constant ionic strength in studying the behavior of ionic species in solution is widely recognized. It is not, perhaps, so widely appreciated that a so-called inert salt introduced for this purpose can exert an effect quite different from that anticipated solely on the basis of ionic strength variation. Recent studies in this laboratory involving the interaction of *N'*-methylnicotinamide cation (NMN) with 8-chlorotheophyllinate anion (CT) have demonstrated quite dramatically that neutral salts can influence observed behavior independently of their contribution to the maintenance of ionic strength. As will be seen, the extent of interaction of NMN with CT, as detected spectrally, was found to vary considerably depending on whether sodium acetate or sodium perchlorate was used to maintain a constant ionic strength. The observed behavior can be reasonably explained on the basis that extensive ion pair formation occurred between perchlorate anion and NMN.

Received December 18, 1967, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication March 18, 1968.

The Computing Center at the State of New York at Buffalo is partially supported by NIH grant FR-00126 and NSF grant GP-7318.

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Ion pairing of quaternary nitrogen compounds is well documented. For example, Azzari and Kraus (1) reported the formation of ion pairs between the tetrabutyl ammonium cation and such anions as iodide, bromide, and nitrate in water at 25°. Fuoss (2) showed that ionic associations could be treated by mass law considerations, and he and his co-workers (3) reported the ion pair formation constant for the tetraethyl ammonium-picrate ion pair to be 0.8 L. mole^{-1} in water. Kosower (4), in a description of charge transfer interactions, asserted that the major contributing form to the ground state complex between 1-methyl-pyridinium cation and iodide anion was the ion pair.

EXPERIMENTAL

Materials—The chloride salt of NMN was prepared by the method of Huff and Perlzweig (7). Nicotinamide was reacted with an excess of methyl iodide in alcohol to yield NMN as the iodide salt, m.p. 204° uncorrected [reported, 205.5–206.5° (7)]. The iodide salt was converted to the chloride salt by reacting the NMN iodide with an aqueous slurry of silver chloride. The resulting aqueous solution of NMN chloride was filtered and concentrated to yield white crystals of NMN chloride. The chloride salt was recrystallized from hot alcohol, and gave a m.p. of 241° uncorrected [reported, 237–238° (7)].

8-Chlorotheophylline was purchased from the Aldrich Chemical Co. and used without further puri-

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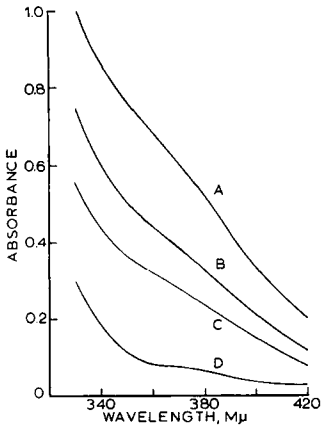

Fig. 1—Plot illustrating the influence of added salts on the spectral characteristics of solutions at a pH of 8.0 containing 0.1 M *CT and 0.005* M *NMN. Curve* A *represents the spectrum of the mixture in the absence of added salt. Curve* B *is the spectrum of the mixture in the presence of 0.9* M *sodium acetate. Curve* C *is the spectrum of the mixture in the presence of 0.9* M *sodium perchlorate. Curve* D *is the spectrum of 0.1* M *CT alone.*

fication. Other chemicals were reagent grade and were obtained from Fisher Scientific Co.

Methods—The influence of sodium acetate and sodium perchlorate on the extent of complex formation between NMN and CT was evaluated by using either neutral salt, or a combination of the two, to adjust the ionic strength of solutions containing the interactants to a value of 1.0. These solutions were prepared by dissolving appropriate amounts of 8-chlorotheophylline, pKa 5.5 (6), and neutral salts in about 35 ml. of water, using enough sodium hydroxide to adjust the pH to a value of 8.0. These were placed in 50-ml. volumetric flasks and aliquots of a stock solution of NMN as the chloride salt were added to the flasks which were then made to volume to gain the desired concentrations. The ionic strength of the resultant solutions was taken as the sum of the concentrations of sodium acetate, sodium perchlorate, and sodium 8-chlorotheophyllinate. The concentration of NMN as the chloride was negligible compared to the concentrations of other ionic species. The resultant solutions were brought to 40° in a water bath. The extent of complex formation was measured spectrally at 40° using a Beckman, model DB, spectrophotometer equipped with a thermostated cell compartment. The large increase in absorbance at 360 mμ of solutions of CT on the addition of NMN, a species with no visible spectrum, was taken as a measure of the extent of interaction between NMN and CT. Absorbance values were corrected for the small contribution due to CT. Representative spectra are shown in Fig. 1.

RESULTS AND DISCUSSION

Spectral evidence for a postulated complex of NMN and CT is given in Fig. 1. It can be seen that the extent of complex formation was decreased by increasing the ionic strength from a value of 0.1 to a value of 1.0 with the addition of sodium acetate or sodium perchlorate. Though a decrease with increasing ionic strength was expected on a theoretical basis, the marked difference in the extent of interaction observed when the different salts were employed was unexpected. The results of a number of experiments are given in Table I, all of which illustrate the same behavior.

That the extent of interaction between NMN and CT is influenced differently by the two salts can be explained on the basis of a rather simple model which assumes that NMN forms ion pairs with perchlorate anion. As a first approximation, based on the much larger influence of sodium perchlorate, it was assumed that acetate anion was not involved in ion pair formation. The model neglects possible differences in activity coefficients, hydration, and ion size parameters of unassociated NMN and CT in the presence of the different salts, and attributes the larger influence of perchlorate to a competitive effect. The model is summarized by

$$\mathrm{NMN} + \mathrm{CT} \overset{K}{=} \mathrm{NMN{:}CT} \qquad \text{(Eq. 1)}$$

$$\mathrm{NMN} + \mathrm{ClO_4} \overset{G}{=} \mathrm{NMN{:}ClO_4} \qquad \text{(Eq. 2)}$$

where NMN:CT = 1:1 complex formed between NMN and CT, K = association constant for the formation of NMN:CT, $\mathrm{NMN{:}ClO_4}$ = 1:1 ion pair formed between NMN and perchlorate, G = ion pair formation constant for $\mathrm{NMN{:}ClO_4}$.

The absorbance, A, due to the complex, NMN:CT can be written as

$$A = \epsilon(\mathrm{NMN{:}CT}) = \epsilon K(\mathrm{NMN})(\mathrm{CT}) \qquad \text{(Eq. 3)}$$

where ϵ = molar absorptivity of the complex.

The absorbance can be stated in terms of total NMN, $(\mathrm{NMN})_t$, as

TABLE I—ABSORBANCE VALUES AT 360 mμ DUE TO COMPLEX FORMED BETWEEN NMN AND CT IN VARIOUS SOLUTIONS ADJUSTED TO AN IONIC STRENGTH OF 1.0

Solution Composition				Absorbance[a]		
(CT)	$(\mathrm{NMN})_t$	$(\mathrm{ClO_4})$	(Ac)	Observed	Calculated	Difference
0.10	0.0050	0.00	0.90	0.359	0.374	−0.016
0.10	0.0050	0.50	0.40	0.289	0.280	+0.009
0.10	0.0050	0.90	0.00	0.238	0.233	+0.005
0.15	0.0050	0.00	0.85	0.515	0.505	+0.010
0.15	0.0050	0.30	0.55	0.415	0.426	−0.011
0.15	0.0050	0.85	0.00	0.341	0.332	+0.009
0.14	0.0025	0.00	0.86	0.241	0.242	−0.001
0.14	0.0025	0.43	0.43	0.193	0.190	+0.003
0.14	0.0025	0.86	0.00	0.142	0.157	−0.015
0.21	0.0025	0.00	0.79	0.303	0.319	−0.016
0.21	0.0025	0.40	0.40	0.253	0.262	−0.009
0.21	0.0025	0.79	0.00	0.214	0.222	−0.008

[a] Corrected for the absorbance contribution due to CT.

$$A = \frac{\epsilon K(\mathrm{NMN})_t\,(\mathrm{CT})}{1.0 + K(\mathrm{CT}) + G(\mathrm{ClO_4})} \quad \text{(Eq. 4)}$$

Equation 4 can be rewritten as

$$\frac{1}{A} = \frac{1}{\epsilon K(\mathrm{NMN})_t(\mathrm{CT})} + \frac{1}{\epsilon(\mathrm{NMN})_t} + \frac{G(\mathrm{ClO_4})}{\epsilon K(\mathrm{NMN})_t(\mathrm{CT})} \quad \text{(Eq. 5)}$$

Equation 5 is essentially the same as that derived by Merrifield and Phillips (8) for a case in which both a solvent and an electron donor interacted with an electron acceptor but the solvent-acceptor complex did not contribute to the absorbance of the system. When $(\mathrm{NMN})_t$ and (CT) are held constant, plots of $1/A$ against $(\mathrm{ClO_4})$ should be straight lines. Such plots are given in Fig. 2. The value of $G/\epsilon K$ can be calculated by multiplying the slopes of the lines by the appropriate $(\mathrm{NMN})_t(\mathrm{CT})$ product. The intercepts of the lines in Fig. 2. multiplied by the appropriate $(\mathrm{NMN})_t$, when plotted against $1/(\mathrm{CT})$ should give a straight line with intercept $1/\epsilon$ and slope $1/\epsilon K$. This plot, which corresponds to a Benesi-Hildebrand (5) plot, is given in Fig. 3. The values for ϵ, K, and G calculated in this manner are 359 L. mole^{-1} cm.$^{-1}$, 2.69 L. mole^{-1}, 0.92 L. mole^{-1}.

Treatments such as the one used here depend heavily upon a knowledge of the value $1/\epsilon$. Since this value is small, a small absolute error in the determination of $1/\epsilon$ can lead to large errors in ϵ, and in the association constants. An attempt was made to evaluate the association constants independently of a determination of ϵ. This can be done by considering two experimental points at one time. It can be shown that

$$\frac{A1}{A2} = \frac{(\mathrm{NMN1})_t(\mathrm{CT1})[1.0 + K(\mathrm{CT2}) + G(\mathrm{ClO_4}2)]}{(\mathrm{NMN2})_t(\mathrm{CT2})[1.0 + K(\mathrm{CT1}) + G(\mathrm{ClO_4}1)]} \quad \text{(Eq. 6)}$$

where 1 and 2 refer to two different experimental systems. The values for K and G were generated, according to Eq. 6, on an IBM 7044 computer. The values for K and G were found to be 2.67 L. mole^{-1}, and 0.86 L. mole^{-1}. The corresponding value of ϵ was calculated to be 355 L. mole^{-1} cm.$^{-1}$.

The calculated absorbance values given in Table I are based upon the computer generated values of K, G, and ϵ. The random distribution of the difference cited in Table I indicates that the observed differences in the effects of sodium acetate and sodium perchlorate can be reasonably explained on the basis of ion pair formation between NMN and perchlorate. The treatment presented here does not preclude the possibility of ion pairing between acetate anion and NMN but does suggest that such ion pairing is small compared with the association between NMN and perchlorate.

It is interesting to note that the definition of a particular ionic behavior may vary quantitatively depending upon the "inert" salt used for the purpose of adjusting ionic strength. In the present investigation, the apparent association constant for the NMN:CT complex in systems in which sodium perchlorate was the salt used was found to be 1.52 L. mole^{-1} by the Benesi-Hildebrand (5) treatment. When sodium acetate was the salt used the apparent association constant was found to be 2.59 L. mole^{-1}. This observation makes it clear that the effects of neutral salts cannot always be judged solely on the basis of their contribution to the maintenance of ionic stength.

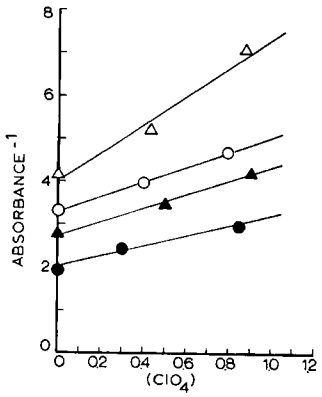

Fig. 2—Plot, based on Eq. 5, illustrating the dependence of complex formation on perchlorate concentration. Solutions contained: 0.14 M CT and 0.0025 M NMN (△), 0.21 M CT and 0.0025 M NMN (○), 0.10 M CT and 0.005 M NMN (▲), 0.15 M CT and 0.005 M NMN (●).

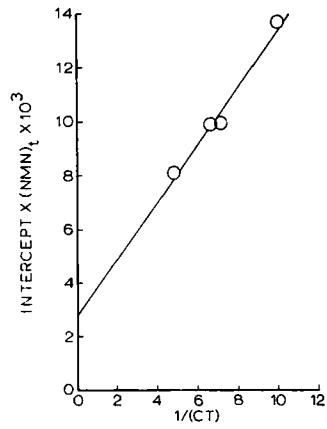

Fig. 3—Plot used to determine K and ϵ. See text.

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Keyphrases

N-Methylnicotinamide—8-chlorotheophyllinate complex
Salt effects—complex formation
Ionic strength—salt maintenance
UV spectrophotometry—analysis

Aminolysis of Benzylpenicillin by Aliphatic Diamines

By MICHAEL A. SCHWARTZ

The kinetics of reaction of penicillin with a series of aliphatic diamines, $NH_2(CH_2)_nNH_2$ (n = 2–6), show terms both first order and second order in diamine. The second-order terms are basicity dependent and account for general base-catalyzed nucleophilic attack by the monoprotonated species. The first-order term includes a contribution by both monoprotonated and unprotonated diamine. An argument in favor of intramolecular general base catalysis by the latter is given and the possible relationship between this mechanism and penicillin antigen formation is discussed.

DIRECT AMINOLYSIS of penicillins by ε-amino groups of lysine residues on proteins has been shown to occur (1, 2) and may be involved in the *in vivo* formation of the penicilloyl-protein conjugate which is considered to be the principal antigenic determinant in penicillin allergy (3). Aminolysis of penicillin by primary amines is however a relatively slow reaction at neutral pH because there is usually only a small fraction of the amine present in the nucleophilic unprotonated form. Investigation of the mechanism of reaction of glycine with penicillins (4) revealed participation by two molecules of glycine anion demonstrating a requirement for general base catalysis. This result indicated that reaction of an amine with penicillin might be more rapid where a second amino group was present on the same molecule and intramolecular general base catalysis could occur. To test this hypothesis studies were conducted of the reaction of benzylpenicillin with a series of aliphatic diamines [$NH_2(CH_2)_nNH_2$ with n = 2 through 6] and the results are reported herein.

EXPERIMENTAL

Materials—Benzylpenicillin was kindly supplied by Bristol Laboratories as the potassium salt. The aliphatic diamines were obtained as, or converted to, the dihydrochloride salts and recrystallized from aqueous methanol. All other materials were reagent grade.

Rate Studies—All measurements were made at 35°. Generally the diamine solution, adjusted to the desired pH with sodium hydroxide, acted as both buffer and reactant. In some cases where the buffer capacity was too low constant pH was maintained by a radiometer TTT-1 pH-stat. Ionic strength was kept constant throughout the study by addition of KCl to the reaction mixture. Initial penicillin concentration was 1.0×10^{-3} M and the diamine concentration was always in excess (above 0.04 M).

The rate of penicillin loss was followed by one of two methods: (*a*) Samples of reaction mixture were removed at appropriate intervals and assayed for residual penicillin by the method of Brandriss *et al.* (5). (*b*) The change in optical rotation of the solution was followed at 313 mμ with the Perkin-Elmer model 141 photoelectric polarimeter with Sargent SR recorder (2.5 mv.). Opening of the β-lactam of penicillins is accompanied by a large decrease in optical rotation. In some instances where both methods were utilized excellent agreement in the results was observed.

The acid dissociation constants (pKa′) of the diamines were determined at 35° and ionic strength 0.5 by potentiometric titration, and the overlapping constants separated by the method of Noyes (6). The values are presented in Table I (see results).

RESULTS

Since the diamine concentration was in excess of penicillin first-order kinetics was observed at constant pH. The rate constants (k_{obs}) were determined either from plots of log penicillin concentration *versus* time when the assay method was employed, or Guggenheim treatment of the optical rotation data. In either case excellent linearity was observed through at least two half-lives and generally through 3–4 half-lives.

Plots of $k_{obs.}$ at constant pH as a function of diamine concentration showed an upward curvature with increasing diamine, indicating an order greater than unity in base. Linearity was observed however when $k_{obs.}/B_T$ was plotted against B_T, where B_T represents the total concentration of all species of diamine present in solution. Figure 1 shows such a plot for 1,3-propanediamine which is typical of those observed with the other diamines. It can be seen from this plot that both the slopes and intercepts vary with pH.

The intercepts, representing terms in the rate law which are first order in diamine, express the sum of rate constants for nucleophilic attack by each of the basic species in solution:

$$\text{Int.} = k_1 f_B + k_2 f_{BH^+} \qquad \text{(Eq. 1)}$$

where f_B is the fraction of the total diamine unprotonated and f_{BH^+} the fraction of monoprotonated species.

Received February 16, 1968, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication March 25, 1968.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

Supported by grant No. AI-06173 from the National Institute of Allergy and Infectious Disease, U. S. Public Health Service, Bethesda, Md.

The competent technical assistance of Mrs. Antoinette DelDuce is gratefully acknowledged.

TABLE I—SPECIFIC RATE CONSTANTS[a] FOR REACTION OF BENZYLPENICILLIN WITH $H_2N(CH_2)_nNH_2$

n	pK_1'	pK_2'	k_1[b]	k_2[b]	k_3[c]	k_4[c]
2	7.07	9.91	5.3	0.1	0.32	14
3	8.70	10.60	47	0.3	4	—
4	9.45	10.77	25	0.1	9	—
5	9.88	10.85	15	—	21	—
6	10.06	10.80	18	—	39	—

[a] $k_{obs} = k_1[B] + k_2[BH^+] + k_3[BH^+]^2 + k_4[B][BH^+]$. [b] Units are M^{-1} min.$^{-1}$. [c] Units are M^{-2} min.$^{-1}$.

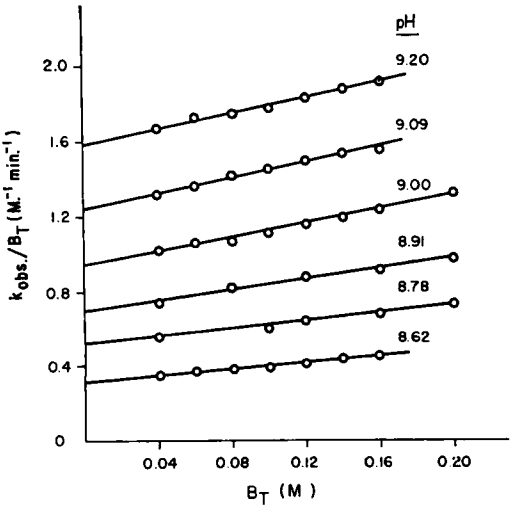

Fig. 1—Plot of k_{obs}/B_T vs. B_T for 1,3-propanediamine as a function of pH.

Dividing by f_{BH^+}:

$$\frac{\text{Int.}}{f_{BH^+}} = k_1 \frac{f_B}{f_{BH^+}} + k_2 \qquad \text{(Eq. 2)}$$

A plot of the left-hand side of Eq. 2 against f_B/f_{BH^+} should be linear with slope k_1 and intercept k_2. A plot of Eq. 2 for 1,3-propanediamine is shown in Fig. 2 and values of k_1 and k_2 were obtained from such plots for all of the diamines. These are given in Table I.

By a similar treatment it was found that the slopes (S) of the lines in Fig. 1 could be fitted by Eq. 3.

$$S = k_3(f_{BH^+})^2 + k_4(f_{BH^+})(f_B) \qquad \text{(Eq. 3)}$$

The values for k_3 and k_4 were determined from plots of $S/(f_{BH^+})^2$ *versus* f_B/f_{BH^+} All of these had zero slope (k_4 = zero) except the line for ethylenediamine where a significant value for k_4 could be determined. The values for k_3 and k_4 are also given in Table I.

Using the constants in Table I slopes and intercepts for lines of the type shown in Fig. 1 were calculated. Table II presents a comparison of these calculated values with those obtained experimentally showing generally good agreement.

DISCUSSION

The rate law for reaction of aliphatic diamines with benzylpenicillin is given in Eq. 4:

$$\text{Rate} = \{k_1[B] + k_2[BH^+] + k_3[BH^+]^2 + k_4[B][BH^+]\}(P) \qquad \text{(Eq. 4)}$$

where P denotes penicillin concentration. With the exception of the term k_2 $[BH^+]$ each of the terms may represent a general base-catalyzed nucleophilic attack by an amine. The k_2 term may denote intramolecular general acid catalysis but makes such a small contribution to the overall rate it is almost negligible. In addition there is considerable error involved in the determination of k_2 from the intercepts of plots of secondary data. The k_4 term is significant only for ethylenediamine and represents general base catalysis of nucleophilic attack by one of the species involved.

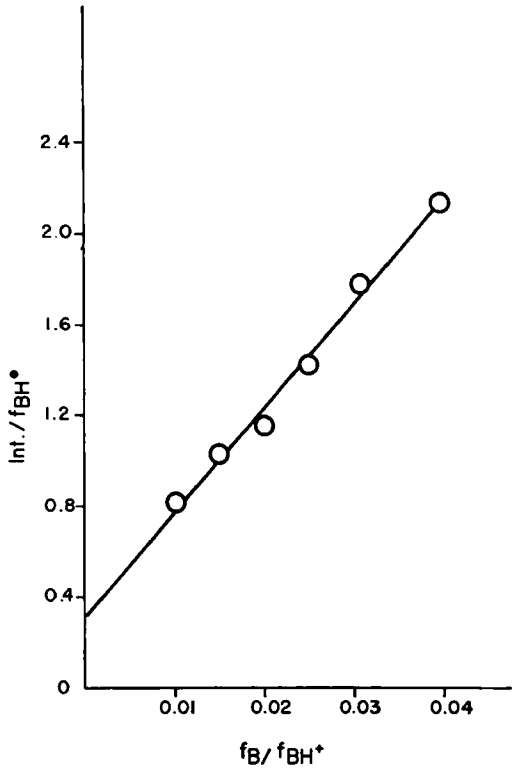

Fig. 2—Plot of equation for 1,3-propanediamine.

The values for k_3 are a function of the basicity of the amine as seen from the typical Bronsted plot in Fig. 3. Comparing the value previously obtained for glycine anion (k = 2.3, pKa = 9.10 at 50°) (4) with this data it may be seen that glycine would fall well below the line. The greater reactivity of the monoprotonated diamines probably reflects the difference in electrostatic charge of the attacking species relative to substrate. The penicillin, which exists as anion in the pH range studied, should attract the amine but tend to repel glycine anion.

There may be some controversy as to whether or not intramolecular general base catalysis does indeed occur in the reaction with the unprotonated diamine.

TABLE II—COMPARISON OF CALCULATED AND EXPERIMENTAL DATA

pH	Intercept Calcd.	Intercept Found	Slope Calcd.	Slope Found	pH	Intercept Calcd.	Intercept Found	Slope Calcd.	Slope Found
	Ethylenediamine					**1,4-Butanediamine**			
7.79	0.12	0.13	0.30	0.27	9.10	0.20	0.20	0.85	0.67
8.09	0.16	0.16	0.43	0.50	9.20	0.29	0.41	1.14	1.26
8.56	0.31	0.41	0.82	0.78	9.43	0.59	0.62	2.06	2.51
9.10	0.79	0.80	1.83	1.63	9.60	1.01	1.03	3.25	3.90
9.27	1.06	1.06	2.31	2.40			**1,5-Pentanediamine**		
9.43	1.40	1.30	2.80	2.80	9.60	0.28	0.25	2.4	2.4
	1,3-Propanediamine				9.80	0.58	0.57	4.0	3.0
8.62	0.36	0.33	0.82	0.75	9.90	0.81	1.06	4.9	5.0
8.78	0.55	0.53	1.18	1.30	10.10	1.5	1.53	6.7	7.5
8.91	0.76	0.70	1.46	1.45			**1,6-Hexanediamine**		
9.00	0.97	0.94	1.72	1.95	9.43	0.14	0.14	1.38	1.30
9.09	1.22	1.24	1.92	2.10	9.60	0.29	0.34	2.50	2.1
9.20	1.59	1.58	2.16	2.15	9.80	0.63	0.59	4.6	4.8
					9.90	0.89	0.85	6.0	6.9

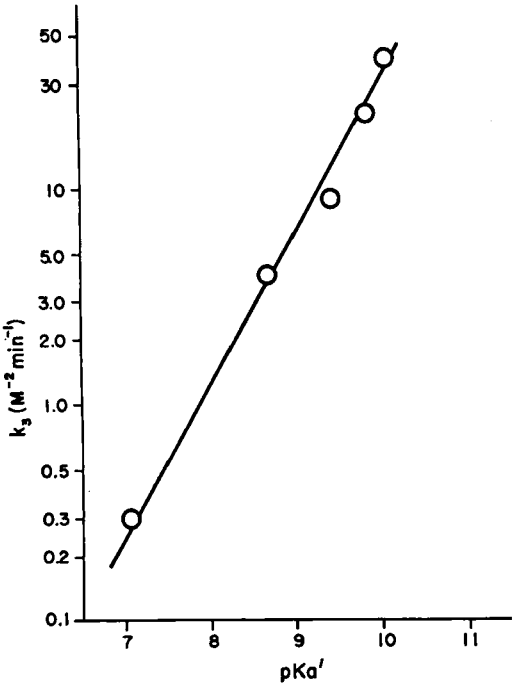

Fig. 3—Bronsted plot of k_3 *(log scale) as a function of* pK_1.

The rate law for reaction of these same diamines with phenyl acetate (7) showed only terms first order in diamine and a conclusion could not be drawn from the data as to whether or not intramolecular catalysis was taking place. On the other hand the kinetics of reactions of glycine with phenyl acetate (8) revealed terms both first and second order in amine while with penicillin as substrate only a second-order term was observed. It appears then that aminolysis of benzylpenicillin requires general base catalysis. These differences between penicillin and phenyl acetate are probably a reflection of the relative susceptibility of these two compounds to nucleophilic attack. Using rates of alkaline hydrolysis as a basis, the ester is much more susceptible, *i.e.*, k_{OH} for penicillin is 12.5 M^{-1}min.$^{-1}$ at 31.5° (9) and for phenyl acetate is 223 M^{-1}min.$^{-1}$ at 30° (10). It has been concluded from studies of aminolysis of phenyl esters that general catalysis becomes of greater significance as the leaving tendency of the phenol decreases (11). The contribution of general base catalysis is probably of more importance with those substrates less susceptible to nucleophilic attack.

It should be noted that it has previously been shown that benzyl penicillin reacts with 1,6-hexanediamine to form a penicilloamide derivative (12).

Intramolecular general base catalysis of aminolysis of penicillins by amino groups on proteins may be one route by which relatively rapid conjugation of the drug to protein may occur, forming antigen through which individuals are sensitized to the drug.

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Keyphrases

Benzylpenicillin—aminolysis
Aminolysis, benzylpenicillin—base catalysis
Optical rotation—reaction rate
Ionic strength—potentiometric titration
Dissociation constants, acid—diamines

TLC Determination of Sulfanilamide as a Degradation Product in Pharmaceutical Preparations Containing Sodium Sulfacetamide

By MURRAY P. GRUBER and ROBERT W. KLEIN

Thin-layer chromatography followed by spectrophotometric analysis of eluted components was successfully employed for the separation and determination of sulfanilamide as a degradation product in sulfacetamide preparations. Significant amounts of sulfanilamide were found to be present as a contaminant in a variety of commercially available ophthalmological and dermatological pharmaceuticals containing sulfacetamide, when subjected to accelerated testing conditions and/or prolonged storage. The procedures used may be applicable to stability testing of other sulfonamides.

FOR SEVERAL DECADES, sulfonamides, especially sulfacetamide and its sodium salt, have been widely and successfully employed in the treatment of ocular and dermatological diseases (1–4). Investigators (5, 6) have observed that pharmaceutical preparations containing sulfonamides or their sodium salts undergo a color change on aging, becoming yellow to red-brown, depending on the sulfonamide(s) present.

Various recommendations (5, 6) were made to eliminate the problem of darkening, but they have not proved completely successful. The replacement of air by nitrogen in filled containers and the introduction of small percentages of antioxidants in the finished sulfacetamide products retard but do not prevent discoloration from occurring, particularly on exposure to elevated temperatures, direct sunlight, or prolonged shelf storage.

Since color changes have historically signified some form of chemical reaction ensuing in a substance, this laboratory undertook an exploratory investigation to determine if this phenomenon was indicative of decomposition.

Aqueous solutions ranging from 5 to 30% of sodium sulfacetamide were prepared. The preservative measures previously suggested were employed in an effort to prevent development of the discoloration. All precautionary measures either alone or in combination failed.

The conventional titrimetric methods of analysis as outlined by USP XVII (7) and NF XII (8) were utilized for the quantitative determination of sulfacetamide in these preparations and in each instance, employing these procedures, no decomposition was observed.

Received February 19, 1968, from the Analytical Research Department, Dermik Laboratories, Inc., Syosset, NY 11791
Accepted for publication April 2, 1968.

Although no subpotency was revealed, since the procedure used is nonspecific for sulfonamides, there appeared to be a possibility that the sulfacetamide was undergoing hydrolysis to yield sulfanilamide. In order to pursue this hypothesis, other techniques which would permit the separation of the sulfonamides prior to analysis were essential.

Chromatographic methods have met with some success in the separation of mixed sulfonamides and related materials. Shepherd, Taft, and Krazinski (9) used descending paper partition chromatography in their work on 4-sulfanilamidopyrimidines. The NF XII (8) describes a similar paper chromatrographic procedure for the separation and identification of sulfamerazine and sulfadiazine. Klein and Kho (10), using a modification of the solvent system described by Wollish, Schmall, and Hawrylyshyn (11) obtained satisfactory separations for various sulfonamides including sulfanilamide from sulfacetamide.

Accordingly, a stability evaluation of commercially available preparations was instituted using a TLC adaptation of the developing system described by Shepherd *et al.* (9). Visualization and quantitation of the developed systems was accomplished using Bratton-Marshall reagent or ultraviolet light followed by spectrophotometric determination of the eluted components.

EXPERIMENTAL

Apparatus and Reagents—Precoated 10 × 20-cm. Silica Gel GF plates, 250 and 500 μ thickness (Analtek, Inc., Wilmington, Del.) and a spectrophotometer, Beckman DU or equivalent, equipped with 1-cm. cells were used. Spray reagents, 1 *N* aqueous hydrochloric acid, 5% aqueous sodium nitrite, and *N*-(1-naphthyl)ethylenediamine dihydrochloride

0.1% in 95% ethanol (Bratton-Marshall reagent) were used. Reagents for colorimetric assay were 0.1 *N* aqueous hydrochloric acid, 2.0% aqueous ammonium sulfamate, and 0.4% aqueous sodium nitrite (freshly prepared). Chromatographic jars were equipped for ascending chromatography of sufficient internal volume for 10 × 20-cm. plates. Suitable delivery pipets for spotting purposes were used. Developing solvents system were (*a*) *n*-butanol, A.R. (*b*) ammonia 28%, A.R.

Preparation of Chromatographic Plates—All plates were activated at 40° for 30 min. and stored in a desiccator over silica gel for 1 hr. prior to use.

Standard Preparation—A 50-mg. quantity, accurately weighed, of each sulfonamide was dissolved in 30 ml. of acetone and diluted to 50 ml. with acetone. Sodium sulfacetamide was first dissolved in 1 ml. of distilled water prior to dilution with acetone.

Sample Preparation—A sample equivalent to 50 mg. of sulfacetamide or sodium sulfacetamide was dispersed in approximately 30 ml. of acetone and shaken for 15 min. The mixture was filtered (if not clear) over Whatman No. 1 paper into a 50-ml. volumetric flask and washed with acetone to volume.

Tablets were first crushed to a fine powder using a mortar and pestle and the procedure continued as above.

Sample Size—(*a*) For screening and quantitative determination of major sulfonamide; 10 μl. of sample (equivalent to 10 mcg. of labeled sulfonamide). (*b*) For quantitation of sulfonamide impurities; 50 μl. of sample applied in a band 1 cm. long for glass plates. Larger samples may be spotted if concentrations lower than 1% of sulfonamide impurities are present. Care must be taken that the applied band be as tight as possible.

Developing System—Add 90 ml. of butanol, 10 ml. of concentrated ammonium hydroxide, and 80 ml. of water to a 250-ml. separator. Shake well and allow the phases to separate. The butanol fraction is used as a developing system. Butanol–ammonium hydroxide–water (9:1:8) by volume.

Tank Preparation—All tanks were lined with Whatman No. 1 paper, filled to a depth of 1.2 cm. with developing solvent, and allowed to equilibrate overnight prior to use. The distance developed was 150 mm.

Procedures and Analysis—Samples and standards (sulfacetamide and sulfanilamide) were applied according to the appropriate procedure. After a solvent travel of 150 mm. the plates were removed and air dried. The component spots were observed under a short wavelength ultraviolet light (257 mμ) and marked.

If the screening procedure was being used, the plates were sprayed with 1 *N* hydrochloric acid followed by 5% sodium nitrite. They were then dried for 10 min. at 100°, cooled, and sprayed with 0.1% *N*-(naphthyl)ethylenediamine forming reddish-purple spots for the sulfonamides. The plates were reexamined to determine the presence of a spot produced by sulfanilamide contamination not detected under ultraviolet light.

If quantitation of the sulfacetamide present was desired, the area marked was completely scraped from the plate into a 25-ml. volumetric flask through a small funnel and the area from which the silica gel was removed carefully washed with approximately 20 ml. of 0.1 *N* hydrochloric acid into the flask. This procedure was repeated for subsequent samples, beginning with samples on the outer portion of the plate and working in. The flask and its contents were shaken for 30 min. and diluted to volume with 0.1 *N* hydrochloric acid. A 12.5-ml. aliquot of the clear supernatant was transferred to a 25-ml. flask, 0.25 ml. of freshly prepared 0.4% aqueous sodium nitrite added, and swirled intermittently for 3 min. A 0.25-ml. volume of a 2% aqueous ammonium sulfamate solution was added, the contents gently shaken for 2 min., and followed by the addition of 2.0 ml. of aqueous 0.1% *N*-(naphthyl)ethylenediamine dihydrochloride. The solution was allowed to stand with occasional shaking for 15 min. The absorbance of the solution was determined in a 1-cm. cell at 545 mμ *versus* a reagent blank prepared from a portion of the plate having the same dimensions and no sulfonamide present. The concentration was determined from an absorbance-concentration calibration curve of sodium sulfacetamide obtained using the same procedure. Results obtained from three replicates of the same sample were averaged and calculated as "% of labeled amount" of sodium sulfacetamide.

An alternate procedure involved elution from the silica gel with 4.0 ml. of 95% ethanol, centrifuging the resulting suspension, and determination of the absorbance of the clear supernatant in a 1-cm. cell at 259 mμ *versus* 95% ethanol eluate obtained from a portion of the plate having the same dimensions and no sulfonamide present. Concentration in the eluate was determined using the absorptivity of sodium sulfacetamide in 95% ethanol at 259 mμ. Calculations were carried out and reported as "% of labeled amount."

Determination of sulfanilamide as a decomposition product was carried out in a similar fashion to the determination of sodium sulfacetamide. Due to the smaller quantity of solvent involved in the direct spectrophotometric technique, this procedure in combination with 500-μ plates was preferred. Where comparatively large amounts of sulfanilamide are noted, the procedures are interchangeable. Results were reported as "% of total sulfonamide found."

RESULTS AND DISCUSSION

Analyses were performed on preparations prior to and following chromatographic separation. It was found that the use of precoated plates significantly reduced variation of the R_f values although standards were run concurrently with samples on each plate to determine the experimental range of R_f values and elution recoveries of the sulfonamides present. These figures are noted in Table I. Some varia-

TABLE I—R_f VALUES AND ULTRAVIOLET RECOVERY ANALYSIS

	R_f	Elution Recovery % UV Analysis
Sodium sulfacetamide	0.14	98.4
	0.14	98.8
	0.16	101.4
	$\bar{x}$ = 0.15	$\bar{x}$ = 99.5
Sulfanilamide	0.50	100.2
	0.50	97.8
	0.49	99.6
	$\bar{x}$ = 0.50	$\bar{x}$ = 99.2

TABLE II—STABILITY ANALYSIS OF SULFACETAMIDE SUSPENSIONS AND SOLUTIONS

Sample	Sulfacetamide (% of Labeled Amount) Initial	6 mos. at RT	4 mos. at 40°	Sulfanilamide (% of Total Sulfonamide) Initial	6 mos. at RT	4 mos. at 40°
A	104.6	98.7	93.6	0.4	4.1	7.8
B	98.4	89.5	82.6	1.2	7.3	12.1
C	102.1	96.1	91.2	0.2	4.6	8.0
D	91.0	87.8	80.2	4.6	7.0	13.0
E	97.4	91.0	83.8	2.2	6.2	11.8
F	105.6	103.0	101.7	0	1.5	2.4

tion in sensitivity and lower detection limit was observed between detection techniques. This, however, posed no particular problem in the overall study.

All of the ophthalmic and dermatologic pharmaceutical samples used in the investigation are commercially available. Stability testing was carried out at room temperature for 6 months and at 40° C. (105° F.) for 4 months. The authors felt that this elevated temperature was not unreasonable since this condition is commonly encountered during storage, particularly during the summer months and for longer duration in the southern areas of the country. With the exception of one preparation, wherein the sodium sulfacetamide was packed separately as a dry powder with instructions to the pharmacist for incorporation at the time of dispensing, none bore an expiration date (Table II).

The separately packed sodium sulfacetamide was also analyzed along with tablets containing sulfacetamide at the same temperatures and for the same durations. No deterioration was detected in any of these materials by any method used (Table III).

TABLE III—STABILITY ANALYSIS OF SULFACETAMIDE TABLETS AND POWDER[a]

Sample	Sulfacetamide (% of Labeled Amount) Initial	6 mos. at RT	4 mos. at 40°
Powder A	100.2	100.8	100.7
Powder B	99.6	100.2	99.8
Tablets A	106.2	104.6	106.1
Tablets B	101.2	102.0	101.1

[a] No sulfanilamide was found in the above preparations.

These results confirm the qualitative findings of Klein and Kho (10) who detected unlabeled sulfanilamide in suspension preparations of mixed sulfonamides containing sulfacetamide but not in tablet formulations of sulfacetamide.

Sulfanilamide was the only significant decomposition product found in sulfacetamide preparations. This was only evident after chromatographic separation. In addition, the amount of sulfanilamide present increased with length of storage. The preparation containing separately packaged dry sulfacetamide powder exhibited degradation characteristics similar to those in the same classification on prolonged storage. However, this pattern emerged only after incorporation of the sodium sulfacetamide into the vehicle and the completed preparation remained stable for the period indicated on the package insert.

SUMMARY

The customary analytical techniques as outlined in USP XVII and NF XII were demonstrated to be inadequate for purity and stability testing of pharmaceutical formulations containing sulfacetamide or its sodium salt.

Pharmaceutical suspensions and solutions containing sodium sulfacetamide were tested *via* TLC for stability under conditions of elevated temperature and prolonged storage at room temperature. All showed significant degradation to sulfanilamide. However, tablets and dry sodium sulfacetamide powder appear to be stable. Since sulfanilamide is a primary sensitizer, occurrence of sensitivity arising from prolonged use of sulfacetamide may be due to the concomitant presence of sulfanilamide as a contaminant.

This study demonstrates that ophthalmological and dermatological preparations of sulfacetamide and its sodium salt have a limited shelf life and should bear an expiration date. However, the possibility that other sulfonamides undergo similar degradation is a consideration which bears further investigation.

Methods for the quantitation of stability data for sulfacetamide are proposed which appear applicable to varied preparations of mixed sulfonamides.

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Keyphrases

Sulfacetamide solutions—stability
Sulfanilamide formation—sulfacetamide solutions
TLC—separation, identity
UV spectrophotometry—analysis
Colorimetric analysis—spectrophotometry

The Analysis of Barium Sulfate

By EVELYN M. COCHRAN, ERVIN B. INSKIP, PERRY KING, and HOWARD W. ZIEGLER

A method suitable for compendial assay of barium sulfate USP has been developed. Barium is separated as the carbonate and precipitated as the chromate. Calcium, strontium, and silica do not interfere. The calcium and strontium content may be determined by atomic absorption or X-ray fluorescence and silica by a colorimetric procedure. An *in vitro* study of the extractability of strontium and lead from barium sulfate into simulated gastric and intestinal fluids was performed.

THE CURRENT USP XVII (1) monograph for barium sulfate does not include an assay procedure. At the suggestion of the USP Committee of Revision, an investigation was initiated and an analytical procedure for the assay of barium sulfate was developed. Norwitz has published methods for the analysis of barium compounds by homogeneous precipitation as barium chromate (2, 3). The procedures were reported to be accurate in the presence of small amounts of strontium and calcium. With some modifications, the Norwitz method has been found satisfactory for the routine assay of barium sulfate. Since the presence of calcium, strontium, and silica was expected, tests for these elements were also developed. The possibility of strontium or lead extraction from barium sulfate was investigated by an *in vitro* experiment.

BARIUM SULFATE ASSAY

Procedure—Weigh accurately between 0.58 and 0.62 g. of sample in a tared platinum crucible. Add 10 g. of anhydrous sodium carbonate and mix by rotating the crucible. Fuse over a Meker burner until a clear melt is obtained and then heat for an additional 30 min. Cool, place the crucible in a 400-ml. beaker, add 250 ml. of distilled water, stir with a glass rod, and heat to dislodge the melt. Remove the crucible from the beaker and wash thoroughly with water, collecting the washings in the beaker. Rinse the inside of the crucible with 2 ml. of 36% acetic acid and then with water, again collecting the washings in the beaker. Continue heating and stirring until the melt is disintegrated. Cool the beaker in an ice bath until the precipitate settles. Decant the clear liquid through a Whatman No. 40 filter paper, taking care to transfer as little precipitate as possible to the paper. Wash twice by decantation as follows: wash down the inside of the beaker with about 10 ml. of cold 2% (w/v) sodium carbonate solution. Swirl the contents of the beaker and allow the precipitate to settle. Decant the supernatant liquid through the same filter paper as before, transferring as little precipitate as possible. Place the beaker containing the bulk of the barium carbonate precipitate under the funnel and wash the filter paper with five 1-ml. portions of 10% hydrochloric acid. Wash the paper well with water. Dilute the solution (which may be slightly hazy) with 100 ml. of distilled water, and then add 5.0 ml. of hydrochloric acid, 10.0 ml. of 40% (w/v) ammonium acetate solution, 25 ml. of 10% (w/v) potassium dichromate solution, and 10.0 g. of urea. Cover the beaker with a watch glass (not ribbed) and digest on the steam bath, or at 80–85°, for at least 16 hr. Filter while hot through a tared *fine* porosity sintered-glass crucible. Scrub the beaker several times with a rubber policeman to insure that all the precipitate is removed from the beaker. Wash with 0.5% (w/v) potassium dichromate solution and finally with about 20 ml. of water. Dry for 2 hr. at 105°, cool, and weigh.

$$\% \ BaSO_4 = \frac{\text{wt. } BaCrO_4 \times 0.9213 \times 100}{\text{sample wt.}}$$

Results and Discussion—In the procedure initially developed, the precipitation was performed by boiling on a hot plate for 1 hr. after a precipitate first appeared. When the method was put into routine use, some difficulties were encountered. Results were not always reproducible and samples were frequently lost due to "bumping" as the precipitate that formed began to settle. The procedure was modified to that given above in which precipitation is carried out by digestion at 80–85° overnight. No difficulties have since been encountered. Replicate results on various samples are listed in Table I.

A very small amount of barium sulfate is reprecipitated with the barium carbonate. This is not dissolved by the hydrochloric acid used to dissolve the barium carbonate, and if it is transferred to the filter paper it will be lost. However, if the barium carbonate is washed by decantation, as directed, a negligible amount of barium is lost. An ignition of a filter paper from the decantation procedure showed less than 0.05% residue based on the initial sample weight. Any barium sulfate remaining in the sample solution will be filtered off and weighed as barium chromate in the final step of the procedure. It was found that the final barium chromate precipitate contains less than 0.5% barium sulfate. This has a negligible effect on the assay result be-

TABLE I—BARIUM SULFATE ASSAY

Sample	Results, %
Ref. Std. laboratory prepared	99.79, 100.06, 100.11, 99.90, 100.09
1	98.57, 98.39, 98.36, 98.49
2	98.40, 98.30, 98.07, 98.22
314	98.23, 98.43, 98.44, 98.57
316	98.49, 98.70, 98.55, 98.74

Received March 28, 1968, from Mallinckrodt Chemical Works, St. Louis, MO 63160
Accepted for publication April 25, 1968.

cause of the small difference in molecular weights of barium sulfate and barium chromate.

An experiment using ^{133}Ba tracer showed about 0.04% of the total barium was lost in the sodium carbonate filtrate, and about 0.06% was lost in the chromate filtrate. Atomic absorption analysis for barium in the chromate filtrate also showed less than a 0.1% loss of barium. Thus total barium losses were found to be about 0.1%.

Calcium and strontium present in barium sulfate do not interfere with the assay since they do not precipitate. Atomic absorption analysis showed that less than 0.05% of the weight of the final barium chromate precipitate is due to calcium and strontium chromates. It was suggested[1] that the ability of the method to separate calcium and strontium at high levels be evaluated. A synthetic "low assay" mixture was prepared containing 70% barium sulfate, 20% strontium sulfate, and 10% calcium sulfate. This mixture was submitted for routine assay by the procedure given above. Duplicate results obtained were 71.6% and 70.6% $BaSO_4$. The slightly high and relatively imprecise results obtained on this mixture may have been due to coprecipitation of calcium and strontium. However, this experiment did demonstrate that essentially barium content is measured.

Other Methods Investigated—As a check of the proposed method, an alternate assay method was developed. The sample was fused with sodium carbonate as in the proposed procedure above. After dissolving the barium carbonate in hydrochloric acid, a slight excess of standardized EDTA solution was added and the pH adjusted to about 10. The excess EDTA was back titrated with magnesium chloride using Eriochrome Black T indicator. In this method, calcium and strontium were also quantitatively titrated. These elements were determined by atomic absorption and the assay corrected for their content. The corrected EDTA results showed about the same precision as the chromate assays and the averages of 10 determinations by each method differed by 0.05%.

DETERMINATION OF SILICA

Procedure—Transfer 0.5 g. of sample to a platinum crucible. Add 5 g. of anhydrous sodium carbonate. Heat over a Meker burner with occasional gentle swirling until the melt is clear and continue heating for an additional 30 min. Cool, rinse the outside of the crucible, and place it in a 250-ml. plastic beaker. Add 100 ml. of water and heat on the steam bath with frequent stirring for 1 hr. Cool to room temperature. Rinse and remove the crucible. Filter through Whatman No. 42 filter paper into a plastic beaker and wash the precipitate and paper thoroughly. Prepare a blank by dissolving 5 g. of sodium carbonate in about 100 ml. of water and carry through the remainder of the procedure. Add to the filtrate 15 ml. of 6 *N* sulfuric acid. Adjust the pH to 4.5–5.0 with 6 *N* sulfuric or silica-free ammonium hydroxide if necessary. Heat on a steam bath to dispel carbon dioxide. Cool to room temperature, transfer to a 200-ml. volumetric flask, and dilute to volume with water. Determine the silica colorimetrically as the reduced silicomolybdate complex as given by Boltz (4), using a 10-ml. aliquot of the solution.

[1] Personal communication, Dr. E. F. Salim, Drug Standards Laboratory.

Results and Discussion—Fusion with sodium carbonate is the method commonly used in analyses of silica-containing materials and was found amenable to barium sulfate. However, the 30-min. fusion time is necessary to convert all of the silica to a soluble form. Samples fused only until the melt was clear gave results which were low and nonreproducible. Fusion times of more than 30 min. were not found to be necessary.

The following experiments were performed to check for losses of silica. The barium carbonate precipitate was checked for silica content on four replicates of a selected high silica sample which averaged 0.83%. The barium carbonate precipitate was dissolved in acid and tested by the colorimetric procedure. The amounts of silica found were 0.02, 0.02, 0.04, and 0.05% on the total sample basis. No silica was found on the filter paper after dissolution of the barium carbonate. This was checked by igniting the paper, fusing the residue with sodium carbonate, and testing for silica colorimetrically.

Silica in solution sometimes polymerizes and in some forms will not form the molybdate complex. Two of the solutions remaining after taking aliquots for the colorimetric determination were tested for silica gravimetrically. The results agreed with those obtained colorimetrically, indicating that all silica in the solution was in a complexable form.

DETERMINATION OF CALCIUM AND STRONTIUM

Procedure—Weigh 0.25 g. of sample into a 150-ml. beaker and add 5 ml. of 70% perchloric acid. Cover with a watch glass and heat on a hot plate until dissolved (15–30 min.). A trace of silica may remain. Cool, wash down the watch glass and sides of the beaker with a small amount of water. The barium sulfate will reprecipitate. Add 50 ml. of 0.1 *M* EDTA solution and adjust the pH to about 10 with 20% sodium hydroxide solution. Cover with a watch glass and boil to redissolve (about 30 min.). Cool, transfer quantitatively to a 100-ml. volumetric flask, and dilute to volume with water. Prepare a reagent blank and carry it through the entire procedure. Determine the calcium and strontium content of these solutions by atomic absorption spectrophotometry using the standard addition-extrapolation technique. Calculate the percent calcium sulfate and strontium sulfate in the sample.

Alternately, the samples can be compared with known mixtures of $BaSO_4$, $CaSO_4$, and $SrSO_4$ by X-ray fluorescence. The two methods were found to give equivalent results.

COMPOSITION OF BARIUM SULFATE USP

Qualitative spectrographic analysis indicated only trace contamination by other cations except sodium and potassium. It is estimated that their combined concentration is less than 0.1%. The composition of barium sulfate indicated by these analyses is summarized in Table II.

THE *IN VITRO* EXTRACTION OF STRONTIUM AND LEAD

The only potentially hazardous substances encountered in this study were strontium and lead. Tests were conducted with simulated gastric and

TABLE II—SUMMARY OF RESULTS, %[a]

Sample	Assay	$SrSO_4$	$CaSO_4$	SiO_2	Loss on Ignition 1,000°C.	Total
1	98.45	0.89	0.36	0.16	0.20	100.06
2	98.20	0.96	0.46	0.16	0.20	99.98
314	98.42	0.88	0.36	0.19	0.15	100.00
316	98.62	0.92	0.29	0.12	0.16	100.11
Precision[b]	±0.32	±0.05	±0.08	±0.04	—	—

[a] Each value reported is the average of four determinations except the loss on ignition which is a single determination.
[b] Percent absolute, single determination, 95% confidence level.

TABLE III—EXTRACTION OF $BaSO_4$ WITH SIMULATED GASTRIC FLUID AND INTESTINAL FLUID—10-g. $BaSO_4$, 30-ml. FLUID, 37°C., 29 R.P.M.

Sample	Hr. Extracted with Gastric Fluid	Hr. Extracted with Intestinal Fluid	p.p.m. Extracted on a $BaSO_4$ Basis, Atomic Absorption Pb	Sr
25	4	—	<1	—
25	—	4	<1	—
25	8	—	<1	—
25	—	8	<1	—
25	24	—	<1	—
25	—	24	<1	—
25	4 additional	16	<1	—
32	24	—	<1	14
32	—	24	<1	0.9
72	24	—	<1	9
72	—	24	<1	1
112	24	—	<1	13
112	—	24	<1	2
21	24	—	<1	10
21	—	24	<1	2
26	24	—	<1	6
26	—	24	<1	0.6
26 plus 1,000 p.p.m. Pb	24	—	<1	8
26 plus 1,000 p.p.m. Pb	—	24	<1	0.9

intestinal fluids T.S. (5) to determine to what extent these elements were extracted from barium sulfate.

Procedure—Ten-gram samples of barium sulfate were placed in 50-ml. bottles with 30 ml. of simulated gastric or intestinal fluids. The bottles were rotated at 29 r.p.m. in a 37° water bath for various periods of time. The mixtures were centrifuged and filtered. The filtrates were tested for lead and strontium by atomic absorption. Standards were prepared with the simulated fluids to provide a constant matrix.

Results—Six different samples of barium sulfate having total lead contents ranging from 15 to 70 p.p.m. were treated by the above procedure using both fluids and extraction times from 2 to 24 hr. Results on all samples showed less than 1 p.p.m. lead (calculated on $BaSO_4$ basis) extracted into the gastric or the intestinal fluids as shown in Table III. As a further check, one sample was spiked with 1,000 p.p.m. lead (on the $BaSO_4$ basis) and carried through the procedure. Again, less than 1 p.p.m. lead was found. The removal of lead from the fluids in this experiment probably is due to adsorption and precipitation since an excess of sulfate is always present in barium sulfate USP.

Fluids from five of the samples were analyzed for strontium by atomic absorption. The strontium extracted into the fluids (in p.p.m. Sr on the $BaSO_4$ basis) is listed in Table III. The extracted residues contained 0.39–0.60% strontium. These results indicate only slight dissolution of strontium in either of the simulated fluids.

SUMMARY

A procedure has been developed for the assay of barium sulfate for compendial use. Replicate analyses in our laboratory on 52 samples indicate a precision of ±0.32% for a single determination at the 95% confidence level. It is felt that the composition of barium sulfate USP has been characterized by this study. *In vitro* studies indicate that no appreciable quantitites of strontium or lead are extracted from barium sulfate by simulated gastric or intestinal fluids.

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Keyphrases

Barium sulfate—analysis
Gravimetric analysis—barium chromate precipitation
Titrimetric analysis—EDTA, $MgCl_2$
Colorimetric analysis—silica
Atomic absorption spectrometry—calcium, strontium analysis
Strontium, lead extraction—gastrointestinal fluids

Determination of Acetylsalicylic Acid, Salicylamide, Acetaminophen, and Caffeine in Tablets or Powders by Independent Methods

By NORMAN SHANE and MARIE KOWBLANSKY

A method, suitable for automation, has been developed for the determination of four compounds commonly found in analgesic tablets. The tablet is dissolved in 85 percent methanol, filtered to remove the insoluble excipients, and three portions are taken. The first portion is analyzed for aspirin (ASA) by titration; the second portion, for acetaminophen (APAP) and salicylamide (SAL) by UV differential spectrophotometry; and the third portion for caffeine (CAF), either by a unique differential spectrophotometric operation, or by an extraction method, where the caffeine content is determined spectrophotometrically in the UV range. The average relative errors for each of the components are: 0.22% for aspirin, 0.51% for salicylamide, 0.80% for acetaminophen, 2.02% for caffeine by method I, and 1.18% for caffeine by method II.

THE ANALYSIS of a multicomponent system of analgesics usually requires many multiple separation steps or complicated UV, IR, or NMR methods, which demand solution of many simultaneous algebraic equations. Column chromatography (1) is a common procedure for the separation of phenacetin, caffeine, and acetylsalicylic acid. Solvent extraction methods for separation of components are described (2, 3). Other methods for determining the various components involve infrared spectrophotometry (4, 9), ultraviolet spectrophotometry (5, 6), titration (2), phosphorimetry (7), and nuclear magnetic resonance spectroscopy (8). Salicylic acid, acetylsalicylic acid, salicylamide, caffeine, and phenacetin in tablets or powders can be analyzed by spectrophotometric determination, requiring the solution of several algebraic expressions (10). Independent methods of analyzing for the constituents in a multicomponent system give greater accuracy, precision, and less computational problems than those which require the solution of simultaneous equations (11).

Recently, work from this laboratory has shown the power of the pH chromophoric procedure for the assay of drugs (12–14). Utilizing this concept, a method has been developed which can independently determine each of the four subject components of a tablet or powder, with no interferences from one another. It is not necessary to separate the components, except in the alternate method for caffeine, where just a simple chloroform extraction is performed. The procedures are very simple, rapid, accurate, precise, and can be easily automated.

Received January 29, 1968, from Bristol-Myers Products, Hillside, NJ 07207
Accepted for publication April 19, 1968.

EXPERIMENTAL

Apparatus and Reagents

Spectrophotometer—A Beckman DU spectrophotometer was used for obtaining quantitative data for SAL, APAP, and CAF. A Beckman DK 2 recording spectrophotometer was used to obtain the spectra in Figs. 5 and 6.

Standard 1-cm. square fused silica cells were employed in all cases.

Titration Apparatus—Experimental data were obtained with a Beckman model No. 1019 research pH meter set to read the millivolt scale, with a Beckman standard combination electrode filled with saturated aqueous KCl solution.

Solvents—Spectral grade, analytical reagent methanol and chloroform (Merck Co.) were used. The solvent system, 85% methanol, was prepared by volume-volume dilution of anhydrous methanol with distilled, deionized water. The titration system was 0.5 *N* alcoholic potassium hydroxide from Hartman-Leddon Co., diluted with 85% methanol and standardized with potassium acid phthalate to be 0.02 *N*.

Buffer Solutions—(*a*) pH 1.3; 250 ml. of 0.2 *M* KCl, 336 ml. of 0.2 *M* HCl, and diluted to 1,000 ml. with distilled water.

(*b*) pH 6; 250 ml. of 0.2 *M* KH_2PO_4, 28 ml. of 0.2 *M* NaOH, and diluted to 1,000 ml. with distilled water.

(*c*) pH 10; 250 ml. of 0.2 *M* H_3BO_3, 250 ml. of 0.2 *M* KCl, 220 ml. of 0.2 *M* NaOH and diluted to 1,000 ml. with distilled water. (In all cases, reagent grade materials were used.) All analgesic compounds *i.e.*, acetylsalicylic acid, USP grade; salicylamide, NF grade; acetaminophen, NF grade; caffeine, USP grade, were checked for purity by infrared spectrophotometry.

Tablets—The standard composition of the synthetic tablets which were analyzed contained 195 mg. of aspirin, 130 mg. of salicylamide, 98 mg. of acetaminophen, and 65 mg. of caffeine.

PROCEDURE

The tablet to be analyzed is crushed to a powder, quantitatively transferred to a 100-ml. volumetric flask, and approximately 4 ml. of chloroform is added (to dissolve the CAF). The solution is brought to volume with 85% methanol, shaken to dissolve all the soluble materials, and filtered to remove the insoluble excipients (Solution *A*). From Solution *A*, a 20-ml. aliquot is transferred into a 250-ml. beaker (Solution *B*), a 4-ml. aliquot is transferred into a 200-ml. volumetric flask (Solution *C*), and a 5-ml. aliquot is transferred into a 200-ml. volumetric flask (Solution *D*).

Acetylsalicylic Acid (ASA)—To determine the ASA content, 100 ml. of 85% methanol is added to Solution *B* and a magnetic stirrer is placed in the beaker. Using 0.02 *N* alcoholic KOH, which has been standardized against potassium acid phthalate, the ASA is titrated potentiometrically with a standard combination electrode filled with saturated aqueous KCl solution. As an alternate procedure, the solution could be titrated to an orange-red end point in the presence of chlorophenol red indicator. (Most tablets containing ASA also contain small quantities of salicylic acid, which are negligible when compared with the amount of ASA present. Since salicylic acid is also titratable by this procedure, the analysis cannot be carried out in the presence of appreciable amounts of salicylic acid.)

Salicylamide (SAL) and Acetaminophen (APAP)—To determine the amounts of APAP and SAL, 5 ml. of 0.5 *M* NaOH is added to Solution *C*; the solution is allowed to stand for approximately 5 min. (to ensure complete conversion of ASA to its sodium salt), and then brought to volume with distilled water. A 20-ml. aliquot of this solution is transferred into each of two beakers. To one beaker, 10 ml. of pH 6 buffer is added, and to the other, 10 ml. of pH 10 buffer is added. Using the pH 6 buffer solution as the reference, and the pH 10 solution in the sample position, absorbance readings are taken on a DU spectrophotometer at 330 mμ (for SAL) and 263.5 mμ (for APAP).

Caffeine (CAF)—*Method I*—To determine the amount of CAF in the tablet, Solution *D* is brought to volume with distilled water, and a 20-ml. aliquot of this solution is pipeted into each of two beakers. To one beaker, 10 ml. of pH 1.3 buffer are added, and to the other, 10 ml. of 1:10 concentrated HCl:H_2O solution. An absorbance measurement is made on a DU spectrophotometer at 283 mμ, with the HCl solution in the reference position.

Method II—An alternate procedure for the determination of the CAF content is to bring Solution *D* to volume with 0.01 *M* NaOH, transfer a 30-ml. aliquot of this solution into a 250-ml. separator, and extract twice with 10-ml. aliquots of spectrograde chloroform. The chloroform extract is collected in a 50-ml. volumetric flask and brought to volume with the same solvent. The absorbance of this chloroform solution is read on a DU spectrophotometer at 276 mμ with chloroform as the reference solution.

RESULTS

Acetylsalicylic Acid (ASA)—Seven synthetic tablets, which contained varied amounts of ASA in the presence of standard amounts of CAF (65 mg.), APAP (98 mg.), and SAL (130 mg.), were titrated for ASA with extremely good results.

Salicylamide (SAL)—Figure 1 depicts three curves of absorbance readings at 330 mμ of solutions containing SAL alone, SAL in the presence of varying amounts of APAP, and SAL in the presence of standard amounts of ASA, CAF, and APAP. Since the three lines representing the relationships of the points coincide, it can be concluded that the determination of SAL in the tablets or powders is independent of the concentrations of the other ingredients, and therefore, no correction term needs to be applied in the calculations. By regression analysis the absorptivity for SAL at 330 mμ has been determined to be 36.5 absorbance units/(g./l.).

Acetaminophen (APAP)—Figure 2 depicts three curves of absorbance readings at 263.5 mμ of solutions containing various concentrations of APAP alone, APAP in the presence of varying amounts of SAL, and APAP in the presence of standard amounts of ASA, CAF, and SAL. Since the three lines representing the relationships of the points coincide, it may again be concluded that the determination of APAP in the tablets is independent of the concentrations of the other ingredients. The absorptivity for APAP at 263.5 mμ, as determined by regression analysis is 38.6 absorbance units/(g./l.).

Caffeine (CAF)—*Method I (Differential Spectro-*

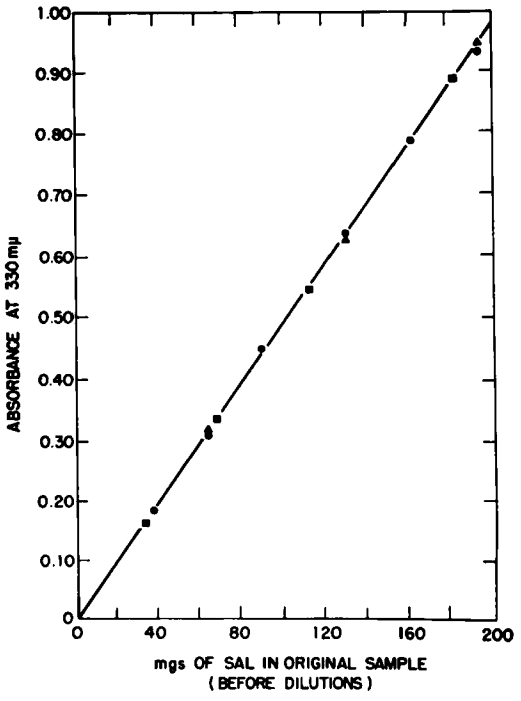


Fig. 1—Differential absorbances of various concentrations of SAL. Key: ■, SAL alone; ▲, SAL in the presence of varying amounts of APAP; ●, SAL in the presence of standard amounts of APAP, CAF, and ASA.

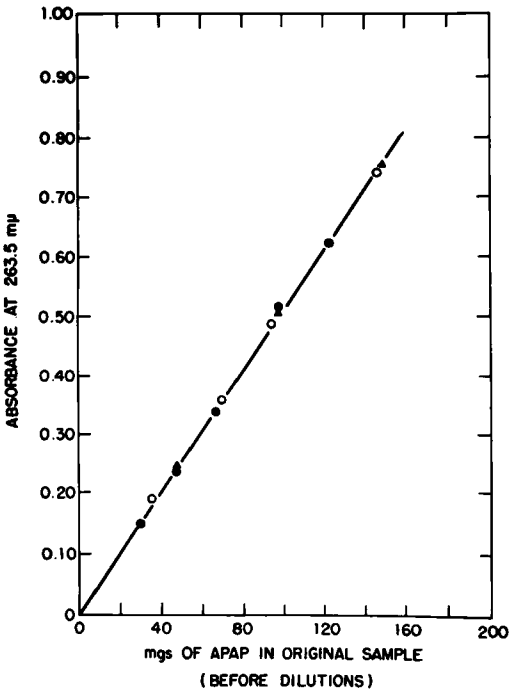


Fig. 2—Differential absorbances of various concentrations of APAP at 263.5 mμ. Key: ○, APAP alone; ▲, APAP in the presence of varying amounts of SAL; ●, APAP in the presence of standard amounts of SAL, ASA, and CAF.

photometry)—Figure 3 summarizes a number of experiments that were performed to determine whether there is any interaction between any of the components in this procedure. Absorbances at 283 mμ are plotted for: (*a*) various levels of CAF alone, (*b*) CAF in the presence of SAL, (*c*) CAF in the presence of APAP, (*d*) CAF in the presence of ASA, and (*e*) CAF in the presence of all three components. As can be seen, curves 1 through 4 are linear and coincide, proving that there is no interaction. The absorbance readings for CAF in the presence of all three components are linear to the 85-mg. region (mg. in the original sample); above this quantity the plot deviates from linearity. This observation may be explained in the following manner. Direct spectrophotometry, where water is used as the reference solvent, indicates that the sample solution containing 65 mg. of CAF plus standard amounts of the other components, reads about 1.2 in absorbance. At 130 mg., the reading is approximately 2.0. At this high absorbance, the phototube response to the small amount of light transmitted through the solution becomes nonlinear. Therefore, due to the high background absorbance of the other three components, readings above 0.125 are unreliable. At 283 mμ, the absorptivity for CAF, as determined by regression analysis, is 8.98 absorbance units/(g./l.).

Method II (*Extraction*)—Figure 4 represents the following relationships: (*a*) absorbances of various concentrations of CAF in chloroform, and (*b*) absorbances of various concentrations of CAF extracted into chloroform from standard amounts of ASA, APAP, and SAL. In both cases the same

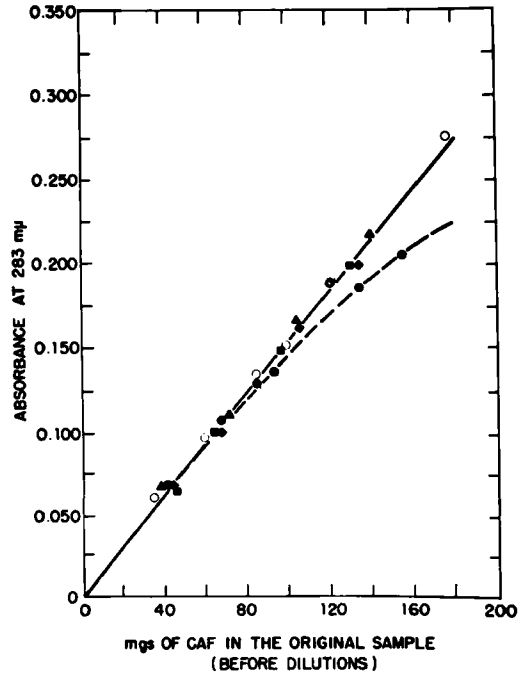


Fig. 3—Differential absorbances of various concentrations of CAF at 283 mμ. Key: ○, CAF alone; ▲, CAF in the presence of SAL; ■, CAF in the presence of APAP; ◆, CAF in the presence of ASA; ●, CAF in the presence of standard amounts of ASA, APAP, and SAL.

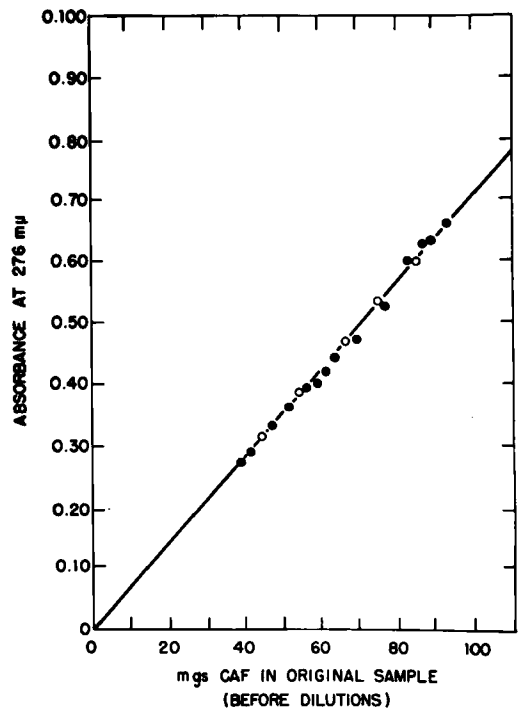


Fig. 4—Standard calibration curve for CAF in chloroform. Key: ○, CAF dissolved in $CHCl_3$; ●, CAF extracted into $CHCl_3$ from standard amounts of ASA, APAP, and SAL.

TABLE I—STATISTICAL RESULTS FOR EACH PROCEDURE

	No. Observations	No. of Analysts	Average Relative Error	Standard Deviation	95% Confidence Limits	95% Confidence Limits, mg.
ASA	7	1	0.22%	±0.30%	±0.60%	195 ± 1.15
SAL	18	4	0.51%	±0.84%	±1.68%	130 ± 2.18
APAP	18	4	0.80%	±1.00%	±2.00%	98 ± 1.96
CAF (method I)	18	4	2.02%	±2.41%	±4.82%	65 ± 3.13
CAF (method II)	24	3	1.18%	±1.43%	±2.86%	65 ± 1.86

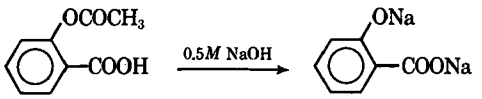
dilutions were made as described in the procedure, thus yielding the same final concentrations; however, for the sake of simplicity, the absorbances are plotted for mg. of caffeine in the original sample. By regression analysis, the absorptivity of CAF in chloroform at 276 mμ has been calculated to be 47.4 absorbance units/(g./l.), with a correction term of 0.013. The CAF concentration, therefore, is calculated using the following relationship: $(A_{276} + 0.013)/47.4$ = g. of CAF/l.

The statistical results for each of the procedures are summarized in Table I.

DISCUSSION

ASA is the only ingredient in the 85% alcoholic solution that is titratable (assuming that the amount of salicylic acid present is negligible). The excipients of the tablet or powder have no effect. For automation, spectrophotometric titration would be more workable than potentiometric titration. At the end point, a rapid color change of the dye indicator could be detected with a colorimeter or spectrophotometer.

In the SAL and APAP procedure, the ASA in the sample is converted to the disodium salt of salicylic acid with 0.5 *M* sodium hydroxide (15). See Scheme I.



Scheme I

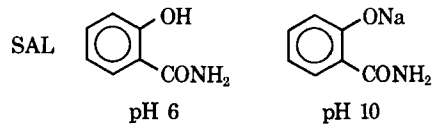
Since the pKa_1 and pKa_2 of salicylic acid are 3.0 and 13.4 (16), respectively, in buffers pH 6 and pH 10, the disodium salicylate converts to the monosodium salt (Scheme II):

Scheme II

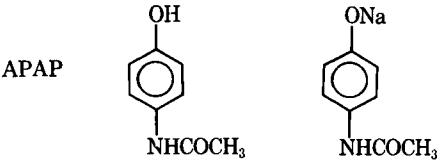
Consequently, since equal amounts of sample are contained in both buffered solutions, the absorbances of the two solutions due to ASA (which has been converted to monosodium salicylate) will exactly cancel each other, and the net absorbance will be zero.

The CAF (pKa = 13.39) (16) is in the same form at both pH's. The predominant species of APAP (pKa = 9.0) (16) and SAL (pKa = 8.4) (16) at the two different pH's are as in Schemes III and IV.

Therefore, at these two pH's each compound will exhibit different absorbance spectra. Consequently, since the pH 6 solution is in the reference position and the pH 10 solution is in the sample position, the spectra will automatically subtract in the spectrophotometer, giving new characteristic spectra for APAP and SAL.



Scheme III



Scheme IV

Figure 5 depicts the Beckman DK 2 differential spectra of samples containing various concentrations of SAL alone, APAP alone, and only CAF plus ASA. The samples were prepared by treating the individual components by the procedure described for SAL and APAP. The composition of the reference and sample solutions are as follows:

Reference pH 6	Sample pH 10	Absorbance 330 mμ	Absorbance 263.5 mμ
Monosodium salicylate (equivalent to ASA) + CAF	Monosodium salicylate (equivalent to ASA) + CAF	none	none
APAP	APAP	none	yes
SAL	SAL	yes	none

Neither CAF nor ASA (which has been converted to the monosodium salicylate) exhibit any absorbance from 255 to 340 mμ. Therefore, the SAL and APAP content of the tablets may be determined differentially at 330 mμ and 263.5 mμ, respectively, since

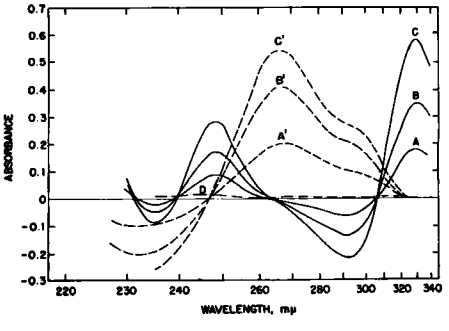


Fig. 5—Differential spectra of SAL (A, B, C), APAP (A', B', C'), and CAF + ASA (D).

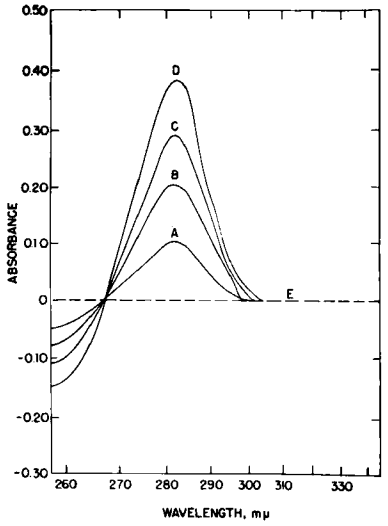


Fig. 6—Differential spectra of CAF (A, B, C, D) *and SAL + APAP + ASA* (E).

APAP shows no absorbance at 330 mμ, and SAL has an isosbestic point of zero absorbance at 263.5 mμ. A detailed explanation on the use of isosbestic point in spectrophotometry may be found (12). To ascertain that the pH 6 and pH 10 buffers cause maximum absorbance for APAP and SAL, pH 11 and pH 12 buffers were tried instead of the pH 10 buffer. As would be expected from the pK values of APAP and SAL, it was found that the pH 11 and pH 12 buffers caused an insignificant increase in absorbance over that of the pH 10 buffer.

In the differential spectrophotometric method for the determination of CAF, two buffer solutions are used—pH 1.3 and 1:10 HCl:H_2O solution. Since the pKa of the conjugated acid of caffeine is 0.61, caffeine exists predominantly as two different species at the two pH's.

Caffeine	Caffeine · H^+ (conjugated acid)
pH 1.3	1:10 HCl:H_2O

Therefore, caffeine should exhibit a differential spectrum between these pH's. Figure 6 demonstrates that CAF shows an absorbance maximum at 283 mμ. As expected from the pK values listed above, the other components exist as the unionized species in both buffers, and, therefore, exhibit no absorbance differentially. Unfortunately, the background effect is too great to permit absorbance readings to be taken in the optimum range of 0.42 absorbance units when the tablets are of standard composition. However, if tablets of different composition are analyzed (where the background absorbances are small), this procedure could prove to be very useful, since one could work at higher absorbances, with no interference from the other components.

In the caffeine extraction method, APAP, SAL, and ASA are converted to their sodium salts with sodium hydroxide and remain in the aqueous layer. The CAF, which is not affected by the sodium hydroxide can then be easily extracted from the other

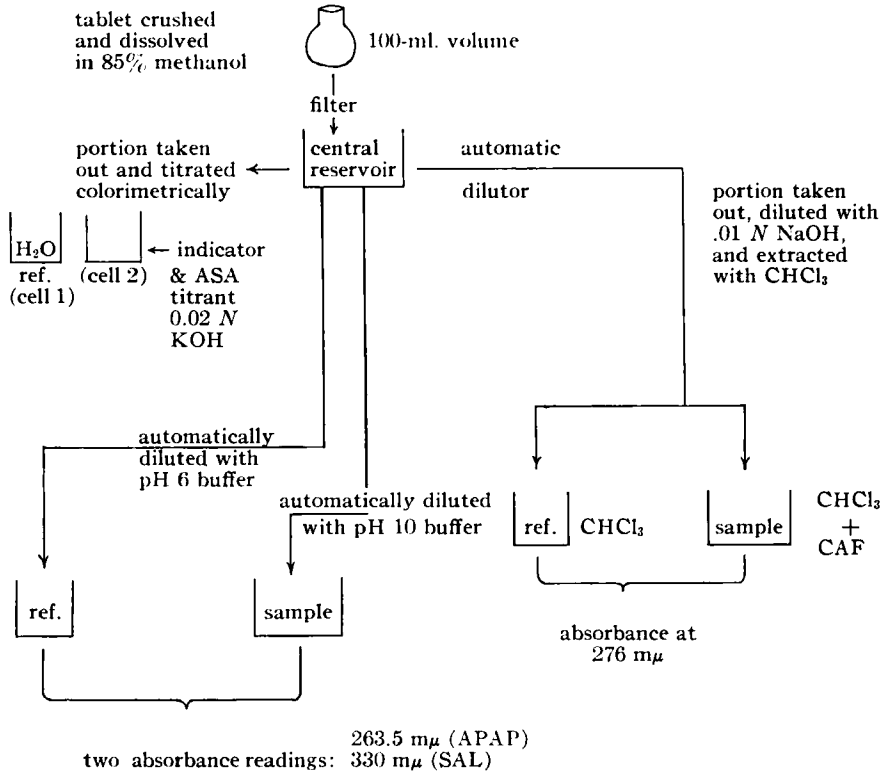


Fig. 7—Proposed schematic diagram for the automated system.

components with chloroform. However, it is imperative to use 0.01 *N* NaOH, because a stronger concentration of the base can change the CAF structure (17), and, therefore, cause erratic results.

Automation for the analysis of the four components can be performed by following the above procedures (extraction, rather than differential spectrophotometry is preferred for CAF). The results would be directly obtainable from the responses of the recorders without need of any computers or calculating devices. The proposed schematic diagram for the automated system is shown in Fig. 7.

The tablet is crushed and dissolved in 85% methanolic solution, which is filtered into a central reservoir. From this container, three portions are taken. One aliquot is colorimetrically titrated for ASA in the presence of dye indicator with 0.02 *N* KOH. A second aliquot is automatically diluted with a given quantity of 0.01 *N* sodium hydroxide, and the CAF is extracted into chloroform, which is read at 276 mμ *versus* chloroform on a spectrophotometer. A third portion is automatically diluted and separated into two segments. To one portion, a pH 6 buffer solution is added and passed through a reference flow cell located in a spectrophotometer. Simultaneously, a pH 10 buffer solution is added to the other segment of the solution and passed through a sample flow cell in the spectrophotometer. The wavelength scale is programmed rapidly to two wavelengths—263.5 and 330 mμ. The APAP and SAL are determined at 263.5 and 330 mμ, respectively.

The advantage of using these methods is that each specific procedure is independent of the concentrations of the other components, and therefore, can be used either in the presence of large quantities or in the absence of any of the other ingredients, with very good accuracy and precision.

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Keyphrases

Analgesic tablets, powders—analysis
Aspirin determination—analgesic mixture
Colorimetric titration—analysis
Salicylamide, acetaminophen determination—analgesic mixture
Caffeine determination—analgesic mixture
UV spectrophotometry—analysis

Application of Sugar Coating to Tablets and Confections by Means of an Automated Airless Spray System I

Investigation of the Direct Coating of Tablets

By G. M. KRAUSE and T. L. IORIO*

The practicability of directly applying sugar coating to unsubcoated tablets has been studied. Initial work performed indicates that such a procedure may be feasible when used in place of current sugar-coating techniques.

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Received May 10, 1967, from the College of Pharmacy, Northeastern University, Boston, MA 02115
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coating is still the principal method used for coating tablets and also confections. Nevertheless, the time involved and the uncertain reproducibility are reflected in high costs of operation. It would seem that an investigation of the practicability of modifying the traditional sugar-coating method of tablets would be desirable.

One of the drawbacks of the traditional sugar-coating procedure has been the necessity of subcoating tablets (6). This procedure is time consuming, and in some instances, an untidy operation. Tablets and confections of odd shapes, having sharp corners and thick edges, generally require subcoating to facilitate the application of a sugar coat. Those with well-rounded edges or corners do not present too much difficulty. This discussion will deal primarily with products of standard round shapes and normal curvatures.

DESIGN AND EQUIPMENT

Hydraulic Systems—A hydraulic system was employed that was capable of developing 1400 psig, although most of the work was done at about 500 psig. The coating solutions were heated, circulated, and sprayed by an airless pump through nozzles as described by Lachman and Cooper (1).

Coating Pan—A 40.6-cm. (16-in.) diameter round stainless steel coating pan, unbaffled, was used because a reasonable quantity of tablets, about 8 kg., could be coated easily, and there was also room to accommodate the spray nozzle. Initial tumbling of the tablets was simplified by previously coating the inner surface of the pan with a thin coat of liquid latex, or syrup, and drying. The speed of the pan was 28 r.p.m. and was constant for all trials.

Ventilation and Air System—A conventional air delivery system was used capable of drying the tablets during the drying cycles. An exhaust system capable of greater air capacity than the air supply was used.

Programmer—The programmer was constructed similar to that described by Mody, Scott, and Lieberman (2), but modified to provide auxiliary controls that could be used to suspend any of the automated functions as desired. A series of neon lights was provided for the timers to indicate sequence of operation, primarily as an instructional aid. All wire connections were made to readily accessible terminal strips for any changes or modifications in circuitry.

EXPERIMENTAL

Tablets and Materials—The initial trials were conducted on ball-shaped, uncoated confections obtained from a commercial producer. They averaged 0.50 in. in diameter and weighed 1.36 g. each. The compressed tablets used during these experiments were prepared from terra alba, using the wet granulation method. They weighed 385 mg./-tablet, with a diameter and gauge of 95.4 mm. (0.375 in.) and 4.43 mm. (0.174 in.), respectively, and were compressed on a Colton 216 single rotary, using extra deep, cup-shaped punches. Approximately 1,250,000 tablets were used during this work. Coating solutions used were 4-lb. cut pharmaceutical glaze (shellac), subcoating solution according to Clarkson (6), and sugar syrup colored with 0.1% F.D. & C. Red No. 3. With the exception of batch series A and I, Table I, all batches used received two coats of glaze, and weighed 8 kg. initially. Batch series A and I received no precoat, and series C was subcoated over the shellac coat. The initial trial coating weight of series C was therefore correspondingly greater, *i.e.*, 9.27 kg. All shellac coats and subcoats were applied by the conventional pan-coating method (6). All sugar coats, with the exception of series B, Table I, were applied by the automated airless-spray method.

Four different kinds of sugar-coated commercial tablets were purchased from local pharmacies. They were in original unopened containers and were of about the same size and weight as the tablets coated by the automated method during this experiment.

Sampling Plan—Samples were removed from the coating pan at the end of 10, 20, 30, and 40 cycles, or 20, 40, 60, and 80 cycles, depending on whether the coating process was for 40 or 80 cycles. The tablets were weighed and measured for diameter and gauge increase individually. The data are seen in Figs. 1 and 2, and Tables II and III.

Coating Procedure—The ball-shaped confections received 60 applications of syrup by means of the automated airless-spray method. The average weight of sugar delivered per confection was 0.350 g., with an increase in diameter of 0.75 mm (0.030 in.), Total coating time was 90 min. The trials were repeated twice, and data are presented in Table I (series I) and in Fig. 3, which represent an average of the three batches.

Subcoated Tablets—Shellac-coated tablets were subcoated and then sugar coated by the automated method. The operation was continued through 40 cycles per batch for the series and sampled according to plan. The average cumulative gain, diameter and gauge are shown in Figs. 1 and 2. Additional data are summarized in Table I (series C).

Shellac-Coated Tablets—These were sugar coated directly by the automated process, with part of the series going through 40 cycles, and the remainder through 80 cycles. In both instances complete coverage took place after 20 cycles, and the process continued through 40 cycles and 80 cycles in order to note uniformity of coating application. Data are presented in Table I (series D, E, F, G, and H).

Uncoated Tablets—This batch series received no precoating, only the usual processing to remove any dust and loose particles. These tablets coated well, and the data are seen in Table I (series A).

Conventional Method—In order to compare the results of sugar-coating tablets by the automated-spray method with those sugar coated by the traditional pan-coating method, several batches of shellac-coated tablets were coated under conditions approximating the spray method as closely as possible. Forty coats were applied, using the same amount of sugar per coat as per cycle in the automated method. Each application was timed with a stopwatch for uniformity of conditions. With the exception of the increased drying time, all other conditions were met, and the results were almost compa-

TABLE I—WEIGHT OF SUGAR ACQUIRED BY TABLETS PER BATCH SERIES USING AUTOMATED AIRLESS-SPRAY SYSTEM

Sample series[a]	A	B	C	D	E	F	G	H	I
Number of cycles	40	40	40	40	40	40	40	80	40
Initial weight/batch, kg.	8	8	9.27	8	8	8	8	8	8
Weight increase/batch, kg.	1.46	1.45	1.46	1.24	1.56	1.56	1.56	3.14	2.06
Sugar delivered/cycle, g.	36.80	36.80	36.80	31.50	39.50	39.50	39.50	39.50	23.00
Total operating time, min.	88	137	104	89.5	88	102	107	201	90

[a] Series A, no precoat; series B, conventional pan sugar coating; series C, subcoat; series I (confections), no precoat; all series except A and I were shellac coated.

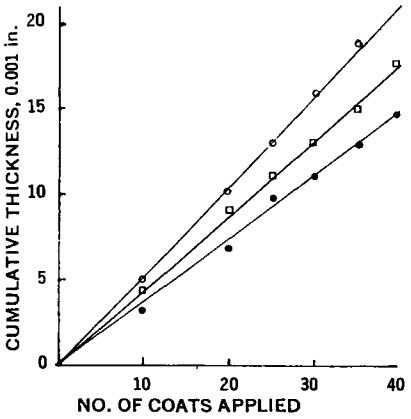

Fig. 1—Comparison of gain in tablet wall thickness of tablets coated by automated method and tablets coated by conventional method. Key: ○, diameter (automated); ●, gauge (automated); □, diameter and gauge (conventional).

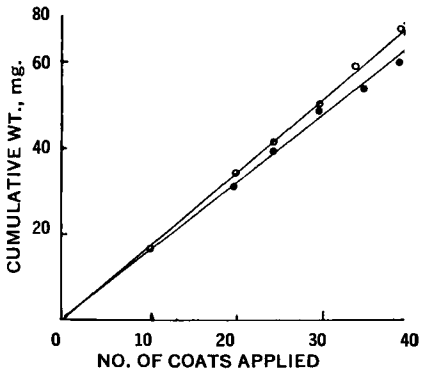

Fig. 2—Gain in weight of tablets coated by automated method and tablets coated by conventional method. Key: ○, automated; ●, conventional.

rable to those of the automated method. Data are presented in Figs. 1 and 2, and Table I (series B).

Total Operating Time—The total operating time for each batch series was dependent on (*a*) the type of tablet coated, *i.e.*, whether it was not precoated, subcoated, or shellac coated, (*b*) quantity of sugar delivered per cycle, (*c*) type of coating method employed, *i.e.*, conventional pan coating or automated airless-spray method, and (*d*) deliberate change in length of drying time increments per cycle in order to determine operating ranges for the production of a satisfactory coat. These changes are reflected in the figures for total operating times in Table I. All sugar coats entered in Table I were judged satisfactory.

Comparison of Coating Variability to Commercial Samples—Twenty tablets were chosen at random from each of four commercial samples and then individually weighed and measured. Thirty tablets each from the lots spray coated by the automated method were also selected at random and weighed and measured. The data are presented in Tables II and III.

RESULTS AND DISCUSSION

The ball-shaped confections were used as the initial samples for coating because objects of such shape roll readily in the coating pan and are rather easy to coat, since they also have no edges or corners that generally cause difficulty in coating. The settings of the programmer for sugar delivery for this ball-shaped confection could be made over a rather wide range, consistent within reasonable limits. The ease of coating experienced with ball-shaped objects would indicate that they could be used for familiarization with the operation of the automated airless-spray system. Normally, baffles are used in the coating pan to produce tumbling with the automated system. However, it was found that the application of a thin coating of liquid latex or syrup to the pan and drying it, would permit good tumbling during the initial stages when tablets generally have a

TABLE II—MEANS ($\bar{X}$), RANGES (R), STANDARD DEVIATIONS (S), AND COEFFICIENTS OF VARIATION (CV) OF TABLET DIAMETER

Type of Sugar Coating	Sample[a]	No. of Tablets	Tablet Diameter, in. $\bar{X}$	R	S	CV
Conventional	A	20	407.5	25	6.38	1.57
	B	20	404.4	16	3.74	0.93
	C	20	430.5	10	2.60	0.60
	D	20	436.6	11	2.78	0.64
Automated airless spray	E	30	408.0	9	2.06	0.51
	F	30	400.8	12	2.52	0.63
	G	30	416.4	10	2.89	0.69

[a] Samples A–D were commercial tablets, and samples E–G, were tablets included in study.

TABLE III—MEANS ($\bar{X}$), RANGES (R), STANDARD DEVIATIONS (S), AND COEFFICIENTS OF VARIATION (CV) FOR TABLET GAUGES

Type of Sugar Coating	Sample[a]	No. of Tablets	Tablet Gauge, in. $\bar{X}$	R	S	CV
Conventional	A	20	225.5	22	5.41	2.40
	B	20	240.7	19	5.14	2.14
	C	20	226.8	11	2.70	1.19
	D	20	253.5	15	3.98	1.57
Automated airless spray	E	30	204.6	11	2.24	1.10
	F	30	194.5	15	4.23	2.18
	G	30	208.5	12	3.19	1.58

[a] Samples A–D were commercial samples, and samples E–G were tablets included in study.

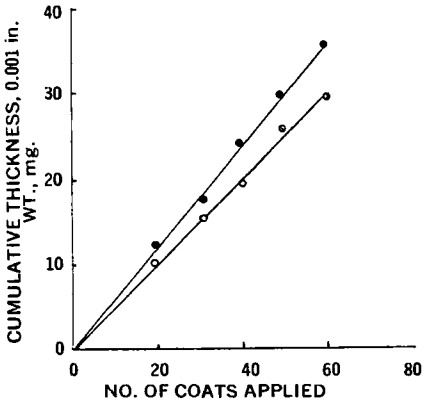

Fig. 3—Gain in weight and tablet wall thickness of ball-shaped confections coated by automated airless-spray method. Key: O, wall thickness; ●, weight

tendency to slide. Difficulty in tumbling is usually not experienced after the first or second cycle.

Each of the four samples obtained from commercial outlets represented a single production lot per sample, and the results therefore cannot be interpreted as being indicative of other lots of the same product. Variations of individual coated tablets within a single lot can be divided into three types: (*a*) those caused by inherent variations in the uncoated tablets, (*b*) those caused by the actual coating operation, and (*c*) the possibility of both *a* and *b* occurring at the same time. The standard deviation of all of the tablets coated by the automated process was equal to or less than that of the four commercial samples. These were used for comparison because they were similar in weight and dimensions to the spray-coated tablets. However, a greater standard deviation in the commercial tablets may not be objectionable if the weight variation is due only to nonmedicinal coating ingredients, and not active materials that might be included in the coats.

Tables II and III show the means, ranges, standard deviations, and coefficient of variations for tablet diameter and gauge, respectively. In all cases, tablets coated by the automated process compare favorably to those coated by the conventional method. The subcoated automated lot had less variation than any of the samples examined.

Batches of tablets weighing 8 kg. each were used because this was equivalent to about 8 l., the optimum working capacity of the 40.6 cm. (16 in.) coating pan. Standard concave-shaped tablets, particularly those with thick edges, are more of a problem than those with a curvature approaching roundness, such as the extra deep cup shape. It is well known to the arts that tablets possessing greater curvature and thin edges are coated with less difficulty than others.

During sampling, it was noted that coverage took place at about 20 cycles, as could easily be seen by examining the samples through a 7× lighted magnifier. The process was initially continued until 80 cycles, but since coverage was complete before this, and prior work indicated that additional coats could be applied readily, the operation was generally stopped at the end of 40 cycles. As was noted before, sugar was applied directly to tablets not precoated, with a reasonable degree of success. This area is being explored further.

Tablets coated by the automated process acquired a greater thickness on the edges (diameter) than on the surface (gauge). At the present time, the reason for this difference is unknown, and work is being done to determine the cause and if it can be controlled. The comparison is shown in Fig. 1.

The build-up of sugar in the coating pan ranged from about 0.08–0.13%, with a mean of 0.10%. This was found to be dependent on the weight of sugar delivered per cycle and the drying time, and could be adjusted readily. Trials were conducted with the 40.6-cm. coating pan to determine the maximum and minimum load of shellac-coated tablets that would slide or tumble in this pan when the latter was coated with latex. As little as 2.5 kg. of these tablets would tumble in the treated pan, and as much as 10–12 kg. would slide in the uncoated pan. It should be pointed out that this latex or sugar coating was used only for the initial start of tumbling of the tablets, because once there is a slight quantity or sugar on the pan walls, tumbling will generally proceed normally.

It would appear that, within specific limitations, direct application of sugar to tablets and confections, with or without precoat, shows promise. Transfering this process to an automated airless-spray system permits greater uniformity of coating and decreased costs of operation because the operator need not be particularly skilled and can control more coating pans with greater precision than one skilled in performing his work in the traditional manner.

Smooth finish coats were obtained by the usual reduction and closed pan method as described in Clarkson (6), however, this requires the removal of the spray gun from the coating pan after the last cycle. The finished tablets can be polished by any conventional means, with the usual materials.

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Keyphrases

Tablets—sugar coating
Spray coating—automated airless system
Automated coating, pan coating—comparison

Semiautomated Assay for the Simultaneous Determination of Propoxyphene Hydrochloride and Paramethasone Acetate in Coated Single Tablets

By C. E. STEVENSON and I. COMER

A semiautomated method for the simultaneous determination of propoxyphene hydrochloride and paramethasone acetate is described for coated tablets. A novel device for the removal and dissolution of tablet coatings is outlined. Solutions are sampled with an automatic sampler at the rate of 20/hr. and fed to two independent systems for analysis. A modified blue tetrazolium procedure is utilized for the determination of paramethasone acetate producing essentially complete color development within 5 min. at room temperature. Working concentrations of 12.5 mcg. paramethasone acetate per ml. can be assayed without resorting to range expansion. Analysis of propoxyphene hydrochloride is accomplished by the formation of a complex with bromocresol purple with subsequent extraction and color measurement in ethylene dichloride.

THE AUTOMATED analysis of single tablets has been reported by Wolski (1) as a useful tool in the concept of quality assurance. The product[1] involved in this study consists of an enteric 500-mg. acetylsalicylic acid (aspirin) core tablet coated with 32.0 mg. of the analgesic propoxyphene hydrochloride (DPH), and 0.250 mg. of the ketosteroid paramethasone acetate (PMA) (6α-fluoro-16α-methylprednisolone 21-acetate). For the quality assurance of this product, an automated single-tablet procedure for the simultaneous determination of DPH and PMA was developed using the automatic analyzer.[2]

A desirable condition involving methods for simultaneous determinations is that each component be capable of measurement in the presence of the other without significant interference. The automated method of Kuzel (2), based on the formation of a complex between tertiary amines and bromocresol purple (BCP), and the automated methods of Greely *et al.* (3) and Beyer (4), utilizing the reducing powers of the ketosteroids on blue tetrazolium (BTZ), meet this condition for the compounds in this study. Slight modifications of these procedures have been made in the development of this automated system.

EXPERIMENTAL

Reagents—*BCP*—A 2% acetic acid–98% deionized water (v/v) solution containing 0.25 mg. of bromocresol purple (5′,5″-dibromo-*o*-cresolsulfonphthalein Na salt, Eastman No. 6266) per ml.; *BTZ*—A solution containing 0.15 mg. blue tetrazolium (Fisher Certified B-410) per ml. SD-3A alcohol; *Ethylene Dichloride*—Fisher Certified E-175; TMAH—A solution of 20% tetramethylammonium hydroxide (Eastman No. 1515)–80% SD-3A alcohol (v/v); *Wash*—A solution of 50% methanol–50% deionized water (v/v); *Theory Standard*—32.0 mg. propoxyphene hydrochloride and 0.250 mg. paramethasone acetate reference standard per 20 ml. of Wash solution.

Sample Preparation—Due to the relatively low steroid concentration in the formulation, it was desirable to limit the amount of solvent used in the dissolution of the active ingredients to as low a volume as possible. Utilization of an automatic[3] module

Received November 20, 1967, from Eli Lilly & Co., Indianapolis, IN 46206
Accepted for publication January 25, 1968.
[1] Marketed as Stero-Darvon with ASA by Eli Lilly and Co., Indianapolis, Ind.
[2] AutoAnalyzer, Technicon Corp., Chauncy, N. Y.
[3] Solidprep, Technicon Corp., Chauncy, N. Y.

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A semiautomated method for the simultaneous determination of propoxyphene hydrochloride and paramethasone acetate is described for coated tablets. A novel device for the removal and dissolution of tablet coatings is outlined. Solutions are sampled with an automatic sampler at the rate of 20/hr. and fed to two independent systems for analysis. A modified blue tetrazolium procedure is utilized for the determination of paramethasone acetate producing essentially complete color development within 5 min. at room temperature. Working concentrations of 12.5 mcg. paramethasone acetate per ml. can be assayed without resorting to range expansion. Analysis of propoxyphene hydrochloride is accomplished by the formation of a complex with bromocresol purple with subsequent extraction and color measurement in ethylene dichloride.

THE AUTOMATED analysis of single tablets has been reported by Wolski (1) as a useful tool in the concept of quality assurance. The product[1] involved in this study consists of an enteric 500-mg. acetylsalicylic acid (aspirin) core tablet coated with 32.0 mg. of the analgesic propoxyphene hydrochloride (DPH), and 0.250 mg. of the ketosteroid paramethasone acetate (PMA) (6α-fluoro-16α-methylprednisolone 21-acetate). For the quality assurance of this product, an automated single-tablet procedure for the simultaneous determination of DPH and PMA was developed using the automatic analyzer.[2]

A desirable condition involving methods for simultaneous determinations is that each component be capable of measurement in the presence of the other without significant interference. The automated method of Kuzel (2), based on the formation of a complex between tertiary amines and bromocresol purple (BCP), and the automated methods of Greely *et al.* (3) and Beyer (4), utilizing the reducing powers of the ketosteroids on blue tetrazolium (BTZ), meet this condition for the compounds in this study. Slight modifications of these procedures have been made in the development of this automated system.

EXPERIMENTAL

Reagents—*BCP*—A 2% acetic acid–98% deionized water (v/v) solution containing 0.25 mg. of bromocresol purple (5′,5″-dibromo-*o*-cresolsulfonphthalein Na salt, Eastman No. 6266) per ml.; *BTZ*—A solution containing 0.15 mg. blue tetrazolium (Fisher Certified B-410) per ml. SD-3A alcohol; *Ethylene Dichloride*—Fisher Certified E-175; TMAH—A solution of 20% tetramethylammonium hydroxide (Eastman No. 1515)–80% SD-3A alcohol (v/v); *Wash*—A solution of 50% methanol–50% deionized water (v/v); *Theory Standard*—32.0 mg. propoxyphene hydrochloride and 0.250 mg. paramethasone acetate reference standard per 20 ml. of Wash solution.

Sample Preparation—Due to the relatively low steroid concentration in the formulation, it was desirable to limit the amount of solvent used in the dissolution of the active ingredients to as low a volume as possible. Utilization of an automatic[3] module

Received November 20, 1967, from Eli Lilly & Co., Indianapolis, IN 46206
Accepted for publication January 25, 1968.
[1] Marketed as Stero-Darvon with ASA by Eli Lilly and Co., Indianapolis, Ind.
[2] AutoAnalyzer, Technicon Corp., Chauncy, N. Y.
[3] Solidprep, Technicon Corp., Chauncy, N. Y.

was therefore undesirable. Since the single-tablet analysis of aspirin was not required, tablet coatings were removed using a stirring device designed by Coffy (5) and fitted with a Styrofoam block to hold twelve 25 × 100-mm. round-bottom centrifuge tubes. This apparatus is shown in Fig. 1. A 20-ml. aliquot of the Wash solution is added to each tube containing a 1.3-cm. Teflon-coated magnetic stirring bar and one tablet. The tube is then stoppered and placed in the stirring apparatus. After about 45 min. of mixing, complete removal of the tablet coatings and dissolution of the active ingredients is achieved. The tablet core and the stirring bar are then removed and the solution centrifuged to settle the undissolved particles. Two 8.5-ml. sample cups are filled with the supernatant liquid for duplicate analysis in the automatic analyzer system.

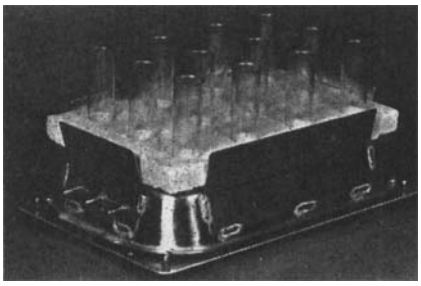

Fig. 1—Tablet dissolution apparatus.

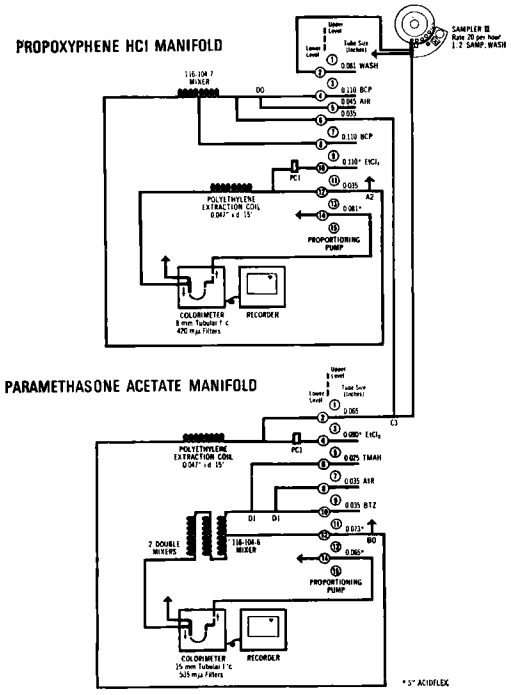

Fig. 2—Flow diagram.

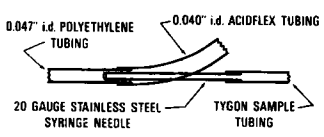

Fig. 3—Special fitting.

Procedure—The complete analytical system is outlined in the flow diagram shown in Fig. 2. The sample solution is aspirated from the liquid Sampler II and flows directly to the C3 debubbler where an unsegmented sample is taken for the PMA manifold. The remaining sample, still isolated between the air bubbles produced during sampling, flows to the DPH manifold where it is injected into an air segmented stream of BCP. This stream is further diluted with BCP and an unsegmented resample is taken at the A2 fitting. This sample is then segmented into a stream of ethylene dichloride ($EtCl_2$) by means of a fitting, previously described by Kuzel (2), and shown in Fig. 3. Extraction of the dye complex is carried out in the polyethylene mixing coil, with subsequent color measurement of the $EtCl_2$ extract after separation from the aqueous stream.

The sample taken at the PMA manifold is immediately segmented into a stream of $EtCl_2$, again using the fitting diagrammed in Fig. 3. Extraction of the PMA into the $EtCl_2$ occurs, and the unsegmented $EtCl_2$ extract is resampled from the bottom of the BO fitting. This sample is injected into an air segmented stream of BTZ and TMAH, mixed through the equivalent of five small mixing coils, and the stream debubbled before measurement in the colorimeter.

Glass wool plugs are inserted in the exit ports of both PCl pulse chambers and in the middle entrance port of the mixing coil 116-104-6 in the PMA manifold. This is done to prevent any flakes of Acidflex tubing from entering the analytical train.

In practice, five theory standards are sampled initially and the standard deviation calculated. Ten samples are then assayed in duplicate followed by standards in duplicate throughout the entire run. If the duplicate standards fall outside the range of 2 σ calculated from the first five standards, the duplicate standards are considered the appropriate values for use in the calculation of the results for the following group of samples. Normally, very little drift is experienced.

RESULTS AND DISCUSSION

The various parameters of the simultaneous methods were investigated. All concentration values listed in the figures are per 20 ml. of the Wash solution and all data were collected from combination standards of DPH and PMA unless otherwise indicated. With the exception of the BCP reagent, the brands of reagents used in this procedure were arbitrarily chosen.

Linearity of the DPH response is shown in Fig. 4 by the straight line connecting the appropriate peaks in the standard curve. All DPH data were collected on a Technicon linearized recorder. The standard curve of PMA is shown in Fig. 5. Since a normal output recorder was used in the collection of the PMA data, the pen response was charted on logarithmic paper. A plot of the absorbance values (Fig. 5) *versus* PMA concentrations results in a straight line passing through the origin. The 60-sec. sampling of the solutions in the procedure produces peak heights which are 77% of the steady state (continuous sampling response) for the PMA system and

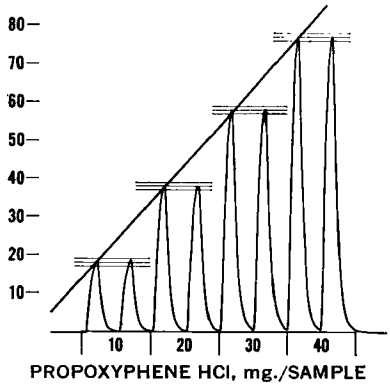

Fig. 4—Propoxyphene HCl standard curve.

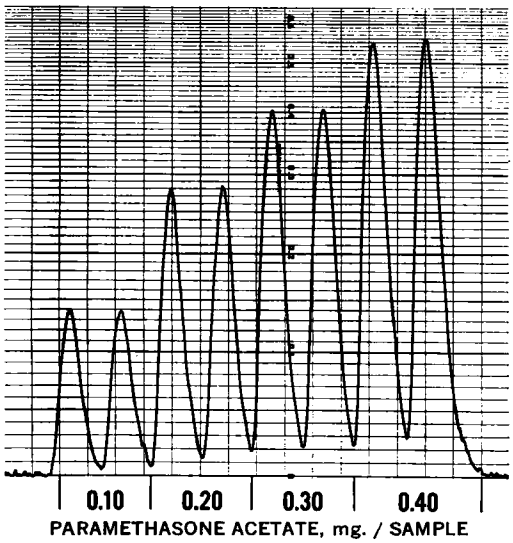

Fig. 5—Parametasone acetetate standard curve.

87% of steady state for the DPH system. Ten repetitive samplings of the Theory Standard solution demonstrated the excellent repeatability of the systems. A relative standard deviation (*RSD*) of only 0.81% was obtained from the DPH system while the PMA system produced a *RSD* of 1.11%. The effect of varying concentrations of PMA on DPH standards and of DPH on PMA standards was studied. No significant interference was observed in either system.

A placebo tablet was assayed by this procedure using 20 ml. of the Theory Standard solution to remove the tablet coating. The supernatant liquid was assayed along with the pure standard solution. An interference of minus 2% for the DPH determination and plus 2% for the PMA determination was observed.

Due to the possibility of a breakthrough of aspirin from the tablet core during coating dissolution, the effect of aspirin upon the response of DPH and PMA standards was studied. No change in DPH response to varying concentrations of aspirin was observed; however, as indicated in Fig. 6, aspirin levels somewhere between 100 mg. and 250 mg. begin to cause a decrease in the PMA response. At the level of 500

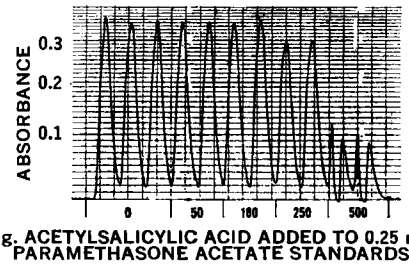

Fig. 6—Effect of acetylsalicylic acid on parametasone response.

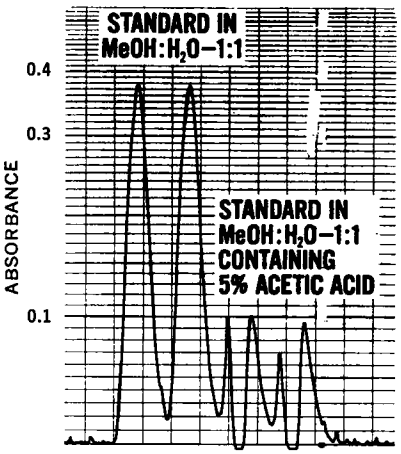

Fig. 7—Effect of acetic acid on parametasone acetate response.

mg., a peak inversion is observed. Since a basic solution is required for the BTZ reduction, it was postulated that the aspirin was producing an acidic condition which inhibited the color development. Addition of acetic acid to the standard solution as shown in Fig. 7, gave the same characteristic peak inversion obtained from the 500-mg. aspirin sample in Fig. 6.

Ten samples were taken at random from an experimental lot of tablets and assayed by the automated procedure. An average of 32.4 mg. DPH and 0.260 mg. PMA per tablet was found with a *RSD* of 5.6 and 6.5%, respectively, including tablet heterogeneity. Results obtained from this lot of tablets by a manual procedure based on a composite sample of 10 tablets were 32.3 mg. DPH and 0.253 mg. PMA per tablet.

As a result of the low PMA concentration in the formulation, the PMA manifold was designed to resample as large an amount of the $EtCl_2$ extract as possible. This in turn was injected into a minimum volume of the BTZ–TMAH reagent. Surprisingly, this resulted in a very rapid color development in the automatic analyzer system. The equivalent of only five small mixing coils was used to develop the color at room temperature. The addition of several more mixing coils before the colorimeter resulted in a relatively small increase in color intensity. Previously automated methods specify either a double holding coil (3) or a 37° heating bath (4) at this point in the flow system. In order to obtain an explanation for this accelerated color development, a study was carried out varying the percentage of

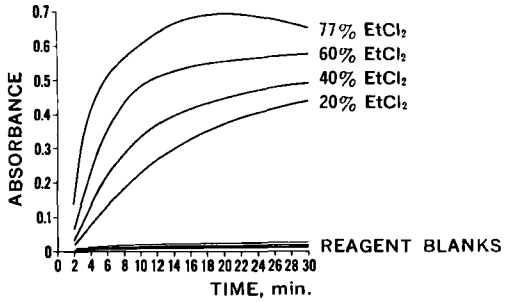

Fig. 8—BTZ–paramethasone acetate color formation.

$EtCl_2$ in the reaction mixture. The reagents used in this study were prepared as follows: 5 ml. of TMAH (10% aqueous solution) was diluted to 100 ml. with SD-3A alcohol; 25 mg. of BTZ was dissolved in 5 ml. of methanol and then diluted to 100 ml. with $EtCl_2$; 30 mg. of PMA was dissolved in 200 ml. $EtCl_2$.

Two milliliters of the PMA solution was added to four 25-ml. volumetric flasks, followed by 3 ml. of the BTZ solution; 0, 5, 10, and 15 ml. of SD-3A alcohol were pipeted into the flasks, followed by $EtCl_2$ to give a volume of about 19 ml. Five milliliters of the TMAH reagent was then added (time zero) and the solution diluted to the mark with $EtCl_2$. Reagent blanks were run substituting $EtCl_2$ for the PMA solution. The absorbance measurements used to plot Fig. 8 were obtained on a Perkin-Elmer Hitachi 139 spectrophotometer. These data show a marked acceleration of color development with increasing concentrations of $EtCl_2$. The same accelerated reaction is obtained by substituting chloroform for $EtCl_2$. Other keto-steroids (cortisone acetate, hydrocortisone acetate, prednisone, prednisolone, and flurandrenolone) assayed in both 20% and 60% $EtCl_2$, gave similar accelerated color development in the solution of greater $EtCl_2$ concentration.

Because the $EtCl_2$ concentration in the automatic analyzer system is approximately 70% and the color measured after only a 5-min. reaction time, it would seem likely from Fig. 8 that the addition of mixing coils to increase the reaction time would substantially increase the color intensity. Since this is not the case, it is speculated that the constant mixing in the automatic analyzer coils may accelerate the reaction even more than that observed by the static manual technique.

SUMMARY

An automated method for the simultaneous determination of DPH and PMA has been described. A technique for the removal of tablet coatings which may be of value in formulations of a similar nature is outlined. A further description of the construction of the stirring apparatus used in this technique was given by Comer *et al.* (6). Various parameters of the simultaneous analysis have been discussed. Due to the functional group specificity of the methods, it is probable they could be used for the simultaneous analysis of most combinations of keto-steroids and tertiary amines. It has also been demonstrated that by carrying out the steroid BTZ reduction in high concentration $EtCl_2$ solutions, an accelerated color development occurs. This knowledge, applied to the existing USP (7) and NF (8) steroid methods, could reduce the time required for these manual determinations.

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Keyphrases

Automated analysis—single tablet
Propoxyphene HCl—paramethasone acetate on aspirin core
Bromocresol purple reagent—propoxyphene HCl
Paramethasone acetate—blue tetrazolium reagent
Diagram—automated apparatus
Colorimetric analysis

Study of the Degradation of Dexamethasone in Certain Pharmaceutical Preparations

By S. K. WAHBA*, S. W. AMIN, and NAZMY ROFAEL

A chromatographic separation of the decomposition products of dexamethasone in tablet form was achieved by using thin-layer chromatography. Spectrophotometric and quantitative colorimetric analyses were run. A stable tablet formula has been suggested.

THE ANTI-INFLAMATORY corticosteroids represented by hydrocortisone and related synthetic analogs have gained an unchallenged position. The therapeutic effects of many steroids depend on their stability (1). It has been found that dexamethasone (Δ'9-fluoro-11β,17α,21-trihydroxy-16-methyl pregna-1,4-diene-3,20-dione) should be protected from light (2) and it has been proved that 50% oxidation of the α-ketol side chain occurs within 6–8 min. in the presence of a base catalyst (3). Dexamethasone has a marked oral potency in range of about 30 times that of hydrocortisone (4–7). The high oral potency is attributed to the protection from metabolic inactivation of the 17,21-dihydroxyacetone side chain by steric hindrance of 16-methyl substituents (8, 9). Identification and investigation of 16-methyl-9-fluoroprednisolone was reported to be carried out by infrared, ultraviolet spectra and triphenyltetrazolium chloride color reaction (10). It has been observed that the direct spectrophotometric analysis of a methanolic extract of dexamethasone tablets yield higher results than the triphenyltetrazolium chloride color assay on aged tablets. A study of the methods of analysis, degradation, and stability of dexamethasone tablets, leads to the finding of a stable tablet formula.

EXPERIMENTAL

Materials—(*a*) Dexamethasone (Roussel). The sample was purified by recrystallization from ether. Purity was confirmed by physicochemical means. m.p. 263°, infrared, ultraviolet absorption. (*b*) Polyvinylpyrrolidone (Kollidon-25, Badische Anilin and Soda Fabrik). (*c*) 2,3,5-Triphenyl tetrazolium chloride (Reanal-Hungary). (*d*) Talc, lactose, starch, magnesium stearate, Cellulose acetate phthalate, carboxymethylcellulose, acetone, ethyl alcohol, methyl alcohol, chloroform, sulfuric acid, Silica Gel G, all from the British Drug House and of analytical grade.

Procedure—*Thin-Layer Chromatography*—Methanolic and chloroformic extracts of dexamethasone tablets were spotted beside methanolic and chloroformic solutions of authentic sample (NF XII) of dexamethasone on 0.3 mm. thick Silica Gel G plates. The plates were developed with chloroform–methanol mixture (9:1). The plates were dried, sprayed either with triphenyltetrazolium chloride reagent (11) (Fig. 1) or sulfuric acid 50% in methanol, and then heated at 80° for 10 min. (12) (Fig. 2). The results obtained are illustrated in Figs. 1 and 2.

From Figs. 1 and 2 it is clear that the methanolic extract of the tablets showed two spots with sulfuric acid reagent; one of them is identical with the spot obtained from the chloroformic extract. On using the triphenyltetrazolium chloride reagent the methanolic extract gave only one spot which was found to be identical with that obtained by the chloroformic extract of the tablets and those of the authentic sample. Hence the lower spot proved to be nonextractable with chloroform.

Quantitative Determination of Standard Dexamethasone, Corresponding Spots, and Its Degradation Products—Dexamethasone authentic sample was assayed by direct ultraviolet absorption at 240 mμ as well as colorimetrically following the official

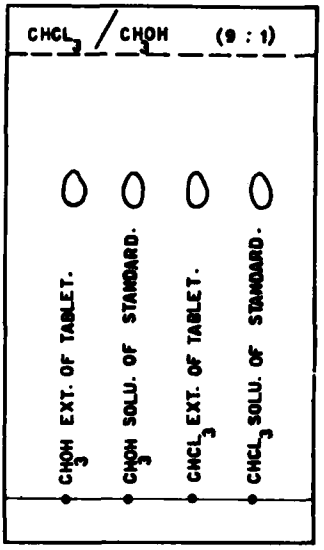

Fig. 1—Dexamethasone chromatograms sprayed with tetrazolium chloride reagent.

Received August 23, 1967, from the Drug Research and Control Center, The Egyptian Organization For Pharmaceuticals, Chemicals and Medical Appliances, Cairo, U.A.R.
Accepted for publication November 7, 1967.
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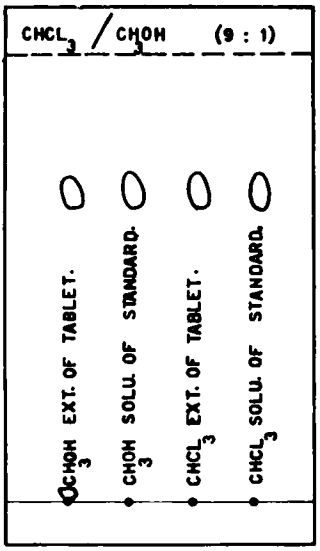

Fig. 2—Dexamethasone chromatograms sprayed with sulfuric acid reagent.

method (13). The tablets were similarly treated when the direct spectrophotometric determination for a methanolic extract gave higher results than the colorimetric method. The chloroformic extract of the same batch of tablets gave the same result as the colorimetric method when assayed spectrophotometrically. Elution of the spots corresponding to the authentic dexamethasone was effected from the plates with methanol. The methanolic eluate was assayed spectrophotometrically for its dexamethasone content. At the same time, the eluate of the lower spot was similarly assayed.

The results obtained are shown in Table I.

Stability of Dexamethasone in Tablet—Preparation of the tablets: four different samples of dexamethasone tablets of the following general formula were prepared: dexamethasone, 0.5 mg., lactose, 88 mg., starch, 15 mg., talc, 3 mg., magnesium stearate, 1.5 mg.

Sample 1: Prepared by the slugging method (14). Sample 2: Prepared by the wet granulation method (16) using 20% solution of polyvinylpyrrolidone in alcohol as a binding agent. Sample 3: Prepared by the wet granulation method using 3% solution of cellulose acetate phthalate in acetone as a binding agent. Sample 4: Prepared by the wet granulation method using 3% mucilage of carboxymethylcellulose in water as a binding agent.

An amount of lactose equivalent to the polyvinylpyrrolidone, cellulose acetate phthalate and carboxymethylcellulose used was reduced from the weight of lactose in the general formula. All tablets were compressed to give a hardness of 3 kg. using a single-punch Korsch machine.

Quantitative Analysis—The four mentioned tablets were assayed using the official colorimetric method every 20 days. Shelf storage at 25° at 60% relative humidity, temperature of 40°, 60°, and humidity of 75% and 100% at 25° temperature were chosen as different factors to compare their stability so as to choose the best tablet formula for making the most stable tablet.

TABLE I—PERCENTAGE OF DEXAMETHASONE IN DIFFERENT ELUATES

Eluate	Ultraviolet Absorption at 240 μ	Dexamethasone, %
Methanolic dexamethasone standard	1.100	100
Methanolic extract of tablets[a]	1.290	117
Methanolic extract of upper spots	0.840	76.3
Methanolic extract of lower spots	0.458	39.09

[a] Direct methanolic extract from tablets.

The results obtained are shown in Table II.

Rate of Degradation—The rates of degradation of the four tablets were found to be a pseudo first-order reaction. Figure 3 shows the relation between the log concentration of dexamethasone and time. The rate of K_{25} and the half-life (16) of the four tablets are shown in Table III.

Observations—(*a*) The high result in the total methanolic tablet extract is due to the decomposition product which absorbs heavily at that wavelength. (*b*) The summation of the absorption of the two spots is equal to the absorption of the direct methanolic extract.

RESULTS AND DISCUSSION

According to the chromatographic analysis the dexamethasone tablet, prepared either by the slugging or the wet granulation method, on aging even at room temperature (shelf) suffered from cleavage of the α-ketol side chain group. This was indicated by the fact that the lower spot was only visible with the sulfuric acid reagent and gave no color with the triphenyltetrazolium chloride reagent which is specific for this group. Hence the direct spectophotmetric determination of a methanolic extract gave higher results than the colorimetric method.

The tablets formulated with polyvinylpyrrolidone as a binding agent showed the greater stability against different humidity and temperature factors and the longest half-life time.

SUMMARY

(*a*) Tablets formulated by slugging or wet

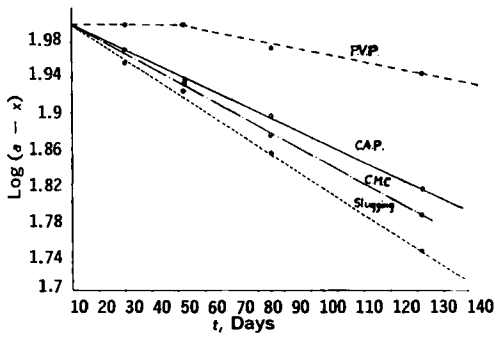

Fig. 3—Relation between the logarithmic concentration of dexamethasone in tablets using different binding agents and time.

TABLE II—STABILITY OF DEXAMETHASONE TABLET AT DIFFERENT TEMPERATURES AND HUMIDITY

Sample No.	Time/Day	Dexamethasone % Shelf[a]	100% Humidity[b]	75% Humidity[b]	40° C.[c]	60° C.[c]
1	0	100				
	20	90.6	85.3	83.9	100	92.3
	42	83.9	55.9	67.1	97.9	92.3
	77	72.6	52.4	55.9	90.9	81.8
	133	55.9				
2	0	100				
	20	99.8	96.4	95.2	95.5	94.6
	42	99.8	94.6	95.2	94.6	94.6
	77	93.8	92.1	93.8	94.6	93.8
	133	88.1				
3	0	100				
	20	93.5	93.5	92.0	79.9	79.9
	42	86.5	78.4	79.9	79.9	76.1
	77	79.3	61.3	74.6	75.3	67.8
	133	65.8				
4	0	100				
	20	94	98.4	87.5	96.9	90.7
	42	86.7	76.9	77.5	90.7	84.6
	77	75.4	61.5	92.4	78.4	69.2
	133	61.4				

[a] at 25° and 60% relative humidity. [b] at 25°. [c] 60% relative humidity.

TABLE III—HALF-LIFE OF THE FOUR DEXAMETHASONE TABLETS

Sample No.	Time/Day	$(a-x)$ %[a]	Log $(a-x)$[a]	Log $\left(\frac{a}{a-x}\right)$[a]	K_{2s}[a]	Half-life, Days
1	0	100	2.0	0.0	0.00436	159
	20	90.6	1.9571	0.0429	0.00436	159
	42	83.9	1.9238	0.0762	0.00436	159
	77	72.0	1.8573	0.1427	0.00436	159
	133	55.9	1.7474	0.2526	0.00436	159
2	0	100	2.0	0.0	0.00139	542
	20	100	2.0	0.0	0.00139	542
	42	100	2.0	0.0	0.00139	542
	77	93.8	1.9722	0.0278	0.00139	542
	133	88.1	1.9450	0.0550	0.00139	542
3	0	100	2.0	0.0	0.00314	220
	20	93.5	1.9708	0.0292	0.00314	220
	42	86.5	1.9370	0.0630	0.00314	220
	77	79.3	1.8993	0.1007	0.00314	220
	133	65.8	1.8182	0.1818	0.00314	220
4	0	100	2.0	0.0	0.00366	189
	20	94	1.9731	0.0269	0.00366	189
	42	86.7	1.9380	0.0620	0.00366	189
	77	75.4	1.8774	0.1226	0.00366	189
	133	61.4	1.7882	0.2118	0.00366	189

[a] a = initial concentration %, x = degradation %, K_{2s} = integrated rate equation.

granulation method and different binding agent except with polyvinylpyrrolidone showed instability toward different humidity and temperature factors.

(*b*) The TLC analysis of the methanolic extract of the tablets showed two spots; one of them is identical with the authentic dexamethasone spot. Meanwhile the other spot could not be localized on using triphenyltetrazolium chloride reagent as a spray which indicates the cleavage of the keto side-chain group.

(*c*) Direct spectrophotometric determination of different degradation products separated by TLC or direct extraction from tablets confirmed the colorimetric assay.

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Keyphrases

Dexamethasone tablets—stability
Degradation rate—dexamethasone tablets
Half-life—dexamethasone tablets
Thick-layer chromatography—separation
TLC—analysis
UV spectrophotometry—analysis
Colorimetric analysis

Problem in the Estimation of Drugs in Biologic Tissue Recovery of Phenol Red from the GI Tract

By STUART FELDMAN and MILO GIBALDI

Recovery of phenol red from homogenates of rat intestinal tissue, using aqueous extraction procedures, was near quantitative. However, significantly poorer recovery was observed after incubation of phenol red with intact intestinal sacs. It appears likely that phenol red binds to tissue or mucosa when in intimate contact, and the bound material is relatively resistant to aqueous extraction. The relevance of this phenomenon to tissue assays is considered.

THE CLASSIC approach to the determination of drug concentration in tissue involves the extraction of the compounds from homogenates. The general principles of this approach have been set forth by Brodie *et al.* (1). Accordingly, experimental standards are based on the recoveries of known amounts of drug from tissue homogenates. The assumption is made that the extent of recovery of standard quantities from the homogenate is equivalent to the recovery from *in vivo* tissue after drug uptake. The authors have recently observed anomalies in the recovery of phenol red from the gastrointestinal tracts of intact rats, rat intestinal sacs, and tissue homogenates, which suggest that the usual assumption of equivalent recovery may lead to significant error.

EXPERIMENTAL

Materials—Phenol red, trichloroacetic acid, and sodium hydroxide were obtained from Fisher Scientific Company as certified reagent grade.

Methods—Male Sprague-Dawley descent rats (Blue Spruce Farms, Altamont, N. Y.) weighing 140–180 Gm. were fasted 24 hr. The animals were then sacrified by decapitation, and the stomach and small intestine were removed. The small intestine was divided into three segments of equal length.

Tissue Homogenates—The stomach and each intestinal segment were homogenized individually for 5 min. in an Eberbach homogenizer with a minimum quantity of distilled water (approximately 3 ml.). The homogenates were placed in Nalgene tubes and 0.5 ml. of a 70 mg. % phenol red solution was added to each tube. The tubes were then agitated slowly for 1 hr. at 37° in a gyrotory water bath shaker.

Gastric Pouch and Intestinal Sacs—Each segment was ligated at both ends and 0.5 ml. of a 70 mg. % phenol red solution was injected in the pouch or sac through the ligated end by means of a 1-ml. tuberculin syringe and blunt needle. No loss of phenol red was observed through the ligatures.

Each segment was then placed in 20 ml. of Ringer's solution in a culture tube. The tubes were agitated for 1 hr. at 37°. The sac or pouch was then removed and homogenized as described above. The serosal fluid was also retained for assay.

Assay—The assay procedure was essentially that of Reynell and Spray (2). Each tissue segment (or homogenate thereof) was homogenized for 5 min. with 1 ml. of 1 *N* sodium hydroxide and about 5 ml. of distilled water. The resulting homogenate was brought to 30 ml. volume with distilled water, centrifuged, and filtered through a Büchner funnel. A 10-ml. aliquot was taken and 1 ml. of a 30% w/v trichloroacetic acid solution was added to the aliquot to precipitate proteins. After centrifugation 5 ml. of the supernatant was removed and 1 ml. of a 1 *N* sodium hydroxide solution was added to develop the color to maximum intensity. The solution was further diluted, passed through a Millipore filter (0.45 μ pore size), and assayed with a Beckman DB-G spectrophotometer at 560 mμ.

RESULTS

Table I shows the recovery of phenol red after addition to homogenates of various gastrointestinal segments. Recovery ranged from 93% to 103% with little difference between homogenates of different segments. The average recovery of phenol

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TABLE I—RECOVERY OF PHENOL RED ADDED TO TISSUE HOMOGENATES

Gastrointestinal Segment	Rat No. 1	Rat No. 2
	%	
Stomach	93	93
Intestine		
Proximal	100	96
Middle	95	103
Distal	100	96

red from all segments of both rats was 97%. These findings are in agreement with those of Reynell and Spray (2) who observed quantitative recovery of phenol red from gastrointestinal tissue homogenates.

The recovery of phenol red after incubation in gastric pouches or intestinal sacs was in marked contrast to the recovery from homogenates. The data in Fig. 1, which represents *total recovery* (*i.e.*, from sac or pouch, its content, and serosal fluid) show a significant *apparent* loss of phenol red from the intestinal segments. The recovery of phenol red from the proximal intestinal segments was significantly different from the recovery from each of the other segments at the 99% confidence level. A progressive increase in recovery is observed from the first to the third intestinal segment. The average recovery of phenol red from all segments was 70%. In each case, 5–10% of the recovered phenol red represents material which permeated the sac to the serosal fluid.

A further experiment was devised to determine whether incomplete mixing of the phenol red with the tissue homogenate was responsible for the excellent recovery from these preparations compared to the recovery from the sacs. In two studies phenol red was homogenized with the intestinal segments for 5 min. to ensure intimate mixing. The homogenates were then incubated as described above for 1 hr. and assayed for phenol red. The results are shown in Table II. An average of 92% phenol red was recovered from the homogenates indicating that mixing does not account for the observed differences.

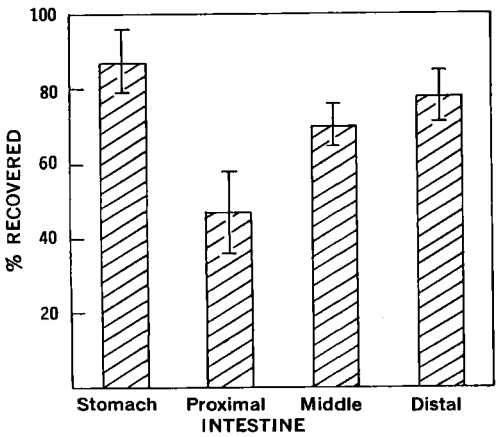

Fig. 1—Phenol red recovery from gastric pouch and intestinal sacs 1 hr. after intubation. Key: bar, average of 5 segments; vertical lines, standard deviations.

TABLE II—RECOVERY OF PHENOL RED HOMOGENIZED WITH TISSUE

Intestinal Segment	Rat No. 1	Rat No. 2
	%	
Proximal	86	81
Middle	94	94
Distal	98	100

DISCUSSION

The reason for the poor recovery of phenol red from intact sacs appears to be related to mucosal or tissue binding, since the possibility of mucosal metabolism has been ruled out by the work of Levine (3). Moreover, the degree of recovery of phenol red from the sacs was site dependent with increasing recovery in the distal direction. This finding is consistent with the amount of mucosal surface available for binding in various portions of the small intestine. If similar binding sites exist throughout the small intestine, then under the conditions of this experiment the greatest amount of phenol red would be bound to the segment having the largest mucosal surface area, *viz.* the proximal portion of the small intestine.

The nature of the binding requires further investigation. However, it would appear to require the mucosa and the phenol red to be in intimate contact. Dilution with fluids or tissue mass, as in the homogenate studies, greatly minimizes the degree of binding. Although the mucosal-bound phenol red is resistant to aqueous extraction, it is unlikely that the binding is covalent since Levine has effected quantitative extraction with ethanolic solutions (3).

The difference in recovery from sac homogenates and intact sacs indicates clearly the possible error involved in assuming the recovery of drug added to homogenates reflects the ultimate efficiency of the tissue assay procedure. This is further emphasized by considering the recovery of phenol red after administration to intact rats.

The authors have recently reported an average recovery of 80% of a dose of phenol red from the gastrointestinal tract, 0.5 hr. after gastric intubation in rats (4). Levy and Jusko (5) report an average recovery of 81% after oral administration of phenol red to rats. In view of the recovery of phenol red from tissue homogenates, one might assume that 15–20% of the dose of phenol red is absorbed from the tract. This value, however, is a significant overestimate. Levine (3) has found that less than 7% of a phenol red dose is absorbed from the gastrointestinal tract. Schanker (6) and Tidball (7) have observed still poorer absorption in the range of 1–2%. On the basis of these reports one must conclude that about 15% of the dose of phenol red is bound to mucosa or tissue *in vivo* and not recovered by the aqueous extraction procedure. The results of these incubation studies with gastric pouches and intestinal sacs are qualitatively consistent with the *in vivo* binding of phenol red.

No previous reports known to the authors have considered the possibility of nonequivalent extraction efficiency after incubation of drug with homogenates or intact tissue. Hence, the extent to which this problem does exist is now known. Nevertheless,

the possibilities and ramifications are manifest. The approach of incubating the drug with intact tissue before homogenization and assay may be a useful one and should be considered even when quantitative extraction from a homogenate is obtained.

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Keyphrases

Drug estimation—biologic tissue
Intestinal homogenates—phenol red recovery
Intestinal sacs, intact—phenol red recovery
Colorimetric analysis—spectrophotometry

Method for Testing the Efficacy of Topical Sunscreen Preparations

By W. M. KOOYERS

A method has been developed for testing the efficacy of topical sunscreen preparations using photosensitive albino rats. The method involves pretreatment of one hind paw with a sunscreen preparation, followed by an oral dose of a photosensitizing agent and exposure to direct sunlight. The delayed reaction and resulting difference between the treated and untreated hind paw weight is an objective index of the protection afforded by the preparation tested.

INTEREST IN topical sunscreen preparations was stimulated by the successful treatment of light-sensitive patients at the University of Minnesota Hospital. Fusaro and Runge (1–3) reported clinical data supporting the hypothesis that topical treatment of the stratum corneum can reduce or minimize the harmful effect of ultraviolet radiation.

The researcher's never-ending quest for new or better means to treat human diseases has led to the development of biological systems which simulate these diseases. As the diabetic rat is used as a screen for antidiabetic drugs (4), and the adjuvant arthritic rat for testing anti-inflammatory drugs (5), this method uses a photosensitive rat to test the efficacy of topical sunscreen preparations. A sunscreen preparation (SSP) is defined as a formulation which, when applied topically, protects the treated area from sunburn.

Albino rats are not hyperphotosensitive; however, rats treated orally with 25 mg./Kg. of 8-methoxypsoralen (8-MOP) become very photosensitive (6–9). Subsequent exposure of 3–7 hr. of direct sunlight will initiate a delayed reaction that progressively worsens. The resulting erythema, edema, and blindness will incapacitate these rats after 5–7 days. Rats photosensitized with 8-MOP, which have only part of their extremities exposed to direct sunlight, will show evidence of photosensitivity on the exposed areas only (9).

This photosensitive reaction is evident 2 or 3 days after exposure and can be visually evaluated by scoring the affected extremities from 0 to 4 depending upon the severity of the erythema and edema. A more objective index is the comparison of hind paw weights of photosensitive rats and normal rats. This is a measurement of the edema caused by the reaction. This objective index is used in this method.

METHOD

Five male albino rats[1] per group, weighing 180–200 Gm., were restrained in stocks while each left hind paw was dipped into the SSP. The restraint was maintained until the treated area was completely dry. This minimized the possibility of systemic effect that could be caused by ingestion if the rats were allowed to clean the treated area.

After application of the SSP, the sunscreen groups and one control group were dosed orally with the photosensitizing agent, 8-MOP[2] at 25 mg./Kg., and were then placed in direct sunlight for 5 hr. Each group was housed in a cage designed to allow maximum exposure to the sunlight. Normal controls were exposed simultaneously.

Three to seven days after exposure, the rats were sacrificed, body weights recorded, and both hind paws were uniformly severed (10) using a suitable apparatus.[3] The weight of each hind paw was recorded. The weight difference between the untreated right hind paw and the treated left hind

Received February 26, 1968 from the Upjohn Company, Kalamazoo, MI 49001
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TABLE I—EFFECT OF SUNSCREEN PREPARATIONS ON HIND PAW WEIGHTS OF PHOTOSENSITIVE RATS

Experiment	Treatment: Day 1 Group No. and Title	Day 2: 8-MOP[b]	Day 2: 5 hr. Sunlight	Av. Wt., in Gm.: 5 Rats/Group: Final Body Weights ± SEM[c]	Right Hind Paw ± SEM	Left Hind Paw ± SEM	Difference ± SEM
No. 1	1 Normal controls	No	Yes	206 ± 2	1.39 ± 0.02	1.40 ± 0.02	−0.01 ± 0.01
	2 Photosensitive controls	Yes	Yes	180 ± 2	2.08 ± 0.03	2.06 ± 0.04	0.02 ± 0.05
	3 Sunscreen A[a]	Yes	Yes	181 ± 1	1.91 ± 0.07	1.35 ± 0.04	0.56 ± 0.05
	4 Sunscreen B[a]	Yes	Yes	180 ± 1	1.92 ± 0.07	1.34 ± 0.02	0.58 ± 0.07
No. 2	5 Normal controls	No	Yes	224 ± 1	1.43 ± 0.07	1.45 ± 0.02	−0.02 ± 0.01
	6 Photosensitive controls	Yes	Yes	185 ± 6	1.80 ± 0.06	1.81 ± 0.08	−0.01 ± 0.02
	7 Sunscreen C[a]	Yes	Yes	178 ± 2	1.93 ± 0.07	1.48 ± 0.05	0.45 ± 0.02
	8 Sunscreen D[a]	Yes	Yes	174 ± 4	1.61 ± 0.07	1.33 ± 0.01	0.28 ± 0.06

[a] Left hind paws dipped into the sunscreen preparation six times on Day 1. [b] 8-Methoxypsoralen 5 mg./ml. suspension given orally at 8:30 a.m. Day 2. [c] SEM = Standard error of the mean. Experiment No. 1 terminated 3 days after exposure. Experiment No. 2 terminated 4 days after exposure.

TABLE II—HIND PAW WEIGHT DIFFERENCE EVALUATED BY THE *t* TEST ON DIFFERENCES

Group No. and Title	Av. Difference	Sum of Difference	Calculated[a] *t*	Allowed *t*[b]: $p = 0.05$	Allowed *t*[b]: $p = 0.01$	Significant
1 Normal controls	−0.01	−0.05	1.00	2.78	4.60	
2 Photosensitive controls	0.02	0.10	0.42	2.78	4.60	
3 Sunscreen A	0.56	2.82	10.84	2.78	4.60	$p = 0.01$
4 Sunscreen B	0.58	2.88	9.31	2.78	4.60	$p = 0.01$
5 Normal controls	−0.02	−0.08	1.55	2.78	4.60	
6 Photosensitive controls	−0.01	−0.03	0.27	2.78	4.60	
7 Sunscreen C	0.45	2.25	15.33	2.78	4.60	$p = 0.01$
8 Sunscreen D	0.28	1.42	4.85	2.78	4.60	$p = 0.01$

[a] Calculation performed by Control Statistical Services, the Upjohn Company. [b] Allowed *t* values are extracted from Table III of Fisher and Yates, Statistical Tables, at 4 *df*.

paw was an objective index of the protection afforded by the SSP. The statistical significance of this weight difference was evaluated by the *t* test on differences.

RESULTS AND DISCUSSIONS

Results presented in Table I are typical for this method. The body weights were routinely recorded to help identify aberrant rats. Normal and photosensitive controls are used to prove the sensitivity of this biological system. The hind paw weights within each control group are uniform; however, the photosensitive controls weigh considerably more than normal controls. This edematous condition of the photosensitive rats occurred 3–7 days after the oral dose of 8-MOP and exposure to direct sunlight.

The efficacy of the SSP is evident in Groups 3, 4, 7, and 8 of Table I. The average weight of sunscreen-treated left hind paws is similar to that of normal controls, while the untreated right hind paws are edematous like the photosensitive controls. The calculated weight difference between the untreated and treated paws of each rat is evidence of the protection afforded the treated paw by the SSP.

Using five rats per group, this weight difference is statistically significant at $p = 0.01$ when evaluated by the *t* test on differences. These statistical data are reported in Table II.

SUMMARY

A method for testing the efficacy of topical sunscreen preparations is reported. The hind paw weight difference of photosensitive rats is a statistically significant index, easily controlled, and objective.

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Keyphrases

Sunscreen preparations—testing
8-Methoxypsoralen—photosensitizer
Protection index—rat paw weights

Studies on the Mechanism of Action of Salicylates III. Effect of Vitamin A on the Wound Healing Retardation Action of Aspirin

By K. H. LEE

Vitamin A and vitamin E reverse the skin-wound healing retardation action of aspirin in rats. Vitamin A alone promotes healing and it also increases acid mucopolysaccharide synthesis in the granulation tissue. The possible mechanisms of action of vitamin A and aspirin on wound healing are discussed.

IN A PREVIOUS PAPER (1), it was shown that aspirin, like cortisone (2, 3), reduces the tensile strength of healing skin wound in rats. Lysosomal enzymes are believed to be involved in inflammation (4). Since aspirin protects lysosomal membrane (5), it was proposed that aspirin retarded healing by its anti-inflammatory action. It is well-known that vitamin A, vitamin E, and a few other fat-soluble compounds can accelerate the release of lysosomal enzymes (6–8). In the present study it was found that both vitamin A and vitamin E reversed the inhibitory action of aspirin on wound healing in rats. It was found that, in the absence of aspirin, vitamin A promoted wound healing.

Mucopolysaccharide formation in the very early phase of wound healing was noticed in many laboratories (9–12). The role of mucopolysaccharides in the formation of connective tissue or collagen has been of interest for many years (12–14). Aspirin and other anti-inflammatory agents inhibited the biosynthesis of mucopolysaccharide and sulfate uptake for chondroitin sulfate synthesis by cartilage slices (15, 16). Vitamin A, on the other hand, increased sulfate incorporation into chrondritin sulfate (17) and increased mucopolysaccharide synthesis (18, 19). In this paper the effect of vitamin A on mucopolysaccharide content of the granulation tissues in rats is reported. The possible mechanisms of action of aspirin and vitamin A on healing are discussed.

EXPERIMENTAL

Wound Procedure—The wound procedure for tensile strength study was essentially the same as described before (1). For mucopolysaccharide study, the wounding technique described by Bentley (12) was followed. Sprague-Dawley rats of either sex, weighing 200–220 Gm., were anesthetized with ethyl ether in an open mask. The hair on the back was depilitated with an electric clipper. One circular piece of skin 5 cm. (2 in.) in diameter, was removed. The wound was left undressed. On the seventh day after wounding, each rat was decapitated and the granulation tissue of the wound was carefully dissected. There were 10 rats in each group. The granulation tissue of all 10 rats of the same group was pooled together.

The body weight of each rat was taken before inflicting the wound and before sacrificing the animal.

Mucopolysaccharide Separation—The mucopolysaccharide separation method described by Bentley (14) was followed with some modification. The granulation tissue was homogenized in a Virtis homogenizer at 45,000 r.p.m. in phosphate buffer pH 8. For every gram of granulation tissue, 5 ml. of buffer was added. Three volumes of boiling acetone was added to the homogenate with stirring. After the precipitate was settled, the supernatant was carefully removed by suction. The acetone extraction was repeated two more times. The fat-free precipitate was separated by suction filtration. The precipitate was washed once with dry ether and then stored *in vacuo* over paraffin wax and phosphorus pentoxide. Three aliquots of the dried fat-free precipitate were digested separately with twice-crystallized papain as described by Antonopoulos *et al.* (20) at 65° for 8 hr. After digestion, the mixture of each sample was centrifuged separately in a Sorvall SS-1 superspeed centrifuge at 10,000× g for 30 min. Aliquot amount of the clear supernatant in each centrifuge tube was dialyzed salt-free in a cellophane tube. Mucopolysaccharide was precipitated from the clear dialysate by the addition of ethanol to a concentration of 80% together with trace amounts of sodium acetate. The precipitate was collected in a centrifuge tube by centrifugation and then redissolved in a small amount of water and reprecipitated by the addition of a small amount of cetylpyridinium chloride (CPC) (21). The CPC-mucopolysaccharide precipitate (CPC-MPS) was redissolved in 60% *n*-propanol and again precipitated by the addition of ethanol and sodium acetate. The precipitate was washed in absolute ethanol, dry ether, and dried *in vacuo* over paraffin wax and phosphorus pentoxide. The dried precipitate was weighed.

Administration of Drugs—Aspirin was administered essentially the same way as described before (1). Crystalline vitamin A acetate (Calif. Corp. Biochem. Research, Los Angeles) was dissolved in cottonseed oil so that each ml. contained 15,000 I.U. One-tenth of a milliliter of this oil containing vitamin A was injected intraperitoneally into each rat on the first, third, and fifth day after inflecting the wound. To the controls, similar amounts of cottonseed oil without vitamin A were given in the same manner. *dl*-Alpha-tocopherol NF (N.B.C., Ohio) was also dissolved in cottonseed oil, so that each milliliter contained 500 I.U. One milliliter of this oil was injected intraperitoneally into each rat on the

Received December 11, 1967, from the University of California School of Pharmacy, San Francisco Medical Center, San Francisco, CA 94122

Accepted for publication February 28, 1968.

Presented at the fall meeting of The American Society for Pharmacology and Experimental Therapeutics, August 1967.

This work was supported by research funds from the Academic Senate and in part by a grant-in-aid from Eli Lilly & Co., Indianapolis, Indiana.

TABLE I—TENSILE STRENGTH OF HEALING WOUNDS

Group	No. of Animals	Aspirin Fed, mg./Kg.	Vit. A[a] —Days after	Vit. E[b] Wounding, i.p.—	Tensile Strength, Gm.	Percent Control
I[c]	21	—	—	—	438 ± 17.7	100
II[c]	13	150	—	—	262 ± 8.7	60
III	9	150	1, 3, 5	—	338 ± 22.7	77
IV	11	150	0, 2, 4, 6	—	325 ± 17.6	74
V		150	—	1, 3, 5	294 ± 14.8	67
VI[c]	12	75	—	—	343 ± 21.0	78
VII	10	75	0, 2, 4, 6	—	447 ± 14.0	100
VIII	18	—	0, 2, 4, 6	—	490 ± 11.0	112

[a] 3,000 I.U./Kg. [b] 1,000 I.U./Kg. [c] Reported before (1).

first, third, and fifth day after wounding. One milliliter of plain cottonseed oil was injected into each of the controls. For the effect of vitamin A on the mucopolysaccharide formation in the granulation tissue studies, the experimental rats received only vitamin A (3,000 I.U./Kg.) intraperitoneally on the first, third, and fifth day after wounding. The controls received cottonseed oil. All of the rats were fed on standard rat feed prepared by Feedstuffs Processing Company, San Francisco, Calif.

Tensile Strength Measurement—The tensile strength of the healing skin wound was measured with the laboratory made centiometer as described earlier (1).

RESULTS

In Table I it is shown that rats receiving an oral dose of 150 mg./Kg. of aspirin daily, starting 1 day before wounding, have an average tensile strength of skin wound of 262 ± 8.7 Gm. on the seventh day which is about 60% of that of the control. When 1,500 I.U./rat (3,000 I.U./Kg.) of vitamin A was given intraperitoneally to rats treated under essentially the same conditions as described above, on the first, third, and fifth day after wounding, the average tensile strength was increased to 338 ± 22.7 Gm., or 77% of the control (Table I, Group III). The increase in tensile strength is significant ($p < 0.001$). When vitamin A was given on the day of wounding and also on the second, fourth, and sixth day after wounding, the average tensile strength was increased to 325.5 ± 17.6 Gm. (Group IV). The improvement was about the same as that of the rats of Group III.

When the dose of aspirin was reduced to 75 mg./Kg. the average tensile strength of the skin wound was 343 ± 21 Gm. or 78% of the control (Group VI). When vitamin A was given to rats receiving 75 mg. of aspirin daily, the average tensile strength was increased to 447 ± 14.0 Gm. (Group VII). Group VII had no significant difference from the control. However, if vitamin A alone was given to rats receiving no aspirin, the average tensile strength was increased to 490 ± 11.0 Gm. or 112% of the control.

When vitamin E (1,000 I.U./Kg.) was given to rats receiving 75 mg. of aspirin daily on the day of wounding and also on the second, fourth, and sixth day after wounding, the average tensile strength on the seventh day after wounding was 294 ± 14.8 Gm. (Group V). Vitamin A significantly reversed the retardation of wound healing action of aspirin.

The results of the studies on mucopolysaccharide formation in granulation tissue of the wound are summarized in Table II. There were 10 rats in each group and at the end of the experiment, in all cases except one, only 8 rats survived. The first 2 days after operation were critical. Rats which survived the first 2 days usually survived to the end of the experimental period. The average amount of CPS-MPS in the granulating tissue of the wound inflicted on the rats receiving vitamin A was 14.10 ± 4.3 mg./rat. The average amount of CPC-MPS of the control was 8.92 ± 1.26 mg./rat. The difference is significant. The Student test was used for evaluating significance in all cases.

TABLE II—EFFECT OF VITAMIN A ON MUCOPOLYSACCHARIDE FORMATION IN GRANULATION TISSUE

Group	No. of Rats	Vit. A, 1,500 I.U./Kg., i.p.	CPC-MPS,[a] mg./Rat	Average
I	8	...	9.25	
II	7	...	9.93	
III	8	...	7.60	
				8.92 ± 1.26
IV	8	+	16.60	
V	8	+	18.74	
VI	8	+	11.67	
VII	8	+	9.42	
				14.10 ± 4.30

[a] Average of triplicate determinations for each group.

DISCUSSION

In a previous study (1), it was proposed that the wound healing retardation action of aspirin is due to its lysosomal membrane protection activity. This suggestion is further supported by the present study. Vitamin A and vitamin E are known to be able to release lysosomal enzymes (22). In the present study, it has been shown that vitamin A, as well as vitamin E, indeed, reversed the wound healing retardation action of aspirin.

Within 6 hr. and increasing for 4 days after wounding, mucopolysaccharide can be demonstrated in the ground substance of the regenerating tissue (23). The direct relation between mucopolysaccharide and collagen formation has been of interest for many years (24). Highberger *et al.* (25), from *in vitro* studies, have shown that a dialyzed acid solution of collagen can be made to precipitate into collagen fiber in the presence of an acid polysaccharide such as chrondroitin sulfuric acid. The collagen fiber formed looked essentially like the natural fiber under electron microscope. The soluble procollagenous substance requires mucopolysaccharide as a template to form insoluble collagen fiber (23). Collagen provides tensile strength to a wound.

Aspirin and a few other anti-inflammatory agents, which inhibited the metabolism of cartilage and other connective tissues *in vitro*, also inhibited the biosynthesis of polysaccharide sulfates by rat

rib cartilage *in vivo* (15). Aspirin (16) also decreased sulfate exchange of chondroitin sulfuric acid.

Wolf and his co-workers (18, 19) have demonstrated the role of vitamin A in the synthesis of mucopolysaccharide in rat colon segments. Recently, it was further shown (26) that a compound which appeared to be an acidic metabolite of vitamin A and vitamin acid was a component of ATP-sulfurylase (ATP: sulfate adenyltransferase, EC2.7.7.4), an enzyme involved in the first step of activation of sulfate. In the present study, it was shown that vitamin A promoted mucopolysaccharide synthesis in the granulation tissue of skin wound in rats. The effect of vitamin A and aspirin on acid mucopolysaccharide synthesis probably is another mechanism of action of these compounds on wound healing.

SUMMARY

The reversal of healing retardation action of aspirin by vitamin A and vitamin E has been demonstrated in rats. In the absence of aspirin, vitamin A promoted healing. It also increased acid mucopolysaccharide synthesis in the granulation tissue.

Inflammation and acid mucopolysaccharide synthesis are two essential features in the early stage of wound healing. Aspirin inhibited both features and vitamin A promoted both features. Two possible mechanisms of action of these two compounds are suggested.

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Tensile strength—healing wound

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By K. H. LEE, LEON THOMPKINS*, and MICHAEL R. SPENCER

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Received February 21, 1968, from the School of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122

Accepted for publication April 1, 1968.

Presented at the 52nd Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 1968.

This work was supported in part by a National Institutes of Health Training grant 5 TI GM 728, U. S. Public Health Service, Bethesda, Md.

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quantitative separation of free ASA and free SA (5). In the present paper the method and also the results of a comparative study on the efficiency of several other types of Sephadex is reported.

EXPERIMENTAL

Chemicals and Materials—The acetylsalicylic acid, ACS grade was obtained from City Chemicals Corporation and the salicylic acid used was Baker analyzed reagent. Sephadex G-25-coarse, G-25-medium, C-25, A-25, G-15, and G-10 were prepared by Pharmacia Fine Chemicals Inc.

Preparation of Column—The column was prepared in a special needle-type Pasteur pipet, 5 mm. i.d. The length of the pipet was about 3 cm. longer than the height of the gel bed. A small amount of Pyrex glass wool was placed in the bottom of the pipet and a slurry of well-soaked Sephadex was introduced into the column. The Sephadex was allowed to settle under gravity and the height of the column was adjusted. A long stainless steel wire was used to stir the column and assist in uniform packing. A standard number 19-G hypodermic needle was then placed on the tip of the Pasteur pipet. The column was washed with phosphate buffer (0.1 *M* at pH 7.4). Every eight drops of effluent is equal to 0.1 ml.

Fractionation of ASA and SA—Two-tenth milliliter of a sample containing freshly prepared ASA (2×10^{-4} *M*) and SA (8×10^{-4} *M*) in phosphate buffer was introduced to the column from a long tip pipet. The concentration of the salicylates used are within the range present in biological fluid. As soon as the sample had disappeared from the surface of the gel bed, phosphate buffer was introduced for elution. A fraction collector (GME product) with drop counter was used to collect 16-drop (0.2-ml.) portions of the effluent. The effluent in each tube was diluted to 4 ml. and assayed in the spectrophotofluorometer (Aminco-Bowman), using an activating wavelength of 305 mμ and detecting fluoresence at 405 mμ (4). All of the samples were saved in their original tubes. Acetylsalicylate does not fluoresce, but can be measured after hydrolysis. The hydrolysis was carried out by adding one drop of concentrated ammonium hydroxide to each sample and then heating in a boiling water bath for 5 min. After cooling to room temperature, the volume in each tube was readjusted to 4 ml. before assaying again in the fluorometer. For each type of Sephadex, the 100-mm. column was usually tried first and from the result obtained the decision was made to choose columns of other lengths. For each type of Sephadex the authors filled three Pasteur pipets for each column length. The types of Sephadex studied included G-25-medium (G-25-m), G-25-coarse (G-25-c), G-15, G-10, C-25 (carboxymethyl-Sephadex), and A-25 (DEAE-Sephadex).

RESULTS AND DISCUSSION

Sephadex G-25, with 100-mm. column length, as shown in Fig. 1, can separate ASA and SA into two distinct fractions, but the fractions are too close to each other. Two fractions that are farther separated as shown in Fig. 2, are of practical importance. For example, if one uses the 200-mm. column of G-25 (Fig. 2) one may just collect the first 4 ml. (the first 20 tubes) and the next 2 ml. (next 10 tubes) of the effluent in two separate tubes, which will contain exclusively ASA or SA, for analysis, either fluorometrically or colorimetrically (6).

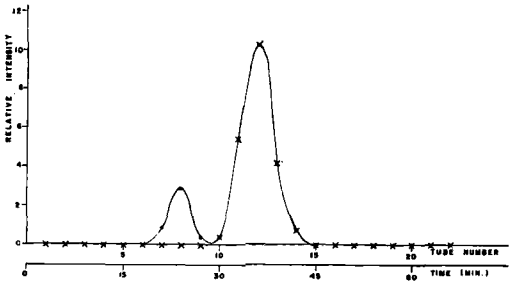

Fig. 1—Separation of ASA and SA by Sephadex G-25-m., 5 × 100 mm. Key: ×, SA before hydrolysis; O, after hydrolysis.

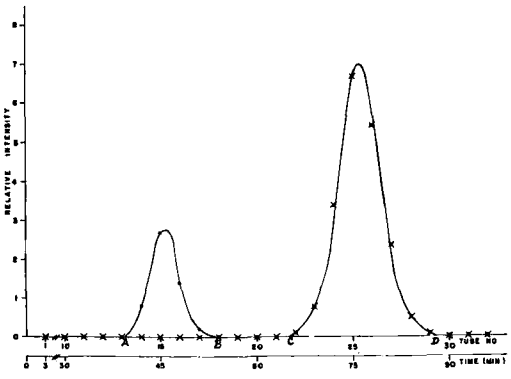

Fig. 2—Separation of ASA and SA by Sephadex G-25-m. 5 × 200 mm. Key: ×, SA before hydrolysis; O, after hydrolysis.

For the convenience of comparing resolution with all of the types of Sephadex of different column lengths studied, the results are summarized in Table I. Let A indicate the volume of effluent collected before the appearance of ASA (see Fig. 2). This volume should be much greater than the void volume (Vo) of the Sephadex bed used, if the biological fluid applied contains proteins. Plasma proteins combine with SA and they should be separated from free ASA. A small volume of effluent that comes out first, which is equivalent to the void volume, carries all of the plasma proteins, whether they are bound with drug or not. For the separation of free ASA and free SA in urine or in other nonprotein fluids, the volume of A is not important. Acetylsalicylic acid fraction is completely eluted at point B and SA begins to come out at point C and is completely eluted at point D. The difference between B and A and the difference between D and C represent the volumes of the effluent containing all of the ASA and SA, respectively. The magnitudes of (D − C) and (B − A) indicate the sizes of the fractions. The difference between C and B (C − B) should be large enough to insure the clear separation of ASA and SA fractions. Point D indicates the total volume of eluent required for the fractionation. This volume should not be too large in order

TABLE I—SEPARATION OF ASA AND SA BY DIFFERENT TYPES OF SEPHADEX GEL FILTRATION

Gel Type	Particle Size Diameter, μ	Bed Dimension, mm.	Volume of Effluent, ml., at[a] A	(Vo)	B	C	(C − B)	D	Time Required, min.
G-25-m	50–150	5 × 100	1.2	(0.79)	1.8	1.8	(0)	3.0	45
G-25-m	50–150	5 × 200	2.6	(1.57)	3.6	4.3	(0.7)	3.9	88
G-25-c	100–300	5 × 100	0.8	(0.79)	5.0	1.0	(−4)	—	—
G-15	40–120	5 × 50	0.6	(0.29)	1.4	1.4	(0)	2.6	12
G-15	40–120	5 × 100	1.2	(0.59)	2.2	3.6	(1.4)	5.8	33
G-10	40–120	5 × 50	0.6	(0.35)	1.9	2.8	(0.9)	7.2	36
G-10	40–120	5 × 100	0.8	(0.71)	2.4	5.8	(3.4)	11.6	76
C-25	40–120	5 × 100	1.2	—	2.0	2.0	(<0)	3.2	32
C-25	40–120	5 × 200	2.7	—	3.9	4.0	(0.1)	5.8	59
C-25	40–120	5 × 300	4.0	—	5.4	6.0	(0.6)	8.2	123
A-25	40–120	5 × 50	1.0	—	3.2	5.2	(2.0)	13.6	47
A-25	40–120	5 × 100	2.4	—	4.8	12.0	(7.2)	>16	—

[a] See Fig. 2 or text for denotations. Each milliliter is equal to the effluent collected in five tubes.

to avoid unnecessary dilution of the drugs and prolongation of fractionation time.

Sephadex G-10 and G-15 are less porous than G-25, and will effect a better resolution of low molecular weight substances. Indeed, Sephadex G-15 of a 100-mm. column and G-10 of only 50-mm. column separated two salicylates as well as G-25 with 200-mm. column length. Sephadex C-25 (cation exchanger) behaved just like G-25-m and Sephadex A-25 (anionic exchanger, DEAE Sephadex) separated salicylates most efficiently. A 50-mm. column of A-25 was sufficient for the separation.

It is always preferable to use a shorter column. The longer column has a slower flow rate and also it is difficult to keep the bed uniform. The flow rate of different types of gel with equal column length may not be the same. The Sephadex G-25-coarse has a higher flow rate, but it has lower resolution power. Both G-10 and A-25 only need 50-mm. column. Sephadex G-10 has a higher flow rate and A-25 gives better resolution.

The recovery of drugs was complete, since there is no irreversible retention of substance on the Sephadex column. When the amounts of ASA and SA were increased, the distribution of the drugs in the effluent was not changed, but the peaks of the fractions were increased. The time required for the filtration was very short and there was no detectable hydrolysis of ASA in the column. Sephadex gel filtration method for the separation of ASA and SA is very simple and practical.

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Keyphrases

Aspirin, salicylic acid—separation
Sephadex gels—aspirin, salicylic acid separation
Fluorometry—analysis

Determination of Silicon in a Commercial Hand Lotion by Atomic Absorption Spectroscopy

By ERNEST MARIO and RALPH E. GERNER

A procedure for the extraction and quantitative determination of silicon by atomic absorption spectroscopy in a commercial hand lotion is described. After the complete removal of water, benzene is used to extract the silicon derivative (dimethylpolysiloxane) from the sample. The benzene extract is then analyzed by atomic absorption spectroscopy utilizing a nitrous oxide/acetylene flame. The method was verified by the addition of known quantities of octaphenylcyclotetrasiloxane to samples of known silicon content prior to extraction. Standard curves obtained by plotting absorbance *versus* p.p.m. silicon were found to be linear in the range of 0 to 120 p.p.m. silicon.

THE RAPIDLY INCREASING utilization of silicone fluids in commercial beauty aids for skin protection has pointed up the need for a more rapid and accurate method to analyze these products for silicon content. The classical acid digestion methods (1) are rather time consuming, and high temperatures are required to convert the silicon completely to silicon dioxide. The infrared methods (2) require considerable sample clean-up and introduce the usual variables encountered with quantitative infrared determinations. This paper describes the suitability of atomic absorption spectroscopy for the determination of silicon in a commercial hand lotion containing approximately 85% water. The method offers both speed and accuracy.

EXPERIMENTAL

Apparatus—A Perkin-Elmer model 303 atomic absorption spectrophotometer was utilized with a fuel mixture of nitrous oxide and acetylene. The high temperatures achieved with this fuel mixture were necessary since silicon is a refractory element. The nitrous oxide flow rate was held at approximately seven at the flowmeter and the pressure regulated to 30 psig at the tank regulator. The acetylene flow rate was held at approximately six using the lower ball in a double ball flowmeter and the pressure regulated to 8 psig at the tank regulator. The flame was considered suitable when the red inner cone attained a height of approximately 1 in. (2.54 cm.). A hollow-cathode silicon lamp was used as the source of emission. The measurement wavelength was approximately 251 mμ (maximized) in the ultraviolet region. The instrument was operated with a slit setting of three and a meter response setting of four. These parameters are dimensionless and will therefore vary from instrument to instrument.

Materials—The commercial samples examined in this study were prepared with dimethylpolysiloxane.[1] The dimethylpolysiloxane used in the specific lot of commercial material discussed in this paper was analyzed by atomic absorption spectroscopy and found to contain 38.25% silicon. Octaphenylcyclotetrasiloxane[2] was used as the internal standard. This material is currently used by the National Bureau of Standards as a primary standard for atomic absorption studies involving silicon. The material was certified to contain 14.15% silicon. Analytical reagent grade benzene was used throughout this study.

Methods—Samples of hand lotion containing approximately 400 mg. of silicon were weighed accurately into a 250-ml. flat-bottom Pyrex flask equipped with a ground-glass neck. Several glass beads were added and a suitable glass elbow adapter affixed to the flask. The mixture was heated gently until *all* water was volatilized. The residue remaining varied in color from light amber to colorless depending on the rate of heating. The sample was allowed to cool and 100 ml. of benzene was added. The flask was stoppered and the sample extracted for 60 min. on a mechanical shaker. The extract was decanted through a cotton pledget into a 200-ml. volumetric flask. An additional extraction using 50 ml. of benzene was performed for 30 min. in the same manner. This extract was added to the 200-ml. volumetric flask as described above and the flask and cotton pledget were rinsed with sufficient benzene to obtain 200 ml. Three milliliters of this solution was pipeted into a 100-ml. volumetric flask and diluted to volume with benzene. Standards, from which a linear calibration curve was obtained, were prepared by dissolving known amounts of octaphenylcyclotetrasiloxane in benzene.

Internal recovery standards were prepared by mixing samples of hand lotion containing approximately 400 mg. of silicon with known amounts of the octaphenylcyclotetrasiloxane standard. These internal recovery standards were heated to remove all water and extracted with benzene as described above.

The samples and standards were read in an alternate manner throughout this study to verify conformance with the standard curve. The solvent (benzene) was aspirated after each test solution. Each test solution was aspirated three times and the absorbance values averaged.

RESULTS AND DISCUSSION

Table I contains the absorbance data obtained

TABLE I—CALIBRATION DATA FOR P.P.M. SILICON *versus* ABSORBANCE

Sample No.	p.p.m. Silicon	Absorbance
1	14	0.0801
2	36	0.2034
3	57	0.3233
4	71	0.4034
5	85	0.4763
6	106	0.5901
7	120	0.6720

Received December 29, 1967, from the Quality Contro Department, Strasenburgh Laboratories, Rochester, NY 14623

Accepted for publication February 12, 1968.

[1] Marketed as SF-96 350 by General Electric Silicone Products Department, Waterford, N. J.

[2] The authors acknowledge with thanks receipt of this standard material from Dr. R. A. Mansfield of General Electric, Waterford, N. J.

TABLE II—P.P.M. SILICON IN COMMERCIAL HAND LOTION

Sample No.	p.p.m. Silicon (Theory)	p.p.m. Silicon Added	Total p.p.m. Silicon (Theory)	Total p.p.m. Silicon (Found)	
1	57.0	None	57.0	56.2	56.8
2	57.0	4.3	61.3	62.0	62.0
3	57.0	10.6	67.6	67.6	67.4
4	57.0	21.2	78.2	80.0	78.0
5	57.0	42.4	99.4	99.0	97.4

from seven samples of octaphenyltetrasiloxane in benzene containing known amounts of silicon (p.p.m.) in the range of 0 to 120 p.p.m. As can be seen in Table I, the relationship of p.p.m. silicon to absorbance is linear in the range described. This linear relationship permits the use of these data in determining the silicon content of unknown samples provided the instrument conditions remain unchanged. Table II contains the data obtained from five samples (each performed in duplicate) of the same lot of hand lotion. In all samples shown, the initial weight of hand lotion was the same. Samples 2 through 5 contain additional known quantities of silicon resulting from the addition of octaphenylcyclotetrasiloxane to the original hand lotion prior to heating and extraction with benzene.

The removal of all water from the sample prior to benzene extraction is necessary since the organic extract exhibits turbidity if water is present during extraction. This turbidity renders the benzene extracts unacceptable for aspiration and results in a ±10% variation for p.p.m. silicon. The ratio of fuel to oxidizer is critical for minimizing background signal and optimizing flame temperature. When the ratio of gases is not optimal, linearity between p.p.m. silicon and absorbance is lost above 75 p.p.m. silicon. This loss of linearity may be due to the formation of refractory oxides of silicon within the flame during aspiration.

As can be seen in Table II, the method is very reliable and accurate. The heating step to remove water (about 85% in the product examined) requires approximately 30 min. The overall procedure including standard preparations can be performed in approximately 3 hr.

SUMMARY AND CONCLUSION

A rapid, accurate method for the determination of silicon in a commercial hand lotion has been developed utilizing atomic absorption spectroscopy. In concentrations up to 120 p.p.m., silicon absorbance is linear with respect to p.p.m. Critical parameters are the flow rates of fuel and oxidizer, the appearance of the flame, the requirement of complete removal of water prior to extraction with benzene, and the need for frequent aspiration of pure solvent (benzene) to prevent instrument memory.

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Keyphrases

Silicon analysis
Dimethylpolysiloxane—determination in lotions
Atomic absorption spectroscopy—analysis
Octaphenylcyclotetrasiloxane—standard

Mannich Bases of Vanillin Oxime

By H. V. MAULDING, Jr.*, M. A. ZOGLIO, P. TURI, and R. LA POFF

Synthesis of a group of 5-cycloalkyl- and 5-dialkylaminomethyl derivatives of vanillin oxime is reported. Results of microbiological tests are cited.

PHENOL AND CRESOLS are well-known antibacterial agents of long standing. It has previously been shown that α-dialkylamino-*o*-cresols exhibit significant activity against both bacteria and protozoa (1, 2). From these reports it was postulated that Mannich bases derived from vanillin oxime might fit the structural requirements necessary for possession of some degree of bacteriostatic effect.

Mannich bases were obtained from reaction of the oxime of vanillin with paraformaldehyde and various secondary amines.

Received February 23, 1968 from the Pharmacy Research and Development and Analytical Department, Sandoz Pharmaceuticals, Hanover, NJ 07936

Accepted for publication March 21, 1968.

* Compounds reported were prepared by H. V. M. at the University of Houston, Houston, Texas.

EXPERIMENTAL

Vanillin oxime was prepared after the general method stated by Shriner and Fuson (3) and recrystallized from ethanol–water, m.p. 115–117°. Synthesis of phenolic Mannich bases was achieved by the procedure of Burckhalter *et al.* (Procedure I) (2). The hydrochloride salts were recrystallized from solvents listed in Table I.

In the case of compound No. 4, Table I, the solid free base was obtained from the reaction mixture and recrystallized from ethanol, m.p. 181–184°. It gave a correct elemental analysis and was converted to its hydrochloride by treating an ether solution of the amine with ethanolic HCl. The other bases were oils.

pKa values were determined by differential plots of Δ pH/Δ *V versus* volume of 0.1 *N* NaOH titrant using approximately 50 mg. of the salt (4).

Antimicrobial activity was measured on *E. coli* (A.T.C.C. No. 8739) after a slight modification of the turbidimetric assay method of Eisman and coworkers (5).

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Keyphrases

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Dimethylpolysiloxane—determination in lotions
Atomic absorption spectroscopy—analysis
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Mannich Bases of Vanillin Oxime

By H. V. MAULDING, Jr.*, M. A. ZOGLIO, P. TURI, and R. LA POFF

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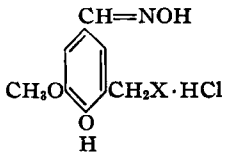
TABLE I—MANNICH BASES OF VANILLIN OXIME



No.	X	Yield, %	M.p.,[a] °C.	Formula	pKa	Analyses[b] Calcd.	Found
1	$N(CH_3)_2$	22	197–199[c]	$C_{11}H_{17}N_2ClO_3$	7.21	C, 50.7	50.5
						H, 6.6	6.7
						N, 13.6	13.5
2	$N(C_2H_5)_2$	56	184–186[d]	$C_{13}H_{21}N_2ClO_3$	7.14	C, 54.1	53.8
						H, 7.3	7.4
						N, 12.3	12.0
3	NC_5H_{10}[e]	47	194–196[c]	$C_{14}H_{21}N_2ClO_3$	7.25	C, 55.9	55.6
						H, 7.0	7.4
						N, 9.3	9.0
4	NC_4H_8O[f]	62	189–192[d]	$C_{13}H_{19}N_2ClO_4$	6.52	C, 51.6	51.4
						H, 6.3	6.3
						N, 9.3	9.1

[a] Melting points are uncorrected. [b] Carbon, hydrogen, and nitrogen analyses are through the courtesy of Mr. Urs Stoeckli. [c] Recrystallized from absolute ethanol–petroleum ether. [d] Recrystallized from absolute ethanol–ether. [e] Piperidino. [f] Morpholino.

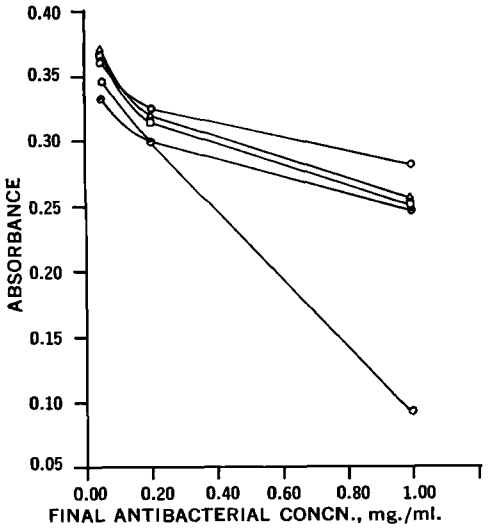


Fig. 1—Plot of absorbance of E. coli *suspension versus concentrations of antibacterial substances from Table I. Key:* ●, *compound 1;* □, *compound 2;* △, *compound 3;* ○, *compound 4;* ○, *chlorobutanol.*

A stock culture of *E. coli* was maintained on trypticase soy agar slants. The 24-hr. growth from these slants was suspended in sterile 0.9% saline and the suspension adjusted to 10–12% transmission at 580 mμ. A 1.5-ml. portion of this saline suspension was diluted with 100 ml. of trypticase soy broth.

Serial dilutions containing 4.0, 0.8, and 0.2 mg./ml. of the test compounds along with chlorobutanol were prepared in a pH 5.8 phosphate buffer (USP XVII). Aliquots of these solutions (2.5 ml.) as well as a 2.5-ml. quantity of the pH 5.8 buffer for control were mixed with 7.0-ml. portions of the inoculated broth. The tubes were incubated at 37° until the controls reached an approximate absorbance of 0.350 at 530 mμ. At this point growth was terminated by addition of 0.5 ml. of formalin in water (1:3) to all tubes and the solutions read at 530 mμ. The graph in Fig. 1 shows computed values obtained from 12 determinations.

RESULTS

The four derivatives of vanillin oxime appear to exhibit some inhibitory effect on the test organism. The slopes of the linear portions (Fig. 1) indicate this inhibition to be similar for all four compounds and they are significantly less active than chlorobutanol.

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Keyphrases

Vanillin oxime derivatives—synthesis
Mannich bases—5-cycloalkyl, 5-dialkylaminomethyl vanillin oxime derivatives
Antimicrobial activity—vanillin oxime derivatives

Hemolysis by Nonionic Surface-Active Agents

By TAMOTSU KONDO and MICHIKO TOMIZAWA

The hemolytic actions of polyoxyethylene glycol monododecyl ethers, $C_{12}H_{25}(C_2H_4O)_nOH$, have been studied. The amount required of these agents to produce lysis was found to be linearly related to the red cell concentration within experimental error. This was interpreted as showing the significant role of the adsorption of nonionic agent molecules onto the red cells prior to lysis. The average amount adsorbed of the agents necessary to cause lysis was estimated.

HEMOLYTIC ACTIONS of nonionic surface-active agents have been described by several authors (1–3). The mechanisms of the hemolysis, however, have not yet been well understood.

Pethica and Schulman stressed the importance of the surface effects of nonionics (4). Thus, they claimed that the nonionics used in their work lysed on attaining surface pressures of 34 dynes/cm. or above. On the other hand, the present authors suggested a significant role of the adsorption of nonionics onto the red cell membranes, because they found a proportionality between the hemolytic concentration of polyoxyethylene carboxylates and the cell count (5). The estimation of the amount adsorbed of the nonionics, however, was not possible owing to the heterogeneity of the materials.

Recently, polyoxyethylene glycol monoalkyl ethers have become available, which are homogeneous with respect to alkyl and polyoxyethylene chain length. This has now made it possible to determine the amount of nonionics adsorbed. The aim of this study has been to confirm the adsorption mechanism and to find a relation between the hemolytic activity and molecular structure of polyoxyethylene glycol monoalkyl ethers.

EXPERIMENTAL

Materials—The nonionic surface-active agents used were polyoxyethylene glycol monododecyl ethers having the general formula, $C_{12}H_{25}(C_2H_4O)_nOH$. These agents were synthesized by a successive condensation reaction between carefully fractionated dodecyl alcohol and diethylene glycol and/or triethylene glycol (6), and were generously supplied by Nihon Surfactants, Co., Ltd., Tokyo. The homogeneity of these nonionics were checked by gas or thin-layer chromatography. In Table I are listed the critical micelle concentrations and cloud points of the nonionics, which are referred to as $C_{12}E_n$ for brevity.

The red cell suspension used was prepared from dog blood as follows. Citrated blood was centrifuged and the cells were washed three times with the phosphate-buffered isotonic saline (pH 7.4). The washed, packed cells were then suspended in the above medium to yield a suspension of a desired concentration. A concentration of 1% v/v of the suspension was found in a hemocytometer to correspond to a cell count of 170–180 $\times$ 10^6/ml.

Hemolysis Techniques—The determination of the degree of hemolysis was done in the following way. Two milliliters of the cell suspension were pipeted into test tubes, into which an equal volume of various concentrations of the nonionics was added quickly by a syringe to prevent any local lysis. The mixtures were then allowed to react for an hour in a water bath controlled at 27° or 37 ± 0.1° with shaking so that the adsorption of nonionic molecules attained an equilibrium as quickly as possible. At the end of this period, the mixtures were immediately centrifuged to remove the unhemolyzed cells. The degree of hemolysis was estimated by determining spectrophotometrically the amount of hemoglobin released in the supernatant liquid.

Received March 12, 1968, from the Faculty of Pharmaceutical Sciences, Science University of Tokyo, Shinjuku, Tokyo, Japan.

Accepted for publication April 18, 1968.

The authors are indebted to Misses Naoko Fukuhara and Tomoko Yoshida for their assistance in this work.

RESULTS AND DISCUSSION

Hemolytic Activity—Effect of Polyoxyethylene Chain Length—Figure 1 shows the relation between the final percent hemolysis and the concentration of the nonionics studied at a cell concentation of 5% v/v.

All the hemolysis curves are of the similar shape and they shift toward the higher concentration region as the chain length of polyoxyethylene becomes longer. This means that the hemolytic activity of $C_{12}E_n$ decreases as the value of n increases. The same tendency was observed at all the cell concentrations studied.

Effect of Cell Concentration—In general, as the cell

TABLE I—CRITICAL MICELLE CONCENTRATIONS AND CLOUD POINTS OF NONIONIC SURFACE-ACTIVE AGENTS

Material	Cloud Point, °C.	C.M.C.	Temp., °C.
$C_{12}E_6$	55	$8.0 \times 10^{-5} M$	30
$C_{12}E_7$	70	$8.8 \times 10^{-5} M$	30
$C_{12}E_8$	82	$1.4 \times 10^{-4} M$	30

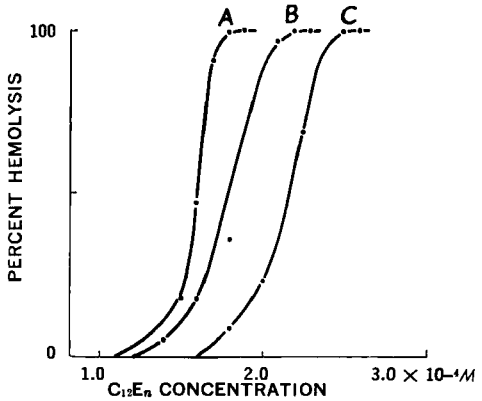

Fig. 1—Percent hemolysis versus *$C_{12}E_n$ concentration curves for 5% red cell suspension at 37°. Key:* A, n = *6;* B, n = *7;* C, n = *8.*

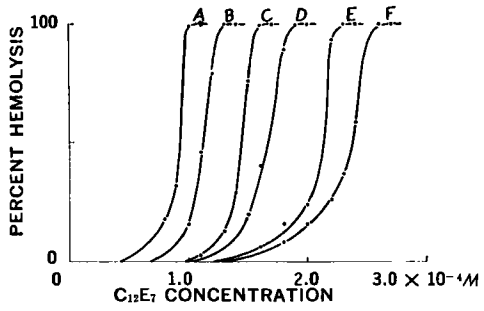

*Fig. 2—Shift of $C_{12}E_7$ hemolysis curves with change in cell concentration at 27°. Key: cell concentration % v/v—*A, *0.5;* B, *1;* C, *2;* D, *3;* E, *4;* F, *5.*

TABLE II—$C_{12}E_7$ CONCENTRATIONS REQUIRED TO PRODUCE 100% HEMOLYSIS FOR 5% v/v RED CELL SUSPENSION

Run	27°C.	37°C.
1	$1.2 \times 10^{-4}M$	$0.96 \times 10^{-4}M$
2	$1.4 \times 10^{-4}M$	$1.2 \times 10^{-4}M$
3	$1.3 \times 10^{-4}M$	$1.1 \times 10^{-4}M$

concentration was raised, the hemolytic curves were shifted to higher concentrations of the nonionics without change in shape. A typical example for $C_{12}E_7$ is given in Fig. 2. This result may rule out the possibility of the surface pressure mechanism, because the hemolytic concentrations are independent of surface tension of solutions.

Effect of Temperature—The rise in temperature appeared to make the red cells more sensitive to the lyzing agents. Thus, the hemolytic concentrations of the nonionics at 37° were lower than those at 27°. Table II gives the $C_{12}E_7$ concentrations to cause 100% hemolysis at both temperatures.

In connection with these findings, it is worth noting that the release of lipids from the red cell membranes was detected after shaking the cell suspension without the presence of surface-active agent in water bath for an hour. Moreover, the amount released of the lipids was observed to increase as the temperature rose. It may be inferred, therefore, that the lipid release from the red cell membranes correlates with the adsorption of the nonionics in the hemolysis, especially at elevated temperatures.

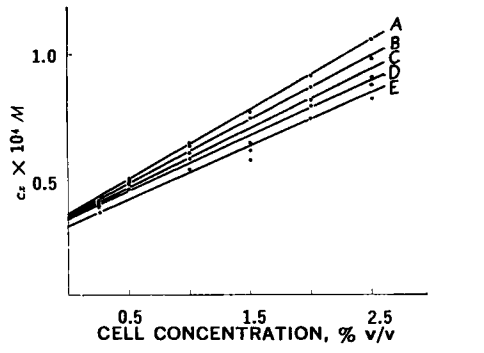

Fig. 3—$C_{12}E_7$ concentration required for various degrees of hemolysis, c_x, versus *cell concentration at 37°. Key: hemolysis percent—*A, *100;* B, *90;* C, *60;* D, *40;* E, *20.*

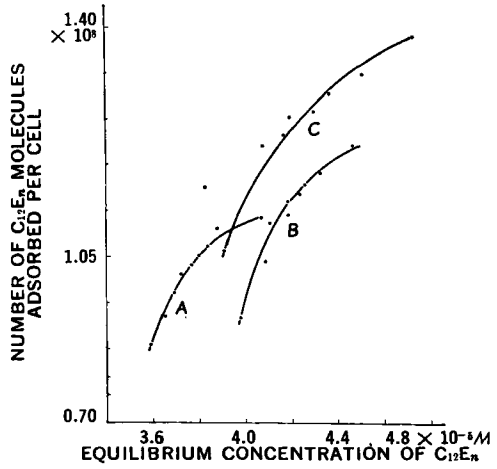

Fig. 4—Adsorption isotherms for $C_{12}E_n$ at 27°. Key: A, n = *6;* B, n = *7;* C, n = *8.*

Adsorption—The amount adsorbed of the nonionics on the red cells was estimated by an indirect method proposed by Thron (8), because several attempts have failed to determine it directly due presumably to the presence of surface-active materials released from the red cells. This method is based on the assumption that an individual cell undergoes lysis if and only if it adsorbs nonionic agent molecules in excess of a certain amount (not necessarily the same amount for different individual cells). If each cell comes into an adsorption equilibrium with its surroundings, then its uptake of the nonionic agent will be a function of the concentration of unadsorbed nonionic agent.

When the free nonionic agent concentration is b_x, where x is a given % hemolysis, the average amount adsorbed per cell has a corresponding value a_x, and the total amount adsorbed by the cells is a_xN, N being the cell count per unit volume of the system. This quantity and the free nonionic agent concentration, b_x, make up the total amount of nonionics, c_x, required to produce x% hemolysis:

$$c_x = a_xN + b_x$$

This equation means that a linear relation should hold between c_x and N. As shown in Fig. 3 the linear relation was obtained within experimental error.

Using the values of a_x and b_x, determined by the least-square method at various degrees of hemolysis, an adsorption isotherm can be set up. The results for the nonionics used at 27° are shown in Fig. 4.

As the polyoxyethylene chain length is decreased, the amount adsorbed necessary to cause lysis is also decreased. This will reflect an enhanced effectiveness in hemolysis of the nonionics with shorter polyoxyethylene chain. Thus, for example, the $C_{12}E_6$ molecules are more easily adsorbed onto the red cells from the solution and more strongly incorporated into the interior of cell membrane than are the $C_{12}E_7$ or $C_{12}E_8$ molecules. In other words, $C_{12}E_6$ causes the complete lysis at lower concentrations than does $C_{12}E_7$ or $C_{12}E_8$.

In Table III are given the amounts adsorbed of $C_{12}E_7$ per cell at 100% hemolysis. A decrease in the

TABLE III—NUMBER OF $C_{12}E_7$ MOLECULES ADSORBED PER CELL AT 100% HEMOLYSIS

Run	27°C.	37°C.
1	1.3×10^8	1.1×10^8
2	1.2×10^8	1.0×10^8
3	1.0×10^8	0.86×10^8

amount adsorbed is clearly seen with a rise in temperature.

As the slope of the adsorption isotherms at 37° was generally steeper than that at 27°, the rise in temperature would result in an increased tendency of the adsorption of nonionic agent molecules onto the red cell surface from aqueous solution owing to the temperature-dependent hydration of the molecules, thereby promoting the hemolysis. A similar adsorption behavior of the polyoxyethylene glycol monoalkyl ethers has been reported (9). The increased release of lipids from the red cells mentioned earlier may also contribute to a certain extent to the enhancement of the hemolysis at elevated temperatures.

As to the attacking site of the polyoxyethylated nonionics molecules, Mima and co-workers have concluded, based on the monolayer experiments, that the cholesterol portion in the cell membrane would be predominant (10). There still remains some doubt on this conclusion. In view of the limited scope of this study, however, any conclusion on the attacking site of the nonionics cannot be drawn.

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Keyphrases

Hemolysis—nonionic surfactants
Polyoxyethylene glycol monododecyl ethers—hemolysis
Temperature effect—hemolysis
Erythrocytes—surfactant adsorption
Spectrophotometry—analysis

Simple Method for Resuscitating Rats from Ether Overdosage

By L. F. SANCILIO, P. KRAUS, C. MYERS, and L. WAGNER

A simple method using compressed air for resuscitating rats from ether overdosage is described. Exposure to ether vapors for 55 and 90 sec., respectively, was lethal to 70 and 100 percent of the animals. The compressed air technique afforded protection to animals exposed to the anesthetic for 55, 90, 120, and 150 sec., while the conventional hand technique was ineffective against the 55- and 90-sec. exposure.

DURING THE PERFORMANCE of various experimental techniques in rats, *e.g.*, intrapleural injection (1), cotton pellet implantation (2), and adrenalectomy (3), using ether as the anesthetic agent, marked respiratory depression leading to death sometimes occurs unless the animal is immediately hyperventilated. This study describes a simple and effective technique used in our laboratory for reviving rats manifesting respiratory arrest following exposure to this anesthetic agent.

METHOD

Compressed air is required in performing this technique. In our laboratory, the air is pressurized at 60 p.s.i., and the tapered outlet (labcock) contains a valve to regulate its flow. The depressed animal is placed in a prone position in the palm of the hand with the head held gently but firmly between the thumb and index finger. The valve is partially opened (approximately 25%), and the air directed toward the rat's nostrils. The animal is moved back and forth rapidly across the stream of air to allow for inflation and deflation of the lungs. Spontaneous breathing is usually restored within 30 sec. An excess of pressurized air must be avoided as this will inflate the gastrointestinal tract.

The following study was undertaken to demonstrate the superiority of the compressed air to the manual technique. In the latter method, intermittent pressure was applied to hyperventilate the animal by placing the thumb on one side and the index and ring fingers on the other side of the rib cage.

Charles River CD rats (100–150 Gm.) of either sex were etherized in the following manner. One hundred milliliters of ether was poured into a 41-oz. glass dressing jar (Aloe Medical), the base of which was immersed in a water bath maintained at 39°. Ether was added periodically to replace that which had evaporated. The animal was placed on a wire mesh platform 7.5 cm. (3 in.) from the floor of the jar. After covering the jar, the exposure time to the ether vapors was determined with a Universal timer.

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Ether Exposure Time, Sec.	No. Animals	% Survival—Treatment—No Treatment	Compressed Air Technique	Hand Technique
		Experiment No. 1		
55	10	20	—	—
	10	—	90	—
90	10	0	—	—
	10	—	100	—
120	10	—	100	—
150	10	—	40	—
		Experiment No. 2		
55	10	30	—	—
	10	—	—	20
90	10	0	—	—
	10	—	—	0

The animal was then removed and either placed in a prone position with its head turned to one side, or subjected to the compressed air or hand techniques. Return of the righting reflex indicated resuscitation from the effects of the anesthetic.

RESULTS AND DISCUSSION

Exposure to ether vapors for 55 sec. was lethal to 70 and 80% of the animals, and for 90 sec. all the animals succumbed to the anesthetic (Table I). Those dying from the 90-sec. exposure showed no visible respiratory movements following removal from the jar, and death ensued shortly as indicated by no palpable heart beat. The compressed air technique resuscitated all animals subjected to the ether vapors for 90 and 120 sec. and partially revived those exposed for 150 sec. In contrast, the hand method afforded no significant protection to animals exposed to the anesthetic for either 55 or 90 sec. (Table I).

These results clearly demonstrate the effectiveness of the compressed air technique and its superiority to the hand method. The compressed air method hyperventilates the animals and perhaps, activates those stimulatory reflexes present in the nasal and pharnygeal areas (4).

The simplicity of this technique is clear, and it requires equipment commonly found in the laboratory.

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Keyphrases

Resuscitation method—rats
Ether vapor—respiratory arrest
Compressed air—resuscitation

A Spectrophotometric Method for the Analysis of Strychnine Phosphate in the Presence of Magnesium Stearate

By JANELLE BARRETT, RAMONA PUCKETT, and R. D. POE

A spectrophotometric technique for the analysis of strychnine phosphate in the presence of certain tablet excipients is reported. Tablets containing the alkaloidal salt are well dispersed in water, basified, and extracted with chloroform. The chloroform solution of the free base is then reacted with bromothymol blue in pH 7.65 buffer and the resultant colored product is quantified by the spectrophotometric technique.

SEVERAL METHODS are available for the analysis of strychnine phosphate, but most of them have disadvantages for use in tablet analysis, such as a large sample size, lack of precision, tedious procedure, or interference from other components present in the tablet. Among these methods are a picric acid method (1), a vanadate method (2), a titration method (3), a bromophenol blue method (4), a reduction and nitrite method (5), and a silicotungstic acid method (6).

In some of the above methods, magnesium stearate gives a negative interference. This has been overcome by a bromothymol blue extraction procedure using chloroform, in which an aliquot of methanol is added to the extract prior to making to volume. This procedure is simple and gives accurate results when applied to tablet assays.

Received December 11, 1967, from the Alcon Laboratories, Inc., Fort Worth, TX 76101
Accepted for publication February 29, 1968.

EXPERIMENTAL

Reagents—Chloroform A.R., methanol A.R., phosphate buffer pH 7.65 were used. Mix 50 ml. of 0.1 *M* sodium dihydrogen phosphate with 450 ml. of 0.1 *M* disodium hydrogen phosphate. Bromothymol blue: Prepare 0.018% in pH 7.65 buffer and wash twice with chloroform before use. Ammonium hydroxide, 10% aqueous. Strychnine phosphate standard: the commercially available salt should be thoroughly investigated by the usual

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TABLE I—RECOVERY OF STRYCHNINE PHOSPHATE FROM LOT 1 BY VARIOUS METHODS

Method[a]	Average Percentage[b] Found
1	73%
2	87%
3	82%
4	74%
5	90%[c], 88%[d]

[a] Code number explanation: 1, bromothymol blue method with no methanol, 2, modified bromothymol blue method with methanol added, 3, bromophenol blue, 4, ammonium vanadate method, and 5, picric acid method. [b] Averages of four to six samples. [c] Calculated from absorbances at the 400-mμ peak. [d] Calculated from absorbances at the 360-mμ peak.

techniques of melting point, infrared, ultraviolet spectroscopy, and thin-layer chromatography to assure its purity and identity as a "house standard."

Preparation of Standard—Accurately weigh approximately 43.2 mg. of dry strychnine phosphate standard. Dilute to 200.0 ml. with water and mix well. Pipet 4.0 ml. of the standard into a 125-ml. separator.

Preparation of Sample—Stir two tablets (0.432 mg. strychnine phosphate per tablet) in 7 ml. of water until well disintegrated. Transfer quantitatively with a small amount of water to a 125-ml. separator.

Procedure—Add 0.5 ml. of the ammonium hydroxide to each separator. Extract four times with 20 ml. of chloroform, combining the extracts in 150-ml. beakers. Evaporate the extracts to about 5 ml. on the steam bath. Cool and transfer to a 125-ml. separator, washing each beaker with about 5 ml. of chloroform, and adding these washings to the corresponding separator. Add 10 ml. of pH 7.65 buffer and 10 ml. of bromothymol blue reagent to each one and to a third separator containing 10 ml. of chloroform. Shake well and allow to separate. Transfer the chloroform layers to 50-ml. volumetric flasks. Add 15.0 ml. of methanol to each volumetric flask.

Extract the buffer layers again with two further 9-ml. portions and one 5-ml. portion of chloroform, and drain the chloroform extracts into the volumetric flasks each time. Make to volume with chloroform. Mix well and determine the absorbance against the reagent blank at approximately 410 mμ on a suitable spectrophotometer, using 1-cm. cells. Calculate against the standard.

DISCUSSION AND RESULTS

Two lots of a tablet containing strychnine phosphate have been analyzed using the above method. The first lot was also analyzed by several other procedures. Comparison of the results by the different procedures is listed in Table I. The second lot, analyzed by the modified bromothymol blue method, gave an average recovery of 100% for three assays.

To ascertain whether lot No. 1 was actually low, or whether all of the techniques were inapplicable to the tablet in question, individual samples of the other ingredients (calcium glycerophosphate, dibasic potassium phosphate, lactose, starch, and acacia) were weighed to approximately the amounts present in the tablet. These samples were added individually to aliquots of the standard and were assayed against the standard. (The procedure used was the bromothymol blue without the addition of methanol.) Only magnesium stearate gave any appreciable difference. The others gave an average recovery of 99%. A precipitate was noticed in the magnesium stearate extract. The addition of methanol cleared the extract.

TABLE II—RECOVERY OF STRYCHNINE PHOSPHATE IN THE PRESENCE OF MAGNESIUM STEARATE

Amount of Magnesium Stearate Added, mg.	Percentage of Strychnine Phosphate Found
2	103
4	102
8	100
12	100
	Average 101

Further work indicated that the addition of methanol to blank, standard, and sample gives good results.

The results in Table II were obtained using the methanol modification of the bromothymol blue method for analysis of strychnine phosphate in the presence of varying amounts of magnesium stearate.

Investigations comparable to the above were conducted using calcium stearate, zinc stearate, and stearic acid. The recovery of strychnine phosphate averaged 101% in the presence of 2–10 mg. of zinc stearate. The recoveries in the presence of calcium stearate and stearic acid were not quantitative by the procedure described above. However, addition of 1.5 ml. of ammonium hydroxide in lieu of the stated 0.5 ml. and filtration (Whatman No. 40) after the dye-alkaloid extraction resulted in an average recovery of 100% in the case of calcium stearate but only 92% in the case of stearic acid.

Although the picric acid method and the bromophenol blue method gave comparable results, the modified bromothymol blue method was found to be preferable for the tablet being analyzed because it was less tedious than the bromophenol blue method and more precise than the picric acid method. (One or more washing steps were absolutely essential to remove interference in the picric acid method.)

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Keyphrases

Strychnine phosphate tablets—analysis
Magnesium stearate interference—strychnine PO_4 analysis
Colorimetric analysis—spectrophotometry
Bromothymol blue—reagent

Synthesis and Antibacterial Activity of Certain β-Aminoketones

By RAJENDRA S. VARMA and W. LEWIS NOBLES

A series of β-aminoketones has been synthesized under the conditions of the Mannich reaction. Most of the compounds have exhibited antibacterial activity.

β-AMINOKETONES (Mannich bases) have been synthesized and screened as antispasmodics, local anesthetics, analgesics, and antibacterial agents (1–11). In a recent article from this laboratory the authors described the synthesis of a group of β-aminoketones derived from 1-(*N*-β-hydroxyethyl-4-piperidyl)-3-(4-piperidyl) propane (12). Several of these compounds have exhibited antibacterial and antiviral activity (13). In this communication the synthesis and antibacterial activity of β-aminoketones obtained from 4-(3-phenylpropyl)-piperidine is described.

BIOLOGICAL DATA

The β-aminoketones listed in Table I were screened *in vitro* against four organisms: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Mycobacterium smegmatis*, and *Klebsiella pneumoniae* by agar diffusion technique. Filter paper disks (6.35 mm. diameter) saturated with the solution (20 mg./ml.) of the test compound were placed on the agar. After 72 hr. of incubation, the zones of inhibition around the disks were measured. The results are recorded in Table I. A negative sign indicates no observable inhibition.

EXPERIMENTAL

Melting points were recorded on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were observed on a Perkin-Elmer model 137 spectrophotometer in Nujol mull. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tennessee.

β-Aminoketone Hydrochlorides—*Procedure A*—4-Phenylpropylpiperidine (5.07 Gm.; 0.025 mole) was dissolved in 5 ml. of ethanol. To this solution concentrated hydrochloric acid (2.5 ml.) was added followed by 5 ml. of 37% formalin and 0.025 mole of the appropriate ketone. The resulting reaction mixture was refluxed for 4 hr. At the end of this period some solvent was evaporated under vacuum. The contents were then diluted with 50 ml. of acetone. Refrigeration of the contents yielded the desired product.

Procedure B—A mixture of 0.025 mole of 4-(3-phenylpropyl)piperidine hydrochloride, 0.025 mole of the ketone, and 1 Gm. of paraformaldehyde in 10 ml. of ethanol containing two drops of concentrated hydrochloric acid was heated under reflux for 2 hr. Another 0.5 Gm. of paraformaldehyde was added, and the refluxing continued for 3 hr. At the end of this period the contents were poured into 50 ml. of acetone, after removing some solvent. The product obtained after overnight refrigeration was recrystallized from a suitable solvent.

4-Phenylpropylpiperidinoethyl-4-bromophenylcarbinol Hydrochloride (Compound 14)—β-4-(3-Phenylpropyl)-piperidine-4-bromopropiophenone hydrochloride (13.5 Gm., 0.03 mole) was neutralized with 10% aqueous sodium hydroxide with cooling. The product obtained was filtered and washed thoroughly with cold water until washings were free from alkali. After air drying, the product was suspended in 100 ml. of methanol in a 1-L. flask. Three and eight-tenths grams of sodium borohydride, dissolved in 50 ml. of methanol, was added slowly with stirring. During the additions the temperature was kept below 50°. The reaction mixture was stirred for an additional 2 hr. and then left at room temperature overnight. It was filtered and the filtrate evaporated to dryness under vacuum. Fifty milliliters of water was then added to the residual solid. The resulting mixture was extracted with ether. The extract, after washing with water, drying over magnesium sulfate, and evaporating the ether, yielded a viscous oil which soon solidified. It was dissolved in 10 ml. of ethanol, acidified with concentrated hydrochloric acid, and poured into 100 ml. of acetone. Overnight refrigeration of the acidified solution gave the desired product. An analytical sample was prepared by recrystallizing from ethanol-acetone, m.p. 173–174°; yield, 4.05 Gm. (30%). IR: 3333 cm.$^{-1}$ (OH).

Anal.—Calcd. for $C_{23}H_{31}BrClNO$: C, 61.00; H, 6.90; N, 3.09. Found: C, 61.29; H, 7.14; N, 3.00.

DISCUSSION

4-(3-Phenylpropyl)-piperidine was converted to the hydrochloride salt and condensed with formaldehyde and several substituted ketones. Paraformaldehyde, as well as aqueous formalin, yielded satisfactory products. The Mannich base hydrochlorides were usually isolated from the reaction mixture

TABLE I—ANTIBACTERIAL ACTIVITY OF β-AMINOKETONES: MICROBIAL SPECTRUM

Compound No.[a]	*S. aureus* K257	*P. aeruginosa*	*K. pneumoniae* ATCC 8052	*M. smegmatis*
1	+	+	+	+
2	+	+	−	+
3	−	+	−	−
4	+	+	+	+
5	+	+	+	+
6	+	+	+	+
7	−	−	−	−
8	−	−	+	+
9	−	−	−	−
10	+	+	−	+
11	+	+	+	+
12	−	+	+	+
13	+	+	+	+
14	+	+	+	+

[a] As in Table II. A negative sign indicates no inhibition.

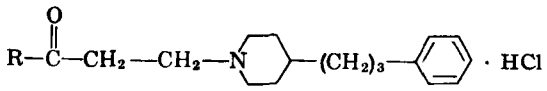
Received January 8, 1968, from Department of Pharmaceutical Chemistry, University of Mississippi, University, MS 38677

Accepted for publication March 11, 1968.

This investigation was supported in part by grant No. AI-04701 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

The authors express their appreciation for the facilities and assistance provided during screening of these compounds to Dr. Lyman A. Magee and Mr. William Bing of the Biology Department of the University of Mississippi.

TABLE II—β-AMINOKETONES



Compound No.	R	Formula	% Analysis Calcd.	% Analysis Found	Yield,[a] %	M.p., °C.	Infrared C=O	Infrared +NH
1	Phenyl	$C_{23}H_{30}ClNO$[b]	C, 74.26 H, 8.13 N, 3.77	74.48 8.25 3.76	53	173–174	1685	2475
2	4-Chlorophenyl	$C_{23}H_{29}Cl_2NO$[c]	C, 67.96 H, 7.19 N, 3.45	67.72 7.22 3.47	64	190–192	1680	2450
3	4-Methylphenyl	$C_{24}H_{32}ClNO$[d]	C, 74.66 H, 8.36 N, 3.63	74.47 8.20 3.62	46	190	1680	2450
4	4-Fluorophenyl	$C_{23}H_{29}ClFNO$[d]	C, 70.85 H, 7.50 N, 3.59	70.87 7.68 3.70	44	179–181	1675	2500
5	4-Nitrophenyl	$C_{23}H_{29}ClN_2O_3$[b]	C, 66.25 H, 7.01 N, 6.72	66.12 7.18 6.56	48	175–176	1680	2400
6	4-Ethoxyphenyl	$C_{25}H_{34}ClNO_2$[b]	C, 72.16 H, 8.24 N, 3.37	71.95 8.22 3.41	60	190–191	1670	2530
7	4-Phenylphenyl	$C_{29}H_{34}ClNO$[c]	C, 77.75 H, 7.65 N, 3.13	77.51 7.49 3.15	44	199–200	1670	2500
8	3-Nitrophenyl	$C_{23}H_{29}ClN_2O_3$[d]	C, 66.25 H, 7.01 N, 6.72	66.46 7.25 6.60	40	159–161	1695	2600
9	4-Hydroxyphenyl	$C_{23}H_{30}ClNO_2$[c]	C, 71.20 H, 7.80 N, 3.61	71.49 7.84 3.49	51	208–209	1670	2600
10	3-Hydroxyphenyl	$C_{23}H_{30}ClNO_2$[b]	C, 71.20 H, 7.80 N, 3.61	71.14 7.99 3.50	49	176–178	1690	2600
11	3,4,5-Trimethoxyphenyl	$C_{26}H_{36}ClNO_4$[d]	C, 67.61 H, 7.86 N, 3.03	67.50 8.02 3.04	52	171–172	1665	2400
12	4-Bromophenyl	$C_{23}H_{29}BrClNO$[c]	C, 61.25 H, 6.48 N, 3.11	61.08 6.50 3.09	51	192–193	1695	2500
13	2-Thenyl	$C_{21}H_{28}ClNOS^1/_2H_2O$[b]	C, 65.16 H, 7.55 N, 3.62	65.55 7.72 3.77	33	165–166	1650	2500

[a] Yields are of the products obtained after first recrystallization. [b] Recrystallized from ethanol. [c] Recrystallized from aqueous ethanol–acetone. [d] Recrystallized from ethanol–acetone.

by adding acetone to it and refrigerating overnight. It was observed that while using 4-phenylacetophenone, the best results were obtained when some of the solvent was distilled from the reaction mixture before the addition of acetone. The period required to refrigerate the reaction mixture in order to get a solid product varied considerably. While using 3-nitroacetophenone, a solid product was obtained after 4 days of refrigeration. Prolonged refrigeration resulted in improved yields. The β-aminoketone from 4-bromoacetophenone was reduced to the corresponding γ-amino secondary alcohol by sodium borohydride. Infrared spectra of the β-aminoketones exhibited characteristic absorption bands for carbonyl and $\overset{+}{N}H$ (14). No carbonyl absorption was observed in the infrared spectrum of the aminoalcohol; instead, absorption due to OH group, as expected, was observed.

SUMMARY

Thirteen β-aminoketones have been synthesized using 4-(3-phenylpropyl)-piperidine and several ketones under Mannich reaction conditions. One β-aminoketone (No. 12, Table II) has been reduced to the corresponding γ-amino secondary alcohol by sodium borohydride. Antibacterial activities have been determined for all compounds described herein.

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Keyphrases

β-Aminoketones—synthesis
Antibacterial activity—β-aminoketones
IR spectrophotometry—structure

Influence of Starch Concentration on the Disintegration Time of Tolbutamide Tablets

By K. C. COMMONS, A. BERGEN, and G. C. WALKER

Disintegration tests were performed on tablets compressed from 16/20, 40/60, and 60/80 mesh granulations prepared by the method of dry granulation to contain 250 mg. of tolbutamide and corn starch concentrations of 6, 7, 8, 9, and 10 percent in each granulation size. It might be expected that disintegration time would decrease as the percentage of starch in the tablet increased. This does not appear to be the case for tolbutamide tablets. Instead, there appears to be a critical starch concentration for different granulation sizes.

In a number of instances, poor tablet formulation has been shown to cause a significant variation in gastrointestinal absorption and physiological availability of the active ingredient (1–3). Variables in the formulation and processing include such factors as particle size and shape, binders, diluents, disintegrating agents, lubricants, compression rate, and compression pressure as well as physical features of the dosage form itself. Alteration of any of these factors may influence the disintegration time, dissolution rate, and clinical effectiveness of compressed tablets.

Disintegrating agents have been widely used in compressed tablets as substances or mixtures of substances added to a tablet to facilitate its breakup or disintegration following administration (4). There have been many classifications of disintegrating agents but one of the more recent is that of Feinstein and Bartilucci (5) who divided them into starches, clays, celluloses, algins, or gums. Fakouhi *et al.* (6) point out that disintegrating agents generally fall into three classes: (*a*) agents that react with moisture to constitute a foam, (*b*) effervescent substances that react with moisture to form a gas, and (*c*) substances that react with moisture to swell (most common). The most commonly used agent has been starch from various sources but particularly corn or potato starch.

A study was undertaken to determine the influence of corn starch concentration on the disintegration of tolbutamide tablets prepared from various size granules by direct compression.

EXPERIMENTAL

Tolbutamide powder (USP XVI, 100 mesh) was dried at 100° for 24 hr. Approximately 1 Gm. of powder at a time was compressed at 10,000 lb. gauge pressure in a Carver hydraulic press using 3/4-in. flat-face punches. The slugs were granulated using a Fitzpatrick mill and the granules sieved for 5 min. through 16, 20, 40, 60, and 80-mesh screens using an automatic sieve shaker. Granule sizes were designated 16/20, 40/60, and 60/80 which refers to a passage through the first sieve size number designation and retention on the second sieve. The tolbutamide granules and corn starch (USP XVI, moisture content 9.2% w/w) were thoroughly mixed to give granulation-starch mixtures containing 6, 7, 8, 9, and 10% corn starch. Compressed tablets of 250-mg. tolbutamide content were prepared from the various granule-starch combinations using 3/8-in. shallow concave punches at 2,000 lb. gauge pressure in the Carver press. Tablets and granules were assayed, and disintegration tests performed, using the methods as outlined in the USP XVII (7).

RESULTS AND DISCUSSION

Table I shows the average disintegration time of compressed tablets prepared from different granule sizes and containing varying percentages of corn starch. This information is presented graphically in Fig. 1.

It might be expected that disintegration time would decrease as the percentage of starch in the tablet is increased. This does not appear to be the case for tolbutamide tablets. Instead, there appears to be a critical starch concentration for different granulation sizes when granulations are prepared by methods involving dry granulation and com-

Table I—Average Disintegration Times in Minutes of Tolbutamide Tablets

Starch Concn., %	Granule Size 16/20	20/40	40/60	60/80
6	>30	>30	—	—
7	2.3	22.3	>30	>30
8	1.1	8.8	28.8	>30
9	0.4	0.3	1.9	2.1
10	—	—	0.7	1.0

Received December 22, 1967, from the Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada.
Accepted for publication February 23, 1968.
This investigation was supported in part by a research grant to one of us from Smith Kline & French, Inter-American Corporation, Montreal, Quebec.

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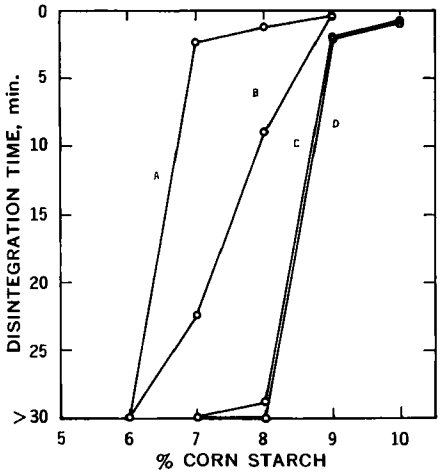

Fig. 1—Disintegration times, determined by the USP XVI method, of tolbutamide tablets prepared from different granule sizes and containing varying corn starch concentrations. Key: A, 16/20 granules; B, 20/40 granules; C, 40/60 granules; D, 60/80 granules.

pression. In most cases, tablets failed to disintegrate at a particular percentage of starch but disintegrated when the starch concentration was increased by about 1%. For example, tablets prepared from 16/20-mesh granules containing 6% corn starch failed to disintegrate within 30 min., whereas tablets containing 7% corn starch disintegrated in an average time of 2.3 min.

Curlin has suggested that the disintegration of tablets containing starch is due to capillary action (8). The more widely held view is that disintegration is caused by absorption of water by the disintegrating agent and development of pressure within the tablet caused by swelling or expansion of the disintegrating agent (8–10). Fakouhi *et al.* (6) in their studies of lactose tablet disintegration with powdered corn cob, powdered redwood bark, wood flour, powdered Douglas fir, methylcellulose, and corn starch point out that a decrease in disintegration time would be expected with an increase in concentration of the agent, but in the case of methylcellulose, redwood bark, and Douglas fir this was not experienced. The authors suggest that these substances possess adhesive or binding properties with a tendency to form adhesive gels when hydrated thus suggesting an optimum or maximum quantity of these substances beyond which tablet disintegration will be retarded. The mechanism of disintegration action for all agents is probably the same, *i.e.*, swelling in contact with water and subsequent rupture but with the necessary absorption of sufficient moisture. Forlano and Chavkin (11) used separate granulations of sodium bicarbonate, lactose, and magnesium trisilicate in mesh sizes ranging from 6–8 to 140–200 in increments of 2 mesh sizes with one at 200 mesh and finer to study the influence of granule size and capping in compressed tablets. In the case of sodium bicarbonate and lactose tablets, granules of 16–60 mesh showed the longest disintegration times. As the granule size decreased below 60 mesh the disintegration time decreased with an unexpected rise at 100–140 mesh, followed by a decrease at 140–200 mesh. In the case of magnesium trisilicate, tablets made using 12–30-mesh granules had longer disintegration times than the smaller mesh sizes. However, tablets made from granules smaller than 30 mesh showed decreased disintegration times. It is possible that the nature of the bonding is different between tablets and that the binders have different actions. In the case of magnesium trisilicate tablets the small hard core together with the nature of the substance probably prevented penetration of the center by water.

When starch is incorporated into a tablet, it is found in the channels between the granules. Starch is a relatively incompressible substance and tends to increase the porosity of the tablet. If there were no starch present in the channels, the tolbutamide granules would be deformed under compression to partially block the channels. Tablets were prepared from corn starch soaked in a saturated aqueous iodine solution and dried. Fracture of the tablet followed by macroscopic and microscopic examination suggested that the starch grains form continuous chains along the channels between the granules even at concentrations below those required to make the tablet disintegrate. However, it appears that as the percentage of starch is increased, thicker chains of starch are formed in the pore channels, thus enlarging the pores. This pore enlargement could allow water to enter the tablet more readily. Patel and Hopponen (12) have shown that where contact of starch grains in the interparticle spaces is continuous, disintegration is most rapid. They have also found that dried corn starch increased in volume by 78% when suspended in water. This increase in volume strongly suggests that swelling is the principal mechanism of action of corn starch as a disintegrator. Wurster and Seitz (13) have demonstrated that a small pore size which could be occluded by air would hinder entry of water into the tablet. This would be particularly true in the case of a hydrophobic substance such as tolbutamide. In this case, disintegration of the tablet would be slowed but could continue as the pores are enlarged by expanding starch grains near the surface of the tablet. Wurster and Seitz concluded that capillarity itself does not appear to have a disintegrating effect although it may be a factor in aiding entry of water into the tablet when pore size is favorable.

The critical starch concentration may have practical significance in both wet and dry granulation procedures, but particularly in view of the increasing tendency to use methods of dry granulation and direct compression in the pharmaceutical industry. Similar results have been secured using acetylsalicylic acid, salicylamide, and phenylbutazone. Preliminary *in vivo* studies using acetylsalicylic acid have shown good correlation between disintegration, dissolution, urinary excretion, and critical starch concentration. These results will be published shortly.

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Keyphrases

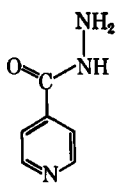
Tolbutamide tablets—disintegration
Corn starch grains—channel formation
Disintegration time—starch concentration

Influence of Metallic Ions on the Antituberculous Activity of Isonicotinoyl Hydrazones

By V. A. E. VOYATZAKIS*, G. S. VASILIKIOTIS†, G. KARAGEORGIOU‡, and IR. KASSAPOGLOU‡

Eleven isonicotinoyl hydrazones were prepared and they were tested *in vitro* as antitubercular agents. The effects of cupric and cobalt ions on their activity were investigated.

THE SPECIFIC activity of isonicotinic acid hydrazide (isoniazid I) against tubercle bacilli suggests interference with an essential metabolite. It has been shown (1, 2) that isoniazid combines with cupric ions to give 1:1 Cu-isoniazid complex (II) and 1:2, respectively. It also has been claimed that the action of isoniazid against *M. tuberculosis in vitro* is increased when copper is supplied in excess of that normally present in the medium (2).



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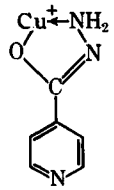


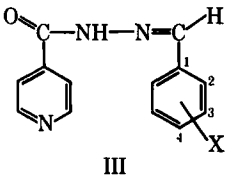
II

Moreover it has been shown (3) that 8-hydroxyquinoline (oxine) and related substances are toxic to bacteria only when traces of iron or copper ions are present in the medium. In this case the 1:1 metal complex has been shown to be the true toxic agent (4). The hypothesis was put forward that oxine is active due to the combination with these metal ions in the medium and that the complexes thus formed catalyze the oxidation of essential cell constituents. Cobalt can prevent injury to the cells, and it was suggested that this ion may be an essential cell constituent whose function is to protect, from oxidative destruction a vitally important chemical group. However, Albert (5) pointed out that while chelation might play some part in the activity of isoniazid, there must be some more important factor since the closely related picolinic acid hydrazide was less effective against tubercle bacilli but showed a metal affinity 10^3 to 10^6 times that of isoniazid.

Youatt (6) who investigated the effect of cupric ions on the uptake of hydrazides, found that copper prevented the development of strains resistant to isoniazid and increased the sensitivity to hydrazides of a strain which was already resistant to isoniazid. Stimulation of isoniazid uptake was still observed if the cells were exposed to copper ions and then washed before the addition of the isoniazid. This suggests that copper may first be bound to the cell and that chelation may occur on or in the cell.

Among the most active derivatives of isoniazid are its hydrazones. Fox and Gibas (7) have shown that 1-isonicotinoyl-2-isopropylidene hydrazone was very active against tubercle bacilli. Shchukina *et al.* (8) synthesized a number of isonicotinoyl hydrazones from aldehydes and ketones. Their biological tests, as antitubercular agents indicated that some of these are more active *in vivo* and far less toxic in mice. Sah and Peoples (9) reported also that a large number of isonicotinoyl hydrazones possess very high *in vivo* activity against *M. tuberculosis*, H 37 Rv, higher than that of streptomycin, and at least of the same order of magnitude as isoniazid. Recently Chakravarty, Bose, and Bose (10) synthesized some isonicotinoyl hydrazones which showed antituberculous activity comparable to that of isoniazid (Table I).

In this paper there is reported: (*a*) the synthesis of 11 isonicotinoyl hydrazones derived from substituted aromatic aldehydes and ketones having the general formula (III); (*b*) their microbiological examination *in vitro* as antitubercular agents, and (*c*) the influence of cupric and cobalt ions on their activity.



III

EXPERIMENTAL

Preparation of Isonicotinoyl Hydrazones—The hydrazones used in this study were synthesized according to the method of Sah and Peoples (9). All of them were recrystallized twice from methanol

Received October 30, 1967, from the University of Thessaloniki, Laboratory of Analytical Chemistry, Thessaloniki, Greece.
Accepted for publication February 20, 1968.
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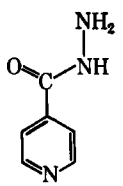
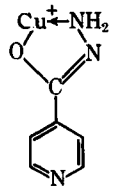
THE SPECIFIC activity of isonicotinic acid hydrazide (isoniazid I) against tubercle bacilli suggests interference with an essential metabolite. It has been shown (1, 2) that isoniazid combines with cupric ions to give 1:1 Cu-isoniazid complex (II) and 1:2, respectively. It also has been claimed that the action of isoniazid against *M. tuberculosis in vitro* is increased when copper is supplied in excess of that normally present in the medium (2).



I



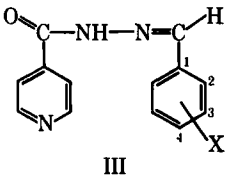
II

Moreover it has been shown (3) that 8-hydroxyquinoline (oxine) and related substances are toxic to bacteria only when traces of iron or copper ions are present in the medium. In this case the 1:1 metal complex has been shown to be the true toxic agent (4). The hypothesis was put forward that oxine is active due to the combination with these metal ions in the medium and that the complexes thus formed catalyze the oxidation of essential cell constituents. Cobalt can prevent injury to the cells, and it was suggested that this ion may be an essential cell constituent whose function is to protect, from oxidative destruction a vitally important chemical group. However, Albert (5) pointed out that while chelation might play some part in the activity of isoniazid, there must be some more important factor since the closely related picolinic acid hydrazide was less effective against tubercle bacilli but showed a metal affinity 10^3 to 10^6 times that of isoniazid.

Youatt (6) who investigated the effect of cupric ions on the uptake of hydrazides, found that copper prevented the development of strains resistant to isoniazid and increased the sensitivity to hydrazides of a strain which was already resistant to isoniazid. Stimulation of isoniazid uptake was still observed if the cells were exposed to copper ions and then washed before the addition of the isoniazid. This suggests that copper may first be bound to the cell and that chelation may occur on or in the cell.

Among the most active derivatives of isoniazid are its hydrazones. Fox and Gibas (7) have shown that 1-isonicotinoyl-2-isopropylidene hydrazone was very active against tubercle bacilli. Shchukina *et al.* (8) synthesized a number of isonicotinoyl hydrazones from aldehydes and ketones. Their biological tests, as antitubercular agents indicated that some of these are more active *in vivo* and far less toxic in mice. Sah and Peoples (9) reported also that a large number of isonicotinoyl hydrazones possess very high *in vivo* activity against *M. tuberculosis*, H 37 Rv, higher than that of streptomycin, and at least of the same order of magnitude as isoniazid. Recently Chakravarty, Bose, and Bose (10) synthesized some isonicotinoyl hydrazones which showed antituberculous activity comparable to that of isoniazid (Table I).

In this paper there is reported: (*a*) the synthesis of 11 isonicotinoyl hydrazones derived from substituted aromatic aldehydes and ketones having the general formula (III); (*b*) their microbiological examination *in vitro* as antitubercular agents, and (*c*) the influence of cupric and cobalt ions on their activity.



III

EXPERIMENTAL

Preparation of Isonicotinoyl Hydrazones—The hydrazones used in this study were synthesized according to the method of Sah and Peoples (9). All of them were recrystallized twice from methanol

Received October 30, 1967, from the University of Thessaloniki, Laboratory of Analytical Chemistry, Thessaloniki, Greece.
Accepted for publication February 20, 1968.
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TABLE I—ISONICOTINOYL HYDRAZONE DERIVATIVES

Compd.	R	X	Compd.	R	X
A-1	H	H	A-7	H	4—OH
A-2	H	2—OH	A-8	H	4—NO_2
A-3	H	2—Cl	A-9	CH_3	4—OH
A-4	H	2—NO_2	A-10	CH_3	4—CH_3O
A-5	H	3—OH	A-11	CH_3	4—CH_3
A-6	H	4—$N(CH_3)_2$			

before use and their melting points were in agreement with those given in the literature (9); their infrared absorption spectra have already been reported (11).

Microbiological Examination—Test organism. Stock strains: (*a*) A strain (M K1) of *M. tuberculosis* isolated from a patient before any treatment. This strain was sensitive to isoniazid in concentration of 0.1 γ/ml. and was catalase and peroxidase positive; (*b*) A strain (M K2) of *M. tuberculosis* isolated from patient treated with isoniazid. It was resistant to isoniazid (10 γ/ml.) and was catalase and peroxidase negative. *In vitro* test: Compounds were incorporated with Lowenstein-Jensen medium before its coagulation at 80°. Their concentration is expressed in γ/ml. of medium. Thereafter, they were inoculated with a culture of *M. tuberculosis* grown in the same medium and incubated at 38° for 28 days. Control tubes containing only strain culture were maintained for comparison. All these specimens were incubated at 38° for 28 days again. In each case cultures of both strains were tested with 0.1 γ, 1 γ, and 10 γ/ml. of compound and then again with the same concentration but with the addition, separately of 1 γ, 10 γ, 20 γ, 40 γ, and 80 γ of cupric or cobalt ions, derived respectively from $CuSO_4$ or $CoSO_4$ solutions. Stability tests were performed to find out the rate at which compounds and isoniazid became inactivated by incubation at 38°. The results suggest that the proportionate loss of activity was of about the same order for all compounds and isoniazid.

RESULTS AND DISCUSSION

Strain M K1 was sensitive to all of these compounds in concentration of 0.1 γ/ml., while strain M K2 was resistant to them even in 10 γ/ml. So the results mainly concern the resistant strain M K2 and the activity of these compounds is comparable to isoniazid. Cupric ion alone, in concentration above 60 γ/ml., inhibited completely the development of cultures in both cases while cobalt ion was ineffective up to 80 γ/ml. In mixtures with isonicotinoyl hydrazones, the first notable influence of Cu^{2+} appeared in the range of 20 γ/ml. added to 0.1 γ/ml. of these compounds. Results are summarized in Table II. There was a complete inhibition, in all cases, by the addition of 40 γ/ml. of cupric ions. About the same results were obtained with 1 γ/ml. and 10 γ/ml. of these compounds.

TABLE II—EFFECT *In Vitro* OF Cu^{2+} (20γ/ML.) ON THE BACTERIOSTATIC ACTIVITY OF ISONICOTINOYL HYDRAZONES (0.1 γ/ML.) BY USING ISONIAZID RESISTANT STRAIN M K2

Compd.	Inhibition
A-1	++[a]
A-2	+++
A-3	+++
A-4	++++
A-5	++
A-6	+++
A-7	++
A-8	++
A-9	—[b]
A-10	+++
A-11	—
Cu^{++} (60 γ/ml.) alone	+++++

[a] Each + represents 20% of inhibition. [b] No inhibition at all.

TABLE III—EFFECTS OF COBALT IONS ADDED TO HYDRAZONES

Compd.	Inhibition	Compd.	Inhibition
A-1	++[a]	A-7	—[b]
A-2	+++	A-8	++
A-3	+++	A-9	++
A-4	++	A-10	+
A-5	++	A-11	++
A-6	+++	Co^{2+} (80 γ/ml.) alone	[c]

[a] Each + represents 20% of inhibition. [b] No inhibition at all. [c] 10–20 colonies observed against ~500 of the control tube.

Effects of cobalt ions (40 γ/ml.) added to a concentration of 0.1 γ/ml. of hydrazones are summarized in Table III.

By adding 80 γ/ml. of Co^{2+}, to the tested compounds, still 10–20 colonies were observed against to 400–500 of the control tube.

In the mixtures used in this study, the concentration of free cupric ions was well below that which would cause inhibition by them alone. There is also a possibility of formation of cupric chelates with hydrazones and then the concentration of free Cu^{2+} will be even lower and will depend of course on its affinity to the ligand, on pH, ratio of ligand to metal, stability constants, and acid dissociation constants of the ligand.

Since the results of the inhibition due to added cupric ions to the strains studied were independent of the concentration of the compound used (between 0.1 γ/ml. and 10 γ/ml.) there is some evidence that free cupric ion may be responsible for the stimulation of isonicotinoyl hydrazone uptake as well as in the case of isoniazid (6).

Thus, development of the resistant strain M K2 was supressed and the critical quantity of cupric ions, under the experimental conditions was 20 γ/ml. even in the presence of 0.1 γ/ml. of the drug. Of course there are variations of the activity of these drugs and research on their Cu complex stability constants is continuing and the results shall be published in due time.

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Keyphrases

Isonicotinoyl hydrazones—synthesis
Metallic ions–isonicotinoyl hydrazones—complexes
Antitubercular activity—cupric, cobalt ions effect

Potential Diazo Reagents for Colorimetric Determination

By TIBOR URBÁNYI and JOSEPH A. MOLLICA

The diazonium derivatives of 27 substituted aromatic amines were investigated as reagents for colorimetric determination. The phenolic moiety used in the study was the estrogen, estradiol. Their applicability was determined on the basis of speed, sensitivity, color stability, and reproducibility of the coupling reaction. Using these criteria, the diazotization product of 4-amino-6-chloro-*m*-benzenedisulfonamide appeared to be the most suitable colorimetric reagent for the compounds containing a phenolic hydroxyl group.

A RAPID AND SENSITIVE method for the determination of estrogens is based on their ability to couple with diazotized amines. This communication considers the results of investigations regarding the suitability of various substituted aromatic amines as analytical reagents for compounds having coupling capability. Under the experimental condition employed, 4-amino-6-chloro-*m*-benzenedisulfonamide appeared to be the most promising reagent. The mechanism for the formation of its diazonium compound and its application to the analysis of several estrogens was reported previously (1, 2).

The following criteria for suitability were established: the coupling reaction should be rapid at room temperature, the color produced should be relatively stable, and the method should be sensitive. Since speed, sensitivity, and reproducibility were the objectives, the same reaction conditions were employed for all amines. Although these conditions were not optimum for all amines tested, this procedure was selected since the goal was to find suitable analytical reagents and not to investigate the reaction for each amine.

EXPERIMENTAL

A Beckman DU spectrophotometer was used to determine the absorbance values, and a Cary model 11 spectrophotometer was used to record the absorption spectra.

All compounds were of reagent grade quality and were used without further purification.

Reagents and Solutions—Aromatic amines: a 7×10^{-3} *M* solution of the amine was prepared in either methanol or 1 *N* hydrochloric acid. Sodium nitrite, 1% aqueous solution; hydrochloric acid, 1 *N*; sodium acetate 2 *N*; sodium hydroxide 0.1 *N* and 1 *N*. The estradiol solution was prepared as follows: about 50 mg. of estradiol, accurately weighed, was dissolved in 50 ml. of methanol and then this solution was diluted 10-fold with 0.1 *N* sodium hydroxide; concentration approximately 0.1 mg./ml.

Procedure—Into a 10-ml. volumetric flask were pipeted 1 ml. of the aromatic amine solution, 1 ml. of sodium nitrite solution, and 1 ml. of 1 *N* hydrochloric acid. The solution was mixed well and allowed to stand for 1 to 2 min. Then, 1 ml. of estradiol solution and 2 ml. of sodium acetate solution were added, mixed, and the resulting solution allowed to stand for exactly 6 min. The contents of the flask were diluted to volume with 1 *N* sodium hydroxide solution. A blank solution was prepared in the same manner except that 1 ml. of 0.1 *N* sodium hydroxide was used in place of the estradiol solution. The absorbance of the sample solution was measured against the blank solution at the wavelength of the absorption maximum in 1-cm. cells.

DISCUSSION

The coupled products yielded absorption spectra with maxima in the range 450–550 mμ. The spectra were all similar to the spectrum of 4-amino-6-chloro-*m*-benzenedisulfonamide and estradiol which was published previously (2). The apparent molar absorptivity values in Table I were obtained by dividing the absorbance of the coupled solution, measured at the wavelength of absorption maximum, by the concentration of the amine employed. The molar absorptivity values reported are only apparent values since for many of the compounds investigated, the absorbance continued to increase after the 6-min. reaction time. This is due to either incomplete coupling and/or side reactions of the diazotized amine (3, 4). As stated in the introduction, the purpose of this study was not to investigate the mechanism of reaction, but to find a suitable analytical reagent. However, some conclusions regarding the effect of substituent groups on color intensity can be drawn. Comparisons of the molar absorptivity values indicate that substitution strongly influences the intensity of the coupled product, *e.g.*, 3 and 4, 13 and 15, 23 and 24; not

Received August 28, 1967, from the Development and Control Department of Ciba Pharmaceutical Company, Summit, NJ 07901
Accepted for publication April 1, 1968.

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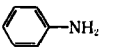
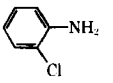
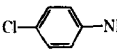
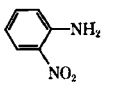
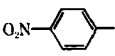
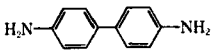
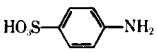
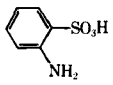
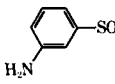
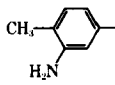
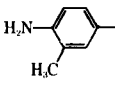
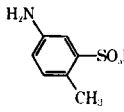
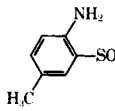
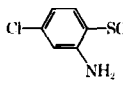
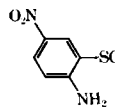
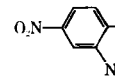
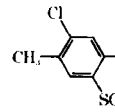
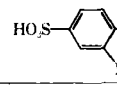
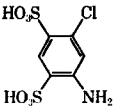
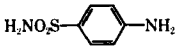
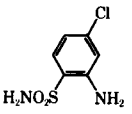
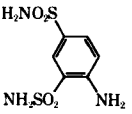
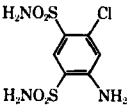
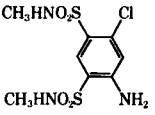
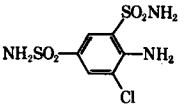
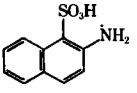
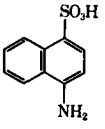
Accepted for publication April 1, 1968.

TABLE I—SUBSTITUTED AROMATIC AMINES FOR DETERMINATION OF ESTROGENS

Compound	Structural Formula	Initial Solvent	Wavelength, Abs. Max.	Apparent Molar Absorptivity[a]
(1) Aniline		MeOH	NC	NC
(2) *o*-Chloroaniline		MeOH	465 mμ	2,040
(3) *p*-Chloroaniline		MeOH	465 mμ	1,500
(4) *o*-Nitroaniline		MeOH	500 mμ	3,130
(5) *p*-Nitroaniline		MeOH	525 mμ	2,940
(6) Benzidine		MeOH	NC	NC
(7) Sulfanilic acid		1 *N* HCl	490 mμ	2,380
(8) *o*-Aminobenzenesulfonic acid		1 *N* HCl	NC	NC
(9) Metanilic acid		1 *N* HCl	490 mμ	2,180
(10) 3-Amino-*p*-toluenesulfonic acid		1 *N* HCl	450 mμ	680
(11) 4-Amino-*m*-toluenesulfonic acid		1 *N* HCl	450 mμ	680
(12) 5-Amino-*o*-toluenesulfonic acid		1 *N* HCl	510 mμ	730
(13) 6-Amino-*m*-toluenesulfonic acid		1 *N* HCl	NC	NC
(14) 2-Amino-4-chloro-benzenesulfonic acid		MeOH	NC	NC
(15) 2-Amino-5-nitro-benzenesulfonic acid		MeOH	537 mμ	8,210
(16) 2-Amino-4-nitro-benzoic acid		MeOH	450 mμ	708
(17) 6-Amino-4-chloro-*m*-toluene-sulfonic acid		MeOH	NC	NC
(18) 2-Amino-*p*-benzenedisulfonic acid		1 *N* HCl	475 mμ	1,020

(*Continued on next page.*)

TABLE I (*Continued*)

Compound	Structural Formula	Initial Solvent	Wavelength, Abs. Max.	Apparent Molar Absorptivity[a]
(19) 4-Amino-6-chloro-*m*-benzenedisulfonic acid		MeOH	500 mμ	6,560
(20) Sulfanilamide		MeOH	487 mμ	4,440
(21) 2-Amino-4-chlorobenzenesulfonamide		MeOH	475 mμ	1,630
(22) 4-Amino-*m*-benzenedisulfonamide		MeOH	480 mμ	4,134
(23) 4-Amino-6-chloro-*m*-benzenedisulfonamide		MeOH	500 mμ	8,170
(24) 4-Amino-6-chloro-N^1,N^3-dimethyl-*m*-benzenedisulfonamide		MeOH	NC	NC
(25) 4-Amino-5-chloro-*m*-benzenedisulfonamide		MeOH	470 mμ	1,768
(26) 2-Amino-1-naphthalenesulfonic acid		MeOH	NC	NC
(27) 4-Amino-1-naphthalenesulfonic acid		MeOH	NC	NC

[a] Determined after a coupling reaction time of 6 min. in pH 5 acetate buffer. NC = No color.

only the substituent group, but also its position relative to the amine function affects the intensity, *e.g.*, 7 and 8, 15 and 16, 23 and 25. In general, the most sensitive amines were those possessing electron-withdrawing groups *ortho* and/or *para* to the amine function. The relative effects of various substituents on the intensity of the coupled product are in general agreement with the observations of Zollinger for various azo dyes (5).

The authors recognize that without more kinetic data the interpretation of the above-mentioned effects is limited. The data are consistent with what one could predict based on substituent effects on spectral properties or reaction rates.

Taking this into consideration, it is still felt that the data in Table I will be of value to other workers who may be desirous of utilizing a coupling procedure. The amines tested should react in an analogous manner regardless of the phenolic moiety. With perhaps slight adjustment of experimental conditions to suit particular cases, amines numbered 15, 19, 20, 22, and 23 should be more suitable as diazonium reagents than those numbered 1, 10, 11, 12, 16, 18, and 21.

SUMMARY

1. Twenty-seven aromatic amines have been investigated as potential diazo reagents for the colorimetric determination of phenolic compounds. The criteria for suitability established were speed, sensitivity, and reproducibility.

2. The compound 4-amino-6-chloro-*m*-benzenedisulfonamide was considered the most suitable based on the *criteria* established. Although compound 15 has a greater apparent molar absorptivity, it was not selected as the most suitable since the color produced was not as stable as that produced with compound 23.

3. Amines with electron withdrawing groups

ortho and/or *para* to the amine group possess the greatest potential.

REFERENCES

(1) Urbányi, T., Pagliery, M., and Rehm, C. R., *Anal. Chem.*, **39**, 1656(1967).
(2) Urbányi, T., and Rehm, C. R., *J. Pharm. Sci.*, **55**, 501(1966).
(3) Kuhn, R., and Löw, I., *Berichte Deutschen Chem. Gess.*, **72**, 1453(1939).
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Keyphrases

Reagents—colorimetric analysis
Amines, substituted—diazonium derivatives
4-Amino-6-chloro-*m*-benzene-disulfonamide—estradiol analysis
Apparent molar absorptivity values—diazonium reagents
Colorimetric analysis—spectrophotometry

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By NORMAN SHANE and DENNIS MIELE

A simple, specific potentiometric method is described for the assay of toothpastes for free fluoride ion in concentrations of about 0.1 percent, equivalent to 0.22 percent sodium fluoride or 0.4 percent stannous fluoride. The ionic fluoride content of the toothpastes is determined by relating the potential obtained with a fluoride electrode in an aqueous dispersion of toothpaste to a calibration curve constructed by adding known scaler amounts of fluoride to a blank toothpaste formulation.

THE DETERMINATION of fluoride ion concentration in toothpastes has ordinarily required steam distillation as a separation procedure and titration by thorium nitrate for the fluoride ion using a colored dye as an indicator. The classical Willard-Winter distillation (1), as modified by Grimaldi *et al.* (2), Singer and Armstrong (3), and Wade and Yamamura (4), has been the basis for most analytical procedures. Following distillation, spectrophotometric methods utilizing the blue complex formed between the fluoride ion and the cerium (III) salt of 3-amino-methyl-alizarin-*N*,*N*-acetic acid have been employed by Bellack *et al.* (5). Also, Harley and Revinson (6) and Calkins (7) have used high-frequency titration techniques, and other methods are described in review articles by Fishman *et al.* (8). Frant (9) explained the theory and operation of the fluoride ion electrode. In contrast to the potentiometric method as explained in this paper, Lingane (10) showed a titration method for fluoride ion employing the fluoride electrode.

The specific method for free fluoride ion described in this paper requires no separation steps. Fluoride ion is detected in toothpastes in the range of 0.1% F^- ion equivalent to 0.22% NaF and 0.40% SnF_2. An important consideration in this analysis is that the fluoride must be present as a free ion. The fluoride ion activity electrode model 94–09, developed by Orion Research, Inc., was used as easily as a conventional pH electrode with an Orion digital meter, model 801. A standard glass sleeve calomel reference electrode was employed in conjunction with the fluoride electrode. The electrodes were immersed in an aqueous mixture of toothpaste so as to measure the activity of ionized (free) fluoride in terms of concentration. By use of an appropriate calibration curve, the concentrations of F^- ion from NaF and SnF_2 were determined.

EXPERIMENTAL

Apparatus and Reagents—Fluoride electrode, model 94-09, obtained from Orion Research, Inc., Cambridge, Mass.; Orion Research meter 801; Beckman calomel electrode (with glass sleeve); 50-ml. Nalgene centrifuge tube; 100-ml. volumetric flask; 150-ml. beaker; Sargent magnetic stirrer.

Stirrer—Made from an electric toothbrush with a special made paddle. This paddle is a 10.1-cm. aluminum shaft and has a 3.2 mm. diameter. Two fins, each 2.5 cm. long and 1.9 cm. wide, are located at the stirring end.

NaF Stock Solution for Calibration Curve—180 mg. NaF/100 ml. aqueous solution (0.18% NaF),[1] 200 mg. NaF/100 ml. aqueous solution (0.20% NaF), 220 mg. NaF/100 ml. aqueous solution (0.22% NaF), 240 mg. NaF/100 ml. aqueous solution (0.24% NaF), 260 mg. NaF/100 ml. aqueous solution (0.26% NaF).

SnF_2 Stock Solution for Calibration Curve—To each solution is added 400 mg. of malic acid as a stabilizer. Deoxygenated water is used so as to further prevent oxidation of the stannous ion. 320 mg. SnF_2/100 ml. aqueous solution (0.32% SnF_2),[2] 360 mg. SnF_2/100 ml. aqueous solution (0.36% SnF_2), 400 mg. SnF_2/100 ml. aqueous solution (0.40% SnF_2), 440 mg. SnF_2/100 ml. aqueous solution (0.44% SnF_2), 480 mg. SnF_2/100 ml. aqueous solution (0.48% SnF_2).

PROCEDURE

For NaF and SnF_2 Calibration—Weigh approxi-

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mately five 1-g. samples of the paste[3] without NaF or SnF_2 into five 50-ml. centrifuge tubes. Add 45 ml. of water to each tube. For the SnF_2 calibration, deoxygenated water is required. For the NaF experiment 1 ml. of 0.18%, 0.20%, 0.22%, 0.24%, and 0.26% stock solutions of NaF are added to the five tubes, respectively. For the SnF_2 experiment, 1 ml. of 0.32%, 0.36%, 0.40%, 0.44%, and 0.48% stock solution of SnF_2 is added. Stir each tube vigorously for 1.5 min. with the stirrer to disperse the paste in the solution. Pour this suspended solution into a 100-ml. volumetric flask and dilute to volume with water. In the case of SnF_2 deoxygenated water must be used. Transfer the solution to a 150-ml. beaker containing a magnetic stirring rod and stir to ensure homogeneity. Immerse the fluoride and calomel electrodes into the beaker and record the millivolt reading obtained from the research meter. Temperature control of the meter should be set to ambient conditions. A working curve is obtained by plotting the mv. reading against % NaF or % SnF_2 on one-cycle semilogarithmic graph paper. A calibration curve should be prepared at least once a day. With the Orion meter, the original calibration curve can be shown on the mv. scale. If the reading on a standard solution changes by a few mv., and experience shows that the slope does not change, readings can then be taken on the REL mv. scale which is adjusted so that the standard still reads the same, and the same curve can be used. The same standard solutions can be used for the calibration curves.

For NaF Samples—Laboratory samples are run in the same manner as explained for the calibration curve. It should be noted that the fluoride electrode should be conditioned to the fluoride concentration that is to be determined. For NaF pastes, the mv. reading will become steady within 1 min.

For SnF_2 Samples—Laboratory samples are analyzed in the same manner as explained for the calibration curve. It takes 4–5 min. to condition the electrode to SnF_2 so as to obtain steady readings. After a long period of time (about 10 min.) the millivolt reading will start to increase, indicating a reduction of the free fluoride ions due to complexation. It is imperative to read the mv. reading at the steady-state condition.

RESULTS

The data obtained for the calibration curves of NaF and SnF_2 pastes are shown in Tables I and II. The results are the average composite of five different experiments; each experiment was done on 5 different days. The toothpastes without the NaF and SnF_2 were used to give the proper background for constant ionic strength conditions. NaF or SnF_2 were then added to the toothpastes and millivolt readings were taken. During a given day, the experiment was repeated and the readings were reproduced. The chart indicates linearity with respect to the semilog plot of mv. *versus* % NaF or SnF_2 for an average composite of five experiments in both cases (Fig. 1). However, because the method depends on direct measurements, the variability of the electrode characteristics, such as change of junction potential, may occur. For this reason, it is advisable to calibrate the meter once every day.

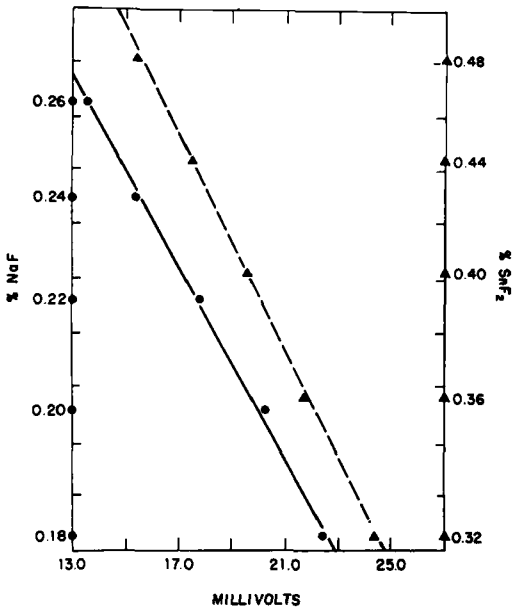

Fig. 1—Semilog plot of the percentage of NaF or SnF_2 versus mv. readings for an average composite of five experiments. Key: ———, NaF; – – –, SnF_2.

Laboratory samples of the cited toothpastes, including NaF or SnF_2 are shown in Tables I and II, which were run by the standard distillation method of Willard-Winter (1) with thorium nitrate titration using chrome azurol S as the indicator and by the fluoride electrode method. The distillation method for total fluoride, including the titration which, in the authors' opinion, gives a poor end point, takes about 40–45 min. The fluoride electrode method can be completed in 5–9 min. Since the chart is based on the % NaF or % SnF_2 for 1 g. of paste, the calculations for the samples are obtained by the graph reading of the respective percentages divided by the weight of the samples.

The statistical data of the corresponding tests, as shown in Tables I and II, indicate that there is no significant difference between the two methods.

DISCUSSION

The fluoride ion electrode is used to measure the fluoride activity regardless of sample ionic composition or total ionic strength. As described by Kielland (15), the relationship of activity to concentration of the ionized (free) fluoride is obtainable by an appropriate calibration curve. The electrode does not respond to fluorides which are bound or complexed. Since these pastes contain no calcium or other ions capable of removing fluoride, the total fluoride is equivalent to the free fluoride ion level.

A concentration working curve is prepared by plotting potential measurements of known NaF or SnF_2 concentration standards on semilogarithmic graph paper. Since the electrode responds to the activity and not to the concentration of the fluoride ion, the working curve is prepared for fluoride solutions which contain other ions, if the standards approximate the unknowns in composition. The

[3] For the NaF paste the formulation is similar to that given in *References 11* and *12* as formulation No. 3, shown to be clinically active as anticaries dentifrice (11–13). For the SnF_2 paste the formulation is that given in *References 11* and *12* as formulation No. 4, accepted as clinically active anticaries dentifrice (11, 12, 14).

TABLE I—SODIUM FLUORIDE[a]

% NaF	mv.
0.18	22.5
0.20	20.2
0.22	17.8
0.24	15.4
0.26	13.6

Samples Analyzed[b]

Sample	Wt. of Sample, g.	mv.	Distillation	Fluoride Electrode
A	1.0144	18.3	0.21	0.21
B	1.0331	16.6	0.22	0.22
C	1.0223	17.1	0.23	0.22
D	1.0541	17.7	0.20	0.21
E	1.1621	13.9	0.23	0.22
F	1.0132	20.1	0.19	0.19
G	0.9997	19.5	0.21	0.20
H	1.1356	16.5	0.20	0.20
I	1.1512	17.2	0.19	0.19
J	1.2141	14.0	0.21	0.21
A	0.9987	19.4	0.21	0.21
B	1.0122	18.8	0.22	0.21
C	1.0141	19.4	0.23	0.20
D	0.0185	17.7	0.20	0.21
E	1.1913	14.5	0.23	0.21
F	1.1382	17.0	0.19	0.20
G	1.0415	19.3	0.21	0.20
H	1.1626	17.0	0.20	0.19
I	1.1718	18.6	0.19	0.18
J	1.0859	15.9	0.21	0.22
K	1.2613	16.0	0.19	0.19
L	1.0172	18.4	0.20	0.21
M	1.0185	20.3	0.18	0.20
N	0.9817	21.3	0.19	0.19
O	1.0831	17.8	0.19	0.20

[a] Calibration based on average composite of five experiments for 1-g. sample of paste. [b] Calculation for samples analyzed: graph reading/wt. of sample = % NaF; $\bar{x}_1$ = 0.2052; $\bar{x}_2$ = 0.2036; $\bar{a}$ = 0.0016; SD = 0.0020; t test = 0.8000 for 25 samples.

TABLE II—STANNOUS FLUORIDE[a]

% SnF_2	mv.
0.32	23.8
0.36	21.8
0.40	19.7
0.44	17.6
0.48	15.5

Samples Analyzed[b]

Sample	Wt. of Sample, g.	mv.	Distillation	Fluoride Electrode
A	1.0142	19.6	0.36	0.37
B	0.9998	20.6	0.37	0.37
C	1.0761	19.6	0.36	0.36
D	1.0150	19.4	0.37	0.39
E	1.1012	17.3	0.39	0.40
A	1.1112	19.5	0.36	0.35
B	1.0815	20.0	0.37	0.35
C	1.0514	19.4	0.36	0.37
D	1.0723	19.1	0.37	0.37
E	1.0615	17.0	0.39	0.41
F	1.0431	18.6	0.40	0.40
G	0.9971	17.6	0.40	0.42
H	1.1341	19.2	0.35	0.36
I	1.1562	19.8	0.36	0.34
J	1.1314	18.1	0.40	0.38
F	1.0516	17.9	0.40	0.40
G	1.0723	17.1	0.40	0.41
H	1.1512	18.1	0.35	0.36
I	1.1823	17.6	0.36	0.36
J	1.2114	16.1	0.40	0.38
K	1.2176	17.2	0.37	0.37
L	1.1152	17.7	0.38	0.39
M	1.1921	18.6	0.37	0.35
N	1.1891	17.2	0.40	0.38
O	1.0117	20.8	0.39	0.38

[a] Calibration based on average composite of five experiments for 1-g. sample of paste. [b] Calculation for samples analyzed: graph reading/Wt. of sample = % SnF_2; $\bar{x}_1$ = 0.3772; $\bar{x}_2$ = 0.3768; $\bar{a}$ = 0.0004; SD = 0.0027; t test = 0.1512 for 25 samples.

activity depends on the total ionic strength of the sample. Errors will result if the standards are prepared with pure NaF or SnF_2, and unknowns containing relatively high concentrations of other ions. The calibration curves for the determination of NaF or SnF_2 were based on adding known amounts of the given NaF or SnF_2 to nonfluoride paste in order to fulfill the ionic strength requirement for the calibration curve. The activity of the working curve is a straight line within the working range.

The mathematical relationship of voltage to activity is shown by the Nernst equation:

$$E = E_s + \frac{2.3RT}{F} \log F^-$$

where E = measured potential, E_s = constant due to the system calibrated, 2.3 RT/F = Nernst factor, 59.2 mv. at 25°, R and F are constants, T = temperature in °K., F^- = fluoride ion activity in the sample solution.

The Orion meter is readable to ±0.1 mv. Since the voltage is proportional to the log of the fluoride activity by the Nernst equation, the instrument is sensitive to about 0.5 mv. change per 0.01% NaF and 1 mv. per 0.01% SnF_2 in the paste in the region of the analysis.

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Keyphrases

Toothpaste—fluoride ion analysis
Potentiometric analysis
Fluoride electrode—ion determination

New Compounds: Nucleosides Derived from D-*Glycero*-D-*gulo*-heptose

By LEON M. LERNER

The preparation of new nucleosides derived from D-*glycero*-D-*gulo*-heptose are described. These nucleosides are distinguished from the naturally occurring nucleosides by their seven carbon chain and pyranose ring. The configurations and anomeric purity of these compounds are unknown.

A NUMBER of nucleosides have been prepared in the last few years, in which the sugar moiety has been altered from that which normally occurs in nature. The reason for this interest was stimulated by the discovery of antibiotics which were identified as nucleosidic substances possessing an altered structure in the carbohydrate. Nucleoside analogs have since been prepared with the intention of obtaining useful agents which would possess antitumor or antimicrobial activity.

The first synthesis of a nucleoside derived from a heptose was described by Kohn *et al.* (1), who prepared 9-β-D-*glycero*-D-*gulo*-heptofuranosyladenine. This compound, as well as several nucleosides derived from hexopyranoses (2), was found to be inhibitory to the multiplication of *Herpes simplex* virus.[1] A natural outcome of these observations was the preparation of some nucleosides derived from a heptopyranose, which is the subject of this communication.

The acetic anhydride–acetic acid reagent described by Montgomery and Hudson (3) was used to acetylate D-*glycero*-D-*gulo*-heptopyranose. This reagent has been used to isomerize sugar acetates to the α anomer. A preponderance of the α hexaacetate was crystallized, but the expected high yield of α anomer did not materialize. Instead, a mixture of α and β anomers was obtained which was separated by fractional crystallization. Such an outcome has been noted previously in the case of D-allose (2), where the β form predominated.

Either anomer could be used in the preparation of the bromide in a hydrogen bromide–acetic acid mixture. Coupling of the bromide with 6-benzamidochloromercuripurine was achieved in refluxing xylene in the usual manner (4). The blocking groups were removed in hot methanolic sodium methoxide and the product, 9-D-*glycero*-D-*gulo*-heptopyranosyladenine, was purified *via* its picrate salt (5). Unfortunately, this procedure resulted in a significant loss of the nucleoside on the resin. When 6-benzamidochloromercuripurine and the hexaacetate were condensed in the presence of titanium tetrachloride, the yield was only about 10% of that obtained by coupling with the bromide. This was in contrast to the results of other workers (6, 7), who have claimed that the yields are increased, often considerably, over that obtained by prior formation of the halogenose. However, Tong *et al.* (8) have had a similar experience to that reported in this paper during their attempt to couple 1,2-di-*O*-acetyl-5-*O*-benzoyl-3-*O*-methyl-D-ribofuranose to a purine.

1-D-*Glycero*-D-*gulo*-heptopyranosyluracil was obtained by the coupling of the bromide with bis(trimethylsilyl)uracil in benzene at room temperature. The intermediate acetate was purified by chromatography on silicic acid and the blocking groups were removed with sodium methoxide. The nucleoside was obtained as a hard, very hygroscopic glass, which migrated as a homogeneous spot in four solvent systems on TLC plates, but did not give a proper analysis. Benzoylation of this substance yielded the pentabenzoate, which served the purpose of being a derivative of the desired product and offered an alternative purification route. However, removal of the benzoate groups yielded a product whose elementary analysis was not much improved over that obtained previously. Application of column and preparative TLC gave further evidence of one component, but the analyses were still relatively poor. The identity of this nucleoside is based upon its ultraviolet spectrum and the preparation of the crystalline benzoate derivative.

The anomeric configuration of 9-D-*glycero*-D-*gulo*-heptopyranosyladenine has been tentatively decided upon as β by reference to the *trans* rule (9). This rule states that a heavy metal salt of a purine or pyrimidine will substitute at the anomeric carbon *trans* to the acyloxy group at C-2 of the sugar. However, the rule is not applicable to the coupling of the halogenose to bis(trimethylsilyl)uracil. Application of nuclear magnetic resonance spectroscopy[2] at 60 Mc. failed to resolve the anomeric configuration of the pentabenzoate derivative of 1-D-*glycero*-D-*gulo*-heptopyranosyluracil.

EXPERIMENTAL

D-*Glycero*-D-*gulo*-heptopyranose was prepared from D-*glycero*-D-*gulo*-heptono-1,4-lactone as described by Wolfrom and Thompson (10). Elementary analyses were performed by the Spang Microanalytical Laboratories, Ann Arbor, Michigan. Thin-layer chromatography (TLC) was performed on Silica Gel HF plates (E. Merck, AG, Darmstadt). Spots were located with an ultraviolet lamp and the plates were routinely exposed to the chromic acid charring method to test for homogeneity. Evaporations were done *in vacuo* at a bath temperature of 40–45°.

Hexa - O - acetyl - D - glycero - D - gulo - heptopyranose—The acetylation of D-*glycero*-D-*gulo*-heptopyranose was carried out directly with the acetylation reagent as described earlier (2, 3). After a reaction time of 5 days followed by the usual workup (2, 3) the α anomer was crystallized from a large

Received March 1, 1968, from the Department of Biochemistry, State University of New York, Downstate Medical Center, Brooklyn, NY 11203

Accepted for publication March 28, 1968.

Supported in part by grant No. T-442 from the American Cancer Society.

[1] Paul Kohn, personal communication.

[2] Obtained by Dr. Harry Agahigian of the Baron Consulting Co.

volume of ethyl ether in an open flask, m.p.[3] 155–158°. Recrystallization in the same manner gave the α anomer in 55% yield, m.p. 164–165°, $[\alpha]_D^{25}$° + 85.5° (c 3.10, $CHCl_3$). Lit. (11) m.p. 164°, $[\alpha]_D^{20}$° + 87°.

The mother liquors from these crystallizations were combined and the solvent was removed *in vacuo*. The β anomer was crystallized from 50% aqueous ethanol in an open beaker. The long needles had m.p. 128–130°. Recrystallization in the same manner gave the pure product in a 20% yield, m.p. 133.5–134°, $[\alpha]_D^{22}$° +3.5° (c 6.03, $CHCl_3$). Lit. (11) m.p. 135°, $[\alpha]_D^{20}$° +4.8°.

When a shorter reaction time was used the product distribution was nearly one α to one β.

9-D-Glycero-D-gulo-heptopyranosyladenine—To 25 ml. of a saturated solution of hydrogen bromide in acetic acid was added 2.0 Gm. of hexa-*O*-acetyl-D-*glycero*-D-*gulo*-heptopyranose. After 2 hr. at room temperature the solvent was evaporated *in vacuo* at a bath temperature below 30°. Dry toluene was added and removed 5 times in a similar manner. The orange syrup which remained was dissolved in 25 ml. of dry xylene and added to a well-stirred refluxing mixture containing 6-benzamidochloromercuripurine (2.03 Gm.), diatomaceous earth[4] (1.95 Gm.), cadmium carbonate (1.03 Gm.), and 150 ml. of xylene (4). After the usual workup (4), an orange glass (2.6 Gm.) was obtained by evaporation of absolute alcohol. This material was dissolved in a mixture of sodium methoxide (350 mg.) in 60 ml. of methanol and the solution was refluxed for 1.25 hr. The methanol was removed by evaporation and the residue was dissolved in 35 ml. of water and neutralization was effected with acetic acid. The aqueous solution was washed with 35 ml. of chloroform, treated with activated charcoal (Darco G-60), and concentrated to a light orange syrup. Since the product could not be induced to crystallize at this stage, the picrate was prepared. The syrup was dissolved in 15 ml. of methanol and 25 ml. of 10% methanolic picric acid was added. The flask was chilled in an ice bath for 2 hr., the yellow precipitate was removed by filtration, and it was washed well with cold methanol and cold water. The picrate (1.11 Gm.) started to decompose at 166° and melted at 170–173°.

The picrate was converted to the free nucleoside with the carbonate form of an ion-exchange resin as described previously (2, 5). Crystallization occurred slowly from water in an open flask after 3 days of standing at room temperature. The flask was chilled for several more days in a refrigerator and 142 mg. of white crystals were collected. This material decomposed slowly starting at about 220° and melted completely with decomposition at 273–275°, $[\alpha]_D^{29}$° −7° (c 0.86, 1 *N* HCl); $\lambda_{max.}^{pH\ 1}$ 256 mμ(ϵ 13,800), $\lambda_{max.}^{H_2O}$ 258 mμ(ϵ 14,000), $\lambda_{max.}^{pH13}$ 258 mμ(ϵ 14,200). This substance was homogeneous upon chromatography on Whatman No. 1 paper with 5% aqueous disodium phosphate as the developing solvent, R_{Ad} 1.91 (R_{Ad} of adenine = 1.00).

Anal.—Calcd. for $C_{12}H_{17}N_5O_8$: C, 44.03; H, 5.25; N, 21.40. Found: C, 44.00; H, 5.25; N, 21.45.

1-D-Glycero-D-gulo-heptopyranosyluracil—To 30 ml. of hexamethyldisilazane and 0.6 ml. of trimethylchlorosilane was added 2.3 Gm. of uracil and the stirred mixture was refluxed at an oil bath temperature of 170° for 3 hr., protected from moisture (12). The clear solution was cooled to below 50° and the solvent was distilled under high vacuum at a bath temperature of 50–60°, leaving a slightly turbid syrup. This syrup was dissolved in 100 ml. of dry benzene and added to a solution containing the bromide (prepared from 4.1 Gm. of hexa-*O*-acetyl-D-*glycero*-D-*gulo*-heptopyranose) in 250 ml. of benzene. Mercuric acetate (2.8 Gm.) was added and the mixture was stirred for 5 days, protected from moisture. The solids were removed by filtration, washed with 100 ml. of chloroform, and the solvents were evaporated. The residue was triturated with 150 ml. of chloroform, the insoluble material was removed by filtration, and the filtrate was washed with 30% aqueous potassium iodide, water, and dried over magnesium sulfate. Removal of the chloroform under vacuum gave a syrup weighing 5.6 Gm., $\lambda_{max.}^{EtOH}$ 255 mμ. The syrup was dissolved in a minimum amount of chloroform and applied to the top of a column of silicic acid (100 Gm., Mallinckrodt 100 mesh). The column was eluted with chloroform (750 ml.), chloroform–ethyl acetate (4:1, 500 ml.), and chloroform–ethyl acetate (1:1, 500 ml.). Various sugar derivatives were eluted with all of these solvent mixtures, but no ultraviolet absorbing material. Upon elution with ethyl acetate (500 ml.), 1.0 Gm. of the nucleoside acetate was obtained, $\lambda_{max.}^{EtOH}$ 255 mμ; $\lambda_{max.}^{smear}$ (cm.$^{-1}$) 1750 (acetate ester), 1700 (—NHCO— of pyrimidinone), 1630 (C=C of pyrimidine ring), 1230 (acetate C—O—C), 1070, 1050 (sugar C—O—). This intermediate pentaacetate could not be induced to crystallize and so the acetyl groups were removed by refluxing for 1 hr. in 50 ml. of 0.1 *N* methanolic sodium methoxide. An ion-exchange resin[5] was added until the pH was 6.5. The resin was removed by filtration and the solvent was evaporated. The syrupy material was partitioned between water and chloroform and the aqueous layer was concentrated to dryness. The clear, colorless syrup which remained would not crystallize, but it did form a hard glass upon repeated evaporation with ethanol. The glass was pulverized, yielding 225 mg. of product, which was homogeneous upon TLC in the following four developing systems: 1-butanol–water (86:14 v/v), 5% aqueous disodium phosphate, 1-butanol–acetic acid–water (5:2:3 v/v/v), and 2-propanol–25% aqueous ammonia–water (7:1:2 v/v/v). This substance had $[\alpha]_D^{25}$° −16° (c 0.51, water) and was extremely hygroscopic; ultraviolet spectrum at pH 1: maximum at 258 mμ (ϵ 7430), minimum at 230 mμ(ϵ 1930); at pH 5–7: maximum at 257 mμ(ϵ 7460), minimum at 229 mμ(ϵ 1870); at pH 13: maximum at 258 mμ(ϵ 5400), minimum at 244 mμ(ϵ 4360).

Anal.—Calcd. for $C_{11}H_{16}N_2O_8$: C, 43.42; H, 5.30; N, 9.21. Found: C, 40.74; H, 5.58; N, 7.22.

Since the pentabenzoate was also prepared (see below) as a pure derivative an attempt was made to obtain the nucleoside from this derivative. The benzoate groups were removed as described above for the acetate and the product was worked up in the usual way. The nucleoside was precipitated from methanol by addition of ethyl ether and the

[3] Melting points were obtained on a Fisher-Johns apparatus and are corrected.

[4] Celite 545, Johns-Manville, New York, N. Y.

[5] Dowex 50-(H$^+$), Dow Chemical Co., Midland, Mich.

white solid obtained was dried at 100° *in vacuo* over P_2O_5.

Anal.—Calcd. as for above. Found: C, 42.08; H, 5.59; N, 7.00.

1 - (Penta-O-benzoyl - D - glycero - D - gulo-heptopyranosyl)uracil—To 19 ml. of dry pyridine was added 88.6 mg. of 1-D-*glycero*-D-*gulo*-heptopyranosyluracil and the mixture was stirred in an ice bath. Benzoyl chloride (2 ml.) was added dropwise and after 0.5 hr. at 0° the mixture was stored at room temperature for 21 hr. It was then heated on a steam bath for 20 min., cooled, 50 ml. of benzene was added, followed shortly thereafter by 50 ml. of water. The mixture was stirred for several minutes and the layers were separated. The benzene solution was washed three times with 50-ml. portions of 2 *N* sulfuric acid solution, once with 50 ml. of water, 4 times with 50-ml. portions of saturated sodium bicarbonate solution, and finally with 50 ml. of water. The dried ($MgSO_4$) solution was evaporated to a thin, orange oil. This was triturated with hot petroleum ether and cooled in an ice bath for 1 hr. The petroleum ether was decanted and the procedure was repeated. A hard syrup remained which was dissolved in warm methanol and placed in a freezer. A solid was filtered off in two crops (203 mg.). Recrystallization from chloroform–ethanol in an open flask at room temperature yielded beautiful, clear, star-like clusters of large rods, 117 mg. in two crops; m.p. 123–124.5° (to a very viscous liquid) with prior softening at 119°. $[\alpha]_D^{28}$° −55.9° (c 2.81, $CHCl_3$). λ_{max}^{film} (cm.$^{-1}$) 1730 (benzoate ester), 1680 (—NHCO— of pyrimidinone), 1600 (C=C of phenyl and pyrimidine ring), 1260 (benzoate C—O—C), 1100, 1090, 1065 (sugar C—O—), 706 (monosubstituted phenyl). The product was homogeneous on TLC. The R_f was 0.84 using chloroform–methanol (9:1) as the irrigating solvent.

Anal.—Calcd. for $C_{46}H_{36}N_2O_8$: C, 66.99; H, 4.40; N, 3.40. Found: C, 67.02; H, 4.48; N, 3.37.

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Keyphrases

D-*Glycero*-D-*gulo*-heptose derivatives—synthesis
Nucleosides—D-*glycero*-D-*gulo* heptose derivatives
TLC—separation
Column chromatography—separation
UV spectrophotometry—identity
Optical rotation—identity

New Compounds: Synthesis of Heterocyclic Estrogens

By LARRY GORUM* and W. LEWIS NOBLES

Several nitrogen analogs of diethylstilbestrol have been prepared by a convenient route from pyridyl lithium and the appropriate ketone. These compounds are expected to have estrogenic activity with possible greater selectivity of action than previous known synthetic estrogens.

THE INVESTIGATIONS of Dodds and co-workers (1) on synthetic compounds possessing the physiological action of the female sex hormone estrone reached a climax with the discovery of diethylstilbestrol (1). Since that time much work with molecular modification has been done in an effort to improve the selectivity of pharmacological action in the therapeutic uses of diethylstilbestrol. Endocrine therapy in metastatic prostatic cancer consists initially of estrogen therapy as first demonstrated by the pioneering investigation of Huggins (2). Oral estrogen preparations such as diethylstilbestrol are effective to a limited extent but elicit side effects such as nausea, gynecomastea, and edema. Because of these undesirable side effects it is necessary to find new compounds which possess a more selective estrogenic action.

Many compounds of this type have been prepared and tested and are described in a review by Grundy (3). However, thus far none of the compounds tested offered sufficient selective antineoplastic activity to replace diethylstilbestrol in the clinic. In the hundreds of compounds reported, several contain

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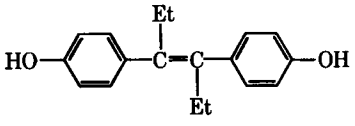
heterocyclic atoms but none in an aromatic arrangement such as pyridine which might incorporate greater antineoplastic specificity in an analog of diethylstilbestrol such as 3-(2-pyridyl)-4-(*p*-hydroxyphenyl)-hexane (II), and 3-(2-pyridyl)-4-(*p*-methoxyphenyl)-3-hexene (III). This report describes the synthesis of II and III by a convenient route.



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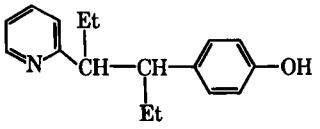

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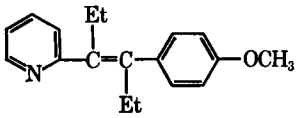


III

DISCUSSION

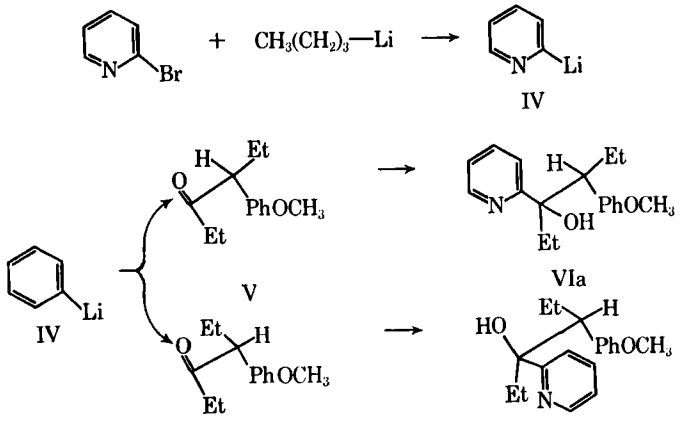
This preparation involved a synthetic route employing 2-pyridyl lithium (IV) and 3-(*p*-anisyl)-4-hexanone (V) in a Grignard-type reaction. A racemic mixture of the resultant alcohol VI*a* and VI*b* was predicted by invoking Cram's rule of asymmetric synthesis (4). This rule states that the asymmetric carbon in the starting ketone is so oriented that the carbonyl function is flanked by the two smaller groups and subsequent attack by the Grignard reagent on the carbonyl carbon occurs over the smallest group.

Unexpected difficulty with the dehydration of the resultant alcohol (VI), necessitated a dehydrohalogenation approach to the olefin (VII). This was accomplished by chlorination with thionyl chloride with subsequent elimination of hydrogen chloride with potassium hydroxide to afford 3-(2-pyridyl)-4-(*p*-methoxyphenyl)-3-hexene (III). Assurance that the reaction afforded the *trans* olefin has strong support from studies by Cram and Greene (5). Reduction of the olefin using 15% palladium on carbon resulted in a good yield of the desired 3-(2-pyridyl)-4-(*p*-methoxyphenyl)-hexane (VIII). Extensive work on similar systems by Wessely (6) demonstrated that a *d, l* pair of *threo* form enantiomers represented by structure VIII resulted from *cis* addition of hydrogen to the olefin (III).

Demethylation of VIII with a mixture of hypophosphoric acid and hydrobromic acid resulted in 3-(2-pyridyl)-4-(*p*-hydroxyphenyl)-hexane(IX). The phenol (IX) was a very hygroscopic solid foam and was converted to α, β-diethyl-4'-benzoyloxydihydro-2-stilbazole hydrochloride (X), which was a white powder suitable for instrumentation studies for structure analysis.

EXPERIMENTAL[1]

3-(2-Pyridyl)-4-(p-methoxyphenyl)-3-hexanol (VI)—A modification of the procedure of Woodward (7) was followed. To a solution of 30 ml. (0.1 mole) of *n*-butyl lithium in 100 ml. of dry ether in a nitrogen atmosphere and dry ice acetone bath was added dropwise a dry ethereal solution of 20 Gm. (0.12 mole) of 2-bromopyridine. After the addition was complete, the mixture was allowed to stir 30 min. at −40° and 10 Gm. (0.1 mole) of 3-(*p*-anisyl)-4-hexanone was added dropwise over a 15-min. period. The reaction mixture developed a dark rust color and was stirred for an additional hr. at −40°. Stirring was continued until the reaction mixture attained room temperature. The dark mixture was then allowed to sit overnight followed by filtration and washing with three 100-ml. portions of water. The early filtration facilitated work-up of the reaction by removing much of the dark reaction



IV

V

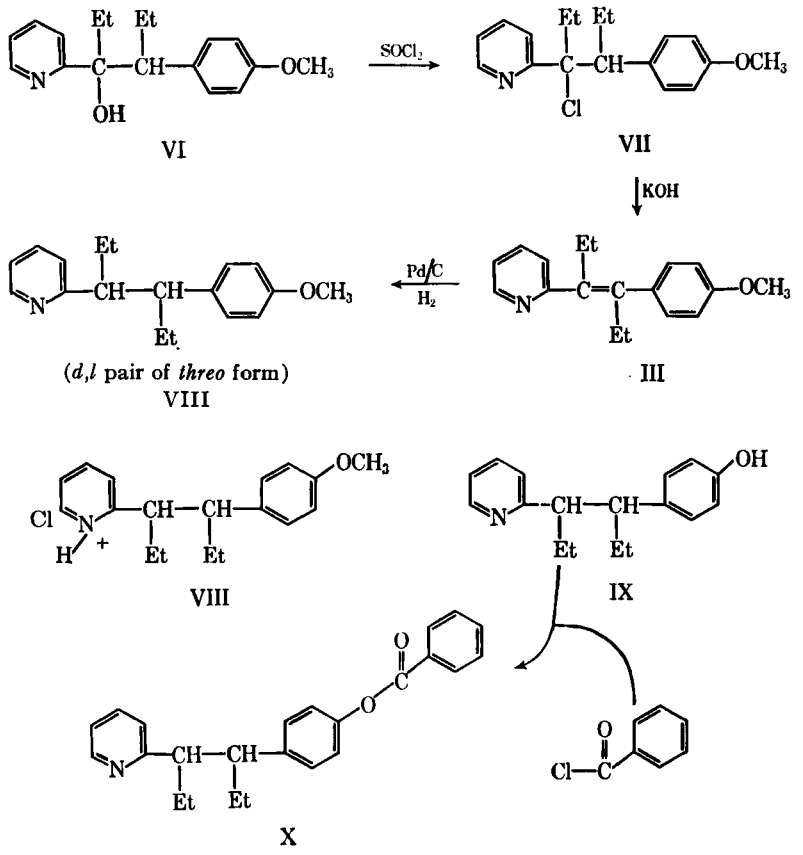
Racemic mixture of starting ketone

VIa

VIb

Racemic mixture of resultant alcohol $PhOCH_3$-*p*-methoxyphenyl

[1] All melting points were taken on a Thomas-Hoover Uni-melt apparatus and are corrected. The NMR spectra were taken using a Varian model A60-A instrument. The elemental analyses were determined by Dr. Alfred Bernhardt, 433 Mulheim (Ruhr), West Germany and Dr. Paul N. Craig, Smith Kline & French Laboratories, Philadelphia, Pennsylvania. The NMR spectra were measured at 60 Mc., and the chemical shifts are reported as τ-values (p.p.m.) from tetramethylsilane (internal standard). The multiplicity is shown by *s* = singlet, *d* = doublet, *t* = triplet, *q* = quartet, and *m* = incompletely resolved multiplet.



by-products. The ether phase was then evaporated *in vacuo* to leave a dark residue which was refluxed in water until crystallization occurred. The water was then decanted off and the solid recrystallized three times from ethanol to yield 7.1 Gm. (40%) of the desired alcohol m.p. 94–95°. The hydrochloride of this compound was prepared by dissolving the free base in anhydrous ether and bubbling dry HCl gas through the solution until precipitation was complete. The precipitate, collected by filtration, had a m.p. of 204–205°.

The infrared spectrum of the free base had hydroxyl group absorption at 3500 cm.$^{-1}$.

Anal.—Calcd. for $C_{18}H_{23}NO_2$: C, 75.76; H, 8.12; N, 4.91. Found: C, 75.92; H, 8.32; N, 5.08.

The NMR spectrum in carbon tetrachloride was δ = 4.79 (*s*, indicated to be OH, the peak disappeared on D_2O exchange), 0.5 (*q*, C—CH_3, 6H), 1.4 (*t*, C—CH_2, 3H), 7.00 (*q*, 2,3,5, and 6H of phenyl ring, 4H), 8.66 (*d*, 3, 4, 5, and 6H of pyridyl ring, 4H).

3 - (2 - Pyridyl) - 4 - (p - methoxyphenyl) - 3-hexene Hydrochloride (III)—Modifications of procedures by Hall (8) and Smith (9) were followed. To 1.0 Gm. (3.5 mmoles) of 3-(2-pyridyl)-4-(*p*-methoxyphenyl)-3-hexanol in a three-necked flask equipped with a stirrer, reflux condenser with calcium chloride tube, and dropping funnel in an ice bath was added dropwise 10 ml. of thionyl chloride. After the addition was complete, the solution was allowed to reach room temperature gradually and then heated to a gentle reflux for an additional hr. The cooled solution was then added slowly, under the hood, to 100 ml. of ethanol cooled in an ice bath in order to decompose the unreacted thionyl chloride. This solution was then evaporated to dryness *in vacuo* to leave a light red oil that was then dissolved in 25 ml. of ethanol. This solution was added dropwise to a cooled 25-ml. solution of 15% KOH in ethanol while the temperature was maintained at less than 50°. After addition was complete, stirring was continued for 4 hr. and the solution was allowed to stand overnight. Addition of water and evaporation *in vacuo* resulted in a light yellow oil which was extracted with three 50-ml. portions of ether. The ether extracts were dried over anhydrous magnesium sulfate and dry HCl gas bubbled through the solution to result in precipitation of a light oil. The oil was dissolved in hot ethyl acetate and refluxed until the oil crystallized into a light yellow solid. After recrystallization twice with ethyl acetate, 0.5 Gm. (42%) of the hydrochloride of the desired olefin resulted as a white powder, m.p. 146–147°. The product gave a positive $Br_2/CHCl_3$ test for an olefin.

Anal.—Calcd. for $C_{18}H_{23}ClNO$: C, 70.93; H, 7.60; Cl, 11.63; N,4.59. Found: C, 70.77; H, 7.40; Cl, 11.77; N, 4.34.

The NMR spectrum in deuterated chloroform was δ = 0.83 (*m*, C—CH_3, 6H), 1.65 (*q*,—CH_2—, 4H), 3.65 (*s*, O—CH_3, 3H), 6.00 (*s*, H of hydrochloride, 1H), 6.75 (*m*, 2,3,5, and 6H of phenyl ring, 4H), 8.81 (*m*, 3,4,5, and 6H of pyridyl ring, 4H).

3 - (2 - Pyridyl) - 4 - (p-methoxyphenyl) - hexane Hydrochloride (VIII)—A modification of the procedure by Horning (10) was followed. To 4.0 Gm. (0.014 mole) of 3-(2-pyridyl)-4-(*p*-methoxyphenyl)-3-hexene hydrochloride in 50 ml. of ethanol was

added 0.2 Gm. of 10% palladium on carbon. This mixture was then allowed to shake on a Parr hydrogenation apparatus under 45 p.s.i. of hydrogen for 48 hr. and then gravity filtered. The solution was then evaporated to dryness *in vacuo* and the residue was taken up in anhydrous ether. The ether solution was dried over anhydrous magnesium sulfate and then saturated with dry HCl gas which yielded a light yellow oil. After prolonged refluxing with ethyl acetate, the yellow oil crystallized into a light yellow solid which was collected by filtration and recrystallized twice from ethyl acetate to result in 2.59 Gm. (57%) of the desired compound, m.p. 154–155°. This product gave a negative $Br_2/CHCl_3$ test for an olefin.

Anal.—Calcd. for $C_{18}H_{24}ClNO$: C, 70.69; H, 7.91; Cl, 11.59; N, 4.59. Found: C, 71.69; H, 7.33; Cl, 11.63; N, 4.34.

The NMR spectrum in deuterated chloroform was δ = 0.85 (*m*, —C—CH_3, 6H), 1.71 (*d*, —CH_2—, 4H), 3.71 (*s*, O—CH_3, 3H), 6.00 (*m*, —CH—, 2H), 6.25 (*s*, H of hydrochloride, 1H), 7.00 (*m*, 2,3,5, and 6H of phenyl ring, 4H), 8.81 (*m*, 3,4,5, and 6H of pyridyl ring, 4H).

α,β - Diethyl - 4′ - benzoyloxydihydro - 2 - stilbazole Hydrochloride (X)—A modification of the demethylation procedure reported by Franck and Schlinghoff (11) was followed. While maintaining an atmosphere of nitrogen, 20 ml. of a mixture of hydrobromic acid and hypophosphorous acid (prepared from 0.5 Gm. of 50% hypophosphorous acid and 50 Gm. of 48.5% hydrobromic acid) was added to 1.0 Gm. of the 3-(2-pyridyl)-4-(*p*-methoxyphenyl)-hexane hydrochloride. The mixture was heated on an oil bath at 125–130° for 8 hr. and then gradually cooled to 0°. To the cold solution was added 15 ml. of ammonium hydroxide which resulted in separation of a light green oil. The oil was extracted with two 25-ml. portions of ether and the ether extracts dried over magnesium sulfate and evaporated to dryness *in vacuo* to yield a clear solid form unsuitable for purification for analysis. Preparation of the hydrochloride resulted in a similar problem. The free base was then dissolved in 15 ml. of benzoyl chloride and allowed to reflux for 2 hr. The solution was then poured into water and made basic with 10% sodium carbonate and stirred for 2 hr. before extracting the solution with three 50-ml. portions of ether. The ether extracts were dried over magnesium sulfate and dry hydrogen chloride gas bubbled through the solution until saturation was complete. The light yellow oil that separated was allowed to reflux in ethyl acetate for several minutes causing the solid hydrochloride product to precipitate as a white powder. The powder was collected by filtration and recrystallized twice from ethyl acetate and ethanol solution to yield 0.5 Gm. (36%) m.p. 187–188°.

The infrared spectrum showed carbonyl absorption at 1740 $cm.^{-1}$.

Anal.—Calcd. for $C_{24}H_{26}ClNO_2$: C, 72.81; H, 6.62; Cl, 8.96; N, 3.53. Found: C, 72.29; H, 6.00; Cl, 8.88; N, 3.54.

The NMR spectrum in deuterated chloroform was δ = 1.0 (*m*, C—CH_3, 6H), 1.8 (*m*, —CH_2—, 4H), 7.2 (*m*, phenyl protons, 9H), 8.5 (protons of pyridine ring, 4H). The spectrum did not show absorption at δ = 3.65 due to methoxy group.

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Keyphrases

Estrogens, heterocyclic—synthetic
Diethylstilbestrol analogs—synthesis
IR spectrophotometry—structure
NMR spectrometry—structure

Trichloroethyl Carbonate: Influence of Particle Size on Oral Toxicity in Mice

Sir:

The authors have reported that the particle size of 4-acetamidophenyl 2,2,2-trichloroethyl carbonate (ATC) has a great influence on its oral toxicity in mice and on the peak blood levels of acetaminophen it produces in mice and humans (1). These studies confirmed that, with prodrugs such as ATC which are relatively insoluble in water, particle size can have a marked influence on the onset and peak magnitude of the biological effects elicited.

Trichloroethyl carbonate,[1] a prodrug of trichloroethanol (2), has been reported to have an aqueous solubility of about 4 mcg./ml. (3). Therefore, it was expected that particle size would affect its oral toxicity in mice in a manner similar to that observed for ATC. The present study was carried out to determine the extent to which particle size influences the oral LD_{50} of trichloroethyl carbonate in mice.

means of a glass-Teflon tissue homogenizer; and coarse particle, prepared by sieving the drug and suspending the U. S. No. 140 to U. S. No. 170 mesh particles in a 0.5% dispersion of gum tragacanth in water. Photomicrographs of the suspensions are shown in Fig. 1.

The LD_{50}'s of the three particle sizes of trichloroethyl carbonate were determined orally in male Carworth Farms mice, weighing 14–21 Gm., in a manner similar to that used for ATC (1). The results (Table I) show that the three particle sizes of trichloroethyl carbonate had significantly different toxicities at the 95% confidence limit.

These results are very similar to those obtained with ATC. In the ATC study (1), the drug, in various particle sizes, was given orally to mice

TABLE I—ORAL LD_{50}'S WITH 95% CONFIDENCE LIMITS[a] FOR FINE, REGULAR, AND COARSE PARTICLE TRICHLOROETHYL CARBONATE POWDERS IN MICE

Particle Size	LD_{50} (95% Confidence Limits), mg./Kg.
Fine	1175 (1074–1502)
Regular	1775 (1573–2268)
Coarse	>2000[b]

[a] Data calculated by the logit chi-square method of Berkson (4). [b] Only 3/10 animals died at this dose.

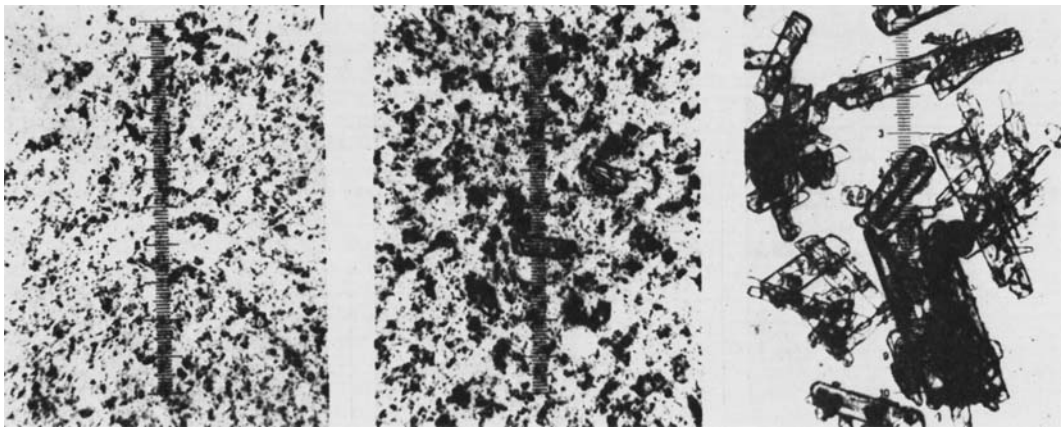

Fig. 1—Photomicrographs of the fine, regular, and coarse particle trichloroethyl carbonate used in this study (scale = 17.4 μ/small division).

The drug was prepared in three particle sizes as follows: fine particle, prepared by ball-milling a suspension of the drug in a 0.5% dispersion of gum tragacanth in water; regular particle, prepared by mixing a suspension of the drug in a 0.5% dispersion of gum tragacanth in water by

and a direct correlation was found between the oral LD_{50}'s and the peak blood levels of acetaminophen in the first 2 hr. Although no trichloroethanol blood levels were determined in this study on trichloroethyl carbonate, one might speculate that there would be a similar correlation between LD_{50}'s and peak trichloroethanol blood levels.

These studies indicate that when biologic mea-

[1] Clorethate, Smith Kline & French, Philadelphia, Pa.

surements are made on compounds that are relatively insoluble in water, the results have little meaning unless the particle size of the administered powder is taken into account.

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Trichloroethyl carbonate—toxicity
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LD_{50} value—particle size effect

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REVIEWS

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It is quite apparent from both the formal papers and the abbreviated versions of the discussions which follow that emphasis was placed on chemical contact or ingestion as the principal etiological factor in carcinogenesis. While this orientation may be justified in a symposium dealing with drugs, it must be recognized that there are many oncologists who subscribe to the view that chemical carcinogenesis is of only minor significance in relation to the incidence of human cancer.

The initial papers in the symposium deal with the present state of methodology for evaluating the potential carcinogenicity of drugs. The statistical assessment of data from the point of view of predictability is then discussed. In view of the law prohibiting the use of carcinogenic substances as food additives, it is interesting to note the view expressed by one of the participants, Prof. I. Berenblum, that "for all practical purposes, a carcinogen is, like any other noxious substance, only harmful above a certain critical dose level." In this connection, the opinion of Prof. H. Druckrey based on his analysis of the dose–time relationships of chemical carcinogenesis, is especially pertinent. He distinguishes between the primary effect of a carcinogen at the cellular or molecular level, and the subsequent multiplication of cancer cells to the point of tumor induction. As far as the primary effect is concerned he holds to the view that "there is no indication for the existence of a subthreshold dose." Nevertheless, he recognizes that a zero tolerance for carcinogens is "not always practicable and is scientifically objectionable" and proposes as a basis for future discussion that "1% of the lowest dosage which, given daily over the whole life span to susceptible experimental animals, produces cancer only at the end of the life span, can be considered as the maximum tolerable dose for human beings."

A number of papers in this volume deal more specifically with the potential carcinogenicity of specific classes of substances such as metal-containing drugs, petroleum hydrocarbons, lactones, and hormones including progesterone. In the two reports dealing with plastics used in orthopedic or surgical practice, as well as in the discussions of these papers, the weight of evidence is in support of a physical rather than chemical explanation of the carcinogenic effect of experimental implants.

In his remarks reflecting the point of view of the pharmacologist, Professor Alastair C. Frazer emphasizes the need for discrimination and judgment in deciding when a drug should be subjected to life-span study for potential carcinogenesis, and questioned the need for identifying "extremely feeble" carcinogenic drugs intended for use over short periods in people whose life expectancy is unlikely to provide time for any effect to be induced.

This monograph is required reading for those who wish to keep up with current thought among the experts in drug safety evaluation. Although the discussions following each presentation reveal the lack of unanimity on many aspects, there is agreement that much remains to be done to get at the root of these problems from both the methodological and interpretative standpoints.

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Advances in Pharmaceutical Sciences. Vol. 2. Edited by H. S. Bean, A. H. Beckett, and J. E.

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Reviewed by Nicholas G. Lordi
Rutgers—The State University
Newark, N.J.

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Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Council

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JANUARY TO DECEMBER, 1968

JOURNAL OF Pharmaceutical Sciences

August 1968 volume 57, number 8

Review Article

Particle-Size Analysis of Emulsion Systems

By M. J. GROVES and D. C. FRESHWATER

BECHER (1) reviewed the definitions of an emulsion and suggested the following as a basis for discussion: "An emulsion is a heterogeneous system, consisting of at least one immiscible liquid intimately dispersed in another in the form of droplets, whose diameters, in general, exceed 0.1 μ. Such systems possess a minimal stability, which may be accentuated by such additives as surface active agents, finely divided solids, etc." Becher's definition is satisfactory on most counts. His limitation on the lower size of the disperse phase particles is clearly not intended to be anything other than a broad generalization. Indeed, Becher himself recognized this since in a later section (p. 44) he stated that: "Emulsions are essentially unstable heterogeneous systems; they are partly dispersions, partly colloids"; the use of the term, colloid, inferring submicron sizes.

The influence of particle size upon the physical properties of emulsions and suspensions has been reviewed by others (1–6). It is generally agreed that stability, viscosity, rate of heat transfer, and optical properties of emulsions are dependent upon the particle size or the particle-size distribution of the disperse phase. However, there is at present no satisfactory correlation of the relationship. For example, Rutgers (5) reviewed the literature (up to 1962) on the relationship between relative viscosity and dispersion concentration. He concluded that absolute particle size was of importance, but not one of the 97 equations discussed by this author contained an expression for size or distribution of size. Sherman (6) also concluded that the bulk of the published literature on the viscosity of emulsions contained little or no reference to the state of the dispersion beyond broad statements to the effect that an emulsion was "fine" or "coarse."

Reexamining the published work that attempts to derive a correlation between some physical property of an emulsion and the state of dispersion, it was noted that many of the size measurements had been obtained by optical microscopy (Table I). However, it is widely accepted that this method of size analysis cannot adequately differentiate between particles of less than 1μ diameter owing to the limited resolution of the light microscope as the particles approach the wavelength of the incident radiation (7–9). Thus even if one accepts Becher's definition of an emulsion and his arbitrary limitation to disperse phase particles exceeding 0.1μ diameter, the accuracy of measurement of the smaller particles that could be present in a system, and the assessment of the significance of the results must at least remain suspect. The purpose of this review is to consider the particle size of emulsion systems and the alternative methods at present available for its measurement.

MEANING OF PARTICLE SIZE

In practice it is rarely found that all the particles in a system under examination have the same size parameter. Accordingly, size measurements on polydisperse particle systems indicate the probability that a given particle picked at random has a specified size. Thus the particle sizes re-

Received from the Research Department, Boots Pure Drug Co., Nottingham, England, and the Department of Chemical Engineering, Loughborough University of Technology, Loughborough, England.

ported for any material are associated with their frequency of occurrence; being the number of particles, or the weight greater or smaller than a stated size, or range of sizes. The purpose of a particle-size measurement technique is to discover the true frequency distribution of particle size, either as a number distribution or a weight distribution.

Form of Size Distribution for Emulsion Systems

Jellinek (10) suggested that it was unlikely that emulsions prepared by different methods and with different materials would have similar mathematical forms of distribution. Nevertheless, Herdan (11) noted that many systems appeared to obey some standard forms of distribution function such as arithmetical or logarithmic forms of normal (Gaussian) distribution, or exponential distributions of the Boltzmann law type.

The earliest attempt to derive a statistical function of this type was by Rossi (12) who considered the mechanism of subdivision as a random process. Rossi's model had certain deficiencies which Dobrowsky (13), van den Tempel (14), and Lederer (15) attempted to overcome. Jellinek found that Rossi's equation fitted experimental data for homogenized bitumen except for the largest particles present.

Troesch (16) applied statistical theory to the breakdown of droplets under turbulent flow conditions, and deduced that the droplet-size frequency followed a normal Gaussian distribution. Brodnyan (17), considering emulsion polymerization processes, found that in some cases radii were distributed normally although in other examples a log-normal distribution occurred.

A Gaussian distribution is absurd in many ways since it implies that a fraction of the particles, even though a small one, has to be expressed as having negative diameters (18). Nevertheless, the concept is useful since moments can be calculated by the use of algorisms (11). Cooper (7, 8) found that the Rossi equation appeared to apply only to the main drops in the emulsion. The presence of small daughter droplets always means that the size distribution is skewed and this also applies to a Gaussian plot. However, empirically, one method of removing this skewness (that is, make the distribution more evenly spaced out) is to plot it as a function of the logarithm of the particle size. Kottler (19), in 1952 reviewed the use of a logarithmico-normal distribution and cited examples back to 1924. Although Kottler's analysis of the statistical aspects of the log-normal distribution was confined to photographic emulsions, he demonstrated that if a distribution were not linear when plotted on log-probability paper it was an indication that

TABLE I—AUTHORS WHO HAVE EXAMINED THE RELATIONSHIP BETWEEN EMULSION AND SUSPENSION PROPERTIES AND PARTICLE SIZE, SHOWING METHOD OF PARTICLE-SIZE ANALYSIS

Authors	Reference[a]	Particle-Size Analysis Method
Axon	(161, 162)	Microscope
Becher	(291)	Microscope
Bredee and de Booys	(163, 164)	Microscope
Chessman and King	(165)	Microscope
Eilers	(166, 167)	Microscope
Elworthy and Florence	(290)	Microscope
Eveson	(168, 169)	Microscope
Gillespie	(170–173)	Microscope, electron microscope
Groot	(289)	Adsorption
Johnson and Kelsey	(174)	Electron microscope
Kelsey and Johnson	(175)	Electron microscope
Knoechel	(54)	Microscope
Knochel and Wurster	(55)	Microscope
Lawrence and Rothwell	(176)	Microscope
Leviton and Leighton	(177)	Microscope
Lyttleton and Traxler	(178)	Microscope
Manley and Mason	(179)	Microscope
Matsumoto	(151–154)	Sedimentation
Mima and Kitamori	(145)	Coulter counter
Nawab and Mason	(180, 181)	Microscope
Orr and Blocker	(182)	Microscope
Rajagopal	(183)	Microscope
Richardson	(3, 184)	Microscope
Rowe	(144)	Coulter counter
Saunders	(185)	Electron microscope
Sherman	(56–58, 186–196, 292, 293)	Microscope
Sheth	(197)	Microscope
Sheth *et al.*	(198)	Microscope
Shotton and Davis	(284)	Coulter counter
Shotton and White	(199, 200)	Microscope
Sibree	(201, 202)	Microscope
Simpson	(203)	Microscope
Srivastava	(204)	Microscope
Sweeney and Geckler	(205)	Microscope
Takano and Kambe	(206, 207)	Electron microscope
Talman *et al.*	(217)	Microscope
Taylor	(208, 209)	Microscope
Traxler	(210)	Microscope
Vand	(211)	Microscope
van den Tempel	(212, 213)	Microscope
Wachtel and La Mer	(127)	Coulter counter
Williams	(214, 215)	Microscope, Centrifuge
Wilson and Parke	(216)	Microscope

[a] Papers not necessarily discussed in the text.

the sample contained at least two different populations.

Mugele and Evans (20) critically compared Rossin-Rammler and Nukiyama-Tanasawa distributions with log-probability plots and showed that the first two were largely unsatisfactory. They found that the log-probability equation predicted the general distribution trend correctly for most data and gave good results for the mean diameter calculations. These authors showed that the emulsion data of Harkins and Beeman (21) and of Cooper (7, 8) fitted this presentation satisfactorily despite the extremely small size of the emulsion particles.

Gwyn, Crosby, and Marshall (22) considered bias in particle-size analyses resulting from the use of a log-normal distribution. Many analyses indicate the existence of maximum size classes but the log-normal distribution is in fact infinite in extent. Thus, the results tend to be biased, an occurrence implicitly recognized by Mugele and Evans. Gwyn *et al.* assumed that the sample was taken from a population with a true log-normal distribution and adjusted the data for bias. They suggested that the analysis could be carried out in two parts, a few particles being first counted to establish the central portion of the distribution, and a larger number of particles falling above, say, 5% of the total distribution based on the central portion. As noted by Gwyn *et al.*, if a single distribution applies over the entire range of sizes the curve for the upper range will exactly, or very nearly, coincide with the extension of the curve for the central portion of the distribution.

Rajagopal (23) derived a rigorous proof of the log-normal distribution by considering the randomness due to violent break-up of the interface during cavitation, the formation of daughter droplets, the disruption due to collisions, and coalescence due to the same cause. The process was considered as a Markoff chain and the empirical applicability of a log-probability law to emulsion particle-size distribution was placed on a firm theoretical basis.

Schwarz (24) introduced a so-called universal distribution law and this was later amplified and confirmed in two papers by Schwarz and Bezemer (25, 26). This equation, derived on statistical grounds, is also a variation upon the truncated log-normal distribution. From the data supplied by these authors, the equation appears to be less universal in application than their claims suggest. However, Sprow (27) found that the equation was useful when investigating droplet sizes produced in turbulent liquid-liquid dispersions.

There are a number of advantages in utilizing a distribution law of the log-normal type to describe a particle-size distribution, as discussed by Smith and Jordan (28). Thus, on a number basis a particle-size distribution is defined uniquely by two parameters, M, the geometric mean particle diameter, and σ_g, the geometric standard deviation. Both these parameters can be readily obtained by plotting the size-distribution data as a cumulative undersize curve on log-probability paper, a point also brought out by Kottler (29). In establishing line of best fit Kottler pointed out that preference should be given to those points lying closest to the midvalue of 50 cumulative percent. Due to the distortion of the probability axis, the distance by which points are displaced from a straight line becomes increasingly significant as one considers points progressively closer to the 50% mark. For this reason some investigators such as Drinker and Hatch (30) fit the best line to those points within the 20–80% limits, ignoring points outside these marks.

Espenscheid *et al.* (31) criticizes the use of log-normal distribution functions obtained from light-scattering data. They suggested that this was a new distribution function with different parameters and physical properties. However, Honig (32) pointed out that the suggested distribution function was readily reduced to the usual log-normal distribution function and this was the only one which could be determined experimentally.

One other approach deserves mention since the method of presenting the size analysis is unique. Broadbent and Callcott (33) suggested that a size analysis could be presented in the form of a matrix, leading to the derivation of a single parameter, the breakage function (π), which was defined as the proportion of particles that are broken during a grinding process. This approach, requiring distributions at different times, was developed in a series of papers (34–36) in the context of coal grinding and assumes that the chance of breakage is the same irrespective of the particle size. Furthermore, in its suggested form it could not be applied to emulsion systems since no account is taken of coalescence which always occurs during an emulsification process. Nevertheless, the concept of presenting the size analysis as a matrix is an attractive mathematical model since it is not necessary to assume any "law" controlling the emulsification process, and it might be possible to derive a single parameter to describe any one distribution. Arbiter and Harris (37) extended the concept to include a time relationship in grinding, but the approach

does not appear to have been used in the context of emulsions and might be a fruitful approach for a future investigation.

To summarize, there is considerable agreement that a size distribution follows a log-normal distribution, being characterized by the mean size and the slope, or standard deviation. This distribution can be plotted on log-probability paper and, although a relatively insensitive method of presenting results, it must be regarded as satisfactory when considering such factors as sampling error and repeatability of a particular size analytical method.

METHODS OF PARTICLE-SIZE ANALYSIS

The Society for Analytical Chemistry in 1963 published a classification of methods for determining particle size (38). This, supplemented by the standard textbooks on the subject by Herdan (11), Orr and Dallavalle (39), Rose (40), and Cadle (41), and review articles such as that by Scarlett (42), are sufficient background. It is not proposed therefore, to discuss the methods in general but rather to select certain aspects for comment and deal with two methods, the Coulter counter and the centrifugal photosedimentometer, in detail.

MICROSCOPE MEASUREMENT OF EMULSION PARTICLES

Most of the work carried out on correlation of properties of emulsions to the particle size of the disperse phase has relied upon optical microscopy for the particle-size measurement (Table I). The microscope has the advantage that it is direct and inexpensive, but the measurements in themselves may be extremely lengthy and tedious.

Mounting techniques for emulsions have been discussed and described by Simmonite (43). The emulsion under examination must first be diluted before mounting, and care is required to ensure that the system is not affected by this treatment. Various dilution media have been proposed including aqueous gelatin, acacia solution, and diluted polyols such as glycerin or propylene glycol (9). Various techniques have been described for examining the particles and classifying them into size groups. The simplest form utilizing a micrometer eye-piece or calibrated graticule has been widely employed (44). Harkins and Beeman (21) used a projection microscope, thereby reducing eye strain and improving the accuracy of the method. Smith and Grinling (45) introduced a counting technique using a hemocytometer slide and Cockton and Wynn (46) improved this by using a shallow Helber counting chamber. Levius and Dromond (9) criticized the previous papers and suggested a camera lucida method which they found to be an improvement over the counting techniques. Photographic methods were employed by, for example, Cooper (7, 8) who measured the particle size from prints. Photomicrography of emulsions is difficult since particles in the micron range exhibit Brownian movement, necessitating very short exposure times with subsequent loss of contrast.

Automatic scanning methods have been described (294) in an attempt to reduce the tedium of microscope analysis and improve speed and accuracy. Collins (295) investigated the sources of error using a Mullard type L188 "flying-spot" analyzer. Fisher (296) described a new scanning instrument which includes a computer to produce secondary information such as total projected area, total numbers of particles, or parameters such as mean Feret's or Martin's diameter.

The necessity of sizing large numbers of particles to obtain meaningful results has received some attention, the most serious attempt being probably that of Fischer and Harkins (47) who counted 50,000 particles in each of their systems However, as discussed by Becher (1), statistically significant counts can be obtained with only 300 particles. A count of 300 particles will result in a cumulative distribution in which the error at any value will be less than 8% ($p = 0.95$) and to lower this to 5% would require a count of 2,960 particles. Kaye (48) also discussed this point and showed that errors arising from sampling were such that a 10-fold increase in count only produced a slight increase of accuracy. The statistics of sampling are discussed (49).

Cooper (7, 8) carried out the classical critical evaluation of microscope sizing methods for emulsions. He pointed out that photographic methods were limited by the depth of focus since no lens is available which can give a clear image of particles of 1 μ or less and yet have a depth of focus of 20 μ, the thickness of a hemocytometer cell. Direct visual counting, in which the focus of the microscope can be continually varied, was found always to show a higher value for the number of particles in the smallest size range, confirmed by van Kreveld (50). As the particle approaches the wavelength of the incident light, the resultant halo makes estimation of the actual particle diameter extremely difficult so that sizing of particles below, say, 2 μ cannot be carried out with 100% efficiency, a point also confirmed by Saylor (51). Lucas (52) noting that rubber lattices contained particles well below 0.5 μ, attempted to improve the light microscope by reducing the wavelength of the radiation to the

ultraviolet range (λ = 2573 Å). The technique was extremely difficult since the source was not stable. van den Tempel (14) showed from Lucas's data that there are a considerable number of particles present in his system below the visibility limit of 0.12 μ, probably more than 60% being below the limit. van den Tempel considered the use of the light microscope for such small particles to be meaningless and favored the ultramicroscope which can be used to detect and count particles down to 0.02 μ diameter but is unable to differentiate particle sizes.

Although Cooper showed that microscope measurement of particles below 2 μ was of limited value, the method has continued to be used up to the present. King (53) implied that misleading results were occasionally obtained when correlating emulsion stability with particle sizes of 0.5–1 μ diameter. Knoechel (54) and Knoechel and Wurster (55), investigating o/w emulsion stability, discussed the inability of the microscope to take into account submicron particles, concluding: "The experimental evidence indicates that the presence of sub-visual particles could lead to unreliable values for various diameters and interfacial areas during the period in which minute globules coalesce to a visible size." This conclusion was echoed by Sherman (56, 57, 292, 293) who proposed that the only way out of this dilemma was to allow all the minute particles to coalesce until they could be measured microscopically. The value of this suggestion for unstable systems may be doubted, but the idea clearly cannot be applied to those which are stable or in which the particles do not coalesce. Sherman pointed out in the discussion of a later paper (58) that submicroscopic particles are "cancelled out" when determining a mean size. He made a tentative proposal for utilizing a reciprocal mean diameter, thereby bringing out the importance of submicron particles in a system.

ELECTRON MICROSCOPE

The early development of electron microscopy and its application to the investigation of materials below the limits of light-microscope discrimination was reviewed by Anderson (59). Walton (60) and Watson (61) reviewed the application of the instrument to particle-size measurement and described some of the limitations of the technique. The method has some advantages compared with light scattering, sedimentation, and other methods. In particular any peculiarities in shape can be observed directly (62). As noted by Walton (60), major difficulties are sampling and the effects of drying under vacuum. Local heating effects in the electron beam are often important when examining soft materials such as emulsion particles. Cravath *et al.* (63) improved the sampling of suspensions by depositing small droplets of the suspension from a mist (aerosol) onto a specimen film treated to promote wetting. The entire residue left by a single droplet could then be examined. Nixon and Fisher (64) used an aerograph gun to produce droplets and dispersed their material in bovine serum albumin which dried out to yield a transparent matrix supporting the particles. The basic technique of Nixon and Fisher was later improved by Craik who used a more concentrated albumin solution and stained the protein with osmium to improve contrast at the edges of the particles where the maxtrix was thickest, described by Groves and Scarlett (65).

Other methods of supporting suspensions for examination have been reported (66, 67). Soft polymeric materials which distort in vacuum have been hardened before examination by bromination (68) or cross-linking with hard polymers using high energy irradiation (62, 69, 70). However, in general these hardening techniques have not been applied to the electron microscopy of liquid-liquid emulsion systems.

Particle-size analysis using the electron microscope requires the measurement of a large number (at least 3,000) of particles by a photographic method since the image is often not sufficiently stable for more than a few minutes. The problems associated with this aspect of the method were described by Crowl (71) who proposed a mechanical method for sizing from photographic plates. The alternative approach of image shearing proposed by Rippon (72) may also offer a solution for future development.

However, the use of electron microscopy for measuring the particle size of emulsion particles below about 1μ diameter would appear to be the only direct method at present available.

ULTRAMICROSCOPE AND LIGHT-SCATTERING METHODS

Recent work on ultramicroscope methods has been devoted to either counting particles in a flow cell (73) or using a flow method to separate particles out according to their size and counting in a flow ultramicroscope (74–76).

Particle sizes can also be measured in principle by optical methods depending on the measurement of the reduction of light directly transmitted through a dispersion (turbidimetric or nephelometric methods) or by light scattered at some definite angle (usually 90°) from the light path.

Krishnan (77) appears to have been one of the first workers to investigate the light scattering in dilute emulsion systems, and Sing (78) showed that for small particles secondary scattering could no longer be neglected. This author utilized the Mie theory and the approach was investigated theoretically by van der Hulst (79).

The relationship between the scattering of light, the wavelength of the light, and particle size is complicated, but for particles small relative to the wavelength of light it assumes the simplified form originally derived by Rayleigh:

$$S = 24\pi^3 \left[\frac{m^2 - 1}{m^2 + 2}\right] \frac{V^2}{\lambda^4} \qquad \text{(Eq. 1)}$$

where S = total amount of light of wavelength, λ, scattered by a sphere of volume, V, and relative refractive index, m, per unit intensity of illumination.

This equation holds for spherical transparent particles when $r \leqslant 0.1\lambda$. When a particle is comparable in size or large relative to the wavelength of the incident radiation the scattered light will be the resultant of light waves originating from various parts of the particle. The phase and intensity of such waves are related in a complicated manner, predicted by the Mie equation (80, 81), written in the form:

$$S = \frac{\lambda^2}{2\pi} \sum_{v=1}^{\infty} \left(\frac{a_v^2 + p_v^2}{2v + 1}\right) \qquad \text{(Eq. 2)}$$

where S = total scattering by one spherical particle per unit intensity and the a_v's and p_v's are functions of X $(2\pi r/\lambda)$ and β $(2\pi rm/\lambda)$, r being the particle radius.

This equation has been discussed in detail by van der Hulst (79) and numerous tables of solutions to the Mie series have been published (80).

When S is divided by πr^2, the scattering per unit cross-sectional area of the particle, known as the scattering coefficient K, is obtained. Equation 2 then becomes

$$K = \frac{s}{\pi r^2} = \frac{\text{light obscured by particle}}{\text{light obscured if laws of geometric optics were valid}}$$

$$= \frac{2}{X^2} \sum_{v=1}^{\infty} \left(\frac{a_v^2 + p_v^2}{2v + 1}\right) \qquad \text{(Eq. 3)}$$

Thus, K is a function of r/λ and when the scattering coefficient is known for a particular value of r and λ its value is established for the ratio regardless of the individual values.

Equations 1–3 have been derived for scattering from single particles, but unless the number concentration of particles in a suspension is very high the scattering from a suspension can be considered to be the sum of the scattering by the individual particles. In practice, fine particles usually have a range of size as well as being of irregular shape. Thus, although the total scattering is the sum of the scattering by individual particles, the scattering coefficient may vary widely from one particle to another. If the ratio of r/λ is greater than 50, the value of K is constant and can be taken as unity. For smaller ratios the value of K becomes a complex function of r/λ. The effect of adding the scattering from a number of particles of varying size would be expected to damp out the wide fluctuations in scattering coefficient with varying size that occur for single particles. Gumprecht and Sliepcevich (82) considered the problem and proposed a quantitative relationship between Mie theory and K which enabled them to obtain a size-frequency distribution. The approach was simplified and does not appear to have gained wide acceptance. Goulden (84) used scattering-coefficient data calculated by van der Hulst (79) to apply the Mie equation to determining the particle size of homogenized milk (85–89). However, his method can only yield a mean particle size, and although useful for control purposes, cannot be employed for other than relatively monosized systems.

When a measure of total scattering is desired, it is much more convenient to measure the percent transmission than to attempt to integrate the intensity of the scattered light. Transmission methods have been used in many fundamental studies of light scattering and form the basis of a number of methods of particle-size determination.

When a beam of light is passed through a suspension of particles, the intensity of the beam is attenuated as a result of the scattering, and the decrease in intensity obeys the exponential law:

$$I/I_0 = \exp.(-\tau l) \qquad \text{(Eq. 4)}$$

The initial intensity = I_0, the intensity after the light has traversed distance 1 is I and τ is the absorptivity, τl being termed the absorbance.

Equation 4 can be rewritten for monosized systems as

$$I/I_0 = \exp.(-K\pi r^2 n l) \qquad \text{(Eq. 5)}$$

where n = the numbers of particles of radius, r, per unit volume of suspension, and K is the scattering coefficient as defined in Eq. 3. For very small particles which obey the Rayleigh relationship Eq. 1 can be rewritten as

$$I/I_0 = \exp.\left[-\frac{128\,\pi^5 r^6}{3\lambda^4}\left(\frac{m^2 - 1}{m^2 + 2}\right)\right] n l \qquad \text{(Eq. 6)}$$

On the other hand, when the particle diameters

are large K approaches a value of 2. Lothian and Chappel (83) discussed the fractional loss of intensity of a beam of light passing through a layer of emulsion particles, and showed that the absorbance of a dilute suspension of spheres was a function of their particle size. After strong criticisms of turbidimetric methods by Skinner and Boas-Traube (90), an improved instrument was described by Branson and Dunning (91). This device was further developed by Rose (40) who narrowed the angle of acceptance to reduce forward scattering. Other investigations were carried out by Kaye (48) and by Hodkinson (92). The latter made measurements of the angular scattering distribution over the range of 0.5–90° using suspensions of powdered flint, diamond, anthracite, and bituminous coal. Smooth curves were obtained which were quite unlike those for single particles or monodispersed spheres. Mie theory was therefore not applicable to these irregular particles, and K was found to rise slowly from 0 to 2. Kaye, and later Kaye and Allen (93) investigated the optical scattering cross-sections of small particles using white light and a wide angle of acceptance. Under these conditions, they also found that fluctuations of K were much reduced and for many purposes it was sufficient to take it as unity. Thus, the claim by Bolton and Marshall (94) that a simple turbimeter could be used to measure wax particles down to 0.06 μ diameter must be viewed with caution. It is not clear if these authors used a white light source, but it is of interest to note that they found values for K as low as 0.04 for their smallest particles.

Trice and Rodger (95) measured interfacial areas using a light-transmittance method similar to that used earlier by Langlois *et al.* (96) for coarse, unstable emulsions.

Reflectance relationships for emulsions are, if anything, more complex. This was noted by Lloyd (97) who incorporated a red dye in the oil phase and found that the reflectance at wavelengths at which the colored internal phase partially absorbed the incident light was inversely proportional to a power of the surface-area particle diameter. This relationship was claimed to be independent of the particle-size distribution. Schulman and Friend (98, 99), and van der Waarden (100) both used the Rayleigh formula to calculate the mean size of very small droplets (up to 400 Å. diameter). La Mer (101) investigated monodisperse aerosols using the higher-order Tyndall spectra method. Heller *et al.* (102) verified experimentally that Mie theory was applicable to light-scattering methods and tabulated the data required to transform light-scattering measurements into particle-size distributions (103, 105). Although initially confined to relatively monodisperse polymer lattices, Wallach (104) claimed that the method was applicable to the study of size distributions of dimethylnaphthalene–water emulsions.

Gledhill (106), Wales (107), and Weber (108) have all described methods for measuring particle-size distributions by measurement of turbidity spectra using methods similar to Heller. La Mer and Plesner (109) described a rapid method of sizing a monodisperse suspension by measuring the light scattering as a function of the angle between the incident beam and scattered light and the method was extended by Dezelic and Kratohvil (110, 111). Kratohvil and Wallace (112) reexamined the claims made by Heller and Tabibian (113), and by Wales (107), and found them unreliable.

To summarize, there is general agreement that light scattering and turbidity methods can give a measure of the mean particle size present in a system although there is some disagreement on the method by which the size distribution can be determined. Simplified techniques, making a number of assumptions, have been applied to determining particle-size distributions by a combination of sedimentation methods and turbidity measurements.

COULTER COUNTER

Coulter described his instrument in 1956 (114) and the device has come to be widely accepted as a convenient and valid method of size analysis. The Coulter counter determines the number and size of particles suspended in an electrically conductive liquid. This is done by forcing the suspension to flow through a small aperture having an immersed electrode on either side. If the suspension is sufficiently dilute, the particles will pass through the aperture one at a time. As each particle passes through, it displaces its own volume of electrolyte thus changing the resistance across the aperture. An electrical current passing between the electrodes converts the resistance change into a voltage pulse, whose amplitude for a given current and electrolyte is proportional to the volume of the particle. The voltage pulses resulting from a known volume of suspension passing through the aperture are fed to an electronic counter fitted with an adjustable threshold level such that all pulses above the threshold are counted. In this manner the number of oversize particles is obtained at different levels of particle volume so that a particle-size distribution by number or by weight can be determined. Detailed descriptions of the in-

strument and its principle of operation have appeared in the literature (114–127) and it is not proposed to repeat them here.

As might be anticipated with any new advance the Coulter counter has been subjected to critical evaluation by many workers in recent years (128–137). However, to date no fundamental flaw in the principle or mode of operation has been detected and it is generally agreed that the instrument is a unique and valuable contribution to the science of particle-size analysis.

Theoretical Response of the Coulter Counter

Batch (115) and Gregg and Steidley (279) have considered the theoretical response of the Coulter counter for various shapes of particles. The current between the electrodes is maintained at a constant level so that any change in the resistance of the cell results in a change of voltage across the cell. The presence of a particle of different resistivity in the aperture will therefore result in a change of resistance, the amount of change being in some way proportioned to the value of the particle.

Consider an orifice of cross-sectional area A, diameter D, filled with an electrolyte of resistivity, r_0, and containing a particle of resistivity, r. If a is the projected area of the particle at the portion of an elemental section of length dl then the resistance of this element of the particle in the direction of the orifice axis is $r\,dl/a$, and the resistance of the annulus of electrolyte is $r_0 dl/(A - a)$. The total resistance across the element is

$$dR = \frac{r_0 r\,dl}{ar_0 + r(A - a)} = \frac{r_0\,dl}{A - a(1 - r_0/r)}$$

and the increase of the resistance of the element due to the particle is

$$d(\Delta R) = \frac{r_0 dl}{A - a(1 - r_0/r)} - \frac{r_0 dl}{A}$$

$$= \frac{(1 - r_0/r)r_0 a\,dl}{A^2[1 - (1 - r_0/r)a/A]} = \frac{ar_0 k\,dl}{A^2(1 - ka/A)} \quad \text{where } k = (1 - r_0/r)$$

Thus, the total resistance change is

$$R = \frac{r_0 k}{A^2}\int \frac{a\,dl}{(1 - ka/A)} \qquad \text{(Eq. 7)}$$

For a rod-shaped particle lying along the axis this equation can be solved simply because a is not a function of 1 so that

$$R = \frac{rkv}{A^2(1 - ka/A)}$$

Batch solved Eq. 7 for spherical and cone-shaped particles by expanding the expression under the integral sign. Gregg and Steidley obtained exact solutions by solving the integral explicitly before expansion. The resulting equations for change in resistance are summarized in Table II.

The equation for a sphere approximates to

$$R = \frac{r_0 v}{A^2}\left[1 + \frac{0.3K^2 + 0.13K^4}{(1 - K^2)^{-1/2}} + + +\right] \qquad \text{(Eq. 8)}$$

Thus, for any shaped particle, provided a/A, or d/D, $= K$ is small, the change in resistance is directly proportional to the volume of the particle. Allen (132) has shown that this assumption is valid for particle–tube ratios of up to 20%. The assumption of the linear response for up to 40% ratio can lead to an error in particle diameter of 7.8% for rod-shaped particles, and 3.6% for spherical-shaped particles.

The error term in Eq. 8

$$\frac{1 + 0.3K^2 + 0.13K^4}{(1 - K^2)^{-1/2}}$$

can be used to assess error due to assuming a linear response, as shown in Table III. Thus it will be seen that, strictly, the response of the Coulter counter is not linear with particle volume and the nonlinearity is dependent upon particle

Table II—Summary of the Equations[a] Relating Particle Shape and Total Resistance Change in an Orifice Containing an Electrolyte

Particle Shape	ΔR[b]	ΔR[c]
Rod length $\gg$ dia.	$\frac{rkv}{A^2}\left(1 + \frac{ka'}{A} + \frac{k^2a'^2}{A^2} + \ldots\right)$	$\frac{r_0 v}{\pi^2 D^4}\frac{1}{(1 - K^2)}$
Rod length $\sim$ dia.	—	$\frac{r_0 v}{\pi^2 D^4}\frac{1}{(1 - K^2)}\left[t\pi r_1^2 + \sigma\pi r_1^3\right]$
Sphere	$\frac{rkv}{A^2}\left[1 + \frac{4}{5}\frac{ka'}{A} + \frac{24}{35}\frac{k^2a'^2}{A^2} + \ldots\right]$	$\frac{2r_0}{\pi D}\left[\frac{\tan^{-1}[K(1 - K^2)^{-1/2}]}{(1 - K^2)^{1/2}} - K\right]$
Cone	$\frac{rkv}{A^2}\left[1 + \frac{3}{7}\frac{ka'}{A} + \frac{3}{7}\frac{k^2a'^2}{A^2} + \ldots\right]$	—

[a] Where A = area of orifice of diameter D, a' = maximum cross-sectional area of particle. The value of k is $(1 - r_0/r)$, where r is the resistivity of the particle and r_0 the resistivity of the electrolyte. Gregg and Steidley assume r/r_0 to be negligible and define K as d/D = particle diameter/orifice diameter, where t is the length of rod and σ is a constant. [b] From *Reference 115*. [c] From *Reference 279*.

TABLE III—MAGNITUDE OF THE ERROR IN ASSUMING A LINEAR RESPONSE FOR THE COULTER COUNTER (EQ. 8)

d/D	0.1	0.2	0.3	0.4	0.5
$\Delta R \cdot A^2/V$	1.008	1.033	1.077	1.145	1.26
Error in volume, %	0.8	3.3	7.7	14.5	26.0
Error in diameter, %	0.3	1.1	2.9	4.8	8.7

shape. However, it should be emphasized that in practical terms the error is not significant if the largest particle is of the order of 40% of the tube diameter. Particles larger than this can be measured providing the "error" is recognized although blockage of the orifice by such large particles generally means that in practice the size analysis would be carried out using a larger orifice tube. Experimentally Eckhoff (283) has found that spherical and comminuted particles of identical volumes give rise to voltage pulses of identical amplitude; the effect may, therefore, prove to be less significant than was earlier thought.

The resistivity of the particle has a theoretical effect upon the response of the instrument. Thus, from Batch's equations (Table II), if $k = (1 - r_0/r)$ is less than 1, the magnitude of the errors shown in Table III will be increased. However, in practice it has been found that the majority of powders, whether conducting or not, behave in the electrolyte as particles with infinite resistance (116). Thus the assumption that $k = 1$ can generally be assumed to be good. It is interesting to note, however, that if the particles showed the same resistivity as the electrolyte there would be no "size" error.

Lower Limit of Size Measurement by the Coulter Counter

Coulter's Laboratory Manual (1966) claims that, with a 20-μ nominal diameter orifice tube, the instrument will detect particles of 0.4-μ equivalent sphere diameter. Kubitschek (120) claimed to detect particles of 0.2 μ diameter with a resolution of 0.04–0.06 μ^3. This claim must be viewed with caution although Princen (private communication) has confirmed it and suggests that it may be due to instrument idiosyncracy. It is widely accepted that the instrument is useful for particles of diameters down to 1 μ, and will detect particles down to 0.5 μ diameter (138). However, in this range the instrumental background caused by electrical effects, especially heating within the orifice, and cavitation by the passage of material through the small orifices which are required for these sizes make actual detection of submicron particles less certain. In addition, the loss of count caused by "shadowing" of small pulses by larger ones makes the detection of small particles difficult in the presence of large ones (129). The practical difficulties in obtaining electrolyte free from particulate contamination are also considerable. The instrument is therefore limited to approximately the same size range as optical microscope methods but has the overwhelming advantage that a vastly greater number of particles is actually counted for any one size distribution.

Coincidence Correction and Dilution of the Sample

The theory of the Coulter counter assumes that only one particle will be found in the volume defined by the orifice at any one instant of time. However, even with very dilute suspensions there is a finite possibility of two or more particles passing through at the same time. Clearly the more dilute the suspension the lower the probability of coincidence. Wales and Wilson (130, 131) recognized two forms of coincidence: (*a*) All pulses from two or more simultaneous particles add so as to produce a single pulse whose maxima is the sum of the two individual pulses, and (*b*) The pulses from two or more simultaneous particles behave as one pulse whose maximum corresponds to the largest particle.

Princen and Kwolek (280) showed that type *a* coincidence should predominate and would result in overcounting of large particles. The methods of correction for the coincidence error were discussed by these authors and Edmundson (135, 281) has proposed an alternative method. At low counts, however, the required correction will be approximately 1–3% of the total count and the method of calculating this relatively small error is not likely to be significant. For this reason, it is better to operate the Counter at dilutions which will give low total counts and this itself is sometimes a source of difficulty. Groves (139) pointed out that dilutions of the order of 1 : 50,000 or more are often required for emulsion systems and great care is required when making these dispersions for analysis.

Effect of Electrolyte on Dilute Emulsion Systems

Parfitt (282) has recently described the theoretical background to the preparation of dispersions and their subsequent stability. Emulsion particles are often stabilized with surface-active agents and emulsion systems diluted with water

will sometimes flocculate on standing. However, the addition of electrolyte to such a dilution will frequently cause flocculation because the energy barriers preventing aggregation and coalescence are reduced. Although electrolyte is necessary for a Coulter counter analysis, few authors appear to have encountered this phenomenon when using the instrument to measure the particle-size distribution of an emulsion system. Singleton and Brown (140) found that the count of the systems in their investigation changed with time. They noted the elapsed time to the beginning of counting for each of the sizes being measured; counts were then corrected for the time effect by extrapolation to zero time. Groves (139) reported the same effect for an emulsion stabilized with cetrimide and found that the flocculation could be minimized by carrying out the dilution in sodium chloride solution containing cetrimide at a concentration in excess of the critical micelle concentration. The results obtained in this manner agreed with those obtained independently using other size analysis methods. Some dangers of extrapolating to zero time in the manner suggested by Singleton and Brown are illustrated by the results shown in Fig. 1. The phenomenon has been noted in systems stabilized with other surface-active agents (278). Aggregation of an emulsion system is therefore possible during a Coulter counter size analysis, and the analyst should be at least aware of the possibility.

Use of the Coulter Counter for Sizing Emulsion Systems

Wachtel and La Mer (127) employed the Counter to size o/w emulsion systems, using the device to measure the degree of dispersion

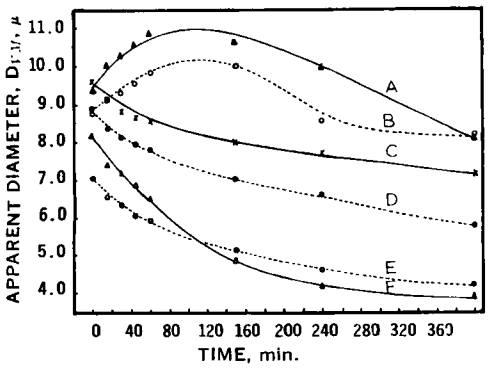

Fig. 1—Change of apparent mean particle size of a cetrimide emulsion measured with a Coulter counter as a function of time. All primary dilutions were made in 0.0003 M *cetrimide in normal physiological saline. Key: secondary dilutions were made in saline containing the following concentrations of cetrimide: A, 0.00003* M; *B, 0.000015* M; *C, 0.0003* M; *D, 0.00015* M; *E, 0.003* M; *F, 0.03* M. [*Reproduced with permission from* J. Pharm. Pharmacol., **18**, *305(1966)*.]

achieved by the use of a Sinclair aerosol generator for making monodisperse emulsions. Higuchi (141, 142) investigated factors influencing the aggregation of emulsions, followed by Lemberger and Mourad (143), and Swift and Friedlander (125). Shotton and Davis (284) investigated the influence of soap concentration on creaming rates of liquid paraffin emulsions in potassium laurate, and Singleton and Brown (140) used the Counter while investigating i.v. fat emulsions. Rowe (144) and Mima and Kitamori (145) studied emulsion-stability parameters as reflected in changes of particle-size distribution. Princen, Stolp, and Kwolek (146) examined the factors controlling the emulsification of linseed oil and Marshall and Taylor (147) described the use of the counter for following changes produced in an emulsion during the evaluation of different homogenizers. In general terms, Schrenzel (148, 149) discussed the use of the Coulter counter for the size analysis of pharmaceutical emulsions. Whittlestone (285) showed that the counter could be used for determining the size distribution of fat globules in cows milk but found no detectable systematic trends in the distribution pattern. However, later Cornell and Pallansch (286) improved the technique and demonstrated the effects of homogenization, pasteurization, and cream separation. The instrument has therefore been applied to many different types of emulsions.

As noted by Edmundson (281), "although simple in principle and reliable in operation, the Coulter Counter is a highly sophisticated apparatus, not to be regarded as a 'black box' that gives the right answer automatically." To summarize this section, therefore, the instrument covers approximately the same size range as the optical microscope but has the overwhelming advantages of improved speed and accuracy; the proper methods for using the instrument and interpreting its results are still being developed.

GRAVITY SEDIMENTATION METHODS

As noted by Becher (1), if an emulsion creams at an observable rate, measurement of the amount creamed per unit time permits the construction of a distribution curve. Most methods which depend on this principle, such as that of Kraemer and Stamm (150), measure the change of density. More recently Matsumoto (151–154) has described a method in which the viscosity changes in a creaming emulsion are used to build up a particle-size distribution of the system. Following the design of a cell to measure emulsion stability using creaming rate monitored by dielectric measurements (155), Kaye and Seager have described a similar device to measure the particle size of o/w

emulsions (156). The method is confined to systems of mean particle size around 100 μ and does not appear to be useful for diameters below 15 μ; it is therefore of limited application to pharmaceutical emulsion systems which have particles well below the lower limit of the method (1). Figurovskii and his co-workers (157–160) examined sedimentation methods and stated that they offered the fastest and most reliable means of determining the degree of dispersion of dispersed systems. Unfortunately, gravity sedimentation methods are not applicable to particles below about 10 μ diameter since the particles "cream" too slowly. In addition convection and displacement currents as well as Brownian movement all tend to disturb the sedimentation pattern. Hence the use of gravity sedimentation is of limited use for most pharmaceutical systems where particle sizes of 1 μ and below are common.

CENTRIFUGAL SIZING TECHNIQUES

One obvious way to increase the settling rate is to centrifuge the suspension. Orr and Dallavalle (39) described a number of centrifugal sedimentation techniques and other devices have been developed in recent years.

The first attempt to use a centrifuge to obtain a size distribution was that of Dumanskii, Zabotinskii, and Evseyev (218). Using an ordinary centrifuge, they determined the amount sedimented by ultramicroscope counts before and after centrifuging, but obtained poor agreement with the ultramicroscope method. Svedberg and Pederson (219) suggested that this was probably due to convection currents caused by serious deviation from ideal sedimentation conditions. Nichols and Svedberg developed a centrifugal densitometer, a two-arm centrifuge in which the boundary of particles was observed by taking photographs at different stages of the analysis. Nichols and Liebe (220) used a much improved version of this early instrument which rotated at 20,000 rpm and had better balancing and freedom from vibration. The obscuring power, related to the cross-sectional surface diameter of the particles, was measured for six colloidal lithopone samples by measuring the absorbance from light absorption measurements. The method was described in detail later by Bailey, Nichols, and Kraemer, (221) and by Bailey (222), the former group showing how it could be applied to the particle size analysis of emulsions. Nichols and Bailey (223) gave a worked example of how to calculate a size distribution of an emulsion using the so-called "low-speed Svedberg ultracentrifuge."

Nichols and Liebe (220) equated the viscous force due to Stokes' law with centrifugal force, ignoring the Coriolis force or accelerating force which is also present. Robison and Martin (224, 225) were able to demonstrate that this assumption would not lead to any significant error. Cheng and Schachman (226), after an intensive investigation of the ultracentrifugal sedimentation behavior of polymer lattices, concluded that Stokes' law was valid under these conditions.

Marshall (227–229) suggested a two-layer technique in which particles are considered to start from substantially the same position. This technique has a superficial attraction but is fraught with many difficulties, including that of irregular sedimentation, a point noted by Keen and Schofield as early as 1930 (230). This technique and methods for overcoming the problems have been discussed (72, 231, 232) and the streaming problem for particulate solids subjected to a two-layer sedimentation technique has been effectively overcome. However, the approach is only applicable to materials which are denser than the sedimentation liquid, *i.e.*, the particles are thrown to the outer periphery of the vessel. Since emulsion particles are usually less dense than water and soluble in organic liquids which have a lower density, the use of a centrifugal sedimentation method is mainly confined to a homogeneous technique. Thus emulsion particles move inward from the outer periphery toward the center of the sedimentation vessel. It is therefore necessary to consider in more detail centrifugation techniques and methods for the solution of a homogeneous system.

Centrifugal Techniques

The early techniques as noted above used centrifugal tubes. Martin (233), and later Robison and Martin (224), reviewed breaker-type centrifugal sedimentation and concluded that it had advantages, not the least being that collisions between the particles and the walls of the vessel are minimized. Robison and Martin developed the theoretical approach and described the application of a modified beaker- or sector-shaped centrifuge cell from which samples were withdrawn at the end of a variable time at a fixed depth (225). Brodnyan (17) used a similar method for polymer lattices but with an ultracentrifuge. Kamack (234) improved the theoretical method and described a similar sector-shaped cell to which was attached a sampling device that removed a sample at a fixed depth while the centrifuge was still running, thereby removing the danger of stirring the suspension during the slowing-down phase. Kamack had to use concentrated suspen-

sions of the order of 1% and only one point on the distribution curve could be obtained from each run. Owing to hindered settling (235, 236) the method was also probably inaccurate. Nevertheless, the method of calculating the experimental results was retained when Donoghue and Bostock (237) introduced the complete disk centrifuge. This particular instrument resulted in the introducion of a centrifugal analog of the Andreasen pipet by Slater and Cohen (238), the Sinko centrifuge. Gupta (239) also described a version of the centrifugal Andreasen pipet technique.

Moser and Schmidt (240) described a centrifugal photosedimentometer using a shallow cylindrical tank or disk rotating in the horizontal plane. The absorbance of the suspension was followed as a function of time by passing a thin beam of light through a fixed point on the tank onto a photoelectric cell. The authors used a nomograph to relate the recorder curve to the percentage of particles of a given size calculated from Stokes' law and stress was laid on the fact that an instrument of this type must first be calibrated by some other method owing to variable factors such as particle shape and opacity. Moser (private communication) stated that the instrument was a prototype which was not developed further.

Later Kaye (241) patented a photosedimentometer which was essentially similar to that of Moser and Schmidt. The new instrument employed a servosystem which compared the attenuated light beam with the unattenuated beam, using the difference signal to rotate an optical wedge to cancel the difference. A potentiometer attached to the optical wedge was used to control the recorder so the instrument plotted out the absorbance of the suspension. The instrument was described in more detail by Kaye (48) and evaluated with Burt (242–244). A simplified instrument, omitting the servo unit, was described and evaluated by Groves, Kaye, and Scarlett (245). The light beam was allowed to fall onto an unbalanced photocell after passing through the centrifugal disk. The instrument was used with the two-layer technique and was insufficiently sensitive to detect the streaming later investigated by Rippon (72). A prototype of an improved version has been described and evaluated (246, 247, 231, 278) and is shown in Figs. 2 and 3.

Other disk centrifuges have also been described. Martin, Brown, and de Bruyn (248) used a hollow aluminum disk centrifuge similar to Kaye's design to size analyze submicron powders containing elements of high atomic density such as lead

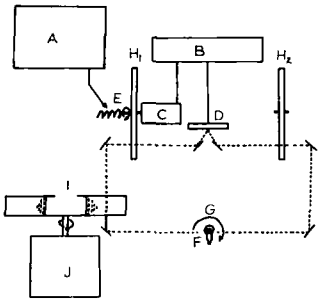

Fig. 2—Diagrammatic cross-section of the Coulter centrifugal photosedimentometer. Key: A, recorder; B, amplifier; C, servomotor; D, photocell (CdS); E, potentiometer; F, light source; G, chopper, H_1, servo-driven optical wedge, H_2, hand-operated optical wedge; I, centrifuge disk containing liquid; J, constant speed motor. The optical path is shown as a discontinuous line.

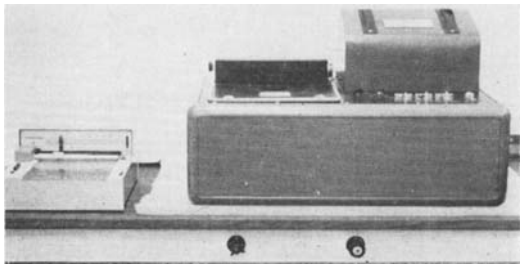

Fig. 3—Photograph of the prototype of the Coulter centrifugal photosedimentometer. The edges of the optical wedges are seen as projections from the right-hand housing. The rotating disk is horizontal and is situated under the lid seen on the left of the main unit.

glass, an X-ray beam passing through it and onto a proportional counter.

Hildreth (249, 250) described a simple photoextinction type of disk sedimentometer, similar in principle to that described by Groves *et al.* An initially homogeneous suspension was used on the variable-time system (*vide infra*), the transmittance *versus* time curve being related to transmittance weight curve by obscuration factors. As noted by Rippon (72), this is an oversimplification of the theory and is not a tenable method.

Atherton, Cooper, and Fox (251), Atherton and Tough (252), and Murley (253) described a small diameter disk centrifuge in which the two-layer technique is used in conjunction with a rotating-probe sampling device, the I.C.I.-Joyce Loebl centrifuge, Figs. 4 and 5. Thus, liquid and suspended contents are removed for subsequent analysis after predetermined centrifugation times at a known radius while the centrifuge is still rotating. This centrifuge has been investigated in our laboratories using a homogeneous suspension method. Irregular sedimentation behavior was observed at the high concentrations of an emulsion required for subsequent analysis. In addi-

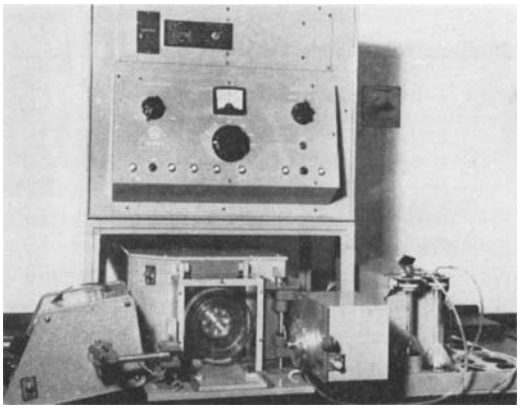

Fig. 4—Photograph of the Joyce-Loebl centrifuge, showing the disk probe, and sampling equipment.

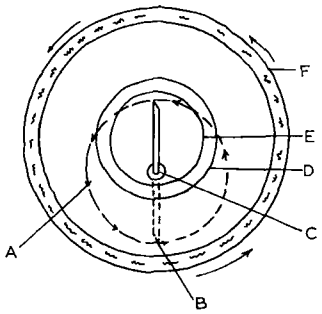

Fig. 5—Sketch of Joyce-Loebl centrifuge disk, showing relationship of probe during sampling. Key: A, path described by probe tip; B, final collection point; C, probe pivot; D, meniscus of liquid before sampling; E, inner edge of disk port; F, outer edge of disk.

tion considerable turbulent disturbance was observed when the sampling probe was inserted into the suspension (Groves, unpublished results). However, Jones (private communication) claims that the instrument has been used to obtain the particle-size distributions of submicron polymer emulsion systems.

Theory of Homogeneous Suspension Methods

When the material to be analyzed is dispersed in the sedimentation liquid to produce a homogeneous dispersion, three main modes of operation are possible as noted by Murley (253). (*a*) The inner radius of the liquid surface and the outer radius of collection are kept constant, samples being collected after varying running times of the centrifuge. (*b*) The running time of the centrifuge is kept constant, as is the inner radius of liquid and the outer collection radius is varied. (*c*) The running time of the centrifuge is kept constant as before, but the outer collection radius is kept constant for successive measurements, while the inner liquid surface radius is varied.

In all cases the centrifuge speed is kept constant. Donoghue and Bostock (237) gave solutions for solving methods *b* and *c*, to which Murley added another proof. However, Donoghue and Bostock also stated that no analytical proof exists for the derivation of the particle-size distribution of a sample when time is varied, unfortunately the precise situation relevant to the centrifugal photosedimentometer. Murley agreed that this was correct but pointed out that iterative methods could now be developed for calculating the required results using modern computer techniques at relatively low cost. The variable time mode of operation offers a number of important experimental advantages and as a result many attempts have been made to find a solution, or the best approximation.

Although the position of a particle relative to its initial position can be described mathematically, it still remains difficult for a distribution function to be derived from the experimental information. This is complicated by the fact that, from the initially homogeneously dispersed particulate system, particles are accelerated proportionally to their position from the axis of rotation. At any given reference or sampling zone, after a certain time t it can be said that all particles greater than a certain size d_t will have passed, where d_t is the Stokes' diameter of the particle which started from the free surface and in time t had transversed to the sampling zone. Subsequently the theory was developed for a disk centrifuge which is an ideal form of a sector-shaped cell since wall collisions are reduced to a minimum. The size distribution is considered to be continuous, containing in an infinitesimal range of diameters a fraction of $F(y)dy$.

Referring to the sector of the disk centrifuge, the distance from the axis to the free surface is S; that to the sampling zone, R.

Hence a particle of diameter d is defined as that particle which will just travel from the surface at S to R during the centrifuging. From a consideration of the centrifugal forces:

$$\ln \frac{R}{S} = \frac{(\rho - \sigma)\omega^2 d^2 t}{18\,\eta} \qquad \text{(Eq. 9)}$$

where ω = angular velocity, ρ = density of particle, σ = density of sedimentaion liquid, and η = the viscosity of the liquid. Thus,

$$\ln \frac{R}{S} = kd^2t, \text{ where } k = \frac{(\rho - \sigma)}{18\,\eta}\,\omega^2 \qquad \text{(Eq. 10)}$$

These particles of diameter d and all larger particles will have been sedimented as well as a fraction of the particles smaller than d (*i.e.*, those which were nearer to the sampling zone than those at the surface) in time t.

If p is the total fraction of material deposited, then p is given by the equation

$$p = \int_d^\infty Fy(dy) + \int_0^d \frac{R^2}{(R^2 - S^2)} [1 - e^{-2ky^2t}] \, Fy \cdot dy \quad \text{(Eq. 11)}$$

Differentiating Eq. 11 with respect to time gives:

$$\frac{\delta p}{\delta t} = \int_0^d \frac{R^2}{(R^2 - S^2)} - 2ky^2 e^{-2ky^2t} \, Fy \cdot dy \quad \text{(Eq. 12)}$$

This equation is not capable of analytical solution although Romwalter and Vendl (254) obtained Eq. 11 and invalidated their solution by an error. This error was detected by Brown (255) who obtained Eq. 11 but solved it by making ($R - S$) variable. Robison and Martin (224) used an iterative method to produce a set of approximate solutions which were claimed to give better accuracy than that due to normal experimental errors. Kamack (234) improved the iterative technique to give an approximate general solution to Eq. 12 in recursive form:

$$F_i = \frac{1}{2}(S + S_{i-1,i})C_i + \sum_{i=1}^{i=1} \left[\frac{S + S_{i-1,i}}{S_{j+1,i} + S_{j,i}} - \frac{S + S_{i-1,i}}{S_{j,i} + S_{j-1,i}} \right] F_j \qquad i = 1,2 \ldots n \quad \text{(Eq. 13)}$$

where $F = F(D)$ = fraction by weight of particles smaller than diameter D. C = concentration of suspended solids, as a fraction of the concentration of uniform suspension prior to settling, and $S = (R/S)^2$.

Others have attempted to obtain a solution of the variable-time equation. Nichols and Liebe (220) published a result which would not, however, give a weight-distribution function. Parkinson (256) overcame the variation due to the centrifugal field by the simple process of ignoring it. More practically, Jacobsen and Sullivan (257) employed a very low value of R/S (1.05), but this was experimentally difficult since only small volumes of material were available for analysis. Dana (258) employed a pipet method using an inclined-tube centrifuge for which mathematical analysis was found to be inapplicable. Other attempts were critically reviewed by Robison and Martin (224). It must be concluded that an exact solution of the theory for deriving a distribution function from an initially homogeneous dispersion centrifuged for various time intervals is not possible. As noted by Murley (253) approximate methods of an order of accuracy better than that due to experimental techniques are, however, available.

Light Scattering as Applied to Centrifugal Techniques

The use of a beam of radiation to measure the concentration of particles in a centrifugal cell has many attractions, especially as it is not necessary to disturb the sedimenting system in order to obtain an analysis which can be made continuous. The pioneer investigations of Svedberg and Nichols and Liebe, and Nichols and Bailey have been mentioned. However, it is usual to employ Schlieren techniques to follow boundary changes in analytical ultracentrifugation, the boundary being detected by changes of refractive index between solvent and suspension. This method was used by Hermans and Ryke (259) to determine the particle size of colloidal silica particles. Bierman *et al.* (260) used the technique to measure particle-size distributions of the chylomicrons, the submicron lipoprotein particles found in plasma after meals of fat, and found that the turbidity of their suspensions sometimes interfered with observation of the boundary. McCormick (261) noted that by increasing the intensity of the light beam with a mercury arc lamp the Schlieren patterns could still be obtained for concentrations up to 0.05% polystyrene latex particles with diameters of 2500 Å. He was therefore able to adapt conventional analytical ultracentrifugal techniques for the size analysis of a number of polymeric emulsions. Averink *et al.* (262) criticized McCormick's method since the turbidity forced him to use such low concentrations that the Schlieren peaks were very small, leading to poor accuracy. Averink *et al.* showed that the applicability of Schlieren optics was restricted to a range of particle diameters ranging from 200 to 2,400 Å. They concluded that with absorption optics the range could be greatly extended and described a method for achieving this. A graphical method was used to relate absorbance to concentration in order to calculate the size distribution. Using monochromatic light this method would appear to be the most satisfactory currently available.

Using a simple beam of light of mixed wavelengths, Groves, Kaye, and Scarlett (245) emphasized that their method could not be absolute for particles whose diameter approximates to the wavelength of the incident light but was useful for the detection of differences between samples. Kaye and Jackson (263) applied a modified centrifuge developed from the earlier models to the size analysis of polymer emulsions of narrow size range, and were able to deduce that these

materials had a wider range than that claimed by the manufacturers.

Thus, although the technique is relatively unsophisticated it may have some use where the particles are still big enough to cause interference with a light beam (>0.01 μ), since these particles are separated according to Stokes' law. The quantitative expression of the actual particle concentration may not be exact and may result in an overemphasis of the fines (277). The relationship between this effect and errors due to, for example, inadequate dispersion of the fine particles remains to be established. An adaptation of the technique described by Schleusener and Read (287) for detecting sedimenting particles with a 6328 Å./He-Ne gas laser may offer some improvement for future investigation.

Other Centrifugal Techniques for Sizing Emulsion Particles

The ultracentrifuge has been used to determine emulsion stability, measuring the free oil and compacted emulsion boundaries as a function of time (264–269). By analogy with gravity sedimentation methods this technique could be applied to particle-size determinations. Pinter and Zilversmit (270), and Zilversmit (271) described a density-gradient method for the ultracentrifugal size measurement of chylomicrons. This technique is used in studies on living materials (272) and the approach might well be applicable to emulsion systems.

ADSORPTION METHODS

Brodnyan and Brown (288) estimated the soap concentration adsorbed onto polymer emulsion particles. Assuming a monolayer of absorbed molecules of known molecular cross-section, they were able to calculate the total interfacial area and an average particle size from a knowledge of the phase volume. A similar method has been described by Groot (289) for paraffin oil emulsions stabilized with sodium lauryl sulfate. Since analytical methods for estimating a wide variety of surface-active agents are now available, the method may be generally applicable.

DISCUSSION AND CONCLUSIONS

The range of particle sizes in operationally usable pharmaceutical emulsions may be within the arbitrary limits 0.01 and 10 μ (297), although some have claimed that particles of up to 100-μ diameter may be found (156). From a practical standpoint it should be pointed out that emulsion droplet sizes well below Becher's (1) limit of 0.1 μ have been reported in the literature. For example, droplet diameters in transparent emulsions investigated by Bowcott and Schulman (273) were of the order 100–500 Å. van der Waarden (100) prepared emulsions with particle diameters varying between 250 and 900 Å. Kiyama *et al.* (274, 275) reported particle sizes varying between 0.08 and 0.2 μ, finding up to 30% w/w of their material below 0.08 μ in some instances. Lucas (52) found that rubber latex particles were generally below 0.1 μ, and chylomicrons have a size range extending down to 150 Å. (260). Schoenholz and Kimball (276) showed that bright-drying wax polish emulsions contained particles in the range of 0.05–0.1 μ, the particle size decreasing if the ratio of emulsifier–wax was increased. Thus, although it seems that emulsion systems may contain particles below the limit of detection by the light microscope, as shown in Table I, many workers have chosen arbitrarily to ignore them. It is true that on a weight basis these particles may only represent a small fraction of the total dispersed phase. On the other hand, when considered in terms of surface area, or number, the small particles could have a disproportionate influence on the behavior of an emulsion system, as noted by Sherman (56, 57, 292, 293). The problem can therefore be resolved into one of deciding which method of particle-size analysis to utilize; it is clear from this review that no one method in isolation is adequate to cover the wide range of particle sizes which occur in emulsion systems.

This problem was considered by Groves, Scarlett, and Freshwater (277) who determined the particle size of a model emulsion system by a variety of experimental techniques. An appreciable amount of submicron material was found to be present and results obtained are shown in Fig. 6. It is useful to remember when comparing results by different methods that different particle-size parameters are often measured. For example the light and electron microscopes both measure a cross-sectional diameter by direct observation or measurement of a photographic image. The Coulter counter measures the volume of the particle, and the result is derived as the diameter of a sphere of equivalent volume. Similarly, sedimentation methods yield a Stokes' diameter, which is the diameter of a sphere of identical density sedimenting under streamline-flow conditions at the identical velocity. Although it is generally assumed that emulsion droplets are spherical this may not always be the case (65, 298, 299). Nevertheless, emulsion particles are approximately spherical and the different derived parameters representing the particle diameter would be expected to show a reasonable measure of agreement. As will be

seen from Fig. 6, in this system there is general agreement above 1 μ, with the light microscope results tending to give a slightly higher size. This may be due to the difficulties previously noted of resolving and measuring particles below 2 μ. There is good agreement on the size of the largest particles present. The electron microscope results diverge but this was explained by sampling difficulties, the area presented by the electron photomicrograph field being 10 × 12 μ. The chance of sampling a 4-μ diameter particle is clearly reduced so that it would require an inordinate number of photographs or samples before this method would give results consistent with those from other methods.

However, it is below 1 μ that the methods show their greatest divergence. The system contained particles down to at least 0.02 μ diameter, well below the limiting discrimination level of the Coulter counter or light microscope. Rajagopal (23), recognizing that submicron particles could exist in an emulsion system, suggested that these could be determined by extrapolation of a log-probability plot and Batch (115) suggested a similar process. However, as can be seen from Fig. 6, extrapolation of both the Coulter counter and the light-microscope results yields a serious under estimate of the size of the smallest particles. The extrapolation techniques are clearly untenable for the model system examined by these authors and the validity of the approach was questioned. Comparing all these results with those obtained by the centrifugal photosedimentometer, it will be seen that this instrument

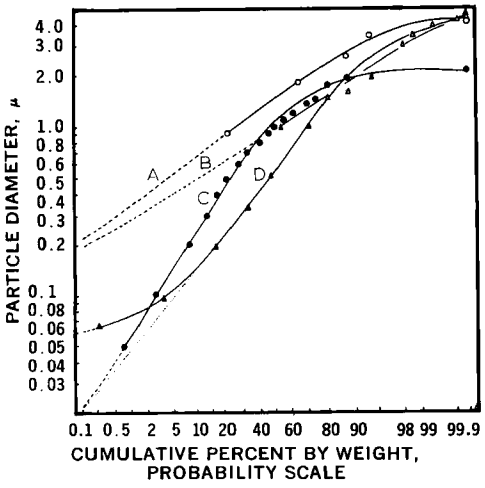

Fig. 6—The particle-size distributions of a model emulsion system determined by: A, light microscope; B, Coulter Counter; C, electron microscope; D, centrifugal photosedimentometer. Extrapolations are shown as broken lines. [Reproduced with permission from Proc. Particle Size Anal. Conf., *Loughborough 1966, paper No. 22, p. 281.]*

was capable of detecting the majority of submicron particles known to be present in the model system. The size range indicated by the instrument depends upon the particle sedimentation velocity and is therefore probably realistic. On the other hand, the quantitative measurement depends on the light-scattering properties of these small particles which are less predictable as they approach the magnitude of the wavelength of the incident radiation, resulting in overemphasis of the fines. In addition, as pointed out by these authors elsewhere (246), there is a marked tendency for small particles to adhere to the larger ones. Divergence from the electron microscope results may therefore be also due to inadequate dispersion of the system before sedimentation.

From this investigation it was concluded that the Coulter counter and centrifugal photosedimentometer offered the best combination at present available for carrying out an adequate measurement of the relatively wide size distributions that can exist in some emulsion systems. This conclusion has now been explored in more detail (278). The centrifugal photosedimentometer is limited to particles below about 10 μ diameter since above this size sedimentation is no longer under streamline-flow conditions, and a realistic lower limit is approximately 0.05 μ. The Coulter counter was used with a 70-μ orifice tube covering the range 1–25 μ. Using the two methods together on emulsion systems stabilized with a wide range of surface-active agents showed that in most cases on a log-probability plot the results were parallel over the common range of particle size. This enabled a log-probability plot to be built up over the whole of the distribution by a normalization process which was shown to be statistically valid. The particle-size distribution was therefore defined as the midpoint of the log-probability distribution lying between 20 and 80% probability (22, 28–30). As noted earlier, this method is a relatively insensitive method of presenting results. However, when considering such factors as sampling error and repeatability of a particular size analytical method it must be regarded as satisfactory and represents a considerable advance over many methods used before.

Examination of the flow behavior of emulsion systems for which the particle sizes had been determined in this manner suggested that the relationship between these two properties may be more complex than has been indicated from earlier studies (6). The ideal situation has not yet been reached whereby it is possible to predict emulsion behavior from a knowledge of the

particle-size distribution. However, with improved methods which will give a reasonably accurate picture of the state of dispersion further investigations may well allow prediction of rheological properties with greater precision. Similarly, as noted by Garrett (297), the study of emulsion stability problems would also be assisted by the use of improved methods. The evaluation of emulsification equipment (147) and control of production batches of pharmaceutical emulsion are other examples where, in the long run, a more complete understanding of the quantitative relationship between the particle size and performance may be beneficial. Similarly, emulsion formulation studies would be materially assisted if the effects of surface-active agents, both quantity and quality, and the concentration and type of disperse phase could be predicted with greater accuracy than the rule-of-thumb calculations which are all too prevalent in everyday practice. Becher's excellent text (1) shows that there is a vast fund of knowledge of emulsions; nevertheless there is still room for improvement in many areas and it has been the purpose of this review to highlight just one of these.

"For out of olde fieldes, as men seith
Cometh al this newe corn froe yeer to yere;
And out of olde bokes in good feith,
Cometh al this new science that men lere." (300).

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Keyphrases

Emulsion systems—particle size
Particle size—analysis methods
Instruments—particle-size determination
Homogeneous suspensions—theory
Light scattering—particle concentration measurement

Drug Release from Compressed Hydrophilic Matrices

By H. LAPIDUS* and N. G. LORDI

This study is concerned with the release of drugs from the planar surface and the whole surface of a hydrophilic polymer tablet matrix. The sustained drug-release characteristics of hydroxypropyl methylcellulose 15,000 cps. previously described was found to be the result of a hydration layer being formed at the tablet surface. As long as the integrity of the hydrated polymer was maintained, the release of drug was diffusion controlled. Environmental conditions have been investigated. Diffusion coefficients for the drugs used were calculated by means of the model equations predicting drug release from inert matrices and ointment bases. Comparison of the rates of drug release from the planar surface of a compressed polymer matrix with theoretically predicted values was made.

A PHYSICAL BARRIER is used in the majority of prolonged-action dosage forms to decrease the rate of drug release to the absorption site. One type of prolonged action tablet can be prepared by compressing a mixture of a hydrophilic polymer and drug (1). In contact with moisture, the tablet swells, effecting delayed release of drug. Huber, Dale, and Christenson (2) proposed that drug release was controlled both by drug diffusion through and attrition of the gel sheath formed around the tablet. However, their data precluded quantitative interpretation of the exact mechanism of release.

Recently, the authors reported data characterizing the release of chlorpheniramine maleate from a hydroxypropyl methylcellulose ether matrix (3). Release patterns measured from one face of flat-surfaced tablets could be linearized when plotted as a function of the square root of time. This suggested to us that equations derived by Higuchi (4) and Higuchi (5) for drug release from insoluble matrices and from homogeneous ointments were applicable to this system. A question was also raised concerning the significance of the rate of water penetration through the hydrated matrix as a factor in controlling drug release.

Further data are presented here to establish the extent of application of the Higuchi equations to the availability of both water-soluble and insoluble drugs from hydratable matrices. In addition, the effect of temperature, added diluent, and polymer type on release patterns measured from plane surfaces and whole tablets is reported. Whole-tablet release data were utilized to estimate the relative contributions of attrition and diffusion to drug availability from this type of dosage form. A technique for measurement of drug release from the fully hydrated polymer was also developed in order to ascertain the relative importance of the rate of water penetration and drug diffusion in the gel matrix.

EXPERIMENTAL

Materials—A hydroxypropyl methylcellulose ether 90 HG,[1] 15,000 cps. was selected for detailed study as a base material because of its demonstrated effectiveness in retarding drug release from tablets (2). The other polymers employed as base materials were hydroxypropyl methylcellulose 90 HG, 25 cps., methylcellulose MC, 4,000 cps., sodium carboxymethylcellulose (Hercules Company, Wilmington, Del.), carpolene,[2] and polyvinylpyrrolidone K30 (General Aniline and Film Corp., New York, N. Y.).

Table I lists the drugs employed in this study. Included are the solubilities measured at 37°, the wavelengths used for their spectrophotometric assay, and the moles of drug required to produce an absorbance value of 1.00 in 1 ml. of water. Beer's law was followed in all instances. Chlorpheniramine maleate (CPM) and sodium salicylate were included as examples of water-soluble drugs; benzoic acid and benzocaine were representative of water-insoluble drugs.

Lactose (USP) and tricalcium phosphate (NF) were added as diluents. Neither the diluents nor gums, with the exception of carpolene, interfered with the spectrophotometric analysis. An absorbing species was extracted from carpolene matrices necessitating blank corrections.

Drug Release from Tablets—Tableting granulations were prepared by mixing the drug with the base material and granulating with USP ethanol, except for tablets where polyvinylpyrrolidone and carpolene were used as bases. In these cases, the powders were blended and compressed directly.

Received February 16, 1968, from the College of Pharmacy, Rutgers—The State University, Newark, NJ 07104
Accepted for publication May 3, 1968.
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[1] Methocel 90 HG, Dow Chemical Co., Midland, Mich.
[2] Carbopol 934, B. F. Goodrich Chemical Co., Akron, Ohio. Carpolene is the title under which this article was provisionally admitted to National Formulary XIII.

TABLE I—SOLUBILITIES AND ABSORBANCE OF DRUGS USED IN THIS STUDY

Drug	Solubility (37°) g./ml.	Wavelength of Maximum Absorbance, mμ	Moles/ml. × 10^7 to Obtain Absorbance of 1.0000
Chlorpheniramine maleate[a]	0.6100	262	1.72
Sodium salicylate[b]	0.6850	294	2.84
Benzocaine[c]	0.0012	282	0.58
Benzoic acid[d]	0.0042	274	13.60

[a] Roselle Labs—USP. [b] Merck Co.—reagent. [c] Merck Co.—NF. [d] New York Quinine Co.—USP.

In some experiments with hydroxypropyl methylcellulose 90 HG, 15,000 cps. hydroalcoholic solutions were used to granulate in order to assess the effect of initial water content on drug-release patterns. Water was added to the USP ethanol in varying concentrations (10 and 20%), and all of the solution was used for the granulation. Increased water concentration in the granulation did not provide any observable effect in drug release from the tablet matrix.

Details of the tableting procedures were previously reported (3). Compression forces of both 3,000 and 10,000 lb. were utilized. However, no significant difference in drug-release patterns was observed from tablets of differing density. This observation has been confirmed by Huber and Christenson (6).

The techniques, equipment, and composition of the solvents used to measure drug release from both the single face of a tablet and whole tablets were previously described (3). Flat-faced tablets of 1.11 cm. ($^7/_{16}$ in.) diameter, weighing 300 mg., were used in all experiments. In cases where the drug concentration was altered or where diluents were added, the polymer concentrations were increased or decreased to produce a 300-mg. tablet.

Two-layer tablets were prepared for some experiments in which the inner layer contained drug and the outer layer was a placebo. This was accomplished by lightly pretamping the drug layer initially placed in the die, adding the placebo granulation, and then compressing the combined granulations.

Drug Release from Gels—Predetermined amounts of drug, diluent, and hydroxypropyl methylcellulose 90 HG, 15,000 or 25 cps. were added to a tared beaker. Approximately 10 ml. of boiling water was added with careful stirring, ensuring complete dispersion of the gum and either dispersion or solution of the drug and diluents. Care was taken to ensure that the stirring did not introduce air into the system. The beaker was then covered with plastic wrap and placed in the freezer compartment of a refrigerator for 15 min., after which time the plastic wrap was removed and the total weight of the beaker and contents recorded. From these values an accurate calculation of total water content was made, thereby determining the exact gel concentration. At this time the gel was completely transparent and contained no air bubbles.

The density of the gel was measured by weighing a quantity of hydroxypropyl methylcellulose 90 HG, 15,000 cps. into a tared graduate having a ground-glass stopper. Approximately 10 ml. of boiling water was then added to the graduate and the contents stirred gently and cooled. From the final weight and volume of the gel, the density was calculated. This figure was used to determine the w/v % drug in the gel.

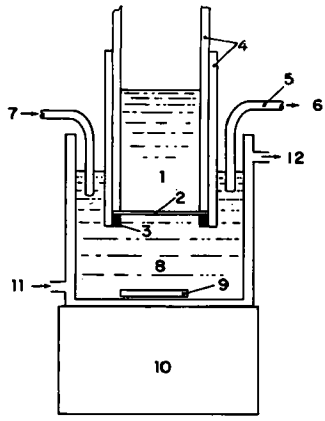

Fig. 1—Sketch of the diffusion cell used to measure drug-release rates from a fully hydrated polymer matrix. Key: 1, hydrated gel; 2, Gelman membrane; 3, Kel-F gasket; 4, Kel-F tubing; 5, Tygon tubing; 6, to Beckman flow cell; 7, from Beckman flow cell; 8, solvent; 9, stirring rod; 10, magnetic stirrer; 11, inlet for water jacket; 12, outlet for water jacket.

The gel was forced into the internal cylinder of the diffusion cell, a sketch of which is shown in Fig. 1, and cut so that it was flush with the bottom of the tube. A precut Gelman Acropor membrane, type AN-3000, pore size 3.0 μ, and 85% porosity (7) was placed over the exposed gel and kept firmly in place by an 0.31-cm. ($^1/_8$-in.) gasket composed of the same Kel-F material. A thin film of silicone grease was applied to the external walls of the inner tube and the tube and gasket forced (telescoped) into the outer sleeve of polymer material. The cylinders were designed to ensure a tight fit in order to prevent any water seepage between the walls. The inner tube was then corked to prevent any water evaporation and the entire cell secured in a jacketed beaker containing 125 or 300 ml. of water maintained at 37°. The volume of solvent was varied, depending on the concentration and absorbancy of the drug being studied. The water level was maintained just above that of the gel plug. The beaker rested on a magnetic stirrer, and a stirring bar was rotated in the beaker at a rapid rate in order to ensure adequate mixing. The release medium was cycled through the flow cell of the spectrophotometer and a continuous recording of the drug release obtained.

Analysis of Results—In applying the Higuchi model to release of drug from a compressed hydrophilic matrix, water penetration is visualized as a front moving into the tablet, hydrating the polymer, and dissolving the active material, which then diffuses out through the swollen matrix. If the drug has limited water solubility such that it has not completely dissolved when the polymer is hydrated, then diffusion will commence from a saturated solution. The expression describing drug release from the single face of a tablet would be (4)

$$\frac{W_r}{t^{1/2}} = S\left[D'\epsilon C_s\left(\frac{2W_0}{V} - \epsilon C_s\right)\right]^{1/2} \quad \text{(Eq. 1)}$$

where W_r is the amount of drug released in time t, W_0 is the dose of the drug, S is the effective diffusional area, V is the effective volume of the hydrated matrix, C_s is the solubility of the drug in the release medium, ϵ is the porosity of the hydrated matrix, and D' is the apparent diffusion coefficient of drug in the hydrated matrix. The following expression relates D' to the actual diffusion coefficient, D, of the drug in the release medium: $D' = D/\tau$ where τ is the tortuosity of the hydrated matrix.

If the drug has completely dissolved when the matrix is hydrated, then the following expression should apply (5):

$$\frac{W_r}{t^{1/2}} = 2W_0\left(\frac{S}{V}\right)\left(\frac{D'}{\pi}\right)^{1/2} \qquad \text{(Eq. 2)}$$

If the assumptions made in the derivation of Eqs. 1 and 2 hold for measurements of drug release from compressed hydrophilic matrices, the right-hand side of these expressions should be constant. The experimental conditions were established so that drug release was measured in a near-perfect sink, since the concentration in the release medium was insignificant compared to that in the tablet. In addition, the percent drug released was significantly less than 30% of the initial dose, conforming to the restrictions placed on Eq. 2 by Higuchi (5). However, the S and V terms are larger than the corresponding surface area and volume of tablet prior to immersion in the solvent, owing to swelling of the matrix when hydrated.

Experimental data in the form of absorbance readings were fitted to a linear function of $t^{1/2}$ using the least mean squares criterion. Absorbance readings at 34 time intervals were obtained over a period of 115 min. for this purpose. The slopes computed in absorbance units were converted to moles/min.$^{1/2}$ using appropriate conversion factors. The variance observed for the slopes in all cases analyzed never exceeded 2% and generally were less than 1% of the slope values, indicating excellent linearity.

Although Eqs. 1 and 2 predict a zero intercept, small negative intercepts were observed in all cases, including both measurements of release from the tablets and gels. This was attributed largely to failure of the systems to immediately attain the state of diffusion described by Eqs. 1 and 2.

RESULTS AND DISCUSSION

Effect of Drug Concentration—Figure 2 shows square root of time plots for representative runs of sodium salicylate, benzocaine, and benzoic acid used as the drug contained within the hydroxypropyl methylcellulose 90 HG, 15,000 cps. polymer matrix. Although the solubilities of all the drugs tested vary, their rates of release were apparently linear with the square root of time.

In the previous publication (3) it was noted that a plot of the slopes of the lines obtained as a function of the dose of chlorpheniramine maleate in the tablet was linear up to a 25% concentration of the drug. This is reproduced in Fig. 3 along with sodium salicylate data. The conclusions drawn in that publication suggesting that Eq. 1 applies to this system were in error. Equation 1 was derived assuming $(W_0/V) \gg C_s$. In the case of chlorpheniramine and sodium salicylate, however, C_s is greater than W_0/V.

Further investigation showed that a portion of the standard tablet containing 14.4% of the tablet mass swelled to a volume of 4.34 times that of its original volume. The estimated value of $2W_0/V$ in Eq. 1, considering the swelling of the hydrated portion of the tablet, is 0.074 g./ml. in a 16.6% chlorpheniramine maleate tablet. This is much smaller than C_s (0.615 g./ml.), therefore, invalidating the application of Eq. 1 for the more water-soluble drugs.

If Eq. 2 applies in this situation, one would also expect a linear relationship as the concentration of drug is increased, all other parameters remaining constant. However, the $S/V(D')^{1/2}$ term is subject to variation as the drug-to-polymer ratio is increased. The surface-to-volume ratio would be a function of the extent to which the polymer swells on hydration and its resistance to attrition. That the hydroxypropyl methylcellulose 90 HG, 15,000 cps. resists attrition under the conditions of measurement is indicated by the linear $t^{1/2}$ relation observed at even 5:1 drug-to-polymer ratios. No significant difference in the surface-to-volume ratio was observed at the different polymer levels studied. The diffusion coefficient, being a function of the drug concentra-

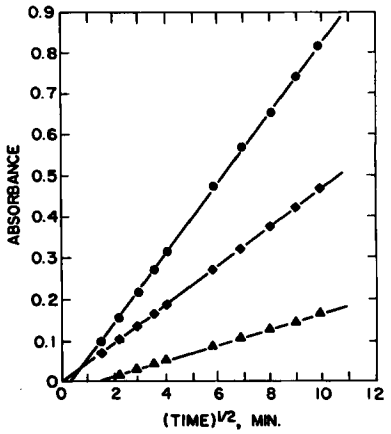

Fig. 2—Drug release as a function of the square root of time from a hydroxypropyl methylcellulose 90 HG, 15,000 cps. tablet matrix. Key: ●, benzocaine 25% w/w; ■, sodium salicylate 8.3% w/w; ▲, benzoic acid 33% w/w.

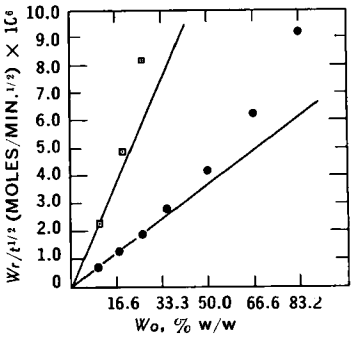

Fig. 3—Rates of drug release in water as a function of drug concentration in a hydroxypropyl methylcellulose 90 Hg, 15,000 cps. tablet matrix. Key: ⊡, sodium salicylate; ●, CPM.

tion, decreases as the concentration is increased (8). Therefore, the positive deviation in linearity shown in Fig. 3 must be the result of a change in tortuosity of the hydrated polymer. The tortuosity of a gel is an inverse function of polymer concentration (9); therefore, as the drug is increased beyond the 25% level, one could expect τ to decrease significantly. At the lower drug concentrations, τ remains essentially constant. Hence, linearity is apparently maintained, and the results conform with those predicted from Eq. 2.

The plot of $W_r/t^{1/2}$ *versus* W_0 for sodium salicylate, Fig. 3, shows a similar relationship to that for chlorpheniramine maleate except that deviation appears to occur at lower drug content. The greater apparent effect of sodium salicylate in decreasing the tortuosity of the gel may be due to the greater ability of sodium ion, compared to the chlorpheniramine ion, to dehydrate the polymer.

The effect of concentration of benzocaine and benzoic acid can be seen in Fig. 4. This result is predicted by Eq. 1. Since the C_s term is very small, *i.e.*, 1.2×10^{-3} g./ml. at 37° in the case of benzocaine, Eq. 1 reduces to:

$$\frac{W_r}{t^{1/2}} = S\left[\frac{2D'\epsilon C_s W_0}{V}\right]^{1/2} \qquad \text{(Eq. 3)}$$

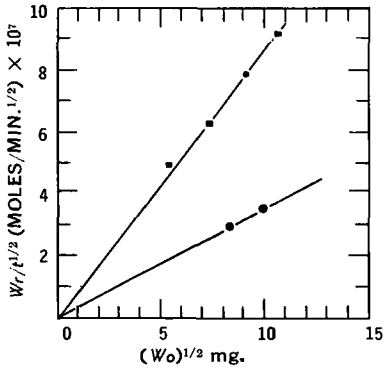

Fig. 4—Rates of drug release from 0.3-g. tablets as a function of the square root of W_0 *assuming the effective volume of the matrix is constant. Key:* ■, *benzocaine;* ●, *benzoic acid.*

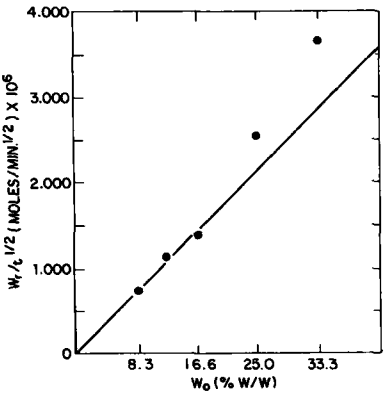

Fig. 5—Rates of drug release as a function of chlorpheniramine concentration in a hydroxypropyl methylcellulose 90 HG, 15,000 cps. tablet matrix at pH 1.5.

A linear relationship should be observed if drug-release rate ($W_r/t^{1/2}$) is plotted *versus* $(W_0)^{1/2}$ assuming that $S/V^{1/2}$ is constant. This is apparently a correct assumption since Fig. 4 shows such linearity.

Limited data were obtained in the case of benzoic acid due to the low degree of sensitivity found in the assay for benzoic acid. The concentrations of the solutions obtained from tablets containing less than 25% by weight of benzoic acid were not high enough to give acceptable absorbance readings.

A plot of drug-release rates for tablets with varying concentrations of chlorpheniramine maleate in a pH 1.5 medium is shown in Fig. 5. Comparison of these results with those shown in Fig. 3, where water was the solvent, showed a deviation from linearity at a lower drug level. Deviation occurred after 16.6% w/w of drug in 0.1 *N* HCl in comparison to 25% w/w of drug in water. These results are understandable if, in addition to the decrease in tortuosity due to the dissolution of drug in tablets with high drug levels, consideration is given to the additive effect of lowered viscosity introduced by protonation (3). In the acid medium the tortuosity has been lowered by both the decreased viscosity of the gel due to less tightly bound water molecules and the effect of drug dissolution.

Effect of Diluents—The effects of the addition of the water-soluble diluent lactose, and the water-insoluble diluent tribasic calcium phosphate, to 16.6% by weight chlorpheniramine maleate in a hydroxypropyl methylcellulose 90 HG, 15,000 cps. matrix (to be referred to as the standard tablet) can be seen in Fig. 6. The increase in specific release rates was similar for both diluents up to the addition of 33% by weight of diluent. At a dilution level of 50%, however, a marked divergence occurred which was accentuated at the 66.6% level.

The observed divergence can be explained by the difference in solubility of the diluents and their subsequent effects on the tortuosity factor. As the water-soluble diluent dissolved, it diffused outward and decreased the tortuosity of the diffusion path of the drug. On the other hand, the tricalcium phosphate did not diffuse outward, but rather became entrapped within the matrix and effected an increase in release

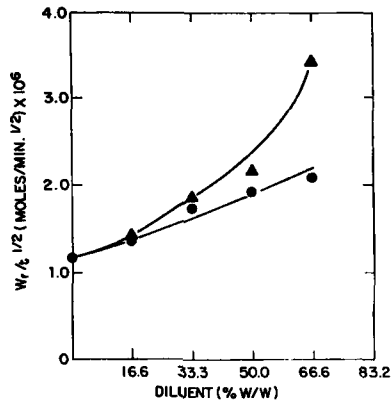

Fig. 6—Effects of addition of tablet diluents on the rate of chlorpheniramine maleate release from a hydroxypropyl methylcellulose 90 HG, 15,000 cps. tablet matrix. Key: ▲, *lactose;* ●, *tricalcium phosphate.*

TABLE II—DRUG RELEASE FROM HYDROXYPROPYL METHYLCELLULOSE HG 15,000 cps. TABLET MATRICES FROM VARIOUS SOLVENTS AND TEMPERATURES

Drug	% Drug (w/w)	Solvent	$W_r/t^{1/2}$ (Moles/Min.$^{1/2}$) × 10^6 25°	37°	50°
CPM	16.6	Water	1.05	1.16	1.24
CPM	16.6[a]	Water	1.48	1.83	2.22
Benzocaine	8.3	Water	0.43	0.47	
CPM	16.6	0.15 *M* Na_2SO_4	0.93	0.95	∞
CPM	16.6	0.20 *M* Na_2SO_4	1.06	∞	
CPM	16.6	0.1 *N* HCl	1.38	1.40	1.66

[a] The tablet base had 33.3% w/w lactose in addition to the polymer.

of drug only by the fact that its presence necessarily decreased the gum concentration.

Further evidence for these conclusions was obtained when the hydrated portions of the following three tablets mentioned were removed from the tablet after 2 hr. of exposure to the solvent, weighed, dried, and the residue reweighed. Measurements were made in triplicate. The results for the standard tablet were 11.8 ± 0.4% residue, 8.0 ± 0.8% residue, for the tablet containing an added 16.6% lactose and for the tablet with 16.6% tricalcium phosphate 9.5 ± 0.7% residue. These results were consistent with the fact that a lower concentration of polymer produced faster drug release, and the addition of a soluble diluent acted to further increase release due to its dissolution and, therefore, complete elimination from the matrix.

Effect of Temperature—The experimental results summarized in Table II show the expected increase in release rate of all drugs tested as the temperature was increased.

For systems governed by Eq. 2, only the diffusion coefficient and the tortuosity term should be affected by an increase in temperature. Furthermore, if the work by Longsworth (10) on the temperature dependence of diffusion coefficients in aqueous solutions is considered, the diffusion coefficient for molecules of somewhat similar properties to those of the drugs used here appears to increase by about 33% when the temperature is raised from 25 to 37°. Since $W_r/t^{1/2}$ is proportional to $D^{1/2}$, a 15% increase in slope measured at 37° over that measured at 25° would result. A less than 33% increase in the diffusion coefficient would be expected when the temperature is raised from 37 to 50° since an Arrhenius-type relationship with temperature should be observed. A 10% increase in slope is observed from 25 to 37° and a 7% increase from 37 to 50° for the standard tablet. The apparent diffusion coefficient would appear to have a smaller temperature dependence than the bulk diffusion coefficient. However, an increase of over 20% in the slopes is observed when lactose is substituted for polymer. This must be attributed to a significant temperature-induced decrease in tortuosity.

In the case of an insoluble drug in suspension which would be characterized by Eq. 2 in addition to the diffusion and tortuosity terms, the solubility term (C_s) is also increased. Although the relative increase in release rate observed for benzocaine is very similar to that observed for chlorpheniramine maleate, this does not seem contradictory if consideration is given to the fact that C_s is very small. The solubility of benzocaine is 9×10^{-4} g./ml. at 25° and 1.2×10^{-3} g./ml. at 37°, both values being negligible in comparison to $2W_0/V$ (0.37 g./ml.).

It is of interest to note the effect of increased temperature on release of chlorpheniramine maleate from hydroxypropyl methylcellulose 90 HG, 15,000 cps. tablets into 0.1 *N* HCl (Table II). In relation to this experiment, it has been shown that as the gel point is approached, small increments of temperature produced greater changes in viscosity than at lower temperatures (11). Therefore, since the gel at 25° has already been disoriented, as indicated by the 30% increase in release rates over that for water, an increase in temperature to 37° showed a small effect. On the other hand, the change from 37 to 50° brought the matrix much closer to its gel point at which temperature-induced discontinuities and diminished tortuosities became exaggerated. These results illustrate the similarity between temperature, salt, and pH effects on the hydroxypropyl methylcellulose 90 HG, 15,000 cps. matrix.

Extremely rapid release of chlorpheniramine maleate was observed within minutes after the standard tablet was placed in 0.2 *M* solutions of either sodium or magnesium sulfate at 37° (Table II). As was reported previously (3), a noticeable elongation of the tablet was observed, similar in appearance to a "gum drop." This same effect was duplicated by immersing a similar tablet into water held at a temperature (95°) above the gelation point of hydroxypropyl methylcellulose 90 HG, 15,000 cps. which was reported to be 85° (12). As the gel point for a polymer of this type is reached, the bound water molecules are removed from the hydration shell.

When the concentrations of both salts reached 0.2 *M*, hydration of the polymer along with almost instantaneous dehydration of the formed gel produced a solid aggregation of polymer particles with massive discontinuities and no inhibition for outward diffusion. The effect is similar to that of decreasing the gel point of the polymer to 37° by aiding the withdrawal of water from the gelled matrix with the use of dissolved electrolytes. Proof of this conclusion was shown by running an experiment in 0.2 *M* Na_2SO_4 at 25° and obtaining a normal release pattern with no gum drop effect. As can also be seen in Table II, a similar effect was observed in 0.15 *M* Na_2SO_4 solution. Whereas 37° was a sufficient temperature to reach the gelation point in 0.2 *M* salt solutions, 50° was necessary in 0.15 *M* solutions.

Effect of Polymer Type—Hydroxypropyl methylcellulose 90 HG, 25 cps. was chosen for investigation in order to determine the effect of polymer molecular weight on drug release. Initially, this tablet was positioned so that its surface was set flush with the edge of the Kel-F holder. A plot of absorbance *versus* the square root of time for a 16.6% by weight chlorpheniramine tablet showed linearity for ap-

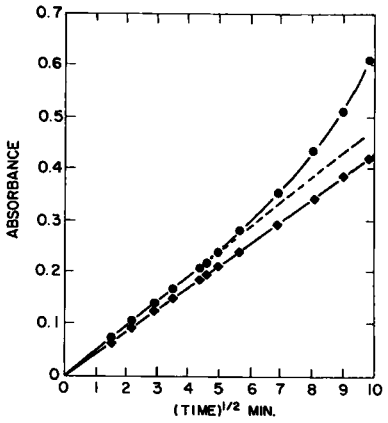

Fig. 7—Release of chlorpheniramine maleate from a hydroxypropyl methylcellulose 90 HG, 25 cps. tablet matrix. Key: ●, planar tablet surface placed flush in holder; ■, planar tablet surface recessed in holder.

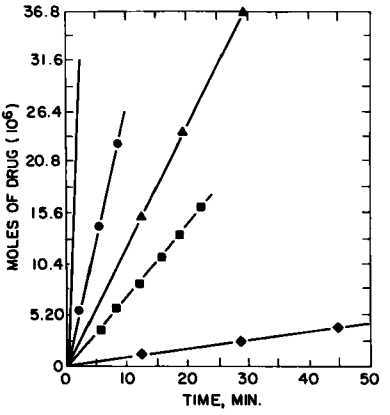

Fig. 8—Drug release from various compressed polymer matrices. Key: ●, CPM from polyvinylpyrrolidone; ▲, sodium salicylate from sodium carboxymethylcellulose; ■, CPM from methylcellulose MC, 4,000 cps.; ◆, CPM from sodium carboxymethylcellulose; ———, CPM from lactose.

proximately 25 min. An upward deviation from linearity was observed at later times as can be seen in Fig. 7. When this same tablet was recessed in the Kel-F holder and the assay procedure repeated, the drug release pattern was shown to remain linear with the square root of time for the entire 115-min. time period.

In the former case, hydration of the lower molecular weight polymer occurred rapidly, and the gum resisted attrition for the first 25 min. After this period, significant erosion of the surface due to the convective movement of the solvent past the tablet was observed. The eroded particles exposed greater surface area for drug release, resulting in a progressive increase in release rate. When the tablet was recessed 0.15 cm. in the holder, convection maintained the hydrated layer within the boundary of the holder.

Figure 8 shows the release of chlorpheniramine maleate from a tablet where lactose, polyvinylpyrrolidone, methylcellulose MC, 4,000 cps., and sodium carboxymethylcellulose had been used as the tablet matrix. The tablets contained 16.6% by weight of drug in all cases, and the release of drug maintained linearity when plotted against time at 37°.

Lactose served as a base line for a matrix which undergoes simple dissolution. The very water-soluble polyvinylpyrrolidone forms a hydrated layer that is very susceptible to attrition. The drug-release rate from this tablet was slightly less than that of lactose but by such a small degree as to indicate the formation of a hydration layer of minor significance.

The release of chlorpheniramine maleate from a methylcellulose MC, 4,000 cps. matrix was slower than that from the polyvinylpyrrolidone matrix but still maintained a straight-line relationship with time. The retarding action can be directly attributed to the formation of a hydration layer by this polymer. This hydration layer, however, did not resist attrition and remain intact. The MC variety of polymer is less hydrostable than the hydroxypropyl derivative. Consequently, attrition becomes more important than diffusion; the hydrated layer dissolved away almost as rapidly as it formed. Recessing this tablet did not alter its linear release pattern but merely reduced the slope slightly.

When the sodium carboxymethylcellulose tablet was removed from the flow cell, it was found to be misshapen and had the distinctive gum drop appearance. The outer area of the hydrated portion was clear and showed greater continuity than had been found in the previous two cases. When a tablet containing 16.6% by weight of sodium salicylate was analyzed, the same linear relationship with time was observed, although a more rapid release was apparent (Fig. 8). The significantly higher release rate of sodium salicylate as compared to chlorpheniramine maleate from the carboxymethylcellulose matrix shown in Fig. 8 indicated that the latter drug interacted with the polymer. Since sodium salicylate was an anionic drug, interaction with sodium carboxymethylcellulose was not likely, while complexation of chlorpheniramine maleate could be expected with the polymer on the basis of the work performed by Kennon and Higuchi (13).

It appears that the polymer which forms the hydration layer least susceptible to erosion and dissolution will show the greatest retarding action on drug release. If the hydrated layer remains intact, the drug diffuses through that layer, and the release pattern is linear with the square root of time. If the hydrated layer does not maintain its integrity, the release of drug will be linear in relation to time as is expected from a system of constant surface area subject to dissolution in a perfect sink.

Another polymer material used as a tablet matrix was carpolene. The advantage of this acidic polymer, as stated by Mayron (14), was that the polymer did not dissolve or hydrate in gastric juice but was neutralized and became water dispersible in the intestinal tract. The result of the neutralization was proported to increase the rate of release of the drug. In this study of carpolene rapid and continued release of chlorpheniramine maleate was obtained in 0.1 *N* HCl throughout the 115-min. period of analysis (Fig. 9). The decreased release rate observed in pH 7.5 buffer could be attributed to the increased gelation of the polymer in an alkaline media and interaction of the cationic chlorpheniramine with the polymer. The pKa of carpolene is 6.6 (15);

therefore, the polymer is almost completely dissociated at pH 7.5.

The rapid release of sodium salicylate in contrast to the slow release of chlorpheniramine maleate observed in water (pH ≈ 5.5) indicated that the latter reacted with the polymer. Precipitation of a carpolene complex with chlorpheniramine was observed when the polymer was added to an aqueous solution of the drug.

Barrier-Layer Effect on Drug Release—Figure 10 shows the release of chlorpheniramine maleate from a standard tablet and a standard tablet with a 25- or 50-mg. barrier layer of compressed hydroxypropyl methylcellulose 90 HG, 15,000 cps. on its surface. The quantity of drug released after 115 min. for the tablet with the 25-mg. barrier layer was 18% of that which was observed with the standard tablet. In addition, a time lag of 2.2 min. was noted before drug appeared in the solvent. The release curve was linear with time rather than with the square root of time. The tablet with a 50-mg. barrier layer showed slower drug release than the tablet with the smaller barrier layer. The time lag was approximately 5 min. for this tablet. The delay in the observance of drug release is clearly due to the time required for the water to pass through the barrier layer and then for the dissolved drug to pass back through this partially hydrated layer. This delay also accounts for the apparent zero-order release rate. The drug is not depleted very rapidly from the barrier layer; therefore, this layer acts as a "buffer" zone of constant concentration and area. Since this is the case, the concentration gradient does not change, and a zero-order release rate is observed. Deviation from zero-order release should be subsequently observed. The rate will ultimately decrease as drug is depleted from the core.

The 2-min. delay before drug release is observed as an indication of the time required for the water to penetrate the additional thickness provided by the barrier layer. A thickness of the standard tablet equivalent to the barrier layer contained about 4 mg. of chlorpheniramine maleate. Since only 4.7 mg. of drug is released from this tablet after 115 min. under standard conditions, it appears that the penetration of water into the tablet is not the rate-determining step for drug release.

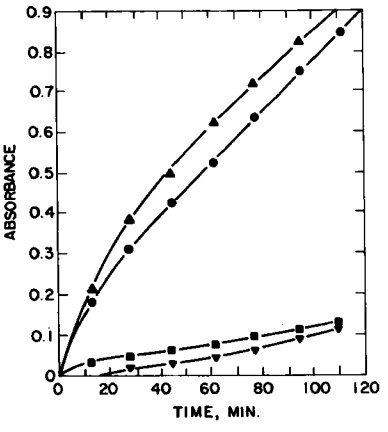

Fig. 9—Drug release from a carpolene compressed tablet matrix in various solvent media. Key: ▲, CPM, in pH 1.5 solution; ●, sodium salicylate in water; ■, CPM in pH 7.5 buffer; ▼, CPM in water.

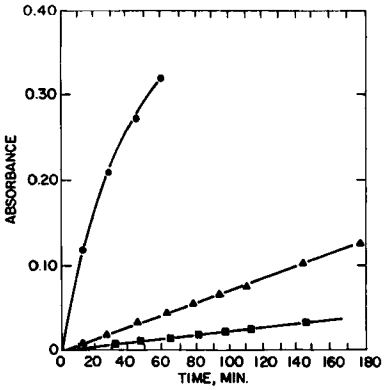

Fig. 10—Effect of a barrier layer on chlorpheniramine maleate release from the planar surface of a standard tablet. Key: ●, standard tablet; ▲, 25-mg. barrier layer; ■, 50-mg. barrier layer.

Another indication that water penetrability is not the rate-determining step is shown by Higdon and Robinson (16) whose calculation of the diffusion coefficient for water through an 8% sodium carboxymethylcellulose gel was 2.06×10^{-5} cm.²/sec. A value for the diffusion coefficient of benzoic acid was given by Higuchi *et al.* (17) as 1.1×10^{-5} cm.²/sec. Therefore, the diffusion coefficient of water in the polymer is about twice the value of a typical drug investigated in this study.

Diffusion Measurements from a Hydrated Polymer—The objective in making diffusion measurements in a fully hydrated hydroxypropyl methylcellulose 90 HG, 15,000 cps. polymer gel was to establish that diffusion was the controlling mechanism in drug release from the tablet. If the diffusion coefficients of the drugs used in this study could be obtained, then by substitution into Eqs. 1 and 2, it would be possible to confirm the application of these equations to drug released from the plane surface of a tablet matrix without attrition.

The data obtained are summarized in Table III. Drug-release rates from a fully hydrated polymer gel were evaluated for all the drugs used in the tablet study. The relative effects were similar to those observed with tablets.

Since $W_r/t^{1/2}$ equals the slope obtained from this particular system, the following equation may be used to calculate the apparent diffusion coefficient of water-soluble drugs in the gel.

$$D' = \frac{\pi}{60}\left(\frac{\text{slope}}{2S \cdot C_0}\right)^2 \qquad \text{(Eq. 4)}$$

where C_0 is the initial concentration of drug in the gel in moles/ml. The cross-sectional area of the diffusion cell was 0.094 cm.². The Gelman membrane had a porosity of 85%; hence, the actual diffusional area (S) was 0.59 cm.².

Extrapolation of a plot of D' as a function of polymer concentration indicated an approximate diffusion coefficient for chlorpheniramine maleate of 8×10^{-6} cm.² sec.$^{-1}$, which seemed reasonable for a molecule of its molecular weight. Approximate diffusion coefficients and tortuosities were calculated using this value and are listed in Table III.

The relative values of the experimentally observed release rates for the soluble drug are understandable in terms of τ values obtained for each diffusion

TABLE III—DRUG RELEASED FROM A FULLY HYDRATED HYDROXYPROPYL METHYLCELLULOSE 90 HG MATRIX IN WATER AT 37°

Drug	Polymer	% Drug (w/w)	% Polymer	$W_r/t^{1/2}\ S$ (moles min.$^{-1/2}$ cm.$^{-2}$)	D' (cm.^{2} sec.$^{-1}$ × 10^6)	τ	D (cm.^{2} sec.$^{-1}$ × 10^6)
CPM	Viscosity grade, cps.						
	15,000	5.27	2.6	3.26	5.37	1.49	8.0
CPM	15,000	5.35	5.4	2.65	3.45	2.32	8.0
CPM	15,000	5.34	8.0	2.34	2.69	2.97	8.0
CPM	15,000	5.28	10.6	2.02	2.04	3.92	8.0
CPM	15,000	5.34	15.7	1.68	1.40	5.70	8.0
CPM	15,000	2.73	10.9	1.13	2.55	3.92	10.0
CPM	15,000	4.08	10.9	1.55	2.07	3.92	8.1
CPM	15,000	5.28	10.6	2.02	2.04	3.92	8.0
CPM	15,000	6.47	10.3	2.43	1.94	3.92	7.6
CPM	15,000	7.99	10.7	2.95	1.89	3.92	7.4
Sodium salicylate	15,000	5.35	10.7	7.54	4.68	3.92	18.3
Benzoic acid	15,000	5.20	10.4	5.37	14.66	3.92	57.5
Benzocaine	15,000	5.20	10.4	5.48	0.94	—	—
CPM	25	5.23	10.5	2.24	2.53	3.16	8.0
CPM	25	5.22	15.7	1.73	1.50	5.33	8.0
CPM	25	5.37	21.4	1.62	1.29	6.2	8.0

measurement. An increase in the polymer concentration showed a corresponding increase in tortuosity for the same concentration of drug. If the τ value is assumed to be independent of drug concentration, the data indicate an overall decrease in diffusion coefficient of about 25% as the chlorpheniramine maleate concentration is increased from 2.7 to 8%. In addition, the lower expected tortuosity of the 25 cps., as compared to the 15,000 cps. hydroxypropyl methylcellulose 90 HG, was confirmed.

An approximate diffusion coefficient of 18.3 × 10^{-6} cm.^{2} sec.$^{-1}$ at 37° was indicated for a 5.35% sodium salicylate solution. This value compares favorably with the value reported by Desai *et al.* (18) which was 10 × 10^{-6} cm.^{2} sec. at 30° for a 6.8% solution.

The following form of Eq. 1 was used to calculate the apparent diffusion coefficients of the insoluble drugs in the gel.

$$D' = \frac{1}{120 \times C_s \times C_0}\left(\frac{\text{slope}}{S}\right)^2 \text{cm.}^2 \text{ sec.}^{-1}$$

The diffusion coefficient of benzoic acid, estimated using the τ value which characterized a 10–11% gel, is too large. Higuchi *et al.* (17) report a value of 11.1 × 10^{-6} at 30°. This suggested that the τ values characterizing release of benzoic acid and benzocaine from the gel were close to unity. This did not seem unreasonable when the length of the diffusional path was considered. A greater amount of drug is concentrated in the surface layer of the gel in the suspension case. In the same 2-hr. period, molecules diffusing from a suspended solid phase had a much shorter distance to travel and, therefore, a less tortuous path than molecules diffusing from a solution. It is also reasonable to assume that as the concentration of the solid phase is increased, the tortuosity value should be progressively lowered.

The estimated diffusion coefficient of benzocaine, 9.4 × 10^{-7}, is too low and cannot be considered the true diffusivity for this drug. Since benzocaine is very insoluble, solution of the drug at the gel interface becomes the rate-determining step rather than diffusion of the drug through the gel matrix. Therefore, the apparent diffusion coefficient is in error.

Correlation of Diffusion Data with Drug Release from a Tablet—Apparent diffusion coefficients derived from gel-diffusion measurements were substituted in Eqs. 1 and 2 and theoretical drug-release rates from hydroxypropyl methylcellulose 90 HG, 15,000 cps. compressed matrices calculated. The dry-tablet volume was 0.311 ml. for the standard tablets. Analysis of the swollen portion of a tablet showed that the polymer matrix swelled 4.3 times its original volume during the 2-hr. test period. The surface-to-volume ratio of the hydrated tablet was assumed constant during the course of the experiment and the lateral swelling of the tablet was ignored. The S term, or the effective diffusional area calculated on a dry basis of flat face, was 0.98 cm.^{2}. A standard tablet recessed 0.15 cm. in the holder so as to prevent lateral swelling, yielded a 10% lower slope than a tablet that was not recessed. The apparent diffusion coefficients estimated in 10.4–10.7% polymer gels were substituted in Eqs. 1 and 2. An average value of 11.8 ± 0.6% polymer was obtained when the hydrated portion of a stan-

TABLE IV—COMPARISON OF THE RATES OF DRUG RELEASE FROM THE PLANAR SURFACE OF A COMPRESSED HYDROXYPROPYL METHYLCELLULOSE 90 HG 15,000 cps. MATRIX WITH THE THEORETICALLY PREDICTED VALUES

Drug	% Drug in Tablet (w/w)	D' × 10^6	Theoretical Release ($W_r/t^{1/2}$) moles/min.$^{1/2}$ × 10^6	Actual Release ($W_r/t^{1/2}$) moles/min.$^{1/2}$ × 10^6	% Theory
CPM	16.6	2.04	1.13	1.16	103
Sodium salicylate	16.6	4.68	4.44	4.85	109
Benzoic acid	33.0	14.66	6.06	4.38	72
Benzocaine	16.6	0.94	0.42	0.47	112

dard tablet after a 2-hr. assay was removed, dried, and weighed. This polymer concentration will vary, however, depending on the drug or diluent content. Some actual and theoretical release rates are summarized in Table IV.

The actual drug-release rates obtained from the tablet containing 16.6% chlorpheniramine maleate and sodium salicylate came within 3 and 9% of theory, respectively, as calculated by Eq. 2. The theoretical value for benzocaine was 12% of the experimental value, while in the case of benzoic acid, a 28% faster release rate was predicted than was observed. This deviation reflects the high D' value calculated for benzoic acid. The agreement between actual drug-release rates and theoretical values appears to confirm the fact that diffusion is the major factor in determining drug release from a planar surface of a tablet prepared from a hydroxypropyl methylcellulose 90 HG, 15,000 cps. matrix.

Although the theoretical values predicted by Eq. 1 for insoluble drug systems are in good agreement with the actual values obtained, the error encountered in determining diffusivity must limit any conclusions that might be drawn.

Drug Release from Whole Tablets—The results obtained when drug release from a plane surface of a standard tablet was analyzed for 5 hr. showed linearity for the entire run (Fig. 11) when percent total drug released was plotted *versus* the square root of time. This established that an intact hydrated layer was maintained over this period of time; therefore, diffusion was the most important factor contributing to the rate of drug release in this system. However, when release from a whole tablet was analyzed rather than from a plane surface, linearity was not maintained, since other factors influenced the drug-release rate.

In Fig. 11 plots of percent of total drug released *versus* the square root of time show the release of chlorpheniramine maleate from the whole standard tablet. The release pattern in 0.1 *N* HCl is also shown together with drug release from a similar tablet containing 33% by weight lactose as a diluent in addition to the drug. It can be seen that the standard tablet showed a linear relationship for approximately 100 min., after which time there was a positive deviation. If diffusion were the only factor involved in drug release from the tablet, linearity would have been maintained as in the release from the plane surface.[3]

Attrition of the tablet surface accounts for the positive deviation. If attrition is considered to be the wearing away of the external surface of the hydration layer, the eroded material will go into solution quickly and provide drug in addition to that already available from diffusion.

When the standard tablet was exposed to 0.1 *N* HCl, the drug-release rate was faster than that observed in water. The tablet containing lactose showed release of drug to be somewhere in between that for the water and acid. These results are similar to those obtained in the flow cell. A whole tablet containing tricalcium phosphate was also

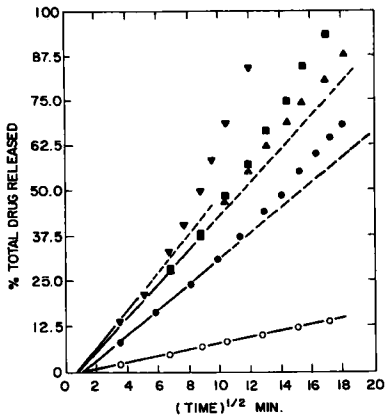

Fig. 11—Chlorpheniramine maleate release from a whole tablet. Key: ▼, hydroxypropyl methylcellulose 90 HG, 25 cps.; ■, standard tablet in pH 1.5 solution; ▲, 33.3% w/w of polymer replaced with lactose in the standard tablet; ●, standard tablet in water; ○, planar surface of a standard tablet.

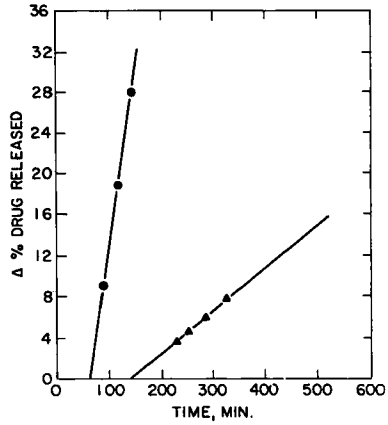

Fig. 12—Difference between the linear extension of the drug plot versus $t^{1/2}$ *and the actual drug release from a whole tablet. Key: ●, hydroxypropyl methylcellulose 90 HG, 25 cps.; ▲, hydroxypropyl methylcellulose 90 HG, 15,000 cps.*

evaluated in this manner but is not graphically illustrated. The release of drug was faster in this tablet than the standard, but this could not be numerically evaluated due to the diluent producing opacity in the circulating media. This was not encountered in measurements from plane surfaces. Therefore, the fact that attrition of the hydrated layer is important in determining release from the whole tablet was confirmed.

The difference between the linear extension of the drug release plot *versus* $t^{1/2}$ and the actual drug-release data on Fig. 11 was plotted for hydroxypropyl methylcellulose 90 HG, 25 cps. and hydroxypropyl methylcellulose 90 HG, 15,000 cps. matrices. A straight line is obtained in both cases (Fig. 12). Assuming that the deviation in drug release from linearity is considered the result of attrition, it is not possible to determine the relative contribution made by this factor drug release. By extrapolating the data in Fig. 11, it was determined that the

[3] Higuchi (4) has described a theoretical treatment of drug release from a spherical pellet of a homogeneous matrix. He concluded that for the first 50% of release there should be no deviation between a sphere and a plane of the same area. Thereafter, the rate of release would be less from a sphere. The swollen standard tablet maintained essentially cylindrical geometry under the conditions of measurement.

hydroxypropyl methylcellulose 90 HG, 15,000 cps. matrix would release 82% of drug through diffusion in approximately 9.5 hr. Attrition, based on the slope obtained in Fig. 12, would contribute 18% drug at 9.5 hr. Similarly, the hydroxypropyl methylcellulose 90 HG, 25 cps. matrix would be totally depleted of drug after 2.8 hr. Attrition accounts for 36% of drug release in this case while diffusion contributes the remainder. It appears from these data that the lower molecular weight polymer is approximately two times more susceptible to attrition than the higher molecular weight species.

The area of the planar surface of the tablet is 0.98 cm.^{2}. The area for the standard whole tablet is 3.06 cm.^{2}. The ratio of experimental drug-release rates between the whole tablet and the planar surface was 4:1, while the ratio for the dry, unhydrated surfaces was 3:1. These results are not unexpected if the hydrated surface is considered in both cases. Hydration of the planar surface results in essentially a projection of the same area. Hydration of the whole tablet, however, changes the tablet diameter resulting in an increase in the area of the planar surface in proportion to the square of the radius. In addition, the increase in the circumference provides more area to the rim of the tablet in proportion to the radius. The added diffusional area resulting from hydration would account for the increased drug release observed over that expected from the unhydrated surface.

Chlorpheniramine maleate release patterns obtained from methylcellulose MC, 4000 cps. tablets appear to be completely dependent on attrition since a straight-line relationship holds when drug release is plotted against time.

It would appear that since the surface of the tablet was diminishing, a nonlinear release rate would be expected. However, erosion was not uniform, and fragmented particles continued to add to the drug concentration.

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Keyphrases

Matrices—compressed, hydrophilic
Polymers—compressed, hydrophilic matrices
Release rates—compressed hydrophilic matrices
Diffusion—drug from matrices
Diluent effect—drug release rates, matrices
Temperature effect—drug release rates, matrices
UV spectrophotometry—analysis
Hydroxypropyl methylcellulose tablets—hydration layer

Renal Metabolizing Activity Studied in Dog and Monkey by the Isolated Perfused Kidney *In Situ*

By G. BENZI, F. BERTÉ, A. CREMA, and E. ARRIGONI

The drug metabolizing power of the renal tissues in an isolated *in situ* kidney preparation has been evaluated in the dog and monkey. Under these experimental conditions, the kidney remained connected to the other parts of the living animal, except for blood circulation which was supplied by a pump-oxygenator system. The disappearance of the tested substances (oxazepam and aminopyrine) from extracorporeal blood was partially replaced by their metabolites. In fact, both the drugs and the metabolites were present at different concentrations in the excreted urine and in the cortical and medullar renal tissues.

IN PREVIOUS PAPERS (1–3) the techniques were described for the perfusion of isolated areas of the body (liver, brain, uterine–fetal complex) to study some of their drug-metabolizing activities, such as demethylation, acetylation, and glucuronide conjugation.

In a systematic investigation (4, 5) on drug

Received March 18, 1968, from the Departments of Pharmacology, Pavia and Pisa Universities, Italy.
Accepted for publication May 2, 1968.
The authors are grateful to P. Mascherpa, Department of Pharmacy, University of Pania, under whose direction the program of the present research was developed.

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Renal Metabolizing Activity Studied in Dog and Monkey by the Isolated Perfused Kidney *In Situ*

By G. BENZI, F. BERTÉ, A. CREMA, and E. ARRIGONI

The drug metabolizing power of the renal tissues in an isolated *in situ* kidney preparation has been evaluated in the dog and monkey. Under these experimental conditions, the kidney remained connected to the other parts of the living animal, except for blood circulation which was supplied by a pump-oxygenator system. The disappearance of the tested substances (oxazepam and aminopyrine) from extracorporeal blood was partially replaced by their metabolites. In fact, both the drugs and the metabolites were present at different concentrations in the excreted urine and in the cortical and medullar renal tissues.

IN PREVIOUS PAPERS (1–3) the techniques were described for the perfusion of isolated areas of the body (liver, brain, uterine–fetal complex) to study some of their drug-metabolizing activities, such as demethylation, acetylation, and glucuronide conjugation.

In a systematic investigation (4, 5) on drug

Received March 18, 1968, from the Departments of Pharmacology, Pavia and Pisa Universities, Italy.
Accepted for publication May 2, 1968.
The authors are grateful to P. Mascherpa, Department of Pharmacy, University of Pania, under whose direction the program of the present research was developed.

metabolism and tissue distribution, the present research was extended to the metabolizing activity of the kidney isolated *in situ* in living animals.

The techniques of the perfusion *in vitro* of isolated kidney suspended in a special box and placed in an extracorporeal circuit have been utilized for about a century (6, 7).

The present research, instead, utilizes the concept of Creech *et al.* (8) that various anatomic regions of the living animal can be isolated from the general circulation, supplied with a separate extraneous system for pumping and oxygenating the blood, and maintained for varying periods of time under these conditions without significant damages.

To test the metabolizing activity of the kidney isolated *in situ*, some transformations of oxazepam (glucuronide conjugation) and of aminopyrine (demethylation and acetylation) have been examined.

METHOD

The simple abdominal technique of kidney perfusion *in situ* was carried out on 39 dogs (9.8–18.8 kg.) and six monkeys (*C. hamadryas:* 7.8–13.8 kg.) preanesthetized with urethan (0.4 g./kg. i.p.). Anesthesia was induced and maintained in closed circuit by nitrous oxide, cyclopropane, or ethyl ether.

The animals were given artificial ventilation by a tracheal Warne tube placed during succinylcholine chloride action (1 mg./kg. i.v.). Arterial blood pressure was measured from a cannula inserted into a femoral artery and connected with a Polygraph-Schwarzer recorder.

After laparatomy, the left kidney and the upper part of the left ureter were exposed; the peritoneal sheath around the left ureter was opened 3–6 cm. from the renal pelvis to expose the ureter and the vessels of the surrounding fat. All the vessels were ligated and a polythene catheter was gently inserted in the ureter, pointing towards the renal pelvis.

The left adrenal gland was removed and the adrenal vessels ligated. The left renal artery and vein were isolated from the surrounding fat and a chain of loose ligatures placed around the vasal connection of the renal area. Both isolated left renal artery and vein were ligated, cannulated, and connected with a pump-oxygenator system; the ligatures around the vasal renal area were tightened to block the communications between the left kidney and the remainder of the body. The time from clamping of the renal artery until the start of perfusion was between 3 and 6 min.

The renal perfusion apparatus employed (9) consists of a venous reservoir, an oxygenator with gas meter, a roller-type pump with flow meter, a glass-wool or a polyester staple blood filter, an apparatus to eliminate blood foam, a blood exchanger with telethermometer, and a perfusion pressure regulator connected with a Polygraph-Schwarzer recorder.

Before the extracorporeal kidney perfusion, the pump-oxygenator system was filled with 400–600 ml. of heparinized blood drawn from donors. The collected blood was filtered through cloth, preserved by ampicillin (1:10,000), and stored in a refrigerator. The perfusions were made with (*a*) whole blood, (*b*) blood diluted with Tyrode solution (3:1), (*c*) blood diluted with Tyrode solution (3:1) and added to glucose (10%), (*d*) blood diluted with Tyrode solution (3:1) and added to both glucose (10%) and cyproheptadine (5–10 mcg./ml.) plus dibenamine hydrochloride (2.5–5 mcg./ml.). When whole blood was used, this was obtained from a donor 20 min. prior to use, to prevent the accumulation of lactic acid which would require a subsequent large neutralization. Before the perfusion, the heparinized blood, compatible with that of the subject, was filtered through glass wool or polyester staple, and adjusted to pH 7.35 using 1 *M* sodium bicarbonate. The priming blood was circulated through the pump-oxygenator system fully oxygenated and warmed. A flow of O_2 or O_2–CO_2 mixture (95:5) into the oxygenator was maintained at the rate of 4–6 l./min.; at the beginning of the extracorporeal renal perfusion, the blood flow was kept at the rate of 5–7 ml./kg./min. related to a pressure equal to the initial systemic pressure of the animal (90–145 mm. Hg). The time of kidney perfusion was limited from 60 to 120 min. Blood samples from extracorporeal circuit were collected every 15 min. for determination of hematocrit, clotting time, arterovenous blood oxygen, pH, and plasma electrolytes (Na, Cl, K). The urine from isolated and perfused kidney was collected and measured every 15 min. for determination of flow, pH, and electrolytes excretion (Na, K, Cl).

The metabolizing activity of the kidney isolated *in situ* was investigated by (*a*) the glucuronide conjugation, by evaluating the transformation of oxazepam to glucuronide (10) after addition of 10–20 mcg./ml. of oxazepam into the extracorporeal circuit, (*b*) the demethylation, by evaluating the transformation of aminopyrine to 4-aminoantipyrine (11) after addition of 40–80 mcg./ml. of aminopyrine into the extracorporeal circuit, (*c*) the acetylation, by evaluating the transformation of 4-aminoantipyrine to *N*-acetyl-4-aminoantipyrine (11) under the conditions mentioned in *b*.

The eventual leakage of perfusate into the systemic circulation was evaluated by taking samples of blood and lymph, to verify the absence of the tested substance or of its metabolites.

RESULTS AND DISCUSSION

The most frequent observation during an extracorporeal kidney perfusion isolated *in situ*, was the steady increase of blood perfusion pressure at constant rates of flow.

In the experimental conditions, the authors preliminarly minimized one of the various responsible factors by filtration of the blood through glass wool or polyester staple to remove both platelet aggregates and fibrin clots that tend to block the microvasculature of the organs (12–14).

Table I shows occurrence and degree of the increase of the renal vascular resistance, in different experimental conditions of dog's extracorporeal kidney perfusion *in situ*. A mixture of 5% carbon dioxide and 95% oxygen, used instead of pure oxygen in the aeration of the extracorporeal blood, reduced the degree of the perfusion pressure increase, in agreement with the results of Bachrack *et al.* (15),

TABLE I—OCCURRENCE IN DOG OF INCREASE OF THE RENAL PERFUSION PRESSURE AT CONSTANT RATE OF BLOOD FLOW (5–7 ml./kg./min.) IN DIFFERENT EXPERIMENTAL CONDITIONS

Aeration in Oxygenator		Medium of Perfusion					Occurrence After 2 Hr. of the Increase of Perfusion Pressure in Comparison with the Initial Values		
				With Addition of					
O_2(100%)	O_2(95%) + CO_2(5%)	Whole Blood	Diluted Blood (3:1)	Glucose (10%)	Cypro heptadine + Dibenamine	No. of Dogs	<10%	10–30%	>30%
yes	—	yes	—	—	—	8	1	2	5
—	yes	yes	—	—	—	8	1	4	3
—	yes	—	yes	yes	—	8	2	5	1
—	yes	—	yes	yes	yes	8	2	3	3

Malette *et al.* (16), and Steyn *et al.* (17). In comparison with the whole blood, the use of the blood diluted with Tyrode solution (3:1) and added to glucose (10%) reduced occurrence and degree of the increase of vascular resistance, in agreement with the data of Dottori *et al.* (18), Harvey (19), and Steyn *et al.* (17). The use of antihistaminic, antiserotoninic, and adrenergic-blocking agents was without any effect in reducing the increase of renal perfusion pressure, in partial agreement with Steyn *et al.* (17) and in contrast with Hollenberg *et al.* (20) and Belzer *et al.* (14). During the extracorporeal renal circulation *in situ*, no significant difference was observed between initial and terminal values of both the plasma electrolytes concentration of the perfusing blood, and the urine pH and electrolytes excretion, as indicated in Fig. 1.

In the extracorporeal circuit of both dog and monkey, the decrease of oxazepam plasma levels was 52% (42–58%) in 60 min.; the drug disappearance was partially replaced by its glucuronide. Both oxazepam and its studied metabolite appeared in the urine, as shown in Fig. 1; the oxazepam urine concentration decreased with the decrease of plasma concentration as the glucuronide urine concentration increased with the increase of its plasmatic concentration.

In the extracorporeal circuit of both dog and monkey, the decrease of aminopyrine plasma levels was 35% (28–41%) in 60 min.; the drug disappearance was partially replaced by its metabolites. Aminopyrine and its demethylated and acetylated metabolites appeared in the urine as indicated in Fig. 2. Figures 1 and 2 show the cortical and medullar concentrations of the drugs and their studied metabolites after the perfusion of the kidney: the affinity for aminopyrine, or instead for oxazepam, seems similar in both animal species.

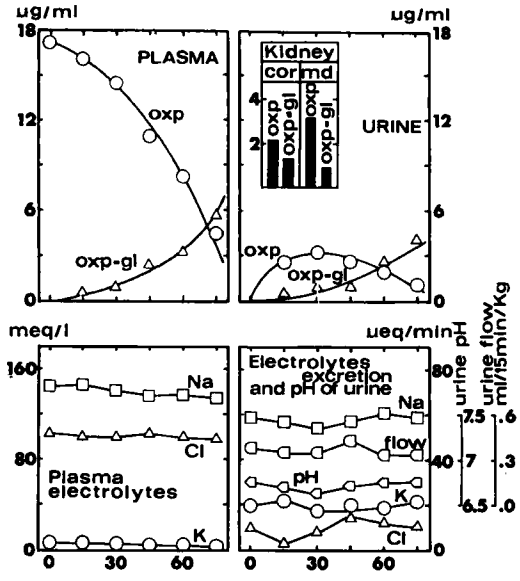

Fig. 1—Oxazepam metabolism studied by the isolated kidney perfusion in situ *in a dog weighing 18.2 kg. Blood in kidney extracorporeal circuit = 520 ml.; blood flow rate of extracorporeal circuit = 110 ml./min.; initial concentration of oxazepam in the plasma of the extracorporeal circuit = 17.5 mcg./ml. Top, at left the plasma concentration curves (mcg./ml., in ordinate) of oxazepam (oxp) and its glucuronide (oxp-gl), assayed every 15 min., for 75 min. At right, the urine concentration curves (mcg./ml., in ordinate) of oxazepam and its glucuronide; insert shows the cortical (cor) and medullar (md) renal concentration (mcg./g., in ordinate) of oxazepam and its glucuronide, evaluated 75 min. after the extracorporeal kidney perfusion with addition of oxazepam. Bottom, at left the plasma electrolytes concentrations (meq./l.) of the extracorporeal circulating blood assayed, every 15 min., for 75 min. At right, the urine pH, flow (ml./15 min./kg., in ordinate) and electrolytes excretion (meq./min., in ordinate) assayed as above indicated.*

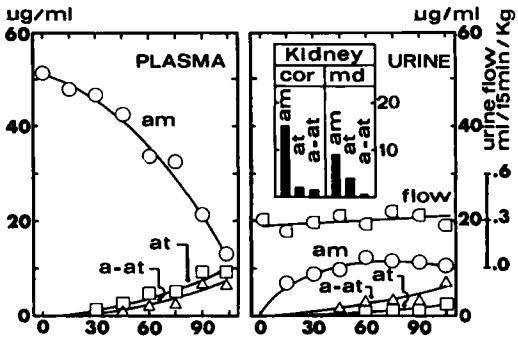

Fig. 2—Aminopyrine metabolism studied by the isolated kidney perfusion in situ *in a monkey* (C. hamadryas) *weighing 12.3 kg. Blood in kidney extracorporeal circuit = 480 ml.; blood flow rate of extracorporeal circuit = 74 ml./min.; initial concentration of aminopyrine in the plasma of the extracorporeal circuit = 51.9 mcg./ml. At left, the plasma concentration curves (mcg./ml., in ordinate) of aminopyrine (am), 4-aminoantipyrine (at), and N-acetyl-4-aminoantipyrine (a-at) assayed every 15 min. for 105 min. At right, the urine flow (ml./15 min./kg., in ordinate) and urine concentration curves (mcg./ml., in ordinate) of aminopyrine and its mentioned metabolites. Insert shows the cortical (cor) and medullar (md) renal concentrations (mcg./g., in ordinate) of aminopyrine and its two metabolites, assayed 105 min. after the extracorporeal kidney perfusion with addition of aminopyrine.*

CONCLUSIONS

The kidney isolated *in situ* shows a good metabolizing power evaluated by studying the glucuronide conjugation of oxazepam and the demethylation and acetylation of aminopyrine, both in the dog and the monkey. The tested substances and their metabolites were present in the excreted urine and in the renal tissues. During the extracorporeal renal perfusion (from 1 to 2 hr.) no significant differences were observed between the initial and final values of plasma electrolytes concentrations and urine pH, flow, and electrolytes excretion.

In comparison with the metabolizing power of other organs isolated *in situ* the kidney shows a lower activity than the liver (1) and a higher activity than the brain (2).

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Keyphrases

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Kidney, perfused—dog, monkey
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Influence of Calcium and Antiarrhythmic Drugs on Palmitate Uptake by Rabbit Heart Slices

By C. M. MOKLER* and P. P. MATHUR

Nonesterified fatty acids diffuse into myocardial cells at a rate which is proportional to the concentration of unbound acid, and are used in intracellular oxidative and synthetic reactions. Quinidine is known to inhibit cellular oxidative processes. The effects of quinidine, disopyramide, and pronethalol on oxygen consumption and palmitate uptake by rabbit ventricle slices were determined by standard Warburg manometric techniques. In the absence of Ca^{++}, all three drugs in concentrations of 10^{-5}–10^{-4} moles/l. induced marked increases in the rate of palmitate uptake from its albumin-bound form in incubation media without significant changes in tissue Q_{O_2}. This effect was not seen at drug concentrations of 10^{-6} or 10^{-3} moles/l., or when 2 m*M* Ca^{++} was present. A relationship is suggested between palmitate, Ca^{++}, antiarrhythmic drugs, and extracellular protein, which may involve the binding of fatty acid to the protein moiety.

PLASMA NONESTERIFIED FATTY ACIDS (NEFA) serve an important function in mammalian myocardial nutrition (1). They apparently diffuse across cell membranes and become available to cytoplasmic and mitochondrial enzyme systems in quantities which are related to the molar ratio of NEFA–plasma albumin (2) because of the reversible manner in which NEFA are bound to plasma albumin. Quinidine is also bound to plasma albumin (3), as is ionic calcium (Ca^{++}) (4), which is known to play an important role in cardiac function (5). Nayler (6) has shown an additional relationship between Ca^{++}, quinidine, and cell lipids. Quinidine will inhibit the movement of Ca^{++} from an aqueous phase into a chloroform phase which contains lipids extracted from myocardial membranes and

Received February 9, 1968, from the Department of Pharmacognosy and Pharmacology, College of Pharmacy, University of Florida, Gainesville, FL 32601
Accepted for publication April 25, 1968.
Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
This research was supported in part by a grant-in-aid from the Florida Heart Association.
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microsomes. A similar effect was demonstrated with pronethalol and propranolol (7) which also possess antiarrhythmic properties. These findings suggest that the antiarrhythmic drugs may modify the affinity of membrane lipids for Ca^{++}, or that they may compete with these lipids for Ca^{++}.

The work described here will show a further interaction of quinidine, pronethalol, and disopyramide with myocardial cells which results in modified uptake of palmitate by these cells, and a reversal of the effect with extracellular Ca^{++}.

METHODS

Standard Warburg manometric techniques were employed. Healthy, male, nonfasting animals were used in this study. New Zealand white rabbits were stunned by blows on the head and hearts were removed and placed in chilled buffer without substrate. Thin longitudinal slices (0.5 mm.) were taken from ventricles with a Stadie-Riggs slicer. Slices were halved and distributed equally between control and drug-containing vessels. Each Warburg reaction vessel contained 150–200 mg. of tissue in buffer containing the drug but no substrate. Each experiment included a thermobarometric control flask (no tissue) and at least four control flasks which contained tissue and buffer but no drug. Calcium levels were the same in all flasks of a given experiment. The low calcium buffers contained 88 m*M* NaCl, 5 m*M* KCl, 2 m*M* $MgCl_2$, 40 m*M* Na_2HPO_4, and either no calcium or 0.2 m*M* $CaCl_2$. The high mmoles calcium buffer differed only by having 2 m*M* Na_2HPO_4, 2 m*M* $CaCl_2$, and 124 m*M* NaCl to keep ionic strength constant. The pH of each solution was adjusted with 1 *N* HCl to 7.4 before use. The substrate solution was prepared by adding warm sodium palmitate solution dropwise with stirring to a solution of bovine albumin (Cohn's fraction V[1]), to give 3 mg. palmitate per ml. of 16% albumin solution. This mixture was refrigerated overnight and filtered at least once before use. When added from the side arm of the Warburg flask, 0.5 ml. gave flask concentrations of 5–8 μmoles palmitate and 80 mg. albumin per 3 ml. of medium, or a molar ratio of about 5:1.

The reaction vessels were gassed for 5 min. with 100% oxygen, equilibrated for 5 min. at 37 ± 0.1° and, after initial manometer readings, substrate was added from the side arm. Manometer readings were made every 15 min. for 2 hr, but calculation of oxygen consumption was based on readings from the first hour.

At the end of the incubation period, flasks were immediately chilled in crushed ice. A 2.0-ml. aliquot of medium was quickly removed from each flask, the fatty acids were extracted, and the concentration of fatty acids in each extract was determined by the microtitration method of Dole and Meinertz (8). To calculate tissue uptake of palmitate, the quantity remaining in the medium of each control and experimental flask was compared with that remaining in the thermobarometric control flask.

Tissue slices were blotted, weighed, and dried overnight at 104° before the final weighing. Oxygen consumption was expressed as Q_{O_2}, or microliters of oxygen used per milligram dry weight of tissue per hour.

Drugs used were quinidine sulfate (USP crystals, Mallinckrodt), crystalline disopyramide,[2] and pronethalol.[3] Drug concentrations ranged from 10^{-6} *M* to 10^{-3} *M*.

The data from drug-treated heart slices were compared with those from untreated control slices by Fisher's *t* test, using the 5% significance level.

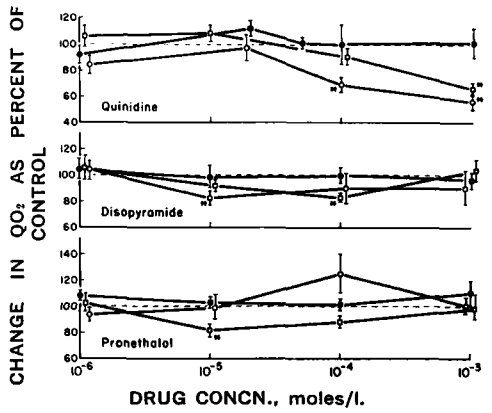

Fig. 1—Comparison of drug-treated heart slices with untreated control slices with respect to the oxygen consumption at varying concentrations of drugs and calcium ions. Asterisk (*) indicates significant difference from controls (p < .05). The vertical bar in each figure indicates the standard error of the change from control found with each treatment. Key: ●, No Ca; □, 0.2 mM Ca; ○, 02.0 mM Ca.

TABLE I—RESPONSE OF HEART SLICES TO CHANGES OF CALCIUM CONCENTRATION IN THE INCUBATION MEDIUM

Ca^{++} (m*M*)	$Q_{O_2} \pm S/E$	*p*	Palmitate Uptake, (μmol per g./hr.) ● S/E	*p*
0	5.6 ± 0.54(8)[a]		0.82 ± 0.20(8)	
0.2	4.0 ± 0.55(6)	>.05	0.75 ± 0.26(6)	>0.5
1.0	3.3 ± 0.29(6)	<.005[b]	0.66 ± 0.18(6)	>0.5
2.0	3.2 ± 0.42(6)	<.005[b]	0.60 ± 0.12(6)	>0.2

[a] Number of flasks. [b] Significant difference from control (0 calcium).

RESULTS

Figure 1 summarizes the effects of varying drug concentrations and Ca^{++} levels on the rate of oxygen consumption by rabbit ventricle slices. Significant changes in Q_{O_2} were found only with 2 m*M* Ca^{++} at quinidine concentrations of 10^{-6} to 10^{-3} *M*, and with 0.2 m*M* Ca^{++} only at 10^{-3} *M* quinidine. That this effect may be due to Ca^{++} is illustrated by Table I, which indicates a significant reduction in Q_{O_2} in untreated heart slices when incubated with 2 m*M* Ca^{++}. This reduction of Q_{O_2} was in general

[1] Palmitate and albumin from Nutritional Biochemicals Corporation.

[2] Kindly supplied by Dr. Donald L. Cook, G. D. Searle & Co., Chicago, Ill.

[3] Kindly supplied by Dr. Alex Sahagian-Edwards, Ayerst Laboratories, New York, N. Y.

not seen when disopyramide or pronethalol was present.

Figures 2–4 show how these factors altered the rate of disappearance of palmitate from the medium of Warburg flasks. The rate of palmitate uptake appears to be inversely related to the Ca^{++} concentration at the intermediate drug levels in each case, but is less dependent on Ca^{++} concentration at higher and lower concentrations of drugs. In the absence of drugs, palmitate uptake was not depressed by increasing Ca^{++} concentrations (Table I).

While reasonably small differences in Q_{O_2} and palmitate uptake were observed between control slices from any one heart, considerable variation between hearts was observed. Thus, the treated slices from any heart were compared only with untreated control slices from the same heart, and results are expressed as the observed change from control values.

Control studies revealed no significant differences between vessels containing 2 m*M* phosphate and those containing 40 m*M* phosphate with respect to either Q_{O_2} or palmitate uptake from the medium (Table II).

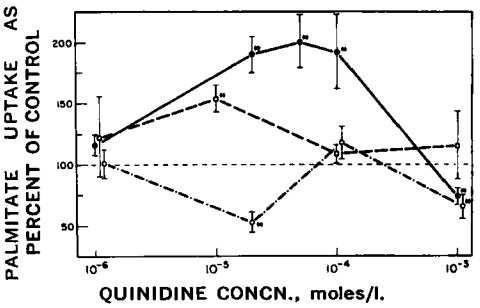

Fig. 2—Changes in palmitate uptake by heart slices as induced by quinidine at various levels of calcium. Changes in Q_{O_2} of these slices are shown in Fig. 1. Asterisk (*) indicates significant difference from controls ($p < .05$). *Key: ●, No Ca; □, 0.2 mM Ca; ○, 2.0 mM Ca.*

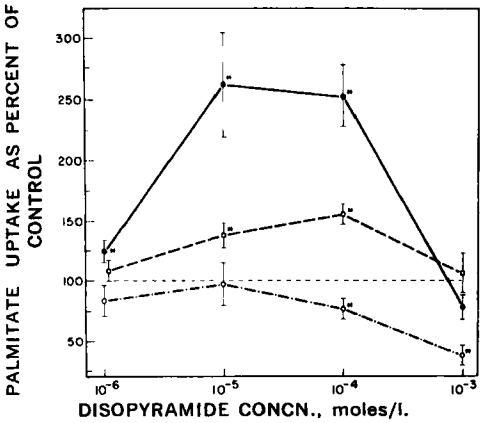

Fig. 3—Changes in palmitate uptake by heart slices as induced by disopyramide at various levels of calcium. Changes in QO_2 of these slices are shown in Fig. 1. Asterisk (*) indicates significant difference from controls ($p < .05$). *Key: ●, No Ca; □, 0.2 mM Ca; ○, 2.0 mM Ca.*

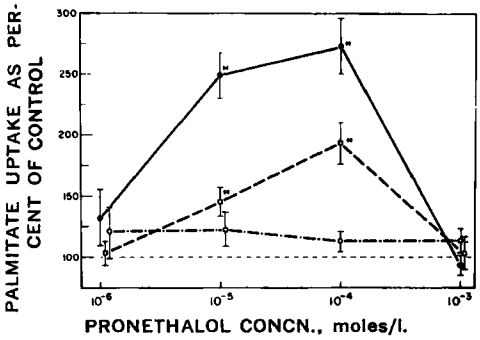

Fig. 4—Changes in palmitate uptake by heart slices as induced by pronethalol at various levels of calcium. Changes in Q_{O_2} of these slices are shown in Fig. 1. Asterisk (*) indicates significant difference from controls ($p < .05$). *Key: ●, No Ca; □, 0.2mM Ca; ○, 2.0 mM Ca.*

TABLE II—EFFECT OF PHOSPHATE CONCENTRATION ON Q_{O_2} AND PALMITATE UPTAKE BY RABBIT VENTRICLE SLICES (No Ca^{++})

Phosphate Concn.	*N*	Q_{O_2}	Palmitate Uptake (μmoles/g. tissue/hr.)
2 m*M*	6	8.2 ± 0.4[a]	2.90 ± 0.25
40 m*M*	6	8.4 ± 0.7	2.44 ± 0.30

[a] Mean ± *SE*.

DISCUSSION

As Evans (2) has indicated, the movement of fatty acids across cell membranes is probably a physical process since the rates of its loss from the medium (9) and its oxidation to CO_2 (10) appear to be dependent on the quantity of unbound or loosely bound NEFA in the medium. The experiments described here show that quinidine, disopyramide, and pronethalol will, at certain concentrations, enhance the rate of disappearance of palmitate from the medium in which rabbit ventricle slices are incubated. The effect is not present when calcium is added in concentrations (2 mmoles/l.) which approach the normal concentrations found in plasma. The increased rate of palmitate uptake is not accompanied by a parallel change in the rate of oxygen consumption by the ventricle tissue. Thus, although additional palmitate appears to be moving into the cells under these conditions, it must be used in nonoxidative or synthetic reactions of a nature which is not revealed by these experiments.

Several possible explanations of this phenomenon exist. One such explanation is related to the binding of fatty acids to plasma albumin. Goodman (11) has shown that palmitate is bound to plasma albumin in molar ratios of up to 27:1, with approximately 20 moles of palmitate per mole of protein having a relatively low association constant. If the antiarrhythmic drugs alter this binding to lower the association constant or to displace fatty acid from this binding, more NEFA will be available to be taken up by intacellular proteins (2). That quinidine is also bound to albumin (3) increases the plausibility of such an explanation, although there is no *a priori* reason to believe that albumin could not bind both fatty acid and quinidine independently.

At the higher drug concentrations where palmitate uptake is reduced to control values or below, explanations for the observed data become more speculative. It is possible that, even though palmitate is made more available for diffusion into the cells, less is taken up because of drug inhibition of intracellular enzyme systems which would normally utilize the fatty acid as a substrate in oxidative or synthetic reactions. The concentration gradient for diffusion of fatty acids into the cells would not be as great as normal under these circumstances. There is evidence that palmitate oxidation is inhibited (12) by quinidine in concentrations (greater than 10^{-4} *M*) at which the rate of uptake (Fig. 2) begins to diminish. Other workers have demonstrated inhibited oxidation of citric acid cycle intermediates (13, 14) in the presence of high concentrations of quinidine.

However, the rate of oxygen utilization (Fig. 1) did not diminish with increased quinidine concentration in those slices (no Ca^{++}) which showed the greatest change in fatty acid uptake. Nor was the Q_{O_2} much altered by disopyramide or pronethalol, both of which induced similar changes in palmitate uptake. These observations could mean that the rate of incorporation of the fatty acid into neutral lipid molecules is diminished by the higher concentrations of the drugs, but additional experiments are needed to support this theory. A more plausible explanation for the quinidine data is that quinidine inhibits endogenous substrate oxidation, but that the absence of calcium permits easier entry of palmitate into the cell where it is available for oxidation and prevents a fall in the oxygen consumption. This possibility also requires additional experimentation for support.

The role of Ca^{++} in cancelling the drug effect is also obscure. Ca^{++} itself is bound to plasma protein, to quinidine (15), and to fatty acids, and thus could significantly affect acid release from albumin in several ways.

A third possible explanation for the phenomenon may be seen in the alteration of cell-membrane permeability by Ca^{++} and the antiarrhythmic drugs. It is known that Ca^{++} and quinidine have definite effects on membrane electrical characteristics which are, in turn, dependent on the relative movements of ions across the membrane. It is possible that Ca^{++} and the antiarrhythmic drugs could alter the permeability of myocardial cell membranes to palmitate and exert their individual effects on NEFA uptake in this manner.

The alteration of palmitate uptake by heart slices which is caused by antiarrhythmic drugs in the absence of Ca^{++} ions reveals a relationship between the three factors which may have some bearing on the action of this class of drugs. The intracellular presence of quinidine and fatty acid is measurable and relatively constant during therapy, and sarcoplasmic Ca^{++} concentrations vary considerably during the cardiac cycle. If fatty acid binding to intracellular proteins varies inversely with the drug concentration, the presence of these drugs may alter the availability of the fatty acids for enzymatic processes, and thus affect the economy of the cell.

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Keyphrases

Palmitate uptake—rabbit heart slices
O_2 consumption—ventricle slices
Drug effect—O_2 consumption
Calcium effect—drug-altered palmitate uptake, O_2 consumption

Thin-Layer Chromatographic Assay of Vitamin D in High-Potency Preparations

Analysis of Fat-Soluble Vitamins IX

By K. H. HANEWALD, F. J. MULDER, and K. J. KEUNING

Attention is called to the reversible thermal isomerization of calciferols (D) to precalciferols (P) as a factor interfering with vitamin D assay. Distinction is made between potential vitamin D content (D + P) and actual vitamin D content (D only). The potential D content can be determined by the well-known Nield reaction. For the determination of the actual D content the Bolliger TLC method was modified to make it suitable for irradiation products: tachysterol is eliminated, the protection against oxidation was improved, P and D are isolated and may be determined separately. Applying this method, the isomerization rates of vitamins D_2 and D_3 were proved to be equal; the effects of boiling in solutions, saponification, and Soxhlet extraction on the ratio of P and D were examined. "Glassy" vitamin D resins appear to isomerize very slowly; "pasty" resins isomerize at the same rate as dilute solutions.

DISCREPANT RESULTS of vitamin D assays are frequent. Though admittedly the analytical techniques of the determination of ergocalciferol (D_2) and cholecalciferol (D_3) are not simple, a very real source of error is the neglect of the slow reversible isomerization, calciferol $\rightleftharpoons$ precalciferol (P) (1). It induced this laboratory to investigate the influence of the isomerization reaction on the results of vitamin D assay methods.

Potential and Actual Vitamin D Content—In vitamin D analysis the first thing necessary is to define what is meant by "vitamin D content." Distinction must be made between the *actual* vitamin D content, that is the amount of calciferol alone, and the *potential* vitamin D content, meaning the sum of calciferol and precalciferol, which is independent of the ratio of both components. The authors posit that the actual vitamin D content alone does not give any useful information and that only the potential vitamin D content is suitable for the evaluation of preparations. Simultaneous determination of both components D and P separately gives additional information, if wanted.

Isomerization of the Calciferols—Only in solutions do the calciferols isomerize reversibly to the corresponding precalciferols, forming an equilibrium mixture (2–4).

Hanewald (5) determined by ultraviolet absorptiometry the first-order rate constants for

$$P_3 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} D_3$$

and their dependence on temperature:

$$\log k_1 = \frac{-4200}{T} + 10.29 \quad \text{and}$$

$$\log k_2 = \frac{-5180}{T} + 12.53$$

(k in min.$^{-1}$; T in °K.).

From these rate constants the fractions P/(P + D) and D/(P + D) can be calculated at a given temperature, as can the time needed to approach the equilibrium (1).

From the literature it is not clear if the isomerization rates of $P_2 \rightleftharpoons D_2$ and $P_3 \rightleftharpoons D_3$ are equal. Using the analytical methods described below this equality was affirmed. Furthermore, the isomerization rates in the crude irradiation products of 7-dehydrocholesterol were investigated, which after evaporation to dryness have a resin-like consistency. Besides the commercial importance of these resins, it is of interest to compare the rates in the resins with those in solutions which seem to be temperature dependent only and with those in the solid state (crystalline state is temperature independent).

Determinations of Potential and Actual Vitamin D Content—Since too little attention has been given to the isomerization of the calciferols, few methods of vitamin D assay give reliable values either of the potential or of the actual vitamin D content. Most methods lead to results between these two values (1).

The *potential* vitamin D content can be determined directly by absorptiometry, at 500 mμ, of the pink color obtained with antimony trichloride (Nield reagent). The result is identical for D, P, and tachysterol (6). Vitamins A and E and many other polyenes interfere and are eliminated beforehand (7).

Received December 13, 1967, from the Philips-Duphar Research Laboratories, Weesp, The Netherlands.
Accepted for publication April 15, 1968.

The *actual* vitamin D content of solutions containing only D and P can be determined by an ultraviolet-absorptiometric two-component analysis. It is calculated from the absorbance at 265 mμ and the potential vitamin D content known from Nield reaction or other sources (5, 6). The P content is obtained simultaneously. Separation is not required. In solutions of irradiated ergosterol or 7-dehydrocholesterol, by-products such as lumisterols and tachysterols interfere.

Biological assay can give the potential vitamin D content, provided that the necessary precautions for equilibration of sample and standard are observed; the actual vitamin D content cannot be determined by biological assay (1).

A reliable and not too vulnerable determination of the actual vitamin D content requires the separation of D and P. Presently this can be done only by thin-layer chromatography (TLC), or in a much longer time by paper chromatography (8). Bolliger and König (9) published a useful TLC method for vitamin D assay in 1965; for previous work, most of it qualitative or semiquantitative, the authors refer to the paper (9) and to Bolliger's chapter in Stahl's handbook on TLC (10). Bolliger succeeds in separating calciferol and precalciferol; unfortunately, in an attempt to correct for its biological activity, only part of the precalciferol is included in the determination which results in values between the potential and actual vitamin D content. Isomerization of samples and standard solutions before and during the analysis is not taken into account, nor is the contamination with tachysterol, often present in vitamin D preparations, which is not separated from calciferol by TLC (11). Tachysterol gives the same absorptivity with Nield reagent but can be eliminated in the analysis of irradiation products by the addition of maleic anhydride (MA) at 75° (6).

As no practical method for the determination of the actual vitamin D content and of previtamin D was available, the Bolliger method was modified for this purpose (*a*) by collecting the P and D spots separately for quantitative analysis, (*b*) by preventing oxidation more thoroughly, (*c*) by elimination of tachysterol (TA) from the D + TA mixture isolated by TLC.

EXPERIMENTAL[1]

Chromatography—On silica plates as described below the R_f values were measured for the calciferols (D), the precalciferols (P), lumisterol (L), and tachysterol (TA), using the solvent media: cyclohexane + ether (1 + 1) (9):0.32 (D), 0.42(P), 0.37 (L), 0.32 (TA); benzene + ether (3 + 2): 0.37 (D), 0.48 (P), 0.43 (L), 0.37 (TA); benzene + ether (4 + 1) (solvent III):0.29 (D), 0.42 (P), 0.34 (L), 0.29 (TA). D and TA are not separated.

BHT (0.01%) in the solvent medium suffices to prevent oxidation during development; squalane is necessary for protection during the application. The protection is demonstrated easily with ^{14}C-labeled cholecalciferol. Using both squalane and BHT no tailing occurs, but with only BHT tailing by deterioration is evident. The developed and dried plate is stable for 1 day.

Elimination of Tachysterol—In the manufacture of vitamins D by irradiation P is formed, which is isomerized to D. The resins are obtained by evaporation of the solvent. Of the by-products, only the tachysterols interfere with the Nield reaction. Tachysterols, calciferols, and precalciferols are converted by addition of maleic anhydride into adducts indifferent to antimony trichloride, but the addition rate of TA is much larger, therefore TA can be eliminated. In the chemical assay this has been done at 75° (6). To avoid side reactions like oxidation and to simplify the analysis, this temperature was lowered to 25°, but the rate was restored by a 10-fold MA concentration. One milligram of tachysterol was dissolved in 1 ml. of 10% MA solution in benzene; 15, 30, 60, and 120 min. after dissolving, the solution was diluted with benzene and the residual tachysterol content determined with Nield's reagent.

No color was observed in any of the four experiments, thus at least 98% of the tachysterol had been eliminated within 15 min. The chosen reaction time for addition at room temperature was 0.5 hr. The same experiments with 1 mg. of pure cholecalciferol gave calciferol recoveries of 98.1, 98.6, 99.7, 99.0%. Recovery experiments with mixtures of tachysterol and calciferol gave the same results (recovery TA = 0%, recovery D = 99%).

Thin-Layer Chromatographic Determination of Actual Vitamin D and Previtamin D in the Presence of Tachysterol

Reagents and Chemicals—Benzene, thiophene-free; ether, free from peroxide and acids; dichloromethane, distilled; ethanol, 96% v/v, free from aldehyde and acids. Solvents: (I) 0.01% BHT in benzene; (II) 1% squalane and 0.01% BHT in benzene; (III) benzene + ether (4 + 1 vol.) + 0.01% BHT. Maleic anhydride (MA), freshly distilled, 10% w/v solution in benzene. Calciferol standard solution, 10 mcg./ml. crystalline cholecalciferol in solvent I (stable for at least 3 months in an amber-colored bottle in the dark, control occasionally by comparing with a fresh standard solution). Nield reagent, 20% $SbCl_3$ and 2% acetylchloride in chloroform; for preparation the authors refer to *Reference 12*.

Apparatus—Chromatoplates, 200 × 100 × 4 mm. with a thin layer (0.25 mm.) of Soxhlet extracted (24 hr. with methanol and 24 hr. with dichloromethane) Silica Gel GF 254 (Merck) and dried at 110°; vacuum collectors with porous-glass disk and ground joints; spectrophotometer, *e.g.*, Coleman Junior with 19 × 105-mm. cells; rapid-delivery pipet, 5 ml.

Procedure—Dissolve *w* g. of sample (containing approximately 50 mg. of D) in 50.0 ml. of solvent I

[1] With the collaboration of Mrs. M. Damen-van't Sant.

(for oils) or solvent II (for resins and for ethanolic solutions after evaporation to dryness *in vacuo* at room temperature with 3 × 10 ml. of benzene). Pipet 0.5 ml. of the solution on a 8-cm. line at 15 mm. from the lower edge of a chromatoplate. Develop with solvent III until the front is 17 cm. past the origin and dry the plate. Mark in ultraviolet light (254 mμ) the P region (R_f = 0.42) and the D + TA region (R_f = 0.29) and remove the light. Scratch (9) or suck (13) the silica from between the marks quantitatively into two dry collectors. Clean both open spaces on the plate with cotton wool moistened slightly with ethanol and put each plug into the corresponding collector. Extract P from its collector with 2 × 25 ml. of dichloromethane (nitrogen pressure). Evaporate extract to dryness *in vacuo* (cold), release vacuum with nitrogen, and dissolve P residue in 5.00 ml. of benzene. [According to USP XVII (14) 1,2-dichloroethane can be used instead.] Extract D + TA similarly but collect and evaporate the extract in a 50-ml. volumetric flask. Dissolve D residue in 1.00 ml. of MA solution. Keep at room temperature for 30 min. Then make up to volume with benzene.

Measurement—Pipet 1 ml. of the final benzene solution (P or D, respectively) into a cell. Add 5 ml. of Nield reagent from rapid-delivery pipet, swirl, and 60 sec. (stopwatch) after addition, measure the absorbance at 500 mμ against air ($=A$). Reagent blank: treat 1 ml. of benzene accordingly (absorbance = A_b). Standard: treat 1 ml. of standard solution accordingly (absorbance = A_s). The cholecalciferol (ergocalciferol) and the precholecalciferol (preergocalciferol) contents of the sample are:

$$\frac{C_s}{C} \times \frac{A - A_b}{A_s - A_b} \text{ mcg./g.}$$

C_s = concentration of standard solution in mcg./ml. (= 10); C = sample in the final benzene solution in g./ml. (= w/500 for P, and w/5000 for D).

Remarks—This modified determination of actual D and P simultaneously by TLC is suitable for samples containing irradiation products and vitamin D resins. However, in the determination of the actual D content the conditions starting from the moment of sampling should be such that isomerization virtually cannot occur, *i.e.*, by handling at low temperature.

By this method the actual D content in oily concentrates can only be determined in samples containing at least 6 mg. (240,000 I.U.) of vitamin D per g. of oil. At lower concentrations the oil must be removed, for which Bolliger and König (9) made suggestions. The problem is to maintain unchanged the ratio of P and D. Generally, high-potency vitamin D preparations do not contain vitamin A. Vitamin A, thus far not separated from P and D and thus contaminating the isolated spots, should be eliminated before the Nield reaction is carried out, *e.g.*, by chromatography on Florex (7, 9). The modified TLC method also gives the potential D content by adding the contents found for D and P. If only the potential D is desired both removed regions can be combined and thereafter treated as a single sample of D + TA.

APPLICATIONS AND RESULTS

In the following experiments determinations were done by the TLC method described above, by absorptiometric two-component analysis, and by Nield reaction.

Absorptiometry—The absorptivity $a(= A_{1\text{ cm.}}^{1\%})$ is independent of the solvents used here; this applies to each of the compounds D_2 (475), D_3 (485), P_2 (208), and P_3 (218). All absorbances were measured at 265 mμ, the absorption maximum of D_2 and D_3, though the absorption maximum of P_2 and P_3 is 260 mμ. The fractions of P and D can be calculated from the difference of the absorptivities (a = sample, a_D = pure D; a_P = pure P):

$$[P] = \frac{a_D - a}{a_D - a_P} \cdot 100\% \quad \text{and} \quad [D] = \frac{a - a_P}{a_D - a_P} \cdot 100\%.$$

Nield Reactions—All were performed in the same way: aliquot parts of the solutions to be analyzed were evaporated to dryness *in vacuo* at room temperature and redissolved in a measured volume of benzene to obtain a concentration near 10 mcg./ml. These solutions were treated as described in the TLC procedure, starting at "Measurement."

Isomerization by Boiling for 0.5 Hour in Various Solvents—Ergocalciferol and cholecalciferol were dissolved in cold methanol, ethanol, and isooctane and refluxed for 0.5 hr. in a nitrogen atmosphere. The absorbances of the cold solutions were measured at 265 mμ, immediately after preparation and again after refluxing. The TLC and the absorptiometric determinations were done in the same solutions. In Table I the fractions of P and D are given in % of potential D content (P + D) found. The recovery is the potential D found in % of submitted calciferol.

Saponification—Crystalline cholecalciferol was dissolved in peanut oil at low temperature. The potential as well as the actual D content = 47.5 mg./g.

Two grams of the oily solution was saponified by heating for 0.5 hr. under nitrogen with 5 ml. of 2 *N* KOH in methanol, in ethanol, or in glycol-monomethylether (purified) at 60, 80, and 100°, respectively. After cooling (nitrogen), 8 ml. of methanol and 20 ml. of water were added. The liquids were extracted with 4 × 40 ml. of ether and washed with 2 × 50 ml. of 0.5 *N* aqueous KOH and 4 × 50 ml. of water.

In all samples the following determinations were done: (*a*) D + P with Nield's reagent (potential D content). (*b*) The apparent vitamin D content, by absorptiometry at 265 mμ (actual D + part of P). (*c*) D and P separately by TLC (actual D content). The effect of the saponification temperature on actual and potential vitamin D contents is seen in Table II.

Soxhlet Extraction—The samples consisted of 1 g. of cotton seed oil, containing 48.8 mg. of cholecalciferol, adsorbed on 5 g. of cellulose powder. These were extracted during 5 hr. under nitrogen, with ether, cis-dichloroethene, *n*-hexane, cyclohexane, or isooctane, each containing 0.01% of BHT, at 35, 60, 68, 80, and 100°, respectively, and the extracts analyzed (see Table III): (*a*) P + D with Nield reagent (potential D content), (*b*)

TABLE I—P AND D CONTENT AFTER BOILING CALCIFEROLS FOR 0.5 HR.

Solvents and B.p., °C.	Ergocalciferol: P_2 + D_2 Recovery, %	By TLC: Fractions, % of Potential D P_2	By TLC: Fractions, % of Potential D D_2	Absorptiometric: a at 265 mμ	Absorptiometric: Fractions, % of Potential D P_2	Absorptiometric: Fractions, % of Potential D D_2	Cholecalciferol: P_3 + D_3 Recovery, %	By TLC: Fractions, % of Potential D P_3	By TLC: Fractions, % of Potential D D_3	Absorptiometric: a at 265 mμ	Absorptiometric: Fractions, % of Potential D P_3	Absorptiometric: Fractions, % of Potential D D_3	Calcd. from k_1, k_2 P_3
Methanol, 65	101.8	4.8	95.2	465	3.7	96.3	100.2	4.2	95.8	473	4.5	95.5	4.3
Ethanol, 78	101.4	13.5	86.5	440	13.1	86.9	101.0	12.2	87.8	452	12.4	87.6	12.2
Isooctane, 100	101.2	26.6	73.4	407	25.5	74.5	101.7	28.8	71.2	408	28.6	71.4	28.3

TABLE II—EFFECT OF SAPONIFICATION AT DIFFERENT TEMPERATURES

Solvent with 2 *N* KOH	Heated for 30 min., °C.	By Nield Reaction Potential D mg./g.	By TLC: Fractions, % of Potential D P	By TLC: Fractions, % of Potential D D	By TLC: Potential D (P + D), mg./g.	By TLC: Actual D, mg./g.	By Absorptiometry Apparent Vitamin D, mg./g.	Calcd. P Fractions, % of Potential D After 0.5 hr.	Calcd. P Fractions, % of Potential D Equilibrium
Methanol	60	47.7	4.5	95.5	46.2	44.2	46.5	2.6	16.1
Ethanol	80	47.7	13.6	86.4	47.0	40.7	44.2	13.9	22.0
Methyl cellosolve	100	48.0	20.2	79.8	48.0	38.2	42.3	28.3	28.5

TABLE III—EFFECT OF SOXHLET EXTRACTION: CONTENTS OF EXTRACTS

5 Hr. Extraction with	B.p., °C.	TLC: Fraction, % of Potential D P	TLC: Fraction, % of Potential D D	P + D, mg./g.	Nield Reaction Potential D, mg./g.	Calcd. Equilibrium P Fraction, % of Potential D
Ether	35	8.5	91.5	48.2	47.8	10.0
cis-$C_2H_2Cl_2$	60	12.5	87.5	48.8	48.8	16.1
n-Hexane	68	18.9	81.1	48.8	48.2	18.4
Cyclohexane	80	21.8	78.2	47.4	47.4	22.0
Isooctane	100	27.7	72.3	49.2	48.8	28.5

D (actual D content) and P separately by TLC.

Storage and Analysis of Resins—In the experiments of Table IV samples of resins were stored at different temperatures during times in which diluted calciferol solutions are equilibrated completely. In Table V stored resins are compared with solutions of the same in oil; in this case the storage time is insufficient to reach the equilibrium for solutions.

P + D + TA was determined by Nield reaction absorptiometry in this solution. P and D + TA were isolated by TLC of 0.5 ml. of the solution (containing approximately 0.5 mg. of D) and P determined by Nield reaction. From D + TA the TA was eliminated by MA addition and the remaining D determined by Nield reaction. The values for P + D in Table IV were obtained by adding those for P and for D. The values for TA

TABLE IV—STORAGE OF A SOLID (GLASSY) VITAMIN D_3 RESIN IN NITROGEN DURING TIMES NEEDED BY DISSOLVED CALCIFEROL TO REACH P-D EQUILIBRIUM

Determined \ Storage Time and Temp. →	% w/w: After 1 Month, 20°	After 1 month, 20° and: 11 Days, 30°	3.8 Days, 40°	1.7 Days, 50°
P + D + TA	68.5	67.3	69.6	69.9
P + D (= potential D)	62.6	59.5	63.0	63.5
D (= actual D)	54.8	51.3	53.9	53.6
P	7.8	8.2	9.1	9.9
TA	5.9	7.8	6.6	6.4
	P and D Fractions, % of Potential D[a]			
D	87.5 (93)	86.3 (91.1)	85.5 (89.9)	84.4 (86.5)
P	12.5 (7)	13.7 (8.9)	14.5 (11.1)	15.6 (13.5)

[a] The numbers in parentheses are the equilibrium fractions in solutions.

TABLE V—COMPARISON OF THE P-D RATIO AFTER STORAGE OF NONCOOLED (LIQUID OR PASTY) VITAMIN D_3 RESIN AND OF THE SAME RESIN DISSOLVED IN OIL

Compd.	P and D Fractions, % of Potential D_3: Start Resin	Start Oil	19 Days at 20°C. Resin	19 Days at 20°C. Oil	6 Days at 30°C. Resin	6 Days at 30°C. Oil	2 Days at 40°C. Resin	2 Days at 40°C. Oil
P	25	23	10	12	9.5	9.5	11	13
D	75	77	90	88	90.5	90.5	89	87
Potential D, % w/w	60.7	5.4	61.5	5.9	59.2	6.0	60.0	5.9

were obtained by subtracting P + D from P + D + TA. Determination of TA or D + TA by Nield reaction after TLC is wrong as TA decomposes during TLC.

The samples of Table IV were obtained by weighing 500 mg. of briefly heated (100°) vitamin D_3 resin into volumetric flasks, sealing the flasks under nitrogen, and storing them for 5 hr. at −25° at which time the resin had solidified and taken its glassy appearance. These samples were stored for the times mentioned in Table IV, then dissolved in solvent III at room temperature and promptly analyzed.

In Table V, vitamin D_3 resin was heated for 2 hr. at 85°. The 500-mg. portions were sealed in volumetric flasks under nitrogen and stored immediately at the temperatures of the storage tests. Ten grams of the same vitamin D_3 resin was heated for 2 hr. at 85° in 90 g. of peanut oil. The solution was stored in completely filled, stoppered 5-ml. flasks.

DISCUSSION

Recovery—The recoveries of the sum of P and D found by the described TLC method (Table I) and the comparison with potential D content found by Nield reaction before chromatography (Tables II and III) are satisfactory.

Isomerization, Equilibrium, and Rate—In the experiment of Table I the equilibrium is reached within 30 min. at 100°, as can be calculated from the rate constants (1); at 65 and 78°, 8 and 3 hr. would be needed. Accordingly at 100°, the P fractions are near the equilibrium value of 28.5%; at 65 and 78° they are much lower than the equilibrium value (4.5 instead of 17.6% and 13 instead of 21.4%, respectively). It is evident that the P and D fractions, even those measured *during* the conversion, are virtually equal for ergocalciferol and cholecalciferol. This means that the equilibrium and the isomerization rates of vitamin D_2 and vitamin D_3 differ only very slightly.

Saponification—From Table II it is clear that the actual D content decreases considerably during saponification and progressively so with increasing temperature; the potential D content remains unchanged. In a comparison of Tables I and II the P and D fractions found after saponifying 0.5 hr. at 60 and 80° agree reasonably with the values found without KOH at 65 and 78° after 0.5 hr., experimentally and calculated. At 100° there is a certain discrepancy. The conclusion is that the rate, and thus the equilibrium P ⇌ D is not altered by the presence of KOH if the temperature does not exceed 80°, but that at 100° KOH causes other effects. Without trying to explain the effect of KOH, the authors advise saponification only at temperatures of 80° or less.

Extraction (Table III)—After extraction at 68, 80, and 100° the P and D fractions are in fair agreement with the equilibrium fractions. Below 68° the agreement fails because 5 hr. is too short a time to reach equilibrium.

Resins—The effect of temperature on glassy resins appears to be very small, which emphasizes their solid character (Table IV). Unlike the glassy vitamin D_3 resin the viscous noncooled vitamin D_3 resin of Table V isomerizes at the same rate as its solution.

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Keyphrases

Vitamin D—analysis
Tachysterol—elimination
Isomerization—equilibrium, rate
TLC—separation
UV spectrophotometry—analysis

Pharmacokinetics of Acetylsalicylic Acid and Salicylic Acid After Intravenous Administration in Man

By MALCOLM ROWLAND and SIDNEY RIEGELMAN

The pharmacokinetics of acetylsalicylic acid (ASA, 650 mg.) and salicylic acid (SA, 500 mg.) were studied following intravenous administration in males. The resultant plasma concentration-time curves were described by bi-exponential equations. The half-life of the first exponent was 2–5 min. for both compounds while that of the second exponent was 13–19 min. and 3.5–4.5 hr. for ASA and SA, respectively. Over the dose range 0.3–1.2 g. ASA, the area under the ASA plasma concentration-time curve was proportional to the dose administered. Also SA was shown to be the exclusive metabolite of ASA. Analysis of the present results, over the dose range and duration of study, showed that the data could best be fitted by conceiving the body to act as a two-compartmental open system with respect to these drugs. The significance of these findings on measurement of the rate constants of metabolism, volume of distribution, and other pharmacokinetic parameters are discussed.

It is an interesting fact that, although acetylsalicylic acid (ASA) has been consumed in tremendous quantities since its introduction in 1899, only scant information is available pertaining to the pharmacokinetics of this drug in man. This is all the more surprising as the salicylates have been the subject of two monographs (1, 2) and a symposium (3). Essentially, the problem has been an analytical one in that previous assays either did not differentiate between ASA and its metabolite, salicylic acid (SA), or estimated ASA as the increase in SA upon hydrolysis of the sample (4, 5), which is at best an insensitive method. More recently attempts have been made to increase the specificity of the assay using paper chromatography or selective extraction (6, 7).

Although ASA is known to be rapidly metabolized to SA *in vivo*, only the mean data of Leonards (8) and Lange and Bell (6) allow any estimate for the apparent elimination half-life, and values of 17 min. and 30 min. are obtained, respectively. However, in both studies, the drug was given orally so that estimates could be influenced by continued absorption of ASA during the decline of drug blood levels. The total urinary recovery of ASA as SA or its metabolites (9) suggests that SA is the only metabolite of ASA while 1.5% has been reported to be excreted unchanged in the urine of man (10). Much more information is available relating to SA owing to its high plasma levels, relatively long half-life, and ease of analysis (1–3). Nevertheless, there are little data on the kinetics of distribution of this drug in man.

In the present paper both ASA and SA have been given intravenously to man as their *N*-methylglucamine salt. The kinetics of distribution, metabolism, and excretion of each drug was then determined. Over the duration of this study it was found that the results can only be adequately explained by proposing as a minimum a two-compartmental open-system model for each drug. A preliminary communication of these results have been reported elsewhere (11).

EXPERIMENTAL

The dose of either ASA or SA was administered as its *N*-methylglucamine salt. Equivalent amount of acid and base were mixed together and dissolved in water for injection so that each dose was contained in 10-ml. final product. The solutions were passed through a bacterial filter into a sterile, multidose container and used immediately after preparation. The solutions were assayed for SA, and ASA, by UV, spectrophotofluorometric, and gas-liquid chromatographic procedures to verify contents.

Five male subjects were used in the study and each received a rapid intravenous injection (over 5–10 sec.) of 650 mg. ASA while on another occasion four of these subjects received 500 mg. SA. Blood samples (5 ml.) were usually taken at 0, 1, 2, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 105, 120, 180, 240, 300, 360, and 480 min. following ASA administration, while slightly less frequent samples were taken in the SA study. In addition one subject was given 325 mg. ASA i.v. and in another study received a zero-order i.v. infusion of 1.24 g. ASA over a 50-min. period using a Harvard infusion pump. In the ASA study the collected blood samples were immediately placed into cooled tubes containing heparin and potassium fluoride (which inhibits further hydrolysis of ASA) and centrifuged. The plasma was then analyzed specifically for ASA using gas-liquid chromatography

Received January 8, 1968, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122

Accepted for publication April 1, 1968.

Supported in part by funds from the University of California, Academic Senate Research Committee, and Sterling-Winthrop Laboratories, Rensselaer, N. Y.

while SA was determined spectrophotofluorometrically (12).

RESULTS AND DISCUSSION

In all subjects the ASA plasma-concentration-time curve following 650 mg. ASA i.v. was described by a bi-exponential equation of the form $C_p = Ae^{-\alpha t} + Be^{-\beta t}$ (*e.g.*, Fig. 1).[1] Both the initial distribution phase and the so-called elimination phase are extremely short with average half-lives of 2.8 and 15 min., respectively (Table I). Consequently, no ASA is present in the body 1 hr. following the intravenous dose. The rapid *in vivo* hydrolysis of ASA to SA causes the SA plasma level to rise sharply (40–50 mcg./ml.) and owing to its metabolic stability, the disposition half-life[2] was 3.0–4.5 hr. in this study, SA remains in the body for a long time after all the ASA has been eliminated.

The bi-exponential plasma curve can be interpreted in terms of a two-compartmental open system depicted as in Fig. 2.

In this model k_{12}, k_{21}, are the first-order rate constants for distribution of the drug between the central and so-called peripheral or tissue compartment while k_{13} is the elimination (metabolism and excretion) rate constant. V_p,C_p are the volume of the central compartment and the plasma concentration of drug, respectively, while V_T,C_T are the corresponding values for the tissue compartment (expressed relative to the central compartment). It follows from this model that dose administered = $V_pC_p^0$, where C_p^0 is the plasma concentration at zero time and can be seen to be numerically equal to the sum of the coefficients A and B in the bi-exponential equation describing the plasma concentration-time curve. The values of k_{12}, k_{21}, and k_{13} are calculated from A, B, α, and β (13). Also the model shows ASA distributing between two compartments while metabolism and excretion occur only in the central compartment, which includes the liver, kidneys, as well as blood. Needless to say, the model is an over-simplification of the body, but it is the minimum model required to fit the observed ASA data. Having assumed such a model, the various parameters were determined for each subject (Table II). As expected the distribution rate constants k_{12}, k_{21} are extemely fast with mean half-lives of 8.6 min. and 5.8 min. (*i.e.*, $0.693/k_{12}$, $0.693/k_{21}$). More noteworthy is the value of the elimination rate constant k_{13}, which is of the same magnitude as k_{12} and k_{21}, and is approxmately twice that of the disposition rate constant β (Table I). In fact, the only reason that the latter phase has a half-life as long as 15 min. ($0.693/\beta$) instead of 6.7 min. ($0.693/k_{13}$) is due to distribution of ASA into the peripheral compartment so that effectively less is available for immediate metabolism.

In further experiments subject A received 325 mg., 650 mg., and 1.24 g. ASA i.v. on separate occasions and the ASA areas (area/g. dose) were 1460, 1460, and 1390, respectively. Thus, proportionality between area and dose exists over the range studied and could only occur if the rate constants do not change with dose. Since the disposition half-life of SA is dose dependent, increasing with higher doses, proportionality between SA area and dose is not expected.

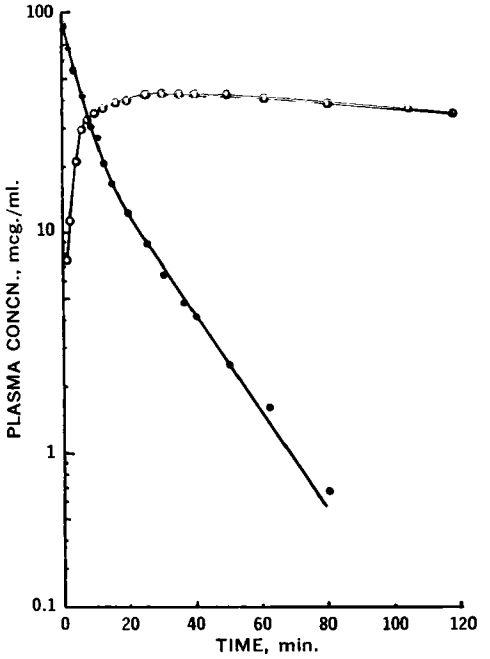

Fig. 1—Graph showing plasma concentrations of ASA and SA following i.v. administration of 650 mg. ASA. Subject D. Key: ●, ASA; ○, SA.

TABLE I—BI-EXPONENTIAL EQUATIONS DESCRIBING THE LOSS OF DRUG FROM PLASMA, THE HALF-LIVES OF THE DISTRIBUTION AND DISPOSITION PHASES OF ASA, TOGETHER WITH THE AREA UNDER THE PLASMA CONCENTRATION-TIME CURVE OF ASA FOLLOWING AN I.V. DOSE OF ASA IN MAN

Subject	Dose, mg.	$C_p = Ae^{-\alpha t} + Be^{-\beta t}$, t in min.	Half-Lives[a] min. 0.693/α	0.693/β	Area ($\int_0^{\infty} C_p dt$), mcg. min. ml.$^{-1}$
A	325	$42e^{-0.26t} + 15.5e^{-0.048t}$	2.7	14.5	480
	650	$90e^{-0.24t} + 29e^{-0.050t}$	2.8	15.0	960
B	650	$56e^{-0.31t} + 37e^{-0.050t}$	2.2	13.7	910
C	650	$60e^{-0.29t} + 40e^{-0.048t}$	2.4	14.5	1000
D	650	$67e^{-0.23t} + 33e^{-0.050t}$	3.0	14.0	960
E	650	$64e^{-0.19t} + 22e^{-0.037t}$	3.7	19.0	950
Mean			2.7	14.9	—

[a] Mean half-life values in this and subsequent tables are harmonic means.

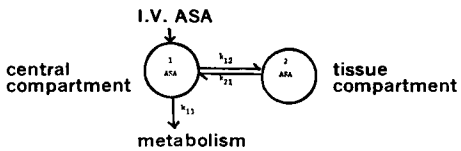

Fig. 2—Schematic diagram showing the distribution and metabolism of ASA following i.v. administration.

[1] As discussed by Riegelman *et al.* (13), the terms α and β are the fast and slow disposition rate constants, respectively, while the term disposition half-life is the value $0.693/\beta$ referred to by others as the elimination or biological half-life.

[2] The authors observed, among others (14,15), that the disposition half-life of SA varies with dose. The half-life values reported here are those obtained following either 650 mg. ASA or 500 mg. SA.

TABLE II—ESTIMATES FOR THE RATE CONSTANTS AND HALF-LIVES OF DISTRIBUTION AND ELIMINATION FOR ASA IN MAN, ASSUMING A TWO-COMPARTMENTAL OPEN SYSTEM

Subject	Dose of ASA, mg.	Rate Constants, min.$^{-1}$ k_{12}^{ASA}	k_{21}^{ASA}	k_{13}^{ASA}	Half-Lives, min 0.693/k_{12}^{ASA}	0.693/k_{21}^{ASA}	0.693/k_{13}^{ASA}
A	325	0.083	0.104	0.117	8.4	6.7	5.7
	650	0.073	0.097	0.123	9.5	7.2	5.6
B	650	0.105	0.157	0.110	6.7	4.2	6.2
C	650	0.095	0.152	0.091	7.3	4.6	7.6
D	650	0.067	0.109	0.105	10.4	6.4	6.6
E	650	0.058	0.074	0.092	12.1	9.4	7.0
Mean		0.081	0.119	0.103	8.6	5.8	6.7

TABLE III—BI-EXPONENTIAL EQUATIONS DESCRIBING THE LOSS OF DRUG FROM PLASMA AND THE HALF-LIVES OF DISTRIBUTION AND DISPOSITION PHASES OF SA FOLLOWING AN I.V. DOSE OF SA IN MAN

Subject	Dose, mg.	$C_p = Ae^{-\alpha t} + Be^{-\beta t}$ t in min.	Half-Lives, min. 0.693/α	0.693/β
A	508	$38e^{-0.17t} + 58e^{-0.0027t}$	4.1	256
B	484	$32e^{-0.22t} + 50e^{-0.0026t}$	3.2	266
C	500	$44e^{-0.23t} + 60e^{-0.0028t}$	3.0	210
D	508	$40e^{-0.14t} + 49e^{-0.0032t}$	5.0	216
Mean			3.8	238

Before attempting to interpret the SA plasma levels resulting from the ASA dose, SA was given intravenously. A dose level of 500 mg. was chosen as this would result from complete hydrolysis of 650 mg. ASA. The resulting plasma levels, like those of the ASA study, showed a bi-exponential curve in the four subjects examined. However, whereas the distribution half-life (3.8 min.) is similar to ASA, the disposition half-life[3] is much longer (Table III). As with ASA, the results were interpreted in terms of a two-compartmental model, and the corresponding rate constants k_{12}, k_{21}, and k_{13} were calculated (Table IV). These analyses confirm that the major difference between ASA and SA is one of metabolic stability since the corresponding k_{12}, k_{21} values of SA and ASA for each subject are similar whereas the k_{13}^{ASA} is 15 times k_{13}^{SA}.

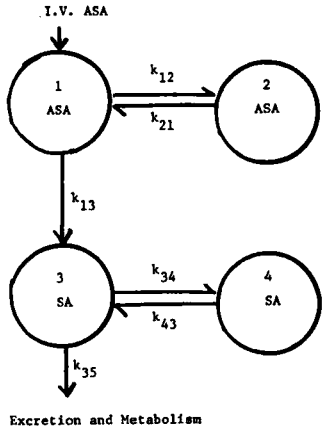

Fig. 3—Schematic diagram for the distribution and elimination of ASA and SA following an i.v. dose of ASA (650 mg.).

this model k_{13} is shown to be the only route of elimination of ASA and to be equal to the rate constant for conversion of ASA to SA. Obviously urinary excretion of unchanged drug and other metabolites are also possibilities. However, the contribution of excretion is small as only 1.5% of the dose has been reported to be excreted unchanged and may be ignored (10). The second possibility was discounted

TABLE IV—ESTIMATES FOR THE RATE CONSTANTS AND HALF-LIVES OF DISTRIBUTION AND ELIMINATION FOR SA IN MAN ASSUMING A TWO-COMPARTMENTAL OPEN SYSTEM

Subject	Dose SA, mg.	Rate Constants, min.$^{-1}$ k_{12}^{SA}	k_{21}^{SA}	k_{13}^{SA}	Half-Lives, min. 0.693/k_{12}^{SA}	0.693/k_{21}^{SA}	0.693/k_{13}^{SA}
A	508	0.070	0.102	0.0042	9.9	6.8	165
B	484	0.085	0.133	0.0040	8.1	5.2	173
C	500	0.097	0.131	0.0056	7.2	5.3	124
D	508	0.061	0.078	0.0058	11.3	8.9	120
Mean		0.078	0.110	0.0049	9.1	6.2	142

From the foregoing results and discussion, the scheme in Fig. 3 is proposed to describe the pharmacokinetics of ASA and SA following an intravenous injection of ASA to man. In this diagram, $k_{34} = k_{12}^{SA}$, $k_{43} = k_{21}^{SA}$ and $k_{35} = k_{13}^{SA}$ as in Table IV. In when calculations showed that the SA area[4] following 650 mg. ASA was equal to that obtained from 500 mg. SA administration (Table V). Since 650 mg. ASA is equivalent to 500 mg. SA the above result can only occur when SA is the sole metabolite of ASA,

[3] After the distribution phase the SA plasma levels for the 8-hr. period over which most studies were conducted could be fitted by a straight line when plotted on semilogarithmic paper. Literature evidence (16) and the authors' observations, however, show that SA plasma levels exhibit a curvature if followed long enough. Hence the β^{SA} and k_{13}^{SA} values calculated are only apparent constants but are adequate for describing the levels observed during the course of these experiments.

[4] In calculating the total area under the SA concentration time curve, it was assumed that the observed apparent disposition half-life (0.693/β) would not change with time so that the extra area subsequent to the 8-hr. study is determined by $C_{8A} \cdot 1/\beta$ where C_{8A} is the last plasma data point. It was reasoned that even if curvature of the plasma SA took place it would be similar for both i.v. SA and SA produced from ASA, so that the error introduced in comparing the relative areas would not be appreciable.

and therefore, k_{13} has the meaning depicted in Fig. 3.

The pharmacokinetics of these salicylates were studied further using a PACE TR 48 analog computer (the differential equations and analog diagram of which are given in the appendix). As expected the computer curves fitted the observed i.v. ASA, and SA data (Fig. 4). Additional evidence in favor of the proposed model (Fig. 3) was obtained when, using the rate constants calculated for each subject (except E which received no i.v. SA), there was an excellent fit between observed and predicted SA levels resulting from i.v. ASA, *e.g.*, Fig.

TABLE V—COMPARISON OF THE AREA UNDER THE SALICYLIC ACID PLASMA CONCENTRATION-TIME CURVE FOLLOWING 650 MG. ASA AND 500[a] MG. SA I.V.

Subject	SA Area After 650 mg. i.v. ASA, mcq./min. ml.$^{-1}$	SA Area After 500 mg. i.v. SA, mcq./min. ml.$^{-1}$	Ratio of Areas × 100
A	22,000	20,600	107
B	20,300	20,100	101
C	21,700	21,400	101
D	14,100	15,000	94
Mean			101

[a] Areas corrected to a 500-mg. SA dose.

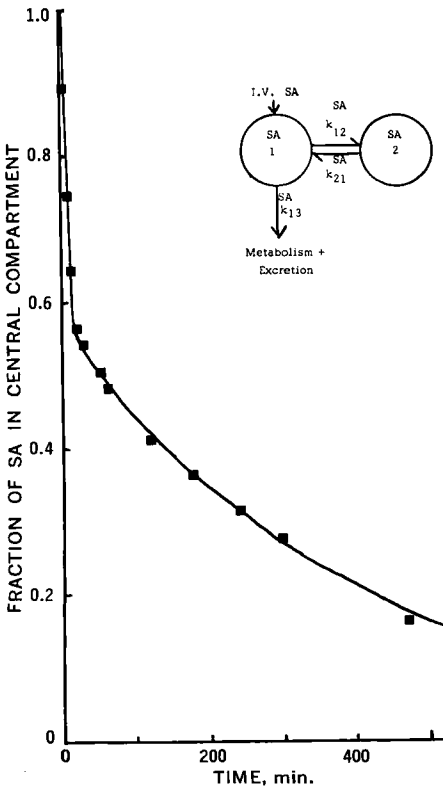

Fig. 4—Graph showing the observed and predicted values for the fraction of SA in the central compartment following 484 mg. i.v. SA administration. Subject B. Key: ■, experimental SA; ———, predicted analog computer curve.

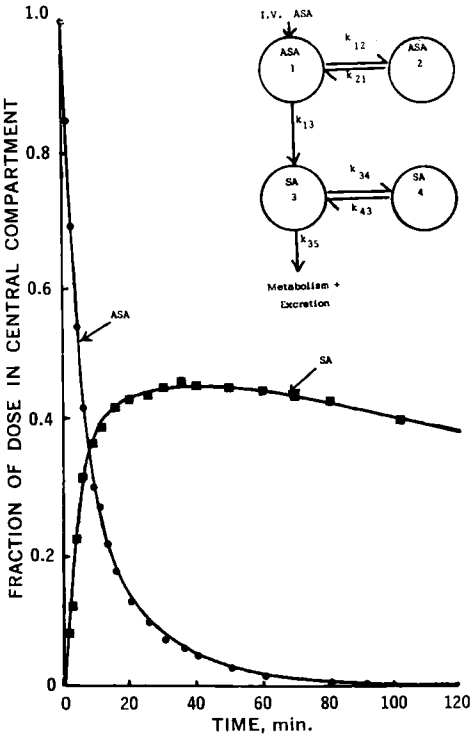

Fig. 5—Graph showing the observed and predicted values for the fraction of the dose as ASA and SA in the central compartment following i.v. administration of 650 mg. ASA. Subject D. Key: ●, experimental ASA; ■, experimental SA; ———, predicted analog computer curves.

5. It should be noted that in Fig. 4 and in subsequent analog computer curves the concentration terms have been replaced by the fraction of each drug present in the central compartment, otherwise overloading of the computer can occur. For the intravenous doses the fraction term is simply obtained by dividing the plasma concentration by the corresponding value of C_p^0. The SA data obtained from i.v. ASA were normalized by making the maximum SA plasma concentration equal to the peak fraction of the dose as SA in the central compartment which had been obtained from the analog computer curves.

It is apparent that an alternative model, in which ASA hydrolysis takes place exclusively in the peripheral compartment or partially in both, may also account for the observed data. The former was eliminated since it is known that ASA is hydrolyzed in whole blood which is an essential part of the central compartment. The latter model was further examined using the analog computer and like the model depicted in Fig. 3 it also fitted the ASA plasma data (*e.g.*, Fig. 6). However, unlike the originally proposed model, the one assuming partial peripheral metabolism resulted in a lower rate of appearance of plasma SA than the experimental data. Any attempts at varying the SA rate constants, while maintaining the same ASA constants determined for the model involving peripheral metabolism of ASA (model B, Fig. 6), in order to increase the rate of appearance of plasma SA but still fitting the i.v. SA data, proved unsuccessful in all subjects. It was,

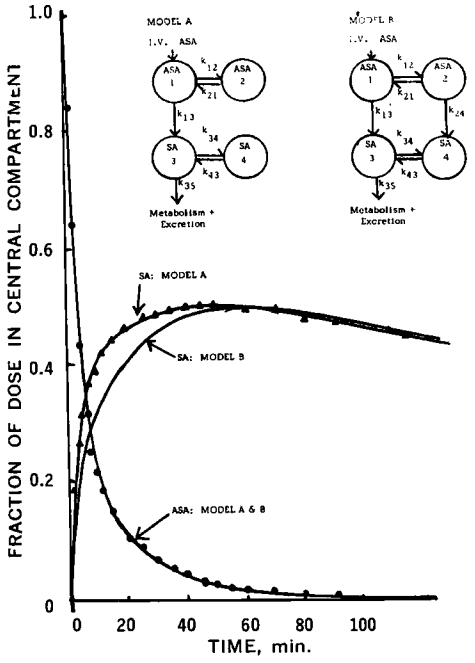

Fig. 6—Graph showing the observed and predicted values for the fraction of the dose as ASA and SA in the central compartment following i.v. administration of 650 mg. ASA. Model A assuming metabolism of ASA only in central compartment while model B assuming metabolism occurs in both compartments. Subject A. Key: ●, experimental ASA; ▲, experimental SA; ———, predicted analog computer curves. Model A, k_{12}, k_{21}, k_{13}, are 0.083, 0.104, 0.117 min.$^{-1}$, respectively. Model B, k_{12}, k_{21}, k_{13}, k_{24}, are 0.128, 0.094, 0.090, and 0.030 min.$^{-1}$, respectively, while k_{34}, k_{43}, and k_{35}, are 0.070, 0.105, and 0.0044 min.$^{-1}$ for both models.

therefore, concluded that the model proposed in Fig. 3 describes the observed kinetics of distribution, metabolism, and excretion of both ASA and SA.

The volume of distribution is another parameter which is used to characterize a drug. In terms of the two-compartmental model this is defined by the relationship (17).

$$V_{d_{ss}} = V_p \left(\frac{k_{12} + k_{21}}{k_{21}} \right) \qquad \text{(Eq. 1)}$$

where $V_{d_{ss}}$ is the volume of distribution at steady state of equilibrium, and is the only definition independent of k_{13}, the elimination rate constant. Assuming a single-compartmental model, the volume of distribution, V_d, is obtained by dividing the dose administered by the extrapolated intercept value for the concentration at zero time which, in terms of the two-compartmental model, is expressed by:

$$V_d = \frac{\text{dose}}{B} \qquad \text{(Eq. 2)}$$

The values of $V_{d_{ss}}$ and V_d for ASA and SA following their respective i.v. doses have been calculated and are listed in Table VI. Comparison of the respective values for ASA shows that the V_d may be twice as large as $V_{d_{ss}}$ due to significant metabolism of ASA occurring during the distribution phase of this drug, which is ignored in the single-compartment

TABLE VI—VALUES OF V_p[a], $V_{d_{ss}}$, AND V_d FOR ASA and SA

Subject	ASA V_p (L)	ASA $V_{d_{ss}}$ (L)	ASA V_d (L)	SA V_p (L)	SA $V_{d_{ss}}$ (L)	SA V_d (L)
A	5.6	10.1	21.0	5.3	8.7	8.8
B	6.9	12.0	17.6	5.9	9.7	9.7
C	6.5	10.6	16.3	5.2	9.1	9.1
D	6.5	10.4	19.7	5.7	10.2	10.4
E	7.6	13.3	30.2	—	—	—
Mean	6.6	11.3	21.0	5.5	9.4	9.5

[a] See text for definitions of terms.

model. Had the V_d term been used to compare these drugs, one could be led to the erroneous conclusion that the volume of distribution of ASA is almost twice that of SA. Contrary to this, the V_p and $V_{d_{ss}}$ for these two drugs are very similar with the ASA values being slightly larger than those of SA in each subject. This may be due to protein binding since SA has a much greater affinity for albumin than ASA (18) so that more SA would be held in the plasma and lower V_p and $V_{d_{ss}}$ would be expected. Other possible factors are differences in pKa and lipid solubility. Nevertheless, the difference between these drugs is not very great.

In contrast to ASA there is only a very small difference between the $V_{d_{ss}}$ and V_d for SA owing to negligible elimination occurring during the distribution phase of this drug. Therefore, with respect to the above calculations for SA either compartmental

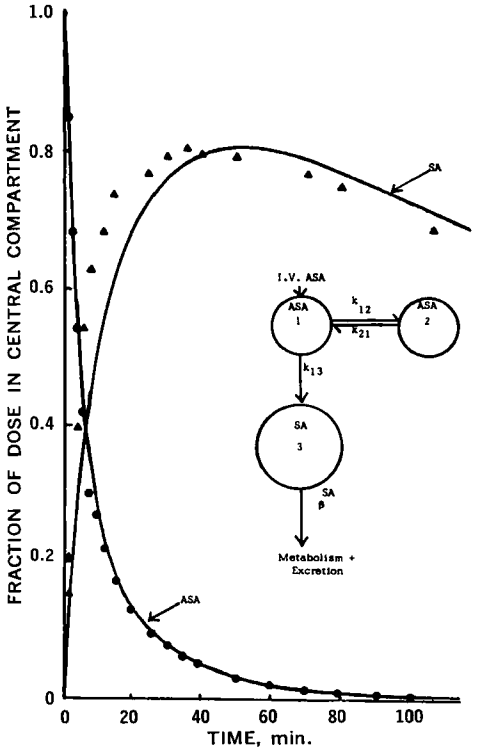

Fig. 7—Graph showing the observed and predicted values for the fraction of the dose and ASA and SA in the central compartment following i.v. administration of 650 mg. ASA and assuming single compartment for SA. Subject B. Key: ●, experimental ASA; ▲, experimental SA; ———, predicted analog computer curve.

model would be satisfactory. However, the two-compartmental model cannot be reduced to the single-compartmental model in all situations. If the latter model were substituted for SA in the schematic diagram describing the pharmacokinetics of ASA and SA (Fig. 3), the resultant predicted SA levels rise much more slowly than the observed values (Fig. 7). This results from the rapid metabolism of ASA to SA, such that SA builds up in the central compartment faster than it distributes. Hence, higher plasma levels are observed than one would expect using the single-compartment SA model which assumes spontaneous distribution of this drug. The V_p is a constant of the model, and although it is approximately 6 l. for either salicylate, it should not be equated to a physiological space such as the blood volume, even though their sizes are similar. Thus had whole blood been used instead of plasma, then as 70–80% of the salicylate is in the plasma (19), V_p values of approximately 10.1 would have been calculated.

Cummings (20, 21) observed that, following 0.65-g. plain aspirin tablets, peak plasma salicylate levels were at 1 hr. while excretion rate reached a maximum after 3 hr. To explain these results he suggested that distribution of SA was extremely slow in man, 3 or more hr. being required for equilibrium and that it was the tissue concentration, rather than the plasma, that controlled the excretion of this drug. Examination of the present data shows that distribution is very rapid and equilibrium (when tissue concentration is at a maximum) can be achieved within 20 min. following i.v. SA (Fig. 8). Probably, the explanation of Nelson and Levy (22) is correct in which they showed that the assay used by Cummings measures both SA and its glycine conjugate, salicyluric acid. Since plasma contains

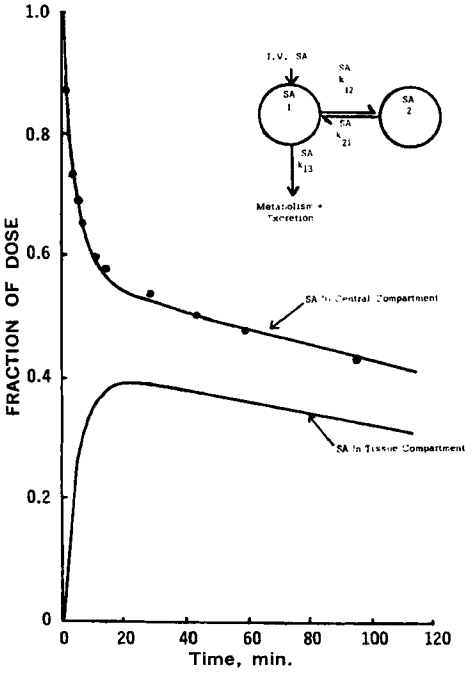

Fig. 8—Graph showing fraction of the dose in the central and tissue compartment following i.v. administration of SA. Subject B. Key: ●, experimental SA; ———, predicted analog computer curve.

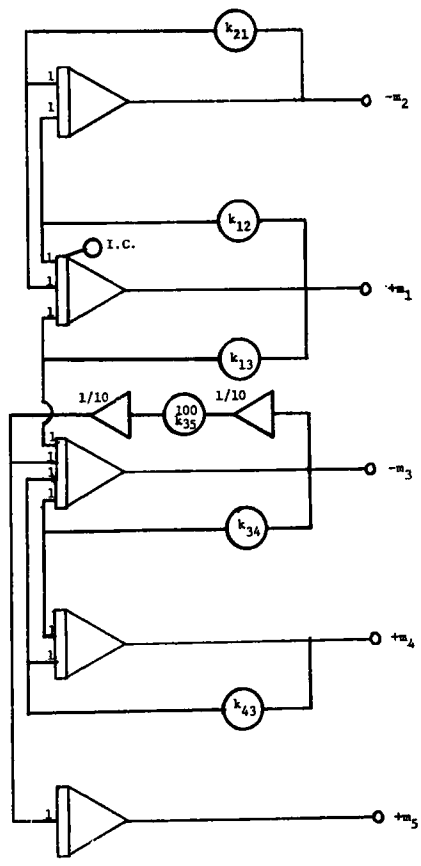

Fig. 9—Analog computer program.

predominately SA and urine mostly salicyluric acid (9) it follows from the precursor–product relationship that the maximum rate of excretion of total salicylate will be later than the peak plasma level.

An estimate of the contribution of whole blood to the *in vivo* hydrolysis of ASA in man can be made from the present data. Since the *in vitro* rate constant for the hydrolysis of ASA in human whole blood at 37° is 0.023 $min.^{-1}$ (23) and the blood volume is 5 l., the expected *in vivo* clearance (volume × rate constant) of ASA by blood is 115 ml./min. From the i.v. data the whole body clearance ($V_p \cdot k_{13}^{ASA}$) of ASA is 660 ml./min. (6,600 × 0.103). Consequently, whole blood only accounts for approximately 20% of total hydrolysis in man, and probably liver and perhaps kidney, are the major sites of ASA esterase activity. The implication that liver is the important metabolizing organ for ASA has been subsequently demonstrated (11). Obviously, as all the hepatic portal blood must pass through the liver this high hepatic clearance of ASA will significantly influence the amount of intact ASA which reaches the general circulation following oral administration of ASA.

Analysis of the present results has allowed a clearer understanding of the pharmacokinetics of ASA in man. Comparison shows that other than metabolic stability the pharmacokinetics of ASA and SA are very similar. However, even though the present model is useful, it must be emphasized that this model only describes the disposition kinetics

of SA at essentially one dose level, and consistency at one level is no test of a model. Since it is known that SA elimination kinetics do change with dose, more information is needed before a model can be proposed which will adequately describe the observed data at all dose levels of this drug.

APPENDIX

The mass equations describing the scheme in Fig. 3 are:

$$-\dot{m}_1 = k_{12} \cdot m_1 + k_{13} \cdot m_1 - k_{21} \cdot m_2 \quad \text{(Eq. 1a)}$$

$$\dot{m}_2 = k_{12} \cdot m_1 - k_{21} \cdot m_2 \quad \text{(Eq. 2a)}$$

$$\dot{m}_3 = k_{13} \cdot m_1 - k_{34} \cdot m_3 - k_{35} \cdot m_3 + k_{43} \cdot m_4 \quad \text{(Eq. 3a)}$$

$$-\dot{m}_4 = -k_{34} \cdot m_3 + k_{43} \cdot m_4 \quad \text{(Eq. 4a)}$$

$$-\dot{m}_5 = -k_{35} \cdot m_3 \quad \text{(Eq. 5a)}$$

$$\text{Dose} = m_1 + m_2 + m_3 + m_4 + m_5 \quad \text{(Eq. 6a)}$$

where m_x is the amount of durg in compartment x. The analog computer program for this scheme is shown in Fig. 9.

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Keyphrases

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Two-compartmental open system—pharmacokinetic model
UV spectrophotometry—analysis
Fluorometry—analysis
GLC—analysis

Differential Ninhydrin Chromatographic Assay for the Gentamicin Complex

By GERALD H. WAGMAN, JANET V. BAILEY, and MORTON M. MILLER

A differential ninhydrin chromatographic assay has been devised to quantitatively determine the proportions of the three gentamicin components in gentamicin preparations. The technique consists of separation of the antibiotics on paper using the lower phase of a solvent system composed of chloroform, methanol, and 17 percent ammonium hydroxide (2:1:1 v/v). After development, strips containing the antibiotic are treated with ninhydrin reagent, developed, and color intensities read on an integrating scanner from which component proportions can be determined. Results are in excellent agreement with the microbiological method.

RECENTLY, Weinstein *et al.* (1) reported that preparations of the gentamicin complex consist of three antibiotic components which have been designated C_1, C_1a, and C_2 and that there are no significant differences in their biological properties. It became desirable therefore to devise methods for detection, isolation, and assay of these components. Thin-layer, paper, and column chromatographic separation techniques have been described by Wagman *et al.* (2) using a solvent system composed of chloroform–

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methanol–17% ammonium hydroxide (2:1:1 v/v).

A differential paper chromatographic microbiological assay has been reported (3) which utilizes this solvent system and is based on bioanalytical methods for determinations of penicillin mixtures. The method consists of chromatographing samples containing gentamicin together with a series of standards on paper strips, bioautographing the strips against *Staphylococcus aureus* on standardized assay plates, and measuring the diameters of the zones of inhibition of the separated components. Standard curves are constructed, and the concentration of each of the components in a sample of unknown composition is determined from an appropriate curve.

A chemical method has now been devised to carry out the differential analysis of the gentamicin components and is described here. While this new technique uses the chromatographic solvent system as reported in the bioanalytical assay, it does not require biological procedures. After chromatographic separation the papers are treated with ninhydrin, heated at 105° to develop the color, and run through an integrating scanner. The quantities of the individual components are determined by the peak intensities of the chromatogram zones. Although the quantity of material required in this method is somewhat greater than in the microbiological assay procedure, the technique is simple and reproducible.

EXPERIMENTAL

Apparatus and Materials

(*a*) Standard rectangular chromatographic tanks with troughs 30.5 × 30.5 × 61 cm. high (12 × 12 × 24 in.); insulate the outsides completely with paper.

(*b*) Schleicher and Schuell No. 589 blue ribbon chromatographic paper cut to size 20 × 58 cm. (along grain).

(*c*) Integrating scanner—instrument used for the determinations to be described was the model RB Analytrol, manufactured by Beckman Instruments, Inc., Fullerton, Calif.

(*d*) Reagents—the purest available reagent grade chemicals were used. The 17% ammonium hydroxide was made by mixing 600 ml. concentrated NH_4OH with 400 ml. water. Gentamicin C_1, C_1a, and C_2 standards were obtained as the sulfates and converted to their respective free bases by use of small columns of IRA 401S resin (Rohm and Haas) in the hydroxyl form and the solutions lyophilized. All standards were dried at 60° under vacuum for 3 hr. prior to use. Ninhydrin reagent (triketo hydrindene hydrate) was prepared fresh daily as a 0.25% solution by dissolving in a 1:1 mixture of pyridine–acetone.

Methods

Chromatographic Solvent and Conditions—The solvent is prepared by mixing together chloroform, methanol, and 17% NH_4OH in a ratio of 2:1:1 (v/v) in a separator and allowing the layers to separate. The lower layer is placed in the bottom of the chromatographic jar, and the upper layer is placed in a beaker with a wick at the bottom of the jar. The jar is equilibrated for several hours prior to use. The lower layer is used for development of the chromatograms. Optimum temperature for development is in a range of from 20 to 25°, and it is important that the temperature at which the chromatograms are run be maintained at a constant level.

Preparation of Standards and Samples—Gentamicin standards consisting of individual solutions of the C_1, C_1a, and C_2 components, either all as the sulfates or all as free bases, are dissolved in water to give concentrations of exactly 50 mg./ml. Equal volumes of each component standard are mixed together to give a solution containing the three components in a 1:1:1 ratio. Three replicate standard solutions are prepared in this manner. Samples in which the ratios of the gentamicin components are to be determined are dissolved in water to give a concentration of about 50 mg./ml.

Preparation and Development of Chromatograms—A line is drawn approximately 7 cm. from the end of each strip and 5-μl. quantities of each standard mixture are pipeted twice on triplicate spots equidistant from each other. This results in three chromatographic sheets containing a total of nine standard mixtures at a level of 500 mcg./spot.

The samples to be assayed are spotted in exactly the same manner as the standards, three spots for each unknown, using 10 μl./spot (two increments of 5 μl.).

The papers are placed in the chromatographic jars and solvent added to the troughs immediately. No equilibration is necessary. The papers are developed, descending, for 18 hr., and air dried for 15 min. to remove solvent. The sheets are pulled through a trough of ninhydrin reagent, dried in a fume hood, and heated for 5 min. at 105° to develop the color. The gentamicin C_1 zone is found nearest to the front, followed by C_2, and the zone closest to the origin is designated as C_1a. A chromatogram showing the relative positions of the components is illustrated in Fig. 1.

Scanning Chromatograms and Computations—The chromatograms are cut into strips 3.2 cm. (1¼ in.) in width and reference lines are marked on both ends of the papergrams beyond the last zone and before the origin. The strips are put through the integrating scanner using a slit width of 1.5 mm., and the zone intensities are automatically plotted and integrated. The chromatogram is lined up with

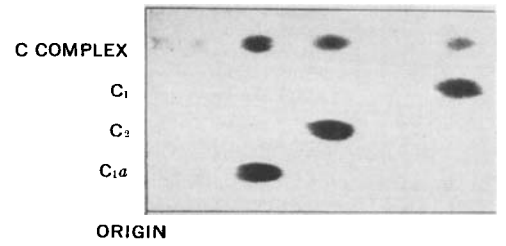

Fig. 1—Ninhydrin-treated chromatogram illustrating separation of gentamicin components using chloroform–methanol–17% ammonia (2:1:1 v/v) solvent system.

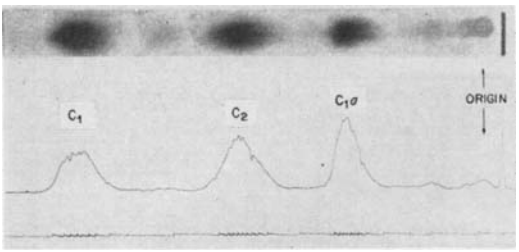

Fig. 2—Integrated scan of ninhydrin reaction following paper chromatography. Instrument used was Beckman Analytrol model RB, slit width = 1.5 mm. Upper curve is densitometer scan; lower, sawtooth line is integrator trace.

its scan using the reference marks, and the total scan area and area under each peak is determined for each chromatographic strip. A typical chromatogram of a gentamicin mixture and its integrated scan is illustrated in Fig. 2. Ratios of ninhydrin intensity of standards in a mixture containing equal weights of components can then be calculated as will be described. Results for each group of nine reference standards are averaged to obtain a final set of values.

Comparison of ninhydrin reactivity for each of the gentamicin base component standards showed that the intensity of color varied with $C_1a > C_2 > C_1$. This was true also for the sulfates, but the ratios were somewhat different. For the free bases, if C_1a is assigned a ninhydrin peak value of 1.00, then an equal quantity of C_2 results in a less intense color reaction and a value of 0.58 (58% of the intensity of color for an equal weight of C_2 compared to C_1a). For the C_1 component, the value is 0.52. Therefore, in order to calculate the correct proportions of components in an unknown mixture, it is necessary to multiply the C_1 and C_2 peak integrations by the appropriate factor (the reciprocal of the color intensity) to compensate for the differences in intensity between those components and C_1a. Similarly, for the sulfates, this compensation must also be made. The peak intensities and correction factors (reciprocals) for the bases and sulfates are summarized in Table I.

TABLE I—COMPARISON OF INTENSITY OF NINHYDRIN REACTIONS ON CHROMATOGRAMS FOR INDIVIDUAL COMPONENTS OF THE GENTAMICIN COMPLEX

Derivative	Component	Relative Intensity of Ninhydrin Spot	Factor, Reciprocal of Intensity
Base	C_1a	1.000	1.00
	C_2	0.578	1.73
	C_1	0.518	1.93
Sulfate	C_1a	1.000	1.00
	C_2	0.485	2.06
	C_1	0.397	2.52

Samples of unknown composition are run in a manner similar to that for the standard mixtures. After integration of component peaks, the peak areas are multiplied by the appropriate factors for each compound and the corrected intensities for each zone are added together. The percentage of each component can be calculated by dividing the number of integrations for each chromatogram zone by the total number for the complex. The three replicate sets of chromatogram data are averaged to obtain final results.

RESULTS AND DISCUSSION

Assay responses using the ninhydrin differential assay were compared to results obtained by the previously described microbiological method on six lots of gentamicin sulfate. Using the antibacterial potencies of C_1, C_1a, and C_2 gentamicins (assayed against the same working standard) it was possible to convert the percentages by weight to percentages based on activity.

Table II indicates the average assay results obtained using the ninhydrin assay both by weight and activity compared to the results obtained for the same samples in the microbiological assay. Assay variation as determined by a series of completely randomized analyses of variance indicates the residual error to be in excellent agreement with the biological procedure.

It has been found that a significantly high correlation ($p < 0.01$) exists between the biological assay and the ninhydrin assay, both by weight and activity. The product-moment correlation coefficient indicates the degree of association between the two assay procedures where −1.0 indicates perfect negative correlation and +1.0 indicates perfect positive correlation. These data are summarized in Table III and are a reflection of the data given in Table II, where each observation is a mean of three values.

TABLE III—CORRELATION OF NINHYDRIN DIFFERENTIAL ASSAY WITH MICROBIOLOGICAL DIFFERENTIAL ASSAY FOR GENTAMICIN SULFATE BATCHES

Component	Correlation Coefficient—Weight	Activity
C_2	0.87	0.94
C_1a	0.89	0.88
C_1	0.87	0.94

TABLE II—AVERAGE ASSAY VALUE OF GENTAMICIN SULFATE BATCHES BY LOT AND METHOD

	Ninhydrin Assay								
	By Weight, %			By Activity, %			Microbiological Assay, %		
Lot No.	C_1	C_2	C_1a	C_1	C_2	C_1a	C_1	C_2	C_1a
GMC-4J-5I	46.9	36.2	16.9	41.2	40.8	18.0	42.2	39.2	18.7
60-1197-65I	46.3	33.1	20.6	40.6	37.4	22.0	41.2	34.1	24.9
60-1197-64I	35.6	32.2	32.2	30.6	35.6	33.8	36.5	32.9	30.6
GMC-4J-1-4C	32.4	42.6	24.9	27.5	46.6	25.9	29.1	40.5	30.5
60-1197-61I	44.8	33.2	21.9	36.0	39.4	24.6	38.7	37.8	23.5
60-1197-60	40.2	41.8	18.0	34.7	46.4	19.0	36.8	43.5	19.7

TABLE IV—AVERAGE ASSAY VALUE OF GENTAMICIN BATCHES CONVERTED TO THEIR FREE BASES BY LOT AND METHOD

Lot No.	Ninhydrin Assay—By Weight, % C_1	C_2	C_1a	Ninhydrin Assay—By Activity, % C_1	C_2	C_1a	Microbiological Assay C_1	C_2	C_1a
GMC-4J-5I	42.5	37.2	20.3	36.9	40.8	22.3	42.2	39.2	18.7
60-1197-65I	43.1	34.0	23.0	37.7	37.2	25.1	41.2	34.1	24.9
60-1197-64I	35.8	35.3	28.8	30.7	38.2	31.1	36.5	32.9	30.6
GMC-4J-1-4C	31.0	41.0	28.0	26.3	43.9	29.9	29.1	40.5	30.5
60-1197-61I	38.8	37.1	24.0	33.5	40.4	26.1	38.7	37.8	23.5
60-1197-60	37.5	42.1	20.3	32.3	45.7	22.0	36.8	43.5	19.7

TABLE V—CORRELATION OF NINHYDRIN DIFFERENTIAL ASSAY WITH MICROBIOLOGICAL ASSAY FOR GENTAMICIN BATCHES CONVERTED TO THEIR FREE BASES

Component	Correlation Coefficient—Weight	Activity
C_1	0.98	0.97
C_1a	0.98	0.98
C_2	0.93	0.96

As a second step, the six samples under consideration were converted to their free bases by means of an ion-exchange resin. The identical assay was performed using factors for the ninhydrin intensities of the compounds determined on known mixtures of the free bases of all of the components (Table I). The average response for these samples by weight and activity are presented in Table IV and are compared to the results obtained in the microbiological differential assay.

The correlations between the two assays using the free base mixtures measured by weight and activity are shown in Table V. All of the correlations are significant ($p < 0.01$).

The differential ninhydrin assay which has been described for the quantitative determination of the components of the gentamicin complex is highly reproducible and easier to perform than is the microbiological method. A significant correlation exists between the results obtained in the two procedures, and this new assay should therefore be very useful in determining proportions of the three gentamicin components in mixtures of the complex.

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Keyphrases

Gentamicin complex—differential analysis
Paper chromatography—separation, analysis
Ninhydrin—color reagent
Integrating scanner—color intensity

Dissolution Kinetics of Drugs in Human Gastric Juice—the Role of Surface Tension

By PER FINHOLT and SISSEL SOLVANG

The kinetics of *in vitro* dissolution of phenacetin and phenobarbital in human gastric juice have been determined and compared to those in hydrochloric acid containing various amounts of polysorbate 80. The surface tension of the dissolution medium is shown to have an appreciable effect on the dissolution kinetics of the drugs studied.

DURING THE LAST YEARS, a number of rate studies on the dissolution of drugs have been conducted. An excellent review of these is given by Wurster and Taylor (1). Various dissolution media have been used, but, surprisingly enough, not that solvent, in which the dissolution process takes place *in vivo*—that is, human gastric juice.

The purpose of this investigation was to study the rate of dissolution of drugs in human gastric juice. In addition, a closer examination of the

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TABLE IV—AVERAGE ASSAY VALUE OF GENTAMICIN BATCHES CONVERTED TO THEIR FREE BASES BY LOT AND METHOD

Lot No.	Ninhydrin Assay—By Weight, % C_1	C_2	C_1a	Ninhydrin Assay—By Activity, % C_1	C_2	C_1a	Microbiological Assay C_1	C_2	C_1a
GMC-4J-5I	42.5	37.2	20.3	36.9	40.8	22.3	42.2	39.2	18.7
60-1197-65I	43.1	34.0	23.0	37.7	37.2	25.1	41.2	34.1	24.9
60-1197-64I	35.8	35.3	28.8	30.7	38.2	31.1	36.5	32.9	30.6
GMC-4J-1-4C	31.0	41.0	28.0	26.3	43.9	29.9	29.1	40.5	30.5
60-1197-61I	38.8	37.1	24.0	33.5	40.4	26.1	38.7	37.8	23.5
60-1197-60	37.5	42.1	20.3	32.3	45.7	22.0	36.8	43.5	19.7

TABLE V—CORRELATION OF NINHYDRIN DIFFERENTIAL ASSAY WITH MICROBIOLOGICAL ASSAY FOR GENTAMICIN BATCHES CONVERTED TO THEIR FREE BASES

Component	Correlation Coefficient—Weight	Activity
C_1	0.98	0.97
C_1a	0.98	0.98
C_2	0.93	0.96

As a second step, the six samples under consideration were converted to their free bases by means of an ion-exchange resin. The identical assay was performed using factors for the ninhydrin intensities of the compounds determined on known mixtures of the free bases of all of the components (Table I). The average response for these samples by weight and activity are presented in Table IV and are compared to the results obtained in the microbiological differential assay.

The correlations between the two assays using the free base mixtures measured by weight and activity are shown in Table V. All of the correlations are significant ($p < 0.01$).

The differential ninhydrin assay which has been described for the quantitative determination of the components of the gentamicin complex is highly reproducible and easier to perform than is the microbiological method. A significant correlation exists between the results obtained in the two procedures, and this new assay should therefore be very useful in determining proportions of the three gentamicin components in mixtures of the complex.

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Keyphrases

Gentamicin complex—differential analysis
Paper chromatography—separation, analysis
Ninhydrin—color reagent
Integrating scanner—color intensity

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relationship between the surface tension of the fluid and the dissolution rate of drugs was felt needed. The reason for this was that previous studies on the rate of dissolution of drugs from powders, granules, and tablets had shown that the surface properties of the solid particles was an important factor (2, 3).

EXPERIMENTAL

Materials—The powdered drugs used were of Pharmacopoea Nordica grade. They were separated into the desired size fractions by sieving.

The gastric juice was delivered from a hospital department engaged in testing the gastric secretion of patients under examination for diseases of the stomach.[1] It would of course have been desirable to use gastric juice from healthy persons; but such large amounts were needed for the experiments that it was quite impossible to get enough from healthy volunteers.

The gastric juice obtained from each patient was collected on an empty stomach, 1 hr. before and 1 hr. after stimulation with large doses of histamine (subcutaneous injection). Immediately after collection the gastric juice was frozen at about −20° and stored at this temperature in a freezer until it was to be used. The time of storage was usually 3–4 weeks. However, the gastric juice could be stored at −20° for months, without changing properties like pH and surface tension.

When gastric juice was to be used as dissolution medium, samples from 25–30 different persons were mixed and then diluted with an equal amount of water. Water was added since powders and tablets normally are swallowed with water and the dissolution process *in vivo* thus takes place in a mixture of gastric juice and water.

Determination of Dissolution Rate—The dissolution rate was determined by the beaker method of Levy (4) with minor changes.

Procedure A—The dissolution assembly consisted of a 1-l. Pyrex beaker immersed in a constant-temperature bath adjusted to 37.5°, and a flat, straight-blade (6 cm. diam. and 1.5 cm. high) stainless steel stirrer attached to an electric stirring motor giving the stirrer a constant and easily adjustable speed of rotation.

Five-hundred milliliters of the dissolution medium was placed in the beaker and permitted to equilibrate to 37.0°. The stirrer was immersed into the middle of the liquid and rotated at a speed of 50 r.p.m. A certain amount of the drug powder (1.0 g. of phenacetin, 2.0 g. of phenobarbital, 2.4 g. of sodium phenobarbital) was gently spread over the surface of the fluid. During the test the powder would partly remain on the surface and partly settle down through the fluid. At appropriate intervals 5-ml. samples were withdrawn by means of pipets provided with short plastic tubings filled with glass wool or fritted-glass immersion filter tubes of medium porosity for filtration of the sample. The samples were analyzed and the drug concentration found was plotted *versus* time.

Procedure B—A 250-ml. beaker, a flat, straight-blade (4 cm. diam. and 1.5 cm. high) stirrer, 100 ml. of the dissolution medium, and 0.5 g. of the drug were used and 2-ml. samples were withdrawn. Otherwise Procedure B was the same as Procedure A.

Analytical Methods—*Determination of Phenacetin*—The samples withdrawn were diluted with water and absorbancies were measured at 245 mμ with a Beckman DK 2A spectrophotometer.

Determination of Phenobarbital—The samples withdrawn were mixed with borate buffer pH 9.5 and diluted with water. Absorbancies were measured at 240 mμ.

Determination of Solubility—The solubilities of the drugs were determined by using the beaker method and following the dissolution process until the dissolution medium became saturated with drug.

RESULTS AND DISCUSSION

Effect of Surfactant on Dissolution Rate—Phenacetin was chosen as model substance because of its hydrophobic properties. Polysorbate 80 was used as surfactant.

Figure 1 shows the rate of dissolution of phenacetin in 0.1 *N* HCl to which different amounts of polysorbate 80 had been added. An increase in the

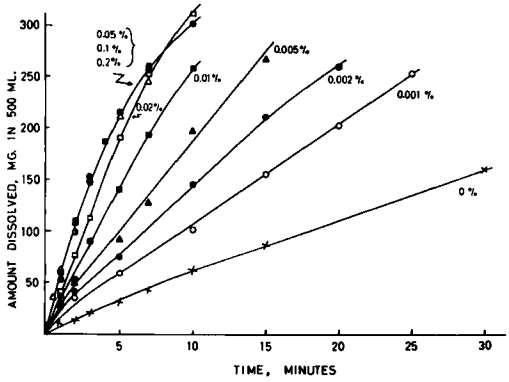

Fig. 1—Effect of polysorbate concentration of the dissolution medium (0.1 N HCl) on dissolution rate of phenacetin (0.21–0.30 mm.).

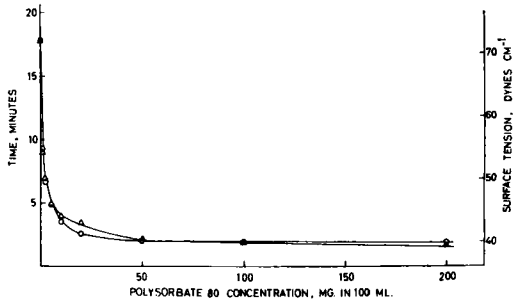

Fig. 2—Relationship between the polysorbate concentration of the dissolution medium (0.1 N HCl) and the time necessary for dissolution of 100 mg. of phenacetin (0.21–0.30 mm.); and the relationship between the polysorbate concentration of the dissolution medium and the surface tension of the same. Key: ○, time; △, surface tension.

[1] The authors want to thank Dr. J. Myren, Medical Department IX, Ullevål Hospital, Oslo, for the gastric juice and for information about the gastric secretion rate of the patients.

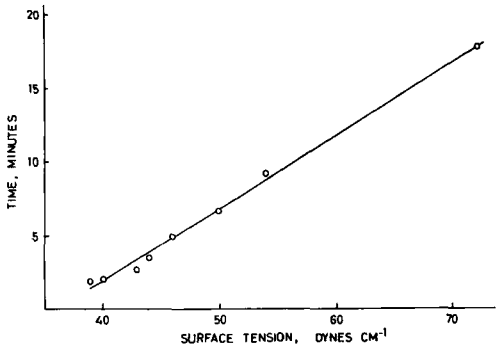

Fig. 3—Relationship between the surface tension, the dissolution medium and the time necessary for dissolution of 100 mg. of phenacetin (0.21–0.30 mm.). Dissolution media: 0.1 N HCl containing different amounts of polysorbate 80.

polysorbate concentration from 0 to 0.01% causes a significant increase in dissolution rate. Addition of further amounts of the surfactant has very little effect.

The times necessary for dissolution of 100 mg. of phenacetin in the different polysorbate–HCl mixtures can easily be picked from Fig. 1. In Fig. 2 these times are plotted *versus* the corresponding polysorbate concentrations. The same figure also shows the surface tension of the dissolution medium in relation to the concentration of polysorbate 80. The surface tension was measured at 20° with a Cenco Du Nouy tensiometer. It will be seen that the two curves are nearly superimposed. This means that a nearly linear relationship exists between the surface tension of the dissolution medium and the time necessary for dissolution of 100 mg. of phenacetin. This relationship is clearly demonstrated in Fig. 3.

The solubilities of phenacetin at 37° in 0.1 *N* HCl containing 0%, 0.001%, 0.01%, or 0.1% of polysorbate 80 were found to be 1.18 g./l., 1.26 g./l., 1.26 g./l., and 1.29 g./l., respectively. Polysorbate 80 in the concentrations used thus has a very small influence on the solubility of phenacetin in HCl. This means that the effect of polysorbate 80 on the rate of dissolution of phenacetin is due only to a small extent to its solubilizing power, but is caused mainly by its ability to decrease the interfacial tension between the substance and the dissolution medium. This result is in accordance with observations by Levy and Gumtow (5), who found that sodium lauryl sulfate enhanced the rate of dissolution of salicylic acid from compressed tablets. This effect was assumed to be due to increased wetting and to better solvent penetration into the tablets and granules as a result of the interfacial tension lowering effect of the surfactant.

Surface Tension of Human Gastric Juice—Since the surface tension of the dissolution medium seems to be a deciding factor as far as dissolution rate is concerned, it is of great interest to know the surface tension of human gastric juice. However, no values could be found in the literature. Therefore, a determination of the surface tension was made using gastric juice collected from a large number of persons under examination for diseases of the stomach. Samples of fasting as well as of histamine-stimulated secretion were tested. Table I shows the gastric secretion rate and the pH and surface tension of the gastric juice from some of the persons tested.

The results of the whole test are shown in Fig. 4, where the surface tension of the samples is plotted *versus* their pH. There is no significant correlation between these properties. The calculated correlation coefficient was less than the theoretical one at the 5% probability level. From these experiments it appears that the surface tension of human gastric juice is nearly independent of pH and secretion rate, having a value between 35 and 50 dynes cm.$^{-1}$.

Dilution of gastric juice with an equal amount of

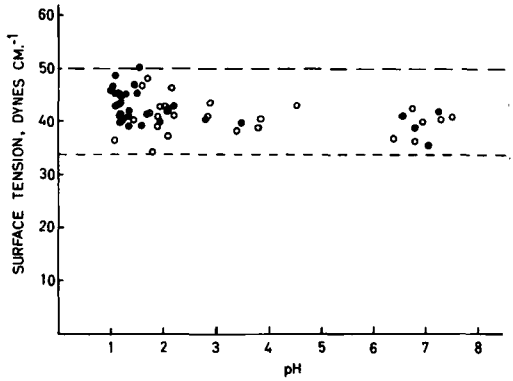

Fig. 4—pH and surface tension of gastric juice from 27 different persons. Key: ○, fasting secretion; ●, histamine-stimulated secretion.

TABLE I—GASTRIC SECRETION RATE AND pH AND SURFACE TENSION OF GASTRIC JUICE

Patient No.	Unstimulated Gastric Juice: Secretion Rate, ml. hr.$^{-1}$	pH	Surface Tension, dynes cm.$^{-1}$	Histamine-stimulated gastric juice: Secretion Rate, ml. hr.$^{-1}$	pH	Surface Tension dynes cm.$^{-1}$
1	5	7.2	—	20	2.2	42.7
2	15	3.4	38.0	100	1.2	39.5
3	25	2.5	40.1	105	1.2	37.8
4	25	6.2	40.2	15	1.6	—
5	30	5.2	38.5	110	1.3	37.5
6	35	6.3	36.5	35	6.1	38.3
7	50	1.9	40.0	230	1.1	40.3
8	60	6.8	36.1	120	1.4	40.5
9	125	4.9	44.0	230	1.2	44.2
10	130	3.8	38.5	130	1.5	46.7
11	125	1.7	42.4	165	1.4	43.2
12	180	1.6	39.0	260	1.1	40.3

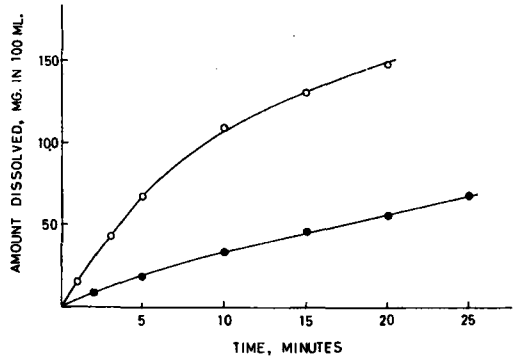

Fig. 5—Rate of dissolution of phenobarbital (0.21–0.30 mm.) in 0.1 N HCl and in diluted gastric juice. Key: ●, 0.1 N HCl; ○, diluted gastric juice.

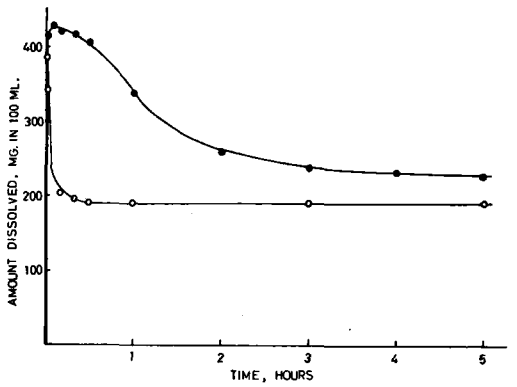

Fig. 6—Dissolution of sodium phenobarbital in 0.035 N HCl and in diluted gastric juice, and precipitation of the free acid from the supersaturated solutions. Key: ○, 0.035 N HCl; ●, diluted gastric juice.

water caused an increase in the surface tension of only 1–3 dynes cm.$^{-1}$.

Rate of Dissolution of Drugs in Diluted Gastric Juice[2]—Figure 5 shows that the rate of dissolution of phenobarbital (0.21–0.30 mm.[3]) in diluted gastric juice is substantially higher than the rate of dissolution in 0.1 *N* HCl. This was to be expected, since the diluted gastric juice has a much lower surface tension than the 0.1 *N* HCl. In addition, the solubility of phenobarbital at 37° was found to be slightly higher in diluted gastric juice (2.00 g./l.) than in 0.1 *N* HCl (1.90 g./l.).

Since phenobarbital is used in medicine not only as the free acid, but also as the sodium salt, the rate of dissolution of the salt in diluted gastric juice was determined and compared to the rate of dissolution of the salt in an HCl solution having the same pH as the diluted gastric juice. Figure 6 shows that initially nearly the same, high drug concentration is obtained in both dissolution media. However, the precipitation of the free acid in excess of solubility takes place at a much slower rate in gastric juice than in hydrochloric acid. Gastric juice obviously contains substances retarding the precipitation of the acid.

[2] A mixture of equal volumes of gastric juice and water.
[3] Powder passing through standard sieve US No. 50 (sieve opening, 0.30 mm.), but not through sieve No. 70 (sieve opening, 0.21 mm.).

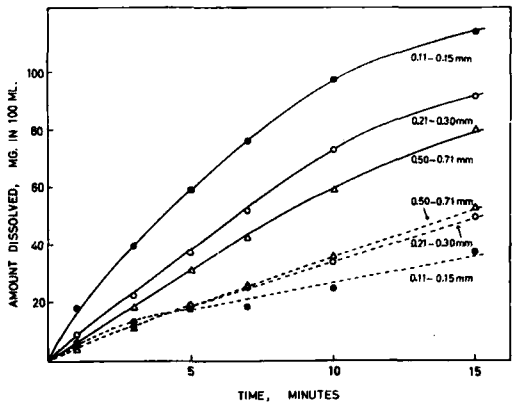

Fig. 7—Effect of particle size on dissolution rate of phenacetin in 0.1 N HCl and in diluted gastric juice. Key: – – –, 0.1 N HCl; ——, diluted gastric juice

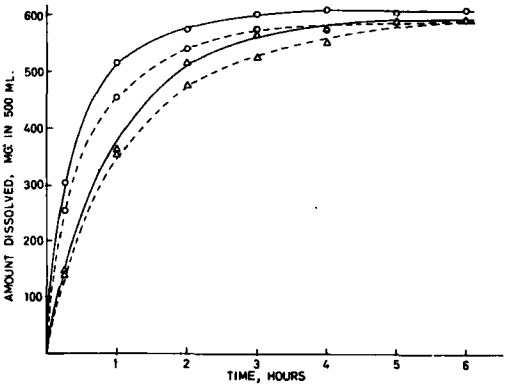

Fig. 8—Rate of dissolution of phenacetin (0.21–0.30 mm.) in diluted gastric juice and in diluted hydrochloric acid at the same pH and surface tension. Key: ——, diluted gastric juice (pH 1.6); – – –, diluted HCl + polysorbate 80 (pH 1.6); ○, surface tension 40.9 dynes cm.$^{-1}$; △, surface tension 50.7 dynes cm.$^{-1}$.

Effect of Particle Size on Dissolution Rate of Drugs in Diluted Gastric Juice—The solid lines in Fig. 7 show the effect of particle size on the rate of dissolution of phenacetin in diluted gastric juice. It is seen that the rate increases with decreasing particle size, whereas the opposite is the case when 0.1 *N* HCl is used as dissolution medium, as will be seen from the dotted lines in the same figure. These results are in agreement with earlier findings (2). Under the conditions of the experiment the rate of dissolution of hydrophobic drugs *increases* with decreasing particle size when the dissolution medium has a low surface tension, *decreases* when the surface tension is high. Figure 7 also shows that all powder fractions tested dissolve faster in diluted gastric juice than in 0.1 *N* HCl. This is due not only to the lower surface tension of the diluted gastric juice, but also to the higher solubility of phenacetin in this solvent (1.38 g./l. at 37°) compared to the solubility in 0.1 *N* HCl (1.05 g./l. at 37°).

Rate of Dissolution of Drugs in Diluted Gastric Juice and in HCl Solution at the Same pH and Surface Tension—According to Figs. 5 and 7

hydrophobic drugs such as phenobarbital and phenacetin dissolve faster in gastric juice than in 0.1 *N* HCl, mainly because of the lower surface tension of the former. The question arises whether there will be any difference between the dissolution rates in the two solvents if an HCl solut on of the same pH and surface tension as the diluted gastric juice is used. This question is important in relation to what kind of dissolution medium should be recommended for an *in vitro* dissolution test in the pharmacopeias.

The problem was studied with phenacetin as model substance. Two dilutions of gastric juice were made, one from samples of gastric juice with a relatively low surface tension (35–40 dynes cm.$^{-1}$) and one from samples with a relatively high surface tension (45–50 dynes cm.$^{-1}$). Rates of dissolution were determined using these two dilutions of gastric juice and two HCl solutions of the same pH and surface tension as the gastric juice mixtures. The surface tension of the HCl solutions was adjusted at the correct value by addition of polysorbate 80.

Figure 8 shows that phenacetin dissolves nearly at the same rate in hydrochloric acid as in gastric juice when the two media are adjusted at the same pH and surface tension. As far as this drug is concerned, an HCl solution adjusted to a surface tension of 40–50 dynes cm.$^{-1}$ is a better dissolution medium for an *in vitro* test than an HCl solution with no surfactant added. To see if this also applies to other drugs further studies are needed.

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Keyphrases

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Phenobarbital—dissolution rates
Phenacetin—dissolution rates
Gastric fluid, HCl solution, compared—drug dissolution
Surfactant concentration—dissolution effect
Surface tension—dissolution effect
UV spectrophotometry—analysis

Evaluation of the Effect of Isomerization on the Chemical and Biological Assay of Vitamin D

Analysis of Fat-Soluble Vitamins X

By J. A. KEVERLING BUISMAN, K. H. HANEWALD, F. J. MULDER, J. R. ROBORGH, and K. J. KEUNING

The reversible thermal isomerization of calciferols (D) to precalciferols(P), leading to a temperature-dependent equilibrium in solutions, interferes with many chemical and biological analytical procedures for the evaluation of vitamin D products. Two quantities should be distinguished: the potential vitamin D content (precalciferol + calciferol) and the actual vitamin D content (calciferol only). Of these two, only the potential vitamin D content is of importance to the buyer of vitamin D. Biological vitamin D assays give the potential vitamin D content, provided sample and reference standard dilutions are equilibrated by heating simultaneously to be sure that all have the same P–D ratio. This equilibration is superfluous in most of the chemical determinations of the potential vitamin D content. The actual vitamin D content can be determined by chemical, but not by biological, assay.

IT IS UNFORTUNATE that the assay of vitamin D preparations still frequently gives rise to difficulties. The reproducibility of biological assays is often poor, especially when assays from different laboratories are compared. The correlation of biological and chemical determinations is often unsatisfactory.

Received April 25, 1967, from the Philips-Duphar Research Laboratories, Weesp, the Netherlands.
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A source of errors which has drawn too little attention is the reversible isomerization of the calciferols in solutions to the corresponding precalciferols, forming an equilibrium mixture (1–3).

In 1931 Reerink and Van Wijk (4) observed that the physical constants of freshly irradiated solutions of ergosterol changed spontaneously. Velluz *et al.* (3) isolated the 3,5-dinitrobenzoate of the responsible intermediate in 1949 and called it

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precalciferol. Legrand and Mathieu (5) used infrared spectrophotometry to prove the mutual isomerization of the dinitrobenzoates of ergocalciferol (D_2) and preergocalciferol (P_2) leading to an equilibrium by first-order reactions. Verloop *et al.* (6, 7) and Velluz *et al.* (8, 9) established the structure of precalciferol. Havinga and coworkers (6, 10) investigated the chemical background of the isomerization of conjugated trienes and established that the isomerization rate is not affected by the solvent, acidity, ultraviolet light, catalysts, and free-radical reaction inhibitors.

Hanewald *et al.* (11) determined by ultraviolet absorptiometry the first-order rate constants of the isomerization of cholecalciferol, and their dependence on temperature. The isomerization rates of ergocalciferol and cholecalciferol are virtually equal (12). This should be a relief for many bioanalysts who have been compelled to standardize vitamin D_2 samples against cholecalciferol standards.

Shaw and Jefferies (13) reported an antirachitic activity of preergocalciferol of only 40% of the activity of ergocalciferol in rats; Bolliger (14) found 56% and Bekemeier and Pfordte (15) found 34%. For precholecalciferol Hanewald *et al.* (11) reported 35% of the activity of cholecalciferol in a chick test. The lower activity of the precalciferols must be due either to poor absorption or to lower intrinsic activity, the latter possibly originating exclusively from calciferols formed within the animal body by conversion of precalciferols (11, 13). A solution prepared from pure calciferol or pure precalciferol will after a time contain both compounds in a ratio dependent on storage time and temperature. This is also true for vitamin D preparations prepared from irradiation mixtures because during the photochemical manufacture of both calciferols, primarily the precalciferols are formed and the calciferols are formed from these by thermal isomerization. Whether the equilibrium will be reached clearly depends on the processing time and temperature, and if these are short or low, respectively, the ratio precalciferol/calciferol in the commercial product may be higher than conforms to equilibrium ratio at the ambient temperature and it will change until equilibrium has been reached.

These facts should be taken into account when assaying vitamin D or standardizing analytical methods.

THEORETICAL CONSIDERATIONS

Actual and Potential Vitamin D Content—When defining the vitamin D content there must be a distinction between the *actual vitamin D content*, *i.e.*, the content of calciferol alone, and the *potential vitamin D content*, meaning the sum of the contents of calciferol and precalciferol. Of these two, only the potential vitamin D content of a sample is independent of conditions during, preceding, and after the analysis and only the potential vitamin D content is a lasting characteristic and as such suitable for the evaluation of preparations. The actual vitamin D content concerns the sample at one distinct moment and it has a limited value, only in connection with the sample's antecedents.

By not distinguishing between potential and actual vitamin D contents a rather chaotic situation has arisen with regard to biological and chemical vitamin D assays, standardization, reference standards and their use, declared potencies, comparison of experimental results, stability tests, *etc.* Moreover, it must be emphasized that considerable errors in the actual vitamin D content may be introduced during analysis unless anything liable to change the ratio of both compounds, *e.g.*, heating, is carefully avoided (12).

Calculation of Equilibrium Compositions and Reaction Times—The isomerization constants for $P_3 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} D_3$ are (11):

$$\log k_1 = \frac{-4200}{T} + 10.29$$

$$\log k_2 = \frac{-5180}{T} + 12.53 \quad (k \text{ in min.}^{-1};\ T \text{ in °K.})$$

At equilibrium the cholecalciferol fraction in % of the potential D content is:

$$f = \frac{100\ D_3}{P_3 + D_3} = \frac{100\ k_1}{k_1 + k_2}$$

The time needed to reach 0.99 × the equilibrium fraction of the formed component, starting from solutions of pure cholecalciferol or pure precholecalciferol, is

$$t_{0.99} = \frac{2 \times 2.303}{k_1 + k_2} \text{ min.}$$

Table I contains the fractions and the times calculated for a series of temperatures. These times vary from years at below 0°C. to some minutes at sterilization temperature (120°).

On the other hand, the time needed in practice to approach the equilibrium at a certain temperature, T may be reduced to a few days if one starts with a mixture equilibrated at some higher temperature T_0, as can be seen in Table I. This time is calculated from

$$t_{0.99} = \frac{2.303}{k_1 + k_2} \log 100 \left(1 - \frac{f_0}{f}\right) \text{ min.}$$

f_0 and f are the equilibrium fractions at the temperatures T_0 and T, respectively; k_1 and k_2 are the rate constants in min.$^{-1}$ at T.

Implications of the Distinction Between Potential and Actual D Content—The constancy of the potential D content and the inconstancy of the actual D content due to isomerization lead to a number of important consequences.

(*a*) Only the *potential* D content is useful for the evaluation of vitamin D preparations, because it is

TABLE I—CALCULATED EQUILIBRIUM FRACTIONS AND TIMES $t_{0.99}$ NEEDED TO REACH 0.99 × THE EQUILIBRIUM FRACTION OF THE FORMED COMPONENT (D_3 or P_3)

Temp. T_0, °C.	Equilibrium Fractions, % of P + D P_3	D_3	Pure P_3 or D_3 at T_0	Time $t_{0.99}$ Needed Starting from Equilibrium Mixture at T_0, Days at T 0°	20°	30°	40°	50°
−20	2	98	16 years	—				
0	4	96	350 days	—				
20	7	93	30 days	85	—	—	—	—
30	9	91	10 days	126	5	—	—	—
40	11	89	3.5 days	153	10	2.0	—	—
50	13	87	1.3 days	173	13	3.0	0.6	—
60	16	84	0.5 days	195	15	4.5	1.5	0.3
80	22	78	0.1 days	225	19	6.0	2.0	0.7
100	28	72	30 min.	250	21	7.0	2.5	0.8
120	35	65	7 min.	270	23	7.5	2.7	1.0

reproducible and independent of the antecedents of the sample.

(*b*) This applies still more to the comparison of experimental results obtained at different places and at different moments. Stability tests should never compare actual but always potential D contents since it cannot be decided whether a decreased actual D content is due to reversible isomerization or to irreversible decomposition.

(*c*) For the analysis of many formulations it is necessary to isolate or concentrate the vitamin D. This can hardly be done without saponification, extraction, and other operations involving an elevated temperature and consequently a change in P–D ratio. In such formulations the potential D can still be determined but the actual D cannot.

(*d*) It must be emphasized that knowledge of the actual D content can only be valuable if the circumstances preceding the analysis are well defined and if the potential D content is known.

(*e*) In a determination of the actual D content the circumstances starting from the moment of sampling should be such that isomerization virtually cannot occur, *viz.*, by handling at low temperature.

These points are illustrated by the following examples.

1. Preparations heated during manufacture (sterilization) and cooled abruptly have a low relative actual D content (D/D + P) which will be maintained by storing at 0° or below. If dosage is based on any actual D assay in the cooled product this will lead to overdosing.

2. Two solutions of pure crystalline calciferol in oil are prepared (*a*) by dissolving in ether, mixing with oil and evaporating the ether at 20° *in vacuo*, and (*b*) by dissolving in ethanol, mixing with oil, and evaporating the ethanol during 30 min. at 100°. The actual vitamin D content of both is determined and each is diluted with oil to make the actual D content 10 mg./ml. (solutions *a* and *b*). The fresh solution *a* contains no appreciable amount of P (0.01 mg./ml.); however, the fresh solution *b* approx. 4 mg. of P per ml.

After storage for 1 month at 20°, these solutions will show actual D contents of: (*a*) 9.3 mg./ml. and (*b*) 13.0 mg./ml. The potential D contents will be unchanged: 10.0 mg./ml. in *a* and 14.0 mg./ml. in *b*.

3. Animals having a body temperature of 40° are injected intramuscularly with dilutions of these fresh solutions *a* and *b*. Suppose half the depot has been absorbed a month after injection. The remaining half depot of *a* will contain at that moment 45% but *b* 65% of the calciferol present in the injected dose.

The conclusion of all this is that the potential D content is the value important for the consumer. Evaluation and assay of vitamin D preparations should be confined to the determination of the potential D content.

METHODS OF EVALUATION

When determining the *actual* vitamin D content (or P content), one should observe the following points. (*a*) The conditions (temperature and time) from the moment of sampling until measurement should be such that an appreciable conversion D ⇌ P does not occur, *e.g.*, <1 hr. at 40°, or <8 hr. at 20°, or <3 days at 0°, or 1 year at −20°. (*b*) D and P should be separated completely without conversion and then determined separately, or (*c*) D and P are not separated but freed from interfering contaminations and then one or both or the ratio are determined, and the sum of both (potential D).

An example of *b* is TLC followed by Nield's reaction. An example of *c* is chromatographic purification (*e.g.*, alkaline alumina), followed by a Nield determination of P + D and ultraviolet absorptiometry for the ratio. D might be determined in the presence of P by infrared absorptiometry at 900 $cm.^{-1}$ [exocyclic methylene band missing in P (5)]. Or P is converted by iodine into tachysterol of which the maleic anhydride adduct does not interfere with the Nield reaction of D (7).

The determination of the *potential* vitamin D content is not limited by the conditions mentioned above. There are three possibilities.

1. Purification without separating P and D and determination by a common reaction (*e.g.*, Nield reaction).

2. Separation and purification; the P and D fractions are analyzed separately and the results added, or the fractions are reunited and analyzed together (*e.g.* TLC, followed by extraction of the separate or combined P and D fractions, and Nield reaction).

3. Comparison of a measurable property of a (purified) sample solution and a potential vitamin D standard solution, after equilibration of both under rigorously identical circumstances (*e.g.*, ultraviolet absorptiometry or biological assay).

Effect of the Isomerization P ⇌ D on Biological Assays—The uncertainty as to the P–D ratio combined with a considerable difference between the biological activities of P and D interferes with the reliability of biological assays.

In biological assays the physiological effects of a sample and of a reference standard are compared and from this the content of the sample relative to that of the reference standard is calculated. A predominant condition for a realistic assay is the availability of a dependable reference standard.

A reference standard prepared by dissolving crystalline ergocalciferol or cholecalciferol will contain, after some time, a mixture of the original calciferol and, as a result of isomerization, precalciferol. The biological response to this standard will now represent the resultant of two compounds differing markedly in biopotency. A further complication is the new shift in isomerization that begins at the moment of administration and is caused by the animal body temperature. Even if only the actual D is biologically active the unknown degree of isomerization of the standard makes it unfit for use in the determination of the actual D (or the P) with a bioassay.

For the same reasons quantitatively equal physiological effects will not be obtained if sample and standard contain equal amounts of actual D but different amounts of P.

In other words an equal antirachitic effect does not prove the equality of the actual D contents of sample and standard unless the P–D ratios are equal.

The situation is quite different, however, in the case of the more important potential vitamin D content. In the first place the amount of potential vitamin D in a standard solution is exactly known, *viz.*, the amount of calciferol used in its preparation, provided the vitamin D has not been destroyed by irradiation or oxidation. In the second place dilutions of sample and standard, containing equal amounts of potential vitamin D (calciferol plus precalciferol) will cause quantitatively the same antirachitic effect, if beforehand any possibly different P–D ratios have been made equal by heating at the same temperature until the equilibrium pertaining to this temperature is reached.

In addition reference standard and sample should be applied in virtually identical formulations, *e.g.*, dissolved in a vegetable oil and both should be administered to the animals in the same way. Complications due to absorption differences of calciferols and precalciferols cannot occur if formulation, equilibration, and administration of all dilutions of sample and reference standard are identical.

Equilibration of Sample and Standard for Bioassay—The following example describes an equilibration of the dilutions of the sample and the reference standard for the bioassay of potential vitamin D in oil samples.

For dilution use a vegetable oil, pure and clear and free from peroxides, acids, and halogens.

Weigh sufficient quantities of sample and reference standard and dilute with vegetable oil and antioxidant solution to obtain all dilutions needed for administering, containing the required vitamin D concentrations and equal concentrations of antioxidant (*e.g.*, 0.02% of BHA). Mix each dilution thoroughly by swirling under nitrogen.

Heat all flasks simultaneously for 2.5 hr. at 80°C. in an oven. Cool simultaneously to room temperature. Proceed with the bioassay according to common practice (16–19).

The simultaneousness is essential and care must be taken to handle *all* dilutions of standard *and* sample in the same way with respect to temperature and time. When not in use all dilutions are stored in a refrigerator.

In addition to the precautions mentioned above to prevent oxidation and other irreversible conversions it is good practice to replace air by nitrogen each time after the flasks have been opened. In case the sample is not a solution in oil, some degree of isolation may be necessary to precede dilution by vegetable oil, *e.g.*, saponification, extraction, evaporation, dissolution. These operations will not alter the potential vitamin D content if they are carried out by reliable methods which prevent losses. The P–D ratio may change if heating is involved, but this will not influence the results because of the subsequent equilibration.

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Keyphrases

Vitamin D—analysis
Isomerization effect—vitamin D assay
Actual vitamin D content—calciferol concentration
Potential vitamin D content—calciferol and precalciferol concentration

Drug Biotransformation Interactions in Man I

Mutual Inhibition in Glucuronide Formation of Salicylic Acid and Salicylamide in Man

By GERHARD LEVY and JOSEPHINE A. PROCKNAL

Co-administration of salicylamide (SAM) and sodium salicylate to healthy adult humans resulted in a pronounced decrease in the formation of the glucuronides of these two drugs. This is thought to be due to a limited capacity for glucuronide conjugation in man and/or to the uncoupling action of salicylate on oxidative phosphorylation. Salicylate *may* inhibit also the formation of SAM sulfate; it has no effect on the excretion rate constants of SAM glucuronide and SAM sulfate in the doses used in the investigation. The results of this study indicate that concomitant use of certain drugs which are readily available to the public may lead to appreciable interactions in biotransformation and to enhanced pharmacologic effects.

CLINICAL PHARMACOLOGIC STUDIES in recent years have led to a recognition of the important role of drug interactions in therapy and in toxicology (1). Most of these studies have dealt with enzyme induction (2), but the role of competitive and noncompetitive inhibitory effects on drug biotransformation and excretion processes is also being recognized. It is now evident that a number of important drug biotransformation processes in man have a relatively limited capacity (3–6). This in itself is not surprising since drug biotransformation involves enzymic reactions, but it was not realized until recently that "saturation" effects may be encountered when drugs are given in the usual therapeutic dose range. It may be expected that co-administration of two or more drugs which share a biotransformation pathway of low capacity will lead to competitive inhibition of the formation of the respective metabolites. Consequently, there will be a change in quantitative composition of metabolites and a decrease in the elimination rate of one or both of the drugs. This can result in enhanced pharmacologic activity or, if the metabolite produced by the capacity-limited process is the pharmacologically active species, in a decrease of the pharmacologic activity of a drug. An exploration of the mutual pharmacokinetic effects of drugs sharing a biotransformation route of low capacity in man is therefore highly pertinent. Such effects have obvious implications with respect to drug safety since they may lead to adverse reactions and intoxications; it should be recognized, however, that the judicious and informed use of such drug combinations may also result in safer and more effective medication. Finally, the study of drug metabolism interactions represents an effective research approach for identifying possible differences in the rate-limiting step in the formation of metabolites of different drugs which apparently share the same biotransformation pathway. This report is the first of a series from this laboratory in which studies of such drug interactions will be described. It is concerned with what is generally considered to be the most important, versatile, and ubiquitous drug biotransformation pathway in man, the formation of glucuronides. The relevance of such studies is emphasized further by the nature of the two drugs utilized in the investigation—salicylate and salicylamide—since they are among the most widely used and most readily available to the public.

Received March 4, 1968, from the Biopharmaceutics Laboratory, Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication April 23, 1968.

Presented at the 52nd Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 1968.

Supported in part by grant No. 1 R01 DS00021-01 from the U. S. Public Health Service, Bethesda, Md.

EXPERIMENTAL

Healthy, male, ambulatory human subjects received orally 0.6 g. salicylamide in one test, and the same dose of salicylamide 2 hr. following oral administration of 2.32 g. sodium salicylate (equivalent to 2 g. salicylic acid) in another test. Both tests were initiated in the morning on an empty stomach. Food was withheld for at least 2 hr. after drug administration. Both drugs were given in aqueous solution. Urine was collected every 30 min. for the first 4–8 hr., then at longer intervals for a total of 24 hr. From 50 to 100 ml. water was ingested after each urine collection to assure adequate urine output. The urine samples were stored in a freezer until assayed.

Assay Methods for Salicylic Acid and Its Metabolites—Salicylic acid and its major metabolites in urine were determined by a further modification of the Smith *et al.* (7) modification of the colorimetric method of Brodie *et al.* (8) for the determination of salicylic acid in plasma. In essence, the procedure as used in the study reported here consists of (*a*) determination of salicylic acid (SA) by extraction of the sample with carbon tetrachloride and reextrac-

tion into ferric nitrate reagent solution, (*b*) determination of the absorbance due to both SA and salicyluric acid (SU) by extraction of the sample with ethylene dichloride and reextraction into ferric nitrate solution, and (*c*) determination of total salicylates [*i.e.*, SA, SU, and salicylic glucuronides (SAG)] after complete acid hydrolysis of these metabolites to SA, followed by step *a*. SU content is calculated by difference of *a* from *b*, and SAG content is obtained by subtracting SA and SU values from *c*.

The major difference between the method of Smith *et al.* (7) and the method described here is based on studies in this laboratory which have shown that SU does not partition to any significant extent into carbon tetrachloride. Partition coefficients of SU between 0.1 *N* HCl and various organic solvents were determined with a pure sample of SU obtained after several recrystallizations from water and ethanol–chloroform mixture and found free of impurities by thin-layer chromatography, elemental analysis, and by examination of the infrared spectrum. These partition coefficients are listed in Table I. The lack of significant partitioning of SU into carbon tetrachloride even in the presence of SA is evident from the data shown in Table II. The apparent extraction of SU by carbon tetrachloride reported by Brodie *et al.* (8) and Smith *et al.* (7) was probably due to contamination of their sample (both groups used material from the same source) by about 5% SA. The other modification of the method of Smith *et al.* (7) was to hydrolyze SA metabolites totally rather than partially, since differences in heating and cooling rates can affect the degree of partial hydrolysis, and thus the reproducibility of the assay results.

Procedure A—The concentration of SA in urine is determined as follows: 2 ml. of the urine sample (diluted with water if necessary), 0.5 ml. 6 *N* HCl, and 30 ml. carbon tetrachloride are placed in a glass-stoppered bottle. After shaking vigorously for 5 min., the mixture is transferred to a centrifuge tube, centrifuged to separate the phases, and the urine phase is aspirated and discarded. Ten milliliters of the organic phase and 5 ml. of ferric nitrate reagent solution are placed in a glass-stoppered centrifuge tube which is shaken vigorously for 5 min. After centrifuging if necessary to separate the layers, a portion of the aqueous phase is removed and its absorbance is determined at 530 mμ. A reagent blank value is obtained by using distilled water in place of the urine sample and applying the same procedure. The ferric nitrate reagent solution is freshly prepared by diluting 5 ml. of ferric nitrate stock solution [1.0% $Fe(NO_3)_3$ in 0.07 *N* HNO_3] with distilled water to 100 ml. All chemicals and solvents should be of reagent grade quality. An aqueous solution containing 10 mg.% SA, when subjected to the above procedure, yields an absorbance in the ferric nitrate reagent phase of about 0.14 in cells of 1-cm. path length.

Procedure B—Salicyluric acid is determined by essentially the same procedure as that described for SA, except that reagent grade ethylene dichloride is used instead of carbon tetrachloride. To account for the contribution of SA to the absorbance of the ferric nitrate reagent phase, 1.06 times the absorbance value obtained in Procedure A is subtracted. Multiplication by 1.06 corrects for the small difference in the partitioning of SA when using carbon tetrachloride and ethylene dichloride, respectively, for the extractions. This difference has been constant and reproducible during the several years that the procedure has been used in this laboratory. A solution containing 10 mg.% SU yields an absorbance of about 0.059 in the ferric nitrate reagent phase (1-cm. cell path length) while a 10-mg.% solution of SA produces an absorbance of about 0.15 in the procedure described here. Table II shows the results of assays of solutions containing known concentrations of both SA and SU.

Procedure C—Total salicylate in the urine is determined by hydrolyzing SU and SAG to SA. Three milliliters of the urine sample and 3 ml. of concentrated hydrochloric acid are heated in a sealed 20-ml. ampul at 100° for 16 hr. This length of time is necessary to bring about an essentially complete hydrolysis of SU to SA. Two milliliters of the hydrolyzed solution is then analyzed for SA by Procedure A. The concentration of SAG in the sample is determined by subtracting the sum of the SA and SU concentrations (the latter corrected for molecular weight by multiplying by 0.708) from the total SA concentration of the hydrolyzed sample.[1] Thus, $(SAG) = (SA_{total}) - [(SA_{free}) + 0.708 (SU)]$ where the terms in parentheses denote concentrations in metric (rather than molar) units, and (SAG) is expressed in terms of (SA). Urine blanks are usually somewhat less than 1 mg./hr. apparent total SA. The recovery of total salicylate following an oral dose of SA or aspirin has been consistently between 95 and 100% under properly controlled experimental conditions (*i.e.*, when complete urine collections could be monitored in the laboratory or in a clinical research center). When the dose of SA exceeds 1 g. in adults (and proportionately less in infants and children), gentisic acid (GA) determinations are necessary to account adequately for the administered dose.

TABLE I—PARTITION COEFFICIENT OF SALICYLURIC ACID IN SEVERAL ORGANIC SOLVENTS[a]

Solvent	Partition Coefficient (Organic/Aqueous)
Carbon tetrachloride	<0.002
Chloroform	0.05
Ethylene dichloride	0.15
Ether	4.0

[a] Solutions of 100-mg. % salicyluric acid in 0.1 *N* HCl were equilibrated with equal volumes of organic solvent at room temperature.

TABLE II—DETERMINATION OF SALICYLURIC ACID IN THE PRESENCE OF SALICYLIC ACID

Composition of Solutions, mg. %		Found, mg. %[a]	
Salicylic Acid	Salicyluric Acid	Salicylic Acid	Salicyluric Acid
0	25	0	24.5
5	25	5.07	24.5
10	25	10.0	24.6
25	25	25.1	24.1
50	25	50.7	24.5

[a] Average of two determinations.

[1] Determination of SAG by enzymatic hydrolysis for 16 hr. with β-glucuronidase yields results which are about 20% lower than those obtained by acid and heat hydrolysis. This appears to be due to the rather slow rate of the enzymic reaction.

TABLE III—VERIFICATION OF ASSAY METHOD FOR SALICYLATE AND ITS METABOLITES AND SALICYLAMIDE AND ITS METABOLITES IN URINE SAMPLES CONTAINING BOTH GROUPS OF SUBSTANCES

		Concentrations, as mg. % SA or SAM Equivalent			
		A	B		
Metabolite[a]	Sample[b]	Urine After Salicylate Admin.	Urine After Salicylamide Admin.	Equal parts, A + B	
				Assay	Theoretical
SAMG	I	0.2	6.9	3.5	3.6
	II	0.3	12.7	6.5	6.5
SAMS	I	0.1	9.1	4.4	4.6
	II	0.2	8.7	4.4	4.4
SA	I	0.6	0.3	0.4	0.4
	III	1.5	0.7	1.1	1.1
	II	2.1	0.8	1.5	1.5
SAG	I	2.7	0.0	0.9	1.4
	III	6.1	0.0	2.5	3.0
	II	8.1	0.0	3.5	4.0
SU	II	17.8	0.6	9.8	9.2
	I	21.3	0.3	11.0	10.8
	III	34.1	0.5	16.8	17.3

[a] SAMG, salicylamide glucuronide; SAMS, salicylamide sulfate; SA, salicylic acid; SAG, salicylic glucuronides; SU, salicyluric acid. [b] Roman numerals refer to a specific urine sample obtained after either SA or SAM administration.

This is due to saturation effects with respect to SU formation, resulting in an increase in the fraction of the dose metabolized to other products. GA is determined by the method of Becher (9). Experience has shown that the concurrent assay of reference solutions containing known concentrations of GA is desirable to achieve satisfactory results.

Assay Methods for Salicylamide and Its Metabolites—The procedures were those of Levy and Matsuzawa (5) with minor modifications. In the determination of total salicylamide (SAM), carbon tetrachloride was substituted for ethylene dichloride. In the assays of SAM glucuronide and SAM sulfate, the pH after enzymic hydrolysis was adjusted to 6.5 with 0.1 *N* sodium hydroxide solution instead of adding phosphate buffer. Gentisamide (GAM) was determined as such (rather than as GA). In this procedure, 3 ml. of urine sample, 2 ml. of pH 4.5 acetate buffer (0.4 *M*), and 2 ml. of beef liver beta-glucuronidase[2] are incubated at 37° for 48 hr. After adjusting the pH to 6.0 with 0.1 *N* NaOH solution, enough water is added to yield a total volume of 10 ml. Twenty milliliters reagent grade ether is added and the mixture is shaken for 5 min. Fifteen milliliters of the ether phase is extracted with 6 ml. of a 5% solution of sodium bicarbonate. To 4 ml. of the aqueous phase are added 1 ml. of concentrated HCl and 1 ml. of Folin-Ciocalteu reagent and the absorbance of this solution is determined 20 min. later at 750 mμ. Reagent blanks (using water instead of a urine sample) and controls are run concurrently.

Determination of Salicylic Acid, Salicylamide, and Their Metabolites in the Presence of One Another—The difference in the pKa of SA, SU, and SAM affords selective extraction of the latter at pH 6.5 from aqueous solution. Total SAM is determined by extraction of free SAM following enzymic hydrolysis with beta-glucuronidase and sulfatase. Absorbance values in the determination of SA and SU (Procedures A and B) are corrected for the contribution by SAM. These corrections are negligible and essentially equal to urine blank values since practically no free SAM appears in the urine. Total SA from salicylate and its metabolites is determined in the presence of SAM metabolites by subtracting the SA equivalent of the total SAM obtained by beta-glucuronidase and sulfatase hydrolysis from the total SA obtained by hydrolysis of both SA and and SAM metabolites to SA. The analytical procedures were checked by separately assaying urine samples obtained following SA administration and samples obtained following SAM administration, and then assaying mixtures of equal parts of these urines. Table III shows results obtained at low concentrations of SA and SAM metabolites, *i.e.*, under the most difficult conditions.

[2] Ketodase, Warner-Chilcott, Morris Plains, N. J.

RESULTS AND DISCUSSION

Salicylic acid is eliminated in man mainly by formation of salicyluric acid, by renal excretion of free drug, and by glucuronide conjugation (10). Salicylamide is metabolized to the sulfate, the glucuronide, and to gentisamide; excretion of free salicylamide is negligible (5). Both drugs are subject to capacity-limited effects in their biotransformation in man even in the usual therapeutic dose range. This involves the conjugation of salicylate with glycine to form salicylurate (3, 11) and the formation of salicylamide sulfate from salicylamide (5). It has been reported that at high levels of salicylamide, saturation of the glucuronide formation process also becomes evident (12). The elimination of salicylate in man is considerably slower than that of salicylamide and the experimental design of the study reported here was based on this consideration. Salicylate was administered 2 hr. before salicylamide; body levels of the former declined by less than 25% in the period of time when salicylamide and its metabolites were present in the body. The effect of salicylamide on the formation of salicylic glucuronides is shown in Figs. 1 and 2 for two subjects. Similar results were obtained in the third subject.[3] The excretion of salicylic glucuronides decreased precipitously shortly after salicylamide administration and returned to normal levels within about 2 hr. The short duration of the salicylamide effect is due to the rapid elimination of this drug. Figure 2 shows also the time course of salicylurate excretion following salicylate administration. The excretion rate of this metabolite was essentially constant during the first 12 hr., reflecting the limited capacity of man to form salicylurate

[3] Graphs of all the individual data are not shown due to space limitations but are available from the authors upon request.

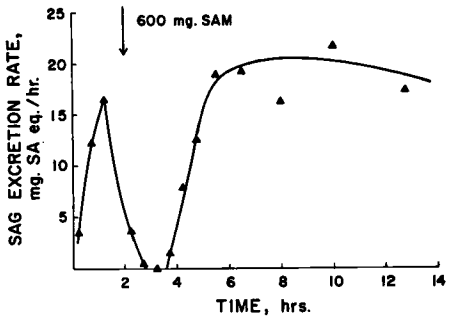

Fig. 1—Excretion rate of salicylic glucuronides following oral administration of 2.32 g. sodium salicylate. Arrow indicates time when 0.6 g. salicylamide was taken. Subject A.

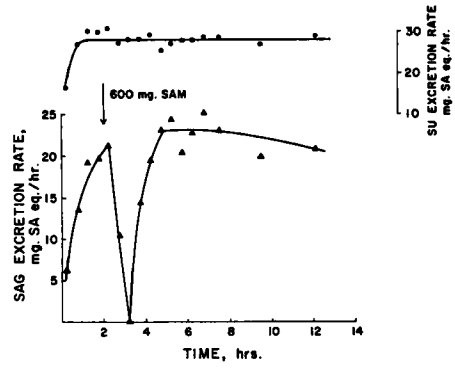

Fig. 2—Excretion rate of salicylic glucuronides following oral administration of 2.32 g. sodium salicylate. Arrow indicates time when 0.6 g. salicylamide was taken. Subject B. Upper curve shows urinary excretion rate of salicyluric acid. Note lack of effect of salicylamide on the latter.

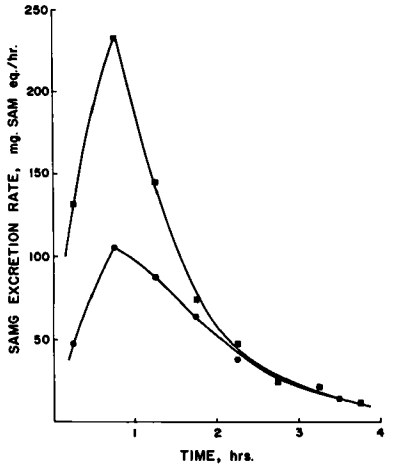

Fig. 3—Excretion rate of salicylamide glucuronide following oral administration of 0.6 g. salicylamide alone (■) and 2 hr. after 2.32 g. sodium salicylate (●). Subject A.

(3, 11). Salicylamide, which is not conjugated with glycine, had no effect on this process in any of the subjects.

The effect of salicylate on the excretion of salicylamide glucuronide is shown in Fig. 3 for one of the subjects and is representative of the data obtained in all three subjects.[3] There was a pronounced decrease in the excretion of salicylamide glucuronide in the presence of salicylate. Since the excretion of salicylamide conjugates is excretion rather than formation rate limited (5, 13), the terminal phase in the excretion rate plot reflects the excretion kinetics of the respective metabolite. Figure 4 is a semilogarithmic plot of salicylamide glucuronide excretion rate in the terminal excretion phase as a function of time. Each of the curves in the figure is displaced on the time axis relative to the others to facilitate comparison of slopes. It is evident that the excretion half-life of salicylamide glucuronide is about 0.8 hr. and that salicylate has no effect on this process. Therefore, the pronounced decrease in the excretion of salicylamide in the presence of salicylate is due to decreased formation of salicylamide glucuronide. This conclusion is substantiated by the lower total recovery of salicylamide glucuronide in the presence of salicylate (Table IV).

Figure 5 shows the excretion rate of salicylamide sulfate in one of the subjects following administration of salicylamide alone or in the presence of salicylate. Similar data were obtained with the other subjects.[3] Figure 6 is a semilogarithmic plot of salicylamide sulfate excretion rate as a function of time in the terminal phase of the process, representing an excretion half-life of about 1 hr. While salicylate has no effect on the process of excretion of salicylamide sulfate, there may be an inhibition of sulfate formation since the maximum excretion rate of salicylamide sulfate was consistently decreased in the presence of salicylate. This inhibition is also suggested by the data in Table IV, which shows the total composition of the urinary excretion products of salicylamide when given alone and with salicylate. While the output of salicylamide glucuronide decreased by about 100-mg. salicylamide equivalent in the presence of salicylate, the output of salicylamide sulfate increased by only about 20-mg. salicylamide equivalent. The major increase was in gentisamide output, which is usually very small when this dose of salicylamide is administered alone. Unfortunately, gentisamide output was determined only when salicylamide and salicylate were given together, but an estimate of

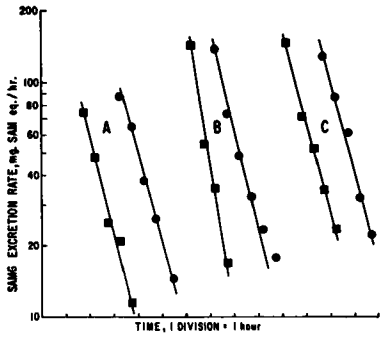

Fig. 4—Semilogarithmic plot of the terminal phase of salicylamide glucuronide excretion following oral administration of salicylamide alone (■) and 2 hr. after salicylate (●). Letters identify data from subjects A, B, and C.

TABLE IV—EFFECT OF SALICYLATE ON THE METABOLIC FATE OF SALICYLAMIDE IN MAN[a]

Subject	Sex	Age, yr.	Weight, kg.	Urinary Excretion Products[b], mg. SAM Equivalent: Total Recovery Control[c]	Total Recovery With SA[d]	SAMG Control	SAMG With SA	SAMS Control	SAMS With SA	GAM Control[e]	GAM With SA
A	M	39	81	569	548	374	226	196	268	<31	54
B	M	31	57	588	577	394	325	195	174	<12	78
C	M	27	86	550	590	326	234	224	233	<50	122
Mean				569	572	365	262	205	225	<31	85

[a] 0.6 g. salicylamide administered orally alone or 2 hr. following 2.32 g. sodium salicylate. [b] SA, salicylate; SAM, salicylamide; SAMG, salicylamide glucuronide; SAMS, salicylamide sulfate; GAM, gentisamide. [c] Excluding GAM. [d] Including GAM. [e] Estimated maximum value, dose − (SAMG + SAMS).

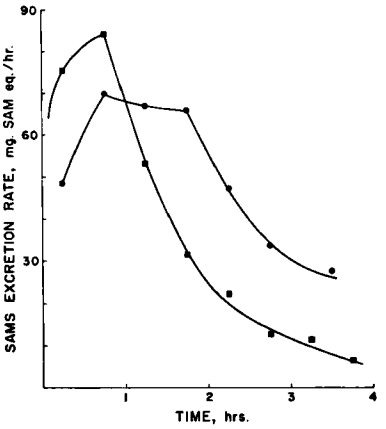

Fig. 5—Excretion rate of salicylamide sulfate following oral administration of 0.6 g. salicylamide alone (■) and 2 hr. after 2.32 g. sodium salicylate (●), Subject A.

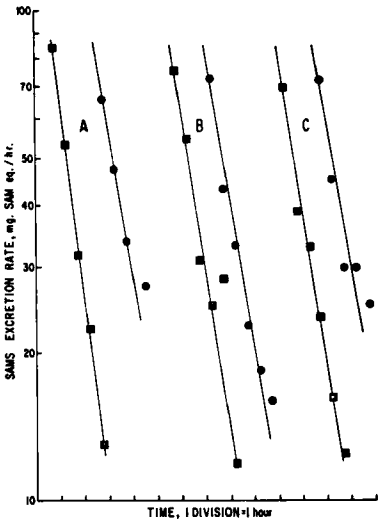

Fig. 6—Semilogarithmic plot of the terminal phase of salicylamide sulfate excretion following oral administration of salicylamide alone (■) and 2 hr. after salicylate (●). Letters identify data from subjects A, B, and C.

the maximum possible gentisamide output from salicylamide in the absence of salicylate has been obtained by subtracting the output of salicylamide glucuronide and sulfate from the total dose. While the lack of a significant increase in salicylamide sulfate formation may be due to the limited capacity of the body to form this metabolite (5), the decreased maximum excretion rate in the presence of salicylate is suggestive of salicylate-induced inhibition of sulfate formation. This problem is presently being studied in more detail.

The most likely reason for the mutual inhibition by salicylate and salicylamide of the formation of their respective glucuronides appears to be competition for uridine diphosphoglucuronic acid (UDPGA) or for UDP glucuronyl transferase, reflecting a limited capacity in man for glucuronide formation. Alternatively, or in addition, there may be a noncompetitive inhibition by salicylate of the glucuronide formation process. It has been suggested that high levels of salicylate inhibit the formation of salicylic glucuronides in mice due to an uncoupling effect of salicylate on oxidative phosphorylation (14); preliminary observations in this laboratory suggest that high levels of salicylate inhibit salicylic glucuronide formation also in man. Aspirin in doses as low as 0.6 g. decreases appreciably the urinary excretion of endogenous corticosteroid glucuronide in man, but not the excretion of free corticosteroid (15). The uncoupling effect of salicylate is thought to be responsible also for the decreased biosynthesis of sulfate esters following salicylate administration to rats (16); it is not unreasonable to suspect that the same effect could result in an inhibition of salicylamide sulfate formation in man. However, the results of the present study are only suggestive of salicylate-induced inhibition of salicylamide sulfate formation; the data can be explained also by assuming salicylate-induced delayed absorption of salicylamide although this latter possibility seems unlikely. The most important finding of this study is the pronounced mutual inhibition in the formation of glucuronides of and by two widely used and commonly available drugs given in usual therapeutic doses. One or both of these drugs is likely to have similar inhibiting effects on the glucuronide conjugation of other drugs; this is now being investigated in this laboratory. There is thus the possibility for frequent occurrences of interactions in drug biotransformation since many individuals taking prescribed drugs are also frequent users of mild analgesics, the latter being taken usually without knowledge of the attending physician. It is interesting also to speculate to what extent the many medicinal preparations consisting of combinations of salicylates with other drugs owe their enhanced effectiveness to a salicylate-induced inhibition of the elimination of one of these other drugs, rather than to the direct pharmacologic effects of salicylate itself.

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Salicylurate excretion—salicylate administration
UV spectrophotometry—analysis

Effect of Complex Formation on Drug Absorption VII

Effect of Complexation and Self-Association on the Absorption of Caffeine

By RICHARD H. REUNING* and GERHARD LEVY†

The effect of the formation of caffeine complexes having a higher apparent lipoid–aqueous partition coefficient than caffeine itself on the absorption of this drug from the rat stomach was investigated. In one such system, caffeine–salicylic acid, the salicylic acid actually decreased the absorption of caffeine due to an effect of the former on the gastric mucosa. In another such system, caffeine–*p*-hydroxybenzoic acid, the *p*-hydroxybenzoic acid did not increase the absorption of caffeine. The intestinal transfer of caffeine alone was studied at low concentrations (where caffeine exists mainly in monomeric form) and at high concentrations (where significant self-association occurs). The intestinal transfer rate constant of caffeine at high concentrations was significantly lower than at low concentrations. The mechanism of this effect was explored.

In the initial studies in this laboratory of the effect of complex formation on drug absorption, it was found that complexation with caffeine decreased significantly the overall[1] absorption of salicylic acid from the stomach of the rat (1). This effect was qualitatively consistent with the apparent lipoid–aqueous partition coefficient (PC) of salicylic acid and of the caffeine–salicylic acid complex; the latter showed an appreciably lower apparent PC than the former. It appeared reasonable to assume that the absorption of caffeine might be enhanced by complexation with salicylic acid, since this complex has a higher apparent PC than caffeine itself (1). Studies were therefore initiated to test this assumption. The investigation was then extended to include a study of the caffeine–salicylate ion complex and the caffeine–*p*-hydroxybenzoic acid complex. The latter was of considerable interest since it has a higher apparent PC than caffeine as well as an appreciably higher stability constant than the salicylic acid–caffeine complex (2). In the course of these studies it became apparent that the self-association of caffeine at high concentrations (3) can affect the absorption of this drug. Therefore, this effect was studied also.

Received April 3, 1968, from the Biopharmaceutics Laboratory, Department of Pharmaceutics, School of Pharmacy State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication May 21, 1968.
This investigation was supported in part by grant R01-AM 08753-03 PET from the U. S. Public Health Service, Bethesda, Md., and by Public Health Service Predoctoral Fellowship No. F1-GM-24005 (for RHR) from the National Institute of General Medical Sciences.
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† To whom requests for reprints should be addressed.
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female Wistar rats, 150–270 g., were anesthetized with urethan (1.25 g./kg. i.p.). After the rat stomach had been exposed, it was washed once with 0.9% sodium chloride solution prior to injection of the drug solution (37°) into the ligated stomach through the pyloric sphincter. All injections were made with a blunt-tipped needle which was withdrawn while tightening the ligature around the pyloric sphincter. The absorption period was 1.5 hr. for the caffeine–salicylic acid solutions and 1.0 hr. for the caffeine–*p*-hydroxybenzoic acid solutions. At the end of the absorption period, the stomach was excised, homogenized,[2] and the homogenate was assayed for unabsorbed caffeine and, when present, for ethanol.

Assay for Caffeine in Stomach Homogenate—A modification of the method of Axelrod and Reichenthal (4) for the assay of caffeine in urine and biologic tissues was used. The stomach homogenate was adjusted to a volume of 50 ml. with water. To 15 ml. of this diluted homogenate was added 25 ml. of 0.1 *M* phosphate buffer, pH 7.5. A 3-ml. aliquot of this dilution (containing 3–5 mg.% caffeine) was placed in a 120-ml. glass-stoppered bottle along with 3 g. sodium chloride and 35 ml. washed benzene.[3] After shaking for 20 min., the phases were separated by centrifuging at about 900×*g* for 20 min. The benzene phase was transferred to another 120-ml. glass-stoppered bottle containing 3 g. sodium chloride and 3 ml. 0.1 *N* NaOH. After the bottle had been shaken for 5 min., the phases were separated as before. Fifteen or 20 ml. of the benzene phase was then pipeted into a 125-ml. separator containing 10 ml. of 5 *N* HCl. After shaking for 5 min., the separator was allowed to stand a few minutes until the phases had separated. The aqueous phase was removed and centrifuged at about 850× *g* for 30 min. to remove any remaining nonaqueous material. An aliquot of the aqueous phase was then assayed spectrophotometrically at 273 mμ. Blank values from tissues comprised about 7% of the usual absorbance and the average tissue recovery, after correction for blank values, was 96%. The amount of caffeine remaining unabsorbed was calculated from the concentration determined spectrophotometrically, the dilution factor of the assay procedure, and the corrections for blank and recovery. Each analysis was done in triplicate and the results were averaged.

Assay for Ethanol in Stomach Homogenate—Ethanol was determined by the method of Hoult and Pawan (5) as modified previously (6). It was found that neither caffeine nor salicylic acid interferes with this assay.

Partition Coefficients—The partition coefficients of *p*-hydroxybenzoic acid and caffeine between 0.1 *N* HCl and isoamyl acetate were determined at room temperature as described previously (1). Assays of the aqueous phase were done spectrophotometrically at 255 and at 273 mμ, respectively, using 0.1 *N* HCl as the diluent. When both drugs were present together, two-component spectrophotometry was used at these wavelengths. In the concentration range of the spectrophotometric assay the absorbances of the two drugs were additive.

The partition coefficients of caffeine between distilled water or Ringer's solution and chloroform 10% (v/v) in isooctane were determined at 30° as described by Guttman and Higuchi (3). The concentration of caffeine was determined spectrophotometrically in each phase.

In Vitro Intestinal Transfer Rate Measurements (Caffeine)—The procedure outlined by Levy and Matsuzawa (7) was used. This is essentially the cannulated everted intestine method developed by Crane and Wilson (8). Male Long-Evans rats, weighing 330–470 g., were anesthetized with ether. The small intestine was removed, rinsed with Ringer's solution, and everted. Two segments, 10 cm. in length when stretched by an 8-g. weight, were obtained from the section of the small intestine just distal to the duodenum. The distal ends of the segments were tied and the proximal ends were attached to the cannula of the apparatus described by Crane and Wilson (8). An equal number of first (from the proximal end) and second intestinal segments was used in each part of the experiment. Each segment was suspended in 80 ml. of caffeine in Ringer's solution (at 30°) contained in a large test tube (mucosal solution). Two milliliters of Ringer's solution was introduced into the intestinal segment (serosal solution).[4] The serosal solution was withdrawn completely every 10 min. After each withdrawal the serosa was rinsed with 2 ml. of Ringer's solution and this rinse was combined with the solution withdrawn initially. Another 2 ml. of Ringer's solution was then placed into the intestine to serve as the serosal solution for the next 10-min. interval. The everted intestinal segment was suspended for 1 hr. in a solution containing a high caffeine concentration, for an additional hour in a solution containing a low concentration of caffeine, and then again for 1 hr. in the solution containing a high concentration of caffeine. A different sequence was used with other intestinal segments. This "pairing" minimizes the effect of variability between segments from different rats which may be due particularly to differences in diameter and surface area of the segments.

Assay for Caffeine in Serosal Solution from the Cannulated Everted Rat Intestine—Caffeine was determined by direct spectrophotometry without prior extraction. At the higher caffeine concentration of the mucosal solution (0.120 mole/l.) the serosal samples were simply diluted with water and their absorbance was determined at 273 mμ. At the 0.005 mole/l. concentration, however, it was necessary to correct for the interference from the blank[5] by two-component spectrophotometry at 273 and 299 mμ. This was done on the basis of a ratio of 1.5:1 for the absorbances of the blank at 273 and 299 mμ. The average recovery of caffeine in the concentration range where corrections were necessary was 101%.

Test for Transport Against a Concentration Gradient—The everted sac method described by Wilson and DeCarlo (9) was used. The intestine of the rat was removed, rinsed, and everted as described in a preceding section. One everted sac, about 6 cm. in length, was prepared from the proxi-

[2] Multi-mix Homogenizer, Lourdes Instrument Corp., Brooklyn, N. Y.

[3] Reagent grade benzene was washed successively with 0.2 vol. of 1 *N* NaOH, 1 *N* HCl, and twice with distilled water.

[4] The pH values of the serosal and mucosal solutions at the end of the absorption period ranged from 5.0 to 6.3. In this range, caffeine is nonionized.

[5] "Blank" refers to apparent caffeine values obtained in experiments in which caffeine-free solvents were used.

mal portion of each rat intestine. The everted sac was filled with 1 ml. of pH 7.4 Krebs-Henseleit Ringer solution (10) containing 0.005 *M* caffeine (serosal solution). The sacs were then placed in 15 ml. of the same solution (mucosal solution) contained in 50-ml. conical flasks. The flasks were flushed with a mixture of 95% oxygen and 5% carbon dioxide, sealed, placed in a 30° water bath, and agitated moderately by means of a reciprocating shaker. Aliquots of the mucosal and serosal solutions were obtained after 1 hr. of incubation and were assayed for caffeine using the two-component spectrophotometric analysis described in the preceding section.

In Vitro Intestinal Transfer Rate Measurements (Caffeine in the Presence of Salicylate)—The procedure was essentially the same as that outlined in a preceding paragraph for caffeine except for the use of female Wistar rats and a Krebs-Ringer buffer (11), adjusted with HCl to pH 4, as the solvent for the mucosal solution. The mucosal solution consisted of 0.1% caffeine, alone or together with 0.3% salicylic acid. Some of the solutions also contained 2% ethanol. The serosal solution consisted of pH 7.0 Krebs-Ringer solution (11). Each experiment was carried out for 60 min. at 37°, with sampling at 15-min. intervals. Caffeine was assayed by the method of Axelrod and Reichenthal (4) and ethanol by the method of Hoult and Pawan (5).

Calculation of Intestinal Transfer Rate Constants—The application of Fick's first law (12) to the transfer of substances across a biologic membrane yields the following equation (13):

$$R = kA \frac{C_1 - C_2}{x}$$

where R is the rate of diffusion across a barrier (*e.g.*, the everted intestine) which may be expressed in units of mg./hr., k is a constant whose units are then cm.²/hr., A is the cross-sectional area of the diffusion barrier in cm.², C_1 and C_2 are the concentrations of drug in the source (mucosal) and receiving (serosal) compartments, respectively, in mg./cm.³, and x is the thickness of the barrier in cm. The transfer rate constant used in this study, k_t, is defined as:

$$k_t = \frac{kA}{x} = \frac{R}{C_1 - C_2}$$

The units of k_t are then cm.³/hr. Total replacement of the serosal solution at short intervals (every 10 min.) prevented any pronounced buildup of C_2 ($<0.17\ C_1$). Also, C_1 remained essentially constant during the experiment due to the large volume of the mucosal solution. Thus, k_t approximates R/C_1 under steady-state conditions.

RESULTS AND DISCUSSION

Caffeine-Salicylic Acid Complex—There are three likely mechanisms by which salicylic acid can affect caffeine absorption from the rat stomach. First, the complex formed by these two drugs (2) may have an absorption rate constant different from that of caffeine itself. Since about 40% of the caffeine in the administered solution was in complexed form, a pronounced difference in the absorption rate constant of free and complexed caffeine would result in an appreciable difference in the overall absorption rate of caffeine when administered alone and together with salicylic acid, respectively. Second, salicylic acid may have a direct effect on the permeability characteristics of the gastric mucosa, and third, it may affect the *in vivo* absorption rate of caffeine by a systemic effect such as a change in the rate of gastric blood flow.

TABLE I—EFFECT OF SALICYLIC ACID ON THE *In Vivo* ABSORPTION OF CAFFEINE AND ETHANOL FROM THE STOMACH OF THE RAT

Initial Composition of Solution[a] % w/v	% Absorbed[b] in 1.5 hr. Caffeine	Ethanol	Ratio of % Absorbed Values, Without Salicylic Acid : With Salicylic Acid Caffeine	Ethanol
Caffeine, 0.2 Ethanol, 2.0	37.1 (4.1)	55.9 (3.8)	1.3	1.2
Caffeine, 0.2 Ethanol, 2.0 Salicylic Acid, 0.275	28.0 (4.0)	46.1 (6.1)		

[a] 0.1 *N* HCl was used as the solvent. [b] Mean of the values from 6 rats, standard deviations in parentheses.

The effect of salicylic acid on caffeine absorption from the *in situ* ligated rat stomach is shown in Table I. It is evident that the absorption rate of caffeine was significantly decreased in the presence of salicylic acid ($p < 0.01$, t test). However, the absorption of ethanol, which was included in the drug solutions as a noncomplexed marker, was also decreased in the presence of this concentration of salicylic acid ($p < 0.02$, t test). For comparative purposes, the effect of salicylic acid on the absorption of caffeine and ethanol was expressed in terms of the ratios shown in Table I. It can be noted that salicylic acid decreased the absorption of both the complexed caffeine and the noncomplexed ethanol to a similar extent. This indicates that the decreased absorption of caffeine in the presence of salicylic acid was not due to complex formation. There was visual evidence of considerable gastric damage due to salicylic acid; the solutions containing caffeine, ethanol, and salicylic acid[6] frequently caused gastric bleeding and ulceration, while no such effects were noted with solutions containing only caffeine and ethanol. There appeared to be a rough inverse correlation between the absorption rates of caffeine and ethanol on one hand, and the extent of gastric bleeding on the other. It seems, therefore, that the decreased absorption of caffeine in the presence of a relatively high concentration of salicylic acid was due to an effect of salicylic acid on the gastric mucosa, and not to an effect of complex formation *per se*. These results demonstrate the need to distinguish between an effect of a complexing agent on the drug and a possible effect on the biologic membrane. They illustrate also the utility of a noncomplexed marker such as ethanol for this purpose.[7] It should be noted, however, that ethanol is not necessarily a suitable marker for the assessment of effects on biologic membranes involving mechanisms other than bleeding and erosions (14).

[6] The drug solution was essentially saturated with respect to free salicylic acid in order to complex as high a fraction of caffeine as possible.

[7] The absorption of ethanol is not decreased at a lower salicylic acid concentration (0.1%) which does not cause visible mucosal damage (1, 6).

TABLE II—EFFECT OF SALICYLATE ON THE TRANSFER OF CAFFEINE ACROSS THE CANNULATED EVERTED INTESTINE OF THE RAT

Composition of Mucosal Solution,[a] %		Transfer Rate[b] Caffeine, mg./hr.	Transfer Rate[b] Ethanol, mg./hr.
Caffeine, 0.1		2.38	67.6
Ethanol, 2.0		2.06	*c*
		1.95	63.8
	Mean	2.13	65.7
Caffeine, 0.1		2.06	69.2
Ethanol, 2.0		1.79	*c*
Salicylic acid, 0.3		1.86	62.0
	Mean	1.90	65.6

[a] The solvent was Krebs-Ringer buffer adjusted to pH 4. [b] Data from individual experiments, each based on 4 samplings at 15-min intervals. [c] Ethanol was not included in these experiments.

TABLE III—PARTITIONING BEHAVIOR OF *p*-HYDROXYBENZOIC ACID AND CAFFEINE, ALONE AND IN THE PRESENCE OF EACH OTHER

Initial Composition of Aqueous Phase, %	Apparent Partition Coefficient[a]
Parahydroxybenzoic Acid	
p-Hydroxybenzoic acid, 0.1	11
p-Hydroxybenzoic acid, 0.1 Caffeine, 2.2	1.6
Caffeine	
Caffeine, 0.194	0.40
Caffeine, 0.194 *p*-Hydroxybenzoic acid, 0.8	0.54
Caffeine, 0.194 *p*-Hydroxybenzoic acid, 4.0[b]	0.65

[a] Isoamyl acetate–0.1 *N* HCl, at 25 ± 2°. [b] In this instance, it was necessary to dissolve the *p*-hydroxybenzoic acid in the isoamyl acetate prior to equilibration because of the limited aqueous solubility of this compound.

TABLE IV—EFFECT OF *p*-HYDROXYBENZOIC ACID ON CAFFEINE ABSORPTION FROM THE STOMACH OF THE RAT

Initial Composition of Solution,[a] %	% Absorbed[b] in 1 hr.
Caffeine, 0.2	32.7 (3.1)
Caffeine, 0.2 *p*-Hydroxybenzoic acid, 0.83	27.7 (4.5)

[a] 0.1 *N* HCl was used as the solvent. [b] Mean of the values from 4 animals, standard deviations in parentheses.

Since caffeine forms a complex with salicylate ion having a stability constant similar to that of the caffeine–salicylic acid complex (2), the study was repeated at pH 4 by means of the everted intestine procedure (8). The results of these experiments are shown in Table II and indicate that complexation with salicylate ion had no appreciable effect on the intestinal transfer of caffeine and certainly did not increase the transfer rate of this drug. Since about 90% of the salicylate was in ionized form, it had no measurable effect on the permeability characteristics of the intestinal membrane, as reflected by the lack of any effect on the intestinal transfer of ethanol. It has recently been observed that caffeine has no effect on the intestinal transfer rate of salicylic acid at pH 5, but decreases the rate of transfer at pH 2 (15).

Caffeine–p-Hydroxybenzoic Acid Complex—Higuchi and Zuck (2) have estimated the stability constant of the caffeine–*p*-hydroxybenzoic acid complex to be at least 100 l./mole. This stability constant and the intrinsic solubility of *p*-hydroxybenzoic acid are considerably higher than the corresponding values for the caffeine–salicylic acid complex and salicylic acid, respectively, and thus permit more extensive complexation of caffeine (at least 83% in this experiment) than was possible with salicylic acid. It was observed also that *p*-hydroxybenzoic acid, unlike salicylic acid, did not visibly damage the gastric mucosa. Complexation of caffeine with *p*-hydroxybenzoic acid increases the apparent partition coefficient of caffeine to a value between that of free caffeine and free *p*-hydroxybenzoic acid, respectively (Table III). Contrary to expectations, this increase in apparent partition coefficient was not accompanied by an increased gastric absorption rate (Table IV). This study was terminated when it was found that caffeine is definitely not more rapidly absorbed in the presence of *p*-hydroxybenzoic acid.[8] The number of experiments was insufficient to determine if the apparent decrease in the absorption of caffeine in the presence of the complexing agent was real ($0.2 > p > 0.1$, t test). A discussion of possible reasons for the observed results will be deferred to a following report which deals with another complexing system and includes data which permit a more definitive interpretation of the type of effects observed with the caffeine–*p*-hydroxybenzoic acid system.

Effect of Self-Association on Intestinal Transfer of Caffeine—It is known that caffeine undergoes considerable self-association except at very low concentrations. Guttman and Higuchi (3) have made an extensive investigation of the equilibria involved in this system. Their data, which show a decrease in the apparent lipoid–aqueous partition coefficient with increasing concentration of caffeine, suggest that caffeine exists only in monomeric form in the organic phase used in their study (chloroform 10% v/v in isooctane), but that it also forms dimers and tetramers in water. These physicochemical characteristics made a study of the effect of concentration on the absorption of caffeine across biologic membranes of interest. The everted small intestine of the rat was used in this study because of the relative simplicity and sensitivity of this procedure.[9]

The results of two experiments, which involved the measurement of caffeine transfer rates from high, low, and high concentrations of caffeine (in that order) across an everted intestinal segment of the rat, are shown in Figs. 1 and 2. The data are plotted in terms of the ratio, transfer rate/mucosal concentration (to be referred to as the transfer ratio), which, in the steady state and under sink conditions, is equivalent to the intestinal transfer rate constant as defined under *Experimental*. At the high concentration of caffeine (0.12 mole/l.), the transfer ratio increased with time until steady state was

[8] This is particularly remarkable since, as is shown in the subsequent section, a reduction in the concentration of uncomplexed caffeine should, by decreasing the degree of caffeine self-association, result in more rapid absorption of the uncomplexed drug.

[9] The absorption studies of the caffeine–salicylic acid and caffeine–*p*-hydroxybenzoic acid complexing systems had to be carried out on the ligated rat stomach rather than the everted rat intestine because of the need to use solutions of very low pH to prevent ionization of the two weak acids.

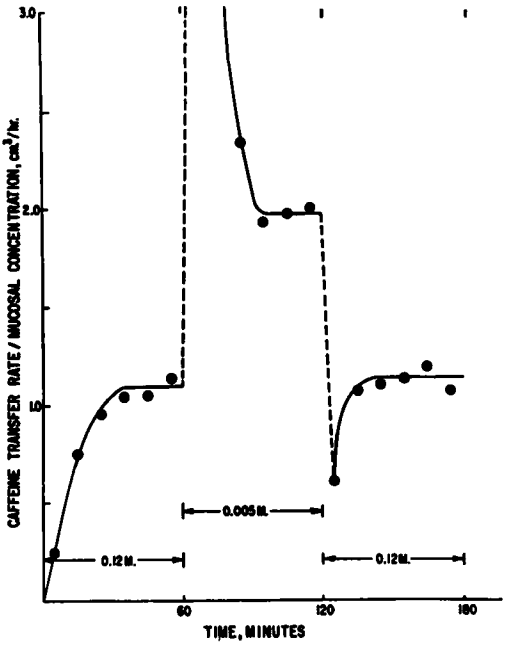

Fig. 1—Effect of concentration on intestinal transfer of caffeine. Transfer rate/mucosal concentration as a function of time, Exp. 1. The data were plotted at the midpoint of each 10-min. sampling period. The concentrations shown in the figure refer to caffeine concentration in the mucosal solution during the various time periods of the experiment. Values which were beyond the scale of the figure were: 17.1 cm.³/hr. at 65 min. and 3.9 cm.³/hr. at 75 min.

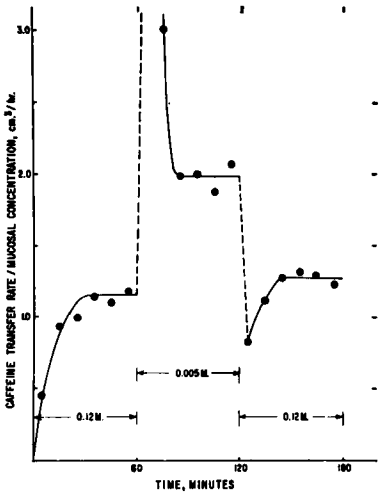

Fig. 2—Effect of concentration on intestinal transfer of caffeine, Exp. 2. Details as in Fig. 1. The value which was beyond the scale of the figure was: 12.4 cm.³/hr. at 65 min.

reached. This is due to a gradual build-up of drug concentration within the intestinal wall (16). At the low caffeine concentration (0.005 mole/l.) following the high concentration, drug which had been retained in the intestinal wall from the preceding period gradually diffused out of the wall until a new apparent steady-state concentration was attained. It is evident that the transfer ratio at the apparent steady state was considerably lower at the high concentration of caffeine than at the lower concentration. The similarity of the apparent steady-state transfer ratios in the first and third time periods indicates that intestinal permeability remained essentially constant during the 3-hr. experiment. The desirability of repeating the conditions of the first time period in a third time period to assess any possible effect of caffeine on the integrity of the intestinal mucosa made it necessary to limit each period to 60 min. Another indication that the intestinal membrane permeability remained essentially unchanged for the duration of the experiments in this study was provided by the results of preliminary experiments in which the plateau transfer ratio attained at about 30 min. (using only 0.005 mole/l. caffeine in the mucosal solution) did not change significantly ($<\pm 7\%$) in the following 3.5 hr.

Figures 3 and 4 show the results of experiments in which the sequence of caffeine concentration changes was reversed, *i.e.*, the segments were suspended for 1 hr. in a 0.005 mole/l. caffeine solution, for a second hour in a 0.12 mole/l. caffeine solution, and finally once more in a 0.005 mole/l. caffeine solution. Again, the apparent intestinal transfer rate constant of caffeine was higher at low concentrations than at high concentrations of the drug. However, the difference between the plateau transfer ratios in the first two time periods was not as pronounced in this series of experiments as in the series shown in Figs. 1 and 2. This may be due to two reasons: the initial exposure of the intestine to a high concentration of caffeine may cause an immediate increase in intestinal permeability which then remains constant throughout the experiment, or, the apparent plateau transfer ratio for caffeine

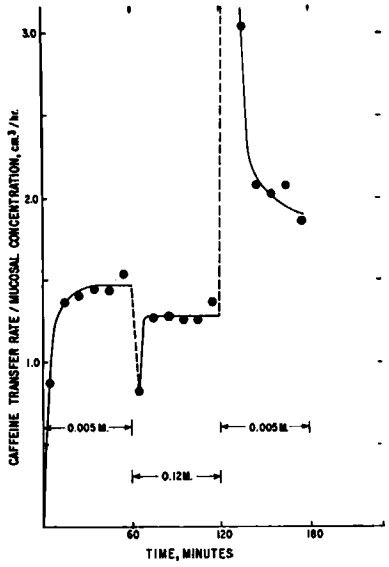

Fig. 3—Effect of concentration on intestinal transfer of caffeine, Exp. 3. Details as in Fig. 1. The value which was beyond the scale of the figure was: 12.7 cm.³/hr. at 125 min.

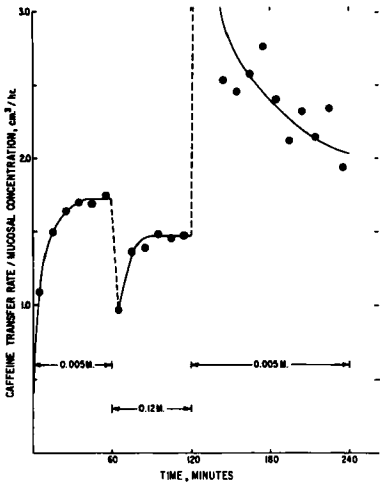

Fig. 4—Effect of concentration on intestinal transfer of caffeine, Exp. 4. Details as in Fig. 1. Values which were beyond the scale of the figure were: 14.2 cm.³/hr. at 125 min. and 3.3 cm.³/hr. at 135 min.

at low concentration following exposure of the intestine to a high concentration of caffeine (Figs. 1 and 2) may not actually reflect steady-state conditions, but rather be the result of a slow efflux of previously accumulated caffeine from the intestinal wall.

Experiments which will be reported in a subsequent publication have shown that the permeability of the everted rat intestine to salicylamide was not affected by exposure of the intestine to a high (0.113 mole/l.) caffeine concentration (17). This would tend to rule out the possibility of an effect of the high concentration of caffeine on intestinal permeability in the present study. In addition, the transfer ratio data for the third time period of the experiments shown in Fig. 3 and particularly in Fig. 4 (where the time period was extended to 240 min.) give a strong indication of a slow efflux following an initial rapid efflux of caffeine from the intestinal wall after the intestine had been exposed to a high concentration of this drug. It appears, therefore, that the transfer rate constants represented by the plateau transfer ratios in the first and second time periods of Figs. 3 and 4 are the most realistic and conservative index of the difference in the intestinal transfer rate constants of caffeine at low and high concentrations of the drug.

The study of Guttman and Higuchi (3) yields information concerning the degree of self-association of caffeine at the concentrations used in this study. It was found that the apparent aqueous–organic partition coefficients of caffeine under the conditions of their study, where distilled water was used as the aqueous phase, were essentially the same as when the aqueous system of the present study, Ringer's solution, was used (Table V). This shows that the self-association of caffeine was not measurably affected by the presence of the components of the electrolyte solution and justifies the direct use of the data from Guttman and Higuchi's study (Table VI).

The results of the intestinal transfer study are summarized in Table VI, which also shows the extent of self-association of caffeine at the two

Table V—Partition Coefficient of Caffeine Between Distilled Water or Ringer's Solution and an Organic Solvent

Initial Concn. of Caffeine in Aqueous Phase, %	Solvent, Aqueous Phase	Aqueous–Organic Apparent Partition Coefficient,[a] This Study	Literature[b]
0.107	Distilled water	26	25
0.107	Ringer's solution	24	
2.14	Distilled water	57	57
2.14	Ringer's solution	56	

[a] The organic phase was chloroform 10% (v/v) in isooctane and the temperature was 30°. [b] See *Reference 3.*

Table VI—Effect of Caffeine Association on the *In Vitro* Rate of Transfer of Caffeine Across the Small Intestine of the Rat

Caffeine Concn. moles/l.[a]	% Total Caffeine Present as a Particular Species[b] Mono-mer	Dimer	Tetra-mer	Transfer Rate Constant cm.³/hr.[c]
0.005	90	10	0	1.67 (0.29)
0.120	39	37	24	1.44[d] (0.23)

[a] Ringer's solution was the solvent. [b] See *Reference 3.* [c] Mean of the values from 4 rat intestinal segments determined at 30°. Standard deviations in parentheses. [d] The difference between the two mean transfer rate constants is statistically significant ($p < 0.01$) as determined by the t test for paired observations.

concentrations used. The transfer rate constants were determined from the steady-state portion of the first two time periods of only the experiments in which the transfer at low (0.005 mole/l.) caffeine concentration was determined first. These transfer rate constants reflect not only the data in Figs. 3 and 4, but also the results of two additional experiments not represented by figures. The intestinal transfer rate constant at the low concentration of caffeine is significantly higher ($p < 0.01$, t test for paired observations) than that at a high concentration. This difference would have been even more pronounced if the experiments shown in Figs. 1 and 2 had been included in the data analysis.

Incubation of everted intestinal sacs containing 0.005 mole/l. caffeine for 1 hr. in solutions containing the same concentration of caffeine in an oxygenated medium yielded no evidence of uphill transport; the mean mucosal–serosal concentration ratio was 0.97 for four experiments. Thus, the concentration dependence of the intestinal transfer of caffeine does not appear to be due to the saturation of an active transport process in the small intestine of the rat. However, other modes of specialized transport cannot be ruled out on the basis of the experimental data. But, since the structural specificity of most specialized intestinal transport processes is quite exacting, it seems unlikely that a drug such as caffeine would be transferred by this type of process. The experimental results also cannot be explained by assuming that only the monomeric form of caffeine is transferred across the intestine; the decrease of the intestinal transfer rate constant of caffeine at the higher concentration is much smaller than the decrease in the fraction of total caffeine which exists in monomeric form (Table VI). The overall diffusivity of caffeine, determined on the basis of the fraction and molecular

TABLE VII—RATIO OF THE INTESTINAL TRANSFER RATE CONSTANTS OF CAFFEINE AT LOW AND HIGH CONCENTRATION COMPARED TO THE THEORETICAL RATIO OF THE OVERALL DIFFUSIVITIES

$k_{0.005\ M}/k_{0.12\ M}$[a]		Diffusivity$_{0.005\ M}$/ Diffusivity$_{0.12\ M}$ Thovert's Equation[b]	Stokes-Einstein Equation[c]
1.14 1.15 1.17 1.18	Mean: 1.16	1.38	1.24

[a] Ratio of intestinal transfer rate constants for caffeine in low and high concentration. [b] D = constant/$M^{1/2}$ (18). In this instance, $M = \Sigma M_i f_i$, where M_i is the apparent molecular weight of a particular species (monomer, dimer, or tetramer) and f_i is the fraction of total caffeine present existing as this species. [c] D = constant/$M^{1/3}$ (19), calculated as in *b*.

weight of each species (*i.e.*, monomer, dimer, and tetramer) is lower at higher concentrations of caffeine. This could affect transfer rate if diffusion of the drug to the membrane surface is transfer rate limiting or if the aggregates remain intact during transfer across the intestine. The ratio of the overall diffusivity of caffeine at low and high concentrations, as calculated by two different equations, is somewhat higher than the ratio of intestinal transfer rate constants determined experimentally (Table VII). It may be possible, however, that diffusion to the mucosal membrane surface affects the transfer rate constant of one or both of the associated species of caffeine but not that of the monomer. The experimental data may also be rationalized by assuming that each of the caffeine species is absorbed as such but that the transfer rate constant of each species is different. This may be complicated further by a possible change in the self-association equilibria at or adjacent to the rate-limiting biologic barrier (7).

The important conclusions of this study are that (*a*) drug complexes with a higher apparent partition coefficient than the free drug are not necessarily more rapidly absorbed, and (*b*) a concentration dependence in the apparent absorption rate constant of a drug does not necessarily reflect the presence of a specialized transport process, but may be due to the physicochemical characteristics of the drug as they affect passive diffusion to and across biologic membranes.

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Keyphrases

Drug absorption—complex formation effect
Caffeine complexes—increased partition coefficients
Salicylic acid retardation—caffeine transfer
Self-association—caffeine
Gastric absorption rate—*in situ* technique
Everted intestine—experimental technique
UV spectrophotometry—analysis

Effect of Complex Formation on Drug Absorption VIII

Intestinal Transfer Characteristics of the Salicylamide–Caffeine System

By RICHARD H. REUNING* and GERHARD LEVY†

Salicylamide and caffeine form a complex in aqueous solution which has an apparent lipoid–aqueous partition coefficient intermediate between the partition coefficients of free salicylamide and free caffeine. The transfer rate of salicylamide across the cannulated everted intestine of the rat is decreased in the presence of caffeine at a concentration sufficient to complex an appreciable fraction of the salicylamide. On the other hand, complex formation with salicylamide has no measurable effect on the intestinal transfer rate of caffeine. These observations suggest that the complex can be transferred as such and that diffusion of the drugs through the bulk solution to the membrane surface is not transfer rate limiting. A theoretical analysis of the simultaneous equilibria involved in the partitioning between an organic and an aqueous phase of two interacting drugs in the presence of one another shows that the apparent partition coefficient of a drug complex does not necessarily represent the ratio of transfer rate constants of the complex itself across the interface. For this reason, it should not be assumed that there will always be a correlation between the apparent partition coefficient and the intestinal transfer rate constant of drug complexes. The results of the reported study support this conclusion.

IN A PREVIOUS STUDY (1) salicylamide and caffeine were found to form a complex in aqueous solution. The stoichiometry and stability constant of the complex were determined at pH 5 where both drugs are essentially undissociated. The purpose of the present study was to investigate the effect of this complex formation on the overall[1] intestinal transfer of both salicylamide and caffeine and to compare the effects of complex formation on the intestinal transfer rates and on the apparent partition coefficients of salicylamide and caffeine, respectively.

The salicylamide–caffeine system was chosen as a model for a study of the effect of complex formation on drug absorption since extensive complexation of either drug is possible under suitable experimental conditions. Such extensive complexation, made possible by the adequate solubility of both drugs in water and by the relatively high stability constant of the complex (41 l./mole at 37°), is desirable because any pronounced effect of complex formation on intestinal transfer will then be reflected by a measurable change in the overall intestinal transfer rates of the two drugs.

Received April 8, 1968, from the Biopharmaceutics Laboratory, Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication May 21, 1968.

This investigation was supported in part by grant RO1-AM-08753-03 PET from the U. S. Public Health Service, Bethesda, Md., and by Public Health Service Predoctoral Fellowship No. F1-GM-24005 (for RHR) from the National Institute of General Medical Sciences.

Previous paper: Reuning R.H., and Levy, G., *J. Pharm. Sci.*, **57**, 1335 (1968).

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[1] In the context used in this paper, "overall" refers to the combined effect or behavior of free and complexed drug.

EXPERIMENTAL

Intestinal Transfer Rate Determinations—The cannulated everted intestine method developed by Crane and Wilson (2) was used with the modifications described previously (3). Each everted intestinal segment, 10 cm. in length, from a male Sprague-Dawley rat (290–380 g.), was suspended in a large test tube containing about 50 ml. of mucosal solution at 37°. Krebs-Henseleit-Acetate Ringer[2] solution (KHAR), pH 5.0, containing caffeine, salicylamide, or both in varying concentrations, was used as the mucosal solution. Two intestinal segments were obtained from each rat and an equal number of first and second segments were used for each experiment. Two milliliters of the serosal solution, Krebs-Henseleit-Bicarbonate Ringer solution (KHBR), pH 7.4 (4), was introduced into the everted intestinal segment. The entire serosal solution was withdrawn every 10 min. After each withdrawal the serosa was rinsed with KHBR and the rinse was combined with the previously withdrawn solution for subsequent assay. Immediately after completion of the rinsing procedure, another 2 ml. of KHBR was placed into the intestinal segment. This frequent replacement of the serosal solution prevented appreciable build-up of drug concentration on the serosal side. In one set of experiments involving the measurement of caffeine transfer rates in the presence of salicylamide in the mucosal solution, salicylamide was also included in the serosal solution.

The serosal samples were diluted with KHAR, pH 5.0, and the amount of caffeine or salicylamide transferred was determined by spectrophotometry at 273 or 299 mμ, respectively. When both drugs were present together, two-component spectrophotometry was used at these wavelengths. The absorbances of the two compounds were additive in the concentration range of the assay procedure. A small correction for blank was necessary only in the first two 10-min. samples, as determined in

[2] This is similar to Krebs-Henseleit-Bicarbonate Ringer solution (4) except that an equimolar amount of acetate buffer was substituted for the bicarbonate buffer.

experiments in which drug-free solutions were used. When known amounts of both drugs were added to KHBR solution which had been incubated for 10 min. with an intestinal segment (*i.e.*, using the cannulated everted intestine method as described above, but without drugs), an average of 101% of added caffeine and 100% of added salicylamide was recovered.

The transfer rates were determined from the slope of a plot of cumulative amount of drug transferred *versus* time. The transfer rate constant was obtained by dividing the slope value by the mucosal drug concentration. This was possible because of frequent replacement of the serosal solution and because the volume of the mucosal solution was large enough so that the drug concentration in that solution remained essentially constant for the duration of the experiment. A derivation from Fick's law of this method of calculating the transfer rate constant has been presented previously (3).

Experimental Design—The study was designed to determine the effect of complex formation on drug absorption under conditions which also permitted an assessment of any possible alteration in the permeability characteristics of the everted intestine as a function of time or upon addition of the complexing agent. The everted intestinal segment was first suspended for 40 min. in a solution containing only the drug, then for 40 min. in a solution containing drug plus complexing agent, and then again for 40 min. in a solution containing only drug. An equal number of experiments was carried out in a different sequence. Comparison of the transfer rates observed in the first and third time periods permitted an evaluation of any possible changes in the permeability of the intestinal segment. Also, the comparison of transfer rates both in the absence and presence of complexing agent in the same intestinal segment eliminates any variability due to possible differences in the surface area, thickness, *etc.*, of different segments.

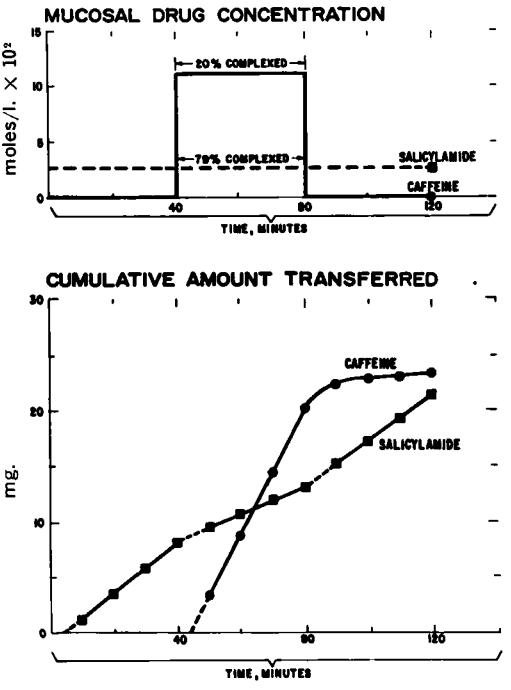

Fig. 1—Effect of complex formation with caffeine on the transfer of salicylamide across the cannulated everted intestine of the rat. In the upper portion of the figure the concentrations of salicylamide and caffeine in the mucosal solution are plotted as a function of time. In the lower portion of the figure, the cumulative amounts of salicylamide and caffeine transferred across the intestine are plotted as a function of time.

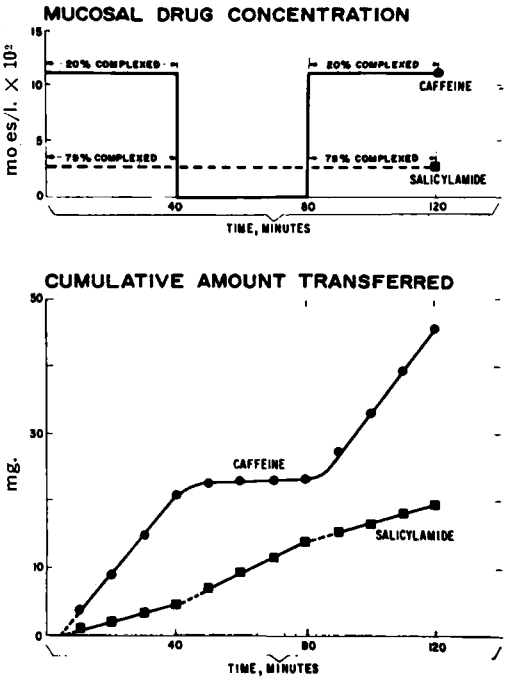

Fig. 2—Effect of complex formation with caffeine on the transfer of salicylamide across the cannulated everted intestine of the rat. Details as in Fig. 1 except that the order of addition of the complexing agent was changed.

Determination of the Extent of Metabolism of Salicylamide by the Everted Rat Intestine—Three 10-cm. segments of everted intestine from a male Sprague-Dawley rat (280 g.) were tied on one end with suture and filled with 2.5 ml. of serosal solution (KHBR, pH 7.4, was the solvent) through the open end which was then also tied off. Each everted sac was placed in a 50-ml. flask containing 15 ml. of mucosal solution (KHAR, pH 5.0, was the solvent). The flasks were flushed with 95% O_2–5% CO_2 and incubated for 1 hr. The mucosal and serosal solutions contained the same concentration of the drug(s), namely either salicylamide, salicylamide and caffeine, or solvent alone. The latter was used as a control to determine the recovery of salicylamide by adding known amounts of this drug, similar to the amounts used in the other two experiments, to the serosal solution, the mucosal solution, and to a homogenate of the intestinal segment. Salicylamide in the mucosal and serosal solutions and in a homogenate of the everted intestinal segment was assayed by a method which is specific for free drug and

excludes glucuronide or sulfate conjugates, if present. This assay, which involves extraction of the drug from a pH 7 buffer into ethylene dichloride and subsequent reextraction into a ferric nitrate reagent solution, has been described elsewhere (5).

Determination of Apparent Partition Coefficients—Apparent partition coefficients were determined (at least in duplicate) with isoamyl acetate as the organic phase and KHAR, pH 5.0, as the aqueous phase. Equilibration was carried out at room temperature for at least 12 hr. using an automatic shaking apparatus. In the determination of salicylamide partition coefficients, the drug in the aqueous phase was assayed colorimetrically as previously described (1). In the determination of caffeine partition coefficients, caffeine alone in the aqueous phase was assayed spectrophotometrically at 273 mμ. When salicylamide was present, both drugs were assayed in the aqueous phase using two-component spectrophotometry at 273 and 299 mμ.

RESULTS AND DISCUSSION

Effect of Complex Formation of Intestinal Transfer of Salicylamide and Caffeine—The effect of caffeine (2.2%) on the overall intestinal transfer rate of salicylamide (0.384%) is shown in Figs. 1 and 2. It is evident that a pronounced decrease in the overall transfer rate of salicylamide occurred when this drug was complexed extensively with caffeine.[3] The figures also show the transfer of the complexing agent, caffeine, and the degree to which it was complexed. The intestinal transfer rate constant for salicylamide alone (0.384% in the mucosal solution) was 3.5 cm.³/hr. as compared to 2.0 cm.³/hr. in the presence of 2.2% caffeine (means of the paired observations from four segments). Subsequent experiments, to be reported in a following paper (6), yielded essentially the same values for the transfer rate constants (3.4 and 2.0 cm.³/hr., respectively) when the serosal solution contained caffeine and was exchanged every 6 min. rather than every 10 min. This shows that practically sink conditions were maintained in the present study.

The transfer rates of salicylamide and caffeine were essentially the same in the first and third time periods (Figs. 1 and 2). This indicates that the permeability of the everted intestine did not change measurably during the time of the experiment, regardless of the presence or absence of a high concentration of caffeine. These results suggest that the effect of caffeine on the intestinal transfer of salicylamide is not due to a modification of intestinal permeability characteristics.

The possibility that the transfer rate data are affected by the formation of salicylamide conjugates in the intestinal mucosa was examined. When salicylamide in a relatively low concentration (0.024% as compared to 0.384% used in the transfer study) was incubated with intestinal tissue for 1 hr., no significant metabolism of salicylamide was found, regardless of the presence or absence of caffeine (Table I). These results are in agreement with those of other workers who have found significant metabolism of salicylamide in rabbit intestinal tissue, but not in that of the rat (7).

[3] The calculations of the extent of complexation in this paper are based on a stability constant of 41.1 l./mole for the salicylamide–caffeine complex (1:1) and are not corrected for caffeine dimerization (1). These calculations indicated 79% complexation of salicylamide in the experiments shown in Figs. 1 and 2; direct experimental determination showed 73% complexation (1).

TABLE I—RECOVERY OF SALICYLAMIDE FROM EVERTED INTESTINAL SACS OF THE RAT AFTER 1 HR. OF INCUBATION[a]

Initial Composition of Mucosal and Serosal Solutions, %	% Recovery[b]
Salicylamide control (no incubation)	99
Salicylamide 0.024	98
Salicylamide 0.024 } Caffeine 2.0 }	99

[a] The everted sacs were incubated at 37° in an atmosphere of 5% CO_2 in O_2. Krebs-Henseleit-Bicarbonate Ringer solution, pH 7.4, was the solvent for the serosal solution and Krebs-Henseleit Acetate Ringer solution, pH 5.0, was the solvent for the mucosal solution. [b] One of each.

The decrease in the intestinal transfer rate of salicylamide in the presence of caffeine cannot be attributed to the decreased thermodynamic activity of salicylamide and an assumption that the complex itself is not absorbed since, in the presence of caffeine, the concentration of free salicylamide was about 21% of that when caffeine was absent while the transfer rate was 58% of that when caffeine was absent. It appears, therefore, that the salicylamide–caffeine complex itself is transferred across the everted intestine but at a rate different from that of free salicylamide.

In contrast to the results for salicylamide, the intestinal transfer rate of caffeine (0.388%) was not appreciably affected when complexed to the extent of about 54% due to the presence of a relatively high concentration of salicylamide (0.543%). The data from two of the four experiments that were carried out are shown in Figs. 3 and 4. The experi-

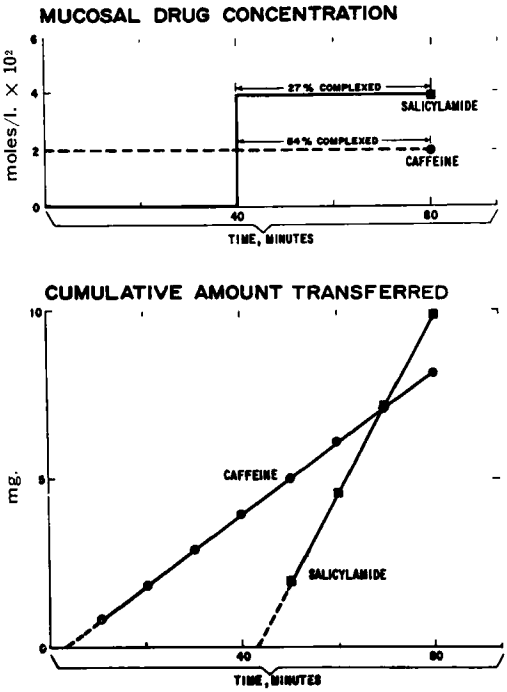

Fig. 3—Effect of complex formation with salicylamide on the transfer of caffeine across the cannulated everted intestine of the rat. Details as in Fig. 1.

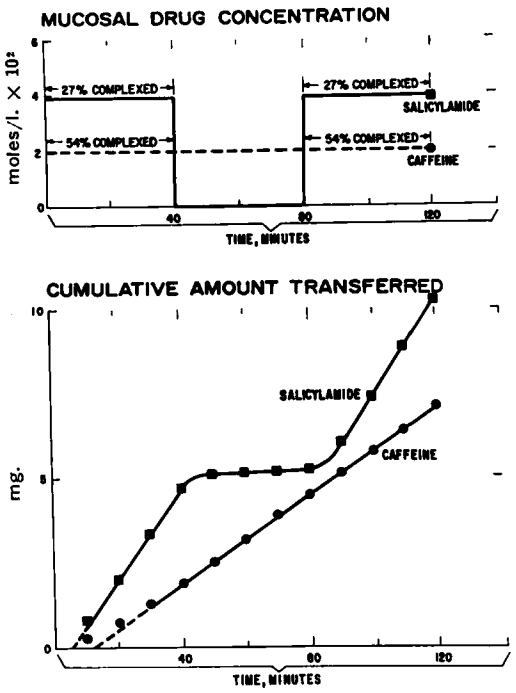

Fig. 4—Effect of complex formation with salicylamide on the transfer of caffeine across the cannulated everted intestine of the rat. Details as in Fig. 1. The order of addition of the complexing agent was different from that in Fig. 3.

ments were repeated under conditions where salicylamide was present in the serosal solution in a concentration equal to that of free salicylamide in the mucosal solution. These results, which are listed in Table II, also show that there was no appreciable difference in the intestinal transfer rate of caffeine alone and in the presence of salicylamide.

In view of the indications from the salicylamide transfer rate determinations that the salicylamide–caffeine complex is transferred as such, and considering the lack of a measurable difference in the overall intestinal transfer of free and complexed caffeine, there is strong indication that the salicylamide–caffeine complex is transferred across the intestine at the same rate as free caffeine. A consequence of this reasoning, which obtains strong support from the results of studies to be reported subsequently (6), is that diffusion of caffeine and the salicylamide–caffeine complex through the aqueous solution to the intestinal mucosa is not

Table II—Effect of Salicylamide in the Mucosal and Serosal Solutions on the Intestinal Transfer Rate Constant of Caffeine

Composition of Mucosal Solution, %	Transfer Rate Constant,[a] cm.³/hr.
Caffeine, 0.388	1.4 (0.05)
Caffeine, 0.388 Salicylamide,[b] 0.543	1.5 (0.32)[c]

[a] Mean of the values from 4 segments, standard deviation in parentheses. [b] Salicylamide was also present in the serosal solution at a concentration equal to the calculated concentration of free salicylamide in the mucosal solution. [c] The larger standard deviation in this set of experiments is due to one unusually high value (2.0 cm.³/hr.). The average transfer rate constant excluding this value is 1.4 cm.³/hr.

Table III—Apparent Partition Coefficients of Salicylamide, Caffeine, and Salicylamide–Caffeine Complex

Amount of Drug in the System,[a] mg. Caffeine	Salicylamide	% Complexation of Caffeine in Aqueous Phase at Equilibrium	Apparent Partition Coefficient
—	10	—	23
19	—	—	0.44
19	100	12	0.57[b]
19	300	27	0.52[b]
19	500	38	0.56[b]

[a] Isoamyl acetate–acetate buffer, pH 5.0, 10 ml. each at 25 ± 2°. [b] Apparent partition coefficient of the salicylamide-caffeine complex itself, calculated as shown in *Footnote 4.*

rate limiting in the intestinal transfer of these two species.

Effect of Complex Formation on the Apparent Partition Coefficients of Salicylamide, Caffeine, and Salicylamide–Caffeine Complex—It is well known that there is a positive relationship between the lipoid–aqueous partition coefficient and the gastrointestinal absorption rate of many drugs (8, 9). It was found that the partition coefficient of salicylamide is higher, and that of caffeine is lower, than the apparent partition coefficient of the salicylamide–caffeine complex itself (Table III). The apparent partition coefficient of the complex was determined[4] at three different salicylamide–caffeine concentration ratios, with essentially the same value being obtained in each case.

An analysis of the simultaneous equilibria which will determine the apparent partition coefficient of a drug complex is appropriate to assess the likely relationship between this property and the rate of transfer of complex across a biologic membrane. These simultaneous equilibria are shown in the following model:

$$\begin{array}{ccc} A_o & + & B_o \overset{K_5}{\rightleftharpoons} A\text{-}B_o \\ \uparrow\downarrow K_1 & & \uparrow\downarrow K_2 \qquad \uparrow\downarrow K_4 \\ A_a & + & B_a \underset{K_3}{\rightleftharpoons} A\text{-}B_a \end{array}$$

where A and B are the two individual components of the drug complex and A-B is the complex itself. The subscript *o* refers to the species in the organic phase and the subscript *a* to the species in the aqueous phase. Both A and B are assumed to partition between the organic and aqueous phases. The definitions of the various constants involved in these equilibria are as follows:

$$PC_{app.} = \frac{[B_o] + [A\text{-}B_o]}{[B_a] + [A\text{-}B_a]} \qquad K_1 = [A_o]/[A_a]$$

$$K_2 = [B_o]/[B_a] \qquad K_3 = [A\text{-}B_a]/[A_a][B_a]$$

$$K_4 = [A\text{-}B_o]/[A\text{-}B_a] \qquad K_5 = [A\text{-}B_o]/[A_o][B_o]$$

[4] The apparent partition coefficient of the complex was calculated as shown below, where A represents salicylamide, B represents caffeine, and where the subscripts and constants have the meanings defined subsequently for the model in the text. (*a*) $[A_a]$, $[B_a]$, and $[A\text{-}B_a]$ are calculated from K_3 and the experimentally determined total salicylamide and caffeine concentrations in the aqueous phase at equilibrium, according to the procedure described in Method I in the appendix of *Reference 1.* (*b*) $[B_o]$ is determined from $[B_a]$ and the partition coefficient, K_2. (*c*) $[A\text{-}B_o]$ = [(total amount B in both phases)-(amount of free B in both phases plus amount of A-B in aqueous phase)] ÷ volume of organic phase. (*d*) Apparent partition coefficient of complex = $[A\text{-}B_o]/[A\text{-}B_a]$.

An increase or a decrease in the apparent partition coefficient of drug B ($PC_{app.}$) when partially complexed with drug A indicates that the ratio $[A\text{-}B_o]/[A\text{-}B_a]$ is greater or less, respectively, than the ratio $[B_o]/[B_a]$, or K_2.[5]

However, the ratio $[A\text{-}B_o]/[A\text{-}B_a]$, the apparent partition coefficient of the complex, reflects only the equilibrium condition and does not give any indication of the relative contribution of each of the several pathways by which this equilibrium can be attained. In fact, it can be shown algebraically that the ratio $[A\text{-}B_o]/[A\text{-}B_a]$ is equal to $K_1K_2K_5/K_3$ whether or not the pathway represented by K_4 actually exists.

It is evident from the above discussion that, unlike the partition coefficient of a pure substance which reflects the ratio of its interfacial transfer rate constants in both directions, the apparent partition coefficient of a complex does not necessarily reflect the rates of transfer of the complex itself from one phase to the other. Yet, it is this direct transfer of the complex itself which would most likely bring about a change in the overall absorption rate of complexed drugs. Herein lies the limitation of the use of partitioning data, which are representative of an equilibrium situation, for correlation with intestinal transfer data, which represent a rate phenomenon. The lack of correlation between the transfer rate constants and partition coefficients of salicylamide, caffeine, and the salicylamide–caffeine complex is consistent with this reasoning.

[5] This statement can be substantiated mathematically, taking as an example the case where complexation results in an increase in the apparent partition coefficient ($PC_{app.} > K_2$). If $PC_{app.} > K_2$, then

$$\frac{[B_o] + [A\text{-}B_o]}{[B_a] + [A\text{-}B_a]} > \frac{[B_o]}{[B_a]}$$

Cross-multiplying yields

$$[B_o][B_a] + [A\text{-}B_o][B_a] > [B_o][B_a] + [B_o][A\text{-}B_a]$$
$$[A\text{-}B_o][B_a] > [B_o][A\text{-}B_a]$$

Therefore,

$$\frac{[A\text{-}B_o]}{[A\text{-}B_a]} > \frac{[B_o]}{[B_a]}$$

It appears that factors which determine the rate of transfer of drug complexes across biologic membranes are likely to be resolved only by a consideration of physicochemical effects at the biologic interface and not on the basis of *in vitro* apparent partition coefficient data or transfer experiments using simple nonbiologic membranes or barriers (10). It is therefore appropriate to direct further attention to the equilibria which exist at the surface of biologic membranes in systems consisting of two interacting drugs. This will be the subject of the following paper.

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Caffeine retardation—salicylamide transfer
Everted intestine—experimental technique
Partition coefficients-transfer rates—comparison
UV spectrophotometry—analysis

Thiopental Pharmacokinetics

By K. B. BISCHOFF* and R. L. DEDRICK

A mathematical pharmacokinetic model, including flow limitations, lipid solubility, protein binding, and metabolism, is used to make *a priori* predictions of the distribution of thiopental in four body regions. Tissue binding is correlated by means of "effective" protein fractions and bovine serum albumin data. Metabolism is represented by a Michaelis-Menten rate equation. Close agreement with existing experimental data lends confidence in the model as a valuable tool for predictions in a variety of therapeutic situations.

MANY PRIOR studies of thiopental pharmacokinetics have been made, but there remains some question concerning the relative importance of metabolism and lean and adipose tissue uptakes. Some years ago, Brodie and co-workers (1, 2) made quantitative measurements of thiopental concentrations in various body regions and concluded that significant amounts were present

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in adipose tissue. The overall rate of metabolism in man was about 15%/hr. and was believed insufficient to account for the observed ultrashort duration of action. Price *et al.* (3) arrived at similar conclusions from mathematical analysis and an analog computer simulation, except that they attributed more of the drug redistribution effects to the lean body mass. Mark and Brand (4) have reviewed much of this work. A somewhat different viewpoint was taken in a recent analog computer study by Saidman and Eger (5) in that a much more important contribution of metabolism was assumed.

BASIS OF MODEL

The purpose here is to devise a mathematical pharmacokinetic model incorporating as many as possible of the variables known to affect drug distribution. Previous work concerning the dynamics of vascular tracers required detailed examination of blood volume distribution and flow rates (6, 7). Since the time scale of interest in the present paper is on the order of a few minutes to several hours, it is permissible to identify certain gross "lumped" regions over which relevant values of perfusion or physical properties may be considered constant. This, then, leads to the same general breakdown used by some previous workers: (*a*) blood, (*b*) viscera (highly perfused lean tissue), (*c*) poorly perfused lean tissue, and (*d*) poorly perfused adipose tissue.

Values of the regional flow and volume parameters representing the normal adult male were taken from Mapleson's detailed data for several body regions (8). Such detail might be of use in future studies, but sufficient thiopental distribution data are not presently available to justify such a complex pharmacokinetic study at this time. Figure 1 illustrates the four body regions and representative numerical parameters.

A useful mathematical model must explicitly include resistances to mass transfer by membrane permeability and constraints caused by flow limitation. Since thiopental is highly lipid soluble, the authors assume, as have earlier workers, that membrane transport is very rapid and the controlling resistance is flow limitation for all regions. A more detailed analysis is required for other solutes which might have lower lipid solubility. For thiopental, then, only the total tissue volumes, quantities of associated equilibrium blood, and blood flows are required, as shown in Fig. 1.

A possible complication that cannot be accommodated by simple compartmental models has recently been discussed by Rackow, Perl, and co-workers (9, 10), in which direct molecular diffusion occurs between two adjacent tissue regions. This would be most likely to occur between adjacent layers of well perfused lean and poorly perfused adipose tissue where the solute is highly lipid soluble. The initial adipose uptake then could be more a result of direct diffusion from the lean tissue than from its own low perfusion. Perl *et al.* (10) considered this effect for fat-soluble gases and showed that certain discrepancies in the data could thereby be explained. For the longer time intervals of interest here, however, and for more slowly diffusing thiopental, it is felt that this effect can be neglected.

Another complication, until recently neglected in the pharmacology and anesthesiology literature, is the phenomenon of protein binding. A good general discussion has been presented by Gillette (11), who illustrated the significance of binding of several drugs. An extensive consideration of binding as it affects pharmacokinetics analyses is found in a series of papers by Krüger-Thiemer (a few of the most recent are 12–14). For cases such as sulfa drugs, only entire body blood and tissue breakdowns were used, and it was clearly shown how binding can alter the features of the mathematical model. For example, the use of an overall distribution coefficient relating blood and tissue concentrations is not possible, since these ratios are a function of the concentration level when nonlinear binding occurs. Also, since the free (unbound) concentration is presumed to be the proper basis for pharmacological action, the extent of binding must be accounted for in order to produce meaningful predictions. The present work includes binding effects; the details will be discussed later.

MATHEMATICAL MODEL

Transient mass balances can be written for each of the body regions considered, assuming flow-limiting conditions. For the blood compartment:

$$\underset{\text{accumulation of unbound drug}}{f_B V_B \frac{dC_B}{dt}} + \underset{\text{accumulation of bound drug}}{(1 - f_B) V_B \frac{dx_B}{dt}}$$

$$= \underset{\text{outflow of unbound and bound drug}}{- Q_B [f_B C_B + (1 - f_B) x_B]}$$

$$\underset{\text{inflow from other regions of unbound and bound drug}}{+ Q_V [f_{VB} C_V + (1 - f_{VB}) x_{VB}] + Q_L [f_{LB} C_L + (1 - f_{LB}) x_{LB}] + Q_A [f_{AB} C_A + (1 - f_{AB}) x_{AB}]}$$

$$\underset{\text{ingestion term}}{+ Mg(t)} \qquad \text{(Eq. 1)}$$

The physical meaning of each term is indicated below it for clarity, and the particular symbols are defined under *Notation*.

A similar mass balance for the viscera gives:

$$\underset{\text{accumulation of unbound drug in equilibrium blood and tissue}}{(f_{VB} V_{VB} + f_{VT} V_{VT}) \frac{dC_V}{dt}} + \underset{\text{accumulation of bound drug in equilibrium blood}}{(1 - f_{VB}) V_{VB} \frac{dx_{VB}}{dt}}$$

$$\underset{\text{accumulation of bound drug in tissue}}{+ (1 - f_{VT}) V_{VT} \frac{dx_{VT}}{dt}}$$

$$= \underset{\text{net inflow of unbound and bound drug}}{Q_V [f_B C_B + (1 - f_B) x_B - f_{VB} C_V - (1 - f_{VB}) x_{VB}]}$$

$$\underset{\text{rate of metabolism of drug}}{+ r_V(C_V)} \qquad \text{(Eq. 2)}$$

The metabolism rate term is written in the simple Michaelis-Menten form for the calculations:

$$r_V(C_V) = -\frac{k_V C_V}{K_{mV} + C_V} \qquad \text{(Eq. 3)}$$

Since the metabolism mainly occurs in the microsomes of the liver (11), the rate of reaction, r_V, appears in the balance for the visceral region. Similar terms could be added to the other balances if required.

The remaining mass balances have a similar form.

For lean tissue:

$$(f_{LB}V_{LB} + f_{LT}V_{LT})\frac{dC_L}{dt} + (1 - f_{LB})V_{LB}\frac{dx_{LB}}{dt} + (1 - f_{LT})V_{LT}\frac{dx_{LT}}{dt} = Q_L[f_B C_B + (1 - f_B)x_B - f_{LB}C_L - (1 - f_{LB})x_{LB}] \qquad \text{(Eq. 4)}$$

For adipose tissue:

$$(f_{AB}V_{AB} + f_{AT}V_{AT})\frac{dC_A}{dt} + (1 - f_{AB})V_{AB}\frac{dx_{AB}}{dt} + (1 - f_{AT})V_{AT}\frac{dx_{AT}}{dt} = Q_A[f_B C_B + (1 - f_B)x_B - f_{AB}C_A - (1 - f_{AB})x_{AB}] \qquad \text{(Eq. 5)}$$

Explicit relations between the various bound and free concentrations are required to solve Eqs. 1–5. For blood plasma protein binding, the results of Goldbaum and Smith (15) are used. They correlated their bovine serum albumin data with the commonly used (see *Reference 11*) two-term relation,

$$x_B = \frac{B_1K_1C_B}{1 + K_1C_B} + \frac{B_2K_2C_B}{1 + K_2C_B} \qquad \text{(Eq. 6)}$$

Goldbaum and Smith (15) also presented data on rabbit tissue binding of a number of barbiturates. In the absence of more information, it is assumed that the relationship for binding by plasma proteins could also be used for tissue protein binding. "Effective tissue protein fractions" are assigned to produce the measured tissue drug concentrations. This assumption is justified on the basis that consistent "effective tissue protein fractions" are found to result for all the barbiturates studied. Further justification of this method with other classes of compounds would be useful.

With this assumed basis, all of the binding relations take on similar functional forms, each with the appropriate free concentration inserted. For example, the viscera binding relations become:

$$x_{VT} = x_{VB} \equiv x_V = \frac{B_1K_1C_V}{1 + K_1C_V} + \frac{B_2K_2C_V}{1 + K_2C_V} \qquad \text{(Eq. 7)}$$

and the lean tissue values,

$$x_{LT} = x_{LB} \equiv x_L = \frac{B_1K_1C_L}{1 + K_1C_L} + \frac{B_2K_2C_L}{1 + K_2C_L} \qquad \text{(Eq. 8)}$$

Adipose tissue must be handled somewhat differently. The equilibrium blood plasma binding has the usual form,

$$x_{AB} = \frac{B_1K_1C_A}{1 + K_1C_A} + \frac{B_2K_2C_A}{1 + K_2C_A} \qquad \text{(Eq. 9)}$$

but the tissue "binding" here is primarily the lipid solubility for which a simple Henry's law formula is used,

$$x_{AT} = B_A C_A \qquad \text{(Eq. 10)}$$

A final simplification assumes that the fractions of protein in all the various blood regions are the same,

$$f_B = f_{VB} = f_{LB} = f_{AB} \qquad \text{(Eq. 11)}$$

These considerations lead to simplifications in Eqs. 1–5 which can now be solved if the input or ingestion function is specified. An approximation to a normalized impulse function similar to that of Bellman *et al.* (16) is used,

$$g(t) = 30\lambda(\lambda t)^2(1 - \lambda t)^2 \qquad \text{(Eq. 12)}$$

where λ is the reciprocal injection duration. This function simulates a smooth injection and is convenient for computation.

RESULTS AND DISCUSSION

Numerical values for the parameters in the above mass balance equations are provided by the data of Mapleson (8) (see Fig. 1) and Goldbaum and Smith (15), except for the metabolism term. In a comprehensive review paper, Mark (17) presents values for the overall rates of metabolism of barbiturates, which for thiopental is about 15%/hr. The parameters k_V and K_{mV} in Eq. 3 are empirically adjusted to provide values of k_V = 26.5 μmole/min. and K_{mV} = 4 (μmole/l.), which gave the desired rate. The recent data of Mark *et al.* (18) can be correlated by a Michaelis-Menten kinetic form to

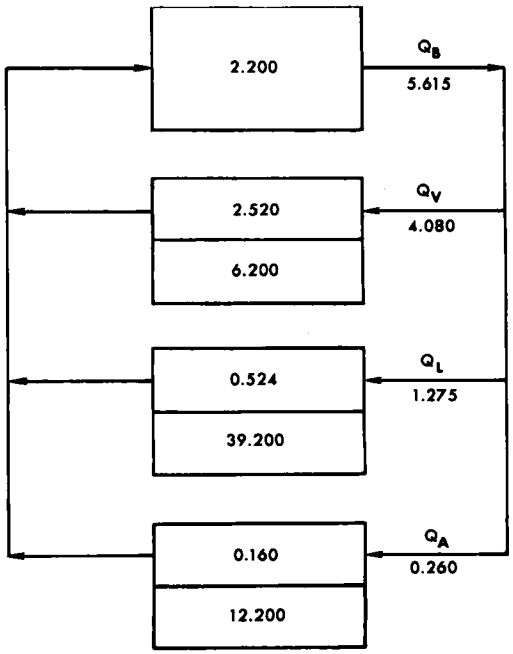

Fig. 1—Body regions and flows. Key: B, blood pool not in equilibrium with tissues; V, viscera; L, lean tissue; A, adipose tissue.

TABLE I—PARAMETER VALUES

Parameter	Numerical Value
Flow distribution, l./min.	$Q_B = 5.615$, $Q_V = 4.080$, $Q_L = 1.275$, $Q_A = 0.260$
Body region size, l.	$V_B = 2.20$, $V_{VB} = 2.52$, $V_{VT} = 6.20$, $V_{LB} = 0.524$, $V_{LT} = 39.20$, $V_{AB} = 0.160$, $V_{AT} = 12.200$
"Effective" fraction water	$f_B = 0.985$, $f_{VT} = 0.963$, $f_{LT} = 0.980$, $f_{AT} = 0.20$
Binding sites, μmole/l.	$B_1 = 18{,}400$, $B_2 = 305{,}000$
Binding equilibrium constant, l./μmole	$K_1 = 0.060$, $K_2 = 0.000625$
Lipid solubility constant	$B_A = 100$

produce parameter values of the same order of magnitude as those required to obtain the 15%/hr. rate. This lends some confidence to the metabolism rate expression, but the need for more quantitative chemical kinetics work remains clear. The lipid solubility constant lies between the value for peanut oil–water from Mark *et al.* (19) and the value from Price *et al.* (3), corrected for protein binding.

Analytical solutions of the differential equations are not feasible because of the nonlinearities caused by the binding terms; the equations have therefore been solved numerically using a digital computer. As a critical test of the pharmacokinetics model, an *a priori* prediction of the experimental results of Brodie *et al.* (1, 2) on thiopental distribution in dogs was attempted. *A priori* here implies that numerical values of all the parameters in the model (except metabolism) were taken from sources other than Brodie *et al.*: human flow distribution and body region size from Mapleson (8); bovine serum albumin and rabbit tissue binding from Goldbaum and Smith (15); lipid solubility from Mark *et al.* (19) and Price *et al.* (3). The numerical values used in the computations are shown in Table I.

In the cited experiments of Brodie *et al.* (1, 2) 25 mg./kg. thiopental was injected i.v. with a 5-min. injection time. Thus, the final two parameters in the model are $M = 6630$ μmoles (for a 70-kg. man) and $\lambda = 0.20$ min.$^{-1}$.

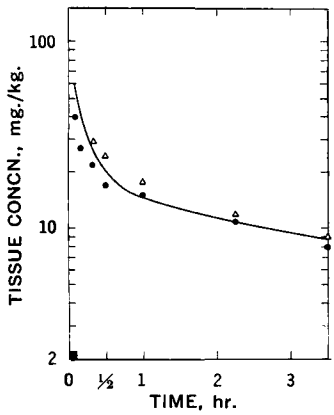

Fig. 2—Comparison of predicted and experimental values for blood. Key: ■, injection duration; ●, dog; Δ, human.

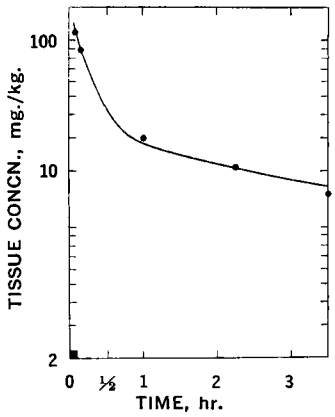

Fig. 3—Comparison of predicted and experimental values for viscera (liver). Key: ■, injection duration; ●, dog.

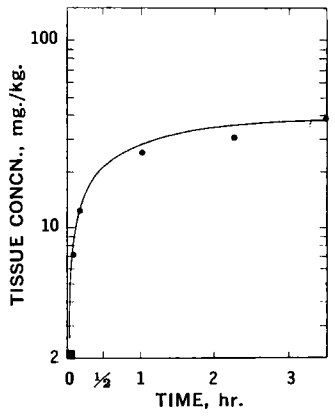

Fig. 4—Comparison of predicted and experimental values for adipose tissue. Key: ■, injection duration; ●, dog.

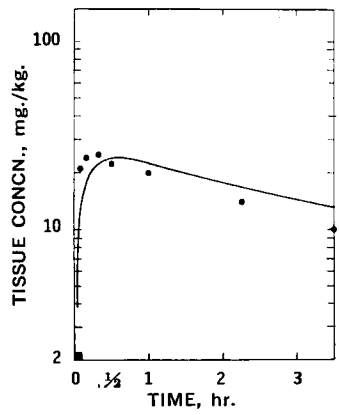

Fig. 5—Comparison of predicted and experimental values for lean tissue. Key: ■, injection duration; ●, dog.

The results of the numerical computations are presented in the graphs. Figure 2 compares the *a priori* predicted and measured values in blood for dogs and for man. The total concentration, bound plus unbound, is plotted. Several features of the graph are worthy of note. First, the pre-

dicted values from the mathematical model agree well with the experimental data. Second, the physical distribution patterns for dog and man are very similar when plotted in a consistent manner. Third, rapid fall in blood thiopental levels is observed for the first half-hour as distribution equilibrium is approached. And finally, the slope of the line at long times principally represents metabolism at the rate of 15%/hr.

Figure 3 compares the computed and experimental (dog) values for the highly perfused or viscera compartment. Again, excellent agreement is obtained. The uptake in adipose tissue is illustrated in Fig. 4, which shows the rapid and continuing solution of the drug in the lipid at the perfusion rate of 2 (ml./min.)/100 g. used (see Table I). The curve for the final compartment, poorly perfused lean tissue, is shown in Fig. 5. The overall predicted trends are correct, but there are some discrepancies between the calculated curve and the experimental data. At short time intervals, the difference can probably be explained by the fact that human flow distribution and perfusion rates are used but that experimental data were obtained on dogs. Since lean tissue of dogs is somewhat more highly perfused than that of humans [value used—3 (ml./min.)/100 g], the measured initial uptake is higher than predicted. Calculations made with a higher lean perfusion rate shift the predicted curves in the correct direction. Since the intent was to see how good an *a priori* prediction could be made, however, these "curve fitting" attempts were not pursued in any detail. The long time portion of the curve also deviates from the data, but slight changes in the lipid solubility produce better agreement; again, a better "curve fit" has not been attempted.

Figure 6 shows the computed behavior over a period of several hours. It is seen that the three nonlipid compartments rapidly approach distribution equilibrium, but that the adipose uptake rises continuously to a maximum at about 3–4 hr. This also corresponds with the observations of Brodie *et al.* (2).

A final very interesting result is exhibited by the plot of computed *free* concentrations in each of the compartments shown in Fig. 7. The blood and viscera free concentrations fall very rapidly and, more important, are essentially equal over the entire time period. This is a good illustration of the concept that blood concentrations are an excellent measure of the viscera concentrations and thus

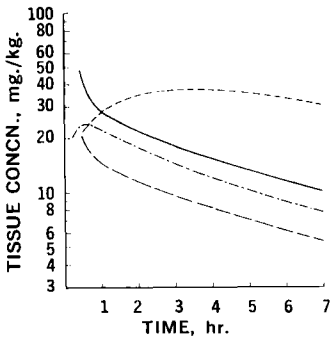

Fig. 6—Long time behavior—total concentration. Key: ———, viscera; – – – –, adipose; — · —, lean; — —, blood.

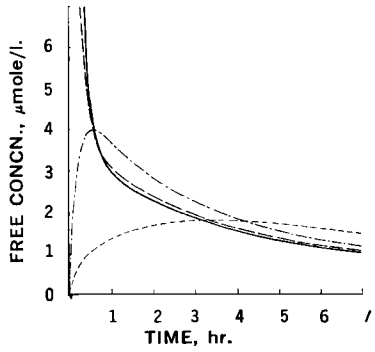

Fig. 7—Long time behavior—free concentration. Key: ———, viscera; – – – –, adipose; — · —, lean; — —, blood.

should provide a basis for correlating pharmacological action. Notice, however, that this is true *only* for the free concentrations (*i.e.*, corrected for binding), since Fig. 6 indicates that the total concentrations in the blood and viscera are not equal.

CONCLUSION

The *a priori* predictions of thiopental distribution resulting from the pharmacokinetic model, including protein binding and lipid solubility, agree closely with published experimental data. Such performance provides confidence in the model as a valuable tool for predictions in a variety of therapeutic situations if it is properly applied and sufficient data are available. One application reported elsewhere (20, 21) considers the physiological solute distributions associated with the extracorporeal treatment of blood with an artificial kidney.

NOTATION

B_A = lipid solubility in adipose tissue (dimensionless)
B_1, B_2 = protein binding sites (μmole/l.)
C = free concentration (μmole/l.)
f = fraction water (dimensionless)
$g(t)$ = injection description function (min.$^{-1}$)
k_V = metabolism rate constant or maximum rate (μmole/min.)
K_{mV} = Michaelis-Menten constant (μmole/l.)
K_1, K_2 = protein binding equilibrium constants (l./μmole)
M = amount of drug injected (μmole)
Q = flow rate (l./min.)
r_V = metabolism rate (μmole/min.)
t = time (min.)
x = bound concentration (μmole/kg.)
V = volume of compartment (l.)
λ = reciprocal injection of time (min.$^{-1}$)

Subscripts

A = adipose
B = blood
L = lean
V = viscera
AB = adipose equilibrium blood
LB = lean equilibrium blood
VB = viscera equilibrium blood
AT = adipose tissue
LT = lean tissue
VT = viscera tissue

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Thiopental—distribution
Protein binding—thiopental
Model, mathematical—thiopental distribution

Effect of Chlordan Pretreatment on the Metabolism and Lethality of Cyclophosphamide

By ROBERT L. DIXON

Cyclophosphamide is converted to a cytostatic agent by enzymes present in the hepatic microsomes. Prior treatment of young rats with the insecticide, chlordan, resulted in an increased cyclophosphamide toxicity. Chlordan pretreatment also increased the *in vitro* metabolism of cyclophosphamide and hexobarbital, increased the excretion of the alkylating metabolite of cyclophosphamide *in vivo*, and decreased the sleeping times of animals treated with hexobarbital *in vivo*. It is most probable that increased amounts of cytostatic metabolite occurred as a result of chlordan induced microsomal drug-metabolizing enzyme stimulation.

CYCLOPHOSPHAMIDE[1] [*N,N*-bis(β-chloroethyl)-*N'*, *O*-propylene phosphoric acid ester diamine monohydrate, NSC 26271] is a potent antineoplastic agent of the nitrogen mustard class and has been used in the treatment of patients with many types of neoplastic diseases. Cyclophosphamide is especially valuable in the treatment of patients with malignancies arising from the hematopoietic tissues. The compound can be administered by all routes (1).

In contrast to the nitrogen mustards ordinarily used in cancer chemotherapy, cyclophosphamide is inert when placed in direct contact with bacteria, leukocytes, and most tumor cells in culture. *In vivo* activation occurs in the liver and perhaps in other sites (2). The subcellular site of enzymic activation in the liver is the microsomes. These hepatic microsomal drug-metabolizing systems require oxygen and NADPH (TPNH) and are capable of altering a number of drugs (3). The microsomal enzyme that activates cyclophosphamide is influenced by a number of various drugs (3). For instance, SKF-525a (β-diethylamino-ethyldiphenylpropylacetate), an inhibitor of microsomal drug metabolism, will decrease serum levels of metabolite capable of alkylation after cyclophosphamide (4), while pretreatment of animals with phenobarbital increases the concentration of this metabolic agent three to fourfold by enzyme induction (5).

The fact that the concentration of metabolite capable of alkylation after cyclophosphamide treatment can be increased by enzyme induction

Received November 6, 1967, from the Department of Pharmacology, School of Medicine, University of Washington, Seattle, WA 98105
Accepted for publication April 18, 1968.
Supported by grants from the National Institutes of Health (HD 02350 and CA 08671), Bethesda, Md.
The author wishes to acknowledge the very able technical assistance of Mrs. Valerie Campell during this project.

[1] Marketed as Cytoxan by Mead Johnson & Co., Evansville, Ind.

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might have therapeutic possibilities. Surely it is of interest to those testing new compounds for toxic effects or action against transplantable tumors in animals. There are a large number of drugs and foreign compounds that have been shown to be effective stimulators of microsomal drug-metabolizing enzymes (2). This paper reports enhancement of cyclophosphamide metabolism and toxicity in rats after exposure to the insecticide, chlordan, which is commonly used in laboratory quarters.

METHODS

Cyclophosphamide was obtained commercially. Technical chlordan, a mixture containing 60–75% of the pure compound and 25–40% of related compounds, was used in these experiments.

Young male rats, 50–60 g., were treated with either 10 mg./kg. of technical chlordan dissolved in corn oil or received corn oil alone. The technical chlordan was dissolved in corn oil to make a 0.1% solution and each chlordan treated animal received subcutaneously 0.01 ml. of this solution per gram of body weight. Animals were pretreated with chlordan twice, 144 and 72 hr. prior to oral administration of cyclophosphamide.

Cyclophosphamide was administered orally to groups of 10–12 rats at a dose of 90, 125, 180, or 250 mg./kg. Cyclophosphamide was administered at a concentration such that each animal received 0.01 ml. of aqueous solution per gram of body weight. Deaths were recorded daily for 30 days following a single dose of cyclophosphamide.

In vitro metabolism of hexobarbital[2] (side-chain oxidation) was determined using methods, conditions of incubation, cofactors, and concentrations described previously (6).

The *in vitro* metabolism of cyclophosphamide was studied using the same conditions described above. The substrate concentration was 10 mg./5 ml. incubation. The active metabolite of cyclophosphamide was estimated by the spontaneous alkylation of α-(4-nitrobenzylpyridine) (NBP) (7, 8). Five milliliters of 6.67% trichloroacetic acid was added to the beaker following incubation. The solutions were mixed and poured into a heavy walled centrifuge tube and centrifuged prior to taking a 3.0-ml. aliquot for assay.

In vivo metabolism of hexobarbital was estimated using sleeping times after hexobarbital treatment. Hexobarbital, 150 mg./kg., was administered intraperitoneally and the time in minutes from loss of righting reflex to recovery of reflex was recorded.

In vivo metabolism of cyclophosphamide was estimated by collecting and analyzing urine for metabolites capable of alkylation after cyclophosphamide treatment. Urine was collected from each of six animals (three chlordan treated, three corn oil treated) for the first 4 hr. following cyclophosphamide treatment. Animals were placed in individual plastic metabolism cages which allowed collection of urine. Each animal received 500 mg./kg. of cyclophosphamide orally. After the 4-hr. collection period, the urine was obtained and the cage rinsed with distilled water. Urine plus cage rinsings were brought to a final volume of 25 ml. with distilled water. Three-milliliter aliquots from each total volume were then assayed for material capable of alkylating the NBP reagent.

Statistical methods used are described by Snedecor (9) and Litchfield and Wilcoxon (10).

RESULTS

Table I demonstrates the effect of chlordan pretreatment on the metabolism of hexobarbital *in vitro* and *in vivo*. It can be seen that chlordan pretreatment increased the rate of metabolism *in vitro*, and decreased the duration of sleep after hexobarbital. These data are in agreement with those of Hart *et al.* (11) and serve only to confirm the effect of chlordan on the injected animals.

In vitro studies of the metabolism of cyclophosphamide by hepatic supernatant fraction from chlordan and corn oil pretreated animals revealed that the chlordan pretreated animals produced 76% more of an alkylating substance during a 30-min. incubation than did corn oil treated control animals. The mean absorbance reading for the metabolite–NBP reagent complex formed by the chlordan treated animals was 0.294 ± 0.096 *A* units/hr./g. of tissue compared to 0.167 ± 0.052 *A* units/hr./g. of tissue for the corn oil pretreated animals. These values are significantly different ($p \leq 0.05$). Five animals were used in the chlordan treated group and four animals were used in the control group. The colored complex was developed from a 3-ml. aliquot taken from the supernatant of the incubation system (5 ml.) plus 5 ml. of TCA, and the color subsequently extracted with 7 ml. of the nonpolar phase. The absorbance readings (*A*) were converted to absolute values by using a standard curve prepared after hydrolyzing cyclophosphamide as described by Philips *et al.* (12). The reactivity of the hydrolyzed solutions of cyclophosphamide averages 99% of that of molar equivalent amounts of nor-NH_2HCl. In these studies a concentration of activated product from 10 mcg. of cyclophosphamide per ml. of the nonpolar extraction phase (acetone-ethyl acetate) had an absorbance of 0.613 when measured at 600 mμ in a 1-cm. square cell. Using this standard curve the above absorbance values represent 112 and 64 mcg. cyclophosphamide metabolized per gram liver per hour, respectively. The absorbance values were multiplied by $^{10}/_{3}$ to correct the value for the aliquot, and by 7 in order to express micrograms of metabolite in the 7 ml. of nonpolar solvent.

In vivo studies of the excretion of a metabolite capable of alkylating the NBP reagent (activated

TABLE I—EFFECT OF CHLORDAN PRETREATMENT ON THE METABOLISM OF HEXOBARBITAL

	Corn oil Treated	Chlordan Treated
In vitro metabolism[a]	3.87 ± 0.85 (4)	5.37 ± 1.17 (5)
In vivo sleeping times[b]	106 ± 14	84 ± 21

[a] Micromoles of hexobarbital metabolized per gram of liver during 45-min. incubation. Values are means ± standard deviation. Number of individual animals is indicated in the parentheses. [b] Minutes of loss of righting reflex after 150 mg./kg. of hexobarbital i.p. Values are means ± standard deviation for six animals per group.

[2] Evipal, Winthrop Laboratories, New York, N. Y.

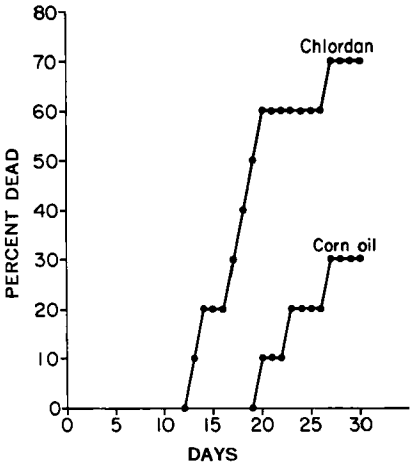

Fig. 1—Percent of animals (10 per group) which died following a single oral dose of cyclophosphamide (125 mg./kg.). The chlordan group was treated prior to cyclophosphamide administration with chlordan. Control animals received only corn oil.

metabolite) after cyclophosphamide treatment revealed that the chlordan pretreated animals excreted more of this substance in 4 hr. than did the corn oil treated controls.

Chlordan pretreated rats excreted 61% more of this metabolite than did corn oil treated controls. The average final readings determined from a 3-ml. aliquot of urine from each total volume of 25 ml. was 0.255 (0.215, 0.283, 0.267) for chlordan pretreated animals compared to a mean reading of 0.158 (0.155, 0.145, 0.173) for the corn oil treated control animals. The metabolite from the 3.0 ml. was extracted into 7.0 ml. of a nonpolar phase prior to reading the *A* with a colorimeter. Using the standard curve referred to in the *in vitro* results, the above values correspond to 242 and 148 mcg., respectively.

Figure 1 expresses the lethality data seen at one dose, 125 mg./kg., of cyclophosphamide. The same trend seen here, that of earlier and greater lethality, was seen with each of the higher doses studied.

Table II presents lethality data for cyclophosphamide for groups of corn oil and chlordan pretreated animals. It can be seen that the LD_{50} for the chlordan pretreated group is lower, 145 (125–166) mg./kg., than that for the corn oil pretreated animals which was 170 (142–204) mg./kg. Neither of the two slopes was significantly heterogeneous and they were parallel. However, the potency ratio of 1.17 was not significant at the 95% level of probability. Nevertheless, it should be pointed out that after chlordan pretreatment a dose of cyclophosphamide normally lethal to 50% of the animals tested resulted in approximately 80% dead due to the very steep dose-response slope associated with this drug.

DISCUSSION

Results presented here demonstrate that exposure to chlordan, an insecticide commonly used in animal quarters, can result in changes in drug metabolism which alter the toxicity (weight loss and diarrhea) and lethality of cyclophosphamide. Animals receiving two subcutaneous injections of chlordan,

TABLE II—EFFECT OF CHLORDAN ON LETHALITY OF CYCLOPHOSPHAMIDE

Oral Dose, mg./kg.	% Alive, 30 days	
	Corn Oil Treated	Chlordan Treated
250	10	0
180	42	20
125	70	30
90	100	100
LD_{50}	170 (142–204)	145 (125–166)

144 and 72 hr. prior to cyclophosphamide, showed signs of toxicity and died earlier and in greater number than the corn oil pretreated animals.

This increase in lethality can be explained by an induction of microsomal enzymes which convert cyclophosphamide to an active form and accounts for the increase in the levels of the cytotoxic form. Data were presented which demonstrated that animals pretreated with chlordan metabolized both hexobarbital and cyclophosphamide to a greater extent *in vitro* than corn oil treated controls. Also, animals pretreated with chlordan excreted more metabolite capable of alkylating NBP reagent in 4 hr. following cyclophosphamide than did corn oil treated control rats. The effect of chlordan was further demonstrated by a decreased sleeping time after hexobarbital administration in the chlordan treated group. This effect was similar to the increased cytotoxic action described for cyclophosphamide after phenobarbital pretreatment (5).

These data indicate that spraying animal quarters with the chlorinated hydrocarbon insecticides can result in spurious testing results concerning toxicity and perhaps effectiveness against transplantable tumors. In most cases increased metabolism of a drug would result in its decreased effectiveness and lethality. However, increased metabolism of cyclophosphamide resulted in an increase of active metabolite and enhanced drug effect.

The clinical implications for therapeutic treatment of such findings is probably less important than possible effects on laboratory animals. However, it might be possible to increase the effectiveness of this drug in patients who for some reason have a less than normal ability to metabolize cyclophosphamide. In such patients cyclophosphamide would be less effective. A decreased metabolism of drugs could possibly occur in patients who are very young, or as a result of various pathological conditions (2). These suggestions are based on findings in animals, and must be extrapolated to the clinical situation with care.

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Keyphrases

Chlordan pretreatment—drug metabolism
Cyclophosphamide metabolism—chlordan effect
Hexobarbital metabolism—chlordan effect
LD_{50} values—cyclophosphamide and chlordan pretreated
Colorimetric analysis—spectrophotometer

Bile Salt Potentiation of Pharmacologic Effects and Drug Uptake in Goldfish

By MILO GIBALDI and CHARLES H. NIGHTINGALE

Concentrations of 1×10^{-4} *M* and 2×10^{-4} *M* sodium taurodeoxycholate (STDC), which demonstrate no intrinsic pharmacologic activity, significantly potentiate the pharmacologic effects (*i.e.*, time required to produce overturn) of pentobarbital and ethanol in goldfish. Quantitatively similar potentiation is noted when the fish is exposed to a bile salt-drug solution or when the fish is pretreated with STDC, rinsed, and immediately exposed to drug solution. The present results suggest that potentiation is independent of the duration of pretreatment of fish with STDC. The effects of STDC are mediated very quickly, reach a maximum level within minutes, and are slowly reversible. The results are consistent with a mechanism involving adsorption of bile salt molecules on the biologic membrane, consequent alteration of membrane permeability, and a resultant decrease in the time required to observe a pharmacologic response to ethanol and pentobarbital. A 1×10^{-4} *M* STDC solution also increases significantly the absorption of 4-aminoantipyrine in goldfish.

BILE SALTS are one of the most important groups of physiologic surface-active agents found in man. Although their role in fat absorption has been studied extensively (1, 2), little attention has been given to the possible effects of bile salts on drug absorption. Recent studies (3–6) have shown that components of bile significantly affect both the solubility and dissolution rate of a number of poorly water-soluble drugs. These studies suggest that bile may serve an important function in dissolution rate-limited drug absorption.

In addition to the solubilizing effects of bile salts, certain biologic effects may also be important in drug absorption. An example is the potential effects of bile salts on the gastrointestinal membrane and on drug transport. Parkinson (7) has observed inhibition of glucose, sodium, and amino acid active transport from the rat jejunum by certain bile salts. However, there is no literature available on the effects of bile salts on passive transport, the usual pathway in drug absorption.

Numerous studies suggest that surface-active agents can affect the integrity of biologic membranes and thereby enhance drug absorption (8). Most recently, Levy *et al.* (9) have observed a significant decrease in the time required for secobarbital-induced death in the goldfish in the presence of polysorbate 80. They attribute this effect to membrane alteration by the surfactant which permits more rapid absorption of secobarbital and decreases the time required to reach a lethal concentration in the fish.

The present study concerns the influence of bile salts on the pharmacologic effects of ethanol and pentobarbital and on the uptake of 4-aminoantipyrine in the goldfish.

EXPERIMENTAL

Goldfish, *Carassius auratus*, common variety, weighing about 3–6 g. were used as the test animal. All fish used in a given experiment were from the same lot. Five different lots of fish were used during the study.

The test drugs, ethanol and sodium pentobarbital, were dissolved in water or aqueous solutions contain-

Received February 19, 1968, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication April 8, 1968.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
This work was supported by grant 50-1077A from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service, Bethesda, Md., and N.I.H. training grant GM-555-06.

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ing 1×10^{-4} or 2×10^{-4} *M* sodium taurodeoxycholate (STDC). The final solutions were adjusted to pH 6.1 with either 1 *N* HCl or NaOH. Solutions were prepared daily as required. All drugs and chemicals were USP or reagent grade. Chromatographically pure STDC, obtained from Maybridge Research Chemicals, Tintagel, N. Cornwall, England, was used.

The measured pharmacologic response was the time required for the fish to lose the ability to maintain itself upright after immersion in test solution, *i.e.*, the overturn time. The use of the overturn end point for the estimation of absorption and elimination kinetics in goldfish was the subject of a previous report (10). After each experiment the pH of the solution was redetermined and found to be 6.1 ± 0.1. Preliminary experiments indicated that the overturn times in ethanol in water and in water adjusted to pH 6.1 were the same.

Overturn time was determined with individual fish immersed in 200 ml. of solution at 26–28°, under the following experimental conditions: (*a*) immersion of fish in drug solutions; (*b*) immersion of fish in bile salt solutions; (*c*) immersion of fish in drug solutions containing bile salt; and (*d*) exposure of fish to bile salt solution, followed by rinsing with water and immersion in drug solutions.

Determination of Uptake of 4-Aminoantipyrine—Goldfish were placed in large beakers containing 250 ml. of solution per fish. The control solutions contained 250 mg.% 4-aminoantipyrine (Eastman) while the test solutions contained the drug and 1×10^{-4} *M* STDC. In each case the solutions were adjusted to pH 6.1. After a 15-min. immersion period, the fish were removed rapidly from the solutions, rinsed, and killed by freezing. The fish were stored in a freezer until assayed.

The analytical method for the determination of 4-aminoantipyrine in goldfish tissue was a modification of the colorimetric assay of Brun (11) as described by Levy and Miller (12). Tissue blanks averaged 22 mcg. 4-aminoantipyrine equivalent per g. of fish tissue. Prior exposure of the fish to 1×10^{-4} *M* STDC for 15 min. yielded identical blank values. Recovery of known amounts of 4-aminoantipyrine (300 mcg./g. of fish) added to homogenates averaged 97.1% (4 determinations, range 96.2 to 97.6%). Recovery of known amounts of 4-aminoantipyrine (400 mcg./g. of fish) added to homogenates of fish which were previously immersed for 15 min. in 1×10^{-4} *M* STDC averaged 94.5% (4 determinations, range 88.5–97.7%). Blank and recovery values were used to correct the analytical results.

RESULTS

Initial studies indicated that STDC showed significant toxicity in the goldfish at concentrations of 1×10^{-3} *M* and above. However, concentrations of 1×10^{-4} *M* and 2×10^{-4} *M* were without apparent effect. Fish from various lots were placed in 2×10^{-4} *M* bile salt solution for 24–48 hr. periods and in no instance was an effect upon the fish observed. Hence, it was concluded that these concentrations of STDC had no apparent intrinsic pharmacologic activity and these levels were used for further drug studies.

The overturn times of goldfish in pentobarbital and ethanol solutions, and the effect of STDC on overturn time, are shown in Table I. A significant potention of the apparent drug activity is observed in each case. The decrease in overturn time appears to be related to the STDC concentration.

The data in Table II represent the results of a number of experiments designed to test the interaction of individual aspects of the bile salt-drug-goldfish system. The following conclusions may be drawn from these results: (*a*) After exposure to pentobarbital to induce overturn, the fish recovers completely within 24 hr. when placed in fresh water and its subsequent response to drug + bile salt is the same as that of fish having no prior exposure. (*b*) After exposure to pentobarbital + STDC to induce overturn, the fish recovers completely within 24 hr. when placed in fresh water and its subsequent response to drug is the same as that of fish having no prior exposure. (*c*) Prolonged exposure to STDC (without observing overturn), followed by rinsing with distilled water and exposure to pentobarbital, results in an overturn time significantly shorter than that observed in Groups A_1 and B_2. In fact, the overturn time of goldfish in 5 mg.% pentobarbital solution after a 24-hr. exposure to STDC is almost identical to the overturn time found when the fish were exposed to drug + STDC.

This latter phenomenon was explored further by investigating the effect of duration of exposure to bile salt on drug-induced overturn time. In each case the fish were exposed to 1×10^{-4} *M* STDC for a period of time, rinsed, placed in either 5 mg.% pentobarbital or 1% ethanol solutions and observed

TABLE II—REVERSIBILITY OF PHARMACOLOGIC EFFECT IN GOLDFISH AND INFLUENCE OF STDC PRETREATMENT ON RESPONSE[a]

Group	Overturn Time,[b] min.	S.E.	Significantly Different[c] ($p < .05$) from Experiment
A_1	18.2	0.42	A_2, B_1, C
A_2	10.4	0.78	A_1, B_2
B_1	9.2	0.50	A_1, B_2
B_2	16.6	2.55	A_2, B_1, C
C	8.8	0.20	A_1, B_2

[a] Group A—Fish exposed to drug (5 mg. % sodium pentobarbital) (A_1); recovered 24 hr.; reexposed to drug plus bile salt (1×10^{-4}*M* STDC) (A_2). Group B—Fish exposed to drug plus bile salt (B_1); recovered 24 hr.; reexposed to drug (B_2). Group C—Fish exposed to bile salt for 24 hr., rinsed and exposed to drug, overturn time determined (C). [b] Values represent mean of 4 fish. [c] Determined by Student *t* test.

TABLE I—EFFECT OF STDC ON APPARENT DRUG-INDUCED OVERTURN TIME (T_0) IN GOLDFISH[a]

STDC, *M* Concn.	Ethanol (1.5% v/v) T_0 (min.)	S.E.	Significance of Difference[b]	Sod. Pentobarbital (5 mg.%) T_0 (min.)	S.E.	Significance of Difference[b]
Control	20.8	1.44	←	18.4	1.06	←
1×10^{-4}	16.0	0.64	←	14.9	1.39	←
2×10^{-4}	14.8	0.97		13.4	0.99	

[a] Values represent mean of 4 fish. [b] Determined by Student *t* test, arrows indicate significance at $p < 0.05$.

TABLE III—EFFECT OF DURATION OF EXPOSURE OF GOLDFISH TO 1×10^{-4} *M* STDC ON DRUG-INDUCED T_0 (MIN.)[a]

Exposure Time, min.	Ethanol (1% v/v) T_0	Ethanol (1% v/v) S.E.	Ethanol (1% v/v) Significance of Difference[b]	Pentobarbital (5 mg. %) T_0	Pentobarbital (5 mg. %) S.E.	Pentobarbital (5 mg. %) Significance of Difference[b]
0[c]	11.0	0.38	← ←	15.0	1.04	← ←
5	4.4	0.26	←	3.9	0.21	←
30	4.1	0.81	←	—	—	
60	—	—		4.9	0.28	←

[a] Values represent mean of 4 fish. [b] Determined by Student *t* test, arrows indicate significance at $p < 0.01$. [c] Control values determined by placing fish in distilled water (adjusted to pH 6.1) for 30 min. The fish were then rinsed and exposed to drug solutions.

for overturn. The results, shown in Table III, indicate that the potentiation of drug-induced overturn is independent of the duration of exposure to STDC. For example, 5-min. exposure to STDC reduces the apparent ethanol-induced overturn time to the same extent as exposure for a 30-min. period. Similar results are observed with pentobarbital.

Figure 1 shows the effect of 1×10^{-4} *M* STDC on the uptake of 4-aminoantipyrine by goldfish after a 15-min. immersion in 250 mg.% drug solution. The presence of bile salt results in a 30% increase in drug concentration in the fish tissue as compared to control values. This difference is statistically significant at the 95% level of confidence. These findings indicate clearly the ability of bile salts to modify membrane permeability.

DISCUSSION

The enhanced response of goldfish to ethanol and pentobarbital may be due to two effects. Firstly, STDC may produce an unspecified pharmacologic effect that is inadequate to promote overturn but makes the fish more susceptible to the pharmacologic action of ethanol and pentobarbital. The second possibility is that the bile salt increases the permeability of the fish membranes to the drugs and permits more rapid absorption. While both effects may be operative, the present findings are consistent with the hypothesis that the principal effect of STDC is alteration of membrane permeability.

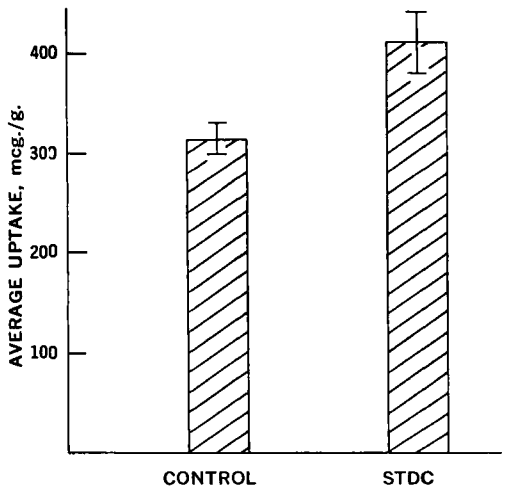

Fig. 1—Effect of 1 × 10⁻⁴ M STDC on the uptake of 4-aminoantipyrine by goldfish immersed in 250 mg.% drug solution. Each bar represents the mean of seven fish; vertical lines indicate standard error of the mean.

For example, if STDC potentiates drug effects in the goldfish by virtue of an intrinsic pharmacologic effect then when fish are first exposed to bile salt solution and immediately exposed to drug solution, one would anticipate a graded response to the drug, which is dependent on the time of exposure to the bilt salt. The data in Table III indicate that this is clearly not the case. The reduction in ethanol-induced and pentobarbital-induced overturn time is independent of the duration of preexposure to STDC solution.

Previous studies (9) of the effects of nonionic surfactants on drug absorption in the goldfish suggest an enhanced permeability to barbiturates but not to ethanol. In the present study this permeability discrimination was not observed. In fact, the observed potentiation was quantitatively similar for both ethanol and pentobarbital. This finding implies a possible difference in the mode of action of the nonionic polysorbate 80 and the anionic STDC.

The potentiation of drug effects in the absence of surfactant from the bulk drug solution, noted in the preexposure studies, has also been observed by Levy and Anello (13). These workers found that an absorption-enhancing effect occurs if the fish are first immersed in polysorbate 80 solution, washed, and then placed in a surfactant-free secobarbital solution. The rapid effects manifested by both polysorbate 80 and STDC are consistent with a mechanism involving adsorption of surfactant molecules on the biologic membrane and alteration of membrane permeability.

The use of the overturn end point rather than a death end point permitted the observation that the effect of STDC is reversible. As noted in Table II, fish exposed to STDC recover within 24 hr. in fresh water and then show the expected response to pentobarbital. Of particular interest is the fact that fish preexposed to 1×10^{-4} *M* STDC for 24 hr. gave the same enhanced response to pentobarbital as fish placed directly in an STDC-pentobarbital solution This suggests that the effect of STDC is mediated very quickly and is maximal within minutes. The persistence of potentiation even in the absence of STDC in the drug solution indicates that the effect of the bile salt is slowly reversible.

Studies with 4-aminoantipyrine suggest that extremely low concentrations of STDC can significantly alter membrane permeability. Levy (12) has previously shown that, under conditions similar to those used in the present study, 4-aminoantipyrine is absorbed by apparent zero-order kinetics. Accordingly the authors have determined an absorption rate of 21 mcg./g./min. in the absence of STDC and a value of 27 mcg./g./min. in the presence of the bile salt. The corresponding first-order absorption rate constants (k_a) were calculated from the relationship: k_a = absorption rate/drug concentration and

found to be 8.4 × 10^{-3} min.$^{-1}$ and 1.1 × 10^{-2} min.$^{-1}$, respectively.

The influence of bile salt on membrane permeability in the goldfish raises the interesting possibility of similar effects on the permeability of the intestinal membranes. The fluid bathing these membranes has significantly higher concentrations of bile salts (in the order of 10^{-2} M) than the concentrations employed in the present study. This possibility is now under investigation.

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Keyphrases

Bile salts—drug uptake, activity potentiation
Taurodeoxycholate, sodium—potentiating effect
Goldfish overturn time—activity analysis
4-Aminoantipyrine uptake—goldfish
Colorimetric analysis—uptake time

Synthesis of Tropine-Labeled Atropine IV

Labeling of the 2, 3, and 4 Positions of Atropine from Citric-^{14}C Acid

By THOMAS E. ELING, JOHN M. McOWEN, and GILBERT C. SCHMIDT*

Using a modification of the Robinson condensation, citric-3-^{14}C acid, citric-2,4-^{14}C acid, and citric-2,3,4-^{14}C acid were converted into correspondingly labeled tropine and atropine. The specific activities of the products varied from 0.62 to 1.0 depending on the labeled compound. This is the first reported syntheses of tropine or atropine labeled with ^{14}C in positions other than the *endo*-methyl group.

THE PROBLEM of selectively labeling the tropine moiety of atropine remains to be solved. For one reason or another, the biosynthetic compounds (1, 2), tritium-labeled atropine (3), and randomly labeled atropine (4) are inadequate for establishing the metabolic fate of the heterocyclic residue in atropine. Classical, microsynthetic methods for selectively labeling tropine and the tropine moiety of atropine are needed badly.

Excluding the authors' work, only Fodor (5) and Werner (6) have used classical methods to label tropine. In both instances, tropine-*N*-methyl-^{14}C and atropine-*N*-methyl-^{14}C were synthesized. Fodor's approach would not permit labeling of other positions, but Werner's use of the Robinson condensation is much more versatile. A lack of suitably labeled Robinson intermediates, other than methylamine hydrochloride, apparently prevented Werner from realizing the full potential of the condensation.

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Abstracted, in part, from a thesis submitted by Thomas E. Eling to the Graduate School of the University of Alabama, Department of Biological Chemistry, in partial fulfillment of Doctor of Philosophy degree requirements.
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* Present address: Department of Chemistry, Northeast Louisiana State University, Monroe, La., to whom requests for reprints should be sent.

found to be 8.4 × 10^{-3} min.$^{-1}$ and 1.1 × 10^{-2} min.$^{-1}$, respectively.

The influence of bile salt on membrane permeability in the goldfish raises the interesting possibility of similar effects on the permeability of the intestinal membranes. The fluid bathing these membranes has significantly higher concentrations of bile salts (in the order of 10^{-2} M) than the concentrations employed in the present study. This possibility is now under investigation.

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Keyphrases

Bile salts—drug uptake, activity potentiation
Taurodeoxycholate, sodium—potentiating effect
Goldfish overturn time—activity analysis
4-Aminoantipyrine uptake—goldfish
Colorimetric analysis—uptake time

Synthesis of Tropine-Labeled Atropine IV

Labeling of the 2, 3, and 4 Positions of Atropine from Citric-^{14}C Acid

By THOMAS E. ELING, JOHN M. McOWEN, and GILBERT C. SCHMIDT*

Using a modification of the Robinson condensation, citric-3-^{14}C acid, citric-2,4-^{14}C acid, and citric-2,3,4-^{14}C acid were converted into correspondingly labeled tropine and atropine. The specific activities of the products varied from 0.62 to 1.0 depending on the labeled compound. This is the first reported syntheses of tropine or atropine labeled with ^{14}C in positions other than the *endo*-methyl group.

THE PROBLEM of selectively labeling the tropine moiety of atropine remains to be solved. For one reason or another, the biosynthetic compounds (1, 2), tritium-labeled atropine (3), and randomly labeled atropine (4) are inadequate for establishing the metabolic fate of the heterocyclic residue in atropine. Classical, microsynthetic methods for selectively labeling tropine and the tropine moiety of atropine are needed badly.

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This report describes the synthesis of atropine-2,3,4-^{14}C, atropine-2,4-^{14}C, and atropine-3-^{14}C from the correspondingly labeled citric acids. These syntheses are the first successful introductions of ^{14}C into other than the *endo*-methyl group of either tropine or the heterocyclic moiety of atropine.

EXPERIMENTAL

Microsynthetic methods for tropine and atropine, described in detail in the first three papers of the series (7–9), were modified only slightly for the synthesis of tropine-^{14}C and atropine-^{14}C from citric-^{14}C acids. In previously reported work, acetone dicarboxylic acid was not a labeled intermediate. The use of this compound as a labeled precursor to atropine required slight modification of the previously described method for handling this intermediate in the Robinson condensation.

The same procedure was used for the synthesis of atropine-2,3,4-^{14}C, atropine-2,4-^{14}C, and atropine-3-^{14}C. To simplify the description of methods, numbers indicating the position of labeling are eliminated. Earlier papers (7–9) should be consulted for additional experimental details.

Synthesis of Acetone-^{14}C Dicarboxylic Acids—Dry citric-^{14}C acid[1] (96 mg. or 0.5 mmole) and unlabeled citric acid (96 mg. or 0.5 mmole) were added while stirring constantly with a thermometer to 0.44 ml. of fuming H_2SO_4[2] (20% excess SO_3), previously cooled to −8° in an ice-salt bath. The labeled citric acid was added at a rate to maintain the temperature at −5° or lower.

After the addition was completed, the flask and content were removed from the cooling bath and stirred on a magnetic stirrer at room temperature until gas evolution ceased. The reaction mixture was again cooled to −8° and 1 g. of chipped ice added in small portions so that temperature did not exceed 0°. The resulting pale yellow solution contained acetone dicarboxylic acid-^{14}C.

Preparation of Succindialdehyde—Succindialdehyde (173 mg. or 2 mmoles) was prepared from 0.34 ml. of 2,5-diethoxytetrahydrofuran as described previously (7).

Synthesis of Tropanone-^{14}C—To the solution of succindialdehyde (2 mmoles) was added the solution of acetone dicarboxylic-^{14}C acid. The round-bottom flask which contained the acetone dicarboxylic acid was washed three times with water and the washings added to the reaction mixture. Saturated aqueous Na_2HPO_4 was added to adjust the pH to 4.5–5.0 using a glass electrode. The buffered solution was transferred to a 250-ml. side-arm conical flask and the system purged with nitrogen gas for 10 min. To the buffered reaction mixture, 135 mg. (2 mmoles) of methylamine HCl was added, the reaction volume adjusted to 50 ml., and the system quickly evacuated to remove the gas phase. The reaction vessel was closed and the mixture allowed to remain under partial vacuum for 12 hr. The slight evacuation was repeated and the reaction mixture allowed to remain at room temperature for an additional 12 hr.

Tropanone-^{14}C was extracted with 100-ml. portions of petroleum ether, exactly as previously described (7), except that extraction was continued until significant amounts of radioactivity no longer were removed. The petroleum ether extracts were dried over Na_2SO_4, transferred to a 250-ml. sublimation flask and the solvent removed, *in vacuo*, on the flash evaporator at 40°. Tropanone-^{14}C, which remained in the flask as a brown oil, was dried *in situ* overnight in a desiccator over $CaCl_2$ and NaOH. The crude product was purified by repeated sublimation at 40° and 1×10^{-4} mm. Hg. After each sublimation, the product was washed from the cold finger with small amounts of absolute ethanol. Sublimation was repeated until the washings no longer contained significant amounts of radioactivity. The combined alcoholic solutions of tropanone-^{14}C were evaporated to dryness on the flash evaporator in a previously tared 25-ml. flask. The product crystallized quickly on cooling, was dried in a desiccator for 12 hr., and was reduced to tropine.

Synthesis of Tropine-^{14}C HCl—Labeled tropanone (60–95 mg., 1 mc./mmole), prepared as described above and dried for 12 hr., was reduced over W-7 Raney nickel, by the modified Van de Kamp procedure described in the third paper of this series (8). The product was dried in a desiccator over NaOH pellets and converted to the hydrochloride in the exact manner described previously (7). Tropine-^{14}C HCl was stored in 10-ml., tared, round-bottom flasks in a vacuum desiccator over NaOH pellets, until used for esterification.

Synthesis of Atropine-^{14}C—Acetyltropic acid (150 mg.) was prepared from 395 mg. of tropic acid and 0.36 ml. of acetyl chloride in the manner described previously (7). The product was dried in a vacuum desiccator over $CaCl_2$ for 12 hr. and converted to *o*-acetyltropic acid chloride (7). To the acid chloride was added well-dried tropine-^{14}C HCl.[3] Using previously described procedures, the reactants then were esterified to form *o*-acetylatropine-^{14}C, the acetylated compound hydrolyzed to the free base, and atropine-^{14}C finally isolated. The crude product was dried in a vacuum desiccator over $CaCl_2$, and finally recrystallized from petroleum ether (7).

RESULTS AND DISCUSSION

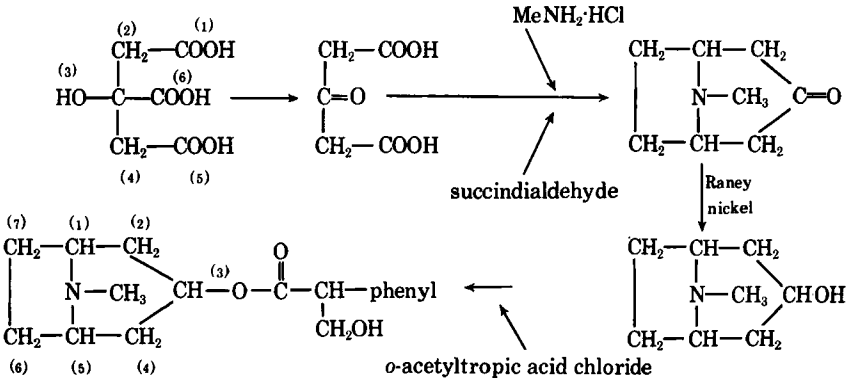
The synthesis of atropine labeled with ^{14}C in the carbon skeleton of tropine has not been accomplished previously, but recent work has simplified the problem. For the synthesis of tropine, the Robinson condensation has been adapted for radioactive synthesis (6, 8, 9). The Wolffenstein and Mamlock esterification of tropine and tropic acid has been adapted for the use of isotopically labeled intermediates (5, 6, 8, 9). Thus the problem of labeling the tropine moiety of atropine, in positions other than the *endo*-methyl group, has become that of obtaining labeled precursors of succindialdehyde or acetone dicarboxylic acid.

Citric acid is a logical precursor of acetone di-

[1] Citric-2,3,4-^{14}C; 2,4-^{14}C; and 3-^{14}C acids, each having a specific activity of 2 mc./mmole, were prepared by Mallinckrodt Nuclear, Orlando, Fla. Citric-^{14}C acids were stored in a desiccator before use.

[2] A 5-ml. round-bottom flask was used routinely.

[3] The yields of tropine-2,3,4-^{14}C and tropine-2,3,4-^{14}C HCl obtained by reduction of tropanone-2,3,4-^{14}C were lower than anticipated. To ensure that sufficient material was present for esterification to atropine-2,3,4-^{14}C, unlabeled tropine HCl was added to increase the combined total weight of labeled and unlabeled tropine HCl to 124 mg. (See *Results and Discussion* for a further explanation.)



Scheme I

carboxylic acid because high yields of the desired product can be obtained by the method of Adams *et al.* (10). Until recently, suitably labeled citric acids were not available. Citric acid must be labeled in positions other than the carboxyl groups because these are lost in conversion to acetone dicarboxylic acid and subsequent condensation to tropanone. For labeling the tropine ring, citric acid must be labeled in one or more of positions 2, 3, and 4. Of the potentially useful citric acids, citric-3-^{14}C, citric-2,4-^{14}C, and citric-2,3,4-^{14}C acid are now available commercially.

Using the commercially available citric-^{14}C acids, the correspondingly labeled atropines were prepared as illustrated in Scheme I.

Using this sequence, tropanone-3-^{14}C, tropine-3-^{14}C, and atropine-3-^{14}C were obtained from citric-3-^{14}C acid. Tropanone-2,4-^{14}C, tropine-2,4-^{14}C, and atropine-2,4-^{14}C were synthesized from citric-2,4-^{14}C acid. From citric-2,3,4-^{14}C acid, tropanone-2,3,4-^{14}C, tropine-2,3,4-^{14}C, and atropine-2,3,4-^{14}C were obtained.

As shown in Table I, the yields of the labeled atropines varied significantly from those predicted on the basis of the authors' previous studies. Previous studies (7, 8) had indicated a 65–70% yield for the formation of tropanone. The deviation from expected yields occurred in this step. In most instances 40–50% yields of labeled tropanone were obtained from labeled citric acids, although an occasional 70% yield was obtained. The critical nature of the citric acid to acetone dicarboxylic acid conversion was not apparent in earlier studies because a known weight of purified keto acid was used in the Robinson condensation. In synthesis, preparation of excess acetone dicarboxylic acid was not practical. Since the quantity of the radioactive acetone dicarboxylic acid from 1 mmole of labeled citric acid was extremely small, no attempt was made to isolate the intermediate, and the entire reaction mixture was used in the Robinson condensation. As a consequence, there are two possible explanations for the observed deviations from expected yields.

Improper labeling or impurity of the labeled citric acids was considered. Using paper chromatography, in a variety of solvent systems, the labeled citric acids were analyzed for impurities. In every instance, an impurity-free starting material was indicated, thus eliminating this potential explanation. That variability of tropanone yields is inherent in the procedure and is not associated with the labeled citric acids is indicated by the following evidence. When unlabeled citric acid was used exactly as described for the labeled compound, tropanone yields were variable and comparable to those from citric-^{14}C acid. Furthermore, yields of labeled tropanones were identical whether calculations were based on weight or on radioactivity. This would not have been the case had there been improper labeling or an isotope effect. It was concluded that the use of microquantities of citric acid made much more critical the quantity of fuming H_2SO_4 used to convert citric acid to acetone dicarboxylic acid. The authors believe this to be the cause of variability and of deviation from previously predicted tropanone yields.

There were no difficulties in the reduction of labeled tropanones to tropine or in the esterification to labeled atropine. The decreased yield of tropine-2,3,4-^{14}C, obtained by reduction of tropanone-2,3,4-^{14}C (see Table I), was due to a known, mechan-

TABLE I—LABELED COMPOUNDS SYNTHESIZED FROM COMMERCIALLY AVAILABLE LABELED CITRIC-^{14}C ACIDS

Labeled Compd.	Predicted Yield, %	3-Labeled Obs. Yield, %	3-Labeled S.A. mc./mmole	2,4-Labeled Obs. Yield, %	2,4-Labeled S.A. mc./mmole	2,3,4-Labeled Obs. Yield, %	2,3,4-Labeled S.A. mc./mmole
Acetone dicarboxylic acid	95[a]	—	—	—	—	—	—
Tropanone	65–70	44	1.0	68	1.0	54	1.0
Tropine	64–69	43	1.0	65	1.0	41	1.0
Atropine	43–49	26	1.0	34	1.0	25[b]	0.62

[a] Predicted yields of ACDA based on isolation, using semimicro amounts of citric acid, unlabeled (7). The intermediate was not isolated when the ACDA was labeled. [b] Yield is based on isotope rather than on weight.

ical loss rather than to the procedure. Since this was the first compound made, unlabeled tropine HCl, sufficient to increase the total weight to the 124-mg. weight normally obtained, was added to the tropine-2,3,4-^{14}C HCl. For this reason the specific activity of the product was correspondingly decreased to 0.62 mc./mmole. In subsequent experiments, the labeled compounds were not diluted.

The radiochemical purity of the resulting labeled atropines was established by paper and thin-layer chromatography as described previously (8). Each of the labeled atropines contained an impurity that chromatographed in all systems as tropine. The impurity, varying from 2 to 6% of total radioactivity (depending on labeled product), could be removed by a second recrystallization from petroleum ether. The yields reported in Table I have been corrected for the impurity and accurately reflect the yields of the products shown.

SUMMARY AND CONCLUSIONS

Using the microsynthetic procedures described previously (7, 9), three different tropine-labeled atropines were prepared. From citric-3-^{14}C acid, tropanone-3-^{14}C (44%), and atropine-3-^{14}C (26%) were synthesized with a specific activity of 1 mc./mmole. From citric-2,4-^{14}C acid was prepared tropanone (68%), tropine (65%), and atropine (34%) having specific activity of 1 mc./mmole and correspondingly labeled. From citric-2,3,4-^{14}C acid was prepared correspondingly tropanone (54%), tropine (53%), and atropine (25%).

The labeled atropine contains a tropine impurity that varied from 2% to 6% of the total radioactivity. This impurity could be removed by recrystallization from petroleum ether.

This report described the first synthesis, by other than biosynthetic method, of tropine and atropine selectively labeled with ^{14}C in the tropine ring.

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Keyphrases

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Atropine—citric-^{14}C acid labeling of 2, 3, 4 positions
Paper chromatography—analysis, radiochemical purity
TLC—analysis, radiochemical purity

Correlation Between *In Vitro* and *In Vivo* Disintegration Times of Enteric-Coated Tablets

By SØREN RASMUSSEN

Investigations were made with enteric-coated tablets on the correlation between the first appearance of a substance in the plasma or the urine and the thickness of the vernix layer, measured by the *in vitro* disintegration time. The content of the tablets is quinine hydrochloride, sodium sulfanilate, or sodium *p*-aminosalicylate. The content of each tablet was either 50 or 500 mg. It was shown that within preparations of the same content a proportionality exists between the *in vivo* and the *in vitro* disintegration times. Between series of preparations with different content the factor of proportionality may differ significantly.

NUMEROUS PAPERS have been published on the correlation between *in vitro* tests and the therapeutic effectiveness of pharmaceutical preparations. The principal aim of such investigations is to establish a predictability of biological properties from simple *in vitro* tests.

With oral preparations of different types, much important work has been done by Levy and his collaborators (1). As to the dissolution rate, it was possible in some cases to show a correlation between the results of *in vitro* and *in vivo* determinations. In such cases it should be possible to predict from *in vitro* tests

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variations in the *in vivo* dissolution rate from batch to batch.

However, with enteric-coated tablets a further problem arises. The time from the intake until the drug appears in the plasma, which is an indication of the beginning of the absorption, may vary independently of the dissolution rate and must be determined separately. Many *in vitro* tests for this disintegration time exist, but the correlation between the *in vitro* and the *in vivo* disintegration time is little investigated.

In some cases a certain connection is found, as appears from Morrison and Campbell (2). Most of the investigation has been done with commercial preparations, and information about the nature and the amount of vernix is lacking. More precise information is given by Wagner *et al.* (3–5) in works with $BaSO_4$-containing tablets coated with increasing amounts of vernix. They followed the *in vivo* disintegration roentgenographically and were able to show a correlation between the *in vivo* and the *in vitro* disintegration times. In a previous paper Rasmussen (6) showed an increase in the *in vivo* disintegration time of enteric-coated tablets of quinine hydrochloride of 50 or 500 mg. proportional to the thickness of the coating layer.

In a subsequent paper Rasmussen (7) demonstrated a rectilinear proportionality between the thickness of the vernix layer and the disintegration time *in vitro*. Measured on tablets of quinine hydrochloride, sodium sulfanilate, or sodium *p*-aminosalicylate of 50 or 500 mg. each, the same factor of proportionality was found, independent of size and content. It was still an open question if a similar constant factor of proportionality could be found between the thickness of the vernix layer and the disintegration time *in vivo*.

This paper reports investigations on the *in vivo* disintegration of tablets of quinine hydrochloride, sodium sulfanilate, or sodium *p*-aminosalicylate of various sizes and compositions and coated with various amounts of vernix.

As will be seen, it was not possible to show a proportionality factor which is independent of the content of the tablets.

EXPERIMENTAL

Preparations

The coated preparations are manufactured from tablets of the following compositions.

Tablets of Quinine Hydrochloride—Preparation No. 6–15: the composition appears from an earlier paper (6). The tablets contain either 50 or 500 mg. of quinine hydrochloride. Preparation No. 46–50: for each tablet take 50 mg. of quinine hydrochloride, 45 mg. of granulatum simplex (8), and 5 mg. magnesium stearate. Granulate by compression, mix, and compress. Spherical punches, 6 mm. diameter.

Tablets of Sodium Sulfanilate—For each tablet take 50 mg. of sodium sulfanilate and 40 mg. of starch. Mix and prepare by moist granulation. To the dried granulate add 10 mg. of talcum. Mix and compress. Spherical punches, 6 mm. diameter.

Tablets of Sodium *p*-Aminosalicylate—For each tablet take 50 mg. of sodium *p*-aminosalicylate, 45 mg. of granulatum simplex (8), 4 mg. of talcum, and 1 mg. of magnesium stearate. Mix and prepare by compression. Spherical punches, 6 mm.

The coating is made with vernix enterosolubilis (9): 10 mg. of cellulose acetate phthalate, 500 mg. of castor oil, and 89.5 g. of acetone. The vernix was applied to the tablets in an open pan. From time to time during the coating, numbers of 500–1,000 tablets were removed from the pan and dried, whereas the rest was coated further. This procedure ensures that subbatches removed during the coating only differ in the amount of vernix applied.

In Vitro Experiments—The alkaline disintegration time was determined by the following modification of the method used by the Nordic Pharmacopoea (10). Place three tablets in a conical flask in a solution of 0.285 g. of sodium phosphate, and 0.055 g. of potassium phosphate monobasic to 30 ml. of water maintained at 40°. Shake the flask gently during the whole of the disintegration. The point of disintegration is defined as the moment when the coating breaks up.

The acidic pretreatment is made as follows: Place the tablets in a mixture of 3 ml. phosphoric acid 1 *M* and 27 ml. water. Shake every 5 min. for 2 hr., pour off, and wash with 5 ml. water.

In Vivo Experiments—Eighteen healthy volunteers served as experimental subjects. The tests were started between 8:30 and 9:00 a.m., 1–1.5 hr. after a light breakfast. The test dose of 0.5 g. was ingested along with 150–200 ml. of water. Blood from the earlobe or urine was collected immediately before and every hour after the ingestion. The difference in time between the ingestion and the first appearance of the substance in the plasma or the urine was defined as the *in vivo* disintegration time. As the samples were collected every hour, the disintegration time was measured in whole hours.

Analytical

Quinine was determined in 0.1 ml. heparinized plasma by the method of Brodie and Udenfriend (11). Sodium *p*-aminosalicylate was determined in the plasma by the following method: 0.10 ml. heparinized plasma was mixed with 1 ml. water. After 15 min. the proteins were precipitated by adding 0.25 ml. 10% trichloroacetic acid. After 20 min. the tubes were centrifuged at $1100 \times g$ for 15 min.; 1 ml. of the clear supernatant was transferred to a tube containing 1.20 ml. of the following solution: 145 ml. of sodium hydroxide 1 *N*; 8.58 g. of sodium borate; to 1000 ml. of water, which gives pH = 11.

The fluorescence was read on an Aminco-Bowman

TABLE I—ALKALINE *In Vitro* DISINTEGRATION TIMES OF ENTERIC-COATED TABLETS WITH AND WITHOUT PRETREATMENT IN ACID FOR 2 HR. AND *In Vitro* DISSOLUTION RATES OF SOME OF THE PREPARATIONS

Content	Preparations No.	Disintegration Time, min. With Pretreatment $\bar{x} + SEM$	(n)	Disintegration Time, min. Without Pretreatment $\bar{x} \pm SEM$	(n)	% Disintegration During the 2 Hr. in the Acid	*In Vitro* Dissolution Half-lives, min.
		Uncoated Tablets					
Quinine hydrochloride	5	7.1 ± 1.3	(26)	1.4 ± 0.3	(30)[a]	13	26.4
	47	2.7 ± 0.4	(55)	1.9 ± 0.4	(30)[a]	8	
	12	**100% Disintegration in the Acid**					
	48	2.66 ± 0.06	(60)	3.25 ± 0.09	(30)[a]	0	
	7	4.5 ± 0.2	(20)	3.97 ± 0.09	(30)	0	31.3
	13	4.7 ± 0.6	(21)	4.6 ± 0.2	(30)	30	
	49	6.36 ± 0.12	(60)	6.20 ± 0.14	(30)	0	51.0
	15	7.41 ± 0.19	(30)	8.1 ± 0.2	(20)	0	53.8
	50	13.2 ± 0.2	(60)	12.4 ± 0.2	(30)	0	56.8
	14	20.9 ± 1.1	(30)	22.5 ± 0.9	(30)	0	
Sodium sulfanilate	22	**Uncoated Tablets**					1.2
	23	2.10 ± 0.14	(20)	2.03 ± 0.07	(30)	0	1.7
	24	2.9 ± 0.2	(20)	3.75 ± 0.12	(30)	0	1.6
	25	6.2 ± 0.3	(20)	6.53 ± 0.13	(30)	0	2.8
Sodium *p*-aminosalicylate	41	**100% Disintegration in the Acid**					7.8
	42	6.26 ± 0.06	(27)	7.8 ± 0.2	(30)[a]	10	14.6
	43	12.5 ± 0.5	(30)	13.5 ± 0.4	(30)	0	11.9

[a] Denotes $p < 0.01$.

spectrofluorometer at 400 mμ, activated at 310 mμ (both uncorrected). Standards and blank were run through the procedure. No differences were found between standards made in plasma and water, and generally aqueous solutions were used. Concentrations down to 0.1 mcg./ml. are detectable with a recovery of 99–100%. The specificity of the method with respect to metabolites has not been investigated, but the appearance of a fluorescence in the plasma is taken as indication of an absorption of sodium *p*-aminosalicylate. Sodium sulfanilate was determined in the urine by the method of Morris (12).

RESULTS

Table I presents the alkaline *in vitro* disintegration times of the coated preparations with and without acid pretreatment.

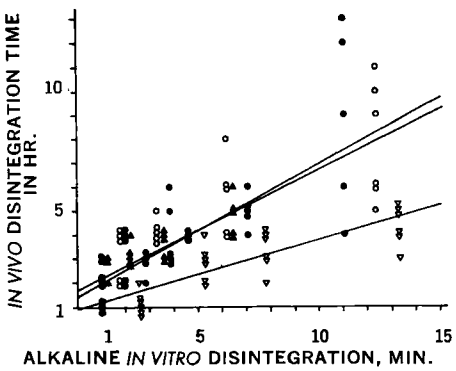

Fig. 1—In vivo *disintegration times of enteric-coated tablets* versus *alkaline* in vitro *disintegration times. Key:* ○●, *quinine HCl preparations;* ▽, *Na-p-aminosalicylate preparations;* ▲, *Na-sulfanilate preparations. The lines are drawn from the equations of regression.*

The *in vivo* disintegration times from all experiments are plotted in Fig. 1 against the means of the *in vitro* disintegration times from Table I. No difference was found between the results with the 50-mg. and the 500-mg. tablets of the preparations No. 6–15, and these preparations are treated as one group.

Regression lines were calculated from the determinations as pairs of numbers. Three instances in which no *in vivo* disintegration was detected are omitted. Tests with uncoated tablets, where the disintegration times are: *in vitro* 0 min., *in vivo* the first hour, are included in the calculations.

The equations of regression are found as follows:

$$Y_Q^{(1)} = 0.60X + 1.14 \quad r = 0.82 \quad n = 40$$
$$Y_Q^{(2)} = 0.50X + 1.96 \quad r = 0.84 \quad n = 26$$
$$Y_Q = 0.56X + 1.47 \quad r = 0.82 \quad n = 66$$
$$b/s_b = 11.6$$
$$Y_{PAS} = 0.26X + 0.98 \quad r = 0.88 \quad n = 22$$
$$b/s_b = 3.18$$
$$Y_{SUL} = 0.50X + 1.70 \quad r = 0.87 \quad n = 30$$
$$b/s_b = 11.6$$

Y is the *in vivo* disintegration in hours of tablets of quinine hydrochloride (Q), sodium *p*-aminosalicylate (PAS), and sodium sulfanilate (SUL), respectively, and X is the *in vitro* disintegration in min.

$Y_Q^{(1)}$ is calculated from tests with the preparations No. 6–15, manufactured differently from the preparations No. 46–50, from which $Y_Q^{(2)}$ is calculated. The two regression coefficients do not differ significantly from each other ($t = 1.00$, $p > 0.3$, $df = 62$), and neither do the positions of the lines ($t = 0.923$, $p > 0.3$, $df = 62$). This permits the calculation of an equation of regression Y_Q, based on all tests with the quinine preparations. The ratios b/s_b show that all regression coefficients differ significantly from zero ($p < 0.005$). The intercepts of the regression lines with the ordinate, do not differ significantly from the expected point (0.1), *i.e.*, the disintegration times of uncoated

tablets in tests with sodium *p*-aminosalicylate and quinine HCl: $t_Q = 1.6$, $p > 0.1$, $df = 64$ and $t_{PAS} = 0.71$, $p > 0.4$, $df = 28$, whereas the difference is significant with sodium sulfanilate: $t_{SUL} = 2.92$, $p < 0.01$, $df = 20$. The comparison between the regression coefficients shows: b_Q/b_{PAS}, $t = 5.25$, $p < 0.001$, $df = 28$; b_{SUL}/b_{PAS}, $t = 3.41$, $p < 0.005$, $df = 20$; $b_Q/_{SUL}$, $t = 0.61$, $p > 0.5$, $df = 84$.

DISCUSSION

The results are in agreement with those of Wagner *et al.* (3–5), who found correlation between the *in vitro* and the *in vivo* disintegration times, but a further important point has to be added. The statistical analysis of the results presented here shows that the lines fitted to tests with quinine HCl and sodium sulfanilate differ significantly from the line fitted to the tests with sodium *p*-aminosalicylate. This means that the factor of proportionality by which the *in vivo* disintegration time increases with increasing thickness of the coating layer may differ significantly with different contents of the tablets. It is thus found that tablets showing an *in vitro* disintegration time of 12 min. take about 8 hr. to disintegrate *in vivo* when the content is quinine or sulfanilate, and about 4 hr. when the content is *p*-aminosalicylate.

The *in vivo* disintegration time, *i.e.*, the time until analytically sufficient concentrations are built up in the plasma or the urine, may be influenced by the rate of dissolution from the preparations in the way that a decreased dissolution rate may increase the time between the *in vivo* break-up of the coating layer and the moment when detectable concentrations are reached, and in this way apparently increase the *in vivo* disintegration time.

If this explanation were valid, one should expect that sodium *p*-aminosalicylate were released at a higher rate than are quinine hydrochloride and sodium sulfanilate from the coated preparations in order to explain the differences found.

However, *in vitro* dissolution half-lives of some of the coated preparations, listed in Table I, show that sodium sulfanilate is released at a much higher rate than sodium *p*-aminosalicylate or quinine hydrochloride, although the former is released faster than the latter.

Another way in which the *in vivo* disintegration time may be altered is by some influence of the acid in the stomach. The influence is tested by determinations of the alkaline *in vitro* disintegration time with and without acid pretreatment. Table I shows that significant alterations are only found with preparations with such a thin vernix layer that some of the tablets disintegrate during the 2 hr. in the acid.

The delay in the *in vivo* disintegration time of the coated preparations could further be explained as a retention in the stomach. Nelson (13) calculated an average stomach retention time of 3 hr., from the results of Blythe *et al.* (14) who worked with coated $BaSO_4$ tablets. If so, the regression lines would intercept the ordinate at a point near to 3 hr., *i.e.*, at a point corresponding to the retention time. This is not the case with any of the preparations in this work. Only the line calculated from tests with sodium sulfanilate tablets has an intercept significantly different from the expected point: (0,1). This, on the other hand, may be explained by the fact that the disintegration times here are determined by the appearance of sulfanilate in the urine, where the limit of detection is not very low because of the variations in the blank readings.

CONCLUSION

Batches of enteric-coated tablets of the same substance show an increase in the *in vivo* disintegration time proportional to the increase in the *in vitro* disintegration time, independently of the size and pharmaceutical composition of the tablets.

Between preparations of different substances the factor of proportionality may differ significantly.

As a consequence, the predictability of the *in vivo* fate of the drug requires determination of the correlation between *in vivo* and *in vitro* dissolution rates, as well as the factor by which the *in vivo* disintegration time changes with the *in vitro* disintegration time. This factor of proportionality has to be evaluated for each substance.

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Keyphrases

Disintegration time—enteric-coated tablets
In vivo, *in vitro* disintegration time—correlation
Tablet formulation effect—disintegration time
Enteric coating thickness—disintegration time
Fluorometry—analysis, plasma

Inhibitors of Monoamine Oxidase II

Syntheses of Some *N*-2(9)-Substituted Tetrahydro-β-carbolines and Evaluation of Their Inhibitory Activities

By BENG T. HO, WILLIAM M. MCISAAC, and K. E. WALKER

Tetrahydro-β-carbolines with alkyl or aralkyl substitution on N-9, alkyl on N-2, and alkyl on both N-2 and N-9 were synthesized and their monoamine oxidase inhibitory activities evaluated. Two different binding conformations were proposed for the 9-methyl and the 9-hydrogen series. The binding of the 9-methyl group hydrophobically to the enzyme would account for the high inhibitory activities of 9-methyl compounds. In the case of 9-hydrogen-2-alkyl series, since there is no methyl group on the indolic nitrogen, hydrophobic bonding would not be possible. Relief of steric hindrance due to the 2-alkyl would probably cause the molecule to assume another binding conformation. Of all the compounds tested, 2,9-dimethyltetrahydro-β-carboline was the most active inhibitor. Its activity was about one-third that of the previously reported 9-methyltetrahydro-β-carboline.

In a previous paper (1) on inhibitors of monoamine oxidase, the influence of methyl substitution on the inhibitory activity of β-carbolines was studied. 9-Methyltetrahydro-β-carboline was found to be a competitive inhibitor of the enzyme, being three times more active than iproniazid, a one-time clinically used antidepressant. Initial findings also indicated that the N-2 nitrogen of the tetrahydro-β-carboline could be substituted by alkyl groups as large as propyl without affecting the inhibitory activity.

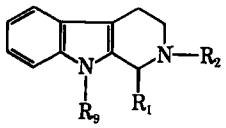
Since a 35-fold increase in inhibitory activity was obtained by the substitution of a methyl group on the N-9 nitrogen of tetrahydro-β-carboline, the synthesis and evaluation of inhibitory effect of some N-9 substituted tetrahydro-β-carbolines has been undertaken, and the studies extended to include some 2,9-disubstituted compounds.

DISCUSSION

A 35-fold increase in inhibitory activity (Table I) was observed when a methyl group was introduced on the N-9 nitrogen of tetrahydro-β-carboline (I). This was attributed to either the increase in the electron density of the indole nucleus of 9-methyltetrahydro-β-carboline (II) thus giving better binding, or the binding of the 9-CH_3 group itself hydrophobically to the enzyme, or both (1). The possibility of the existence of a hydrophobic region on the enzyme in proximity to the 9-CH_3 of II was then explored. Extension of the 9-CH_3 by one carbon resulted in a 50-fold decrease in inhibitory activity of 9-ethyl compound (III). This unexpected decrease in activity was at first thought to be caused by the projection of the terminal CH_3 of the ethyl group into a possible hydrophilic region of the enzyme. In order to investigate this possibility, a hydrophilic group such as hydroxyl was chosen to replace the terminal hydrophobic CH_3 of the ethyl group, anticipating that the binding of OH to the hydrophilic region of the enzyme would result in increased inhibition.

Synthetically, it seemed more appropriate to protect the N-2 nitrogen of the tetrahydro-β-carboline by substitution so that the addition of the hydroxymethyl to the N-9 nitrogen would be exclusive. Alkylation on the N-2 position was assumed not to interfere with studies on the N-9 position, since a previous finding (1) revealed no change in inhibitory activity when alkylating the N-2 nitrogen of 1-methyltetrahydro-β-carboline (XIII) with methyl or *n*-propyl (see XIV and XV). Since

TABLE I—INHIBITION OF MONOAMINE OXIDASE BY TETRAHYDRO-β-CARBOLINES



Compound	R_1	R_2	R_9	I_{50}[a] (m*M*)
I[b]	H	H	H	0.34[c]
II[b]	H	H	CH_3	0.01[c]
III[b]	H	H	C_2H_5	0.46
IV[b]	H	H	*n*-C_3H_7	0.34
V[b]	H	H	*i*-C_5H_{11}	0.50
VI[b]	H	H	$CH_2C_6H_5$	0.30
VII[b]	H	H	C_6H_5	0.32
VIII	H	C_2H_5	H	1.40
IX	H	*n*-C_3H_7	H	1.37
X[b]	H	C_2H_5	CH_3	0.13
XI[b]	H	C_2H_5	CH_2OH	0.74
XII[b]	H	CH_3	CH_3	0.035
XIII	CH_3	H	H	1.50[c]
XIV[b]	CH_3	CH_3	H	1.50[c]
XV[b]	CH_3	*n*-C_3H_7	H	1.30[c]

[a] Concentration of an inhibitor giving 50% inhibition of the enzyme. [b] Hydrochloride salt. [c] Data from *Reference 1*.

Received March 14, 1968, from the Biological Research Division, Texas Research Institute of Mental Sciences, and the Baylor University College of Medicine, Houston, TX 77025

Accepted for publication April 18, 1968.

This work was supported by grants MH-11168 and MH-12959, U. S. Public Health Service, Bethesda, Md., and by the Britton Fund.

The authors would like to thank Miss Patricia Kralik for her technical assistance in providing the enzyme assay data.

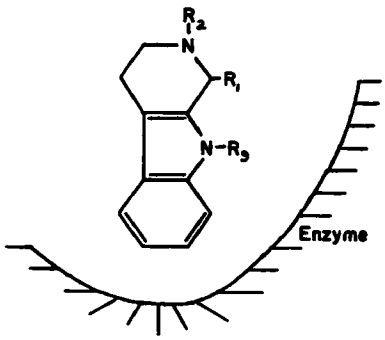
an N-2 ethyl substituent would be easily obtained by the reduction of the N-2 acetyl compound, 2 - ethyl - 9 - hydroxymethyltetrahydro - β - carboline (XI) was synthesized. For comparison with the 2-hydrogen series, 2-ethyl-9-methyltetrahydro-β-carboline (X) and 2-ethyltetrahydro-β-carboline (VIII) were also prepared.

The 2-ethyl compound (VIII) was first found to be a weak inhibitor, being four times less active than I. The corresponding 9-methyl compound (X) was 13-fold less active than II. Thus, it seemed that the steric hindrance by the 2-ethyl group could account for the decrease in inhibitory activity of 2-ethyl compounds when compared with the 2-hydrogen series. Of greater interest was to determine whether or not the enzyme could tolerate a smaller N-2 substituent like the methyl group. The 2,9-dimethyltetrahydro-β-carboline (XII) was indeed a very good inhibitor, being about one-third as active as II and about four times more active than X. This phenomenon was, however, not observed in the 9-hydrogen series. Here, the activity remained the same when the 2-alkyl chain was reduced from propyl to ethyl (IX → VIII). This, along with the previous finding that in the 1-methyl series XIV was equally as active as XV, suggested that it was not at all impossible to have two different modes of binding for substituted tetrahydro-β-carbolines on monoamine oxidase.

Judging from the similarity in inhibitory activities between XIV and XV, and VIII and IX, it was postulated that the 2-ethyl and 2-propyl groups of the above two series might have projected into a "noncontact" area away from the binding site. The conformation A (Fig. 1) proposed for the four compounds was different from conformation B (Fig. 2) proposed for all the 9-methyltetrahydro-β-carbolines. That a class of compounds can assume different conformations of binding to an enzyme has been presented (2). The assumption for the mode of binding of different series of substituted tetrahydro-β-carbolines is as follows.

(*a*) Since a compound always assumes a binding position giving a maximum inhibitory effect, the increase in inhibitory activity of all 9-methyl compounds was thought to be due to the hydrophobic bonding of their 9-methyl group to the enzyme (conformation B). In this conformation, any substituent on the N-2 position larger than hydrogen would cause a decrease in activity. This decrease was observed in inhibitory activity as the length of the 2-alkyl side chain was increased, therefore resulting in increased steric hindrance. This was true for XII and X.

(*b*) When there is no methyl group on the indolic nitrogen, hydrophobic bonding would not be possible, therefore relief of steric hindrance due to the 2-alkyl groups (see conformation B) became an important factor. The preferable binding conformation for both VIII and IX more likely was the conformation A, where the 2-alkyl groups could be projected to the noncontact area and away from the binding site. This could account for the unchanged activity from 2-ethyl to 2-propyl. A similar rationalization could be applied to the 1-methyl series. Apparently, in the conformation B the enzyme would not tolerate a 1-methyl group, and a "twist" to the conformation A not only relieved this steric hindrance, but also allowed substituents



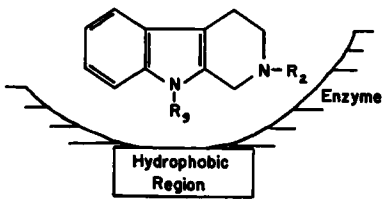
Conformation A

R_1 = H or CH_3

R_2 = CH_3, C_2H_5, or $n-C_3H_7$

R_9 = H

Fig. 1—Proposed conformation of 2-alkyl tetrahydro-β-carbolines.



Conformation B

R_2 = H

R_9 = CH_3

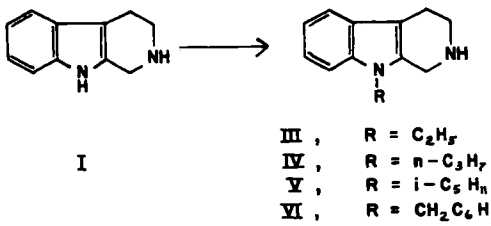
Fig. 2—Proposed conformation of 9-methyltetrahydro-β-carbolines.

on N-2 nitrogen to be projected to the noncontact area. Note that the inhibitory activities of the 1-methyl-2-alkyl series (XIII–XV) were the same as those of the 1-hydrogen-2-alkyl series (VIII and IX).

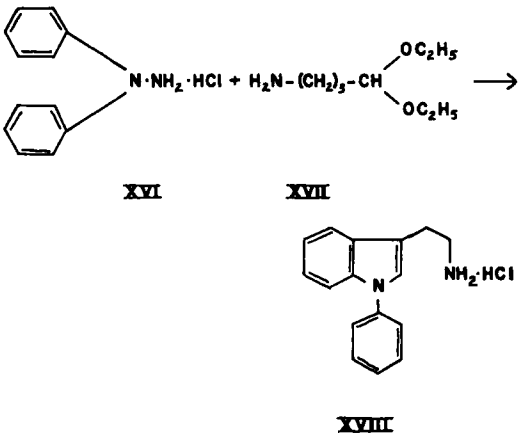
The data on the inhibitory activities of 9-alkyl and 9-aralkyl series indicated that the 9-ethyl compound (III) was 45-fold less active than the 9-methyl compound (II). Since further increase in the length of alkyl chain did not result in large decreases in inhibitory activities, it was assumed that for tetrahydro-β-carbolines any 9-alkyl or 9-aralkyl group larger than methyl would not bind in the conformation B. However, whether the binding conformation of 9-alkyl- or 9-aralkyl-tetrahydro-β-carbolines assumed the conformation A or whether there was a third conformation for the binding of these 9-substituted compounds remains to be determined. The observation that the 9-hydroxymethyl-2-ethyl compound (XI) was not as active as 9-methyl-2-ethyl compound (X) was then not to be unexpected, in view of the apparent steric requirements for conformation B. Apparently, the steric hindrance due to the terminal OH group caused XI to assume a binding conformation which was similar to that for tetrahydro-β-carbolines with N-9 substitutions larger than methyl.

CHEMISTRY

9-Alkyltetrahydro-β-carbolines were prepared by treating the sodium salt of the tetrahydro-β-



Scheme I



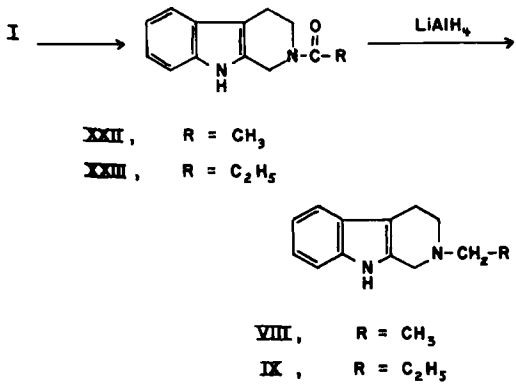
Scheme II

carboline (I) with the appropriate alkyl halides (Scheme I). Alkylation in liquid ammonia gave 9-ethyltetrahydro-β-carboline (III), 9-propyltetrahydro-β-carboline (IV), and 9-isoamyltetrahydro-β-carboline (V) in 56, 20, and 30% yields, respectively. It was found that the reaction of 1-iodo-3-methylbutane with I required longer time than with the lower alkyl halides. In a previous run, when the reaction was allowed to proceed for only 20 min. after the addition of the alkyl halide, as in the preparation of III and IV, a mixture of product and starting material was isolated.

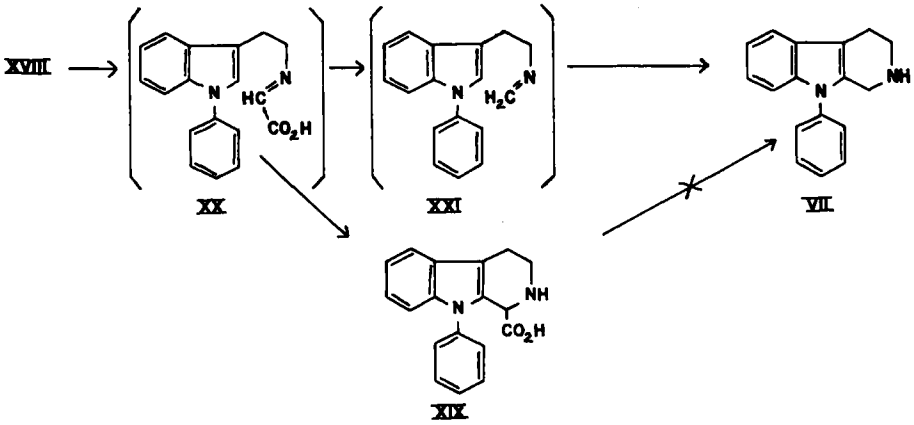
The preparation of 9-benzyltetrahydro-β-carboline (VI) was carried out by the reaction of I with benzyl chloride in dimethylformamide in the presence of sodium hydride.

Condensation of 1,1-diphenylhydrazine hydrochloride (XVI) and α-aminobutyraldehyde diethyl acetal (XVII) at 95° in the presence of hydrochloric acid according to the patent procedure (3) gave 1-phenyltryptamine hydrochloride (XVIII) (Scheme II).

For the preparation of some tetrahydro-β-carbolines the corresponding tryptamine derivatives have been previously treated with glyoxylic acid to form the 1-carboxytetrahydro-β-carbolines which were then decarboxylated yielding the desired products (1). When the same method was employed in the preparation of VII, the intermediate 1-carboxyl-9-phenyl-tetrahydro-β-carboline (XIX) failed to decarboxylate. A final solution to this was to carry out the reaction of XVIII and glyoxylic acid in refluxing aqueous solution instead of at room temperature. At the elevated temperature 1-phenyltryptamine most likely first condensed with glyoxylic acid to give the Schiff base, XX, which then decarboxylated and subsequently cyclized to 9-phenyltetrahydro-β-carboline (VII) (Scheme III). This mechanism has been proposed for the reaction of tryptamine with α-keto acids at elevated temperature (4). Since from the reaction mixture a product that had identical infrared spectrum with 1-carboxy-9-phenyl compound (XIX) formed at pH 4, it was assumed that the cyclization of XX to



Scheme IV

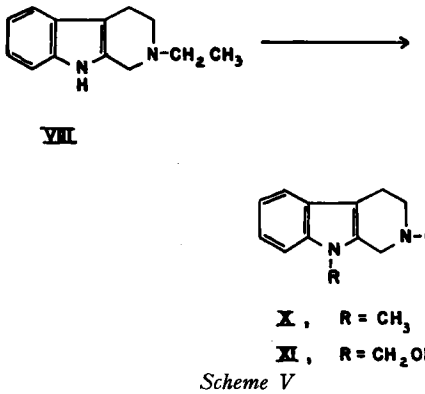


Scheme III

XIX competed with the decarboxylation of XX to give XXI and then VIII. Compound VII had been prepared *via* the condensation of 1-phenyltryptamine with formaldehyde (3).

Acylation of I without application of heat gave 2-acetyltetrahydro-β-carboline (XXII), and 2-propionyltetrahydro-β-carboline (XXIII) in 87.6 and 88.2%, respectively. These two 2-acyltetrahydro-β-carbolines were then reduced with lithium aluminum hydride to give a 66.3% yield of 2-ethyltetrahydro-β-carboline (VIII) and 71.4% of 2-propyltetrahydro-β-carboline (IX) (Scheme IV).

2-Ethyl-9-methyltetrahydro-β-carboline (X) was obtained in a 60% yield by the methylation of VIII with methyl iodide and sodium amide in liquid ammonia. Hydroxymethylation of VIII was performed in aqueous solution with formaldehyde at pH 5.5–6.0. Four days at room temperature gave 9-hydroxymethyl-2-ethyltetrahydro-β-carboline (XI) in 36% yield (Scheme V).



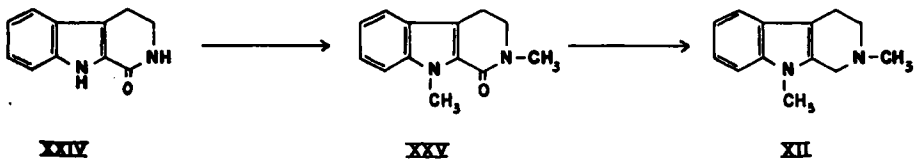
Scheme V

An incomplete methylation of 1-oxotetrahydro-β-carboline (XXIV) resulted when the reaction was carried out in liquid ammonia in the presence of sodium amide. Treatment of XXIV in boiling tetrahydrofuran with methyl iodide and sodium hydride, however, gave the desired product (XXV) in 72% yield. XXV was reduced by lithium aluminum hydride to 2,9-dimethyltetrahydro-β-carboline (XII) in 54% yield (Scheme VI).

sodium with 60–70 ml. of liquid ammonia in the presence of a catalytic amount of ferric nitrate. After all the amine had dissolved, 6.8 g. (0.04 mole) of 1-iodopropane was added slowly over a period of 30 min. Stirring was continued for an additional 20 min. The mixture was evaporated to dryness by a stream of helium while the flask was immersed in hot water. Water (15 ml.) was added to the residue and the product was extracted with toluene (2 × 200 ml.). After filtration of the combined toluene extracts with anhydrous sodium sulfate, the solvent was evaporated *in vacuo* leaving 6.3 g. of orange oil. The product was dissolved in 30 ml. of benzene, and after removal of suspended solid by filtration, the solvent was again evaporated *in vacuo* giving 6.0 g. (93.7%) of orange oil. The extraction process was repeated with 70 ml. of boiling heptane to yield a colorless oil, which was converted to 3.8 g. (50%) of yellow hydrochloride by the addition of ether–HCl to an ethereal solution of the oil. One recrystallization from ethanol gave 1.5 g. (20%), m.p. 231–232°; $\lambda_{max.}$ (KBr) 3.30, 3.88, 3.43, 3.50, 3.60, 3.70, 3.80, 3.85, 3.94, 4.10–4.15 (CH, NH_2^+); 6.20, 6.35 (C=C); 13.45 μ (indole CH); $\lambda_{max.}$ (EtOH) 204, 224, 277, 282, 292 (s) mμ.

Anal.—Calcd. for $C_{14}H_{19}ClN_2$: C, 67.0; H, 7.63; N, 11.2. Found: C, 67.2; H, 7.64; N, 11.3.

9-Isoamyl-1,2,3,4-tetrahydro-β-carboline (V)—1,2,3,4-Tetrahydro-β-carboline (6.88 g., 0.04 mole) was treated with a mixture of 1.04 g. (0.045 g. atom) of sodium in liquid ammonia and 8.91 g. (0.045 mole) of 1-iodo-3-methylbutane as in the preparation of 9-*n*-propyltetrahydro-β-carboline, except after the addition of the alkyl halide the reaction was allowed to proceed for 1 hr. After the evaporation of liquid ammonia, about 50 ml. of water was added to the residue, and the product was extracted with ether (2 × 150 ml.). The ethereal solution was shaken with 150 ml. of 15% hydrochloric acid and the aqueous layer was separated. During an extraction of the aqueous solution with about 100 ml. of ether, the hydrochloride salt precipitated. When the hydrochloride salt was neutralized by sodium hydroxide, the solid free amine was liberated, which became an oil upon drying *in vacuo*. Conversion of the free amine again into the hydrochloride gave 3.5 g. (31.2%) of yellow solid. The hydrochloride



Scheme VI

EXPERIMENTAL

Melting points are corrected and were taken on a Fisher-Johns apparatus. Infrared spectra were obtained with a Perkin-Elmer spectrophotometer model 237 B. For qualitative ultraviolet spectra a Beckman spectrophotometer model DB-G was used.

9-*n*-Propyl-1,2,3,4-tetrahydro-β-carboline (IV)—1,2,3,4-Tetrahydro-β-carboline (5.16 g., 0.03 mole) was added slowly with stirring to a sodamide solution, prepared by reacting 0.92 g. (0.04 g. atom) of salt was recrystallized from ethanol–ether to give 2.5 g. (22.3%) of white solid. A second recrystallization from the same solvent mixture yielded 1.5 g., m.p. 229–230°; $\lambda_{max.}$ (KBr) 3.28, 3.38, 3.43, 3.49, 3.60, 3.65, 3.70, 3.77, 3.83, 3.91, 4.08 (CH, NH_2^+); 6.20, 6.35, 6.80, 6.85 (C=C, CH); 7.20, 7.22 [C-$(CH_3)_2$]; 13.60 μ (indole CH).

Anal.—Calcd. for $C_{16}H_{23}ClN_2$: C, 68.9; H, 8.31; N, 10.0. Found: C, 68.9; H, 8.56; N, 10.1.

9-Ethyl-1,2,3,4-tetrahydro-β-carboline (III)—In a similar manner as in the preparation of 9-*n*-propyltetrahydro-β-carboline, 9-ethyltetrahydro-β-carbo-

line was obtained as a yellow oil. It was converted into the hydrochloride salt, m.p. 233.5–235° (ethanol). On the silica gel chromatogram in *n*-propanol–ammonium hydroxide (95:5) this hydrochloride traveled as a single spot which was different from the starting material. A portion of the yellow oil was converted into its picrate salt. The melting point (225–226° dec.) of the picrate agreed with that reported (224–225°) by Leonard and Elderfield prepared from 1-ethyltryptamine and formaldehyde (5).

9-Benzyl-1,2,3,4-tetrahydro-β-carboline (VI)—To a stirred suspension of 0.576 g. (24 mmoles) of sodium hydride (50% suspension in mineral oil) in 30 ml. of dimethylformamide (DMF) was added over a period of 20 min. a solution of 3.78 g. (22 mmoles) of tetrahydro-β-carboline in 60 ml. of DMF. After stirring for 1 hr., the resulting solution was cooled in ice while a solution of 2.9 g. (23 mmoles) of benzyl chloride was added dropwise over 10 min. At the end of the addition the mixture was stirred for 1 hr. at ice temperature. Water (150 ml.) was added and the mixture was extracted with chloroform (4 × 75 ml.). The combined chloroform extracts were washed with water (4 × 150 ml.), dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was extracted with 50 ml. of heptane. After the removal of insoluble solid by filtration, the heptane extract was evaporated *in vacuo* leaving an oil that was converted into 3.5 g. (53%) of hydrochloride salt, m.p. 252–258°. The hydrochloride was dissolved in 100 ml. of hot water, and after cooling the gummy insoluble substance extracted with ether (3 × 100 ml.). During the last extraction the hydrochloride precipitated from the aqueous solution and was collected on a filter; yield, 0.462 g. (7%), m.p. 277–279°; $\lambda_{max.}$ (KBr) 3.28, 3.31, 3.40, 3.45, 3.60, 3.67, 3.70, 3.77, 3.83, 3.90, 3.92, 4.09, 4.15 (CH, NH_2^+); 6.20, 6.25, 6.30, 6.35, 6.69, 6.72, 6.88 (C=C, CH); 13.45, 14.25 μ (indole and phenyl CH). $\lambda_{max.}$ (EtOH) 207, 223, 275, 282, 295 mμ. No attempt has been made to recover more product from the aqueous solution.

The m.p. of this hydrochloride agreed with that reported (276°) in a patent (3) prepared from the condensation of 1-benzyltryptamine with formaldehyde.

9-Phenyl-1,2,3,4-tetrahydro-β-carboline (VII)—To a refluxed solution of 1.2 g. (4.4 mmoles) of 1-phenyltryptamine hydrochloride (3) in 30 ml. of water was added dropwise a solution of 0.524 g. (6 mmoles) of glyoxylic acid monohydrate in 10 ml. of water. After being refluxed for an additional 1 hr., the mixture was cooled in ice and the solid (XIX) was removed by filtration. The filtrate was first made basic with 30% aqueous potassium hydroxide and the gummy precipitate was extracted with ether (2 × 30 ml.). The combined ether extracts were dried over anhydrous sodium sulfate, filtered, and mixed with ethereal HCl. The hydrochloride was collected on a filter; yield, 0.41 g. (32.5%), m.p. 230–240°. One recrystallization from ethanol gave 0.175 g. (13.8%). The melting point (260–261°) of the pure product agreed with that reported (260–262°) in the literature (3) prepared from the reaction of 1-phenyltryptamine with formaldehyde.

9-Phenyl-1,2,3,4-tetrahydro-β-carboline-1-carboxylic Acid (XIX)—To a solution of 0.898 g. (3.3 mmoles) of XVIII·HCl in 80 ml. of water was added 0.304 g. (3.3 mmoles) of glyoxylic acid monohydrate. After being adjusted to pH 4 with 5% aqueous potassium hydroxide, the mixture was stirred at room temperature for 17 hr. Concentrated hydrochloric acid (5 ml.) was added and the stirring was continued for an additional 1 hour. The resulting "jell-like" mixture was centrifuged. The precipitate washed with ethanol (2 × 40 ml.), and again centrifuged. The precipitate was finally suspended in 80 ml. of ether and collected on a filter; yield, 0.30 g. (27.7%), m.p. 225–226° (with evolution of CO_2). $\lambda_{max.}$ (KBr) 3.29, 3.38, 3.42, 3.50, 3.65, 3.85 (CH, NH_2^+), 6.16 (COO^-), 6.26, 6.66 (C=C), 13.50 μ (indole CH).

Anal.—Calcd. for $C_{18}H_{16}N_2O_2$: C, 74.0; H, 5.51; N, 9.6. Found: C, 73.7; H, 5.64; N, 9.6.

2-Propionyl-1,2,3,4-tetrahydro-β-carboline (XXIII)—Tetrahydro-β-carboline (5.16 g., 0.03 mole) and 15 ml. of propionyl anhydride was thoroughly mixed, then allowed to stand at room temperature for 10 min. After cooling briefly in ice, the mixture was poured with stirring into 100 ml. of water, and the solid was collected after 30 min.; yield, 6.0 g. (88.2%), m.p. 204–206°; $\lambda_{max.}$ (KBr) 3.08 (NH); 6.13, 6.18, 6.30 (C=O, C=C); and 13.40 μ (indole CH).

Anal.—Calcd. for $C_{14}H_{16}N_2O$: C, 73.6; H, 7.06; N, 12.3. Found: C, 73.4; H, 7.09; N, 12.3.

2-Acetyl-1,2,3,4-tetrahydro-β-carboline (XXII)—In a similar manner as in the preparation of 2-propionyltetrahydro-β-carboline, acetylation of tetrahydro-β-carboline gave 2-acetyl product in a 87.6% yield, m.p. 238–239° [lit. m.p. 237–238° (6)].

2-*n*-Propyl-1,2,3,4-tetrahydro-β-carboline (IX)—A suspension of 1.52 g. (0.04 mole) of lithium aluminum hydride in 100 ml. of tetrahydrofuran was refluxed with stirring while 4.56 g. (0.02 mole) of 2-propionyltetrahydro-β-carboline was extracted from a Soxhlet thimble. After 14 hr. of heating the cooled mixture was decomposed with water and filtered. The filtered cake was washed with tetrahydrofuran (3 × 50 ml.), and the combined tetrahydrofuran solution was concentrated under reduced pressure almost to dryness. Addition of about 100 ml. of water caused the precipitation of product; yield, 4.1 g. (97.6%), m.p. 132–136°. This crude product was dissolved in 100 ml. of 10% hydrochloric acid. After clarification by filtration through diatomaceous earth,[1] the free base was liberated by the addition of excess 10% potassium hydroxide. The free amine was recrystallized from aqueous ethanol to give 3.0 g. (71.4%) of needles, m.p. 136.5–137.5°. For analysis a hydrochloride salt was prepared; m.p. 242–243° dec. $\lambda_{max.}$ (KBr) 3.10 (NH), 3.70, 3.76, 3.80, 3.82, 3.88, 4.00, 4.10 (NH^+); 6.15, 6.25 (C=C); 13.35 μ (indole CH).

Anal.—Calcd. for $C_{14}H_{19}ClN_2$: C, 67.0; H, 7.64; N, 11.2. Found: C, 67.2; H, 7.70; N, 11.1.

2-Ethyl-1,2,3,4-tetrahydro-β-carboline (VIII)—In a similar manner as described above, a mixture of 10.6 g. (0.049 mole) of 2-acetyltetrahydro-β-carboline and 4.0 g. (0.1 mole) of lithium aluminum hydride in 550 ml. of tetrahydrofuran was refluxed for 3.5 hr. to yield 8.0 g. (81.6%) of 2-ethyltetrahydro-β-carboline. One recrystallization from aqueous ethanol gave 6.5 g. (66.3%), m.p. 153–154° [lit. m.p. 137–138° (6)]. The hydrochloride salt of the

[1] Celite, Johns-Manville Corp., New York, N. Y.

β-carboline melted at 229–230° [lit. m.p. 227–229° (6)].

2 - Ethyl - 9 - methyl - 1,2,3,4 - tetrahydro - β-carboline (X)—In a similar manner as in the preparation of IV, 2 g. (0.01 mole) of 2-ethyltetrahydro-β-carboline was treated with a mixture of 0.345 g. (0.015 g. atom) of sodium in liquid ammonia and 2.13 g. (0.015 mole) of methyl iodide. After evaporation, the residue was dissolved in 50 ml. of water, and extracted first with 100 ml. then 50 ml. of ether. The combined ethereal extracts were dried with anhydrous sodium sulfate, decolorized with charcoal, filtered, then evaporated *in vacuo*. The oily residue was dissolved in 25 ml. of heptane, filtered, and mixed with ethereal HCl to precipitate the hydrochloride salt. Recrystallization of this crude hydrochloride with ethanol–ether gave 1.5 g. (60%) of white solid, m.p. 234–235°; $\lambda_{max.}$ (KBr) 3.28, 3.32, 3.40, 3.44, 3.47, 3.51 (CH); 3.65, 4.01, 4.12, 4.31 (NH^+); 6.20 (C=C); 13.35 μ (indole CH). $\lambda_{max.}$ (EtOH) 225, 275, 282, 293 mμ.

Anal.—Calcd. for $C_{14}H_{19}ClN_2$: C, 67.0; H, 7.63; N, 11.2. Found: C, 67.2; H, 7.82; N, 11.2.

2 - Ethyl - 9 - hydroxymethyl - 1,2,3,4 - tetrahydro-β-carboline (XI)—To a suspension of 1.0 g. (5 mmoles) of 2-ethyltetrahydro-β-carboline in 10 ml. of water was added dropwise concentrated hydrochloric acid until a solution was obtained (about 0.1 ml.). The pH of the solution was adjusted to 5.5–6.0 with aqueous sodium acetate and 5 ml. of 38% (60 mmoles) of formaldehyde was added. The mixture was allowed to stand at room temperature for 4 days. When the solution was made basic with 10% potassium hydroxide, an oil separated. The oil was extracted into 50 ml. of ether, and evaporation of ether gave a gummy product which was then dissolved in ether and converted into 0.5 g. (36%) of hydrochloride salt by mixing with ethereal HCl. The hydrochloride was hygroscopic; it decomposed when heated to 180°. $\lambda_{max.}$ (KBr) 3.10 (OH); 3.20, 3.25, 3.35, 3.45 (NH^+); 6.18 (C=C); 9.60–9.70 (C–O); 13.40 μ (indole CH); $\lambda_{max.}$ (EtOH) 204, 224, 272, 279, 289 mμ.

Anal.—Calcd. for $C_{14}H_{19}ClN_2O$: C, 63.0; H, 7.17; N, 10.5. Found: C, 62.8; H, 7.20; N, 10.5.

2,9 - Dimethyl - 1 - oxo - 1,2,3,4 - tetrahydo - β-carboline (XXV)—To a suspension of 1.44 g. (0.06 mole) of sodium hydride (50% suspension in mineral oil) in 25 ml. of tetrahydrofuran was added dropwise a solution of 3.72 g. (0.02 mole) of 1-oxotetrahydro-β-carboline in 50 ml. of tetrahydrofuran over a period of 1 hr. After being refluxed for 45 min., the mixture was cooled in an ice bath while 14.2 g. (0.1 mole) of methyl iodide was added dropwise over a period of 15 min. The resulting mixture was allowed to stand overnight at room temperature, then refluxed for 30 min. After the addition of 3 ml. of water, the mixture was heated under a stream of helium until the volume reduced to about 25 ml. A large amount of water was added and an oil separated. The oil was extracted into 150 ml. of ether and the aqueous layer was again extracted with 100 ml. of ether. The combined ethereal extracts were dried with anhydrous sodium sultate and evaporated *in vacuo* leaving an oily residue. When the oil was boiled with 15 ml. of heptane for 10 min. then allowed to cool, it solidified to give 3.1 g. (72%), m.p. 77.5–79°; $\lambda_{max.}$ (KBr) 6.10 (C=O); 6.20, 6.47, 6.70 (C=C); 13.55 μ (indole CH), $\lambda_{max.}$ (EtOH) 207, 230, 242 (s), 303. 330 (s) mμ.

Anal.—Calcd. for $C_{13}H_{14}N_2O$: C, 72.9; H, 6.58; N, 13.1. Found: C, 73.1; H, 6.63; N, 13.0.

2,9-Dimethyl-1,2,3,4-tetrahydro-β-carboline (XII)—To a stirred suspension of 0.76 g. (0.02 mole) of lithium aluminum hydride in 30 ml. of ether was added dropwise a solution of 2.14 g. (0.01 mole) of 2,9-dimethyl-1-oxo-tetrahydro-β-carboline in 30 ml. of ether. After being stirred at room temperature for 2 hr., the mixture was decomposed with water, filtered, and the filtered cake was washed with ether (3 × 20 ml.). The combined ether solution was dried with anhydrous sodium sulfate and evaporated *in vacuo* leaving a white solid. The solid product was dissolved in ether, filtered to remove small amounts of undissolved product, then mixed with ethereal HCl. The hydrochloride salt, m.p. 242–244°, weighed 2 g. (83.3%). One recrystallization from ethanol gave 1.3 g. (54.1%), m.p. 246–247°; $\lambda_{max.}$ (KBr) 3.74, 3.83, 3.89, 3.98, 4.03, 4.12, 4.20 (NH^+); 6.20 (C=C); 13.18, 13.30 μ (indole CH), $\lambda_{max.}$ (EtOH) 204, 227, 276, 282, 292 mμ.

Anal.—Calcd. for $C_{13}H_{17}ClN_2$: C, 65.9; H, 7.23; N, 11.8. Found: C, 66.1; H, 7.30; N, 12.0.

Assay

Mitochondrial monoamine oxidase from beef liver was isolated and purified as previously described (1). All the stock solutions of the hydrochloride salts of inhibitors were prepared in water. Compound VIII was dissolved in 0.01 *N* HCl and compound IX in propylene glycol. Incubation was carried out with tryptamine-2-^{14}C hydrochloride according to the previously described procedure (1).

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Keyphrases

Monoamine oxidase inhibitors
Tetrahydro-β-carbolines, *N*-2(9)-substituted—synthesis
Inhibition, MAO—screening
TLC—separation, identity
IR spectrophotometry—structure

Studies on Protein Binding I

Interaction of *para*-Hydroxybenzoic Acid Esters with Bovine Serum Albumin

By NAGIN K. PATEL, PAI-CHANG SHEEN, and KATHLEEN E. TAYLOR*

The binding of methyl, ethyl, propyl, and butyl esters of *p*-hydroxybenzoic acid (parabens) by bovine serum albumin (BSA) was investigated by an equilibrium dialysis procedure. All the compounds were found to interact with BSA. The association constants were ascertained from the dialysis data, and free energy change, ΔF^0, enthalpy change, ΔH^0, and entropy change, ΔS^0, of the interaction were computed from the data at two temperatures. The possible mechanism of the interaction is discussed. The spectrophotometric study of the interaction revealed hypochromic effects in the absorption spectra of all the parabens in the presence of BSA. The *in vitro* antifungal activity of methyl- and propylparaben was found to be related to the concentration of free or unbound drug.

INTERACTION OF NUMEROUS small molecules with serum proteins has been extensively reviewed by Goldstein and most of the drugs studied have been found to bind primarily to the albumin fraction of plasma proteins (1). Although a majority of the earlier reports were of a qualitative nature, attempts have been made to quantify the data with an ultimate aim of elucidating the nature of equilibria and forces involved in the drug-macromolecular interaction (2, 3). With the advancement of the theoretical knowledge of these interactions, considerable research has been conducted on the binding of salicylates (4, 5), sulfonamides (6–8), penicillins (9), xanthines (10), steroids (11), and other therapeutic agents with serum albumins.

In their extensive reviews Goldstein (1) and Brodie and Hogben (12) have discussed the importance of drug–protein association in the transport, distribution, and elimination of medicinal agents. In addition, it is generally recognized that the specificity of pharmacologic agents lies in the structure of complexes which they form with macromolecular systems. The investigation of the factors involved in such interactions may shed some light on the understanding of drug action.

Interactions of some benzoic acid derivatives (5) with serum albumins have been investigated. The degree of ionization and the relative size of the anion of the interacting molecule influenced the protein binding, and Boyer *et al.* (13) concluded that an anion with a fairly large nonpolar group imparted an increase in its binding capacity for BSA. Both textbook and reference works (14–16) indicate a significant loss of biological activity of some antifungal agents in the presence of protein materials and Alto *et al.* observed that the antimicrobial activity of *p*-hydroxybenzoates was diminished in the presence of protein (17). No reports could be found of experimentation on the direct correlation of binding data with the *in vitro* antimicrobial activity of *p*-hydroxybenzoates. The purpose of this work was to evaluate quantitatively the binding of a series of *p*-hydroxybenzoic acid esters by bovine serum albumin (BSA) and to show that the *in vitro* antifungal activity of methyl- and propylparaben was primarily a function of free or unbound drug.

EXPERIMENTAL

Materials—Bovine serum albumin, fraction V (BSA) purchased from the Armour Pharmaceutical Company (lots B24011 and D25901), was used throughout this study. The albumin[1] content of lots B24011 and D25901 were 97 and 95%, respectively. Both lots of albumin exhibited similar binding tendencies for methyl *p*-hydroxybenzoate. The moisture content of protein was determined and appropriate corrections were made in weighing it; a molecular weight of 69,000 was assumed. Reagent grade monobasic potassium phosphate and dibasic potassium phosphate were employed to prepare the buffer solutions. Reagent grade potassium chloride was used to obtain an equilibrium ionic strength of 0.16 where necessary. The *p*-hydroxy-

Received December 4, 1967, from the Faculty of Pharmacy, University of Alberta, Edmonton, Canada, and from the School of Pharmacy, University of Manitoba, Winnipeg, Manitoba, Canada

Accepted for publication April 5, 1968.

Abstracted from a thesis submitted by Mr. P. C. Sheen to the Faculty of Graduate Studies, University of Alberta, in partial fulfillment of the Master of Science degree requirements.

Presented in part to the Canadian Conference of Pharmaceutical Research, St. John Meeting, August 1966.

This investigation was supported by a research grant A-2058 from the National Research Council of Canada and a research grant from the Smith Kline and French Inter-American Corporation, Canada.

* Recipient of the E. L. Woods Memorial Competition Award.

[1] The analytical results were kindly supplied by Dr. T. Douglas of the Armour Pharmaceutical Company, Kankakee, Ill.

benzoic acid esters (parabens) and the synthetic culture medium were the same as described in a previous paper (18). All paraben and protein solutions were prepared immediately prior to use.

Dialysis Studies—The general approach, technique, and treatment of data of this study have been described previously (19, 20). Dialysis cells were the same as those employed by Patel and Foss (20) with the exception that seamless cellulose membranes (Fisher Scientific Company) were employed in the present work. The membranes were placed in distilled water and heated to 90° for 1 hr., and they were washed with distilled water several times in order to remove any contaminating substances in the cellophane (7). The membranes were permeable to *p*-hydroxybenzoates and impermeable to BSA. Binding data of an extensively dialyzed sample of the BSA yielded results similar to those from an undialyzed commercial material. Thus undialyzed BSA was used in the entire investigation. Binding of parabens by the membranes and the dialysis cells was found to be insignificant. Equilibrium was established at the end of 16 hr. by agitation on a rotational shaker in an Aminco constant-temperature laboratory bath (American Instrument Co. Inc.).

The dialysis studies were performed on 0.1 m*M* BSA, with the exception that in the case of propylparaben–BSA interaction, an additional 0.25 m*M* BSA was included. All parabens were studied at pH 7.4 and their initial concentrations ranged from 5.15×10^{-4} *M* to 1.32×10^{-2} *M*. Binding data were also collected for the methyl- and propylparaben–BSA systems at pH 6.6 for the purpose of approximating the pH of the synthetic culture medium (18).

Spectrophotometric Studies—Spectrophotometric examination of paraben solutions in the absence and presence of BSA was made at room temperature on a Hilger Ultrascan recording spectrophotometer, model H999. The concentration of paraben used was 6.4×10^{-5} *M*. Any interference due to the protein was eliminated by using the appropriate concentration in the reference cell.

Analysis After Equilibration—At the end of equilibrium time 1- or 2-ml. aliquots were removed from both sides of the membrane and were diluted with 0.1 *N* HCl to suppress the dissociation of parabens. The concentrations of parabens were determined using a Beckman DU spectrophotometer at a wavelength of their maximum absorbance, 256 mμ. Any interference due to the protein was eliminated by using the appropriate concentration in the reference cell and it was found that at very high dilutions employed for the analysis of parabens, there was no effect on the absorbance of parabens in the presence of BSA. Any absorption contribution by the dialyzable components in BSA was assumed to be negligible in lieu of the high dilutions employed in the analysis. The pH values of the solutions were recorded with a Beckman zeromatic pH meter, model 76, after each dialysis experiment, with no appreciable change noted.

Microbiological Studies—Observations were made on the influence of BSA on the antifungal activity of methyl- and propylparaben for *Aspergillus niger*.[2] The culture medium and methods as described previously (18, 21) were employed with the following exception in sterilization procedure. In order to avoid denaturation of protein at the high autoclave temperature, the solutions were sterilized by Millipore filtration using a hypodermic syringe, Swinney adapter, and Millipore filter.[3] Inhibitory concentrations for methyl- and propylparaben, in the presence and absence of BSA, were obtained through visual observation of samples each day for a period of 2 weeks.

RESULTS

Treatment of Data—The mathematical analysis of binding data from equilibrium dialysis studies has been described and discussed by Klotz (2, 22) and Scatchard (23). If the intrinsic association constants for all polymer sites are identical, the data can be plotted according to the equation of Scatchard (23),

$$\frac{r}{(A)} = nk - rk$$

where r = moles of bound drug/mole of protein, (A) = free drug concentration, n = number of binding sites available on each protein molecule, and k = association constant. Plotting $r/(A)$ *versus* r will give a straight line with slope = $-k$, and ordinate and abscissa intercepts of nk and n, respectively. By the method of least squares the quantities nk and k were calculated using an IBM 360/67 computer. From the value of k, the thermodynamic functions were calculated according to the following equations (2, 24):

$$\Delta F^0 = -RT \ln k$$

where ΔF^0 = the free energy change, R = gas constant, and T = absolute temperature.

$$\ln \frac{k_2}{k_1} = \frac{\Delta H^0}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

where ΔH^0 = the enthalpy change and k_1 and k_2 are binding constants at temperatures T_1 and T_2.

$$\Delta F^0 = \Delta H^0 - T\Delta S^0$$

where ΔS^0 = the entropy change.

Dialytic Data of the Paraben-Protein Interaction—The data for the interaction of methyl-, ethyl-, propyl-, and butylparaben at pH 7.4 and at two temperatures are plotted in Figs. 1–4. Within the concentration ranges of the drugs and the protein, a linear relationship of $r/(A)$ *versus* r, as described by Scatchard's equation, was noted. The k and n values were obtained by linear extrapolation to the ordinate and abscissa axes.

Absorption Spectra in the Presence and Absence of Protein—The absorption spectra of certain molecules are altered in the presence of proteins and this spectral change is indicative of an interaction between two compounds. This change usually consists of a decrease in the absorption coefficient. The pronounced hypochromic effects of methyl-, ethyl-, propyl-, and butylparaben in the presence of increasing concentrations of BSA are depicted in Figs. 5–8. The method has been employed by var-

[2] UAMH No. 1456, kindly supplied by Miss Marjorie Dineen, of the Provincial Laboratory of Public Health, Edmonton, Canada.

[3] Mixed esters of cellulose, marketed as MF-Millipore, Type HA, Millipore Ltd., Montreal, Canada.

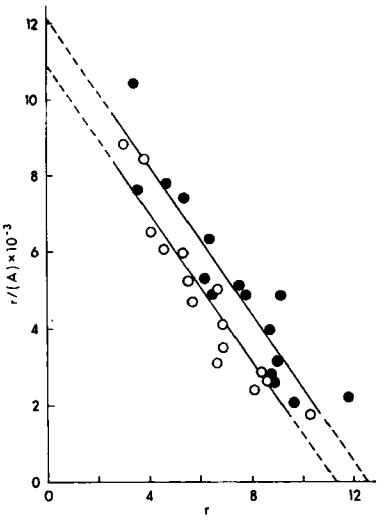

Fig. 1—Binding of methylparaben by BSA, pH 7.4. Key: ○, 30°; ●, 10°.

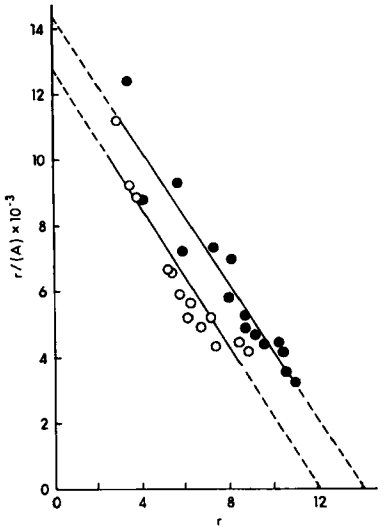

Fig. 2—Binding of ethylparaben by BSA, pH 7.4. Key: ○, 30°; ●, 20°.

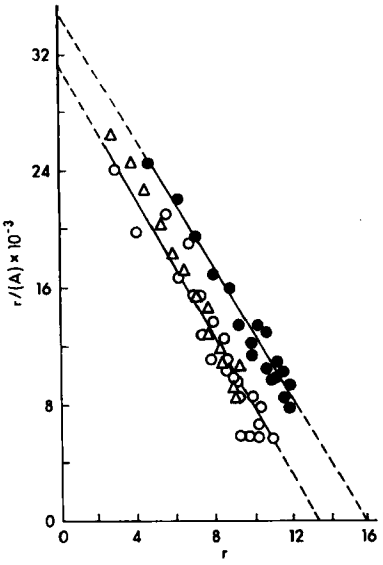

Fig. 3—Binding of propylparaben by BSA, pH 7.4. Key: ○, 30°; ●, 20°; △, data collected employing 0.25 mmole BSA, pH 7.4, at 30°; all other data of Figs. 1–4 were obtained using 0.1 mM BSA.

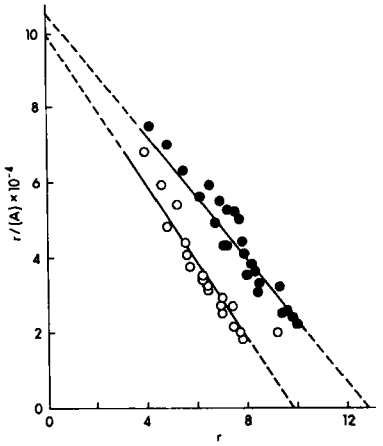

Fig. 4—Binding of butylparaben by BSA, pH 7.4. Key: ○, 30°; ●, 20°.

ious investigators (25–27) to study the binding of some drugs by proteins.

Inactivation by BSA Related to Binding—It was shown in earlier studies that the ratio, R, of total to free preservative for a given concentration of polysorbate 80 and cetomacrogol 1,000 was fairly constant (18–21). If the antifungal activity of parabens can be assumed to be due to the free or unbound form, the R value may be used to predict the minimum inhibitory concentration of antifungal agent in the presence of a definite quantity of BSA by a procedure described previously (18, 21). It is recognized that in the case of BSA the R value would not be constant over a wide range of concentrations of the parabens. However, within a narrow range of concentrations, especially around the minimum inhibitory concentration of the drug, it can be assumed to be reasonably constant, as outlined below. The R values, as obtained employing 0.1 mM BSA solution, at pH 6.6 and 30°, were 1.19 (range 1.16–1.23 with standard deviation of 0.026) and 1.75 (range 1.60–1.95 with standard deviation of 0.12) for methyl- and propylparaben, respectively. The total paraben concentrations on the protein side at the end of the dialysis experiment were 0.062–0.110% and 0.030–0.05% in the cases of methyl- and propylparaben, respectively. The results of the *in vitro* correlation of binding data with the antifungal activity of methyl- and propylparaben for *Aspergillus niger* in the presence of 0.1 mM BSA are summarized in Table I. It is evident from these data that there is a good correlation between the predicted minimum inhibitory concentration and the experimental antifungal concentration for both the parabens.

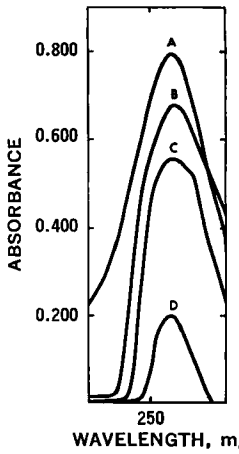

Fig. 5—Absorption spectra of methylparaben at pH 7.4. Key: A, buffer; B, 0.025 mM BSA; C, 0.050 mM BSA; D, 0.10 mM BSA.

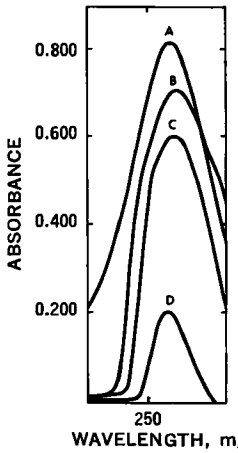

Fig. 6—Absorption spectra of ethylparaben at pH 7.4. Key: A, buffer; B, 0.025 mM BSA; C, 0.050 mM BSA; D, 0.10 mM BSA.

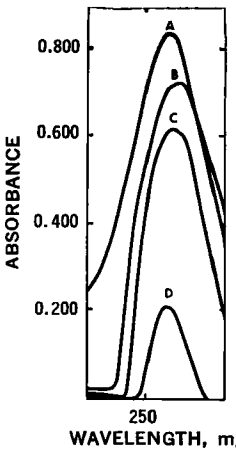

Fig. 7—Absorption spectra of propylparaben at pH 7.4. Key: A, buffer; B, 0.025 mM BSA; C, 0.050 mM BSA; D, 0.10 mM BSA.

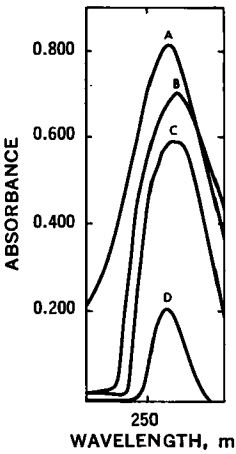

Fig. 8—Absorption spectra of butylparaben at pH 7.4. Key: A, buffer; B, 0.025 mM BSA; C, 0.050 mM BSA; D, 0.10 mM BSA.

DISCUSSION

The ability of *p*-hydroxybenzoic acid esters to interact with several macromolecules has been well documented (18, 19, 28, 29). Based upon this observation, it was expected that BSA might provide binding sites for the parabens. From Figs. 1–4, it is evident that all the parabens interacted with BSA. The values for the association constants, k, and number of binding sites, n, together with the thermodynamic parameters of the interaction, are summarized in Table II. From this table it is noted that the values of n are not integers. This might be either due to the experimental error in dialytic studies as explained by Klotz (30) or due to the view that protein might not be molecularly homogeneous with respect to binding properties as theorized by Karush (31).

The exact mechanism of binding of *p*-hydroxybenzoates by BSA cannot be elucidated from this study, since the results were obtained primarily from the binding of methyl-, ethyl-, propyl-, and butylparaben. However, certain tentative conclusions may be drawn. A comparison of association constants for various parabens at a definite temperature (Table II) reveals that the binding increases from methyl- to ethyl- to propyl- to butylparaben. Due to the limited solubility of hexyl- and heptylparaben it was not possible to evaluate the association constants for these compounds by dialysis studies. An increase in the solubility of these compounds in aqueous BSA solution at pH 7.4 over their water solubility at the same pH indicated that hexyl- and heptylparaben would be bound to a greater extent than butylparaben (32). About 40% of the total amino acids in the protein are nonpolar residues (33) and it is quite possible that the association between these residues and the nonpolar moieties of the paraben molecule (aromatic ring and hydrophobic chain of ester) may contribute to the overall interaction. An examination of the data of Table II shows that the magnitude of binding increased with an increase in temperature, demonstrating that the binding process was endothermic (unfavorable positive enthalpy). More significantly, the binding was accompanied by an increase in entropy term which is typically a thermodynamic behavior in common

TABLE I—CORRELATION OF PROTEIN BINDING DATA WITH *In Vitro* ANTIFUNGAL ACTIVITY OF PARABENS, OBSERVED FOR TWO WEEKS

Concn., BSA	Minimum Inhibitory Concn. Methylparaben, g./100 ml. Pred.[a]	Expt.[b]	Propylparaben, g./100 ml. Pred.	Expt.
0		0.060		0.020
0.1 m*M*	0.071	0.074	0.035	0.035

[a] Predicted from the binding data. [b] Experimental values.

TABLE II—ASSOCIATION CONSTANTS AND THERMODYNAMIC PARAMETERS OF PARABEN–PROTEIN INTERACTION IN AQUEOUS SOLUTION AT pH 7.4

Paraben	Temp.	Binding Constant (k), l./mole × 10^{-3}	nk × 10^{-3}	No. of Sites, n	Free Energy Change (ΔF^0), kcal./mole	Enthalpy Change (ΔH^0), kcal./mole	Entropy Change, (ΔS^0) e.u.
Methyl-	30°	0.987	10.9	11.2	−4.15	0.0845	14.0
	10°	0.968	12.1	12.5	−3.87		14.0
Ethyl-	30°	1.05	12.8	12.2	−4.19	0.529	15.6
	20°	1.02	14.4	14.1	−4.04		15.6
Propyl-	30°	2.33	31.3	13.4	−4.67	0.876	18.3
	20°	2.22	34.9	15.7	−4.49		18.3
Butyl-	30°	10.1	99.4	9.9	−5.56	4.00	31.5
	20°	8.11	105	12.9	−5.24		31.5

with that described by Schachman (34) for hydrophobic bonding. The positive entropy change for this type of interaction has been ascribed by Kauzman (35) and others (36, 37) as resulting from the energy change accompanying the disruption of the "iceberg" structure of water molecules around hydrocarbon groups in aqueous solution (hydrophobic bonding). The large difference in the values of ΔH_0 and ΔS_0 of propyl- and butylparaben was probably due in part to the experimental error which resulted from a significant scatter in the experimental points in the case of butylparaben (Fig. 4), especially at 30°.

In Figs. 5–8, the degree of hypochromism increases with an increase in concentration of protein. The decrease in the absorption has been interpreted by Klotz (25) as an effect due to the formation of drug–protein complex, if the complexation involves a chromophor system of drug. Although spectrophotometric study can often yield information as to the magnitude of the interaction as well as to the nature of forces responsible for the interaction, and such study is in progress, this part of the work was limited to the qualitative nature of the interaction.

The results of correlation of binding data with the inhibitory concentration of methyl- and propylparaben for *Aspergillus niger* in the presence of 0.69% BSA are in good agreement. This part of the study shows that the protein-bound paraben is devoid of *in vitro* antifungal activity.

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Keyphrases

Protein binding
Bovine serum albumin interaction—*p*-hydroxybenzoic acid esters (parabens)
Dialysis—bovine serum albumin-parabens
Parabens, antimicrobial activity—binding effect
IR spectrophotometry—analysis

Human Urinary Excretion of the Quaternary Ammonium Compounds Anisotropine Methylbromide and Propantheline Bromide

By MORRIS PFEFFER, JOSEPH M. SCHOR, SANFORD BOLTON, and RALPH JACOBSEN

Urinary excretion was studied after the administration of anisotropine methylbromide p.o. and i.v. and propantheline bromide p.o. to a group of volunteers. Orally administered material was excreted in the urine for periods of 2–5 days while i.v. administered material was excreted for 16 days. In all cases, periodic excretion peaks were observed. The urinary excretion of anisotropine methylbromide and propantheline bromide seems similar.

A PRELIMINARY STUDY of the excretion of a tablet formulation of the quaternary ammonium antispasmodic anisotropine methylbromide[1] (8-methyltropinium bromide 2-propylpentanoate), in six subjects was the subject of a previous publication (1). The observation of prolonged excretion with discrete periods of peak excretion of the compound has been confirmed and extended with a larger group of subjects. Several oral formulations and an intravenous administration are included. Similar results have been obtained for propantheline bromide[2] (β-diisopropylaminoethyl 9-xanthene carboxylate methobromide) tablets, indicating that the observed pattern of excretion might be a general property of quaternary ammonium compounds (QAC's).

EXPERIMENTAL

Reagents—Anisotropine methylbromide originated and was compounded at Endo Laboratories, Inc. Propantheline bromide was obtained as a commercially available 15-mg. tablet and 30-mg. ampul. Tropaeolin 00 (orange IV) was purchased from Matheson, Coleman and Bell. The grades and sources of supply of all other reagents were indicated previously (1).

Analytical Procedures—All spectrophotometric analyses were performed with a Hitachi Perkin-Elmer 139 spectrophotometer.

The analytical methods employed for both anisotropine methylbromide and propantheline bromide are similar in that they depend upon the formation of a salt between the cationic agent and an anionic dye. The dye salt is extracted from an aqueous medium into an immiscible nonpolar solvent. The analytical procedure for anisotropine methylbromide, employing picric acid as the anionic dye, has been reported previously (1).

Propantheline bromide did not yield an extractable dye salt in the picric acid assay system, presumably due to the presence of a hydrophilic hydroxyl group on the quaternary hydrolysis product of the ester. Hydrolysis was due to the highly alkaline aqueous medium necessary for this assay system. Since Biles *et al.* (2) had successfully analyzed for propantheline bromide with tropaeolin 00, this dye was used here, and a suitable assay was developed for the estimation of propantheline cation in human urine. Absorption spectrum for the propantheline-tropaeolin 00 dye salt in chloroform following extraction from an aqueous solution is given in Fig. 1. Five milliliters of urine or neutral aqueous solution containing propantheline cation was mixed with 0.5 ml. of a saturated aqueous solution of tropaeolin 00 and this mixture was extracted with 5.0 ml. of chloroform by agitation on a Vortex Genie mixer for 15 sec. Longer agitation on a wrist-action shaker for periods up to 30 min. did not improve extraction. A plot of the absorbance at 409 mμ (A_{409}) of the chloroform-extracted dye salt *versus* the original aqueous concentration of propantheline bromide was linear over the range of 0–114 nmoles/ml. The molar absorptivity was 1.76×10^7. The sensitivity

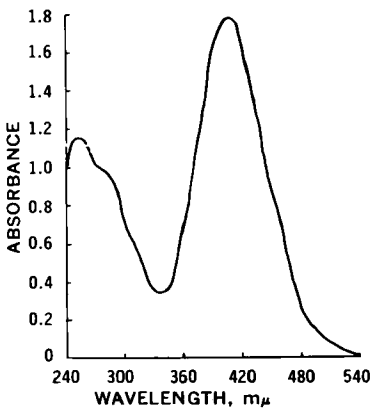

Fig. 1—Absorption spectrum of the propantheline-tropaeolin 00 dye salt in chloroform after extraction from aqueous solution. Propantheline bromide concentration 100 nmoles/ml. H_2O.

Received January 15, 1968, from the Departments of Biochemistry and Medicine, Endo Laboratories, Inc., Garden City, NY 11530

Accepted for publication April 10, 1968.

The authors gratefully acknowledge the assistance of Dr. Solomon Griboff. Thanks are also due to Messrs. Martin Semmel and Norman Gluck for their skilled technical assistance.

[1] Marketed as Valpin by Endo Laboratories, Inc., Garden City, N. Y.

[2] Marketed as Pro-Banthine by G. D. Searle & Co., Chicago, Ill.

of the tropaeolin 00 analytical method for propantheline cation is almost identical to the sensitivity of the picric acid method for methylanisotropinium cation (1).

The naturally occurring QAC's choline chloride, acetylcholine chloride, phosphocholine chloride, and thiamine did not yield discernible dye salt absorption spectra in chloroform extracts when tested in the tropaeolin 00 assay at concentrations up to 0.3 μmoles/ml. H_2O. These compounds had previously been found not to interfere in the picric acid assay (1).

Divatia and Biles (3) reported that the response of QAC's to tropaeolin 00 decreases as the molecular weight of the QAC decreases.

It has been reported that in the picric acid assay for methylanisotropinium ion in human urine there is a component of blank absorption as well as a systematic analytical loss (1). It was demonstrated that both these factors remained constant for any given individual, although they varied from one to another. It developed that this was equally true for the tropaeolin 00 assay for propantheline cation in urine. The blank absorptions and analytical recoveries for both assay systems in the 12 subjects used in this study were calculated by comparing analyses of 0, 5, 10, and 15 nmoles/ml. of each QAC when in deionized water and when added to their normal urines. These control urines were collected for consecutive 8-hr. periods, starting 30 min. before breakfast, over three 24-hr. periods—a collection schedule corresponding to that used during the QAC excretion studies. The resultant correction factors are listed in Table I. The magnitude of their variations are indicated by the standard deviations (*SD*).

Previously (1) the correction values for methyl anisotropinium cation in urine had been determined for 24-hr. collections. In the present series of experiments, correction values were calculated separately for each of the three 8-hr. periods to determine if there were any diurnal variations in blanks and recovery values. Analysis of variance of the blank and recovery values did not indicate significant variation, over the three 8-hr. periods, of the values for any given individual. However, there were significant differences between individuals for blanks and recoveries. There was no apparent correlation between the blank and recovery values.

The values for methylanisotropinium and propantheline cations were determined by correcting the respective A_{365} and A_{409} values obtained from experimental urine samples, for corresponding time intervals, according to the equation

$$B = (A - D)100/C$$

where B = corrected absorbance, A = observed absorbance, D = blank absorbance values for that time period, and C = calculated percentage analytical recovery for that time period. Concentrations were calculated in terms of nanomoles that were then converted back to their equivalents in mcg. of the bromide salts of the QAC's.

TABLE I—ANALYTICAL CORRECTION VALUES FOR HUMAN SUBJECTS

Subject No.	Time Interval, Hr.	Anisotropine Methylbromide		Propantheline Bromide	
		Mean Blank $A_{365} \pm SD$	Mean Recovery % ± *SD*	Mean Blank $A_{409} \pm SD$	Mean Recovery % ± *SD*
1	0–8	0.024 ± 0.004	83.5 ± 11.2	0.037 ± 0.005	81.0 ± 1.6
	8–16	0.033 ± 0.002	80.5 ± 7.1	0.024 ± 0.002	77.4 ± 0.3
	16–24	0.023 ± 0.005	81.0 ± 5.7	0.029 ± 0.000	84.2 ± 1.0
2	0–8	0.061 ± 0.004	80.0 ± 2.3	0.073 ± 0.004	48.8 ± 0.3
	8–16	0.035 ± 0.004	86.0 ± 0.6	0.035 ± 0.003	70.7 ± 0.5
	16–24	0.025 ± 0.003	91.2 ± 4.0	0.026 ± 0.001	73.1 ± 0.4
3	0–8	0.029 ± 0.006	58.4 ± 5.7	0.033 ± 0.000	37.8 ± 1.6
	8–16	0.023 ± 0.004	60.1 ± 1.7	0.034 ± 0.003	58.8 ± 4.1
	16–24	0.018 ± 0.007	64.7 ± 7.7	0.028 ± 0.001	24.3 ± 1.4
4	0–8	0.030 ± 0.009	59.6 ± 5.4	0.042 ± 0.019	22.6 ± 4.7
	8–16	0.026 ± 0.007	71.6 ± 4.9	0.040 ± 0.007	64.1 ± 0.2
	16–24	0.027 ± 0.002	63.7 ± 3.8	0.026 ± 0.002	31.2 ± 4.7
5	0–8	0.026 ± 0.001	57.5 ± 1.6	0.056 ± 0.001	58.9 ± 0.3
	8–16	0.024 ± 0.003	63.0 ± 4.4	0.017 ± 0.004	72.6 ± 1.2
	16–24	0.027 ± 0.003	62.2 ± 5.2	0.022 ± 0.003	57.4 ± 0.3
6	0–8	0.013 ± 0.002	71.7 ± 1.5	0.021 ± 0.003	58.5 ± 2.7
	8–16	0.014 ± 0.000	68.6 ± 3.6	0.010 ± 0.003	62.8 ± 7.1
	16–24	0.016 ± 0.004	78.1 ± 4.6	0.013 ± 0.002	70.7 ± 4.5
7	0–8	0.026 ± 0.006	72.4 ± 2.2	0.017 ± 0.000	49.4 ± 4.9
	8–16	0.027 ± 0.004	73.5 ± 1.4	0.029 ± 0.005	56.7 ± 3.2
	16–24	0.020 ± 0.001	79.0 ± 2.3	0.022 ± 0.003	64.2 ± 6.0
8	0–8	0.020 ± 0.002	73.9 ± 2.5	0.018 ± 0.002	49.3 ± 1.7
	8–16	0.011 ± 0.003	79.0 ± 5.3	0.029 ± 0.000	51.3 ± 2.0
	16–24	0.013 ± 0.000	79.3 ± 3.8	0.022 ± 0.000	66.4 ± 0.9
9	0–8	0.024 ± 0.002	90.3 ± 0.4	0.019 ± 0.002	64.1 ± 0.8
	8–16	0.022 ± 0.002	88.9 ± 0.4	0.021 ± 0.004	60.9 ± 0.9
	16–24	0.032 ± 0.005	91.3 ± 2.6	0.021 ± 0.004	59.6 ± 2.0
10	0–8	0.018 ± 0.002	81.3 ± 0.5	0.031 ± 0.001	69.9 ± 2.5
	8–16	0.023 ± 0.001	71.0 ± 8.2	0.049 ± 0.006	74.7 ± 0.7
	16–24	0.023 ± 0.002	82.0 ± 5.6	0.055 ± 0.001	72.7 ± 0.7
11	0–8	0.038 ± 0.006	80.0 ± 6.5	0.094 ± 0.001	86.7 ± 0.4
	8–16	0.032 ± 0.004	75.6 ± 1.7	0.044 ± 0.006	77.6 ± 1.7
	16–24	0.022 ± 0.004	87.7 ± 5.0	0.038 ± 0.003	85.9 ± 1.6
12	0–8	0.018 ± 0.005	44.1 ± 1.3	0.014 ± 0.002	34.3 ± 1.9
	8–16	0.044 ± 0.005	46.8 ± 5.8	0.011 ± 0.000	39.9 ± 4.1
	16–24	0.036 ± 0.001	35.4 ± 5.0	0.023 ± 0.006	17.0 ± 1.5

TABLE II—COMPLETE THREE-WAY ANALYSIS OF VARIANCE

Source	df[a]	SS[b]	MS[c]	F
People	11	3.97		
People × formulations	31	19.6	0.632	
Times	20	71.0		
Times × formulations	60	15.0	0.251	$F(60,260) = 1.27$
Times × people	220	57.8		
Times × people × formulations	620	123	0.198	
Formulations	3	3.10	1.03	$F(3,31) = 1.63$

[a] *df* = degrees of freedom (see no. of experimental subjects for each formulation in Table III). [b] *SS* = sum of squares. [c] *MS* = mean square.

Creatinine concentrations in urine specimens were determined by the method of Taussky (4). Neither anisotropine methylbromide nor propantheline bromide interfered in this determination.

Excretion Study—Twelve males were employed. None took any other medication during these experiments. None showed any abnormalities of urine specific gravity,[3] blood,[4] protein,[4] glucose,[4] or pH.[4]

The formulations administered are listed in Table III. These were taken 30 min. before breakfast, except the intravenous injection which was given at 11 a.m. Complete dosage of the liquid formulations was ensured by also administering washings of the containers.

Consecutive 8-hr. collections of urine were started immediately upon dosing and were not terminated until nine consecutive 8-hr. samples gave negative results.

The urines were preserved by the addition of SFT tablets[5] and refrigerated until analyzed. All analyses were performed within 48 hr. of sample collection. The SFT tablets did not interfere with determinations of either QAC in water or urine or with the creatinine assay.

RESULTS

The term fractional excretion represents that portion of a QAC dose excreted during any single 8-hr. interval. The mean fractional excretions reported here have been averaged for the 12 subjects for each of the different formulations for each corresponding 8-hr. period and are expressed as percent of total dose. Excretion after intravenous administration is considered separately from oral administration.

After intravenous administration of anisotropine methylbromide, approximately 50% of the dose is excreted in the first 8-hr. period (Fig. 2). Excretion continues for 16 days with a pattern of fluctuating excretion rates until an average of 108% of the administered drug is excreted.

The mean fractional excretions of the orally administered QAC's are presented in Fig. 3. The same pattern of periodic excretion peaks is seen. Although excretion from these formulations is not as prolonged as excretion from the intravenous dose, duration of excretion is still appreciable. An analysis of variance (Anova) of this data was performed (Table II). Neither *F* value obtained in the complete 3-way Anova is significant. Overall QAC excretion levels do not differ. The times × formulations interaction shows no evidence for lack of parallelism between the various fractional excretion plots. It would seem, therefore, that the correspondence in time of occurrence of the excretion peaks in Fig. 3 is real and is indicative of a fundamental similarity in excretion behavior of the two QAC's under study. Superimposition of the corresponding section of the plot for fractional excretion of i.v. administered anisotropine

[3] Adams Midget Urinometer, Clay-Adams Inc., New York, N. Y.
[4] Labstix, Ames Co., Elkhart, Ind.
[5] Sodium fluoride-thymol tablets, Cambridge Chemical Products, Detroit, Mich.

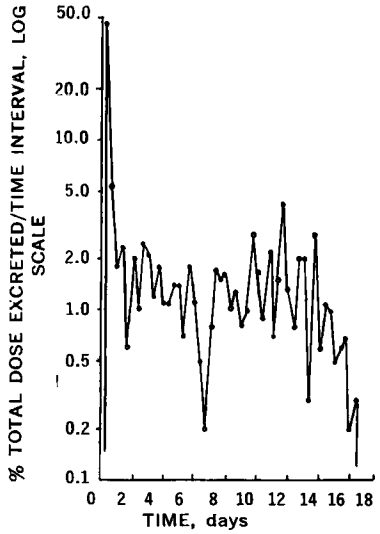

Fig. 2—Fractional excretion of anisotropine methylbromide by human subjects, after i.v. administration Total dose, 5 mg.

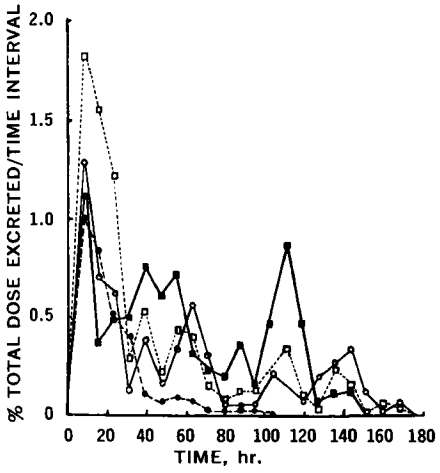

Fig. 3—Fractional excretion of QAC's by human subjects after oral administration. Dosages: anisotropine methylbromide, 25 mg.; propantheline bromide, 30 mg. Key: ●, anisotropine methylbromide tablets; ○, anisotropine methylbromide elixir; □, anisotropine methylbromide kaolin-pectin suspension; ■, propantheline bromide tablets.

TABLE III—TOTAL EXCRETION OF QAC'S

QAC	Formulation[a]	n	Dose, mg.	Mean Total Excretion, mg.	Mean Total Excretion, as % of Dose	Mean Total Excretional Time, hr.
Anisotropine methylbromide	Tablets	12	25	0.76	3.1	55
	Tablets[b]	6	20	1.42	7.1	91
	Elixir	12	25	1.42	5.7	125
	Elixir	3	20	0.99	4.9	51
	Kaolin-pectin suspension	11	25	1.49	6.0	64
	i.v. Injection	12	5	5.4	108	400
Propantheline bromide	Tablets	11	30[c]	2.20[d]	5.7	95
Anova[e]	F_1				1.63	7.48[f]
	F_2				0.47	2.96

[a] Formulation potencies: anisotropine methylbromide, 10-mg. tablet, 2 mg./ml. elixir, 0.33 mg./ml. kaolin-pectin suspension, 5 mg./ml. for i.v. injection, propantheline bromide, 15 mg./tablet. [b] Data from Pfeffer *et al.* (1) included for purposes for comparison. [c] Thirty mg. of anisotropine methylbromide is the molar equivalent of 24.2 mg. of anisotropine methylbromide. [d] Equivalent to 1.73 mg. of anisotropine methylbromide. [e] Anova excludes data from i.v. injection and the anisotropine methylbromide tablets and elixir administered at dosages of 20 mg. $F_1 = F$ for differences among all four oral dose forms. $F_2 = F$ for differences between means of the three oral anisotropine methylbromide dose forms and the propantheline bromide tablets. Significance at $p = 0.05$; $F_1 = 2.91$; $F_2 = 4.16$; at $p = 0.01$; $F_1 = 4.48$, $F_2 = 7.53$. [f] $p = 0.01$.

methylbromide (Fig. 2) onto Fig. 3 would reveal that a parallelism in excretion peaks is maintained between oral and intravenous dosage although the levels excreted differ. There was no obvious relationship between urine volume, weight or age of the subjects, and the variables reported upon here.

The mean values for total excretion time and total excretion are presented in Table III. The statistical analysis indicated that there are no significant differences between the observed QAC levels excreted after administration of the various oral formulations, either when they are compared to each other or when propantheline bromide is compared to the pooled mean excretion level of all the oral anisotropine methylbromide formulations.

The observed total excretion times of the anisotropine methylbromide in its various oral formulations also are not significantly different from the total excretion times for propantheline bromide administered as tablets.

In previous studies QAC excretion has been found to be highly variable (5–11). Despite these normally accepted variations, it is apparent that the several formulations of anisotropine methylbromide are equivalent. It is also of deep interest that the observed excretions of these two antispasmodics are very similar.

DISCUSSION

In a previous paper (1) it was indicated that, due to the nature of the picric acid assay, metabolized anisotropine methylbromide was not likely to be detected. The total mean recovery value of 108% after intravenous administration of anisotropine methylbromide has, therefore, three interesting implications: (*a*) all anisotropine methylbromide in the bloodstream may be eventually excreted through the kidneys, (*b*) this drug is apparently not metabolized to polar compounds in the human body, (*c*) if this QAC is undergoing enterohepatic recycling, the efficiency of intestinal reabsorption of the material excreted in the bile must approach 100%.

Rowland and Beckett (12) have observed successive peaks during the excretion, by humans, of amphetamine and methylamphetamine. This they attributed to the diurnal urinary pH rhythm. The degree of ionization of these weak bases shifts as the urinary pH shifts, altering the degree of reabsorption from the distal tubule to the kidney. This would not, however, appear to serve as an explanation for the periodic excretion peaks seen with QAC's. These strong quaternary bases, for which pKa's are not determinable, will not alter their degree of ionization at any physiologically attainable pH. Furthermore, the timing of the excretion peaks for these QAC's does not correspond to the timing of the diurnal pH changes reported by Rowland and Beckett.

Retention of QAC's in an intracellular tissue reservoir would not seem a likely explanation either. Although the QAC might be slowly released it should not be released in "pulses." If there is an upper limit to the amount of methyl anisotropinium cation the kidney can excrete in a given time period, it has not been approximated in these experiments. After intravenous injection of 5 mg. of anisotropine methylbromide, approximately 2.5 mg. can be excreted in an 8-hr. period and the average amount excreted in any succeeding peak is appreciably larger than the amount excreted in any peak after oral administration. Thus, in these studies, the amount excreted through the kidney would seem to be a function of the amount available to the kidney.

Enterohepatic cycling of the QAC seems to offer the best explanation of the observed phenomenon. Bile, after its formation, is stored in the gall bladder and is periodically released into the intestinal tract (13). This periodicity of bile release might correspond to the periodicity of QAC excretion. QAC excreted into the bile would therefore be held in the gall bladder to eventually be released with the bile. The QAC would be reabsorbed and a portion excreted in the kidney while the balance would be reexcreted into the bile.

By inference, the total excretions after oral dosage represent the total gastrointestinal absorption of anisotropine methylbromide. The amounts absorbed are retained *in vivo* for a prolonged period of time.

The excretion pattern and amount excreted for orally administered propantheline bromide is almost exactly the same as that of anisotropine methylbromide. The question of whether this could be a common excretion pattern for QAC's is raised.

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Keyphrases

Urinary excretion—quaternary ammonium compounds
Anisotropine methylbromide—human urinary excretion
Propantheline bromide—human urinary excretion
IV, oral administration—excretion times
Colorimetric analysis, urine—spectrophotometer

Significance of Kinetic Aspects in the Simultaneous Administration of Drugs

By MILDRED J. TARASZKA and ARLINGTON A. FORIST

Kinetic aspects, such as half-lives for absorption and elimination, as well as limiting solubilities, which should be considered in the simultaneous administration of drugs are discussed. Two simple hypothetical cases are presented: the selection of the ratio of two drugs with different rate constants for absorption and elimination to obtain similar average asymptotic serum levels of each drug on multiple-dose administration, and the selection of the ratio of two drugs with different rate constants for absorption and elimination and different solubilities to minimize the risk of crystalluria. Extension of the latter to the triple sulfas, on the basis of solubility and human blood level data in the literature, has given a "best" ratio of 1:3:4 for sulfadiazine:sulfamerazine:sulfamethazine, respectively, rather than the 1:1:1 now used.

WHEN TWO or more drugs are combined in a single formulation or are given in separate dosage forms at the same time, several factors should be considered in choosing the individual drugs and the amounts of each drug to be used: (*a*) biological spectra, (*b*) minimum effective circulating concentrations for biological activity, (*c*) possibilities of reactions between the combined drugs, (*d*) possibility of one drug affecting the biological response to the other drug(s), (*e*) rate constants for absorption or absorption half-lives, (*f*) rate constants for elimination or biological half-lives, (*g*) rates of production and elimination of metabolites (especially if the metabolites may be active or toxic), (*h*) intrinsic solubilities of the individual drugs and/or metabolites if crystalluria is a potential side reaction.

It is the authors' opinion that drug combinations could be more beneficial if all of the above factors are considered. However, only kinetic aspects that affect drug combinations are considered in this paper; that is, all the calculations are based on the assumption that the individual drugs have the correct spectra of biological activity, similar minimum effective circulating concentrations, and that they are compatible in the *in vitro* and *in vivo* systems. The importance of the kinetic aspects can be seen if the selection of the optimum dosage schedule is considered for multiple-dose therapy with a combination of two drugs which have biological half-lives of largely different magnitudes.

Received January 18, 1968, from the Pharmacy Research and Physical and Analytical Chemistry Units, Upjohn Company, Kalamazoo, MI 49001
Accepted for publication May 2, 1968.
Special acknowledgement goes to Jack I. Northam for solving the complex equations and programming the problem for the digital computer.

DISCUSSION

Example 1—When two or more drugs are combined in a single formulation, or the drugs are given in separate dosage forms at the same time, the multiple-dose serum levels attained with each drug will depend on each biological half-life plus *other* factors. The ratio of two drugs given in combination, which should be used to attain similar serum levels on multiple dosing, can be estimated in the following manner, assuming that the intrinsic drug solubilities, biological activities, and production of metabolites do not have to be considered. Furthermore, it is assumed that the single-dose serum level and urinary data for each drug fit the following model:

$$A \xrightarrow{k_1} C \xrightarrow{k_2} D \qquad \text{(Model 1)}$$

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$$A \xrightarrow{k_1} C \xrightarrow{k_2} D \qquad \text{(Model 1)}$$

where k_1 is the first-order rate constant for absorption, k_2 is the first-order rate constant for excretion and/or metabolite production, C is the serum compartment for the drug, and D is the excretion and/or metabolite compartment for the drug. Since more than one drug is involved, the rate constants and serum concentrations are labeled k_{1i}, k_{2i}, and C_i where $i = 1$ or 2 and denotes which drug is being considered.

According to Wagner *et al.* (1), the average asymptotic serum level of a drug on a multiple dose schedule is given by

$$\bar{C} = \frac{FD}{V_d k_2 \tau} \qquad \text{(Eq. 1)}$$

where F = fraction of dose absorbed, D = dose, V_d = volume of distribution, τ = dosage interval, and k_2 = first-order rate constant characterizing the biological half-life. This equation holds for any model as long as a series of simultaneous linear differential equations can be written describing the model; transfer from the serum is first order; and F, D, V_d, k_2, and τ are constants for each dose of the multiple-dose regimen in a given subject (1). Since two drugs are being considered, the following equations can be written corresponding to Eq. 1.

$$\bar{C}_1 = \frac{F_1 D_1}{V_{d1} k_{21} \tau_1} \qquad \text{(Eq. 2)}$$

$$\bar{C}_2 = \frac{F_2 D_2}{V_{d2} k_{22} \tau_2} \qquad \text{(Eq. 3)}$$

The same average asymptotic serum levels for the two drugs means $\bar{C}_1 = \bar{C}_2$. The ratio of the two drugs in the fomulation is found by rearranging and setting $\tau_1 = \tau_2$ since both drugs are given together.

$$\frac{D_1}{D_2} = \frac{F_2 V_{d1} k_{21}}{F_1 V_{d2} k_{22}} \qquad \text{(Eq. 4)}$$

The parameters on the right-hand side of Eq. 4, or the ratio D_1/D_2, can be calculated from single-dose data if it is assumed each drug reacts independently and will be unaffected by the presence of the other. The dosing interval τ is adjusted according to the smaller biological half-life.

Figures 1*a* and 1*b* show the effect on multiple-dose-serum levels of adjusting the ratio of two hypothetical drugs with differing absorption and biological half-lives, which are given in combination. Serum levels in Fig. 1 are computer calculated. Both drugs, 1 and 2, obey Model 1 with the listed assumptions and have the following values for their kinetic parameters: $k_{11} = 0.693$ hr.$^{-1}$, $k_{21} = 0.174$ hr.$^{-1}$, $F_1/V_{d1} = 0.025$ 1.$^{-1}$, $\tau_1 = \tau_2 = 6$ hr., $k_{12} = 1.39$ hr.$^{-1}$, $k_{22} = 0.087$ hr.$^{-1}$, and $F_2/V_{d2} = 0.0350$ 1.$^{-1}$. In Fig. 1*a*, drugs 1 and 2 are combined in the ratio of 1:1, respectively. In Fig. 1*b*, drugs 1 and 2 are combined in the ratio of 1:0.36, as predicted by Eq. 4. Identical serum-level curves of the two drugs cannot be obtained by altering the combination ratio of the two drugs; only the average asymptotic serum levels can be adjusted to be the same as seen in Fig. 1. The amount of variation about this average asymptotic serum level depends on the model, the dosing interval, and the magnitude of the absorption rate constant (1).

Example 2—The best ratio of two drugs which are given in combination to assure a minimum probability of crystallization of drug in the kidneys can be calculated, assuming the six following conditions: (*a*) each drug has an intrinsic solubility, S_1 and S_2, unaffected by the presence of the other drug; (*b*) the serum level data and urinary excretion data for each individual drug follow Model 1; when given as a single dose (the rate constants k_1 and k_2 are first order); (*c*) the absorption and elimination of each drug is unaffected by the presence of the other; (*d*) metabolites do not have to be considered; (*e*) the appearance of drug crystals in the urine is dependent on the concentration of the drug in the urine; (*f*) the urine flow is independent of the drug given.

According to Model 1, the rate of appearance of the drug in the urine is $d[D]/dt = k_2[C]$. The kinetic equations for Model 1 can be easily derived (2), and it is found that

$$[C] = \frac{[A_o]\ k_1}{k_2 - k_1} (e^{-k_1 t} - e^{-k_2 t}) \text{ when } k_1 \neq k_2 \qquad \text{(Eq. 5)}$$

where $[A_0]$ is the initial concentration in compartment A and equals FD/V_d.

The concentration of the drug in the urine at the time of saturation will equal the solubility of the drug. This time of saturation can be found from

$$[D]\Big]_0^S = \int_0^{t_s} \frac{d[D]}{dt}\, dt \qquad \text{(Eq. 6)}$$

where $[D]$ = concentration of drug in the urine, t_s = time of saturation, and S = saturation concentration (solubility at a given pH).

Substituting for $d[D]/dt$ and integrating Eq. 6 gives

$$S = \frac{[A_o]\ k_1 k_2}{k_2 - k_1} \left(\frac{-e^{-k_1 t_s}}{k_1} + \frac{e^{-k_2 t_s}}{k_2} \right) \qquad \text{(Eq. 7)}$$

The two equations corresponding to Eq. 7 can be written by using the second numerical subscript to indicate which drug is being considered and substituting FD/V_d for $[A_0]$.

$$S_1 = \frac{F_1 D_1 k_{11} k_{21}}{V_{d1}(k_{21} - k_{11})} \left(- \frac{e^{-k_{11} t_{s1}}}{k_{11}} + \frac{e^{-k_{21} t_{s1}}}{k_{21}} \right) \qquad \text{(Eq. 8)}$$

$$S_2 = \frac{F_2 D_2 k_{12} k_{22}}{V_{d2}(k_{22} - k_{12})} \left(- \frac{e^{-k_{12} t_{s2}}}{k_{12}} + \frac{e^{-k_{22} t_{s2}}}{k_{22}} \right) \qquad \text{(Eq. 9)}$$

Also,

$$D_1 + D_2 = D_T \qquad \text{(Eq. 10)}$$

where D_1 = dose of drug 1, D_2 = dose of drug 2, and D_T = total dose given.

To have the least chance for crystalluria, D_1 and D_2 have to be adjusted so the time of saturation for each drug is the same, $t_{s1} = t_{s2}$. Equations 8 and 9 are too complex to solve for t_{s1} and t_{s2} explicitly. However, they can be solved for D_1 and D_2 and substituted into Eq. 10 with $t_{s1} = t_{s2} = X$ to give:

$$\frac{S_1 (k_{21} - k_{11})\ V_{d1}}{(k_{11} e^{-k_{11}X} - k_{21} e^{-k_{11}X})\ F_1} + \frac{S_2 (k_{22} - k_{12})\ V_{d2}}{(k_{12} e^{-k_{12}X} - k_{22} e^{-k_{12}X}) F_2} = D_T \qquad \text{(Eq. 11)}$$

or

$$\sum_{i=1}^{2} \frac{S_i\,(k_{2i} - k_{1i})\,V_{di}}{(k_{1i}\,e^{-k_{2i}X} - k_{2i}\,e^{-k_{1i}X})\,F_i} = D_T \quad \text{(Eq. 12)}$$

In Eq. 11 everything is known from individual single-dose experiments except X and D_T. For a given value of D_T the value of X, which satisfies the equation, is determined by trial and error with a computer. Once X is determined the values of D_1 and D_2 are just equal to the individual terms in the summation.

When $X = \infty$ the terms on the left-hand side of Eq. 12 will become infinite. Therefore, for any value of D_T, a finite value of X exists and a given ratio of drug 1:drug 2 can be obtained to assure a minimum probability of crystalluria.

If the rate constants of absorption and elimination, the fraction absorbed, and the volume of distribution were the same for both drugs, then the best ratio of drug 1:drug 2 would be the ratio of their solubilities at the "mean" pH of human urine, $S_1:S_2$.

Application of Example 2—Example 2 has been extended to determine what ratio of sulfadiazine, sulfamerazine, and sulfamethazine gives the least chance of crystalluria in man.

According to the literature, the serum level and urinary excretion data indicated Model 2 for the absorption and elimination of sulfonamides.[1]

Both the excreted sulfonamide and the excreted acetylsulfonamide may precipitate in the urine and have to be considered. The times of saturation of the acetylsulfonamides, t_{sai}, can be found from

$$[D_i]_0^{sai} = \int_0^{t_{sai}} \frac{d[D_i]}{dt}\,dt \quad \text{(Eq. 13)}$$

where i denotes which acetylsulfonamide and S_{ai} their solubilities. Similarly, the times of saturation of the sulfonamides, t_{si}, can be found from

$$[E_i]_0^{si} = \int_0^{t_{si}} \frac{d[E_i]}{dt}\,dt \quad \text{(Eq. 14)}$$

According to Model 2, $d[D_i]/dt = k_3C_i$ and $d[E_i]/dt = k_4B_i$ and the corresponding integrated Eqs. for 13 and 14 become, respectively,

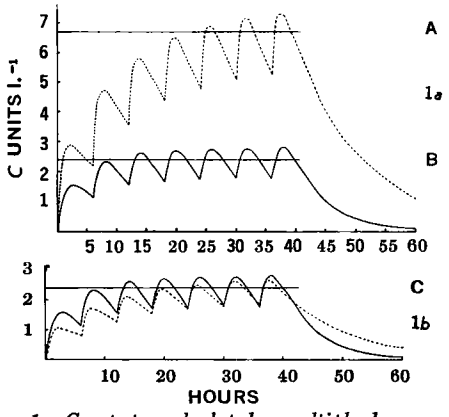


Fig. 1—Computer-calculated, multiple-dose serum levels of a combination of two hypothetical drugs with different kinetic constants. Key: ---, drug I with $k_1 = 0.693\ hr.^{-1}$, $k_2 = 0.174\ hr.^{-1}$, $F/V_d = 0.025\ l.^{-1}$ *and* $\tau = 6\ hr.$; ———, *drug 2 with* $k_1 = 1.39\ hr.^{-1}$; $k_2 = 0.087\ hr.^{-1}$, $F/V_d = 0.035\ l.^{-1}$ *and* $\tau = 6\ hr.$; *curves 1a represent 100 units each of drug 1 and drug 2 administered simultaneously; curves 1b represent 100 units of drug 1 and 35.7 units of drug 2 administered simultaneously.* A, $C_2 = 6.7$ *units* $l.^{-1}$; B, $C_1 = 2.4$ *units* $l.^{-1}$; C, $C_1 = C_2 = 2.4$ *units* $l.^{-1}$.

$$S_i = \frac{k_{1i}k_{4i}A_{oi}}{k_{2i} + k_{4i} + k_{5i} - k_{1i}}\left[\frac{e^{-(k_{2i}+k_{4i}+k_{5i})t_{si}}}{k_{2i} + k_{4i} + k_{5i}} - \frac{e^{-k_{1i}t_{si}}}{k_{1i}}\right] + \frac{k_{4i}A_{oi}}{k_{2i} + k_{4i} + k_{5i}} \quad \text{(Eq. 16)}$$

For ease of manipulation, Eq. 15 may be written as

$$S_{ai} = A_{oi}\,f_i\,(t_{sai}) \quad \text{(Eq. 17)}$$

where $f_i\,(t_{sai})$ is a function of t_{sai}, and Eq. 16 as

$$S_i = A_{oi}\varphi_i\,(t_{si}) \quad \text{(Eq. 18)}$$

Substituting $A_{oi} = F_iD_i/V_{di}$ and rearranging, Eq. 17 becomes

$$D_i = \frac{V_{di}\,S_{ai}}{F_i\,f_i\,(t_{sai})} \quad \text{(Eq. 19)}$$

and Eq. 18 becomes

$$D_i = \frac{V_{di}\,S_i}{F_i\varphi_i\,(t_{si})} \quad \text{(Eq. 20)}$$

$$S_{ai} = k_{1i}k_{2i}k_{3i}A_{oi}\left[\frac{-e^{-k_{1i}t_{sai}}}{k_{1i}\,(k_{3i} - k_{1i})\,(k_{2i} + k_{4i} + k_{5i} - k_{1i})} + \frac{e^{-(k_{2i} + k_{4i} + k_{5i})t_{sai}}}{(k_{2i} + k_{4i} + k_{5i})\,(k_{3i} - k_{2i} - k_{4i} - k_{5i})\,(k_{2i} + k_{4i} + k_{5i} - k_{1i})} + \frac{e^{-k_{3i}t_{sai}}}{k_{3i}(k_{3i} - k_{1i})\,(k_{2i} + k_{4i} + k_{5i} - k_{3i})}\right] + \frac{k_{2i}A_{oi}}{k_{2i} + k_{4i} + k_{5i}} \quad \text{(Eq. 15)}$$

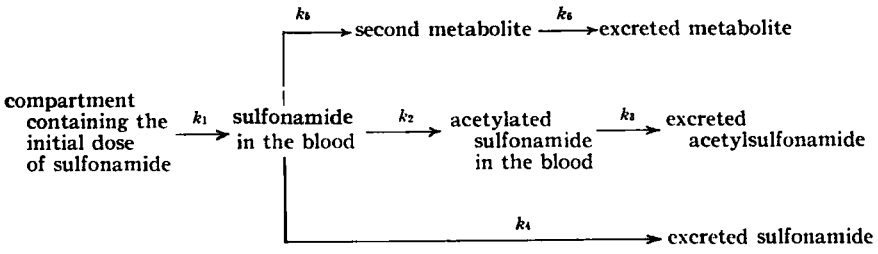
[1] Model 2 for sulfonamides does not include discrete steps for tubular reabsorption or protein binding since the problem only requires characterization of how rapidly the drug concentrates in the urine. All the estimated rate constants are really composite values representing several processes since all models are only approximations for what is really taking place *in vivo*. According to the principle of microscopic reversibility, each step in a mechanism should be reversible, but experimental techniques are not sophisticated enough to determine all discrete rate constants, only "overall" macroconstants are available. The value for k_4 characterizes how fast the sulfonamide is eliminated, and it includes effects from several processes as tubular reabsorption, the distribution of drug into various tissues according to their perfusion and partition characteristics, protein binding in these various tissues and blood as well as the discrete elimination process.

The total dose of sulfonamides, D_T, must equal the sum of the doses of the individual sulfonamides, ΣD_i. For a mixture of three

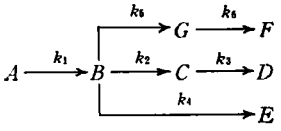
$$D_T = D_1 + D_2 + D_3 \quad \text{(Eq. 21)}$$

and three equations can be written for Eq. 19 and three for Eq. 20.

Since the parent compound and the acetylated derivative have to be considered for each sulfonamide, eight possibilities exist for Eq. 21; that is, all the possible combinations of the six equations corresponding to Eqs. 19 and 20.



in simple notation



(Model 2)

$$\text{Case A: } D_T = \frac{V_{d1}\,S_{a1}}{F_1 f_1 (t_{sa1})} + \frac{V_{d2}\,S_{a2}}{F_2 f_2 (t_{sa2})} + \frac{V_{d3}\,S_{a3}}{F_3 f_3 (t_{sa3})}$$

$$\text{Case B: } D_T = \frac{V_{d1}\,S_{a1}}{F_1 f_1 (t_{sa1})} + \frac{V_{d2}\,S_{a2}}{F_2 f_2 (t_{sa2})} + \frac{V_{d3}\,S_3}{F_3 \varphi_3 (t_{s3})}$$

$$\text{Case C: } D_T = \frac{V_{d1}\,S_{a1}}{F_1 f_1 (t_{sa1})} + \frac{V_{d2}\,S_2}{F_2 \varphi_2 (t_{s2})} + \frac{V_{d3}\,S_3}{F_3 \varphi_3 (t_{s3})}$$

$$\text{Case D: } D_T = \frac{V_{d1}\,S_{a1}}{F_1 f_1 (t_{sa1})} + \frac{V_{d2}\,S_2}{F_2 \varphi_2 (t_{s2})} + \frac{V_{d3}\,S_{a3}}{F_3 f_3 (t_{sa3})}$$

$$\text{Case E: } D_T = \frac{V_{d1}\,S_1}{F_1 \varphi_1 (t_{s1})} + \frac{V_{d2}\,S_{a2}}{F_2 f_2 (t_{sa2})} + \frac{V_{d3}\,S_{a3}}{F_3 f_3 (t_{sa3})}$$

$$\text{Case F: } D_T = \frac{V_{d1}\,S_1}{F_1 \varphi_1 (t_{s1})} + \frac{V_{d2}\,S_{a2}}{F_2 f_2 (t_{sa2})} + \frac{V_{d3}\,S_3}{F_3 \varphi_3 (t_{s3})}$$

$$\text{Case G: } D_T = \frac{V_{d1}\,S_1}{F_1 \varphi_1 (t_{s1})} + \frac{V_{d2}\,S_2}{F_2 \varphi_2 (t_{s2})} + \frac{V_{d3}\,S_3}{F_3 \varphi_3 (t_{s3})}$$

$$\text{Case H: } D_T = \frac{V_{d1}\,S_1}{F_1 \varphi_1 (t_{s1})} + \frac{V_{d2}\,S_2}{F_2 \varphi_2 (t_{s2})} + \frac{V_{d3}\,S_{a3}}{F_3 f_3 (t_{sa3})}$$

The kinetic constants and solubilities used for the sulfonamides under consideration are listed in Table I and were taken from the indicated literature.

To have the least chance for crystalluria the time of saturation for each drug has to be the same; that is, the times of saturation in each of the individual cases (*A* through *H*) have to be equal (for Case *A*, $t_{sa1} = t_{sa2} = t_{sa3} = X_A$). The case which gives the minimum total dose, D_T, for saturation will be the limiting case, and the ratio of the terms in the summation will be the best ratio for the drugs in the formulation.

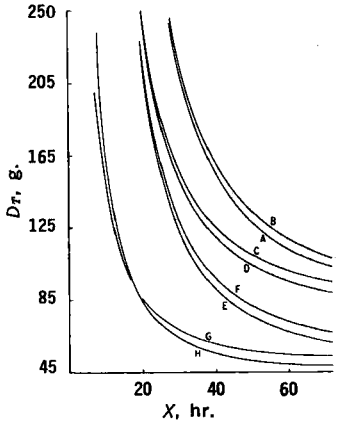


Fig. 2—Plot of D_T versus X *for the 8 cases*, A–H.

The values of D_T for various X_j's are plotted in Fig. 2 for the eight different cases (*A* through *H*). A digital computer was used to calculate the values. For a given D_T greater than the minimum asymptotic D_T for Case *H* either Case *H* or Case *G* will be limiting. Since Case *H* is limiting at lower values of the total dose, D_T, Case *H* is the one of interest. The fact that Case *H* is limiting means that for sulfadiazine and sulfamerazine the parent compounds will cause crystalluria if the dose is

TABLE I—KINETIC CONSTANTS AND SOLUBILITIES FOR THE TRIPLE SULFAS

Parameter	Sulfadiazine, $i = 1$	Sulfamerazine, $i = 2$	Sulfamethazine, $i = 3$
S_i[a] mg. ml.$^{-1}$	0.16	0.34	0.62
S_{ai}[b] mg. ml.$^{-1}$	0.33	0.35	0.74
k_{1i} hr.$^{-1}$	0.65[c]	0.60[d]	0.66[e]
$k_{2i} + k_{4i} + k_{5i}$ hr.$^{-1}$	0.0414	0.0295	0.095
k_{2i}[f] hr.$^{-1}$	0.15(0.0414) = 0.0062	0.35(0.0295) = 0.0103	0.6(0.095) = 0.057
k_{4i}[f] hr.$^{-1}$	0.85(0.0414) = 0.0352	0.45(0.0295) = 0.0133	0.4(0.095) = 0.038
k_{3i} hr.$^{-1}$	0.06	0.04	0.20
V_{di}/F_i ml.	30,000	23,000	18,000

[a] Sulfadiazine equivalent solubilities at 37° and pH = 6 for the sulfas. Taken from *Reference 3*. [b] Sulfadiazine equivalent solubilities at 37° and pH = 6 for the acetylated sulfas. Taken from *Reference 3*. [c] V_{d1}/F_1 and k_{11} were estimated from Fig. 1a of *Reference 6*. The other kinetic constants for sulfadiazine are from Table II of *Reference 5*. [d] V_{d2}/F_2 and k_{12} were estimated from Fig. 1 of *Reference 4*. The other kinetic constants for sulfamerazine are from Table II of *Reference 5*. [e] The kinetic constants for sulfamethazine are calculated from data in *Reference 4*. [f] The kinetic constants k_{2i} and k_{4i} were estimated by multiplying the overall elimination rate constant, $k_{2i} + k_{4i} + k_{5i}$, by the fraction of the sulfonamide excreted as the acetylsulfonamide or the unchanged sulfonamide, respectively. The values used for these fractions were taken from Table IV of *Reference 7*.

large enough, while for sulfamethazine the acetyl derivative will cause crystalluria (this can be seen from the equation for Case H). In order to have the largest total concentration of sulfonamides without crystalluria, the ratio of sulfadiazine–sulfamerazine–sulfamethazine should be 1:3.1:3.9 (on a sulfadiazine equivalent). This is the ratio of the terms in the equation for Case H when $t_{s1} = t_{s2} = t_{s3} = \infty$. The ratio in terms of the individual compounds instead of the sulfadiazine equivalent is sulfadiazine–sulfamerazine–sulfamethazine = 1:3.2:4.3. On the average this ratio should have the least chance for crystalluria, if the kinetic constants are the best average ones. Since the kinetic constants in some cases were only estimated from blood level curves in the literature, they may not be the best.

It should be noted in Fig. 2 that the numerical values for D_T are large. This is a result of using the best ratio of sulfas for the given set of conditions and equations which assume a constant urine volume. The effect of varying urine volume or flow rate was not considered because the objective was only to calculate the best ratio of triple sulfas which gives the least chance of crystalluria. Case H will always be limiting whatever the flow rate.

The solubilities of the sulfonamides depend on pH. Above pH 7 the solubilities increase rapidly. The solubilities at pH = 6 of the three sulfonamides in question were chosen because this approaches the normal human urine pH, and the ratio of the solubilities of the three sulfonamides is almost constant over the pH range 5.6–6.5 (3). Therefore, the calculated ratio of 1:3.2:4.3 for the three sulfonamides should be the best ratio over the pH range 5.6 to 6.5.

As the solubility and/or the biological half-life of a drug increases, the incidence for crystalluria will become less. If the three sulfonamides in a combination have similar long biological half-lives, similar half-lives of acetylated derivative production and elimination, and similar fractions absorbed and volumes of distribution, then the ratio of the sulfonamides in the formulation should be the ratio of their solubilities.

The calculations indicate that kinetic aspects can be important in the simultaneous administration of drugs. The triple sulfas was used as an example for the application of kinetics and limiting solubilities to obtain a formulation with the best margin of safety. To show this increased margin of safety, a large-scale clinical test would be needed.

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Keyphrases

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Sulfadiazine-sulfamerazine-sulfamethazine—co-administration
Absorption-elimination rates—simultaneous administration
Solubility, drug—simultaneous administration
Kinetic equations—multiple drug dosage forms

Griseofulvin Absorption in Man After Single and Repeated Treatments and Its Correlation with Dissolution Rates

By SAMSON SYMCHOWICZ and BERNARD KATCHEN

Absorption of griseofulvin from six different preparations was studied in man. Plasma levels were followed for 1 day after a single oral 500-mg. dose, and for 7 days after daily 500-mg. doses. A high correlation was found between dissolution rates in simulated intestinal fluid and absorption during 0–25 hr., 49–173 hr., and 0–173 hr.

Griseofulvin is widely used for treating fungal infections in man and animals. Its relatively high dose and meager water solubility (15 mcg./ml. at 37°) might contribute to its poor and variable absorption. Because certain minimal drug-plasma levels are needed for an effective cure (1–3), griseofulvin preparations must be carefully designed to ensure maximum absorption.

The authors have shown previously (4) that

Received February 27, 1968, from the Schering Corporation, Departments of Physiology and Biochemistry and Pharmaceutical Research, Bloomfield, NJ 07003
Accepted for publication May 2, 1968.
The authors thank Mr. M. Miller for the statistical analysis and Miss La Reine La Mar for her excellent technical assistance.

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dissolution rates in simulated intestinal fluid correlate well with drug absorption following a single 500-mg. oral dose. The present communication shows there is also a correlation between dissolution rate and absorption following 7 daily 500-mg. doses of griseofulvin. Thus, the absorption characteristics of griseofulvin preparations can be conveniently predicted on the basis of dissolution rate data.

EXPERIMENTAL

In the first part of this study, four different griseofulvin preparations (No. 1–4) were given at weekly intervals to eight normal humans in a replicated Latin square crossover design (5). Before breakfast, each subject ingested one 500-mg. tablet (preparation 2) or four 125-mg. tablets (preparations 1,3,4).

In the second part of this study, three different griseofulvin preparations (No. 2,5,6) were given to 18 normal subjects. Each subject ingested one 500-mg. tablet (preparation 2) or two 250-mg. tablets (preparations 5,6). Preparation 5 has the same composition as 1, and preparation 6 the same composition as 3.

All subjects were told to avoid foods and drugs which interfere with the griseofulvin plasma assay (6). Blood was drawn at specified time intervals (Tables I, IV), and plasma samples were assayed fluorometrically (7) in duplicate. All plasma levels were corrected for their 0-hr. values.

Mean plasma level, which is the absorption index, was calculated by dividing the area under the plasma level curve (calculated by the trapezoidal rule) by the appropriate time interval.

Dissolution rates (Table III) were measured in simulated intestinal fluid as described previously (4). Single 125-mg. tablets, half of the 250-mg., and one quarter of the 500-mg. tablets were assayed.

RESULTS AND DISCUSSION

Single-Dose Study—Table I shows the individual plasma levels at different times following ingestion of the four preparations. Plasma levels vary widely, but, in general, peak at 4 hr. There are large variations in drug absorption between subjects on all preparations. This agrees with the findings of other investigators (1, 8).

Table II—Analysis of Mean Griseofulvin Plasma Levels by Duncan's Multiple-Range Statistic

Treatment[a]	Area/25
1	1.02
4	0.93
2	0.83
3	0.74

[a] Values not connected by a solid line differ significantly. $1 > 3$ ($p < 0.05$); $1 > 2$ ($p < 0.10$); $4 > 3$ ($p < 0.10$).

An analysis of variance procedure for Latin square crossover designs (5), applied to the plasma levels in Table I, shows that treatments, time, and the treatment × time interaction are statistically significant factors ($p = 0.05$). Duncan's multiple-range test, applied to the area/25 values in Table I, shows (Table II) that preparation 1 is significantly better than preparations 2 and 3, and that preparation 4 is significantly better than 3.

Figure 1 shows a good correlation between the mean plasma levels (area/25) in Table I and the logarithm of the 30-min. dissolution values in Table III. The correlation coefficient is 0.94 ($p = 0.04$). The 15- and 10-min. dissolution values have correlation coefficients of 0.96 ($p = 0.03$) and 0.92 ($p = 0.05$), respectively. These results agree well with the authors' previous single-dose study (4).

Because griseofulvin therapy requires prolonged drug administration, the correlation between dissolution rates and drug absorption during prolonged treatment was studied. This was also of interest

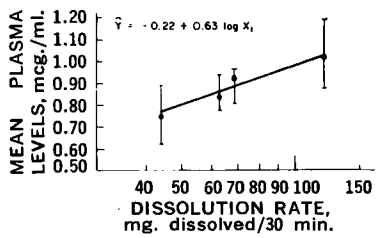

Fig. 1—Correlation of dissolution rate and mean griseofulvin plasma levels. Correlation coefficient = 0.94 (p = 0.04). Brackets enclose 95% confidence intervals of regression line.

Table I—Griseofulvin Plasma Levels (mcg./ml.) Following an Oral 500-mg. Dose

Preparation	Time, Hr.	Subject 1	2	3	4	5	6	7	8	Average	Area/25
1	2	1.73	2.20	1.23	1.23	0.62	1.13	0.35	1.00	1.18	1.02
	4	1.46	1.60	1.58	1.18	1.18	1.51	0.87	1.13	1.17	
	8	0.70	1.15	1.45	0.83	0.82	2.32	0.94	0.84	1.13	
	25	0.71	1.06	0.77	0.49	0.99	1.00	1.11	0.86	0.86	
2	2	0.72	1.07	0.29	0.82	0.43	1.49	0.29	0.75	0.73	0.83
	4	0.82	1.08	1.62	1.13	0.59	1.58	0.53	0.83	1.02	
	8	0.64	0.94	2.20	0.58	0.60	0.86	0.77	0.80	0.92	
	25	0.78	0.62	0.58	0.85	0.65	0.59	1.00	1.03	0.76	
3	2	0.29	0.67	0.49	0.56	0.47	0.75	0.35	0.48	0.51	0.74
	4	0.76	0.71	1.40	0.79	0.52	1.46	0.80	1.00	0.93	
	8	0.96	0.54	1.17	0.68	0.51	1.10	0.51	1.08	0.82	
	25	0.66	0.61	0.53	0.76	0.94	0.69	0.82	0.79	0.72	
4	2	1.00	0.66	0.94	0.64	0.15	1.07	0.50	0.27	0.65	0.93
	4	1.81	0.97	1.58	1.00	0.84	1.20	1.02	1.52	1.24	
	8	1.71	0.71	1.38	1.01	0.93	0.93	0.93	1.10	1.09	
	25	0.84	0.64	0.68	0.54	1.03	0.58	1.00	1.00	0.79	

TABLE III—DISSOLUTION RATES OF GRISEOFULVIN PREPARATIONS

Time, min.	% Dissolved Preparation[a] 1	2	3[b]	4	5	6
5	24	17	7	13	21	11
10	54	30	15	31	48	17
15	77	37	24	42	70	22
30	97	50	35	55	94	33
45	99		45	65	98	42
60	99	65	52	75	100	44

[a] Preparations: 1, 3, 4—125-mg. tablets; 5, 6—250-mg. tablets; 2—500-mg. tablets. [b] This preparation is the same as preparation 2 in the authors' previous study (4).

in view of a report (9) urging caution when interpreting data from a single-dose study with griseofulvin.

Multiple-Dose Study—Table IV shows the individual griseofulvin plasma levels obtained with three preparations, in 18 subjects treated daily for 7 days with 500 mg. of griseofulvin. Table V compares the mean plasma levels in the same eighteen subjects. The data in Table IV show that the peak and residual plasma levels for each treatment group and also within most of the subjects are remarkably constant after the second or third dose. Griseofulvin mean plasma drug levels plateau after the third daily dose with all preparations (Table V).

Analysis of variance of the mean plasma levels in Table V does not show any statistically significant differences between preparations. This is probably due to the small number of subjects, large intersubject variation, and lack of a crossover design. Despite the lack of demonstrable statistically significant differences between preparations, de-

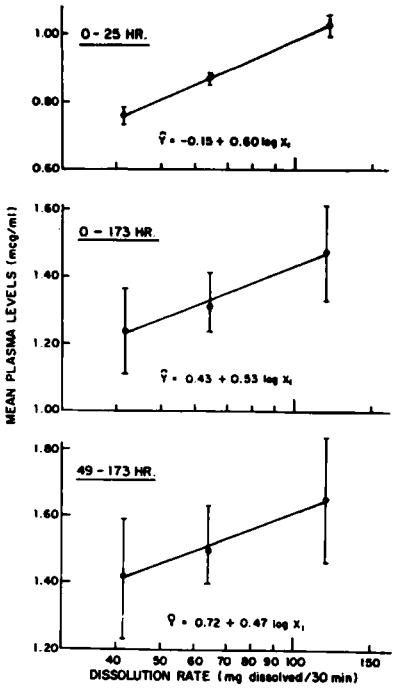

Fig. 2—Correlation of dissolution rate and mean griseofulvin plasma level. Correlation coefficients are: 0.999 (p = 0.015) for 0–25 hr., 0.992 (p = 0.077) for 0–173 hr., and 0.995 (p = 0.056) for 49–173 hr. Brackets enclose 95% confidence intervals of regression lines.

TABLE IV—GRISEOFULVIN PLASMA LEVELS (mcg./ml.) FOLLOWING AN ORAL 500-mg. DOSE DAILY FOR 7 DAYS[a]

Preparation	Subject	Time, hr. 2	4[b]	8	25[c]	49[c]	53[b]	97[c]	101[b]	169[c]	173[b]
2	1	0.97	0.92	0.78	0.74	0.89	1.60	0.96	1.82	1.16	2.16
	2	0.49	1.68	1.12	0.61	0.85	1.44	1.03	2.44	0.97	1.86
	3	0.54	0.71	0.65	0.43	0.96	1.65	0.85	1.79	1.12	2.20
	4	0.41	1.68	1.12	0.92	1.29	2.11	1.34	2.48	1.03	2.40
	5	0.76	1.87	1.42	0.53	0.76	2.49	0.74	2.30	0.85	2.39
	6	0.45	1.31	1.03	0.87	1.09	1.87	1.15	1.60	0.94	1.37
	Average	0.60	1.36	1.02	0.68	0.97	1.86	1.01	2.07	1.01	2.06
5	7	1.43	1.14	0.98	0.90	1.19	1.85	1.23	2.35	1.30	1.96
	8	1.19	0.97	0.83	0.52	0.81	1.69	0.77	1.54	0.75	1.47
	9	0.90	1.36	0.97	0.83	0.83	1.26	1.20	1.98	1.10	1.72
	10	1.27	1.54	1.17	0.93	1.55	2.66	1.57	2.14	1.44	2.33
	11	2.08	2.22	1.89	1.23	1.35	2.58	1.99	2.98	1.35	2.41
	12	0.81	1.14	0.56	1.14	1.27	1.77	1.25	1.65	1.30	1.88
	Average	1.28	1.39	1.07	0.92	1.16	1.97	1.33	2.11	1.21	1.96
6	13	0.41	0.70	0.63	0.80	1.12	1.17	1.38	1.79	1.18	1.61
	14	1.20	1.90	1.75	0.82	1.25	2.70	1.36	2.38	1.36	3.00
	15	0.68	0.51	0.68	0.61	1.18	1.23	0.97	1.43	0.93	1.04
	16	0.44	0.98	0.62	0.36	0.84	1.38	0.85	1.46	0.69	1.32
	17	0.67	0.80	0.77	0.74	0.84	2.31	0.91	2.27	0.88	1.99
	18	0.78	1.07	0.69	0.60	0.77	2.01	0.75	2.15	0.65	1.16
	Average	0.70	1.00	0.86	0.65	1.00	1.80	1.04	1.91	0.95	1.68

[a] Drug was given at 0, 25, 49, 73, 97, 121, 149, and 169 hr.; plasma was analyzed at the indicated time intervals. [b] Peak plasma levels. [c] Residual plasma levels (blood drawn just prior to drug ingestion).

TABLE V—MEAN GRISEOFULVIN PLASMA LEVELS, mcg./ml.[a]

Preparation	Hr. 0–25	25–49	49–53	53–97	97–101	101–169	169–173	49–173	0–173
2	0.87	0.83	1.42	1.44	1.54	1.54	1.54	1.50	1.31
5	1.03	1.05	1.57	1.65	1.72	1.67	1.58	1.65	1.48
6	0.76	0.84	1.40	1.42	1.47	1.43	1.32	1.42	1.24

[a] Areas under curve divided by periods as indicated.

tailed inspection of the data reveals some interesting points. For example, the ranking of preparations based on mean plasma levels during the first 25 hr. (single dose) remains the same throughout the entire experiment (except for the 25–49-hr. period). Also, if the residual plasma levels are considered (values 25 hr. after the first dose, and 24 hr. after the second, fourth, and seventh doses), the following statistically significant differences emerge: at 25 hr., preparation 5 > 6 (p = 0.1, least significant difference), at 97 hr., preparation 5 > 6 (p = 0.1, Duncan's multiple-range statistic), and 5 > 2 (p = 0.05, Duncan's multiple-range statistic). Residual plasma levels may be important in griseofulvin therapy since a minimum level of 1 mcg./ml. appears to be needed for an effective cure (1).

When the dissolution values (Table III) are correlated with the mean plasma levels (Table V), a good correlation is seen (Fig. 2) between the logarithm of the 30-min. dissolution values and mean plasma levels after 25 hr. (effect of a single dose), 49–173 hr. (the plateau region), and 0–173 hr. (total experimental period). The correlation coefficients for the 3 time periods are 0.999 (p = 0.015), 0.992 (p = 0.077), and 0.995 (p = 0.056). The slope of the 0–25-hr. regression line compares favorably with the slope of the single-dose treatment (0.60 in Fig. 2 *versus* 0.63 in Fig. 1). The slopes for the later time intervals (Fig. 2) are lower than the 0–25-hr. slope because differences between the mean plasma levels of the preparations are smaller for the 49–173-hr. period.

Since dissolution rates correlate well with griseofulvin absorption after a single dose, as well as after repeated drug administration, they should serve as an effective tool for selecting therapeutically useful griseofulvin preparations.

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Keyphrases

Griseofulvin absorption—human
Absorption-dissolution rate correlation—griseofulvin
Plasma levels, griseofulvin—single, multiple doses
Fluorometry—plasma griseofulvin analysis

Further Applications of Diazotized 4-Amino-6-chloro-*m*-benzenedisulfonamide

Colorimetric Determination of Pyridoxine Hydrochloride, 17β-Hydroxy-7aα-17α-dimethyl-B-homo-A-norestrane-3,6-dione and Morphine Sulfate

By TIBOR URBÁNYI and SUSAN BUDAVARI

The diazotization product of 4-amino-6-chloro-*m*-benzenedisulfonamide has been found to be a useful reagent for the colorimetric determination of a vitamin, steroid, and alkaloid. A general method, with slight modification, can be applied to those compounds which contain a phenolic or similar-to-phenolic hydroxyl group. Pyridoxine hydrochloride, 17β-hydroxy-7aα-17α-dimethyl-B-homo-A-norestrane-3,6-dione, and morphine sulfate were used as coupling components in this study. The optimum reaction conditions for coupling and color formation have been determined and the applicability of the method in the presence of commonly used ingredients is demonstrated.

A REPORT ON the colorimetric determination of estrogenic hormones by coupling with diazotized 4-amino-6-chloro-*m*-benzenedisulfonamide reagent has already been published (1). The purpose of the present investigation was to extend the application of that method to the quantitative determination of a phenolic alkaloid,

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Further Applications of Diazotized 4-Amino-6-chloro-*m*-benzenedisulfonamide

Colorimetric Determination of Pyridoxine Hydrochloride, 17β-Hydroxy-7aα-17α-dimethyl-B-homo-A-norestrane-3,6-dione and Morphine Sulfate

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a nonphenolic steroid, and similar-to-phenolic vitamin; morphine sulfate, 17β-hydroxy-7aα-17α-dimethyl - B - homo - A - norestrane - 3,6 - dione (steroid); and pyridoxine hydrochloride, respectively. Although the structures of these three compounds differ considerably, each has a similar functional group upon which the proposed method of determination is based.

A search of the literature reveals numerous assay methods for both morphine and pyridoxine. This attests to the importance of the compounds, as well as the difficulty involved in developing satisfactory analytical procedures which are quantitative and sensitive. For example, many alkaloids and vitamins can be determined by thin-layer chromatography (2, 3) with extreme sensitivity; but the results are not always quantitative. The numerous gas chromatographic methods available (4, 5) require great care in column preparation to secure reliable results. Ion-exchange methods (6, 7) generally suffer from the lack of reproducibility. Colorimetric techniques (8–10) can be quite sensitive and quantitative but they, as in the case of the USP procedure for pyridoxine in decavitamin capsules (11), are often time consuming and require rigidly controlled conditions. Among other colorimetric methods are the coupling reactions with diazonium salts. Diazotized sulfanilic acid reagent (12, 13) is often used, but this procedure fails to yield reproducible quantitative results. Aliev (14) proposes a method for pyridoxine determination which is based on the formation of a red-violet zinc complex with the stabilized diazo salt of norsulfazole. This method, as well as the others, proves less satisfactory than the proposed method for reasons of either poor reproducibility, length or complexity, or lack of sensitivity.

Alternate procedures for the analysis of the steroid cannot be cited since no previous references appear in the literature.

The need for a quantitative and sensitive method for the determination of pyridoxine and morphine as pure substances and in pharmaceutical formulations was apparent. Since pharmaceutical interest in steroids similar to the one presented here can be expected, the method for its analysis was included. The aim of this report, therefore, was to propose a practical and general colorimetric procedure for the analysis of these compounds.

EXPERIMENTAL

A Beckman model DU spectrophotometer was used to determine the absorbances, and a Cary model 11 recording spectrophotometer was used to record the spectra.

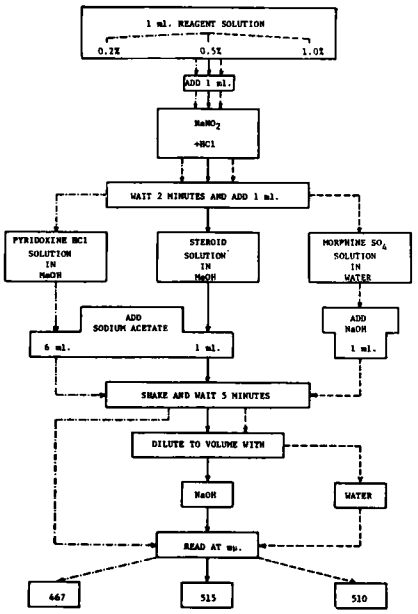


Fig. 1—Schematic diagram.

Reagents and Solutions—4-Amino-6-chloro-*m* benzenedisulfonamide (disulfonamide), 0.2, 0.5, and 1.0% methanolic solutions; sodium nitrite, 1.0% aqueous solution; hydrochloric acid, 1 *N;* sodium acetate, 1 *N* solution; sodium hydroxide, 1 *N* aqueous solution.

Preparation of Assay Solution—The compounds used in this study were dissolved in either methanol or water, whichever provided the optimum conditions for the color measurements. Solutions containing approximately 0.0025% of pyridoxine hydrochloride, 0.015% of steroid in methanol, and 0.13% morphine sulfate in water were prepared.

General Procedure for Color Development—Into separate 10-ml. volumetric flasks 1.0 ml. each of disulfonamide, sodium nitrite, and hydrochloric acid solutions were successively pipeted and mixed well. The solutions were allowed to stand 1–2 min., then 1.0 ml. of assay solution and 1.0 ml. of solvent (blank) were added. The solutions were buffered and allowed to stand approximately 5 min. at room temperature. Each flask was diluted to volume with the proper solvent, and its absorbance was determined at the given wavelength in 1-cm. cells against its respective blank.

The three compounds considered in this study belong to significantly different categories which precludes the use of a rigorous general method which can be applied to each with equal success. Since individual adaptations were made in order to obtain optimum conditions for each analysis, a schematic diagram (Fig. 1) is provided which lists the step-by-step procedure proposed.

DISCUSSION

The absorption spectra of the final coupled products of pyridoxine hydrochloride, steroid, and morphine sulfate with diazotized disulfonamide are shown in Fig. 2.

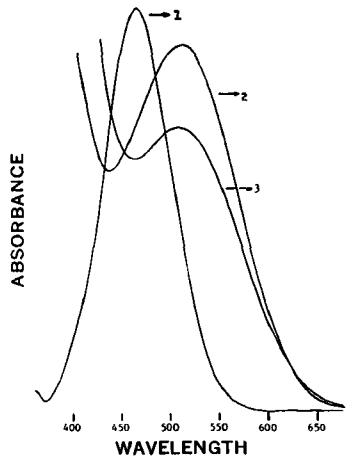


Fig. 2—Absorption spectra of coupled products. Key: 1, pyridoxine hydrochloride; 2, 17β-hydroxy-7aα-17α-dimethyl-B-homo-A-norestrane-3,6-dione; 3, morphine sulfate.

Pyridoxine Hydrochloride—It is apparent that while both the alkaloid and steroid exhibit broad absorption maxima in the 510–515-mμ region, the vitamin absorption maximum appears considerably lower at 467 mμ. This hypsochromic displacement of the absorption maximum in the case of vitamin, along with the much greater color intensity of its coupled product, can be attributed to the fact that the pyridoxine has the most favorable coupling position which is *para* to the phenolic hydroxyl group as well as *ortho* to the ring nitrogen, and thus exhibits strong electron density. Only coupling *ortho* to the hydroxyl group is possible for the morphine and the steroid. The intensity of absorbance was significantly affected by the choice of diluents and the pH of the solution. The color intensity of the final solution was increased 5–10% when the pyridoxine to be determined was dissolved in methanol rather than water. Furthermore, the fact that the intensity was significantly greater when acetate buffer was chosen as the diluent rather than sodium hydroxide is consistent with the observation of Stamm and Zollinger (15). They reported that a 10-fold increase in the concentration of acetate ions catalyzes the coupling reaction and produces five times more *para* coupling.

The most intense and reproducible color was obtained when the final pH was between 5.3 and 6.0. Therefore, 1 *N* sodium acetate, which brought the solution to the suitable pH upon dilution, was utilized as the diluent. Although the orange color developed immediately upon dilution with sodium acetate, it was found advisable to wait about 3 min. before reading the solution to avoid interference from the bubbles which develop.

TABLE I—MOLAR ABSORPTIVITIES

Compd.	ε ($\lambda_{max.}$)
Morphine sulfate	2.1×10^4
Steroid	8.7×10^4
Pyridoxine hydrochloride	35.7×10^4

The relationship between the concentration and the absorbance at 467 mμ was found to be linear over a range of 0–0.04 mg./assay. The molar absorptivity value is considerably larger than the values for morphine and steroid as seen in Table I.

Steroid—The synthetic steroid used in this study is significantly different from the phenolic estrogens considered in the previous publication (1). It is nonaromatic and has the structural formula in Scheme I.

Experimental data have indicated that the enol form of the steroid predominates in the enol–keto equilibrium, thereby making its determination as a coupling component with diazonium salts possible. Based on the above, it was evident that the pH of the solution at the coupling stage should be critically controlled. Sodium acetate was found to be the best buffering agent for this purpose. Although a stable color was obtained omitting the sodium acetate, the results were not reproducible, and a bathochromic shift was observed. An attempt was made to vary the sodium acetate buffer concentration and also to replace it with sodium bicarbonate, sodium carbonate, and sodium hydroxide solutions. Color instability was noted, and the Beer's law plots did not intersect the origin. The most reproducible values were obtained when coupling was carried out with 1 *N* sodium acetate solution at pH 5 ± 0.3, and the coupling time was 5 min. Color developed immediately upon the addition of 1 *N* sodium hydroxide and remained constant for at least 30 min. (Fig. 3). No absorption peak was obtained when the solution was diluted with sodium acetate. Beer's law was valid for this steroid at 515 mμ over a range of 0–0.25 mg./assay.

Morphine Sulfate—The rate of coupling and the color intensity of the coupled product with diazo-

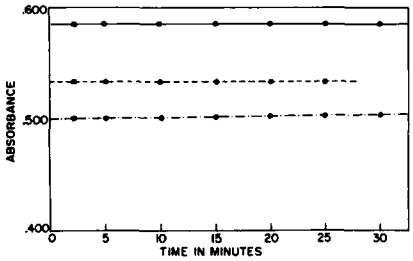
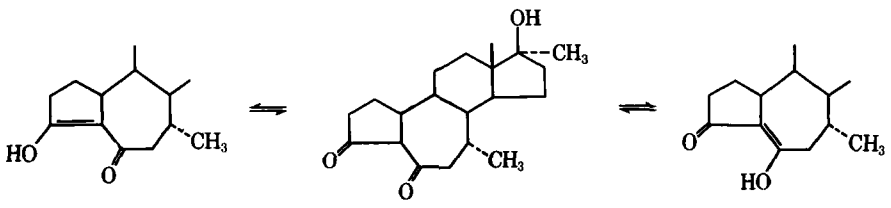
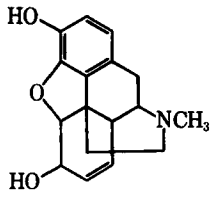


Fig. 3—Absorbance of final solution versus *time. Key: ———, pyridoxine hydrochloride; ---, steroid; ·-·, morphine sulfate.*

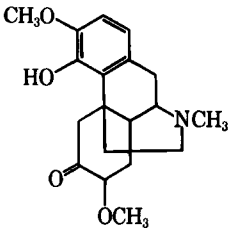


Scheme I

nium compounds are strongly affected by the structural formula of the coupling agent. Alkaloids, having *ortho* or *para* positions to the phenolic hydroxyl unsubstituted, give diazo reactions; however, the intensity of the reaction is dependent upon whether the free coupling site is at the *ortho* or *para* position. One notes that sinomenine (16), with the free *para* coupling position, gives a forty times stronger coupling reaction with diazobenzenesulfonic acid than morphine, where only *ortho* coupling can occur.



morphine



sinomenine

The experimental conditions in this study were carefully investigated to obtain the optimum coupling reaction with morphine sulfate. The concentration of disulfonamide solution was increased to 1% to provide sufficient reagent for the amounts of morphine sulfate added. An increased amount of sodium nitrite raised the color intensity of the coupled product, but the color stability was diminished. Morphine sulfate was dissolved in water, rather than methanol or 1 *N* hydrochloric acid, to obtain linear absorbance *versus* concentration plots. Sodium hydroxide (1 *N*) was abandoned as a solvent despite good color stability and satisfactory adherence to Beer's law because reproducible results were not obtained.

The morphine sulfate was allowed to couple in sodium hydroxide solution. Although acetate at coupling intensified the final color, acetate was not used since plots of absorbance *versus* concentration did not intersect the origin and color fading was noted. An intense red color developed immediately upon addition of sodium hydroxide at coupling, and the 5-min. waiting time ensured the stabilization of absorbance values upon dilution with water. Water was selected as the diluent because the final absorption of the coupled product was about 10–15% higher than when sodium hydroxide was employed. No absorption peak was observed when sodium acetate was used as diluent. Stable and reproducible results were obtained when the pH of the solution was 10.5 ± 0.5.

The absorbance was linear at 510 mμ for concentrations up to 1 mg. morphine (as the sulfate) per assay. As seen in Fig. 3, the plot of absorbance against time was quite constant and showed only about 2% increase in color intensity after 30 min.

Mixtures—The data in the tables confirm that the method already described may be satisfactorily applied even though the compounds studied are in the presence of similar vitamins or alkaloids and excipients commonly used in pharmaceutical preparations. In each of the synthetic mixtures analyzed, the ratio of the active ingredient to the additive was that of the average found in commercial preparations (if existing) rather than the optimum for analysis procedures.

It can be seen from the data in Table II that a 10- to 100-fold increase in excipient concentration resulted in a 100–101% recovery of active ingredient in the presence of sucrose, lactose, and starch.

One can conclude from Table III that pyridoxine hydrochloride can be accurately determined though compounded with a considerable amount of any of the other common B vitamins and without the need for prior separation. However, interference due to ascorbic acid, which is also often found in vitamin B complex formulations, should be first eliminated by a simple oxidation process (14).

As evident from Table IV, morphine may be assayed in the presence of certain related opium alkaloids since those alkaloids without a phenolic group, or with a substituted phenolic group, do not interfere.

The unique character of the steroid investigated here illustrates the extended applicability of this method. Since steroids with similar structural formulas are not found in the literature, 12 steroids with alcoholic or ketonic groups at the 3 position were chosen to check the applicability of the proposed method. Androsterone, ergosterol, dehydrocholic acid, *etc.*, did not give coupling reactions with diazotized disulfonamide. Of course, steroids with phenolic hydroxyl group interfere; however, such combined formulations would not normally be expected.

TABLE II—DETERMINATION OF MORPHINE, PYRIDOXINE, AND STEROID IN THE PRESENCE OF COMMON EXCIPIENTS

Additives	Morphine	Pyridoxine	Steroid
Sucrose			
Added, mg./g.	90.0	120.0	10.0
Found, mg./g.	90.4	120.0	10.0
% Recovery	100.4	100.0	100.0
Starch			
Added, mg./g.		120.0	
Found, mg./g.		121.0	
% Recovery		101.0	
Lactose			
Added, mg./g.	90.0	120.0	10.0
Found, mg./g.	90.5	120.0	10.1
% Recovery	100.6	100.0	100.7

TABLE III—DETERMINATION OF PYRIDOXINE HYDROCHLORIDE IN THE PRESENCE OF RELATED VITAMINS

Additives	% Pyridoxine Recovered[a]
Thiamine hydrochloride	100.1
Riboflavin	100.0[b]
Niacinamide	99.6
Cyanocobalamin	99.9

[a] The compound–additive ratio 1:5. [b] Determined against sample blank.

TABLE IV—DETERMINATION OF MORPHINE SULFATE IN THE PRESENCE OF RELATED ALKALOIDS

Additives	% Morphine Recovered[a]
Papaverine hydrochloride	101.5
Codeine sulfate	100.8
Dionin	100.4

[a] The compound–additive ratio 1:1.

This method with its sensitivity, rapidity, and color stability provides a significant improvement over the procedures mentioned in the literature.

SUMMARY

Pyridoxine hydrochloride, steroid, and morphine sulfate which contain either phenol c, or similar-to-phenolic hydroxyl groups, form colored azo dyes with diazotized disulfonamide.

The color intensity was significantly increased when coupling occurred *para*, rather than *ortho*, to the phenolic hydroxyl group. Certain compounds showing group similarities, as well as commonly used excipients in pharmaceutical formulations, do not interfere in this determination.

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Keyphrases

Pyridoxine HCl—analysis
Morphine—analysis
17β-Hydroxy-7aα-1,7α-dimethyl-B-homo-A-norestrane-3,6-dione—analysis
Colorimetric analysis—spectrophotometer
4-Amino-6-chloro-*m*-benzenedisulfonamide, diazotized—color reagent

In Vivo and *In Vitro* Activity of a Vasoactive Drug 9-(3,5-Dimethylpyrazole-1-carboxamido)-7-methyl-4,6,6*a*,7,8,9,10,10*a*-octahydroindolo[4,3-*fg*] Quinoline Maleate

By PAUL W. WILLARD and CLARENCE E. POWELL

An indoloquinoline compound prepared from lysergic acid base has been found to have an α-blocking action on *in vivo* and *in vitro* preparations. The compound reverses the blood pressure response to epinephrine from pressor to depressor in pithed and decerebrate cats. In renal hypertensive dogs, the compound produced a significant sustained decrease in blood pressure. On rat vas deferens preparations, the spasmogenic effect of epinephrine and norepinephrine was blocked by the compound similar to, but not in the same degree as that observed with dibenamine. The primary effect of the compound appears to be an interference with sympathetic transmission postsynaptically at the α-receptor sites resulting in peripheral dilatation.

COMPOUNDS DERIVED from lysergic acid are known to have a variety of pharmacological properties. Among these properties are effects on blood pressure and other cardiovascular parameters (1). Recently, a chemically unique series of indoloquinoline compounds prepared from the lysergic acid base[1] produced a sustained lowering of blood pressure in unanesthetized and anesthetized animals. This study was made to determine the mechanism of actions whereby one of the members of the series lowered blood pressure.

The compound, 9-(3,5-dimethylpyrazole-1-carboxamido)-7-methyl-4,6,6*a*,7,8,9,10,10*a*-

Received February 19, 1968, from the Division of Pharmacology, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46206
Accepted for publication April 25, 1968.

[1] Compounds were prepared by Dr. William L. Garbrecht of the Process Research and Development Division, Eli Lilly and Co., Indianapolis, Ind.

This method with its sensitivity, rapidity, and color stability provides a significant improvement over the procedures mentioned in the literature.

SUMMARY

Pyridoxine hydrochloride, steroid, and morphine sulfate which contain either phenol c, or similar-to-phenolic hydroxyl groups, form colored azo dyes with diazotized disulfonamide.

The color intensity was significantly increased when coupling occurred *para*, rather than *ortho*, to the phenolic hydroxyl group. Certain compounds showing group similarities, as well as commonly used excipients in pharmaceutical formulations, do not interfere in this determination.

REFERENCES

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In Vivo and *In Vitro* Activity of a Vasoactive Drug 9-(3,5-Dimethylpyrazole-1-carboxamido)-7-methyl-4,6,6*a*,7,8,9,10,10*a*-octahydroindolo[4,3-*fg*] Quinoline Maleate

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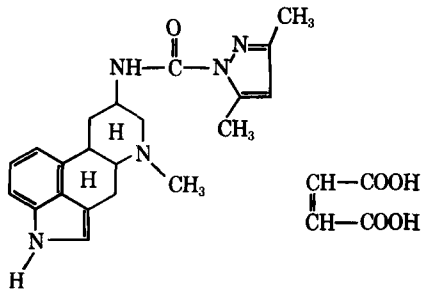
octahydroindolo [4,3-*fg*] quinoline maleate (compound PAI), has the following structural formula:



METHODS

In Vivo Preparations of Unanesthetized Animals—Renal hypertension was produced in adult male dogs by the Goldblatt procedure (2). Blood pressure responses were measured from a direct arterial puncture by a mercury manometer. Following control blood pressure and heart-rate recordings, the drug was administered orally to each of three hypertensive dogs at 5.0, 7.5, and 10.0 mg./kg., respectively. Blood pressure and heart rate were measured at 30-, 60-, 120-, 180-, and 360-min. intervals.

Subacute toxicity studies of the compound were made in adult white roosters (3). One group of three roosters received 2.0 mg. of compound PAI intramuscularly, b.i.d. for 21 days. Another group of three roosters was given 5.0 mg. of compound PAI intramuscularly b.i.d. for 31 days. In a third group of nine roosters, 5.0 mg. of the drug was given intramuscularly daily 30 min. prior to a 1.0-mg. dose of ergotamine tartrate intramuscularly for 21 days. An additional group of nine roosters received only 1.0 mg. of ergotamine tartrate intramuscularly for 21 days.

Anesthetized Animals—Ganglionic inhibition and blood pressure effects were evaluated in four cats anesthetized with chloralose (50 mg./kg.) following administration of progressively larger doses of compound PAI (0.1, 0.25, 0.5, 1.0, 2.0, 5.0, and 10.0 mg./kg.). The superior cervical sympathetic nerve was stimulated for 30 sec. by a 2- or 20-c.p.s. (9.0 v.-5 msec.) square wave from a Grass stimulator. Four minutes were allowed between stimulations for recovery of the preparation. Epinephrine (2.5 mcg./kg.) and norepinephrine (2.5 mcg./kg.) were administered to chloralose anesthetized cats following intravenous administration of compound PAI (5.0 mg./kg.).

In Vitro Preparations—The adrenergic blockade of compound PAI was compared with dibenamine using a method modified from that described by Leach (6). Isolated vas deferens from adult rats, 250–600 g., were suspended in either 2-ml. or 10-ml. muscle chambers containing Tyrode's solution warmed to 36° and gassed with 95% oxygen and 5% carbon dioxide. Responses of the muscles were recorded electronically by either isometric or isotonic myographs with 0.5-g. tension. Epinephrine and norepinephrine were used as the agonists. Doses of the antagonists which would block muscle response to the agonists by 50% were determined. The β-receptor action of dichloroisoproterenol against epinephrine- and norepinephrine-induced contractions of the rat vas deferens was also investigated.

In another series of experiments, the protective action of PAI against dibenamine blockade of norepinephrine-induced contractions of rat vas deferens was studied by a method modified from that of Furchgott (7). In protection experiments a Vickers biological assay apparatus was used and muscle responses were recorded electronically by isotonic myographs with 0.5-g. tension.

The test procedure was to first give a series of norepinephrine doses (1.0 mcg./ml.) at 5-min. intervals until the muscle response was quantitated. The muscle strip was then treated with a dose (100 mcg./ml.) of PAI and 5 min. later dibenamine (0.1 mcg./ml.) was added. Following a 5-min. exposure the muscle was washed three times and allowed to rest 10 min. Norepinephrine in the same dose as control was then given at 5-min. intervals for a period of 30–60 min.

The contractile force and coronary flow was tested on four rabbit hearts suspended in an Anderson-Carver perfusion chamber (4). Chenoweth's solution was used as the perfusion fluid (5). Doses of compound PAI were injected directly into the perfusate as it entered the aortic cannula. The heart-muscle response was recorded electronically with an isometric myograph.

RESULTS

In Vivo Preparations of Unanesthetized Animals—When compound PAI was administered orally to renal hypertensive dogs, blood pressure was lowered within 1 hr. of dosing (Table I). The blood pressure returned to near normal 3 hr. following the 5.0-mg./kg. dose. With 7.5 and 10.0 mg./kg., the blood

TABLE I—CARDIOVASCULAR RESPONSES OF RENAL HYPERTENSIVE DOGS FOLLOWING TREATMENT WITH PAI

	Days			
	1	2	1	2
Dog	ZH116	ZH116	ZH120	ZH120
Dog weight, kg.	8.5	—	14.2	—
Dog dose, mg./kg.	5	10	7.5	—
Control blood pressure	210	210	190	185
Control pulse	120	128	100	112
Compound 39985 oral (capsule), mg.	42.5	85.0	106.5	—
Blood pressure, 30 min.	215	200	175	—
Pulse, 30 min.	120	160	112	—
Blood pressure, 1 hr.	190[a]	170	140[b]	—
Pulse, 1 hr.	128	200	136	—
Blood pressure, 2 hr.	175	130	110	—
Pulse, 2 hr.	120	200+	176	—
Blood pressure, 3 hr.	195	120	140	—
Pulse, 3 hr.	136	200+	138	—
Blood pressure, 6 hr.	200	165	150	—
Pulse, 6 hr.	112	176	144	—

[a] Vomited. [b] Diarrhea (slight).

pressure was still reduced significantly at 6 hr. However, in dog ZH-120, there was no residual effect 24 hr. following drug administration. The heart rate increased in each instance and appeared to be a function of the dose. The dogs vomited after receiving the drug, and a slight diarrhea was evident with the 7.5-mg./kg. dose. Emesis was a frequent occurrence with the drug when administered orally or i.v. to other dogs, however, there was no apparent relationship to dose or to blood pressure decreases. Emesis generally only occurred once about 1 hr. following treatments. Emetic or other apparent central nervous system activity was not produced in unanesthetized cats.

The rooster combs appeared normal following 21 days of treatment with compound PAI (2.0 mg. b.i.d.) and the general physical condition of the birds was good. During the treatment period, the roosters had an average weight gain of 0.3 kg. All roosters receiving 5.0-mg. b.i.d. doses were also in good condition with normal combs.

Seven of nine roosters dosed only with ergotamine tartrate (1.0 mg.) had definite bluing of the comb followed by dry gangrene on the sixth day of treatment. One rooster died on the seventh day and the remaining bird survived with no bluing of the comb.

In the group of roosters receiving 5.0 mg. of compound PAI 30 min. prior to ergotamine treatment, none of the six surviving birds developed gangrenous combs. One rooster died on the third day, one on the tenth day, and one on the twentieth day following initiation of the regimen. Cause of death was not apparent. There was no weight change in this group.

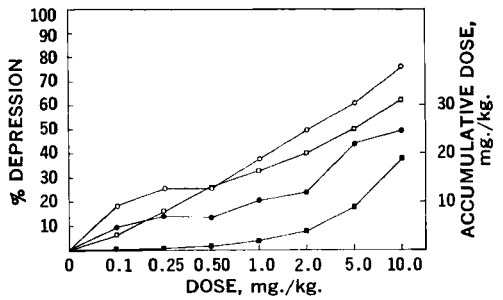


Fig. 1—Four ether–chloralose anesthetized, normotensive cats. Effect of accumulation of larger doses of PAI on blood pressure and nictitating membrane contractile response. Stimulation of nictitating membrane preparation with a square wave (9 v.) stimulation at 2 and 20 c.p.s. following each dose. Key: ○, 2 c.p.s.; ●, 20 c.p.s.; □, B.P.; ■, accumulative dose.

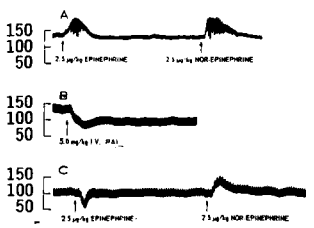


Fig. 2—Example of in vivo *effects of PAI (5.0 mg./kg.) on equal doses of (2.5 mcg./kg. i.v.) of epinephrine and norepinephrine in an ether–chloralose anesthetized cat.*

Anesthetized Animals—The action of compound PAI administered in graded doses of 0.1, 0.25, 0.50, 1.0, 2.0, 5.0, and 10.0 mg./kg. to chloralose anesthetized cats is shown in Fig. 1. A maximum decrease of 62% in blood pressure was produced with 10.0 mg./kg. (accumulated dose = 18.85 mg./kg.). The nictitating membrane was blocked 76% with the 2 c.p.s. stimulation and 50% with the 20 c.p.s. stimulation. "Neural fatigue," defined as a gradual decay of the sustained contractile response, was not observed with any dose of compound PAI.

Following a 5.0 mg./kg. dose of compound PAI to chloralose anesthetized cats, the pressor response to epinephrine was reversed to depressor (Fig. 2). Blood pressure response to norepinephrine was only slightly inhibited.

In Vitro Preparation—Doses of 0.1, 0.2, and 0.3 ml. of a 1-mg./ml. stock solution reduced the isolated heart contractile force by 5.0, 7.0, and 75%, respectively. A dose of 0.5 mg. (0.5 ml.) completely blocked the heart beat with no recovery. The heart rate was not altered nor was the coronary flow changed by smaller doses.

Compound PAI was found to antagonize epinephrine- and norepinephrine-induced spasms of the rat vas deferens. Figure 3 shows a typical response of the muscle to 10 mcg./ml. of the compound. Following matching contractions from doses of 0.3 mcg./ml. of epinephrine, compound PAI (10 mcg./ml.) was injected into the tissue chamber and allowed to act 1 min. Following this procedure, an 0.3-mcg./ml. dose of epinephrine was added to the bath. The stimulating action of epinephrine was reduced 50%. Even with repeated change of bathing fluid and exposure to epinephrine, the muscle failed to recover the pretreatment response after five trials. Thus, compound PAI has a sustained blocking action on epinephrine. The lower tracing on

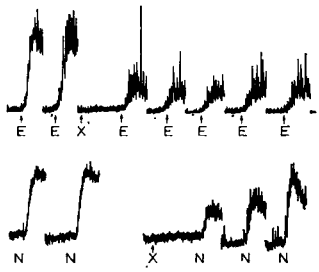


Fig. 3—Comparison of the stimulating action of epinephrine (E) (0.3mcg./ml.) and norepinephrine (N) (0.3 mcg./ml.) on isolated vas deferens of the rat, before and after compound PAI (X) (10 mcg./ml.).

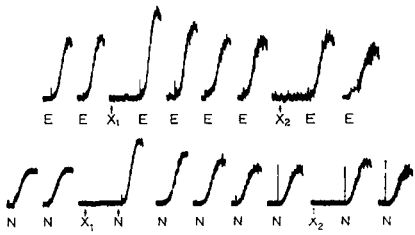


Fig. 4—Potentiation of the spasmogenic activity of epinephrine (E) (0.4 mcg./ml.) and norepinephrine (N) (0.4 mcg./ml.) by DCI (X_1 = 5.0 mcg./ml.) and (X_2 = 10 mcg./ml.) on the isolated vas deferens of the rat.

Fig. 3 shows a similar treatment of the muscle using norepinephrine in doses of 0.25 mcg./ml. Ten mcg./ml. of compound PAI blocked the muscle response to norepinephrine by 50%, but the time of recovery was somewhat less than was required for epinephrine.

In the study with DCI on spasms induced by epinephrine or norepinephrine of the rat vas deferens, it was found that an additive or enhancing action resulted (Fig. 4). DCI was added to the muscle chamber in doses of 5.0 and 10.0 mcg./ml. following matching contractions to 0.4-mcg./ml. doses of epinephrine and norepinephrine. When DCI had been in contact with the tissue for 1 min., a 0.4-mcg/ml. dose of either epinephrine or norepinephrine was added to the chamber. As shown in the tracing, the muscle contracted higher following both catecholamines, indicating an enhancing effect, since DCI in the doses given produced no activity alone.

Although it could be demonstrated in isolated rat vas deferens preparations that compound PAI left in contact with the muscle strip partially blocked norepinephrine-induced contractions (Fig. 3), such was not the case in protection experiments. From control protection tests in which compound PAI alone (100 mcg./ml.) was in contact with the muscle strip 5 min. then washed three times and dosed with norepinephrine (1.0 mcg./ml.), no significant adrenergic receptor blockade was noted. An average of results on three muscle-strip preparations showed response to norepinephrine to be reduced by less than 20%. However, when muscle strips were treated in the same manner with dibenamine (0.1 mcg./ml.), contractile responses to norepinephrine (1.0 mcg./ml.) were reduced by more than 95% through eight doses over a period of 40 min. (Fig. 5).

The protection experiments indicate compound PAI partially protects adrenergic receptors from dibenamine blockade. This confirms the suggestion that PAI reacts with α-adrenergic receptors.

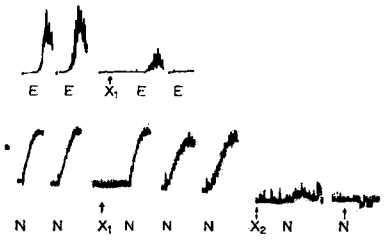


Fig. 5—Antagonism of dibenamine (X_1 = 0.4 mcg./ml.) and (X_2 = 1.0 mcg./ml.) on the stimulation action of epinephrine (E) (0.2 mcg./ml.) and norepinephrine (N) (0.2 mcg./ml.) on the isolated vas deferens of the rat.

DISCUSSION

The *in vivo* and *in vitro* activity of compound PAI suggests that the hypotensive response can be related or ascribed to α-adrenergic blockade. Epinephrine reversal occurs in pithed and decerebrate cats (Fig. 2) and inhibition of epinephrine and norepinephrine contraction of the vas deferens is a prominent feature of the drug (Fig. 3).

It has been clearly demonstrated that compound PAI, when left in contact with rat vas deferens 5 min., protects adrenergic receptors against the contractile action of norepinephrine (Fig. 3). However, when large doses of the compound (100 mcg./ml.) are incubated 5 min. and washed 3 times, most of the blocking action to norepinephrine stimulation disappears. This would indicate the compound does not adhere to the adrenergic receptors as tightly as that of dibenamine. It is well known that dibenamine blockade of epinephrine stimulation of isolated muscle persists up to 2 or more hr. (7). In this study control experiments show dibenamine (0.1 mcg./ml.) to inhibit almost 100% the contractile action of norepinephrine through three initial washes and eight doses of the agonists given at 5-min. intervals (total time 55 min.).

Compound PAI does protect adrenergic receptors against dibenamine blockade of the contractile response of smooth muscle to norepinephrine as demonstrated when compound PAI was incubated with dibenamine and then washed three times. From these experiments it was estimated that PAI protected 38% of the adrenergic receptors from the effectiveness of dibenamine.

The hypotensive response in renal hypertensive dogs was accompanied by an increased heart rate which indicates an attempted compensation by the unblocked β-receptors at the terminals of the cardiac sympathetic nerves (Table I). This response is consistent with the peripheral dilating action of α-blockade (8). Blockade of ergotamine-induced gangrene of rooster combs by PAI further indicates a competitive block at the site of peripheral vasoconstriction. In anesthetized dogs, the heart rate generally decreased. The anesthetic probably depressed the cardiac center and effectively inhibited a compensatory chronotropic response. Compound PAI does produce a blockade of the sympathetic nervous system (Fig. 1). From the data obtained in the above studies, α-receptor blockade probably accounts for a large portion of the hypotensive activity.

SUMMARY

1. Compound PAI produced a significant sustained decrease in blood pressure orally in hypertensive dogs (5.0, 7.5, and 10.0 mg./kg.). Emesis occurred in one hypertensive dog and slight diarrhea in a second.

2. On the rat vas deferens preparation, PAI was observed to block the spasmogenic effect of epinephrine and norepinephrine similar to, but not in the same degree as that observed with dibenamine. Protection experiments show that PAI partially protected the α-receptors from dibenamine blockade of the contractile response to norepinephrine. Thus, compound PAI is primarily an α-blocking agent, since DCI potentiated the spasmogenic effect of epinephrine and norepinephrine. Additional evidence in favor of α-blockade is provided by the fact that epinephrine is reversed in spinal cats by compound PAI.

3. In anesthetized cats, sympathetic block is evidenced following PAI administration. Ergotamine toxicity is inhibited in roosters following prophylactic treatment with PAI.

4. It has been concluded that a primary effect of PAI is interference with sympathetic transmission postsynaptically at the α-receptor which results in peripheral dilatation.

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Keyphrases

Indoloquinoline derivatives of lysergic acid
Hypotensive activity—lysergic acid derivatives
Mechanism—hypotensive activity
α-Receptor blockade—lysergic acid derivative

Micellar Solubilization of Testosterone II

In Aqueous Solutions of Some Ionic Surfactants

By ARVIND L. THAKKAR* and NATHAN A. HALL

The solubility of testosterone at 25° was determined in aqueous solutions containing varying concentrations of dodecyltrimethylammonium bromide (DTAB), hexadecyltrimethylammonium bromide (HTAB), and potassium laurate (KL). After the initial stages of solubilization, a linear relationship was observed between the amount of testosterone solubilized and the molar concentration of the surfactant. The order of increasing solubilizing capacity was DTAB < HTAB < KL. The environment of solubilized testosterone, as investigated by the *Z*-value method, was found to be quite polar in all cases. At low concentrations of DTAB and HTAB the *Z* value was similar to that in water. As the concentration of the quaternary ammonium bromides increased, a precipitous drop in *Z* value, corresponding to a sudden decrease in environmental polarity, was observed in the region of the critical micelle concentration. With further increase in the concentration of DTAB and HTAB the *Z* value remained reasonably constant. In low KL concentrations the *Z* value of the testosterone environment was higher (more polar) than in water. With increasing concentrations of KL the *Z* value displayed behavior similar to that in DTAB and HTAB. In general, solutions of ionic surfactants showed higher *Z* values than those of the nonionic surfactants examined previously.

THE MICELLAR solubilization of testosterone by solutions of the nonionic surfactants, polysorbates 20, 40, and 60, was the subject of a prior report from this laboratory (1). The solubilizing capacities of the three polysorbates were determined and the polarity of the chromophore of testosterone examined in these systems. A description of the *Z*-value method for empirically measuring the environmental polarity of the solubilized testosterone was included and the results were discussed in the light of current micellar theories.

In 1949 Ekwall and Sjöblom (2) prepared clear aqueous solutions of testosterone in 10% sodium oleate, 20% sodium myristyl sulfate, and 20% sodium cholate solutions, but they did not study the micellar solubilization of this steroid in detail. Recently Lach and Pauli (3) reported the solubilizing action of aqueous sodium desoxycholate solutions upon this steroid. These workers attributed the solubilizing action of sodium desoxycholate, at least in part, to channel-like inclusion complex formation. Besides these two reports no data regarding the solubilizing action of other anionic or cationic agents for testosterone are available. The investigations reported here include the solubilizing capacities of dodecyltrimethylammonium bromide, hexadecyltrimethylammonium bromide, and potassium laurate, as well as the effect of varying concentrations of these surfactants upon the environmental polarity of solubilized testosterone as determined by the *Z*-value method.

Received January 12, 1968, from the College of Pharmacy, University of Washington, Seattle, WA 98105
Accepted for publication April 2, 1968.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
Abstracted in part from a thesis presented by Arvind L. Thakkar to the Graduate School, University of Washington, Seattle, Washington, in partial fulfillment of Doctor of Philosophy degree requirements.
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Keyphrases

Indoloquinoline derivatives of lysergic acid
Hypotensive activity—lysergic acid derivatives
Mechanism—hypotensive activity
α-Receptor blockade—lysergic acid derivative

Micellar Solubilization of Testosterone II

In Aqueous Solutions of Some Ionic Surfactants

By ARVIND L. THAKKAR* and NATHAN A. HALL

The solubility of testosterone at 25° was determined in aqueous solutions containing varying concentrations of dodecyltrimethylammonium bromide (DTAB), hexadecyltrimethylammonium bromide (HTAB), and potassium laurate (KL). After the initial stages of solubilization, a linear relationship was observed between the amount of testosterone solubilized and the molar concentration of the surfactant. The order of increasing solubilizing capacity was DTAB < HTAB < KL. The environment of solubilized testosterone, as investigated by the *Z*-value method, was found to be quite polar in all cases. At low concentrations of DTAB and HTAB the *Z* value was similar to that in water. As the concentration of the quaternary ammonium bromides increased, a precipitous drop in *Z* value, corresponding to a sudden decrease in environmental polarity, was observed in the region of the critical micelle concentration. With further increase in the concentration of DTAB and HTAB the *Z* value remained reasonably constant. In low KL concentrations the *Z* value of the testosterone environment was higher (more polar) than in water. With increasing concentrations of KL the *Z* value displayed behavior similar to that in DTAB and HTAB. In general, solutions of ionic surfactants showed higher *Z* values than those of the nonionic surfactants examined previously.

THE MICELLAR solubilization of testosterone by solutions of the nonionic surfactants, polysorbates 20, 40, and 60, was the subject of a prior report from this laboratory (1). The solubilizing capacities of the three polysorbates were determined and the polarity of the chromophore of testosterone examined in these systems. A description of the *Z*-value method for empirically measuring the environmental polarity of the solubilized testosterone was included and the results were discussed in the light of current micellar theories.

In 1949 Ekwall and Sjöblom (2) prepared clear aqueous solutions of testosterone in 10% sodium oleate, 20% sodium myristyl sulfate, and 20% sodium cholate solutions, but they did not study the micellar solubilization of this steroid in detail. Recently Lach and Pauli (3) reported the solubilizing action of aqueous sodium desoxycholate solutions upon this steroid. These workers attributed the solubilizing action of sodium desoxycholate, at least in part, to channel-like inclusion complex formation. Besides these two reports no data regarding the solubilizing action of other anionic or cationic agents for testosterone are available. The investigations reported here include the solubilizing capacities of dodecyltrimethylammonium bromide, hexadecyltrimethylammonium bromide, and potassium laurate, as well as the effect of varying concentrations of these surfactants upon the environmental polarity of solubilized testosterone as determined by the *Z*-value method.

Received January 12, 1968, from the College of Pharmacy, University of Washington, Seattle, WA 98105
Accepted for publication April 2, 1968.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
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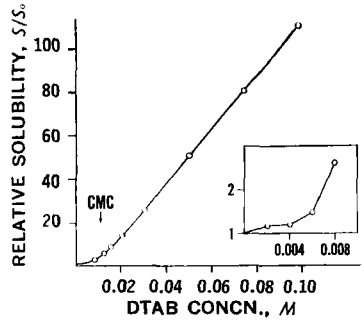

Fig. 1—Relative solubility of testosterone versus *concentration of dodecyltrimethylammonium bromide. (Relative solubility,* S/S_0 = *solubility in surfactant solution,* S/ *solubility in water,* S_0.) *The CMC indicated is the reported (4) value determined in the absence of any solubilizate.*

EXPERIMENTAL

Materials—Dodecyltrimethylammonium bromide (DTAB) and hexadecyltrimethylammonium bromide (HTAB) (K and K Laboratories, Plainview, N. Y.) were recrystallized twice before they were used, DTAB from acetone and HTAB from a chloroform–acetone mixture. The critical micelle concentration (CMC) of DTAB at 25° has been reported as 1.6×10^{-2} mole/l. by Scott and Tartar (4). The CMC of HTAB was calculated from the linear plot of log CMC *versus* carbon atom chain length from the data of these authors as 8.4×10^{-4} mole/l. A 0.1 *M* stock solution of DTAB was readily prepared at room temperature. When preparing a 0.01 *M* stock solution of HTAB, however, it was necessary to apply gentle heat. The dissolved HTAB did not precipitate from solution when cooled to room temperature. From the stock solutions a series of solutions were prepared by appropriate volumetric techniques.

The preparation of solutions of potassium laurate (KL) has been described previously (5). The CMC of KL has been reported as 2.34×10^{-2} mole/l. (6).

Testosterone NF,[1] obtained as micronized crystalline powder, was used in this investigation.

Solubility Determinations—The solubility of testosterone in aqueous solutions of DTAB, HTAB, and KL at 25° was determined by the procedure described earlier (1). As in the case of solubilization of testosterone in solutions of nonionic surfactants, initial supersaturation was found to occur in all the cases. Following this supersaturation, equilibrium solubility was attained within 48 hr. All the solutions, however, were equilibrated for 96 hr. The seemingly anomalous phenomenon of initial supersaturation in solubilized systems containing testosterone has been investigated in detail and will be the subject of a future paper (7).

Z-Value Determinations—This method for determining the environmental polarity of solubilized molecules has been described previously (1) and was utilized in this study. In most micellar solutions of DTAB, HTAB, and KL, however, the quantity of testosterone solubilized was so large that it was not possible to carry out spectral measurements using undiluted solutions. The effect of solubilizate concentration upon the wavelength of maximum absorbance ($\lambda_{max.}$) and *Z* value has been investigated before, for testosterone in solutions of a nonionic surfactant (1), and for camphor in solutions of both ionic and nonionic surfactants (8, 9). No significant differences were found in the values of these parameters. In view of these studies and the fact that these parameters reflect the solvent polarity, it seemed reasonable to assume that dilution with the appropriate surfactant solution would not alter the values of $\lambda_{max.}$ and *Z*. Dilutions were kept to a minimum and, even with the largest dilution, the amount of testosterone in solution was well above its aqueous solubility. This would ensure that the testosterone molecules would be associated with surfactant micelles. In all the cases where dilutions were necessitated, cells of shorter (1 mm.) path length were used.

[1] Generously supplied by Schering Corp., Bloomfield, N. J.

RESULTS AND DISCUSSION

Solubility Determinations—Figures 1, 2, and 3 show the relationship between relative solubility of testosterone and concentration of DTAB, HTAB, and KL, respectively. As in the case of polysorbates,

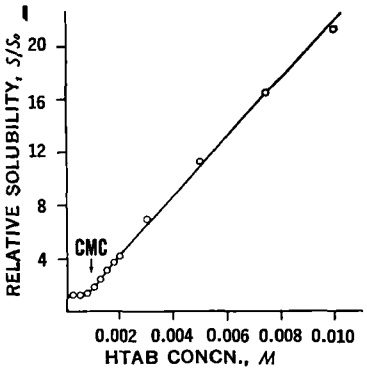

Fig. 2—Relative solubility of testosterone versus *concentration of hexadecyltrimethylammonium bromide. (Relative solubility,* S/S_0 = *solubility in surfactant solution,* S/ *solubility in water,* S_0.) *The CMC indicated is the reported (4) value determined in the absence of any solubilizate.*

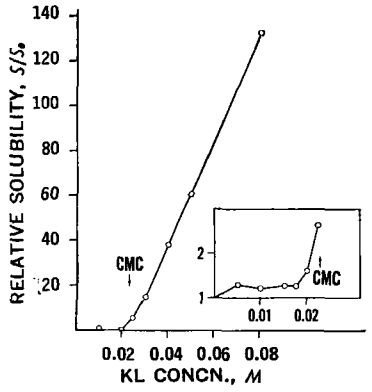

Fig. 3—Relative solubility of testosterone versus *concentration of potassium laurate. (Relative solubility,* S/S_0 = *solubility in surfactant solution,* S/*solubility in water,* S_0.) *The CMC indicated is the reported (6) value determined in the absence of any solubilizate.*

Table I—Solubilizing Capacity of Various Surfactants for Testosterone at 25°

Surfactant	Solubility, Mole Testosterone/Mole Surfactant
Dodecyltrimethylammonium bromide	9.01×10^{-2}
Hexadecyltrimethylammonium bromide	16.26×10^{-2}
Potassium laurate	17.75×10^{-2}
Polysorbate 20	3.529×10^{-2}[a]
Polysorbate 40	3.984×10^{-2}[a]
Polysorbate 60	4.385×10^{-2}[a]

[a] From *Ref. 1.*

the plots are linear after the initial stages of solubilization. The solubilizing capacities were calculated from the slopes of the linear plots and are listed in Table I. In order to facilitate comparison, the previously reported (1) solubilizing capacities of polysorbates 20, 40, and 60 are also listed in Table I.

It may be seen from Table I that among the ionic surfactants the order of increasing solubilizing capacity is DTAB < HTAB < KL. Where surfactant molecules have lipophilic portions of comparable length, the ionic agents are more efficient solubilizers mole for mole than polysorbates, *i.e.*, DTAB and KL are better than polysorbate 20 and HTAB better than polysorbate 60. The anionic KL has the greatest solubilizing capacity of all. Not only is KL more efficient than DTAB and polysorbate 20, but also it is a better solubilizer than HTAB, which is a molecule with a longer lipophilic moiety.

An explanation of the greater efficiency of the fatty acid soap, KL, than that of the quaternary ammonium bromides may lie in the fundamental difference between micelles of the two ionic types. The ions of anionic micelles bear their charges on the exposed oxygen atoms of their polar head groups, the carboxylate groups. For a molecule such as testosterone which has polar–nonpolar characteristics, it is conceivable that hydrogen bonding would be one of the factors in solubilization. The hydroxyl group at C-17 in testosterone would be the site for such bonding. The electron-rich oxygen atoms in the carboxylate ions of KL micelles should be good acceptors for hydrogen. The quaternized nitrogen atom in the polar heads of DTAB and HTAB since it has no unshared electrons would be incapable of hydrogen bonding. The trimethylammonium polar groups will, however, be capable of association with testosterone by ion–dipole and van der Waals type of interactions.

It is interesting to take a closer look at the solubilizing capacities of KL, DTAB, and polysorbate 20. These three surfactants have identical lipophilic moieties within their molecules. In an idealized picture of spherical micelles these lipophilic portions may be considered as forming the relatively nonpolar micellar core which should be similar for micelles of KL, DTAB, and polysorbate 20. If the mechanism of solubilization involved association of testosterone with the nonpolar micellar core, then similar solubilizing capacities would be expected for these three surfactants. However, the solubilizing capacities indicate that KL is about twice as efficient as DTAB and about five times as efficient as polysorbate 20. A similar situation exists for HTAB and polysorbate 60 which have identical lipophilic moieties; HTAB is more than three times as efficient as polysorbate 60. From these differences, it seems logical to believe that solubilization of testosterone may not involve penetration into the micellar interior.

The results of this study and of an earlier one by Bjaastad, Hall, and Thakkar (5) point out that for molecules such as testosterone and camphor which possess a partially polar nature, anionic surfactants are more efficient solubilizers than cationic surfactants. This observation is in apparent disagreement with the generalization that cationic surfactants are better solubilizers than anionic surfactants (6, 10). It is important to take into consideration the nature of the solubilizate and the mode of solubilization before making a generalization regarding the relative solubilizing capacities of different surfactant types. For a nonpolar solubilizate which is solubilized in the lipophilic interior of micelles, for example, cationic agents would be more efficient than anionic agents because the micelles of cationic agents have a greater degree of disorder due to the nature of their ionic head groups (11).

If the concentration of surfactant at which the relative solubility of testosterone begins to increase is considered to be the CMC, then it will be seen from Figs. 1, 2, and 3 (where the CMC's shown are reported values, determined in the absence of any solubilizate) that these values are below the literature values. One might postulate that this early increase in solubility probably results from the formation of mixed micelles between testosterone and surfactant molecules at surfactant concentrations below their usual CMC's and a consequent lowering of the CMC in the ternary system. That such an association between testosterone and the surfactants can occur seems plausible in view of the fact that testosterone also possesses surface activity (12, 13). Wurster and Taylor (14) have shown that the steroid prednisolone is also solubilized in solutions of sodium lauryl sulfate below the binary water-surfactant CMC.

Z-Value Determinations—The relationships among the concentration of DTAB, solubility of testosterone, and *Z* value are shown in Fig. 4. Similar plots for HTAB and KL are shown in Figs. 5 and 6.

In case of the cationic surfactants at low concentrations (up to 0.006 *M* for DTAB, 0.008 *M* for HTAB) in which the solubility of testosterone is not

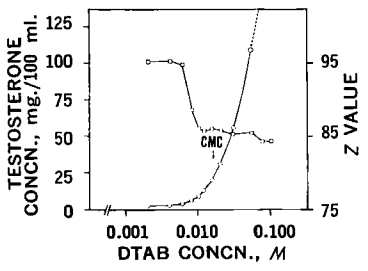

Fig. 4—The relationship between concentration of dodecyltrimethylammonium bromide (logarithm), solubility of testosterone (○), and Z value (□). The CMC indicated is the reported (4) value determined in the absence of any solubilizate.

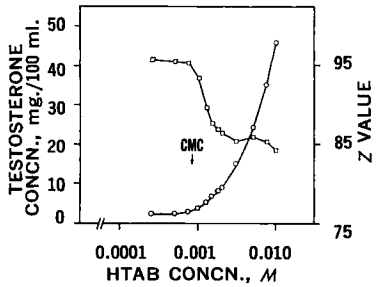

Fig. 5—The relationship between concentration of hexadecyltrimethylammonium bromide (logarithm), solubility of testosterone (○), and Z value (□). The CMC indicated is the reported (4) value determined in the absence of any solubilizate.

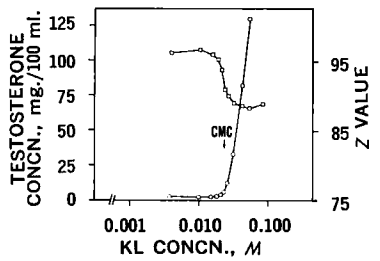

Fig. 6—The relationship between concentration of potassium laurate (logarithm), solubility of testosterone (○), and Z value (□). The CMC indicated is the reported (6) value determined in the absence of any solubilizate.

appreciably greater than in water, the Z value is similar to that in water. However, as the solubility of testosterone begins to increase, the Z value drops sharply over a narrow range of surfactant concentration. After this sudden drop the Z value appears to attain a plateau. It is interesting to note that the relatively precipitous drop in Z value takes place as the solubility of testosterone begins to increase. The solubility of testosterone increases steadily with increasing surfactant concentration in both DTAB and HTAB solutions. Unlike the situation with the polysorbates (1), the increase in the solubility of testosterone does not parallel the change in Z value.

The anionic surfactant, KL, displays a Z-value behavior similar to the cationic surfactants. A sudden drop in Z value takes place in the region of the reported CMC just as the solubility of testosterone begins to increase. The Z value in low concentrations of KL (0.005 M and 0.01 M) is slightly higher than that in water.

As pointed out earlier (1, 8), the decrease in Z values with increase in surfactant concentration may reflect the increase in concentration and organization of the micelles. That this decrease in Z value takes place at or near the CMC is significant. The rather abrupt formation of micelles would indeed lead to an increased structural organization within the solution.

The relative regions of solubilization in or on the micelle may be deduced by a comparison of the changing Z values in the ionic surfactant solutions and in the nonionic polysorbate solutions (1). Higher Z values are attained with all the three ionic surfactant solutions than with polysorbates. The concentrations of ionic surfactants employed in this investigation are much higher on a molar basis than those of the polysorbates used in the previous investigation. The quantity of testosterone solubilized by ionic surfactants is also greater than that solubilized by the polysorbates and yet the solubilized testosterone is in an environment of higher polarity.

The Z value, when it becomes reasonably constant in more concentrated ionic surfactant solutions, compares with the Z value in methanol–water or ethanol–water mixtures. This indicates that the environment of the carbonyl chromophore of the solubilized testosterone is more polar than methanol or ethanol. In view of this high polarity, it seems logical to believe that the carbonyl chromophore is associated with the polar head groups of the surfactant. In the case of the anionic, KL, for which hydrogen bonding between the ionized carboxylate group and the hydroxyl group at the C-17 of testosterone may be postulated, this would mean that the carbonyl chromophore at C-3 is also associated with this polar region of the micelle. If idealized spherical micelles are considered, then this region would be the micellar surface. Such a simplified picture is not easy to visualize for the cationic agents DTAB and HTAB where hydrogen bonding of the hydroxyl group of testosterone cannot take place with the quaternary ammonium polar head groups. From the available Z-value data it can only be concluded that the carbonyl chromophore is in an environment more polar than methanol–water or ethanol–water mixtures. It is not impossible in case of the cationic surfactants that the nonpolar portion of the testosterone molecule could be associated to some extent with the relatively nonpolar palisade region of the micelles. However, as the high Z values indicate the carbonyl chromophore must remain in the polar environment around the polar head groups.

The observation that solutions of KL provide an environment of higher polarity than do the solutions of DTAB and HTAB is in agreement with the findings of Bjaastad and Hall (8) in the case of solubilized camphor and 2-heptanone. An explanation of this observation could lie in the nature of the polar head groups of KL on the one hand and of DTAB and HTAB on the other.

The surface of the KL micelles would be rich in carboxylate ions, and since these ions arise from a weak acid, some hydrolysis would be expected in an aqueous medium. The resulting removal of protons should contribute to the polarization of the environment of the solubilizate (elevated Z value). Hydrogen bonding, possible with KL but not with DTAB or HTAB, may also alter the polarity of the medium at the polar head groups. Furthermore, the carboxylate ions have their charges exposed for direct interaction with the solubilizate chromophore, while the ionic charge on the nitrogen atom of the quaternary ammonium ions is shielded from close contact with the solubilizate by the presence of methyl group substituents. Thus, it appears that these several factors could result in KL solutions producing a more polar environment for the solubilizates than the quaternary ammonium surfactants.

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Keyphrases

Testosterone micellar solubilization
Ionic surfactants—testosterone solubilization
Z values—determination
Surfactant concentration effect—Z values

Interaction of NO_2 with Monolayers of Phospholipids Extracted from *E. coli* at 15 and 37°

By N. D. WEINER, M. AMANAT, D. BLONDO, R. CAPRIOLI, N. DINERMAN, and A. FELMEISTER*

Phospholipids were extracted from *E. coli* grown at 15 and 37°. The fatty acid residues of the 15° phospholipids were found to be considerably more unsaturated than the 37° phospholipids. These phospholipids were spread as monomolecular films and exposed to NO_2-containing atmospheres. Whereas the 37° phospholipid films showed no interaction, NO_2 was found to expand considerably the 15° phospholipid films. The results demonstrate that simple changes in environmental conditions may affect markedly the interaction of air pollutants such as NO_2 with biological membranes.

ISOLATION and characterization of membrane components from a wide variety of organisms indicate that the compositions of the membrane phospholipids differ significantly, not only from organism to organism, but even from tissue to tissue within a single organism (1). The phospholipid composition of the membranes of a number of microorganisms has also been shown to vary considerably with varying growth conditions. For example, Engleman, Terry, and Morowitz (2) point out that the fatty acid residues of the membrane phospholipids of *Mycoplasma laidlawii* are a function of the fatty acids included in the growth medium. Marr and Ingraham (3) demonstrated that the degree of unsaturation of membrane fatty acids of *E. coli* is dependent on growth temperature. These latter workers pointed out that the increase in unsaturation of the fatty acids of *E. coli* grown at lower temperatures is part of an expected adaptive process. The degree of fluidity exhibited at low temperatures by these unsaturated acids compares to that of their saturated counterparts at normal growth temperatures. Thus, the organism is able to maintain the level of membrane transport and other essential processes even at temperatures well below the normal optimum level.

In previous studies of the interaction of air pollutants with monomolecular films, it was noted that NO_2 expanded monolayers of egg lecithin (where 50% of the fatty acid groups contain at least one unsaturated bond), but did not affect monolayers of synthetic dipalmitoyl lecithin. The expansion apparently is the result of a chemical interaction of NO_2 with the double bonds of the unsaturated fatty acid groups of egg lecithin rather than a simple physical penetration into the film (4).

In view of these latter results and Marr and Ingraham's report, it was of interest to determine whether the membrane phospholipids extracted from *E. coli* grown at 15 and 37°, respectively, would exhibit similar differences in their interaction with an air pollutant such as NO_2.

Received March 29, 1968, from the Department of Pharmacy, College of Pharmaceutical Sciences, Columbia University, New York, NY 10023
Accepted for publication June 6, 1968.
This study was supported by the Research Grants Branch, National Center for Air Pollution Control, Bureau of Disease Prevention and Environmental Control (AP 00487-02). One of the undergraduate students, D. B., was partially supported by the National Science Foundation Undergraduate Research Participation Program (GY-2875).
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EXPERIMENTAL

All inorganic salts were reagent grade; the glucose was USP quality. All organic solvents were spectrograde and distilled prior to use.

The growth media used for both the 15 and 37° studies consisted of: 3 g. glucose; 2 g. NH_4Cl; 6 g. Na_2HPO_4; 3 g. KH_2PO_4; 3 g. NaCl; 85 mg. $MgCl_2 \cdot 6H_2O$; 115 mg. Na_2SO_4; 1,000 ml. distilled water.

E. coli (ASTM No. 9637) was inoculated into a flask containing 1,000 ml. of media and the flask was incubated at 37° until the absorbance of the mixture at 600 mμ was about 0.5. Five-hundred milliliters of the resultant mixture was then added to each of two flasks containing 950 ml. of media. The flasks were then placed in water baths at 15 and 37°, respectively. Incubation was allowed to proceed, in the presence of continuous aeration, until the absorbance of the mixture at 600 mμ was 0.7–0.8. The time required to reach this absorbance was about 72 hr. for the 15° batch and about 24 hr. for the 37° batch.

The cells were harvested by centrifugation at 5,000 r.p.m. at 4° for 30 min. The lipids were extracted from the bacterial cells by a modification of the procedure of Kanfer and Kennedy (5). The cells were added to flasks containing 300 ml. of chloroform–methanol (2:1, v/v), and the contents were stirred with a magnetic stirrer for 24 hr. The mixture was then filtered through a thick plug of glass wool to remove most of the cellular debris. The filtrate was then washed three times with equal volumes of 2 *M* KCl and once with an equal volume of water. The solvent was then evaporated by the use of a flash evaporator until a viscous slurry was obtained. The slurry was then redissolved in a minimum amount of chloroform–methanol (2:1, v/v).

The resultant yellow solution was fractionated by column chromatography using a modification of the method of Huston and Albro (6). A slurry of 60 g. of silicic acid (Mallinckrodt, 100/200 mesh) in *n*-hexane was packed into a 2 × 40 cm. column, and the flow rate was adjusted to 1.5 ml./min. The column was then washed with 50-ml. portions each of acetone, ethyl ether, *n*-hexane, and chloroform. The sample, contained in 2–3 ml. of solvent, was then added to the column.

The crude hydrocarbon fraction was eluted with 150 ml. of *n*-hexane–benzene (49:1, v/v), and the simple lipids were eluted next with 300 ml. of chloroform. The complex lipids were next removed by eluting with 300 ml. of chloroform–methanol (1:1, v/v), followed by 100 ml. of methanol. This complex lipid fraction was collected in increments of 10 ml. Each of these 10-ml. fractions was tested with ninhydrin reagent, and only the first 10 fractions, *i.e.*, the first 100 ml. of chloroform–methanol (1:1, v/v) eluate, yielded positive ninhydrin tests. The next five fractions, when concentrated by partial evaporation, also yielded positive ninhydrin tests, while all the remaining fractions gave negative tests. It was therefore decided to use the first 150 ml. of the chloroform–methanol (1:1, v/v) eluate as the total phospholipid fraction.

The identification and purity of this phospholipid fraction was determined by a modification of the method of Parker and Peterson (7). Both 15 and 37° fractions were spotted on Eastman Chromogram sheets (Silica Gel G) and developed using the Eastman Chromogram developing apparatus.

The solvent system of Parker and Peterson proved unsatisfactory in that the phospholipids traveled with the solvent front. A modified solvent system of chloroform–methanol–acetic acid–water (65:10:4:2) proved quite satisfactory. The samples were spotted 2.5 cm. from the bottom of the sheet and the solvent front was allowed to rise to within 5 cm. from the top of the sheet. The average running time was 4–5 hr.

The 15 and 37° samples, as well as a sample of bovine phosphatidyl ethanolamine (Applied Science Laboratories), gave single spots with identical R_f values as determined by development with ninhydrin reagent. No other spots were detected upon development with iodine vapors and concentrated sulfuric acid spray.

Qualitative iodine determinations of the 15 and 37° phospholipids demonstrated that the 15° samples were significantly more unsaturated than the 37° samples. This was confirmed by the method described by Weiner, Felmeister, and Amanat (8) where the surface-pressure increase of monolayers of phospholipids spread on an iodine solution subphase over that of an aqueous subphase was used to measure the degree of unsaturation.

A phosphorous determination according to the method of King (9) was performed on both the 15 and 37° solutions. On the basis of the molecular weight of phosphatidyl ethanolamine, it was calculated that the authors' extraction procedure yielded approximately 80 mg. of phospholipid per 10 l. of *E. coli*, grown at both 15 and 37°.

Both the 15 and 37° samples were then spread as monolayers and subjected to standard and NO_2-containing atmospheres by the method previously described by Felmeister *et al.* (4). The gases were metered by individual flowmeters into a glass tube equipped with baffles, which served as a premixing tube. Mixing was completed by passing the gases into a 500-ml. round-bottom flask. The gas mixtures were then led through a 0.9-m. length of glass tubing connected to a short length of Teflon tubing. The latter was affixed to the underside of the Lucite trough cover which served to maintain the desired gaseous atmosphere over the film. The Teflon tubing within this enclosure was formed into a loop, and a series of small perforations was made in the wall of the tubing. The shape of the loop and the positions of the perforations gave a uniform flow of the gases over the film surface.

The gas mixtures were permitted to flow through the system for at least 4 hr. before the start of each experiment to ensure steady-state conditions.

A Film Balance (Frater Instrument Co., Corona, N. Y.) was used to study the surface pressure-surface area (π-A) characteristics of the films. The balance consists of a Teflon-coated removable trough, totally free of metal contacts. Two variable-speed reinforced rigid Teflon stirrers are set into the trough to facilitate subphase mixing and temperature control. The temperature of the subphase was maintained at 25 ± 0.1° by circulating water from a constant-temperature bath through the water jacket around the trough. The precision lead screw, which drives the reinforced Teflon barrier, allows for changes in surface area of the

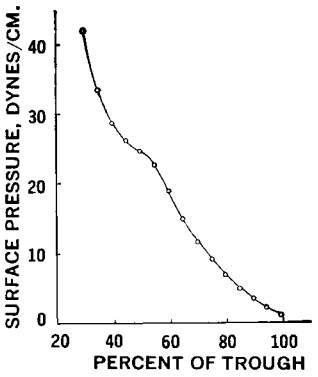

Fig. 1—π-A curve of 37° phospholipid exposed to standard atmosphere (air) and to NO_2-containing test atmosphere.

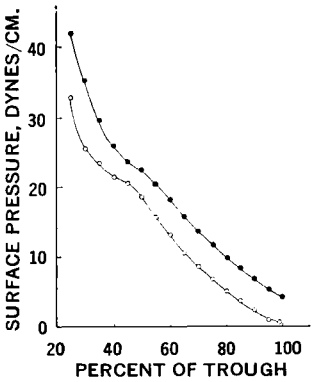

Fig. 2—π-A curves of 15° phospholipid exposed to standard atmosphere (air) (○); and to NO_2-containing test atmosphere (●).

trough as small as 0.0125 cm.^{2}. A quick disengage mechanism permits rapid sweeping of the film for cleaning the surface. The film balance was enclosed in a dust-protective cabinet.

Surface pressures were measured by the Wilhelmy plate method. A thin platinum plate, roughened to ensure complete wetting, was used.

Both the 15 and 37° samples were spread as monolayers from 1:1, v/v, methanol–chloroform solvent mixtures. It was found that when 0.05 ml. of solution was spread onto a subphase containing 0.9% roasted sodium chloride in double-distilled water, a surface pressure of about 1 dyne/cm. was observed, in the case of both the 15 and 37° samples.

These films were studied while exposed to a standard atmosphere (air flowing at the rate of 300 ml./min.) and to a test atmosphere (0.33% nitrogen dioxide in air) flowing at this same rate of 300 ml./min.

In all cases, the films were permitted to stand with the gases flowing for 1 hr. before manual compression of the film was initiated. Surface-pressure readings were then obtained at various film areas.

RESULTS AND DISCUSSION

The surface pressure-surface area (π-A) curve for the 37° phospholipid film exposed to the standard atmosphere is shown in Fig. 1. Exposure of the 37° phospholipid film to the NO_2-containing test atmosphere yielded a π-A curve identical to that of the standard (within ±0.4 dyne/cm. at all areas), indicating no film–NO_2 interaction. Figure 2 shows the π-A curves for the 15° phospholipid films exposed to the standard and test atmospheres. Exposure of 15° phospholipid films to the NO_2 test atmosphere resulted in a π-A curve that was considerably more expanded than that of the standard curve. This interaction, once it occurred, could not be reversed by replacing the NO_2 test atmosphere with the standard atmosphere.

It should be noted that all the curves in Figs. 1 and 2 show a shoulder at π values of about 20–25 dynes/cm. This effect was apparent only at very slow compression rates. Van Deenen *et al.* (10) also reported a shoulder for a synthetic phosphatidyl serine layered on a pH 4 subsolution.

It appears that the 15 and 37° materials differ from one another primarily in the degree of unsaturation of their fatty acid groups. The observed effect of NO_2 on the 15° phospholipid film is most probably the result of the interaction of NO_2 with the double bonds of the unsaturated fatty acid groups.

The results demonstrate that simple changes in environmental conditions may affect markedly the interaction of air pollutants, such as NO_2, with membranes. *In vivo* work now in progress in these laboratories on the effect of NO_2 on *E. coli* grown at different temperatures should serve to determine the relevance of this phenomenon to living systems.

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Keyphrases

Phospholipids—*E. coli* extracted
NO_2 atmosphere—phospholipid film
Film surface, pressure, area—determination
Column chromatography—separation
TLC—identity

Evaluation, Control, and Prediction of Drug Diffusion Through Polymeric Membranes III

Diffusion of Barbiturates, Phenylalkylamines, Dextromethorphan, Progesterone, and Other Drugs

By EDWARD R. GARRETT and PRAMOD B. CHEMBURKAR

Silastic membrane demonstrated the greatest permeability among the nine polymeric films studied. The apparent diffusion constants through silastic membrane were determined by steady-state studies for a series of barbiturates and phenylalkylamines and correlated with the determined chloroform/acetate buffer partition coefficients in the former case. The apparent diffusion constants did not correlate with the determined solubilities of the barbiturate series in the diffusing solvent since the solubilities within the membrane have to be considered. Only the uncharged species diffuse through the polymeric membrane and half of the maximal diffusion rate occurs at the pKa′. Diffusion rates were proportional to concentrations in all cases up to the solubility of the diffusing species and Fick's law was fully applicable. Diffusion rates can be varied by changing the diffusing and desorbing solvents and membrane thickness and area. Solvent variation (*e.g.*, water, mineral oil, peanut oil, alcohol) affects the apparent diffusion coefficients of dextromethorphan and progesterone. The rates of diffusion of the studied compounds decrease with their increasing solubility in the diffusing solvent which can be assigned to the preferential partitioning in the solvent rather than the membrane. Diffusion from capsules repeats the rates and is dependent on the same factors as diffusion through films. Solid particles in contact with silastic membrane dissolve in and are transported through the membrane. Release rates from the membrane into surrounding solvents should depend on whether these dissolution rates were of higher or lower orders of magnitude than diffusion through the membrane.

THE APPLICABILITY of Fick's law to the transfer of uncharged drugs through polymeric membranes has been substantiated with respect to aminophenones (1, 2). The apparent rates of diffusion, dA/dt, through a typical solid polymeric membrane, the silicone silastic, have been shown to be proportional to the concentration of the uncharged drug concentration in the diffusing solution, C_2, and inversely proportional to the thickness, X, of the membrane in accordance with the steady- and quasi-steady-state expression of Fick's law,

$$dA/dt = V_1 dC_1/dt = D'S(K_2 f_2 C_2 - K_1 f_1 C_1)/X \quad \text{(Eq. 1)}$$

where C_2 and C_1 are the concentrations of the drug in volumes V_2 and V_1 of diffusing and desorbing solutions, respectively; where f_2 and f_1 are the fractions of the drug concentrations, C_2 and C_1, that are uncharged, respectively; where K_2 and K_1 are the respective partition coefficients for the uncharged species between the membrane and solvents; and where S is the area and D' the intrinsic diffusion constant within the membrane. In general the concentration C_1 of the V_1 volume of desorbing solution was monitored as a function of time.

The silastic membranes are impermeable to HCl, phosphate buffer salts, and protonated aminophenones (1, 2). The apparent diffusion constants $D = D'K$ of various aminophenones through silastic membrane from and to equivalent solvents, *i.e.*, $K_1 = K_2 = K$, where, from Eq. 1,

$$dA/dt = V_1 dC_1/dt = D'KS(f_2 C_2 - f_1 C_1)/X \quad \text{(Eq. 2)}$$

appear to be proportional to chloroform/water partition coefficients. The apparent diffusion constants, $D = D'K$, for 4′-aminopropiophenone from and to solvents of the same composition (or when $f_1 = 0$) were inversely proportional to the solubility of the 4′-aminopropiophenone in the diffusing solution.

The purposes of this investigation were to screen various polymeric films for their permeability to various drugs and to investigate in detail the diffusion of barbituric acid derivatives, phenylalkylamine, dextromethorphan, and progesterone through silastic membrane and potential dosage forms based on this principal. The effects of temperature, pH, and variation of diffusing and desorbing solvents were determined. The relations between the apparent

Received March 22, 1968, from the College of Pharmacy, University of Florida, Gainesville, FL 32601
Accepted for publication May 3, 1968.
Presented in part to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
Abstracted in part from a thesis submitted by P. B. Chemburkar to the University of Florida in partial fulfillment of Doctor of Philosophy degree requirements.

diffusion constants with solubilities and oil/water partition coefficients were also investigated.

EXPERIMENTAL

Chemicals—The following compounds were supplied by Abbott Laboratories, North Chicago, Ill. The molar absorptivities, ϵ, are given for the designated wavelengths at which the absorbances were measured: amobarbital, $C_{11}H_{18}N_2O_3$, eq. wt. 226, 236 found, ϵ = 6,520 (238 mμ), pKa′ 7.40; barbital, $C_8H_{12}N_2O_3$, eq. wt. 184, 197 found, ϵ = 10,310 (238 mμ), pKa′ 7.45 [lit. value 7.91 (3)]; butabarbital, $C_{10}H_{16}N_2O_3$, eq. wt. 212, 215 found, ϵ = 10,410 (238 mμ); cyclobarbital, $C_{12}H_{16}N_2O_3$, eq. wt. 236, 245 found, ϵ = 9,880 (238 mμ), pKa′ 7.27 [lit. value 7.50 (3)]; diallylbarbituric acid, $C_{10}H_{12}N_2O_3$, eq. wt. 208, 202 found, ϵ = 9,690 (238 mμ), pKa′ 7.30 [lit. value 7.79 (3)]; mephobarbital, $C_{13}H_{14}N_2O_3$, eq. wt. 246, 250 found, ϵ = 5,730 (238 mμ), pKa′ 7.45; metharbital, $C_9H_{14}N_2O_3$, eq. wt. 198, 198 found, ϵ = 6,540 (238 mμ), pKa′ 7.90; pentobarbital, $C_{11}H_{18}N_2O_3$, eq. wt. 226, 232 found, ϵ = 11,070 (238 mμ), pKa′ 7.65 [lit. value 8.11 (3)]; phenobarbital, $C_{12}H_{12}N_2O_3$, eq. wt. 232, 257 found, ϵ = 10,880 (238 mμ), pKa′ 7.40 [lit. value 7.41 (3)]; secobarbital, $C_{12}H_{18}N_2O_3$, eq. wt. 238, 230 found, ϵ = 9,860 (238 mμ), pKa′ 7.45 [lit. value 8.08 (3)]; thiamylal, $C_{12}H_{17}N_2O_2S$, eq. wt. 254, 290 found, ϵ = 18,660 (304 mμ), pKa′ 6.80; thiopental, $C_{11}H_{18}N_2O_2S$, eq. wt. 242, 246 found, ϵ = 12,100 (304 mμ), pKa′ 7.12.

The following compounds were supplied by Smith Kline & French Laboratories, Philadelphia, Pa.: α-methylphenethylamine hydrochloride, $C_9H_{13}N \cdot HCl$, eq. wt. 172, 165 found, pKa′ 9.07; α-ethylphenethylamine hydrochloride, $C_{10}H_{15}N \cdot HCl$, eq. wt. 186, 203 found; 2-amino-4-methyl-4-phenylpentane hydrochloride, $C_{12}H_{19}N \cdot HCl$, eq. wt. 214, 205 found, pKa′ 9.42; 3-amino-1-phenylbutane sulfate, $(C_{10}H_{15}N)_4 \cdot H_2SO_4$, eq. wt. 198, 242 found, pKa′ 9.30; 1-methyl-5-phenylpentylamine hydrochloride, $C_{12}H_{19}N \cdot HCl$.

The following compounds were supplied by the Upjohn Company, Kalamazoo, Mich. The molar absorptivities, ϵ, are given for the designated wavelengths at which the absorbances were measured: progesterone, ϵ = 16,470 (248 mμ), m.p. 128–130° [lit. value 127–131° (4)]; cortisone, ϵ = 16,000 (238 mμ), m.p. 230–236° [lit. value 236–240° (4)]; hydrocortisone, ϵ = 16,200 (242 mμ), m.p. 217–220° [lit. value 217–220° (4)]; prednisolone, ϵ = 15,200 (242 mμ), m.p. 239–240° [lit. value 240–241° (4)].

Dextromethorphan, pKa′ 8.25, ϵ = 3,002 (277 mμ) was supplied by Vick Divisions Research and Development, Richardson-Merrell Inc., Mt. Vernon, N. Y.

The following compounds were purchased from National Biochemical Corp., Cleveland, Ohio: sulfathiazole, m.p. 200–203° [lit. value 200–204° (4)]; sulfisoxazole, m.p. 195–198° [lit. value 194° (4)]; sulfadiazine, ϵ = 5,370 in alkaline solution (255 mμ), m.p. 256–257° [lit. value 252–256° (4)]; sulfabenzamide, ϵ = 4,480 in alkaline solution (255 mμ), m.p. 181–183° [lit. value 181.2–183.3° (4)].

The peanut and mineral oil used were USP. All other reagents were of analytical grade. The 4′-aminopropiophenone used has been discussed previously (2).

Polymer films tested were an 11-aminoundecanoic acid polymer[1] (5), a polyamide[2] (6), cellulose acetate,[3] cellulose triacetate,[4] and cellulose acetate butyrate,[5] thermoplastic cellulosic films (7), polyethylene type B (8), and polyethylene terephthalate.[6] Polypropylene (10) was supplied by the Avisun Corp., Philadelphia, Pa.; silastic medical grade sheeting H-0169, HO 293 is a dimethylsiloxane polymer (11) and was supplied by the Dow Corning Center for Aid to Medical Research.

Analytical Methods—Spectrophotometric measurement of absorbances at 24.0 ± 1.0° was used for the quantitative estimation of barbituric acid derivatives, steroids, and dextromethorphan in a Beckman model DU spectrophotometer using 10-mm. cells and 0.1 slit width at the wavelengths stated above. The linear relationship between absorbance and concentration was verified in all cases. The barbituric acids were measured in pH 10.1 borate buffer as the molar absorptivities are much higher than in the uncharged form. The absorbances of dextromethorphan and steroids were measured in pH 6.8 phosphate buffer solutions. The solutions of the sulfonamides were made alkaline with NaOH solution. A Cary recording spectrophotometer model 14 was used in screening.

The phenylalkylamines were analyzed by a modification of the method of Gettler and Sunshine (12). A 1.00-ml. aliquot of 1.5–10 × 10^{-5} *M* solution in pH 6.8 phosphate buffer was made alkaline with 0.1 ml. of 2 *N* NaOH, 5.00 ml. of chloroform added, and the mixture agitated. After centrifugation at 3,200 r.p.m. for 3 min., 4.00 ml. of the chloroform solution was removed and 0.20 ml. of freshly prepared methyl orange reagent (1:1 saturated solution of methyl orange and boric acid) was added to this sample. The mixture was again centrifuged, 3.00 ml. of the chloroform solution was removed, and 0.20 ml. of absolute ethanol containing 2% concentrated sulfuric acid was added to this sample. The absorbance of this solution was read at 520 mμ on a Beckman DU spectrophotometer against a phosphate blank prepared similarly. Calibration curves were prepared daily.

The cited pKa′ values were obtained by potentiometric titration at 24.0 ± 1.0° on a Sargent model D automatic titrator and standardized at pH 4.0, 7.0, and 10.0 and are accurate to ±0.05 pH unit. The difference method of Parke and Davis (13) was used and the pKa′ determined from the half-neutralization value of the difference between the titers of equivalent volumes of sample solution and blank necessary to give the same measured pH value. Alkaline solutions of the barbiturates were titrated with standard $HClO_4$; the acid solutions of the phenylalkylamines and dextromethorphan were titrated with standard NaOH.

[1] Rilsan (Nylon 11), May Industries, Inc., Atlanta, Ga.
[2] Polypenco(Nylon 101), Polymer Corp., Reading, Pa.
[3] Kodacel A29, Eastman Chemical Products, Inc., Kingsport, Tenn.
[4] Kodacel TA 401, Eastman Chemical Products, Inc., Kingsport, Tenn.
[5] Kodacel B 298, Eastman Chemical Products, Inc., Kingsport, Tenn.
[6] Mylar polyester type S, E. I. du Pont de Nemours & Co., Wilmington, Del.

Solubility Studies—Saturated solutions of the barbituric acid derivatives in pH 4.7 acetate buffer, ionic strength 0.1, were prepared at 50° and equilibrated at 25° for 48 hr. in a thermostated shaker bath in the presence of excess solids. The solutions were then filtered by suction through electrode isolation tubes, which are fitted with a finely porous fritted-glass membrane (E. H. Sargent and Company, Chicago, Ill.). Aliquots of the filtered solutions were appropriately diluted with pH 10.1 borate buffer and the absorbances measured at the pertinent wavelengths.

Partition Coefficients—Aqueous pH 4.7 acetate buffer and chloroform were saturated with respect to each other. An approximately 5×10^{-4} *M* solution of the barbituric acid derivatives was prepared in the acetate buffer presaturated with chloroform. One milliliter of this solution was appropriately diluted, generally with 10 ml. of pH 10.1 borate buffer, and its absorbance measured at the pertinent wavelength against a blank treated similarly. Five milliliters of the acetate solution and 5.0 ml. of the chloroform presaturated with aqueous acetate buffer in a capped, stoppered vial were mixed on a Vortex Junior mixer for 3 min., centrifuged for 3 min. at 3,200 r.p.m., and 1.00 ml. of the aqueous phase was removed, diluted with 10 ml. of pH 10.1 borate buffer, and spectrophotometrically analyzed against an acetate buffer blank treated similarly. The ratio of the differences in absorbances of the aqueous layer before and after partitioning to the absorbance of the aqueous layer after partitioning was taken as the partition coefficient of the drug in the $CHCl_3$–acetate buffer system.

Screening of Polymer Films for Permeability to Drugs—The cited polymer films in 3 and 5 mil thicknesses were washed with distilled water and dried. Saturated solutions of the cited materials in 0.1 *N* HCl, 0.1 *N* NaOH, pH 6.8 phosphate buffer, propylene glycol, peanut oil, ethanol, mineral oil, ethylene glycol, and polyethylene glycol 200 were prepared. Two milliliters of each saturated solution was put into a serum vial (10-ml. capacity) and the vial stoppered with a holed rubber stopper. A small piece of each membrane was laid upon the stopper and an aluminum cap was then crimped to sandwich the membrane. The serum vial was then inverted and placed into a 60-g. ointment jar containing about 10 ml. of pH 6.8 phosphate buffer. The jars were capped, properly labeled, and set aside for at least 1 week, when the phosphate buffer solutions were analyzed for drug by the methods described. A drug was said to have permeated through a membrane when the peaks at wavelengths corresponding to the $\lambda_{max.}$ values of the drug were observed on the Cary model 15. Experiments with positive results were repeated to avoid erroneous conclusions from leaking vials.

Diffusion Studies—The steady-state diffusions of the barbituric acid derivatives were studied through 3-mil silastic membrane from their solutions in pH 4.7 acetate buffer into pH 10.1 borate buffer at 25 and 37.5°.

Weighed amounts of barbituric acid derivatives were dissolved in small volumes of 1 *N* NaOH and immediately diluted with pH 4.7 acetate buffer solution to 2 l. to obtain solutions which were approximately 5×10^{-3} *M* in barbituric acid derivatives. These were used as reservoir solutions and circulated through the steady-state diffusion cells fitted with 3-mil silastic membranes. The system has been described previously (1). The solutions used on the desorption side of the membrane in the beakers were 200 ml. of pH 10.1 borate buffer. Absorbances of samples were measured as a function of time on the Beckman DU spectrophotometer and the samples were returned to the beakers.

The steady-state diffusion of barbital and pentobarbital through 3-mil silastic membrane from their solutions in acetate, phosphate, and borate buffer solutions of different pH values at 37.5° was also studied.

The steady-state diffusion of the phenylalkylamine salts from borate buffers through 3-mil silastic membrane into 120 ml. of pH 6.8 phosphate buffer was studied at 25°. Samples of the phosphate buffer solutions (1 ml.) were removed every 15 min. and stored in a refrigerator. All the samples were analyzed simultaneously with the standard solutions used to prepare the calibration curve.

The steady-state diffusions of dextromethorphan from peanut oil, mineral oil, and pH 10.1 borate buffer; and progesterone from peanut oil, and a saturated solution in phosphate buffer were studied through 3-mil silastic membrane into pH 6.8 phosphate buffer at 37.5°.

The diffusion of dextromethorphan was also studied from its saturated solutions in peanut oil and mineral oil. The concentration of dextromethorphan in peanut oil was determined by diluting a weighed amount of the filtered solution with chloroform and measuring the absorbance of the solution at 277 mμ against a chloroform blank containing an equivalent amount of peanut oil where the absorbance of a similar solution of a known weight of the drug had been determined. The saturated solution of the dextromethorphan in the mineral oil was filtered and its absorbance was measured against a mineral oil blank. The concentration was calculated from the previously determined absorptivity. The steady-state diffusion apparatus used has been described previously (1).

Silastic capsules (Dow Corning Center for Aid to Medical Research) were small silastic pouches prepared by sealing pieces of tubes (about 6 mm. in diameter and about 2.4 cm. in length) at both ends. These were cleaned externally with water and air dried.

Saturated solutions of 4′-aminopropiophenone in phosphate buffer, solutions of barbital, pentobarbital, and phenobarbital in acetate buffer, and solutions of dextromethorphan in borate buffer, peanut oil, and mineral oil were prepared. The silastic capsules were cut open at one corner and were filled with these saturated solutions. The hole in each of the capsules was sealed by forcing a portion of silastic medical grade adhesive type A (Dow Corning Center for Aid to Medical Research) into and around the hole. The capsules were set aside for 1 day and were tested for leaks by pressing them lightly between fingers. Similarly, capsules containing solvents without drugs were also prepared.

The capsules containing barbital, pentobarbital, and phenobarbital were washed with water and put into 50 ml. of pH 10.1 borate buffer in glass vials. These vials were preequilibrated at 37.5° for about 10 hr. Similarly capsules of 4′-aminopropiophenone and dextromethorphan were put in glass vials containing 50 ml. of 0.12 *N* HCl and pH 6.8 phosphate buffer, respectively. The samples from the vials were assayed spectrophotometrically for the respective compounds.

Diffusion of Solid Progesterone Through Silastic Membrane—One window of the stainless steel steady-state diffusion cell was used. One membrane was placed face down and the available area was covered with powdered, dry progesterone. Another membrane was placed atop and the resultant sandwich was stretched tautly while it was clamped into the window. The cell was immersed in 120 ml. of normal saline and vibrated in the shaker bath at 37.5°. Samples of the solution were removed at intervals and significant absorbance due to progesterone, $\lambda_{max.}$ 248 mμ, was observed on the Cary ultraviolet recording spectrophotometer.

RESULTS

Screening of Polymer Films for Permeability—The spectrophotometric curves of the desorbing solutions were checked for the peaks characterizing the compounds whose diffusion was under test. This method of analysis was sensitive to 10^{-4} *M* concentration in all cases and much more sensitive in the case of steroids. When no drug in the desorbing solution was observed at these sensitivities, the membrane is stated to be impermeable to that drug.

Polyethylene membrane was permeable to 4′-aminoacetophenone in aqueous solutions and in peanut oil and to 4′-aminopropiophenone in ethylene glycol. Both compounds permeated an 11-aminoundecanoic acid polymer membrane[1], from ethanolic solutions. A polymide membrane[2] was permeated by 4′-aminopropiophenone and 3′-aminoacetophenone from 0.1 *N* NaOH and ethanol. Polypropylene was nonpermeable to all the drugs tested.

Substances were leached by phosphate buffer from cellulose triacetate and cellulose butyrate membranes that interfered with the spectrophotometric analyses of the drugs. After compensation for the absorbances of these interfering substances, it was concluded that 4′-aminopropiophenone permeated cellulose triacetate from propylene glycol, ethanol, and polyethylene glycol 200. Positive results were recorded also for barbital in polyethylene glycol 200 and sulfabenzamide in ethanol. Polyethylene terephthalate polyester film[6] was available only in small quantity and was impermeable to all the drugs tested with the available films.

Silastic membrane was permeable to 4′-aminopropiophenone and 3′-aminoacetophenone from all the solvents tested except mineral oil, to progesterone from ethanol, to barbital from ethanol and ethylene glycol, and to phenobarbital from peanut oil, ethanol, polyethylene glycol 200, phosphate buffer, and 0.1 *N* HCl. Silastic membrane allowed permeation of the majority of the compounds screened in measurable quantities and was investigated in further detail.

Steady-State Diffusion Studies—The steady-state diffusion of drugs through silastic membranes was studied with the equipment and by the techniques previously described in detail (1, 2). The concentration, C_2, of the diffusing solution was held constant as was the volume, V_1, of the desorbing solution. The linear slopes, dC_1/dt, of the increase of concentration, C_1, of the desorbing solution with time were determined. The concentrations, f_1C_1, of the uncharged species in the desorbing solution were kept at zero by choosing a pH where the fraction of the drug uncharged, f_1, was effectively zero.

The specific rate of diffusion was obtained by dividing this slope, the rate of increase of total concentration, C_1, of the desorbing solution by the concentration, f_2C_2, of the uncharged species in the diffusing solution. When the slope was obtained from the linear increase in absorbance, A_1, of the desorbing solution with time, this slope, dA_1/dt, was divided by the product of the absorbance, A_2, of the diffusing solution and the fraction, f_2, of the drug uncharged therein since

$$R_S = \text{specific rate of diffusion} = (dA_1/dt)/f_2A_2 = [d(\epsilon C_1)/dt]/f_2\epsilon C_2 = (dC_1/dt)/f_2C_2 \quad \text{(Eq. 3)}$$

Typical plots of the increase of absorbance or concentration with time in the desorbing solutions after diffusion through 3-mil silastic membrane are given in Figs. 1 and 2 for various barbituric

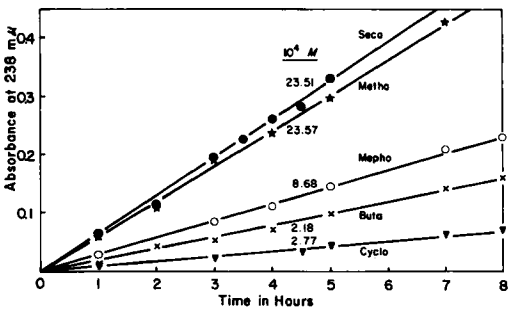

Fig. 1—Absorbance at 238 mμ of 200 ml. of pH 10.1 borate buffer desorbing solution as a function of time on the steady-state diffusion of the stated concentrations of the cited barbitals from pH 4.7 acetate buffer through 3-mil silastic membrane at 37.3°.

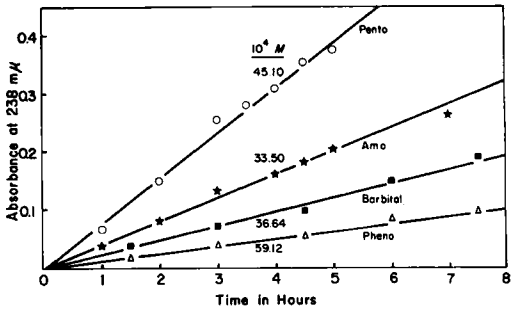

Fig. 2—Absorbance of 238 mμ of 200 ml. of pH 10.1 borate buffer desorbing solution as a function of time on the steady-state diffusion of the stated concentrations of the cited barbitals from pH 4.7 acetate buffer through 3-mil silastic membrane at 37.3°.

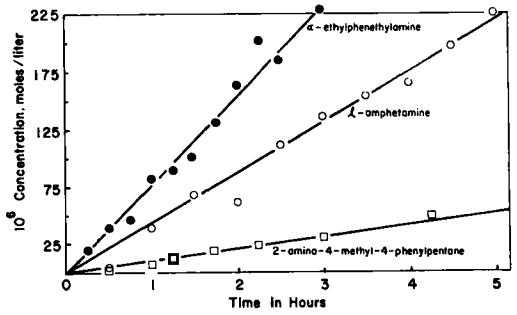

Fig. 3—Concentration of pH 6.8 phosphate buffer desorbing solution as a function of time on the steady-state diffusion of several phenylalkylamines from borate buffer adjusted to the pH corresponding to their pKa' values through 3-mil silastic membrane at 25°. (See Table I.)

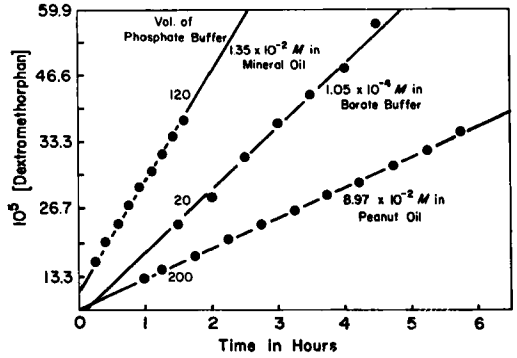

Fig. 4—Concentration of 6.8 phosphate buffer desorbing solution of the stated volumes as a function of time on the steady-state diffusion of dextromethorphan at the cited concentrations from different solutions through 3-mil silastic membrane at 37.5°. The pH of the diffusing solution borate buffer was 10.1.

acid derivatives diffusing from pH 4.7 acetate buffer, where they are unionized, $f_2 = 1$, to pH 10.1 borate buffer where they are ionized, $f_1 = 0$. Typical plots of the diffusion of phenylalkylamines from their partially nonprotonated forms in borate buffers into pH 6.8 phosphate buffers where they are charged, $f_1 = 0$, are given in Fig. 3. Typical plots of the diffusion of dextromethorphan diffusing in its nonprotonated form, $f_2 = 1$, from mineral oil, peanut oil, and pH 10.1 borate buffer into pH 6.8 phosphate buffer, where it would be completely protonated, $f_1 = 0$, are given in Fig. 4.

The apparent diffusion constant, D, was calculated from the rearrangement of Eq. 2 and the determined specific rate of diffusion, $(dC_1/dt)/f_2C_2$ or $(dA_1/dt)/f_2A_2$ as defined in Eq. 3 where $f_1 = 0$,

$$D = XV_1(dC_1/dt)/f_2C_2S \qquad \text{(Eq. 4)}$$

where $X = 7.52 \times 10^{-3}$ cm. for the 3-mil thick silastic membrane, $S = 10.4$ cm.^{2} for the area of the membrane, and V_1 was the maintained volume in liters of the desorbing solution. The fractions of molecules uncharged in the diffusing and desorbing solutions were effectively $f_2 = 1$ and $f_1 = 0$, respectively, for the barbiturates and dextromethorphan. The fraction, f_2, of the phenylalkylamines that were uncharged in the borate buffer diffusing solutions were calculated from the pKa' values of the compounds and the pH values of the borate buffers in accordance with (1),

$$f_2 = [H^+]/([H^+] + K_a) = 10^{-pH}/(10^{-pH} + 10^{-pKa'}) \qquad \text{(Eq. 5)}$$

The concentrations of uncharged phenylalkylamines in the diffusing solutions, their pKa' and pH values, and the observed specific rates of diffusion and apparent diffusion constants are given in Table I.

The determined apparent diffusion constants, solubilities, and partition coefficients for the various barbituric acids are given in Table II. The apparent energies of diffusion, ΔE_a, are calculated from the apparent diffusion constants at the two temperatures studied by

$$\Delta E_a(\text{kcal./mole}) = \frac{10^{-3}(\log D_{37^\circ} - \log D_{24^\circ})2.303R}{(1/T_{24^\circ} - 1/T_{37^\circ})} \qquad \text{(Eq. 6)}$$

where T is the absolute temperature and R is 1.987 cal./degree/mole.

The determined apparent diffusion constants for dextromethorphan from various diffusing solvents are given in Table III.

Further verification of the fact that only nonionized materials diffuse through silastic membranes (1) was obtained from the variation of the apparent diffusion constant, D, on steady-state diffusion into pH 10.1 borate buffer with pH of the diffusing solution on the assumption that $f_2 = 1$ in Eq. 4 (Table IV). The plots of these apparent diffusion constants against pH are shown in Fig. 5. The pKa' values may be considered as the pH values at half the maximum diffusion constant and where dD/dpH is a maximum. These pKa' values for barbital and pentobarbital are 7.50 and 7.72,

TABLE I—APPARENT DIFFUSION CONSTANTS,[a] D, AND SPECIFIC RATES OF DIFFUSION,[b] R_s, OF PHENYLALKYLAMINES THROUGH 3-MIL SILASTIC MEMBRANE AT 25.0° FROM BORATE BUFFER SOLUTIONS INTO pH 6.8 PHOSPHATE BUFFER

Compd.	pKa'	pH	C_2 10^3 M	f_2C_2[c] 10^3 M	R_s[b] 10^2 hr.$^{-1}$	D[a] 10^{10} l./cm.-sec.
α-Methylphenethylamine	9.07	8.93	3.18	1.55	2.84	6.84
3-Amino-1-phenylbutane	9.30	9.40	2.47	1.647	5.00	12.05
1-Methyl-5-phenylpentylamine	9.50	9.60	0.875	0.537	13.41	32.32
α-Ethylphenethylamine	9.30	9.28	1.79	0.917	7.80	18.80
2-Amino-4-methyl-4-phenylpentane	9.42	9.45	0.325	0.190	5.74	13.83

[a] Calculated from $D = XV_1(dC_1/dt)/3{,}600 f_2C_2S$ since $f_1 = 0$, where $X = 7.52 \times 10^{-3}$ cm., and $S = 10.4$ cm.^{2} where $(dC_1/dt)/f_2C_2$ is in hr.$^{-1}$ and $V_1 = 0.120$ l. is the maintained volume of the desorbing solution. [b] $(dC_1/dt)/f_2C_2$, where dC_1/dt is the linear slope of the total concentration in the phosphate buffer solution against time in hours. [c] Calculated from f_2C_2 where C_2 is the maintained concentration of the diffusing solution and $f_2 = [H^+]/([H^+] + Ka') = 10^{-pH}/(10^{-pH} + 10^{-pKa'})$.

TABLE II—APPARENT DIFFUSION CONSTANTS, SOLUBILITIES, AND PARTITION COEFFICIENTS OF BARBITURIC ACID DERIVATIVES

	°C.	C_2 10^3 M	R_S[a] $10^4(dC_1/dt)/C_2$	D[b] 10^{11} l./cm.-sec.	ΔE[c] kcal./Mole	Solubility[d] 10^3 M	Partition Coefficient[e] $CHCl_3$/Acetate Buffer
Amobarbital	24.7	2.97	13.4	5.76	5.1	3.9	20[f]
	37.0	2.80	20.2	8.12			
Barbital	24.1	29.6	0.541	0.216	6.7	38.6	0.80 (0.7)
	37.0	29.6	0.835	0.33			
Butabarbital	24.7	1.96	6.00	2.40	7.2	8.0	9.98
	37.2	2.18	9.87	3.95			
Cyclobarbital	24.7	2.17	2.06	0.82	10.1	35.0	1.36 (13.9)
	37.2	2.44	4.23	1.69			
Diallylbarbituric	24.7	2.34	3.14	1.26	3.6	8.5	3.00
	37.5	2.37	4.04	1.62			
Mephobarbital	24.7	1.01	23.2	9.28	13.6	—	56[f]
	37.2	0.92	61.0	24.4			
Metharbital	24.7	1.32	22.5	9.00	5.5	11.5	56[f]
	37.2	2.78	33.0	13.2			
Pentobarbital	24.1	3.71	11.8	4.72	6.8	3.0	26[f] (28)
	37.0	3.71	19.2	7.68			
Phenobarbital	24.1	4.08	1.64	0.66	6.4	4.6	4.48 (4.7)
	37.0	4.08	2.61	1.04			
Secobarbital	24.7	1.87	22.2	8.88	6.8	7.25	51 (51)
	37.2	1.83	35.6	14.24			
Thiamylal	24.7	0.26	102	40.6	18.2	0.348	high[f]
	37.2	0.42	370	148			
Thiopental	24.1	0.82	216	86.4	6.4	0.82	high[f]
	37.0	0.88	342	137			

[a] Rate of increase per hour of concentration, C_1, of 200 ml. of desorbing solution, pH 10.1 borate buffer, divided by the concentration, C_2, of the diffusing solution of 4.7 pH acetate buffer. This was actually determined from the linear slopes, dA_1/dt, of the plots of absorbance A_1 of the desorbing solution at the wavelengths specified in *Experimental* against time, t, in hours which were divided by the maintained absorbance A_2 of the diffusing solution since $(dA_1/dt)/A_2 = [d(\epsilon C_1)/dt]/\epsilon C_2 = (dC_1/dt)/C_2$ in hr.$^{-1}$. [b] Apparent diffusion constant for the specified barbituric acid derived from steady-state diffusion studies from pH 4.7 acetate buffer diffusing solutions of constant concentrations, C_2 through silastic membrane of thickness $X = 7.52 \times 10^{-3}$ cm. or 3.0 mil and available surface area $S = 10.4$ cm.^{2} into a desorbing pH 10.1 borate buffer solution of volume $V_1 = 0.200$ l. so that D (l./cm. − sec.) = $[(dC_1/dt)/C_2]\ (XV_1)/3600\ S$, where $(dC_1/dt)/C_2$ is given in hr.$^{-1}$. [c] Calculated from $\Delta E_a = [10^{-3}\ (\log D_{37^\circ} - \log D_{24^\circ})\ 2.303R/(1/T_{24^\circ} - 1/T_{37^\circ})]$, where T is the absolute temperature. [d] In pH 4.7 acetate buffer at 25.0°. [e] Parenthetical values are from the literature (14). The acetate buffer was at pH 4.7. [f] These values have large error since the absorbance readings in the acetate buffer were small.

TABLE III—APPARENT DIFFUSION CONSTANTS,[a] D, AND RATES OF DIFFUSION,[b] R, OF DEXTROMETHORPHAN FROM PEANUT OIL, MINERAL OIL, AND pH 10.1 BORATE BUFFER THROUGH 3-MIL SILASTIC MEMBRANE INTO pH 6.8 PHOSPHATE BUFFER AT 37.5°

Dextromethorphan Solution in	Peanut Oil		Mineral Oil	Borate Buffer
	Subsaturated	Saturated	Saturated	Saturated
C_2, 10^2 M	8.97	32.90	1.35	0.0105
R[b], 10^4 M l./hr.	0.6062	2.76	2.10	1.31
V_1, ml.	200	120	120	20
D[a], 10^{11} l./cm.-sec.	2.72	2.02	37.5	501.0

[a] Calculated from $D = XV_1(dC_1/dt)/3600\ f_2C_2S$ since $f_2 = 1$ and $f_1 = 0$, where $S = 7.52 \times 10^{-3}$ cm. and $S = 10.4$ cm.^{2}, where $(dC_1/dt)/f_2C_2$ is in hr.$^{-1}$ and V_1 is the maintained volume of the desorbing solution in liters, and C_1 and C_2 are the total concentrations of the desorbing and diffusing solutions, respectively. [b] dC_1/dt is the linear slope of total concentration in the phosphate buffer solution against time in hours.

respectively, whereas the values from titration were 7.45 and 7.65.

Quasi-Steady-State Diffusion Studies—When nondissociable or nonprotonable compounds such as progesterone are diffused through silastic membrane, there is no way of using buffers to effectively maintain the concentration of the diffusing species as zero in the desorbing solution, *i.e.*, $f_1 = 0$, in the manner that was possible with the previously cited amines and acids. When the same system was used to study the diffusion of progesterone, Eq. 2 is valid where C_2 is maintained constant but C_1 varies with time as $f_2 = f_1 = 1$. Plots of concentration, C_1, of desorbing solution of constant volume, V_1, show decreasing rates of increase with time and approach an asymptotic value as shown in Fig. 6 and the pertinent information is given in Table V.

Diffusion of Drugs from Silastic Capsules—The average area of the silastic membrane in the formulated capsules was 5.58 cm.^{2} with an average membrane thickness of 3.13×10^{-2} cm. However, in this preliminary formulation, the possibilities that some of the silastic adhesive spread over various areas of the capsule modified the thickness and that the capsules were not completely filled must be realized. The observed apparent diffusion constants for various drugs in various solutions from these formulated silastic capsules, on the assumption that the stated available area and thickness are valid, are given in Table VI and compared to those values obtained under the more controlled conditions in all diffusion studies. The comparisons are of the same order of magnitude. When the apparent diffusion constants are related to that of barbital as unity for each method, the

TABLE IV—APPARENT DIFFUSION CONSTANTS,[a] D, OF BARBITAL AND PENTOBARBITAL THROUGH 3.0-MIL SILASTIC MEMBRANE AT 37.3° AS A FUNCTION OF pH

pH	Barbital, D, 10^{13} l./cm.-sec.	pH	Pentobarbital, D, 10^{11} l./cm.-sec.
4.70	33.80	4.60	7.71
6.65	26.08	6.99	7.54
7.30	21.67	7.57	5.58
7.60	15.01	7.90	4.05
8.12	8.83	8.18	3.12
9.15	5.02	8.65	1.53
9.75	2.95	9.05	0.50
10.55	0.65	9.53	0.57

[a] Calculated from $D = XV_1(dC_1/dt)/C_2S$ since $f_1 = 0$, where $X = 7.52 \times 10^{-3}$ cm. and $S = 10.4$ cm.^{2} where V_1 is the maintained volume of the desorbing solution in liters, C_2 is the total concentration of the diffusing solution, and dC_1/dt is the linear slope of total concentration in the desorbing pH 10.1 borate buffer solution against time in seconds.

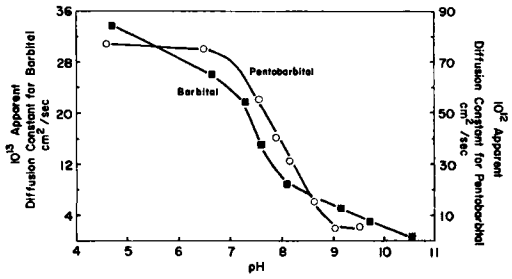

Fig. 5—Apparent diffusion constants of barbital and pentobarbital through 3-mil silastic membranes at 37.3° as a function of pH where no correction has been made for the concentration of uncharged species.

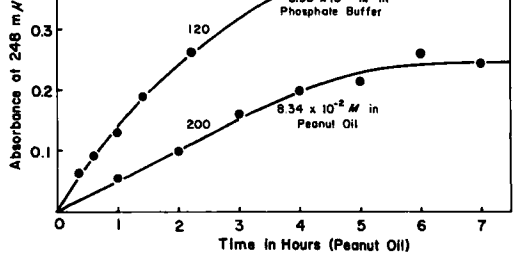

Fig. 6—Absorbance at 248 mμ of the stated volumes of pH 6.8 phosphate buffer desorbing solutions as a function of time on the quasi-steady-state diffusion of progesterone from peanut oil and pH 6.8 phosphate buffer of the stated concentrations through 3-mil silastic membrane at 37.5°.

high degree of correlation of these ratios (Table VI) are truly indicative that the factors affecting diffusion from solution in silastic capsules are the same as those affecting diffusion from solution in the cell.

DISCUSSION

Solid polymeric organic membranes are permeable to uncharged organic molecules which are

TABLE V—APPARENT DIFFUSION CONSTANTS,[a] D, OF PROGESTERONE FROM PEANUT OIL, AND pH 6.8 PHOSPHATE BUFFER THROUGH 3-MIL SILASTIC MEMBRANE INTO pH 6.8 PHOSPHATE BUFFER AT 37.5°

Solution in	Peanut Oil	Phosphate Buffer (Saturated)
Concn., C_2, M	8.34×10^{-2}	5.98×10^{-5}
Volume of phosphate buffer, V_1, ml.	200	120
Apparent diffusion constant, D, 10^{12} l./cm.-sec.	0.38	630

[a] Estimated from the slope of the plot of log $(C_{eq.} - C_1/C_0)$ against time in accordance with the expression log $(C_{eq.} - C_1/C_0) = \log (V_2/V_1 + V_2) - DSt(V_1 + V_2)/(V_1V_2)/2.303X$, where C_1 is the concentration of the desorbing solution of volume V_1 at any time, t, where $C_{eq.}$ is its concentration after final equilibration, C_0 is the concentration of the diffusing solution of volume V_2, S is the area of the 3-mil silastic membrane, and X is the thickness in cm.

lipophilic. In particular, silastic membrane is permeable to the uncharged species of the aminophenones such as *p*-aminoprophenone (1, 2), phenylalkylamines such as amphetamine, steroids such as progesterone, alkaloids such as dextromethorphan, and the barbiturates. These polymeric membranes are impermeable to the charged organic drug and the inorganic ions studied.

The pKa′ values of protonable or dissociating drugs can be determined from the study of rates of steady-state diffusion as a function of the pH of the diffusing solution where half of the maximum diffusing rate at that total concentration is obtained when pH = pKa′ (Fig. 5).

Water and other solvents such as mineral oil do not appear to be readily transported by silastic, although ethanol is transported without modifying the membrane (2).

The classical laws of diffusion appear to hold for silastic membranes so that transport rates in steady-state and quasi-steady-state diffusion can be predicted for particular diffusing and desorbing solutions, their concentrations and pH values from a knowledge of the thickness and area of the membrane, the pKa′ of the diffusing species, and the apparent diffusion constant, $D = KD'$, of the uncharged species.

The latter have now been determined for the several classes of drugs (Tables I–III, V). Only if studies are conducted on thick membranes to determine the intercept value in nonsteady-state diffusion (2, 15–17) can the intrinsic diffusivity constant, D', within the membrane and the actual partition coefficient, K, be separated.

The fact that the rates of transfer (Eqs. 1 and 2) are directly proportional to the concentrations of the diffusing solution up to the limit of the drug's solubility, implies that there is no selective binding to these membranes, no limitations as to sites, and that at all times the concentration in the monolayer on the diffusing side of the membrane is in instantaneous equilibrium with the solution in contact therewith. It also implies and has been confirmed that saturated solutions of drugs diffuse through the membranes at a constant rate.

The apparent diffusion constant, D, can be formulated as a product of the intrinsic diffusion

TABLE VI—APPARENT DIFFUSION CONSTANTS,[a] D, AND SPECIFIC RATES OF DIFFUSION,[b] R_S, OF DRUGS FROM SILASTIC CAPSULES AT 37.5°

Compound	Internal Solution	R_S[b] 10^4 hr.$^{-1}$	D,[a] 10^{11} l./cm.-sec. Silastic Capsules	D,[a] 10^{11} l./cm.-sec. Diffusion Cell	Relative Diffusivities[c] Silastic Capsules	Relative Diffusivities[c] Diffusion Cell
Barbital	Acetate buffer	2.18	0.170	0.338	1.00	1.00
Phenobarbital	Acetate buffer	9.55	0.745	1.05	4.382	3.11
Pentobarbital	Acetate buffer	4.89	3.81	7.71	22.41	22.81
4'-Aminopropiophenone	Phosphate buffer	29.8	38.9	48.2	251.24	142.60
Dextromethorphan	Borate buffer	19.7	154.0	501.0	905.88	1,482.24
Dextromethorphan	Peanut oil	0.967	0.754	2.02	4.38	5.98
Dextromethorphan	Mineral oil	25.0	19.5	37.5	114.71	110.95

[a] Calculated from $D = XV_1 Rs/3600\ S$, where X is the thickness of the membrane in cm. and S is the available surface area in cm.^{2}. [b] The specific rate of diffusion $Rs = (dC_1/dt)/f_2C_2$ is obtained from the slope of the concentration—time in hours plot of the desorbing solution divided by the concentration of the uncharged species in the diffusing solution. [c] Apparent diffusion constant divided by that for barbital from silastic capsules and diffusion cell, respectively.

constant, D', within the membrane, and the operational partition coefficient for the uncharged species between the membrane and the solvent when the diffusing and desorbing solvents are the same (Eq. 2). Another confirmation of this hypothesis is the linear relation between the apparent diffusion constant, D, of a series of barbituric acids for diffusion through silastic membranes and their partition coefficients between acetate buffer solution and chloroform (Fig. 7). Knowledge of rank order of such partition should permit prediction of relative diffusion through such membranes. No significant correlation exists for apparent diffusion constants with solubility or its reciprocal in the diffusing solution for the barbiturate series. This is readily understandable since

$$D = D'K = D'C_m/C_i = D'S_m(1/S_i) \quad \text{(Eq. 7)}$$

where C_m and C_i are the concentrations in the equilibrated monolayer of the membrane and the concentration in the contacting solution, respectively; and where S_m and S_i are the respective solubilities. The apparent diffusion constant would be inversely proportional to the reciprocal of the solubility in the solvent only if the solubility of each drug of a homologous series were the same in the membrane. The fact that the apparent diffusivities, D, are proportional to partition coefficients but not to the solubilities in the case of barbiturates (Table II) denies this assumption.

An interesting result of this analysis is the conclusion that a species in solution can be separated

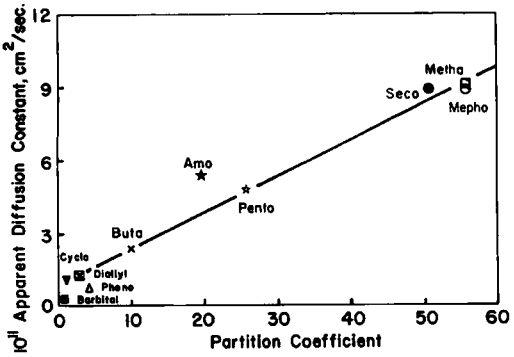

Fig. 7—The apparent diffusion constants of various barbitals diffusing through 3-mil silastic membrane at 25.0° as a function of their partition coefficients between 4.7 pH acetate buffer and chloroform.

from ionic or salt contaminants and concentrated in a desorbing solvent wherein it has a greater activity or solubility than the diffusing solvent. At final equilibrium, the rate of diffusion $dA/dt = 0$ and from Eq. 1.

$$K_2f_2C_2 = K_1f_1C_1 \quad \text{(Eq. 8)}$$

so that, when Eq. 7 is considered,

$$\frac{C_1}{C_2} = \frac{K_2f_2}{K_1f_1} = \frac{f_2}{f_1}\cdot\frac{S_1}{S_2} \quad \text{(Eq. 9)}$$

so that the ratio of the concentrations in both desorbing and diffusing solutions at equilibrium is proportional to the ratio of their solubilities, S_i, in these solutions. In the specific cases of immiscible solvents, these are equivalent to the partition coefficient of the compound in these solvents. In the general case of miscible solvents, such as was shown for ethanol and water (2), these ratios may be considered as hypothetical partition coefficients.

These studies on diffusion through silastic membranes and the fact that solid materials such as progesterone and other steroids in contact with the membrane do permeate (18–21) permits one to conceive of such membranes as isotropic media capable of dissolving drugs in a solid solution with all the operational properties of true solutions. The rates of diffusion within the membrane are dependent on the concentration gradients within them and the distance, X, of traversal. The overall rates of diffusion from solid particles of drug should be dependent on whether or not the rates of dissolution of these particles in contact with the membrane are rate determining. Such particles suspended in a polymer matrix should have rates of dissolution proportional to their surface area in contact. Release rates from the membrane into surrounding solvents would depend on whether these dissolution rates were of higher or lower orders of magnitude than diffusion within the membrane.

SUMMARY

Various polymer films were screened for permeability to various drugs dissolved in several solvents. Detailed attention was placed on the diffusion of a series of barbituric acid and phenylalkylamines, and apparent diffusivities could be correlated with chloroform–acetate buffer partition coefficients. It was ascertained that Fick's law for solid poly-

meric membranes is operative with respect to the concentrations of uncharged organic compounds. Apparent diffusion constants from various solvents were obtained from steady-state and quasi-steady-state studies. Solid materials in contact with such membranes modified to act as potential pharmaceutical dosage forms were studied and demonstrated that basic diffusional information could be used to evaluate, predict, and control such diffusion.

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Keyphrases

Polymeric membranes—drug diffusion
Silastic membranes—diffusion rates
Capsules, silastic—drug diffusion
Solid particles—membrane transport
Diffusion—steady state
Potentiometric titration—pKa values
UV spectrophotometry—analysis
Colorimetric analysis—spectrophotometer

Effect of a Dimethylpolysiloxane Fluid on the Stability of Menadione

By RONALD T. TURNBULL* and KENNETH E. AVIS

The nonaqueous solvents presently in use as parenteral vehicles possess certain disadvantages or have limited application. The silicone fluids have certain physical, chemical, and biological properties which would appear to make them suitable for parenteral formulations. Accelerated thermal and light stability studies were carried out on a dimethyl silicone fluid of 20 centistokes viscosity and corn oil using menadione as the medicinal compound for study. Based on predictions from the accelerated thermal studies, it was found that the dimethyl silicone fluid was superior to corn oil. Corn oil was found to be superior to the dimethyl silicone in retarding photodegradation of menadione at room temperature. Since greater emphasis must be placed on the ability of the vehicle to retard thermal changes in a chemical compound, the experimental results indicate that the dimethyl silicone fluid of 20 centistokes viscosity is superior to corn oil as a vehicle for menadione.

AQUEOUS SYSTEMS are normally preferred for liquid dosage forms intended for parenteral administration because body systems are aqueous and absorption of the drug generally occurs more readily from an aqueous system. However, a number of therapeutic agents, because of their solubility characteristics, can only be formulated in nonaqueous solvent systems. Although the number of such agents is considerably less than those requiring aqueous systems, they are vital to our therapeutic armamentarium.

The nonaqueous vehicles most commonly used are the fixed oils. All of these, however, have disadvantages in use, such as: the development of rancidity with aging, potential allergenic reactions in sensitive individuals and local tissue reac-

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Accepted for publication May 15, 1968.
Presented to the Parenteral Drug Association, Chicago meeting, April 1967, and to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
Abstracted in part from a thesis submitted by Ronald T. Turnbull to the Graduate School, University of Tennessee, Memphis, in partial fulfillment of the Master of Science degree requirements.
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meric membranes is operative with respect to the concentrations of uncharged organic compounds. Apparent diffusion constants from various solvents were obtained from steady-state and quasi-steady-state studies. Solid materials in contact with such membranes modified to act as potential pharmaceutical dosage forms were studied and demonstrated that basic diffusional information could be used to evaluate, predict, and control such diffusion.

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tions, including cysts, foreign body granulomas, and occasional nerve injury (1). All of these and other solvents currently in use possess certain disadvantages or have limited applications, therefore, a need exists for other nonaqueous vehicles.

The dimethylpolysiloxane fluids appear to possess most of the characteristics an ideal nonaqueous solvent should have (1), namely, they possess very great chemical and oxidative stability, they have low viscosity-temperature coefficients, their viscosity can be tailored to meet the desired physical needs, their boiling points are high enough to permit heat sterilization, they are nonflammable, and they are very low in toxicity (2–8).

The use of silicone fluids as nonaqueous vehicles for the intramuscular administration of drugs has been reported in the literature (9). This brief account is the only known record of work that has been done in exploring such use for this potentially valuable series of solvents (10).

The objective of this study was to explore the physicochemical effects of dimethylpolysiloxane fluid, 20 centistokes, when utilized as a vehicle for a particular drug, menadione. The observed effects were compared with those obtained using one of the commonly used nonaqueous vehicles, corn oil.

EXPERIMENTAL

Materials—Menadione, USP; silicone fluid[1], 20 centistokes (supplied by Dow Corning Chemical Co., Midland, Mich.); 2,4-dinitrophenylhydrazine; corn oil.

Equipment—Beckman DU spectrophotometer; type I amber glass vials (supplied by Kimble Products, Division of Owens-Illinois, Toledo, Ohio); rubber vial closures, No. 342 Red, P and H coated (supplied by Carron Products Co., Philadelphia, Pa.); Gossen Luna Six light meter; light stability cabinet.

Assay Method—A modified form of the assay procedure official in the NF XII (11) was utilized. The official assay is a colorimetric procedure reacting menadione with 2,4-dinitrophenylhydrazine, and depends on the formation of the 2,4-dinitrophenylhydrazone of the naphthoquinone portion of the molecule, and the interaction of the latter compound with ethanolic ammonia to yield a blue-green colored solution. The concentration is determined spectrophotometrically at a wavelength of 635 mμ. In the official assay, an original sample of approximately 25 mg. in solution is used, the spectrophotometric reading being taken on a 1:100 dilution.

In preliminary trials, it was found that the absorbance of menadione did not conform to Beer's law, when percent transmission was plotted against concentration, throughout the range of concentration tested, *i.e.*, 2.5–25 mg., from either corn oil or silicone fluid, although it did conform to Beer's law from the standard ether–alcohol vehicle. The deviation in the slope occurred at the original concentration of between 12.5 and 15 mg. Since this deviation did exist, had a 25-mg. sample been used, as recommended in the official assay, the assay results would have been unreliable up to a degradation of 50%. Conformity to Beer's law was demonstrated between 2.5 and about 15 mg. in the test sample, therefore, a 10-mg. sample was used in this study.

Thermal Stability—Four temperatures were chosen to study the thermal stability of menadione in each vehicle: room temperature, 35, 55, and 70°. Solutions of menadione in corn oil and the dimethylpolysiloxane fluid, 20 centistokes, were prepared at a concentration of 1 mg./ml. Three samples were assayed for each storage interval and each assay was performed in triplicate. The solutions stored at 55° were assayed every 7 days for 3 weeks. The solutions stored at room temperature, 35, and 70°, were assayed every 14 days for a period of 9 weeks.

Light Stability—A light stability cabinet suggested by Lachman *et al.* (12) for ordinary illumination was used for the light study. From preliminary trial data a light intensity of 750 ftc. was found to be sufficient to cause degradation within a few hours. Solutions of menadione in both vehicles were prepared at a concentration of 1 mg./ml. Ampuls of type I colorless glass containing the solutions were laid horizontally in the center of the shelf in the cabinet to allow for maximum and uniform exposure.

Three samples were tested for each exposure period and assayed in triplicate. The samples were assayed at zero-storage time and after 2, 6, 10, and 12 hr. exposure to light at an intensity of 750 ftc.

RESULTS AND DISCUSSION

Assay Modification—As indicated previously, the absorbance from menadione did obey Beer's law in the standard ether–alcohol vehicle. Figure 1 shows the results of the same assay procedure when menadione was placed in the dimethylpolysiloxane fluid. This curve and the one obtained for menadione in corn oil are essentially identical. As can be seen, a linear relationship occurs only from 25 mcg./50 ml. to a point somewhere between 125 and 150 mcg./50 ml. Therefore, the assay procedure was modified to use an original sample of 10 mg. (100 mcg./50 ml. in dilution) so that degradation at least up to 75% could be measured by this test procedure.

Thermal Stability—The effects of temperature on the degradation of menadione in both vehicles tested

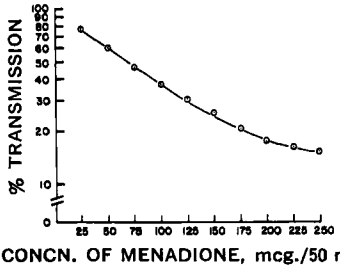

Fig. 1—Standard curve for menadione in dimethyl silicone fluid, 20 centistokes.

[1] Medical Fluid 360.

TABLE I—COMPARATIVE EFFECTS OF TEMPERATURE ON MENADIONE

Temperature, °C.	Vehicle	Days 0	7	14	21	35	49	63
Room temp.	Corn oil	100.0[a]	100.0	...	96.9	95.2	85.6	84.0
	DMSF[b]	100.0	—	100.0	91.8	103.4	105.2	105.6
35	Corn oil	100.0	100.3	...	92.2	87.5	82.9	81.0
	DMSF[b]	100.0	95.5	...	95.5	94.8	95.2	94.1
55	Corn oil	100.0	100.0	91.0	88.1	—	—	—
	DMSF[b]	100.0	85.7	88.0	91.1	—	—	—
70	Corn oil	100 0	92.5	—	74.4	64.6	57.6	53.3
	DMSF[b]	100.0	82.1	—	69.8	51.5	42.3	28.3

[a] Percent remaining. [b] DMSF, dimethyl silicone fluid, 20 centistokes.

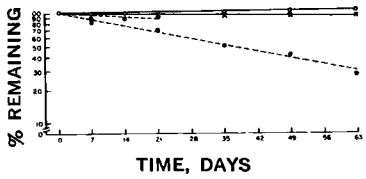

Fig. 2—Effect of temperature on the stability of menadione in dimethyl silicone fluid, 20 centistokes. Key: —○—, room temperature; --×--, 35°; --◑--, 55°; --●--, 70°.

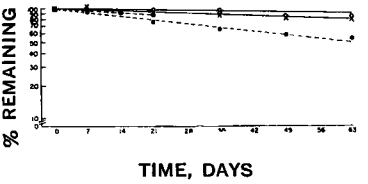

Fig. 3—Effect of temperature on the stability of menadione in corn oil. Key: —○—, room temperature; —×—, 35°; --◑--, 55°; --●--, 70°.

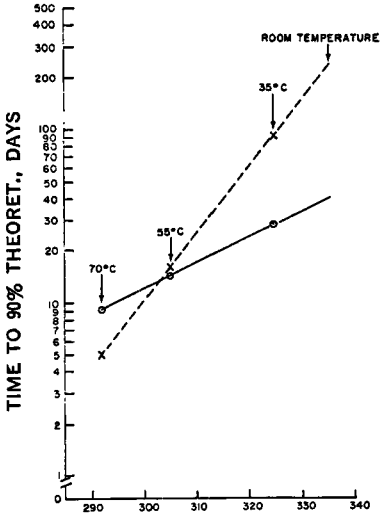

Fig. 4—Comparison of the theoretical shelf life of menadione in corn oil and dimethyl silicone fluid, 20 centistokes. Key: —⊙—, corn oil; --×-- silicone.

are summarized in Table I. When these data are plotted as in Figs. 2 and 3, the straight lines obtained indicate that the rate of degradation in both vehicles follows that of a first-order reaction. The rate of degradation at a storage temperature of 70° was found to be considerably greater in dimethyl silicone fluid than in corn oil, but at room temperature and 35°, considerably greater in corn oil than in dimethyl silicone fluid. In both fluids, the rate of degradation was greater at higher temperatures.

Utilizing these data to prepare an Arrhenius plot, as proposed by Free and Oyer (13), it is possible to predict the stability of menadione at room temperature. By this method, the times at which the product, stored at selected elevated temperatures, reached 90% of its original concentration was plotted against $1/T$ (absolute temperature). Extending the line to the intercept of $1/T$ equal to room temperature indicated the predicted time of stability to 90% of original concentration. The authors' data for menadione in corn oil and in dimethyl silicone fluid have been plotted in this manner and the results shown in Fig. 4. As can be seen, there is a significant difference in the effects of the two vehicles. The predicted shelf life for menadione in corn oil was about 40 days, while that for menadione in the dimethyl silicone fluid was about 230 days. The experimentally determined value for menadione in corn oil was found to be 45 days. Therefore, the predicted value of 40 days was confirmed quite closely by experimental data.

Light Stability—The experimental results from the light stability study are plotted in Fig. 5. As may be seen, there is a difference in the rate of degradation of menadione in the dimethyl silicone fluid as compared with corn oil. The dimethyl silicone fluid was somewhat less effective than corn oil in retarding the photodegradation of menadione. The rate of degradation in both vehicles was rapid up to about 20% of the original concentration remaining. Subsequently, there was a pronounced reduction in the rate, particularly for the dimethyl silicone fluid. This leveling off of the rate of degradation agreed with the results reported by Collins and Kirch (14).

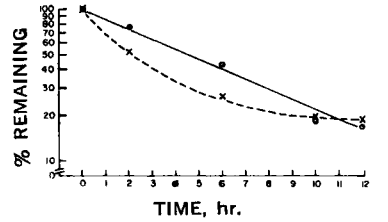

Fig. 5—Effect of light at an intensity of 750 ftc. on the stability of menadione in corn oil and dimethyl silicone fluid. Key: —⊙—, corn oil; --×--, silicone.

SUMMARY

The silicone fluids appear to have certain physical, chemical, and biological properties which would make them useful as nonaqueous solvents for the administration of medicinal agents.

Based upon data from accelerated thermal studies, it was found that the dimethyl silicone fluid was superior to corn oil in preventing the thermal degradation of menadione.

The photodegradation studies indicated that, although corn oil was somewhat superior to the dimethyl silicone fluid in preventing the degradation of menadione, photodegradation was rapid in both vehicles. Since photodegradation can be controlled during normal storage by protective packaging, its practical significance is less than the relatively uncontrollable thermal storage conditions.

In consideration of these findings, it may be concluded that the dimethyl silicone fluid is superior to corn oil as a vehicle for menadione.

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Colorimetric analysis—spectrophotometer

Drug Standards

Assay of Terpin Hydrate and Codeine Elixir by Gas Chromatography

By HAROLD J. WESSELMAN

Terpin hydrate and codeine after extraction from the elixir are separated and determined with the aid of two internal standards and the use of temperature-programmed gas chromatography. Using the same method, elixir of terpin hydrate can also be determined. A precision and accuracy study for both elixirs is included.

THE QUANTITATIVE determination of terpin hydrate, *cis-p*-menthane-1,8-diol hydrate, has always presented problems. Indeed the National Formulary XII (1) does not include an assay for terpin hydrate or codeine in either the elixir of terpin hydrate or the elixir of terpin hydrate and codeine. In 1921 Murray (2) used a gravimetric method for terpin hydrate. Harrison (3), Carol (4, 5), and Lund and Ameiss (6) improved this method. Later, in 1932, Perelmann (7) proposed a colorimetric method which was modified by Platt and James (8) and Vadodaria, Parikh, and Mukherji (9).

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gomery, Jennings, and Weinswig (15) applied ion-exchange chromatography followed by ultraviolet absorption analysis.

Domange and Longuevalle (16) suggested that terpin hydrate could be determined by gas chromatography but provided no tangible evidence. Recently Kurlansik, Damon, and Salim (17, 18) and Mahn, Viswanathan, and Senkowski (19) devised gas chromatographic methods for terpin hydrate in elixir of terpin hydrate and elixir of terpin hydrate and codeine.

The gas chromatography of codeine was investigated qualitatively by Parker, Fontan, and Kirk (20) and Massingill and Hodgkins (21) and made quantitative by Mule (22) and Schmerzler *et al.* (23).

The literature does not record the use of gas chromatography for the simultaneous determination of terpin hydrate and codeine. These compounds are difficult to separate by isothermal gas chromatography because of their widely spaced boiling points. Another difficulty in the case of the elixir of terpin hydrate and codeine is that terpin hydrate being the more volatile of the two is present in the greatest amount, 17.0 mg./ml., while the higher boiling codeine has only a concentration of 2.0 mg./ml.

The present work overcomes these problems by using a combination of isothermal and temperature-programmed gas chromatography when both compounds are present. By using isothermal gas chromatography, terpin hydrate in elixir of terpin hydrate can be determined easily.

EXPERIMENTAL

Equipment—A linear programmed-temperature gas chromatograph, F & M Scientific Corp., model 402, equipped with a flame-ionization detector, was used for the experimental work. The detector signal was fed to a Honeywell Electronic 16 1-mv. recorder with a chart speed of 15 in./hr. and a 1-sec. full-scale response. Samples were injected with a 10-μl. Hamilton, No. 701, syringe.

Materials—Helium was used as a carrier gas, while electrolytic hydrogen and oxygen were used in the detector. The stationary phase was 3.8% Linde W-98 silicone gum applied by the solution technique of Horning *et al.* (24) to Diatoport S (80–100 mesh) and packed in dual borosilicate glass columns 0.91 m. × 0.64 cm. (3 ft. × 1/4 in. o.d.). A mixture of chloroform and ethanol (1:1) was used to dissolve the analytical reagent grade benzoic acid, terpin hydrate, codeine, and cholestane.

Operating Conditions—For the elixir of terpin hydrate and codeine, the columns were operated isothermally at 140° for 4 min. and then programmed to 275° at a heating rate of 10° per min. At the end of each run, the oven was cooled for exactly 10 min. and then equilibrated at 140° for exactly 10 min. before injecting the next sample. The electrometer range was 1,000 with an attenuation of 64 for terpin hydrate and benzoic acid and 4 for codeine and cholestane. In the case of the elixir of terpin hydrate, the columns were operated isothermally at 135° with an attenuation of 32 on range 1,000.

The helium flow rate was 55 ml./min. with an inlet pressure of 40 psig. Oxygen and hydrogen flow rates were 250 and 35 ml./min., respectively. The sample injection port was maintained at 240° and the detector block at 215°. One-microliter injections of all samples were used throughout.

Quantitative Analysis—This method uses two internal standards—benzoic acid for terpin hydrate and cholestane for codeine.

Elixir of Terpin Hydrate and Codeine—Prepare a standard solution by adding exactly 85.0 mg. benzoic acid, 85.0 mg. terpin hydrate, 10.0 mg. codeine alkaloid, and 10.0 mg. cholestane to a 5-ml. volumetric flask and diluting to volume with a 1:1 mixture of chloroform and ethanol.

Prepare a sample solution by transferring exactly 10.0 ml. of the elixir to a 125-ml. separator and adding 3 ml. sodium hydroxide solution (1:10 distilled water). Extract with four 20-ml. portions of chloroform, collecting the chloroform extracts in a 150-ml. beaker. Carefully evaporate the chloroform, using a stream of dry air and a water bath, to a volume of about 2 ml. Transfer the concentrated extract to a 10-ml. volumetric flask with the aid of chloroform. Carefully evaporate the chloroform to dryness. Dilute to volume with the internal standard solution which is prepared by diluting 850 mg. benzoic acid and 100 mg. cholestane to 50 ml. with a 1:1 mixture of chloroform and ethanol. Chromatograph the standard and the extracted sample solutions and measure the peak height of each component.

Elixir of Terpin Hydrate—Prepare a standard solution by adding exactly 85.0 mg. benzoic acid and 85.0 mg. terpin hydrate to a 5-ml. volumetric flask and diluting to volume with a 1:1 mixture of chloroform and ethanol.

A 10-ml. sample of the elixir is extracted as discussed above. The final dilution is made with an internal standard solution prepared by adding exactly 850.0 mg. benzoic acid to a 50-ml. volumetric flask and diluting to volume with a 1:1 mixture of chloroform and alcohol. Chromatograph the standard and the extracted sample solutions and measure the peak height of each component.

Calculations.

$$\frac{\text{peak height standard benzoic acid}}{\text{peak height standard terpin hydrate}} = A$$

$$\frac{\text{peak height sample benzoic acid}}{\text{peak height sample terpin hydrate}} = B$$

$$A/B \times 17 = \text{mg. terpin hydrate/ml. elixir.}$$

$$\frac{\text{peak height standard cholestane}}{\text{peak height standard codeine}} = C$$

$$\frac{\text{peak height sample cholestane}}{\text{peak height sample codeine}} = D$$

$$C/D \times 2 = \text{mg. codeine/ml. elixir.}$$

Precision and Accuracy—To determine the precision and accuracy of the methods, freshly prepared solutions of terpin hydrate and codeine and terpin hydrate in distilled water were used as standard solutions. The sample solutions were prepared

TABLE I—PRECISION AND ACCURACY STUDY FOR ELIXIR TERPIN HYDRATE AND CODEINE

	Terpin Hydrate	Codeine
Standard solution, mg./ml.	17.00	2.00
Sample solution, mg./ml.	16.47	2.00
	16.53	2.03
	16.88	2.01
	17.29	2.01
	17.32	1.99
$\sum x$	84.49	10.04
$\bar{x}$	16.90	2.01
$\sum x^2$	1428.3627	20.1612
s	0.2500	0.0132
RSD	±1.48%	±0.66%
Mean error	−0.10	+0.01
Relative error	−0.59%	+0.50%

TABLE II—PRECISION AND ACCURACY STUDY FOR ELIXIR TERPIN HYDRATE

	Terpin Hydrate
Standard solution, mg./ml.	17.00
Sample solution, mg./ml.	16.83
	16.81
	16.68
	16.66
	16.81
$\sum x$	83.79
$\bar{x}$	16.76
$\sum x^2$	1404.18
s	0.24855
RSD	±1.48%
Mean error	−0.24
Relative error	−1.41%

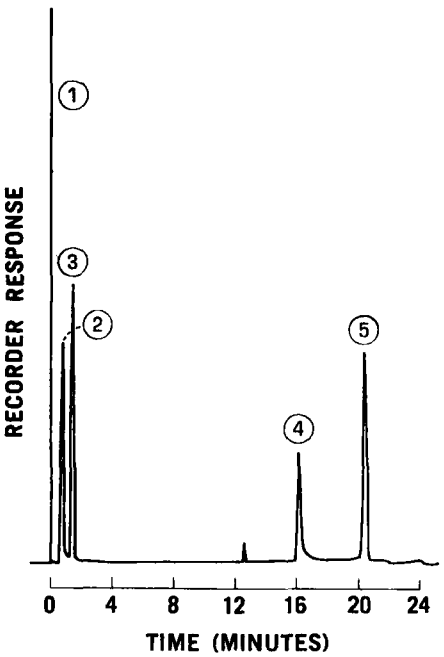

Fig. 1—Typical chromatogram of chloroform-ethanol (1), benzoic acid (2), terpin hydrate (3), codeine (4), and cholestane (5).

by dissolving the same components in freshly made elixir blanks. These studies were carried out in accordance with the suggestions of the Advisory Board of Analytical Chemistry (25) and the recommended nomenclature is used. Tables I and II show the results of these studies.

RESULTS AND DISCUSSION

A typical chromatogram of a mixture of terpin hydrate, benzoic acid, codeine, and cholestane is shown in Fig. 1. Table III gives the results of the assays of three production lots of elixir of terpin hydrate and codeine. Table IV shows the results obtained from three production lots of elixir of terpin hydrate. Triplicate assays in each case show good agreement.

TABLE III—RESULTS OF ASSAY OF ELIXIR TERPIN HYDRATE AND CODEINE

	Terpin Hydrate	Codeine
Theory, mg./ml.	17.00	2.00
Lot A	16.34	2.00
	16.04	2.00
	16.19	1.99
Lot B	17.32	2.00
	17.13	2.02
	17.29	2.01
Lot C	16.83	2.03
	16.84	2.03
	16.88	2.01

TABLE IV—RESULTS OF ASSAY OF ELIXIR TERPIN HYDRATE

	Terpin Hydrate
Theory, mg./ml.	17.00
Lot D	16.39
	16.30
	16.41
Lot E	17.62
	17.50
	17.45
Lot F	17.36
	17.12
	17.45

CONCLUSION

A new method for determining terpin hydrate and codeine simultaneously using a combination of isothermal and programmed-temperature gas chromatography has been developed. The use of two internal standards permits a high degree of precision and accuracy. The relative standard deviations for terpin hydrate and codeine are ±1.48% and ±0.66%, respectively. The procedure is fast and accurate and easily carried out. The overall time for determining the standard and the sample is approximately 1 hr.

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Keyphrases

Terpin hydrate-codeine elixir—analysis
GLC—analysis
Benzoic acid—internal standard, terpin hydrate
Cholestane—internal standard, codeine
Isothermal, programmed temperature—GLC

Technical Articles

Automated Colorimetric Analysis of Ethinyl Estradiol and Mestranol in Pharmaceutical Tablets

By WILLIAM F. BEYER

An automated colorimetric procedure is described for the determination of ethinyl estradiol and ethinyl estradiol-3-methyl ether in pharmaceutical tablets. Chloroform solutions of the estrogen are automatically extracted with alcohol–sulfuric acid and analyzed at a rate of 20 samples/hr. The relative standard deviation of repetitive sampling of tablet extracts was less than 1 percent, and Beer's law was obeyed over the range of 0.40–8.00 mcg./ml. of sample solution. The procedure is applicable to unit dosage assays and to assays requiring composite samples of pulverized tablets or numerous whole tablets.

NUMEROUS METHODS have been reported for the determination of ethinyl estradiol (EE) or its 3-methyl ether derivative (EE-3ME) from tablets (1–5). The majority of these procedures depends upon the spectrophotometric analysis of sulfuric acid-induced color, and involve somewhat lengthy separative techniques.

A recent publication of Khoury and Cali (6) describes an automated assay for EE and EE-3ME based on the fluorescence exhibited by the estrogens in 90% sulfuric acid. The necessity of assaying large numbers of tablets for EE in combination with nonestrogenic steroids also prompted the investigation of automation in these laboratories. A relatively simple, automated method has been developed with instruments normally found in laboratories employing automatic analysis—sampler, proportioning pump, heating bath, spectrophotometer, and recorder. The procedure is based on a manual method developed in these laboratories (7), a modification and quantitation of the USP XVII sulfuric acid identification test for EE (1). The red color formed when sulfuric acid is added to a chloroform solution of EE is extractable with sulfuric acid and exhibits a maximum absorption at 518 mμ. The color develops rapidly and is

Received April 3, 1968, from Control Division, The Upjohn Company, Kalamazoo, MI 49001
Accepted for publication May 14, 1968.
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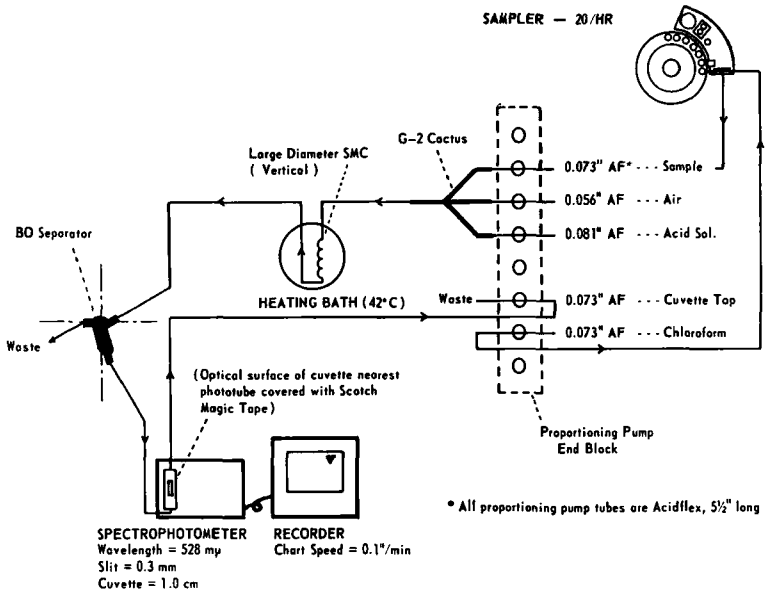

Fig. 1—Flow diagram for the automated determination of ethinyl estradiol and ethinyl estradiol-3-methyl ether, at wavelengths of 528 and 538 mμ, respectively.

stable at room temperature for at least 15 hr. Although the reaction is not influenced by the volume of chloroform, the color is enhanced by the small amount of alcohol used as a preservative in chloroform. The interference of nonestrogenic steroids present in tablets in combination with the estrogen is eliminated by correcting for absorbances due to nonestrogenic steroids.

Details of the automated procedure and a comparison of assay results with the manual procedure are the subject of this report.

EXPERIMENTAL

Equipment—Automatic sampler, proportioning pump, and assorted tubing and glass fittings[1]; heating bath[2]; spectrophotometer[3]; recorder[4]; and 12 × 35-mm. glass shell vials.

Reagents—Chloroform, analytical grade; 10% 3A alcohol[5] in 90% sulfuric acid, analytical grade (v/v).

Standards—Chloroform stock solutions containing 100 mcg./ml. of USP ethinyl estradiol or ethinyl estradiol-3-methyl ether are refrigerated in tightly sealed volumetric flasks. The stock standards are brought to room temperature and diluted with chloroform to approximately 4 mcg./ml. just prior to use.

Interfering Nonestrogenic Steroids—Chloroform stock solutions of steroids present in tablet formulation in combination with EE or EE-3ME are refrigerated in tightly sealed volumetric flasks. Chloroform solutions of nonestrogenic steroids are prepared from the stock solutions, maintaining the ratio of estrogen to nonestrogen that exists in the tablets.

Tablets—Tablets are disintegrated by gentle shaking in polyethylene-stoppered conical flasks, containing a small amount of water and several glass beads. To completely disintegrate the tablets, the stoppers are loosened and the flasks are heated on a steam bath for approximately 15 min. After cooling to room temperature, chloroform is added at a ratio of 10 ml. for each ml. of water so that the final estrogen concentration is similar to that of the standard. For example in the assay of 0.05-mg. EE tablets, eight tablets are disintegrated with 10.0 ml. of water and extracted with 100.0 ml. of chloroform. The flasks are shaken vigorously for approximately 30 min. A portion of the chloroform phase is filtered directly into shell vials using a rapid flow filter paper such as Whatman No. 41. A layer of water (approximately 0.4 ml.) is added to each vial immediately after filtration to suppress evaporation of chloroform.

Procedure—The sampling probe of the automatic sampler is aligned so that satisfactory aspiration from the shell vials and from the rinsing compartment occurs. A zero base line is established with all instruments operating using the flow system of Fig. 1 with sample and rinse lines in chloroform and the remaining line in acidic alcohol. Sampling rate is set at 20/hr. with a sample–rinse ratio of 1:1. Before aspiration of samples, the flow cell is inverted and tapped to release chloroform or water droplets that may be trapped. Vials of the nonestrogenic steroid are sampled first if that particular steroid is present in the tablets in combination with the estrogen. Vials of duplicate EE or EE-3ME standards follow, with approximately 0.4 ml. of water added to vials of both the nonestrogenic steroid and standard. Additional standards are interspaced among test samples to compensate for instrumental variation. EE is measured at a wavelength of 528 mμ, EE-3ME at 538 mμ.

Calculations are based on the relative absorbance of standards and test samples with corrections made for the absorbance of the nonestrogenic steroid present in the tablet formulation.

RESULTS AND DISCUSSION

Virgorous shaking of tablets in chloroform follow-

[1] Technicon, Ardsley, N. Y.
[2] Tamson heating bath, Witt Sales, Cleveland, Ohio
[3] Hitachi-Perkin-Elmer 139 spectrophotometer with 1.0-cm. Thomas flow cell No. 9120-NO5, A. H. Thomas Co., Philadelphia, Pa.
[4] Sargent SRL recorder
[5] US Industrial Chemical Co., New York, N. Y. (100 gal. ethanol denatured with 5 gal. methanol).

ing disintegration with a small amount of water, gave both a simple and efficient extraction of EE. The use of chloroform samples introduced difficulties; however, satisfactory procedures were developed that permitted their use. An attachment to hold glass shell vials in the sample plate of the automatic sampler and a glass cover plate for the sampler were constructed. Two metal disks were attached to the standard sample plate, below the plastic holder; one with forty 1.3-cm. (0.5-in.) holes to center the shell vials, the other without holes as a support for the containers. An exact duplicate of the original plastic cover plate was made using plate glass, to eliminate the effect of chloroform on the plate. Placing a layer of water on the vials of chloroform, eliminated the problem of chloroform evaporation. In-stream extraction of the estrogen in chloroform with sulfuric acid was aided by adding 10% water and 10% alcohol to the acid and by using a 42° water bath. Alcohol added to diluted sulfuric acid decreased the viscosity of the acid, giving a uniform flow of liquids and a satisfactory separation of acid–chloroform phases. The effect of striations caused by sulfuric acid passing through the flow cell was eliminated by applying Scotch Magic tape[6] to the optical surface of the flow cell nearest the photo tube in the manner described by Anderson *et al.* for the automated assay of vitamin A (8). Maximal absorption of EE and EE-3ME occurred at 528 and 538 mμ, respectively, verified with a recording spectrophotometer[7] after manually collecting acid from the exit port of the flow cell while sampling continuously.

Recordings of repetitive and continuous sampling of chloroform solutions of EE at a concentration of 3.0 mcg./ml. are shown in Fig. 2. A relative standard deviation for the 12 replicate analyses was 0.54% even though peak heights of individual samples did not reach that of continuous sampling and the base line was not reached during the rinse cycle. Recordings of various concentrations of EE (Fig. 3), with duplicate sampling at each level, show minimal interaction between subsequent samples. Beer's law was obeyed and a y intercept of −0.004 was calculated. The values gave a calculated slope of 0.999 when plotted on the ordinate against theoretical values on the abscissa.

The recovery of EE from pooled chloroform extracts of oral contraceptive tablets containing 0.05 mg. EE in combination with 10.0 mg. medroxyprogesterone acetate per tablet was tested. EE standard was added to varying quantities of tablet extracts, giving an average recovery of 100.2% (Table I), with a low of 99.6% and a high of 100.7%. Medroxyprogesterone acetate, the progestin in the tablet, did not interfere with the assay. Recovery studies of EE from tablets containing 0.02 mg. EE and 1.0 mg. fluoxymesterone, in which the anabolic androgen contributed approximately 3% of the absorbance, were carried out. Samples were prepared to contain tablet excipients, varying quantities of EE, and fluoxymesterone at a concentration equivalent to 50 times the level of EE used for assay purposes. Table II shows that it is possible to assay EE at a concentration of 75–125% of the labeled amount in the presence of the nonestrogenic steroid at label strength.

Numerous assays for EE in tablets containing medroxyprogesterone acetate or fluoxymesterone were carried out using automated and manual

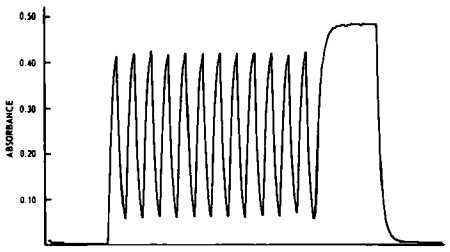

Fig. 2—Absorbance recordings of 3.0-mcg./ml. chloroform solution of ethinyl estradiol sampled continuously and repetitively.

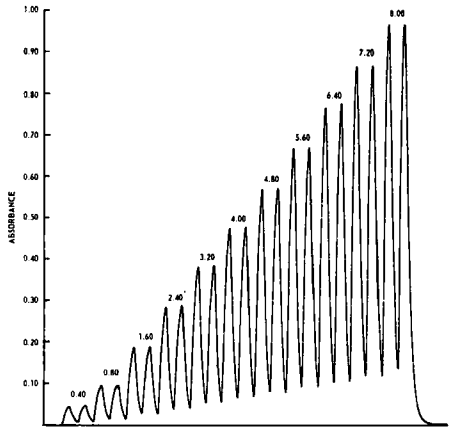

Fig. 3—Absorbance recordings of various mcg./ml. levels of ethinyl estradiol in chloroform.

TABLE I—RECOVERY OF ETHINYL ESTRADIOL FROM CHLOROFORM EXTRACTS OF TABLETS CONTAINING ETHINYL ESTRADIOL AND MEDROXYPROGESTERONE ACETATE

EE Standard Added, mcg./ml.	EE from Tablets, mcg./ml.	Theoretical EE Present, mcg./ml.	Assayed EE, mcg./ml.	Recovery, %
2.60	0.25	2.85	2.87	100.7
2.60	0.50	3.10	3.13	100.6
2.60	1.00	3.60	3.61	100.3
2.60	1.50	4.10	4.11	100.2
2.60	2.00	4.60	4.59	99.8
2.60	2.50	5.10	5.08	99.6
			Av.	100.2

TABLE II—RECOVERY OF VARYING QUANTITIES OF ETHINYL ESTRADIOL FROM SAMPLES SPIKED WITH FLUOXYMESTERONE

EE Present, mcg./ml.	Fluoxymesterone Added, mcg./ml.	EE Assayed, mcg./ml.	Recovery, %
3.0	200	3.05	101.6
3.5	200	3.53	100.9
4.0	200	4.01	100.3
4.5	200	4.46	99.1
5.0	200	4.86	97.2
		Av.	99.8

[6] Minneapolis Mining and Manufacturing, St. Paul, Minn.
[7] Cary model 11, Cary Instruments, Applied Physics Corp., Monrovia, Calif.

Table III—Automated and Manual Ethinyl Estradiol Assays of Tablets Containing Ethinyl Estradiol and a Nonestrogenic Steroid

Lot No.	EE, mg./Tab. Automated	EE, mg./Tab. Manual
0.05 mg. Ethinyl Estradiol + 10.0 mg. Medroxyprogesterone Acetate		
1	0.050	0.050
2	0.049	0.050
3	0.050	0.049
4	0.048	0.049
5	0.047	0.051
6	0.050	0.048
7	0.049	0.049
8	0.047	0.050
9	0.049	0.048
10	0.048	0.050
0.02 mg. Ethinyl Estradiol + 1.0 mg. Fluoxymesterone		
11	0.019	0.019
12	0.019	0.020
13	0.021	0.020
14	0.021	0.022
15	0.020	0.020
16	0.020	0.019
17	0.019	0.020
18	0.017	0.020
19	0.020	0.021
20	0.021	0.021

procedures. Table III shows that satisfactory agreement occurred for the two methods of assay.

Progestins present in formulations of oral contraceptive tablets, in combination with EE-3ME interfered with the automated assay to varying degrees. The interference, however, was eliminated by correcting for the absorbance at 538 mμ, due to the progestin alone. Table IV gives results of tablet assays from five manufacturers.

SUMMARY

An automated colorimetric method has been described for the assay of ethinyl estradiol and ethinyl estradiol-3-methyl ether in tablets. Interferences due to nonestrogenic steroids also present in the tablets are eliminated by correcting for the absorbance due to the steroids. Following manual extraction of test preparations, chloroform samples are analyzed at a rate of 20/hr. The relative standard deviation is less than 1% and Beer's law is obeyed in the range of 0.4–8.0 mcg./ml. Satisfactory agreement of assay results was obtained for the automated and manual procedures.

Table IV—Ethinyl Estradiol-3-methyl Ether Assays of Oral Contraceptives from Various Manufacturers

Manufacturer	Progestin and Labeled Amount, mg.	EE-3ME Assay, mg./Tab.	% of Claim
A	Norethindrone, 1.0	0.047	94
B	Norethindrone, 2.0	0.077	96
C	Norethindrone, 2.0	0.096	96
D	Norethynodrel, 5.0	0.082	109
E	Chlormadinone acetate, 2.0	0.075	94

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Keyphrases

Mestranol—analysis
Automated analysis—tablets, steroid mixtures
Diagram—automated apparatus
Colorimetric analysis—spectrophotometer

Continuous Flow Apparatus for the Determination of the Dissolution Characteristics of Tablets and Capsules

By M. PERNAROWSKI, W. WOO, and R. O. SEARL

The apparatus described in this paper consists of a closed dissolution container, a basket-stirrer assembly, and a variable speed pump. It may be automated by connecting the pump to a flow cell in a suitable spectrophotometer. Two or more test fluids can be used interchangeably for the determination of dissolution characteristics.

THE DISSOLUTION characteristics of a tablet or capsule may be determined by using any one of the many methods described in the literature (1–4). Several different types of dissolution containers, agitation methods, test media, and sampling procedures have been used to determine dissolution profiles or values. However, most methodology is based on either the USP disintegration apparatus (5–7) or on the use of a beaker-stirrer assembly (8–10).

It is now generally accepted that *in vitro* results should be correlated to some physiological parameter. This can only be done if the dissolution method yields results which reflect the physiological differences between chemically similar drug products. This implies that the apparatus should be so designed that its operating characteristics can be easily changed. The apparatus described in this paper is a nodification of the beaker-stirrer method used by Levy (8) and others. A tablet-capsule container has been built into the assembly and provisions have been made for change of test fluids during a dissolution study.

Automated assemblies have been used by several investigators (11–14). The continuous flow apparatus used in this laboratory is easily automated but, at the same time, may be used as a simple flask-stirrer assembly with an auxiliary system for media transfer.

EXPERIMENTAL

Equipment—The continuous flow dissolution apparatus is illustrated in Fig. 1. A diagram of the stainless steel basket-stirrer assembly is shown in Fig. 2. The assembly is connected to a Fisher model 12 Stedi-Speed adjustable stirrer. Ten-mesh stainless steel wire cloth is used in the construction of the main part of the basket.

The dissolution container is a 1-l., three-neck flask. The main neck is 35 mm. in diameter; the secondary necks are 20 mm. in diameter. The total volume of the container is slightly more than 1l.

If fluid flow or changeover is necessary, the container is connected (*via* a suitable filtering device and short lengths of latex tubing) to a Cole-Palmer Series A-76910 pump. The dial readings on this pump are proportional to the pumping rate in ml./min. Flow rates of up to 70 ml./min. have been used in this laboratory. Test fluids may be pumped directly to a collection container. Alternatively, they may be circulated through a 1-cm. flow cell in a Spectronic 505 recording spectrophotometer to the collection flask. Tubing lengths are kept to a minimum.

Dissolution profiles are graphed externally on a previously calibrated Varicord model 43 recorder.

Procedure—Dissolution characteristics may be determined by using exactly 1 l. of fluid or by operating the apparatus as a continuous flow system. If the apparatus is operated continuously, the volume of fluid in the dissolution container is slightly more than 1 l. Dissolution characteristics are based, therefore, on the amount of fluid pumped through the dissolution container.

One of the procedures used in this laboratory for the determination of the dissolution characteristics of phenylbutazone tablets NF is given below and illustrates the continuous flow method of operation with fluid changeover.

Place one phenylbutazone tablet into the basket

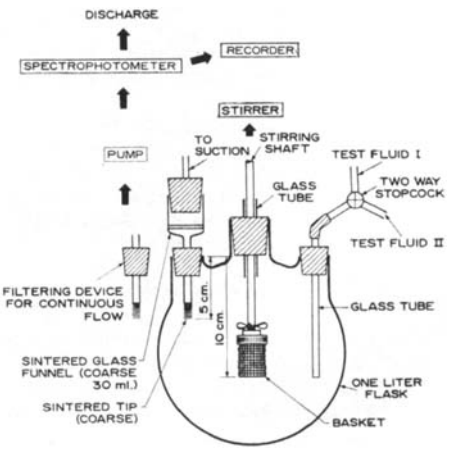

Fig. 1—Continuous flow dissolution apparatus.

Received March 14, 1968, from the Faculty of Pharmacy, University of British Columbia, Vancouver 8, British Columbia, Canada.

Accepted for publication May 27, 1968.

Supported by National Health grant No. 609-7-128 and by the Canadian Foundation for the Advancement of Pharmacy.

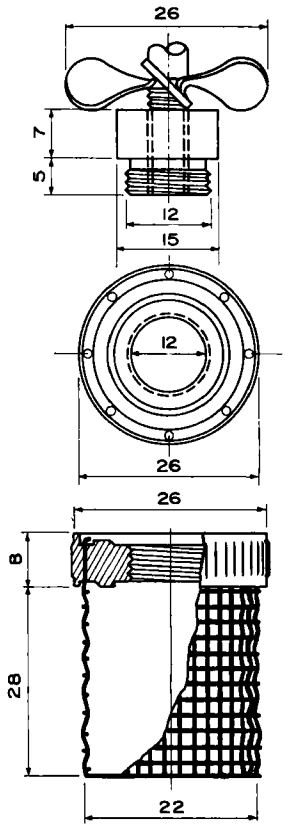

Fig. 2—Specifications (in mm.) for the basket-stirrer assembly used in the dissolution apparatus. The four impeller blades of the stirrer are set at an angle of 45°.

and attach the stirrer. Fill the dissolution container with 1 l. of simulated gastric fluid USP. (The fluid contains no enzyme and is preheated to 37°. Temperature is maintained by immersing the dissolution container in a water bath held at 37°.) Insert the basket-stirrer assembly into the dissolution container and rotate clockwise at 100 r.p.m. Set the pump to deliver 60 ml. of fluid per minute. Allow simulated gastric fluid USP to flow through the container for 30 min. Turn the stopcock connecting the media reservoirs to the dissolution container and allow simulated intestinal fluid USP (no enzyme) to flow through the system for the remainder of the test.

Determine the amount of phenylbutazone in the collection flask at hourly intervals by diluting aliquots with simulated intestinal fluid USP (no enzyme) and reading the absorbance of the solution at 240 mμ (an isosbestic point). Record dissolution profiles on the external recorder.

DISCUSSION

A basket-stirrer assembly similar to that illustrated in Fig. 2 was used to determine the dissolution characteristics of phenylbutazone tablets (15). The apparatus diagrammed in Fig. 1 is currently being used to investigate phenylbutazone tablets using two test media and bishydroxycoumarin tablets using one test fluid.

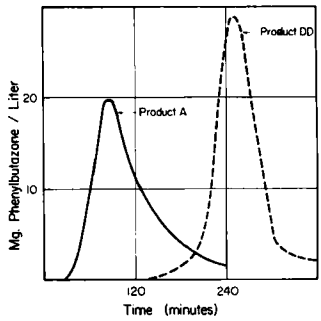

Fig. 3—Dissolution profiles for sugar-coated (Product A) and enteric-coated (Product DD) phenylbutazone tablets.

Dissolution profiles for sugar-coated (Product A) and enteric-coated (Product DD) phenylbutazone tablets are shown in Fig. 3. For the first 30 min., the pH of the fluid is 1.2. Simulated intestinal fluid USP (no enzyme) is then introduced into the flask. At a pumping rate of 60 ml./min., the pH of the fluid changes from that of simulated gastric fluid USP to that of simulated intestinal fluid USP (pH = 7.5) in 30 min.

The dissolution profiles show that both products release drug to a test fluid quickly. However, Product A released most of its phenylbutazone content in 3 hr. Product DD, which failed to comply with the disintegration specification for enteric-coated tablets, released, in 3 hr., only 4.9 (average of three determinations) of the 100 mg. claimed on the label. The apparatus does, therefore, differentiate between two different types of dosage forms. Moreover, these *in vitro* differences appear to be related to physiological availability. Product A was administered to three subjects. The area under the plasma concentration *versus* time curve for Product A was 20 sq. in. units or better. Under the same conditions of administration, the area value for Product DD varied from 4.6 to 11.2 in four subjects.

The basket-stirrer assembly has been used in this laboratory (both in the manner described in this paper and in conjunction with 1, 2, or 3-l. beakers) for approximately 2 years. It is a convenient assembly for tablets but is particularly useful when the dissolution characteristics of a capsule are being determined. Without such a container, capsules tend to float on the surface of the test media. Visual inspection during the test suggests that the tablets and capsules are subjected to much less abrasion than that observed in the USP disintegration test. A 10-mesh wire cloth is used in the construction of the basket and it is possible, therefore, to measure "disintegration" times. Although such values are routinely recorded, no relationship between this time and dissolution characteristics has been established.

This apparatus can be easily adapted to the routine control of tablets and capsules. Although dissolution profiles may be required in certain instances, total amount of drug eluted in a certain period of time would be a satisfactory criterion for many products. For example, the specification for phenylbutazone tablets NF would state that the total quantity of drug eluted from the tablet under specified conditions shall be not less than $x\%$ of that claimed on the label.

Slight changes in pumping rate do not materially change dissolution characteristics. With Product A, the amount of drug in solution at the end of 3 hr. was approximately the same (85 to 90 of the 100 mg. claimed on the label) even though the pumping rate was varied from 50 to 70 ml./min.

The operating characteristics of this apparatus can be easily altered. However, when conditions are fixed, similar dissolution profiles are obtained when drug or tablets from a uniform lot are subjected to the procedure. Repeated tests with Product A[1] produced dissolution profiles similar to that shown in Fig. 3. Moreover, the basket-stirrer assembly positions the tablet in the same way from run to run and helps, therefore, to produce results which are characteristic of the product rather than the apparatus.

[1] Butazolidin tablets, Geigy, Ardsley, N. Y.

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Keyphrases

Continuous flow apparatus—tablet, capsule dissolution
Dissolution profile determination—apparatus
Diagram—continuous flow dissolution apparatus

Some Measurements of Friction in Simple Powder Beds

By EVERETT N. HIESTAND and CHARLES J. WILCOX

An apparatus and procedure for measuring the static friction coefficient of powders in simple beds are described. The relative merits of three variations of the shear cell are considered. A sandwich of powder between two surfaces covered with sandpaper is satisfactory under most experimental conditions. Examples of the effect of relative humidity, time under load, mechanical vibration of the powder bed, and variations in the experimental procedure are presented. Reproducible values of the friction coefficient can be obtained readily if the experimental and environmental conditions are standardized. The results of the experiments suggest that a single value of the friction coefficient applies only to a specific powder bed. In practical applications the entire range of values that may be encountered must be within acceptable limits.

FRICTION COEFFICIENTS and cohesion values have been used to characterize the flow properties of powders. In a recent review (1), the methods used and the problems encountered have been discussed. In the opinion of the authors, there are limitations on the usefulness of such data because neither the friction coefficient nor the cohesion is a single valued property of the powder. As stated in the review article, "The properties of a powder bed depend on the cumulative effect of the previous history of all the portions of the bulk being considered. Isolated regions of shear, vibration, or compaction may have produced high bulk density regions. These may remain intact in subsequent flow of the powder or may fragment into macroscopic regions mixed throughout the less dense bed. The forces acting on the top of the bed may be quite different from those at the bottom of the bed." In the studies reported here, considerable data have been collected that reveal the large number of factors that cause changes in the properties of powder beds as well as indicating the magnitude of these effects.

The basic measurements of friction coefficient and cohesion may be obtained from a simple shear cell described by Nash *et al.* (2). Though

Received March 29, 1968, from the Upjohn Company, Kalamazoo, MI 49001
Accepted for publication June 6, 1968.
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modified in detail, the apparatus used in these studies retains the advantages of simplicity and the use of a thin layer of powder that may be formed by gentle sifting into place. Deep powder beds are not necessary and may be undesirable since uniform bulk density is difficult to maintain in forming deep beds. Other shear cells have been used successfully but some of the observations reported here could not be made using them because of the pretreatment, packing, *etc.*, of the powder bed (3).

FRICTION COEFFICIENT AND COHESION

The friction-cohesion equation used here is basically the same as suggested by Deryagin (4).

$$\tau = \mu (mg + c) \qquad \text{(Eq. 1)}$$

where τ is the force required to shear the powder, μ is the friction coefficient, mg is the load in dynes applied normal to the shear plane, and c is the apparent cohesion force of the powder.

Since friction is said to be the shear strength of the areas of true contact, an increase in friction coefficient should reflect either an increase in true contact area or an increase in force of interaction in the regions of true contact. Either of these should produce a corresponding increase in cohesion. In real cases the cohesion value obtained by applying Eq. 1 often decreases when the friction coefficient increases and vice versa. These experimental values vary in such a way that the authors have been unable to determine their significance. Other workers have observed that values of c based on Eq. 1 are larger than values obtained by direct tensile strength tests (5).

APPARATUS

Shear Cells—The basic shear cell is in the form of a sandwich of powder between upper and lower plates. The powder bed is prepared by sifting it into the 6.2-cm. diameter hole of a thin template placed over the bottom plate. The thickness of the template used varies with the type of upper and lower plates employed. A spatula serves to level the surface by gently scraping away excess powder. Care is taken to accomplish this with minimum disturbance of the powder bed. The template is lifted off and a disk-shaped bed of powder remains. The top plate is gently lowered onto the powder bed to bring its surface into contact with the powder. Additional weights are added to produce the desired applied load. Figures 1 and 2 show the cell in place in the apparatus. Normal loads less than the weight of the top plate are produced by suspending the top plate from one side of the analytical balance holding the desired counterbalance weights (not shown in Figs. 1 and 2). In this case the powder bed is gently raised to meet the top plate and to zero the balance.

Several different designs of top and bottom plates have been used. For some experiments at smaller applied loads, the bottom plate was a rectangular piece of "harrow" assembled from file cards[1] after reducing the bristle length. The top plate was a 5-cm. diameter "harrow" of the same material. This shear cell "sandwich" will be referred to as the HPH cell (harrow-powder-harrow). At higher loads, the harrow teeth may touch during a determination. Consequently, the upper and lower plate materials were changed. The first experiments done in this laboratory used metal disks coated with sandpaper,[2] called the SPS cell. In most of the recent studies the plates have been made from compressed tablets[3] prepared from the same powder used in the shear test, the TPT cell. Figure 3 shows the parts of these three types of cells. The top plate has been removed and placed upside down beside the powder bed.

In a few cases, a direct tablet-to-tablet measurement was made, the TT cell. This would not be a

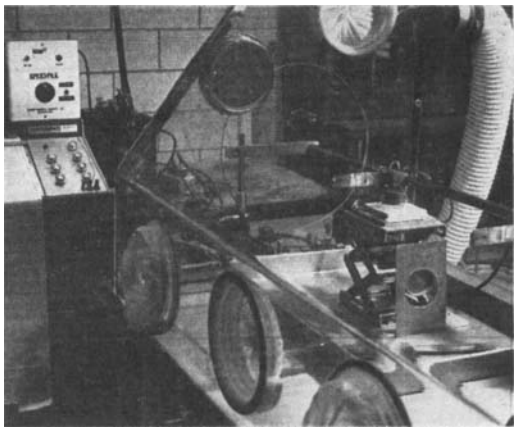

Fig. 1—Apparatus, TPT shear cell in place.

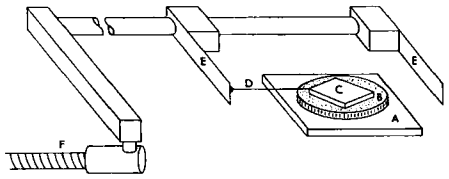

Fig. 2—Schematic arrangement of apparatus, Key: A, bottom plate of cell; B, powder bed; C, top plate of cell; D, tow line; E, strain gauges (only one used at a time); F, jack.

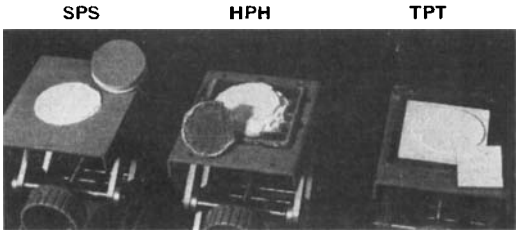

Fig. 3—Three varieties of shear cells used.

[1] Coulton's No. 10 File Cleaner, E. C. Knudson, Mfg., Chicago, Ill. Facing removed and cemented to flat plate. Surface grinder used to reduce length of steel bristles to 0.16 cm.

[2] The grade of sandpaper is not a critical factor. A satisfactory paper is garnet finishing paper, open coat, No. 100, Minnesota Mining and Mfg. Co., St. Paul, Minn.

[3] The top element is a tablet 3.7-cm. square and about 0.64-cm. thick. The bottom element is four of these tablets cemented to a rigid base plate and sanded smooth. Each tablet was made from 10 g. of powder A special die was used with the Carver press to form the tablets. Care was taken to make all tablets in a set as nearly alike as possible.

powder study unless indigenous powder was present. However, a comparison of TPT and TT data was desired.

Force Detector—A cantilever beam strain gauge was connected to a horizontal screw jack. The jack was driven through a gear box by an electronically speed-controlled, reversible electric motor. The upper plate of the shear cell was connected to the strain gauge by a small diameter tow line or wire including a fish-line swivel. The strain gauge output was connected directly to the input of a Sargent model SR recorder and resulted in a sensitivity of 1.315×10^5 dynes/mv. These parts are shown in Fig. 1 and diagrammatically in Fig. 2.

The usual linear speed of the gauge assembly was 4 cm./sec. Only static friction or "breakaway" force values were used. The shear force was insensitive to the rate of application of force in this range of gauge velocities.

The usual operating procedure was to assemble the cell and connect the linkage to the gauge. The screw jack was operated until the top plate moved. Then the direction of the jack was reversed until the tow line became slack. The forward motion of the jack was reestablished and a second pull was made. This sequence was repeated until the same shear force was observed for several sequential pulls. This was called the plateau value of the shear force and the powder bed was considered to have attained a reproducible steady-state condition referred to as the plateau condition.

The frequency of the pulls was arbitrarily selected as approximately 2/min. The loading and unloading times vary with the magnitude of the shear force and therefore with the applied load. The time interval between the "breakaway" peak force and the unloading is determined by the backlash of the jack. The exact magnitude of these factors is not critical but uniformity of practice is desirable to reduce the variability of results.

Environment—The shear cell assembly was contained in a controlled-humidity chamber. The humidity chamber draws from dry and wet air reservoirs to cycle the relative humidity over a range of about ±1% every 2 min. The air in the chamber is circulated rapidly through the chamber and a mixing box beneath it which contains the humidity controller and the dry and wet air-injector systems.

EXPERIMENTAL MATERIALS

With a few exceptions, the materials used were selected from the common chemical entities used in pharmaceutical dosage forms. They were preconditioned in the controlled humidity environment for no less than 24 hr. and usually for more than 80 hr. before use in the shear cell. The dynamic nature of the properties of powders will be illustrated by observations of the changing of a sample over very long periods of time.

CHANGES OF SHEAR STRENGTH IN A SERIES OF PULLS

The series of pulls used to establish the plateau condition will provide a pattern of shear values typical of the powder bed at the applied load. In general, one of three patterns is obtained. These

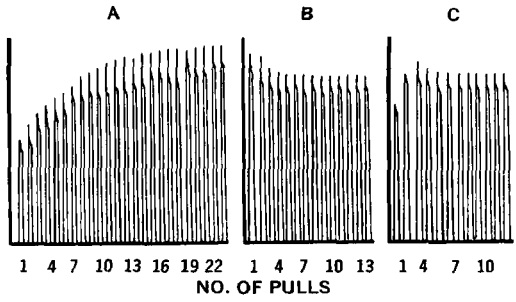

Fig. 4—Recorder chart traces. Patterns developed by peaks prior to reaching plateau condition.

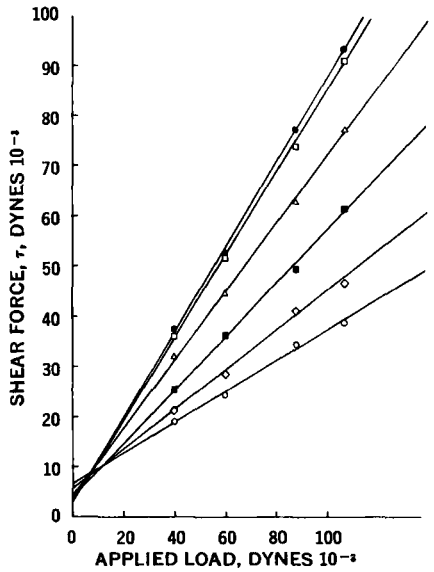

Fig. 5—Repeated shearing of powder bed changing its properties. Each line represents the shear force for a given number of pulls on the top disk obtained at various applied loads. The HPH cell was used in 50% relative humidity environment with calcium acid novobiocin powder. Key: ○, 1st pull; ◇, 2nd pull; ■, 4th pull; △, 8th pull; □, 16th pull; ●, plateau.

are shown in the recorder chart traces in Fig. 4. The peak value of the leading edge of each individual "spike" is the breakaway force. Once shear occurs, the measurement becomes one of dynamic friction and not static friction. The "spike" ends when the force is removed by reversal of the jack. The regular spacing of these spikes shows the uniform schedule of the pull cycles. This is necessary to obtain reproducible results.

One of the patterns shown in Fig. 4 is obtained at each individual applied load. The usual friction plot used in this work is obtained by plotting the plateau values of the shear force against the applied load. Others have preferred to use the first pull data (2). Figure 5 shows both results with calcium acid novobiocin as well as plots obtained by using other selected values, *e.g.*, only second pull values for each series, only fourth pull values, *etc.* At all loads used, the pattern of pulls was like those in Fig. 4A. All strongly cohesive powders produce patterns of the type in Fig. 4A. These

changes with shear may be produced by changes in packing density, changes in bed thickness, or a development of structure that resists particle movement in a given direction. It is not the result of powder piling-up in front of the top element of the cell since removal of the powder in front has negligible effect on the shear force for the following pull. The types of patterns in Figs. 4B and 4C probably result from powders in which particle orientation and/or dialation are dominant factors associated with shearing of the bed. The bed thickness of choice depends on the selection of the shear cell. Obviously the HPH cell must use thicker beds than might be used with the TPT arrangement. Separate studies using beds of different initial thickness gave very similar results, though not always identical values of τ and c. With the HPH cell, the plateau values remained constant until the teeth of the harrow made contact with each other. With the other cells the plateau values remained constant over many pulls. Therefore, changes in bed thickness are not believed to be major factors in these measurements, especially when plateau conditions are used. Probably, the plateau condition is a steady-state situation in which the rate of introduction of new powder at the leading edge remains nearly constant with each successive movement of the top plate (see section on the elasticity of the tow line).

If one concludes that the changes observed in a series of pulls is the result of changes in the bed other than thickness *per se*, then one may conclude that in some applications the friction and cohesion values may vary over at least the range observed in the series of pulls. Certainly a single value of friction and cohesion cannot be characteristic of the powder but only of the specific powder bed. It would be interesting to relate these values to other properties such as bulk density, *etc.* However, it is not simple to obtain both measurements on the powder in the cell under the conditions of each individual pull.

TIME-DEPENDENT EFFECTS

Duration of Application of Static Load—It has been reported that the static friction coefficient between solids varies with the logarithm of the time of contact (6). There is no reason to expect the friction coefficient of powder beds to be independent of time of contact. Lidocaine base illustrates an extreme case as shown in Fig. 6. When the load was placed on the top plate and left for 30 min. before the first pull was made, the static friction force was much larger than when the usual time schedule was followed. However, the next pull, made immediately after the time-delayed pull, is only very slightly different from the first pull made on the usual schedule without the time delay.

Similar results were obtained when the time studies were applied to powder beds brought to the plateau condition. Figure 7 shows data obtained with chlorphenesin carbamate. The plateau condition was established and then the cell was left undisturbed for the chosen time interval. The magnitude of the first pull after the time interval was higher than the plateau value. The lowest line in Fig. 7 shows the magnitude of a second pull made immediately after the time-delayed one for the 30-min. case. Similar results were observed

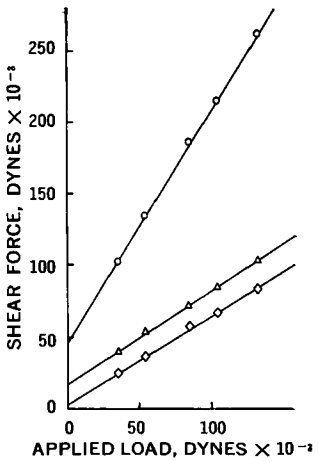

Fig. 6—Lidocaine base, SPS data, 70% relative humidity; influence of time of static loading on magnitude of shear force for first pull. Key: ○, 1st pull after 30 min.; ◇, 2nd pull, immediately after 30-min. delay pull; △, plateau by usual method.

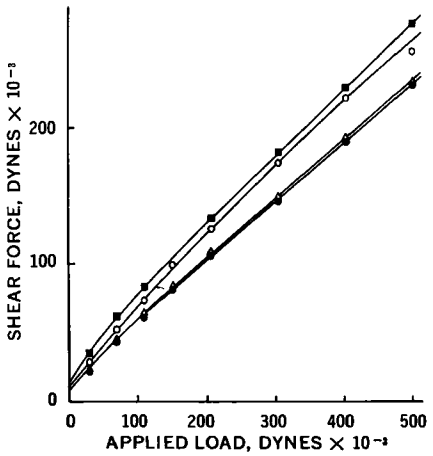

Fig. 7—Chlorphenesin carbamate, TPT data, 50% relative humidity; influence of time of static loading on magnitude of shear forces after plateau condition has been established. Key: ■, 1st pull after 60-min. wait; ○, 1st pull after 30-min. wait; △, plateau; ●, 2nd pull immediately following 1st pull after 30-min. wait.

after each of the time-delay intervals. The shear forces have returned to approximately the same as the plateau condition.[4] This indicates that the changes in shear force with time of static load application are not due to irreversible changes in the power bed.

Figure 8 shows a longer interval of time lapse than shown in Fig. 7 but for only one applied load. Note that changes still are occurring after 30 hr. of standing with the load applied.

Again the conclusion is that a single value of the friction coefficient and the cohesion is not adequate to describe the powder properties.

Effect of Storage Time in the Controlled-Humidity Chamber—Changes in the properties of powders

[4] The fact that this line is slightly below the plateau value in both examples is consistent with an observation made in other cases, *viz.*, whenever a preceding pull results in an unusually high shear force the next pull will be smaller than the average value.

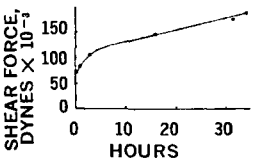

Fig. 8—Chlorphenesin carbamate in the same study as in Fig. 7 but shows extended time scale for the application of a 110-g. applied load.

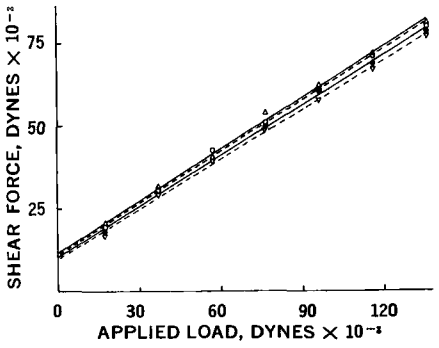

Fig. 9—Changes in the properties of a placebo mixture stored in the controlled-humidity chamber. Key: △, 0 days; ○, 3 days; ●, 14 days; ▽, 25 days.

lengths of time in the controlled-humidity environment. The option of ignoring the effects of time of storage of each material was adopted, obviously to save time. This expediency detracts from the absolute precision of a given value but should not mask the important effects.

Table I shows the differences in plateau values of the friction coefficient obtained in various relative-humidity atmospheres using the TPT cell prepared from β-sitosterol. A minimum value in the 25–65% relative humidity range was expected. The dramatic changes in the shear forces observed in the 30 and 50% relative humidity range were not expected.

EFFECT OF VIBRATION ON SHEAR FORCE

One possible cause of changes of shear force with time is the change in area of true contact induced by the ever present ambient vibrations of the laboratory. If so, an increase of shear force with time should be accelerated by subjecting the shear cell to forced vibration.

An electric vibrator[6] was attached to the lab jack supporting the shear cell. The motion orientation was parallel to the direction of shear. A plateau value was attained in the same manner as before except that the vibrator was turned on for a few seconds between each pull Figure 10 compares the plateau condition results with spray-dried lactose (TPT) subjected to a 4-sec. vibration period with the unvibrated case. On the same graph is shown this same comparison for the TT cell. Figure 11 shows

TABLE I—CHANGES IN THE FRICTION COEFFICIENT OF β-SITOSTEROL POWDER, TPT CELL DATA

Relative humidity, %	30	40	50	72	87	94
Friction coefficient	0.91	0.76	0.87	0.93	0.94	0.89

occur during the storage of the material in the controlled-humidity environment. Figure 9 shows the results of a series of measurements[5] with the HPH cell on a powder mixture. The mixture was used for a placebo tablet preparation and its primary constituent was lactose. The powder was not preconditioned in the controlled-humidity environment. Zero time is taken as the time when the powder was spread on a tray in a layer not in excess of 1.2 cm. thick and was placed in the controlled-humidity chamber for the duration of the experiment. The results of the study indicate that it did not attain an equilibrium with its environment in 25 days.

The controlled environment limits the humidity fluctuations to a range of ±1% relative humidity. Moisture on the surface and especially fluctuations in the amount of moisture could promote surface changes of the solid. However, the environmental changes in relative humidity are rapid enough that one would expect very little effect. Certainly one would not expect all powders to continue to change over such long periods of time but this example illustrates that the possibility must be considered.

EFFECT OF RELATIVE HUMIDITY OF THE ENVIRONMENT

As indicated earlier, most measurements were made after powders had been stored for reasonable

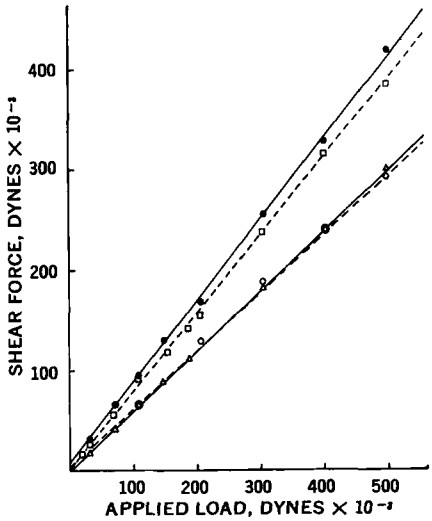

Fig. 10—Lactose, spray dried, 50% relative humidity, TPT and TT data; effects of vibration on the values at plateau conditions. Key: ●—, TPT vibrated 4 sec.; △—, TPT not vibrated; □---, TT vibrated 1 sec.; ⊕---, TT not vibrated.

[5] These data are taken from a study in which all measurements were made using a standard powder bed that reached a plateau condition with 138 g. applied load prior to shearing at the desired load.

[6] Syntron Electric Vibrator, type V-4, style 1518, Syntron Co., Homer City, Pa.

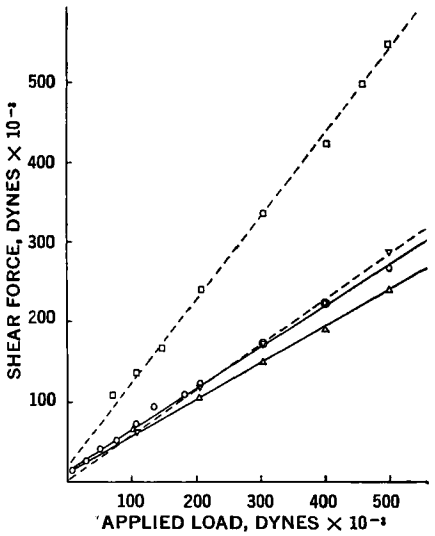

Fig. 11—Chlorphenesin carbamate, 50% relative humidity. TPT and TT data. Effect of vibrations on the values at plateau conditions. Key: □---, TT vibrated; ▽---, TT not vibrated; ○——, TPT vibrated; △——, TPT not vibrated.

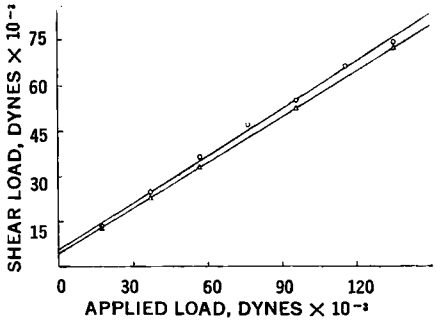

Fig. 12—Changes in the shear forces at plateau conditions resulting from the repeated sifting of the same sample of the powder in a placebo mixture. Key: ⊙, new powder; ◬, sifted 5 times.

TABLE II—EFFECT OF ELASTICITY OF TOW LINE ON PLATEAU SHEAR VALUES FOR 140-g. APPLIED LOAD

Linkage Material	Shear Value, τ_p, Dynes × 10^{-3}	Average Distance Moved per Pull, mm.
Small nylon thread	87.1	1.45
Nylon, No. 15 fish line	92.5	0.53
Wire	93.2	0.44

the analogous case using chlorphenesin carbamate.

TT data for materials such as lactose may be misleading because some powder is formed at the shear plane between the tablets. No evidence of powder was found for the TT case with chlorphenesin carbamate. Forced vibrations produced, in a few seconds, an effect similar to that produced by standing for a much longer time; however, the phenomenon occurring in the powder bed could have been different in the two cases. Possibly the main effect of vibrations is to produce packing changes. Standing for longer periods may permit only the relaxation of elastic stresses at points of true contact. Either could increase the friction force. Again it is difficult to make measurements that would define unequivocally the mechanism producing the effect.

SOME RESULTS RELATED TO EXPERIMENTAL PROCEDURES

Sifting—The same lactose placebo mixture used in the study of changes during storage was used to demonstrate the effects produced by repeated sifting. Figure 12 shows the result; the HPH method was used. Since the sifting to form a shear cell normally is done in the air bath, *i.e.*, the controlled-humidity chamber, in which there is rapid circulation of the air, some fine particles are swept away by the air currents. This is believed to be the cause of the observed changes. These results led to the use of a fresh sample of powder each time a powder bed was formed.

Elasticity of Tow Line—It is obvious that the factors that produce variation in the observed values of the friction coefficient are multitudinous. The influence of the design of the apparatus cannot be eliminated. Table II shows data obtained using three different tow lines between the cell and the strain gauge. The nylon line stretches as tension is applied. When the static friction is overcome, the top plate of the cell will move forward until the tension is reduced to the dynamic force. Obviously this distance of travel depends on the elastic properties of the tow line and the cantilever beam strain gauge assembly. In this study only the tow line was changed. Apparently, the structure of the bed of powder is dependent on the extent of movement of the top plate. Therefore, the plateau value changes. Possibly the differences result from the change in the rate of introduction of new powder at the leading edge.

Differences in Values with Various Shear Cells—It must be noted that the three cells, HPH, TPT, and SPS do not yield identical results in the shear cell studies. The HPH design was developed to assure that the shear was within the powder bed. In concept it is essentially the same as a cell used by Dawes (7) but of a much simpler design. Dawes compared the results obtained using a cell with sandpaper-coated surfaces with those using a cell with vanes extending into the powder bed. The absolute values were found to be significantly different.

The results of these studies confirm this observation. Table III gives examples. Each cell fits a specific need and also has specific limitations. The HPH cell produces shear in the powder bed but cannot be used to obtain plateau conditions if a large applied load is used because the teeth of the harrow-like plates will touch before the plateau

TABLE III—COMPARISON OF VALUES OBTAINED WITH LACTOSE USING DIFFERENT SHEAR CELLS

	HPH	TPT	SPS
Friction coefficient, μ	0.608	0.608	0.602
Cohesion (dynes/cm.²)[a]	+59	−57	+260

[a] These units are obtained by dividing the intercept on the σ axis by (μ × area of top plate).

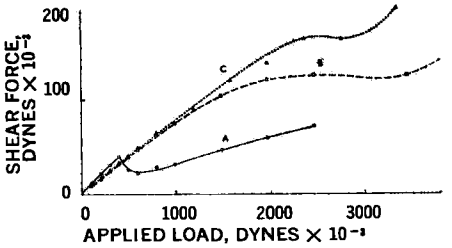

Fig. 13—Anomalous results obtained for plateau conditions with magnesium stearate. TPT and lower SPS data were obtained by pulling top plate in one direction. Top SPS data were obtained by reversing direction of pull each time. Key: A, TPT data for τ_p, all pulls in same direction; B, SPS data for τ_p, all pulls in same direction; C, SPS data for τ_p, pull direction reversed after each pull.

condition is established. The TPT cell is useful with the larger applied loads and does not introduce a foreign material at the interface with the powder. Obviously it cannot be used when the powder will not form a sufficiently strong compact by compression. Sometimes shear may occur at the tablet-powder interface more readily than within the powder bed. With magnesium stearate the surface of the tablet changed during a single run. Reproducible results were obtained by removing the surface layer between each run. Figure 13 shows three friction plots obtained with magnesium stearate. The conditioning of the tablet surface and shifting of the shear plane to the interface is believed to account for the irregular shape of the plot. The SPS cell required larger loads to produce the effect. After shearing to the plateau condition under these larger loads, the sandpaper was plated with magnesium stearate and appeared to be just like the compressed tablet surface. Pulling the top plate alternately in opposite directions diminished this effect and delayed the onset of gross deviations in the plot. The SPS cell seems to be the most versatile assembly.

Differences Produced by Direction of Shear—If after a series of pulls in a given direction the direction of pull is reversed, the observed shear force changes to be more nearly like the first pull, *e.g.*, powders producing patterns like Fig. 4A would require a smaller shear force when the direction of shear is reversed, if like Fig. 4B a larger force would be required. Table IV lists plateau values for several powders. The usual one direction of pull values are given and also the values obtained when the direction of pull is reversed after each individual pull. The magnitude of the plateau values seems to follow the same general rule as for the case of a single pull in the opposite direction.

The failure of these experiments to produce values independent of the detailed design of the apparatus and independent of the procedure followed in its use could prompt extensive criticism of the entire study. However, many of the conclusions have been made using a carefully standardized procedure in which given values may be reproducibly obtained. Therefore, changes in these values produced by humidity, time, vibrations, *etc.*, are believed to be meaningful observations. The discussion of the observations relating to the experimental design or procedure may assist other investigators interested in conducting similar experiments.

TABLE IV—COMPARISON OF PLATEAU VALUES OF SHEAR FORCE BETWEEN CASE OF ALL PULLS IN SAME DIRECTION, τ_p, AND CASE OF PULL DIRECTION REVERSED AFTER EACH PULL, $\tau_p \rightleftharpoons a$ (SPS CELL, 50% RELATIVE HUMIDITY)

Material	Plateau Value of Shear Force τ_p	$\tau_p \rightleftharpoons a$
β-Sitosterol[a]	0.90	0.79
Lactose (spray dried)	0.63	0.65
Erythromycin base	0.82	0.72

[a] Different lots of drugs were used than for studies reported in Tables I and II.

CONCLUSIONS

A relative simple apparatus and procedure are described for obtaining reproducible powder bed conditions. Experimental results have been obtained that indicate some of the factors in the environmental history of a powder bed that produce changes in the friction and cohesion within the bed. In practical applications, the entire range of values of friction coefficients and cohesion should be within acceptable limits. The experimental design used to establish the significant range of values must include the use of powders subjected to the environmental variations that will be encountered by the powder in actual use.

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Keyphrases

Friction measurements—powder beds
Shear strength—powders
Static friction—powder beds
Vibration, effect—shear force
Humidity, effect—friction in powders
Diagram—friction measurement apparatus

Effect of Chronic Poisoning by Emetine on Oxidative Process in Rat Heart III. Effects on Oxidation of Pyruvate and Lactate

By GLENN D. APPELT and HAROLD C. HEIM*

The effect of chronic emetine poisoning on the oxidation of pyruvate and lactate by rat heart and liver homogenates was investigated. Oxygen consumption by heart homogenates was lower in the emetine-poisoned animals when compared to sham-injected controls. No significant difference was noted in liver homogenates. Heart slices from emetine-poisoned rats consumed less oxygen than slices from sham-injected controls. When diaphragm sections were employed, this difference was not noted.

MUCH EVIDENCE has been accumulated to show that the myocardium utilizes, as a source of energy, a variety of substrates including glucose, lactate, pyruvate, and fatty acids. In earlier communications, data were presented which described an inhibitory effect elicited by chronic emetine poisoning on the oxidation of fatty acids (1), and of certain citric acid intermediates (2) by rat heart homogenates. These inhibitory effects were not noted in the oxidation of these substrates by rat liver homogenates from emetine-poisoned animals. The heart contains a small amount of emetine after intraperitoneal injection, although a relatively high concentration of the drug is found in the liver (3). Cardiotoxicity, however, has been observed with emetine (4–6), and this effect may be related to a rather specific aberration of biochemical processes in the myocardium.

Since pyruvate and lactate may be utilized by the myocardium, and apparently represent substrates for the heart (7–9), it appeared of interest to study the effect of chronic emetine poisoning on the oxidation of these compounds by rat heart and liver homogenates. In addition, oxygen consumption by heart slices and diaphragm sections from emetine-poisoned rats in the presence of pyruvate was measured.

EXPERIMENTAL

To study the effects of chronic poisoning by emetine on the oxidation of pyruvate and lactate by heart and liver homogenates, young adult Sprague-Dawley rats of both sexes were employed as test animals. The animals weighed approximately 150 g. at the beginning of the experiment. The animals were separated into two groups of six animals each and given intraperitoneal injections daily for 14 days as follows: Group A, 0.2 mg. emetine HCl in 0.1 ml. water; Group B, 0.1 ml. water. Food and water were allowed *ad libitum*. After 14 days, the animals were decapitated, the heart or liver tissue immediately removed, washed quickly in cold water, blotted dry, and weighed. The tissue was then transferred to a chilled Ten Broek tissue grinder containing sufficient cold 0.1 *M* potassium phosphate buffer, pH 7.4, so that the homogenates contained 80 mg. of fresh tissue per milliliter. The homogenates were pipeted into chilled Warburg vessels containing substrates and cofactors listed below.

When diaphragm sections were employed, the animals were treated for 14 days in the same manner as above, sacrificed, and the diaphragm removed rapidly and cut into segments of approximately 100 mg. each. Ventricle slices were prepared using the tissue slicer described by Stadie and Riggs (10). The individual slices or sections were quickly transferred to a torsion balance, the exact weight determined, and one slice or section placed in each iced Warburg vessel containing the reaction medium.

The main compartment of each flask contained 0.5 ml. of homogenate or a tissue slice, 0.1 ml. of 5×10^{-4} *M* cytochrome c; 0.1 ml. of 1.5×10^{-2}*M* malate; 0.1 ml. of 2×10^{-2}*M* $MgCl_2$; 0.1 ml. of 2×10^{-2}*M* ATP; 0.1 ml. of 1.5×10^{-2}*M* nicotinamide; 0.3 ml. of 0.1 *M* pyruvate or lactate; 1.2 ml. of 0.1 *M* potassium phosphate buffer, pH 7.4, in flasks which contained homogenates, or 1.7 ml. in flasks which contained a diaphragm section or heart slice; and 0.3 ml. of distilled water. In all experiments the center well of each flask contained 0.2 ml. of 10% potassium hydroxide solution. The total volume of the flask contents was 3 ml. in all the oxidative studies. After a 10-min. equilibration period, oxygen consumption was measured according to conventional manometric techniques (11).

Oxygen uptake readings were taken at 15-min. intervals for 90 min. All values represent the average of data obtained with at least six animals, and each determination was performed in duplicate. The vertical line through each point designates the standard error of the mean for each set of values.

RESULTS AND DISCUSSION

Figures 1 and 2 illustrate the effect of chronic poisoning by emetine on the oxygen uptake by rat heart homogenates in the presence of pyruvate and lactate, respectively. The total oxygen consumption at the end of 90 min. was significantly lower ($p < 0.001$) in the preparations from emetine-poisoned rats when compared to values obtained from sham-

Received September 21, 1967, from the College of Pharmacy, The University of Texas, Austin, TX 78712
Accepted for publication May 13, 1968.
This work was supported in part by grant H-5476 (C-3), U. S. Public Health Service, Bethesda, Md.
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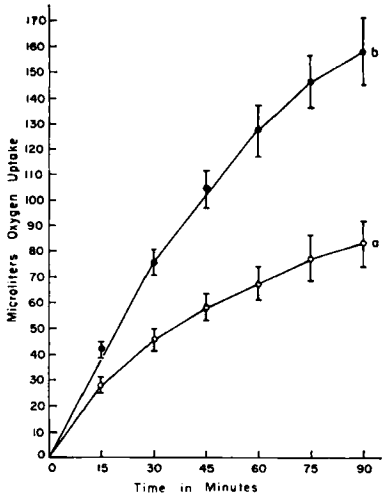

Fig 1—Oxidation of pyruvate by heart homogenates from sham-injected and emetine-treated rats. Key: Curve a, emetine-treated animals; Curve b, sham-injected animals.

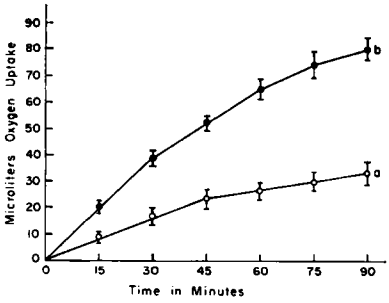

Fig. 2—Oxidation of lactate by heart homogenates from sham-injected and emetine-treated rats. Key: Curve a, emetine-treated animals, Curve b, sham-injected animals.

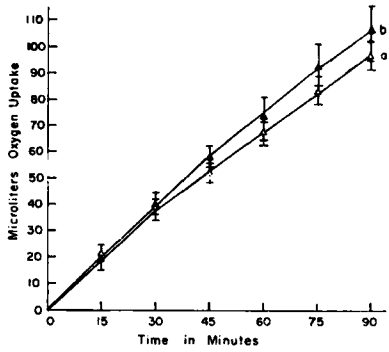

Fig. 3—Oxidation of pyruvate by liver homogenates from sham-injected and emetine-treated rats. Key: Curve a, emetine-injected animals; Curve b, sham-injected animals.

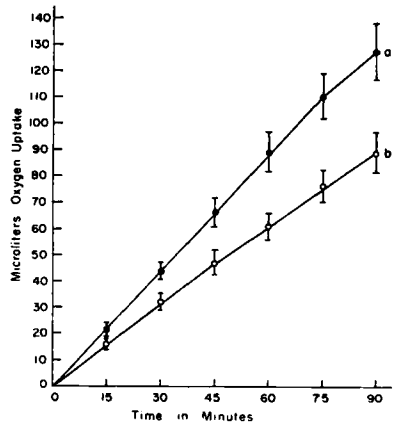

Fig. 4—Oxidation of lactate by liver homogenates from sham-injected and emetine-treated rats. Key: Curve a, sham-injected animals; Curve b, emetine-treated animals.

injected animals. This inhibitory effect represents a 48% decrease for pyruvate and a 59% reduction when lactate was the substrate.

Figures 3 and 4 depict oxygen consumption by rat liver homogenates when pyruvate and lactate were utilized as substrates. The 90-min. *r* readings from emetine-poisoned animals in this instance, however, were not significantly lower than the values from the sham-injected animals.

These data indicate that heart homogenates from emetine-poisoned rats were less efficient in the oxidation of these substrates than homogenates from control animals. Such a difference, however, was not noted in liver homogenates from emetine-poisoned rats.

Figure 5 shows a comparison of the respiration by diaphragm sections from emetine-poisoned rats with that of the sham-injected controls. Pyruvate was the substrate and, under the conditions of the experiment, no significant difference was observed between the test groups. Figure 6 illustrates that the total oxygen consumption of ventricle slices from emetine-poisoned rats was about 40% lower than that obtained with ventricle slices from the sham-injected controls. These results extend the idea that this inhibitory effect may be fairly specific for the myocardium, by including a muscle preparation for comparison.

It has been reported that pyruvate and lactate are utilized less efficiently in certain thiamine-deficient situations (12–14). Thiamine pyrophosphate is necessary in the biotransformation of pyruvate to acetyl-coenzyme A which represents a pathway whereby these substrates may be metabolized *via* the citric acid cycle. Diamant and associates (15) have reported that emetine-treated rats stored smaller amounts of thiamine in the liver than did control animals. It is conceivable that a similar situation exists in cardiac muscle resulting in a diminished utilization of pyruvate and lactate.

Nicotinamide adenine dinucleotide (NAD) functions as a coenzyme in the lactic dehydrogenase system, as well as a cofactor in the conversion of pyruvate to acetyl-coenzyme A. Deitrich and Heim (16) have noted that emetine decreased the oxygen uptake of rat heart homogenates in the presence of pyruvate and malate, however, an enhancement of the oxidation of succinate, an FAD-linked sub-

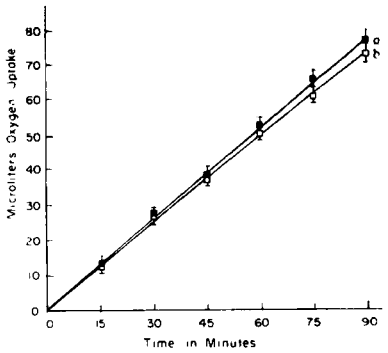

Fig. 5—Oxidation of pyruvate by diaphragm slices rom sham-injected and emetine-treated rats. Key: Curve a, sham-injected animals; Curve b, emetine-treated animals.

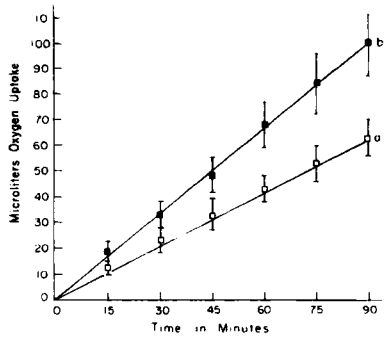

Fig. 6—Oxidation of pyruvate by heart slices from sham-injected and emetine-treated rats. Key: Curve a, emetine-treated animals; Curve b, sham-injected animals.

strate, was observed. A similar inhibitory activity of the oxidation of NAD-linked substrates in emetine-poisoned rats has previously been reported, although no alteration in NAD metabolism was noted (2).

At least one investigator (17) has suggested that emetine is most toxic to those tissues which involve contractility as the principal function. One might expect, therefore, that metabolic processes in tissue such as diaphragm would be altered by emetine. However, it was found that pyruvate was oxidized as rapidly by diaphragm sections from the emetine-poisoned animals as from the sham-injected animals. With heart slices it was found that chronic poisoning by emetine elicited an impaired ability to oxidize pyruvate. Such evidence tends to indicate that the effect of emetine, as far as oxidative processes are concerned, is not common only to those tissues in which contractility is the principal function, and further supports the view that the toxic effect of emetine is rather specific to the heart.

SUMMARY

1. The effects of chronic poisoning by emetine on the oxidation of pyruvate and lactate by various tissue preparations was studied.

2. Oxygen consumption by heart homogenates prepared from emetine-poisoned rats was lower than that observed with homogenates prepared from sham-injected controls.

3. The oxygen uptake by liver homogenates prepared from emetine-poisoned rats did not differ significantly from that observed with homogenates prepared from sham-injected controls with pyruvate and lactate as substrates.

4. Heart slices prepared from emetine-poisoned rats consumed less oxygen in the presence of pyruvate than did slices from the sham-injected controls. With diaphragm sections this difference was not observed.

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Keyphrases

Emetine poisoning, chronic—effects
Heart, liver homogenates—oxidative determinations
Pyruvate, lactate oxidation—emetine poisoning effect

Synthesis, Purification, and Influence of Chain Length on Toxicity of Two Quaternary Ammonium Compounds

By THEODORE H. EICKHOLT, GARY K. BRASHIER*, MICHAEL D. HIGGINS, and U. B. MARTINEZ

The synthesis and purification of two quaternary ammonium compounds was accomplished. The influence of chain length upon toxicity of the two compounds was determined using oral LD_{50} values, in fasted animals, and intraperitoneal LD_{50} values in male Swiss-Webster mice and male Wistar rats except where limited by the critical micelle concentration and the volume required for administration. The larger molecular weight compound was more toxic i.p. in rats than mice while the lower molecular weight compound was more toxic orally in mice than rats. The i.p. LD_{50} values in mice for both compounds were nearly identical and these values in both rats and mice after 24 hr. for the larger weight compound were practically the same. The usual greater toxicity with i.p. administration *versus* oral was not seen with these compounds in mice but was observed in rats.

QUATERNARY AMMONIUM COMPOUNDS are capable of a variety of pharmacologic activities, from antihypertensive to antiseptic. Many surface-active agents are quaternary ammonium compounds and have received considerable investigation as bactericidal agents (1, 2). Cetylpyridinium chloride and benzalkonium chloride are common examples, the latter of which is not a pure, single compound. The LD_{50} of cetylpyridinium chloride i.p. in the mouse is stated to be 10 mg./kg. (3). The LD_{50} in the rat orally is 200 mg./kg., subcutaneously 250 mg./kg., intraperitoneally 6 mg./kg., and intravenously 30 mg./kg. (3). The LD_{50} of benzalkonium chloride, calculated as commercial solution, in the mouse i.p. is 10 mg./kg., and i.v. 10 mg./kg. (3). For none of these figures are times of death indicated.

A mixture of triethyl ammonium bromide homologs[1] is commercially available.

It was, therefore, the purpose of this study to synthesize in a pure form dodecatriethylammonium bromide (DTEAB) and tetradecatriethylammonium bromide (TTEAB) and to determine the influence of chain length on the toxicity of these two compounds, for which little work on the pure systems has been done.

EXPERIMENTAL

The quaternary ammonium bromides were prepared by a method similar to that of Scott and Tartar (4), an established synthesis procedure for making surfactants of the cationic type. The essential differences were ones of degree, *e.g.*, (*a*) reflux times were longer, up to 3 days, (*b*) solvents were not exclusively ethanol, (*c*) surfactants were not limited to only trimethyl head groups on the monomer. Varying the conditions in *a* and *b* the extent of reaction was controlled, thereby producing more surfactant by shifting the equilibrium to the right. The position of equilibrium is most certainly affected by the nature of the monomer formed (*c* above) consequently the reflux conditions were improved as dictated by the specific synthesis.

The long-chain alkyl bromide was mixed with a 35–40% excess of the appropriate trialkylamine and this solution was added to a particular volume of the reaction solvent (CH_3OH or CH_3CH_2OH). The resulting solution was heated under total reflux from 7 hr. to 3 days as, in general, reaction time increases as molecular weight of the quaternary increases.

After the reaction mixture cooled, most of the alcohol and excess amine were boiled off, in some cases, under reduced pressure. Ethyl ether was added to precipitate the quaternary crystals.

Purification was effected by (*a*) repeated recrystallization from alcohol (CH_3OH) by adding ether, (*b*) Soxhlet extraction using ether for as long as 20 hr., or (*c*) a combination of the first two methods. The ether was then filtered from the crystals. The last traces of ether were removed by heating under vacuum for about 24 hr. at approximately 35° (5).

When ethanol was substituted for methanol as the reaction solvent, the reflux temperature was increased sufficiently to enhance the quaternary yield, in some cases by as much as 100%.

Starting materials were reagent grade products of either Eastman Organic Chemicals or Matheson, Coleman, and Bell. In those cases in which the gas chromatogram of the alkyl bromide showed more than one large peak, the compound was purified by vacuum distillation.

The primary criterion for purity of the quaternary compounds was the absence of a minimum in the surface tension-concentration curve as reported by Miles and Shedlovsky (6, 7) and verified by others (8, 9).

Stoichiometric equation:

$$RBr + R_3'N \xrightarrow[\text{reflux}]{\text{alcohol}} R\overset{+}{N}R_3'\overset{-}{Br}$$

$(R = C_{12}, C_{14})$ $(R' = Me, Et, Pr, Bu)$

Example:

$$C_{12}H_{25}Br + Et_3N \rightarrow C_{12}H_{25}\overset{+}{N}Et_3\overset{-}{Br}$$
(DTEAB)

To determine the influence of chain length on the toxicity of the two pure compounds, animals were treated as follows.

Male mice of the Swiss-Webster strain received 7.5, 15, and 30 mg./kg. of DTEAB and TTEAB i.p. Thirty animals were used at each dosage and the LD_{50} after 24, 48, and 72 hr. was determined using the Reed-Muench method (10).

Male rats of the Wistar strain received 30, 60, and

Received July 17, 1967, from the Department of Pharmacology and Toxicology, School of Pharmacy, Northeast Louisiana State College, Monroe, LA 71201

Accepted for publication May 15, 1968.

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[1] Diaper Pure, Boyle-Midway, Division American Home Prod. Corp., New York, N. Y.

TABLE I—SUMMARY OF LD_{50} STUDIES OF DTEAB AND TTEAB ORALLY (IN FASTED ANIMALS) AND INTRAPERITONEALLY IN MALE SWISS-WEBSTER MICE AND MALE WISTAR RATS

		No. Dead/Total No. Treated After			LD_{50} Determined mg./kg.		
	Dose, mg./kg.	24 hr.	48 hr.	72 hr.	24 hr.	48 hr.	72 hr.
				DTEAB			
Mice							
i.p	7.5	7/30	17/30	19/30	19.60 ± 2.07		
	15.0	4/30	13/30	19/30		11.25 ± 1.58	
	30.0	29/30	30/30	30/30			8.38 ± 1.16
p.o.	5.0	9/40	12/40	15/40	12.84 ± 1.42		
	10.0	14/40	25/40	29/40		7.95 ± 0.82	
	20.0	28/40	39/40	40/40			7.03 ± 0.61
Rats							
i.p.	30.0	5/10	8/10	9/10	30.00 ± 6.28		
	60.0	10/10	10/10	10/10		—	
	120.0	10/10	10/10	10/10			—
p.o.	62.5	0/20	0/20	0/20	—		
	125.0	2/20	3/20	5/20		—	
	250.0	2/20	2/20	2/20			—
				TTEAB			
Mice							
i.p.	7.5	6/30	18/30	20/30	20.07 ± 2.17		
	15.0	5/30	13/30	17/30		11.14 ± 1.61	
	30.0	27/30	29/30	30/30			8.45 ± 1.26
p.o.	12.5	18/35	27/35	29/35	15.63 ± 1.83		
	25.0	22/30	23/30	23/30		—	
	50.0	28/30	28/30	28/30			—
Rats							
i.p.	7.5	1/10	1/10	1/10	21.67 ± 3.49		
	15.0	1/10	1/10	1/10		20.83 ± 3.35	
	30.0	9/10	10/10	10/10			20.83 ± 3.35
p.o.	10.0	1/16	1/16	1/16	—		
	20.0	1/20	1/20	1/20		—	
	40.0	1/20	1/20	1/20			—

120 mg./kg. of DTEAB and 7.5, 15, and 30 mg./kg. of TTEAB i.p.

Ten animals were used at each dosage and the LD_{50} after 24, 48, and 72 hr. was determined where possible using the Reed-Muench method (10).

Male mice, Swiss-Webster strain, were fasted at least 20 hr. but not longer than 24 hr., water was given *ad libitum*. The animals were fasted in screen-bottom cages such that there was no access to feces or litter.

The fasted mice in groups of 30–40 received 5, 10, and 20 mg./kg. of DTEAB and 12.5, 25, and 50 mg./kg. of TTEAB orally by means of stomach intubation. The oral LD_{50} was calculated where possible after 24, 48, and 72 hr. using the Reed-Muench method (10).

Male rats, Wistar strain, were fasted similarly to the mice with water *ad libitum* in screen-bottom cages such that there was no access to feces or litter.

The fasted rats in groups of 16 or 20 received 62.5, 125, and 250 mg./kg. of DTEAB and 10, 20, and 40 mg./kg. of TTEAB orally by stomach intubation similarly to obtain the oral LD_{50}.

The doses in all animals were calculated on a mg./kg. basis and all animals used were approximately 10–12 weeks of age with average weights for mice and rats 19 and 128 g., respectively. All surviving animals were observed for a period of 30 days to include any possibility of latent effects.

RESULTS AND DISCUSSION

The synthesis of the expected products was indicated by elemental analysis. Similar confirmation was reported by Venable (11) on related compounds made by this method.

Anal.[2]—Calcd. for (DTEAB) $C_{18}H_{40}BrN$: C, 61.94; H, 11.50; N, 3.99. Found: C, 61.40; H, 11.73; N, 3.97.

Anal.[2]—Calcd. for (TTEAB) $C_{20}H_{44}BrN$: C, 63.46; H, 11.71; N, 3.70. Found: C, 61.81; H, 11.67; N, 3.86.

The possibility of isomers still exists; however, based on studies of organic syntheses of this nature and on the surface and micellar properties of aqueous solutions of these compounds, it would appear identity of the products was established.

The synthesis of the pure quaternary compounds was shown by the lack of a minimum in the surface-tension concentration curve. A purity of 99% plus was obtained. The absence of minima in these curves reflected a high degree of purity and agrees with earlier reports (6, 9)

The critical micelle concentration (CMC) in water at 30° for DTEAB was 5×10^{-3} g./ml. and for TTEAB was 1.06×10^{-3} g./ml.

No particular observance of any autonomic effects typical of other quaternary ammonium compounds was made. A few of the mice injected i.p. with DTEAB first appeared hyperexcitable but soon returned, as were all other animals, to their normal, grouped together, daytime positions.

The results of the LD_{50} studies of DTEAB i.p. and p.o. in mice and rats are listed in Table I. The LD_{50} values of DTEAB i.p. in mice calculated from the results in Table I using death after 24, 48, and 72 hr. as the end points are 19.60 ± 2.07, 11.25 ± 1.58, and 8.38 ± 1.16 mg./kg., respectively. Similarly, the LD_{50} values of DTEAB orally in mice

[2]Analyses performed by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

are 12.84 ± 1.42, 7.95 ± 0.82, and 7.03 ± 0.61 mg./kg. after 24, 48, and 72 hr., respectively.

In the rats, only the i.p. LD_{50} for DTEAB after 24 hr. could be calculated and was found to be 30.00 ± 6.28 mg./kg.

In the mice with DTEAB the i.p. and p.o. LD_{50} values were reversed from what is normally expected when comparing toxicities with these two routes of administration. Orally, DTEAB appeared more toxic than i.p.

In the rats, comparing the i.p. and p.o. DTEAB doses administered, the normal toxicity relationship between the routes of administration was seen. In preliminary work no deaths i.p. were obtained below 30 mg./kg., 50% mortality at 30 mg., and 100% mortality above this, making further calculations impossible. Orally, larger doses were prevented due to the CMC and the maximal volume allowable which could be intubated. In preliminary work, larger doses and volumes, were attempted with no greater mortality rate resulting.

Larger doses of DTEAB were required for toxicity in rats than in mice by either route of administration, being less toxic in the rat and especially appearing much less toxic orally.

The results of the LD_{50} studies of TTEAB i.p. and p.o. in mice and rats are listed in Table I. The LD_{50} values of TTEAB i.p. in mice calculated from the results in Table I using death after 24, 48, and 72 hr. as the end points are 20.07 ± 2.17, 11.14 ± 1.61, and 8.45 ± 1.26 mg./kg., respectively. Only the 24-hr. p.o. LD_{50} for TTEAB in mice could be calculated and was found to be 15.63 ± 1.83 mg./kg.

In the rats, the i.p. LD_{50} for TTEAB was found to be 21.67 ± 3.49, 20.83 ± 3.35, and 20.83 ± 3.35 after 24, 48, and 72 hr., respectively. No p.o. LD_{50} for TTEAB in rats could be obtained again due to CMC and volume limitations.

Comparing i.p. LD_{50} values in mice for DTEAB and TTEAB, the two-carbon increase in chain length and the resulting increase in molecular weight with TTEAB influenced the LD_{50} values obtained very little.

In mice with TTEAB the i.p. and p.o. LD_{50} values were again reversed from what is normally expected when comparing toxicities *via* these two routes of administration; oral administration being more toxic than i.p.

The oral LD_{50} in mice for TTEAB was larger than the oral LD_{50} for DTEAB indicating lesser toxicity for the larger molecular weight compound.

In rats, the i.p. LD_{50} for TTEAB did not change much over 24, 48, or 72 hr., whereas, in mice the i.p. LD_{50} for TTEAB changed considerably. However, the 24-hr. LD_{50} values for both rats and mice for TTEAB were nearly the same.

The i.p. LD_{50} for TTEAB in rats was smaller than the i.p. LD_{50} for DTEAB in rats indicating greater toxicity for the larger molecular weight compound.

The larger oral dose of TTEAB in rats indicates lesser toxicity than i.p. The variations observed in this study again demonstrate the problem of species variation although the data from the rats correlate with increase in molecular weight and toxicity increases. The lesser toxicity of these compounds i.p. than orally in mice is interesting to note as oral absorption of quaternary ammonium compounds is usually stated as difficult, only up to 15% of an oral dose being absorbed due possibly to formation of nonabsorbable complexes (12).

The surviving animals after the 30-day observation period appeared in good health and without deleterious effects.

SUMMARY AND CONCLUSIONS

Synthesis of very pure DTEAB and TTEAB was accomplished.

The influence of chain length or increase in molecular weight of the compounds on toxicity proved to be species variable.

The i.p. LD_{50} values of DTEAB in mice were found to be 19.60 ± 2.07, 11.25 ± 1.58, and 8.38 ± 1.16 mg./kg. after 24, 48, and 72 hr., respectively. The oral LD_{50} values of DTEAB in mice were 12.84 ± 1.42, 7.95 ± 0.82, and 7.03 ± 0.61 mg./kg. after 24, 48, and 72 hr., respectively. Only the 24-hr. i.p. LD_{50} value of DTEAB in rats could be determined and was found to be 30.00 ± 6.28 mg./kg. The usual greater toxicity from i.p. administration *versus* p.o. was not seen with DTEAB in mice but was in rats. DTEAB appeared more toxic in mice than rats, particularly orally.

The i.p. LD_{50} values of TTEAB in mice were found to be 20.07 ± 2.17, 11.14 ± 1.61, and 8.45 ± 1.26 mg./kg., after 24, 48, and 72 hr., respectively. Only the 24-hr. p.o. LD_{50} value of TTEAB in mice could be determined and was found to be 15.63 ± 1.83 mg./kg. The i.p. LD_{50} values of TTEAB in rats were found to be 21.67 ± 3.49, 20.83 ± 3.35, and 20.83 ± 3.35 mg./kg., after 24, 48, and 72 hr., respectively. No. p.o. LD_{50} of TTEAB in rats could be obtained due to limitations of critical micelle concentration and volumes required for administration. The usual greater toxicity from i.p. administration *versus* p.o. was again not seen with TTEAB in mice.

The i.p. LD_{50} values in mice for DTEAB and TTEAB were nearly identical. Chain length appeared to have little influence on toxicity in mice i.p. Orally, the LD_{50} values in mice indicated DTEAB was more toxic than TTEAB, the lesser molecular weight compound being the more toxic. The i.p. LD_{50} values in rats and mice after 24 hr. for TTEAB were practically the same. However, the i.p. LD_{50} values indicated that the higher molecular weight compound, TTEAB, in rats was more toxic than DTEAB which is what usually would be expected. TTEAB was less toxic p.o. than i.p. in rats again demonstrating the variability of absorption seen with quaternary compounds.

General comparisons of the LD_{50} values for DTEAB and TTEAB with those of cetylpyridinium chloride and benzalkonium chloride indicated lesser toxicity for the pure DTEAB and TTEAB compounds.

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Keyphrases

Dodecatriethylammonium bromide—synthesis
Tetradecatriethylammonium bromide—synthesis
Quaternary ammonium compounds chain length—toxicity effect
Surface tension-concentration curves—purity determination
LD_{50} values—quaternary ammonium compounds

Determination of the pKa′ Value for 5,5-Diphenylhydantoin

By SURAJ P. AGARWAL and MARTIN I. BLAKE

The pKa′ value for diphenylhydantoin was found to be 8.31 by ultraviolet spectrophotometry and 8.33 by potentiometric titration.

A NONAQUEOUS titrimetric procedure for determining combinations of phenobarbital and sodium diphenylhydantoin was recently reported by Agarwal and Blake (1). In considering the feasibility of a differentiating titration for mixtures of phenobarbital and diphenylhydantoin, it was apparent that the pKa value for the latter component had not been reported in the literature. Preliminary studies (2) with a variety of solvents, titrants, and electrode systems indicated that a differentiating titration was not possible. It was concluded that the pKa values for the two components were probably too close together to permit an effective differentiating nonaqueous titration. This study was undertaken for the purpose of establishing the pKa′ for diphenylhydantoin. The spectrophotometric procedure described by Albert and Sergeant (3) was applied in this investigation. The pKa′ was also determined potentiometrically.

EXPERIMENTAL

Apparatus—All spectrophotometric measurements were made with a Carl Zeiss model PMQII spectrophotometer, equipped with matched 1.0-cm. silica cells. Potentiometric titrations were performed with a Beckman pH meter (Expandomatic) model 76A equipped with a glass electrode (Beckman No. 41263) and a calomel electrode (Beckman No. 39170). A 5-ml. buret (Kimax) graduated in 0.01 ml. was used for delivery of the titrant.

Reagents and Solutions—Reference standard diphenylhydantoin and sodium diphenylhydantoin were supplied by Parke-Davis and Co. Tris-(hydroxymethyl)aminomethane (THAM), primary standard grade, was obtained from Fisher Scientific Co. Buffer solutions were prepared by combining appropriate volumes of 1.0 *N* HCl and 0.01 *M* THAM solution to give the desired pH. All other chemicals were reagent grade.

Received March 18, 1968, from the Department of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612
Accepted for publication May 31, 1968.

Spectrophotometric Procedure—The absorption spectra of diphenylhydantoin were obtained in 0.01 *N* sodium hydroxide and in 0.01 *N* hydrochloric acid. Since the maximum difference in absorption for the ionized and unionized species occurs at 236 mμ, this was the wavelength selected for all absorbance measurements. The spectra are shown in Fig. 1.

A stock solution of diphenylhydantoin (0.01 *M*) in alcohol was prepared. One milliliter of this solution was transferred by pipet to each of seven 100-ml. volumetric flasks and the volume was brought to the mark with THAM buffer solutions having pH values of 7.7, 7.9, 8.1, 8.3, 8.5, 8.7, and 8.9, respectively. A similar series of solutions was prepared which contained 1 ml. of alcohol and the corresponding buffer solution. These served as blanks for the absorbance measurements. The average ionic strength was 0.005 (0.002–0.008).

Potentiometric Procedure—A series of stock solutions, 0.01 *M* in sodium diphenylhydantoin, was prepared in aqueous alcohol solution containing 20, 30, 40, and 50% alcohol by volume, respectively. Fifty milliliters of each solution was titrated potentiometrically with 1 *N* hydrochloric acid. The ionic strength was 0.01.

DISCUSSION

The pKa′ of diphenylhydantoin was determined spectrophotometrically by the procedure described by Albert and Sergeant (3). The absorbance of the

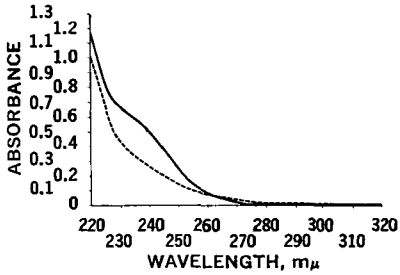

Fig. 1—Ultraviolet absorption spectra of 5,5-diphenylhydantoin in 0.01 N *HCl, solid line; and in 0.01* N *NaOH, broken line.*

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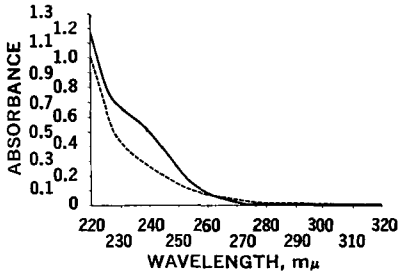

Fig. 1—Ultraviolet absorption spectra of 5,5-diphenylhydantoin in 0.01 N *HCl, solid line; and in 0.01* N *NaOH, broken line.*

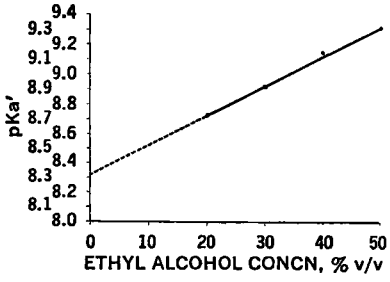

Fig. 2—The pKa' of 5,5-diphenylhydantoin in hydroalcoholic medium plotted against the concentration of ethyl alcohol.

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where A = absorbance of diphenylhydantoin in 0.01 N NaOH, B = absorbance of diphenylhydantoin in buffered solution, C = absorbance of diphenylhydantoin in 0.01 N HCl. For the 7 buffers used in this study an average pKa' of 8.31 ± 0.04 (SD) was obtained.

The pKa' was also determined potentiometrically by titrating sodium diphenylhydantoin with standard hydrochloric acid. Since diphenylhydantoin is soluble in alcohol but not in water, and the reverse is true for the sodium salt, a series of titrations was performed in solvent mixtures containing varying concentrations of alcohol in water. The average pKa' value was determined at each alcohol level from the data obtained for at least five points in each titration curve using the expression,

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Keyphrases

5,5-Diphenylhydantoin—pKa' value determination
UV spectrophotometry—procedure
Potentiometric titration—procedure

Chemistry and Biochemistry of Polyvalent Iodine Compounds VII. The ARP (Apparent Reduction Potential) of 1,3-Dihydro-1-hydroxo-3-oxo-1,2-benziodoxole

By A. EISENMANN* and W. WOLF

Iodoso compounds are strong oxidizing agents. In order to define an order of magnitude for this oxidizing action, the ARP (apparent reduction potential) of 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole has been measured using the method of Conant and Lutz. At pH 7.4, the ARP of benziodoxole was 0.327 ± 0.005 v., indicating that this heterocyclic iodine derivative, under the conditions used, is approximately as strong an oxidizing agent as ceric ion. The usefulness of this technique is discussed.

THE KINETICS (1) and the mechanism (2) of the reduction of 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole (I) to *o*-iodobenzoic acid have been studied in this laboratory. This process is irreversible, and thus, a normal oxidation-reduction potential cannot be measured. As it had been suggested that the difference between the two classes of trivalent iodine (iodoso, iodonium) is more one of degree and rate of reaction than of nature (3), it was of

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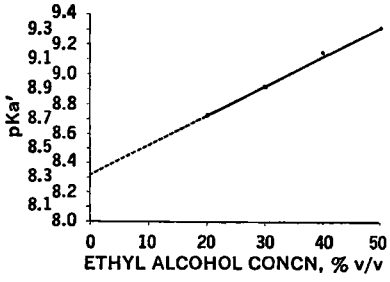

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interest to see if a value could be assigned to the "oxidizing power" of such compounds.

One method that is appropriate for a study of irreversible systems is that of Conant and Lutz (4). Instead of a normal potential, Conant (5) has defined an "apparent reduction potential" (ARP) as a pseudo-redox potential. The ARP is the potential of a reversible redox system which will cause 20–30% reduction within 30 min. of the oxidized form of the irreversible system. A more precise definition is derived from the use of the rate equation for a monomolecular reaction:

$$\frac{ds}{dt} = kx(c - s) \qquad \text{(Eq. 1)}$$

where c is the initial concentration of the compound studied, s is the amount which has been irreversibly reduced at any given time, and $x(c - s)$ is the concentration of the substance undergoing the irreversible change. If the system contains a large excess of a reversible redox system, with an equimolar amount in the reduced and oxidized form, x (fraction of compound reduced irreversibly by the reaction) will be essentially constant during the reaction. Then, integration of Eq. 1 yields Eq. 2:

$$k_1 = kx = \frac{2.3}{t} \log \frac{1}{1 - s} \qquad \text{(Eq. 2)}$$

where t is the time in min. for the reaction and s is the fraction which has been reduced of the irreversible substance. If, according to Conant, t is set at 30 min., use a $k_1 = 0.01$, then, by inserting these values of t and k_1 in Eq. 2 one defines a value of 25.9% reduction for s, instead of the vague 20–30% previously suggested. Using this value, a more accurate ARP could be determined; as shown by Conant (4), the ARP is a general concept and is not restricted to monomolecular reactions.

EXPERIMENTAL METHOD

The apparatus consisted of a glass cell with an i.d. of 65 mm. and a capacity of 125 ml., closed with a loose-fitting rubber top with openings for two electrodes, a gas bubbler, and admittance of the irreversible agent. A platinum electrode and a saturated calomel reference electrode (both Beckman) formed the cell circuit; both were located a few centimeters above the bottom of the cell. Nitrogen gas from a commercial tank was bubbled through using a Corning gas-dispersion fixture. The entire cell was mounted on a magnetic stirrer with adjustable rotational speeds. The potential was measured by a Corning 10 pH meter and also recorded constantly on a Texas Instruments recorder.

A solution of 0.05 M phosphate buffer (pH = 7.4 ± 0.1), 90 ml., was placed in the glass cell at the beginning of each trial. Equimolar amounts of the reducing agent and its oxidized form (each being about 0.001 M in the buffer solution) were then added, thus forming the reversible reference system. The stirring speed was adjusted and nitrogen bubbled in. When a steady potential had been recorded for about 5 min., 10.5 ml. of a solution of I was rapidly introduced into the redox cell. This solution was prepared by dissolving the weighed sample of I in 0.6 M NaOH with 10.0 ml. distilled water. The change in potential was followed for 30 min. As I was approximately 0.002 M in the buffer solution, it was in about 100% excess of the reducing agent.

Not all reversible systems are equally well suited for these experiments. The systems used formed a graded series of potentials, going in steps of about 50 mv. or so. This gave a good range for a quick determination of an approximate ARP. Since the measured change in cell potential was really the change in potential of the reversible redox system, these systems also had to come quickly to equilibrium. A sluggish system would not accurately reflect the amount of reduction which had taken place at any given moment. However, the 30-min. observation period compensated for this, as well as for the different rates of reaction.

RESULTS AND DISCUSSION

The data obtained are summarized in Table I.

The E.M.F. values of the reference systems used ranged from 77.2–599 mv. A redox system with a normal potential higher than the ARP did not appreciably reduce I, while one with a lower potential reduced it almost completely and very rapidly.

After the cell E.M.F. values were corrected for the calomel electrode to a hydrogen standard (E_h), a plot of these E_h values against the change in cell potential after 30 min. showed a linear relationship (Fig. 1). Points 1 and 4 represent two trials of the

Table I—Summary of Data

Run No.	E.M.F.,[a] mv.	E_h, mv.	mv. (min. After) 1	5	10	15	20	30	% Reduction[b]
6	77.2	−164.0	63.9	136.8	166.8	179.8	193.8	200.8	99+
8	82.2	−160.6	138.3	203.8		(after 8 min.)			
9	134.9	−107.9	111.9	153.1	164.1	171.1	174.1	179.1	99+
3	172.0	−71.3	3	37.8	76.2	115.9			99+
4	176.8	−67.1	0.2	0.3	0.3	0.4	0.7	1.4	5
1	185.5	−58.9	0	0	−0.6	−1			0
10	211	−31.8	45	98	122	135	143	150	99+
5	354	110.1	13	50	57	62	69	75	99+
12	366	123.2	3	17	25	30	34	39	91
7	403	160.2	24	53	66	68	68	68	99+
11	534	290.1	−10	−5	0	5	12	21	67
14	575	333.8	−9	−6	−5	−4	−3	−1	0
13	577	334.8	−8	−3	−1	0	2	5	19
16	586	344.8	−3	−2	−2	−1	1	2	8
17	591	348.8	−5	−3	−2	−1	−1	0	0
15	599	356.8	−6	−4	−3	−3	−2	0	0

[a] Experimental value. The experimental E.M.F. values for mixtures of the same ions are not exactly identical, since no system was exactly equimolar. [b] As calculated graphically.

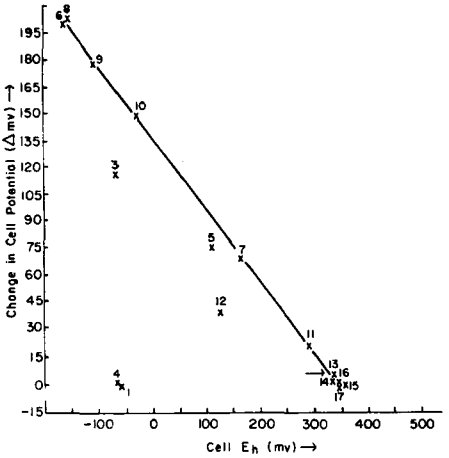

Fig. 1—Change in cell potential (Δmv.) as a function of the corrected E.M.F. (E_h).

ferricyanide/ferrocyanide system; the values are seen to agree well with each other. This apparently anomalous result suggests that the ferricyanide ion is somehow hindered in reacting with *I*, and raises the interesting possibility that *I* may be a sterically selective oxidizing agent.

Some systems were used in one ratio, and the ceric/cerous system was used in different ratios (runs 8, 9, 12). Using a linear least-squares program on a Mathatron 848 computer, the equation of this line was found to be:

$$Y = 136.79 - 0.397X$$

where *Y* is the change in potential and *X* is E_h. Using a theoretical Δmv. of 6.85 for 25.9% reduction, the apparent reduction potential of 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole under the conditions of these experiments was found to be 327 ± 5 mv. (Room temperature, pH 7.4, concentrations given.)

The linear relation shows that the amount of benziodoxole which is irreversibly reduced is a function of the electromotive force of the reversible system tested. This, of course, is the basis for the quantitative study employed here. The graph of percentage reduction *versus* E_h (Fig. 2) shows resemblance to a log curve, which is not surprising since the change of cell potential is logarithmically related to the percentage reduction. Values of E_h much greater than the ARP would show a 0% reduction on Fig. 2.

Extension of this method to other iodoso and

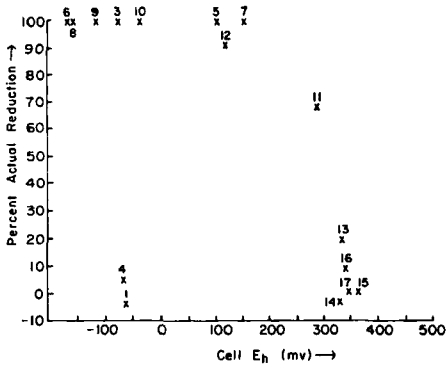

Fig. 2—Percent reduction of 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole as a function of cell E_h.

iodonium compounds is indicated, in order to verify Beringer's suggestions and to determine if a real difference exists or not between these two types of polyvalent iodine compounds.

It would also be advantageous to devise some new method for the determination of the ARP. Although this method has been updated and is relatively simple, a faster method is desirable. Polarography shows some hints of being applicable to the problem, but potentiometric titration seems to be possibly the most promising direction.

The authors consider that the present modification of the method of Conant and Lutz allows for an easy and accurate determination of the ARP of organic compounds suffering irreversible reduction. This method should also be adequate for the determination of the apparent oxidation potential.

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Keyphrases

Iodine compounds, polyvalent—oxidizing agent

1,3-Dihydro-1-hydroxy-3-oxo-1,2-benziodoxole—apparent reduction potential

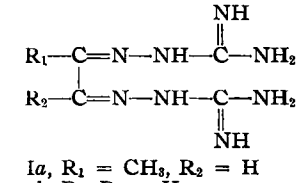
Potentiometric measurements—reduction potential

Methylglyoxal Bis(guanylhydrazone) Analogs VI. Methylglyoxal Bis(guanylhydrazone) Pamoate

By EUGENE G. PODREBARAC and C. C. CHENG

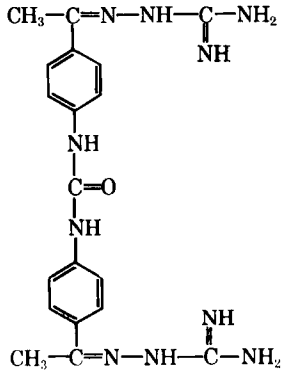
The pamoate of methylglyoxal bis(guanylhydrazone) was prepared. This compound, on a molar basis, is more active and less toxic than the corresponding dihydrochloride salt against leukemia L-1210.

THE ANTITUMOR ACTIVITY of methylglyoxal bis(guanylhydrazone),[1] [methyl-GAG, *Ia*], against leukemia L-1210 in mice and adult acute myelocytic leukemia (1, 2) in man is now well known. This drug, however, was found to be quite toxic (3, 4).



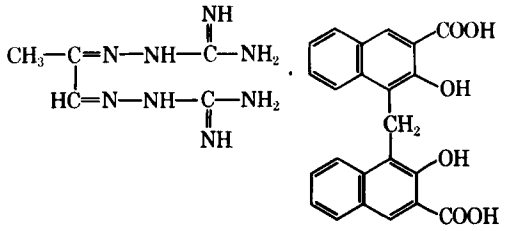
Ia, $R_1 = CH_3$, $R_2 = H$
b, R_1, $R_2 = H$
c, $R_1 = CH_2OH$, $R_2 = H$
d, R_1, $R_2 = CH_3$

Subsequent work revealed that, except for the unmethylated compound *Ib* which is almost as active (1) as *Ia*, other modifications of the basic structure failed to yield compounds active against leukemia L-1210 (5–9). The reported activity of hydroxymethyl-GAG (*Ic*) was proved to be erroneous since the originally synthesized *Ic* (10) was found to be actually hydrated methyl-GAG (11–13). The authentic hydroxymethyl-GAG did not show the expected activity (11–13). Although the compound 4,4′-diacetyldiphenylurea bis(guanylhydrazone) [DDUG, II], was found to have marked activity against leukemia L-1210 (14), no cross-resistance between methyl-GAG and DDUG was observed (14–16). In addition, a structurally related compound, dimethyl-GAG (*Id*), is entirely devoid of antileukemia activity (6). Compound II is believed to be an analog of the terephthalanilide-type (15).



II

In view of the aforementioned information, an attempt was made to modify the absorption and excretion pattern, rather than the basic structure, of *Ia*. Since salts of pamoic acid, due to their very limited solubility, are known to provide slow release of basic drugs and thus maintain a low level of drug in the blood stream,[2] the pamoate salt of methyl-GAG (III) was prepared in this laboratory.



III

The title compound was readily obtained by the reaction of methylglyoxal bis(guanylhydrazone) dihydrochloride and the disodium salt of pamoic acid. Equivalent amounts of pure starting materials gave desired product of analytical purity in 91% yield.

Preliminary tests of III indicated activity in the 9KB primary screen. Tests in the leukemia L-1210 system revealed that, on a molar basis, compound III is much more active and less toxic than the corresponding dihydrochloride salt (see Table I). For comparison, pamoic acid or its sodium salt possessed no anticancer activity against either sarcoma 180, carcinoma 755, or leukemia L-1210 in mice (21).

That compound III is more than a simple acid-base compound was demonstrated by titration studies. The addition of two equivalents of base to an aqueous suspension of the compound, even after prolonged stirring, failed to yield a solution (a solution was obtained after the addition of four equivalents of base). Since these conditions should give freely water-soluble materials—the free base of methyl-GAG and the disodium salt of pamoic acid—it appears that the phenolic functions of the pamoic acid may play an important role in the composition of III. As such, the pamoic acid salt of methyl-GAG is very different from the simple methyl-GAG salts.

EXPERIMENTAL

One mole (257 g.) of the purified dihydrochloride

Received May 5, 1968, from the Midwest Research Institute, Kansas City, MO 64110
Accepted for publication June 5, 1968.
This investigation was supported by the Cancer Chemotherapy National Service Center (CCNSC), National Cancer Institute, of the National Institutes of Health, Public Health Service, contract PH-43-65-94.

[1] According to *Chemical Abstracts*, the name for this compound is 1,1′-[(methyl)ethanediylidenedinitrilo]diguanidine.

[2] In the treatment of malaria, intramuscular injection of an oil suspension of cycloguanil pamoate was found to curtail the effects of malaria in man for extended periods (17–19). In cancer chemotherapy study, it was reported that the pamoates of various alkylating agents had significantly better actions in prolonging the survival time of mice with ascites tumors than their corresponding hydrochloride salts (20).

TABLE I—COMPARISON OF ANTILEUKEMIA ACTIVITY OF THE DIHYDROCHLORIDE AND PAMOATE OF METHYLGLYOXAL BIS(GUANYLHYDRAZONE) AGAINST LEUKEMIA L-1210 IN MICE[a]

Dose (i.p.), mg./kg.	Survivors	Cures	Animal Wt. Diff.	Percent T/C[b]
	Me-GAG·2HCl			
400.0	0/7			
200.0	5/7		−3.8	89
100.0	7/7		−2.0	144
60.0	6/6		−2.3	168
60.0	6/6		−1.2	138
60.0	5/6		−1.4	114
40.0	6/6		−0.1	139
25.0	6/6		−1.2	160
25.0	6/6		−0.5	135
18.0	6/6		−0.4	115
16.0	6/6		−0.8	124
11.1	6/6		−0.3	118
	Me-GAG·Pamoate			
1000.0	10/10		−1.6	208
800.0	10/10		−1.2	170
800.0	10/10	1	−2.4	257
400.0	10/10		−1.7	196
200.0	10/10		−1.3	157
200.0	10/10	1	−1.6	264
100.0	10/10		−1.1	210
50.0	10/10		−0.8	166
25.0	9/9		−1.2	136
19.0	6/6		0.3	123
15.6	6/6		−1.8	135
12.0	6/6		−1.7	125
7.8	6/6		−1.1	116

[a] Biological testing was performed by contract screeners of CCNSC. [b] T/C = survival time: test/control.

salt of methyl-GAG was dissolved in 2,500 ml. of distilled water and the solution warmed to 80°. A disodium pamoate solution was prepared by dissolving 1 mole of disodium pamoate monohydrate (450 g.) in 2,800 ml. of distilled water. The solution was warmed to 70°, treated with Norite, then filtered (still had red-brown color).

The Me-GAG·2HCl solution was stirred vigorously at 80–90° while the disodium pamoate solution at 70° was added portionwise during approximately 45 min. A solid began to separate immediately. The reaction mixture was stirred at 90° for 1 hr. then filtered. The filter cake was washed well with hot water then dried in a vacuum oven at 80–85° at approximately 20 mm. for 20 hr. to give 536 g. (91% yield) of the pamoate salt of methylglyoxal bis(guanylhydrazone), m.p. 262–263° dec.; $\lambda_{max.}^{pH\ 1}$ 282 mμ (ε 61,800); 273 mμ (ε 63,600). $\lambda_{max.}^{pH\ 11}$ 327 mμ (ε 34,100); 235 mμ (ε 94,200). Minor absorption peaks at 300 and 288 mμ and a shoulder at 277 mμ were noted in pH 11.

Anal.—Calcd. for $C_{23}H_{28}N_8O_6 \cdot H_2O$ (mol. wt. 590.6): C, 56.94; H, 5.12; N, 18.97; H_2O, 3.05. Found: C, 56.98; H, 4.71; N, 19.27; H_2O, 3.00.

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Keyphrases

Methylglyoxal bis(guanylhydrazone) pamoate—synthesis
Antileukemic activity—methylglyoxal bis(guanylhydrazone) pamoate
UV spectrophotometry—structure

Monomolecular Film Properties of Protective and Enteric Film Formers I. Effect of Polymer Side Chain on Surface Pressure-Area Isotherms

By JOEL L. ZATZ*, NORMAN D. WEINER, and MILO GIBALDI†

Polymeric film formers of pharmaceutical interest have been investigated using surface monolayer techniques. Surface pressure-area curves were obtained and evaluated for monomolecular films of several half-esters of poly (methyl vinyl ether/maleic anhydride) at the air-water interface. The ester side chain was found to contribute significantly to the surface properties of the polymer.

FILM COATINGS are widely used on pharmaceutical dosage forms as protectives and as diffusion barriers to control drug release. A number of studies has been reported on the physical-chemical properties of free polymeric films (1–7). Although free films represent a useful model for evaluating the gross properties of applied films, they provide limited information of molecular interactions which in fact dictate the properties of the film.

An interesting approach to the study of polymeric films is the use of techniques devised for investigation of insoluble monomolecular films at the air-water surface. Monomolecular films or monolayers have been used as models for a number of biologic and physical-chemical processes. Goddard and Schulman (8) studied the penetration of cholesetrol films by surface-active agents and were able to relate their data to the ability of these surfactants to hemolyze red blood cells. More recently Schaubman and Felmeister (9) discussed the biologic significance of the penetration of monolayers of lecithin by chlorpromazine, and Torosion and Lemberger (10) considered the physical-chemical interactions between lecithin and lipids in mixed monolayers.

Crisp (11) studied a number of synthetic polymers on a surface balance and concluded that many polymers are spread completely at the air-water surface, a condition which must be met if the experimental results are to be properly interpreted (11–13). It would appear that the polymer molecule must have a degree of hydrophilic character but possess very limited solubility to form a stable monolayer (13). The applicability of monolayer techniques for studying polymeric material has been confirmed by a number of other workers (14–20). The polymer is applied to the aqueous surface or substrate in the usual fashion by dissolving it in a suitable solvent which can spread over the surface and subsequently evaporate or dissolve.

However, the area per chemical segment or monomeric unit, rather than the area per molecule, is the preferred parameter to characterize the system since the force-area curves of polymers have been shown to be independent of the degree of polymerization (11, 14).

Received April 1, 1968, from the Department of Pharmacy, College of Pharmaceutical Sciences, Columbia University, New York, NY 10023

Accepted for publication May 10, 1968.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

This investigation was supported by a grant from Merck, Sharp and Dohme, West Point, Pa.

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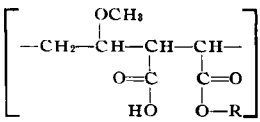
Monolayers of film-forming polymers may provide a useful model for the investigation of polymer structure and orientation, as well as the effect of pH and additives, on the properties of the polymer. The present report concerns the determination and evaluation of surface pressure-area curves for several half-esters of poly (methyl vinyl ether/maleic anhydride). These polymers have been evaluated previously as sustained-action coatings and appear to have potential for controlling drug release (5, 21). The structure of these polymers may be represented by



where R is the hydrocarbon side chain.

EXPERIMENTAL

Preparation of Polymer Derivatives—The various half-esters used in this study were prepared from the same lot of poly (methyl vinyl ether/maleic anhydride)[1] (designated as PVM/MA) by slurrying the polymer in the appropriate organic alcohol (previously purified by repeated passage through an alumina-silica gel column), adding 0.1% HCl, and allowing the mixture to reflux at its boiling point for a specified period of time, as indicated in Table I. The resulting half-esters were purified by precipitation from ethyl ether or *n*-hexane and repeated washings with these solvents, and subsequently dried at 30° *in vacuo* to constant weight. The degree of esterification was confirmed by titrating free acid groups with NaOH. All acid value determinations agreed within 6% of the theoretical values.

TABLE I—PREPARATION OF POLY (METHYL VINYL ETHER/MALEIC ANHYDRIDE) HALF-ESTERS

Derivative	Segment Formula Weight	Reflux Time, hr.
Ethyl	202	4
n-Propyl	216	6
n-Butyl	230	7.25
n-Pentyl	244	8
n-Hexyl	258	9

Determination of Surface Pressure-Area (π-A) Curves—The surface balance employed was made of Teflon and has been described in a previous report (22). Water was triple distilled in a glass still from an alkaline permanganate solution.

[1] Gantrez AN 119, General Aniline and Film Corp., New York, N. Y.

The half-esters were dissolved in a mixture of isopropanol and hexane and spread on the aqueous subphase by means of an Agla micrometer syringe. Preliminary experiments indicated that the addition of 10^{-3} *M* HCl to the subphase was sufficient to suppress ionization of the free carboxyl groups of the polymer derivatives. Surface pressure was determined at room temperature (24.0° ± 0.5°) as a function of area using the Wilhelmy plate method (23, 24).

RESULTS AND DISCUSSION

The surface pressure-area (π-A) isotherms determined with each half-ester are shown in Fig. 1. Each curve represents the average of three determinations. Data points have been eliminated to attain clarity. When the films were spread with other spreading solvents, *e.g.*, ethanol–chloroform–hexane, 1:1:3, or at different initial surface areas, curves identical to those in Fig. 1 were obtained indicating complete spreading (13).

The π-A isotherm obtained with the hexyl half-ester of PVM/MA is presented in Fig. 2 to depict the various regions of the curve which may be defined. In the expanded region (α) the molecules are relatively far apart and do not interact. Compression (*i.e.*, decreasing the surface area occupied by the film) produces a "squeezing out" of water molecules and the segments approach each other. This phenomenon is reflected by the transition region (β) in the π-A curve. In the condensed region (γ) the segments are in close contact. In this region of the curve, a small change in area results in a comparatively large change in surface pressure. The marked increase in π in the condensed region probably reflects changes in orientation or configuration of the polymer. Crisp (11) has suggested that, in the condensed region, there is a gradual expulsion of hydrophobic groups from the surface into an "overfilm" above the monolayer. The polymer molecule, however, remains anchored to the surface probably because of hydrogen bonding between hydrophilic groups and the water molecules.

As compression is continued, a point is reached where the film can no longer be accommodated as a monomolecular layer and the film collapses to form a three-dimensional bulk phase. The surface pressure at which this phenomenon occurs is termed the collapse pressure (Point A in Fig. 2). The collapse pressure is a function of both cohesional forces between polymer segments and adhesional forces between the polar groups of the polymer and the subphase (15).

Point B in Fig. 2 represents the limiting area per molecular segment at zero surface pressure. It is obtained, as indicated, by extrapolating the condensed region of the curve to the abscissa. At the limiting area the film is in a hypothetical state in which it is condensed under no external pressure.

Examination of the π-A isotherms in Fig. 1 indicates a number of interesting relationships. For example, the collapse pressure is found to increase with increasing chain length of the ester. This suggests that cohesional forces, due to van der Waals' interactions between the ester chains, contribute significantly to the physical-chemical properties of the polymer.

Previous workers (16, 17) have reported that ester groups of polymers are oriented in the *cis*-configuration at the air-water surface. Hence, the ester chain protrudes through the surface toward the gas phase. It is likely that the side chains of the PVM/MA half-esters are oriented in a similar fashion since the limiting area for all five derivatives is 48–50 Å.²/segment. This observation implies identical configuration in the limiting case and thereby, the lack of contribution of the ester side chain to the limiting area. If the ester chain were oriented in the plane of the surface, then a direct relation between limiting area and the length of the ester chain would be apparent.

The interactions between polymers and plasticizers, as well as the relationships between polymer chain length, plasticizer, and water vapor transmission are presently under investigation in an effort to elucidate the molecular considerations which control the properties of polymeric films. Finally, the results obtained using monomolecular

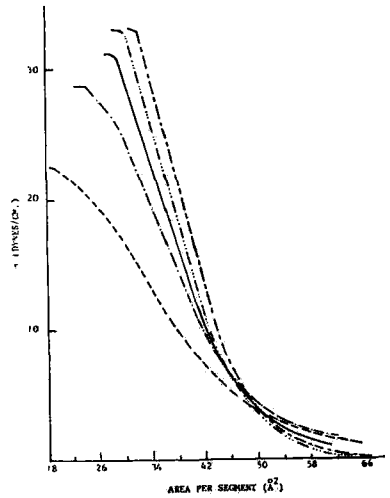


Fig. 1—Surface pressure-area (π-A) isotherms for various half-esters of PVM/MA. Key: — - —, n-*hexyl;* ——·——, n-*pentyl;* ———, n-*butyl;* ---, n-*propyl;* ---, *ethyl.*

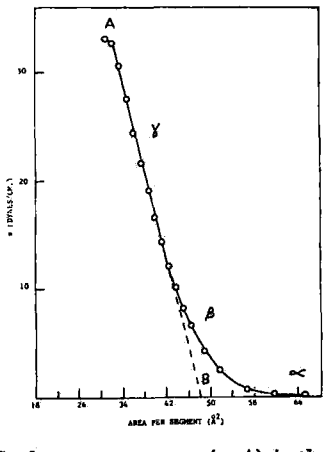


***Fig. 2—Surface pressure-area (π-A) isotherm** for the n-hexyl half-ester of PVM/MA. The plot denotes the expanded (α), transition (β), and condensed (γ) regions of the film upon compression, as well as the collapse of the film (A) and the theoretic limiting area (B).*

films will be compared with literature data on free films in order to evaluate the utility of the model.

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Abrus precatorius (also called Jequirity, Gunja, Kunch, and Rati) is a perennial twiner belonging to the family *Leguminosae* that grows throughout India and other tropical countries (1). It contains some of the most lethal plant poisons in the tropics. Many children have been attracted to its color and have accidentally swallowed the seed. Jequirity beans are frequently used as eyes in dolls, toys, necklace beads, and clothing ornaments. Some of the preparations from this plant induced severe cytotoxicity in concentrations of less than 0.01 mcg./ml. of solid material. Extracts of *Abrus precatorius* initially suppressed the growth of cancer cells and prolonged the survival of laboratory animals (2).

The biological effects of extracts of the seed have stimulated the need for further information concerning the identity of the compounds contained in *Abrus precatorius*.

EXPERIMENTAL

Source and Preparation of Material—Ripe seeds of *Abrus precatorius* were obtained from Herbst Brothers, Seedsmen, Inc., Brewster, N. Y. Approximately 65 g. of seeds dispersed in powdered dry ice were ground to a powder in a Wiley mill.

Extraction of Seeds—The powdered seeds were extracted successively with two 200-ml. portions of *n*-hexane, ethyl ether,[1] and distilled water at room temperature, and finally with two 500-ml. portions of 95% ethyl alcohol in a Soxhlet for 6 hr. Evaporation of 250 ml. of the hexane and ether fractions yielded, respectively, 372 and 177 mg. of yellow oils.

Saponifiable and Unsaponifiable Fractions—The saponifiable and unsaponifiable fractions were extracted (3). The alkaline soaps obtained were acidified with 1 + 1 HCl, extracted three times with petroleum ether, and then converted to the methyl esters (4).

Gas-Liquid Chromatography of Methyl Esters[2]—The mixture of methyl esters was subjected to GLC, using a Wilkins Aerograph model 1520 instrument fitted with a flame-ionization detector. The 2.7 m. × 0.6 cm. i.d. (9 ft. × 1/4 in.) aluminum column was packed with 20% polydiethylene glycol

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TABLE I—UNSAPONIFIABLE COMPONENTS OF *Abrus precatorius*

Fraction	Ethyl Ether Extract 2.2 mg.	Hexane Extract 18.4 mg.
Hydrocarbon	C_{16}–C_{24}	C_{16}–C_{26}
	Squalene, $C_{21}OH$–$C_{32}OH$	C_{27}–C_{35}
Alcohol-methyl sterol	$C_{20}OH$–$C_{28}OH$	$C_{10}OH$–$C_{28}OH$
	$C_{29}OH$, $C_{33}OH$ (?)	β-Amyrin
	Campesterol (?)	Cycloartenol
	β-Amyrin	
	Cycloartenol	
Sterol	Cholesterol	Cholesterol
	Brassicasterol	Brassicasterol
	Campesterol	Campesterol
	Stigmasterol	Stigmasterol
	β-Sitosterol	β-Sitosterol
	Methyl sterols (?)	Methyl sterols

TABLE II—PERCENT OF TOTAL FATTY ACIDS[a] EXTRACTED FROM *Abrus precatorius*

Fatty Acid	Hexane Fraction, %	Ether Fraction, %
Octanoic (8:0)	trace	—
Dodecanoic (12:0)	trace	0.2
Tetradecanoic (14:0)	0.1	0.7
Hexadecanoic (16:0)	4.0	10.5
9-Hexadecenoic	0.2	1.3
Unidentified[b]	0.2	0.7
Octadecanoic (18:0)	1.0	1.9
9-Octadenenoic (18:1)	23.0	22.3
9,12-Octadecadienoic (18:2)	7.7	30.7
Eicosanoic (20:0)	0.6	—
11-Eicosenoic (20:1)	5.2	16.8
9,12,15-Octadecatrienoic (18:3)	10.6	
Docosanoic (22:0)	3.9	2.2
13-Docosenoic (22:1)	33.6	3.4
Tetracosanoic (24:0)	1.9	9.4
15-Tetracosenoic (24:1)	2.9	—
Unidentified[c]	4.8	—

[a] As methyl esters; not corrected for detector response. [b] Retention time *versus* 16:0 = 1.47. [c] Retention time *versus* 16:0 = 9.07.

succinate (DEGS) on 60–80 mesh Chromosorb W. The gas chromatographic conditions were as follows: helium gas flow 70 ml./min.; column temperature 207°; injection port 253°; cell temperature 268°.

Identification of Esters—The retention times of the sample components were compared with that of highly purified esters on polar (DEGS) and nonpolar columns (2.7 m. × 0.6 cm. i.d.; 3% SE-30 on 60–89 mesh Chromosorb W). Semilogarithmic plots of retention times *versus* carbon number were used to identify 15-tetracosenoic acid and to confirm assignments.

Column Chromatography of Unsaponifiables—Magnesium silicate[3] was heated to 260° for 2 hr., then deactivated with 10 g. of water per 100 g. of magnesium silicate and allowed to equilibrate overnight. The 18.4 mg. and 2.2 mg. of unsapouifiable material from the hexane and ether fractions, respectively, were chromatographed on a 2.5 × 30 cm. column containing 20 g. of the prepared magnesium silicate. The hydrocarbons were eluted with 40 ml. of 10% anhydrous ethyl ether in hexane and the alcohol-methyl sterols with an additional 140 ml. of the same mixture. The sterols were then eluted with 150 ml. of 50% ether in hexane.

Gas-Liquid Chromatography of the Hydrocarbon, Alcohol-Methyl Sterol, and Sterol Fraction—The three fractions eluted from the column were subjected to GLC on a Barber-Colman model 5000 instrument utilizing the hydrogen-flame detector. The instrument was fitted with a 1.8 m. × 0.6 cm. i.d. (6 ft. × 1/4 in.) glass column filled with 3% JXR on 100–120 mesh Gas Chrom Q. Nitrogen was used as the carrier gas at 30 p.s.i. Detector and sample temperatures were maintained at 262°. The column temperatures were controlled at 180° and 240° (see Table I). For quantitative estimation of various components, areas beneath gas chromatographic peaks were determined by the peak height retention time method (5).

RESULTS AND DISCUSSION

The fatty acids detected in *Abrus precatorius* in this study differ from an earlier chemical investigation (1) of the lipid fraction which did not report the presence of trace amounts of octanoic (8:0), dodecanoic (12:0), or tetradecanoic (14:0) acids, in addition to larger amounts of 9-hexadecenoic (16:1) acid (see Table II).

GLC revealed that 13-docosenoic (22:1) acid was the third most prevalent component in this preparation. However, Mandiratta and Dutt (1) did not indicate the presence of this lipid.

Examination of the chromatogram of the hydrocarbon fraction and plots of retention time *versus* carbon number (Fig. 1) indicate the presence of three homologous series in addition to squalene. The first series consists of normal straight chain hydrocarbons in the range C_{16}–C_{26}. The second series consists of iso and/or anteiso hydrocarbons (*e.g.*, iso = 2-methyl; anteiso = 3-methyl). Pre-

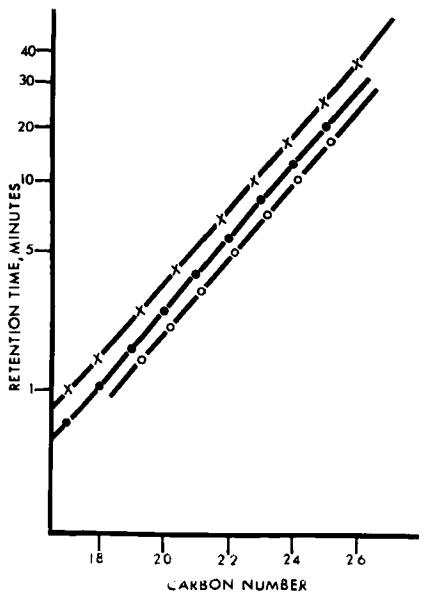

Fig. 1—Semilogarithmic plot of carbon number versus retention time of hydrocarbons obtained from hexane extract. Key: ●, iso/anteiso; O, multi-branched; ×, normal.

[3] Florisil PR, Floridin Co., Pittsburgh, Pa.

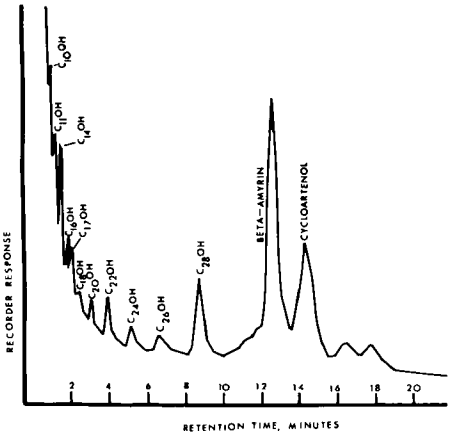

Fig. 2—Chromatogram of alcohol–methyl sterols from hexane extract.

TABLE III—PERCENT[a] OF TOTAL STEROLS EXTRACTED FROM *Abrus precatorius*

Sterol	Hexane Fraction, %	Ether Fraction, %
Cholesterol	1.3	2.3
Brassicasterol	2.8	1.7
Campesterol	21.0	16.5
Stigmasterol	46.8	50.8
β-Sitosterol	24.8	26.9
Unidentified[b]	2.4	1.8
Unidentified[c]	0.9	—

[a] Not corrected for detector response. [b] Retention time *versus* cholesterol = 1.84. [c] Retention time *versus* cholesterol = 2.22.

vious examination of standards indicated that iso and anteiso hydrocarbons with the same molecular weights could not be resolved. Iso and anteiso compounds have been reported to be present in vegetable and animals oils (6–9). The third series probably consists of multiple-branched hydrocarbons, which Mold *et al.* (6) have reported to be present in tobacco leaf wax.

The alcohol–methyl sterol fraction contained mainly a series of C_{10}–C_{28} normal alcohols, β-amyrin, and cycloartenol (Table I and Fig. 2). Campesterol and the C_{29} and C_{33} alcohols may be present as minor components.

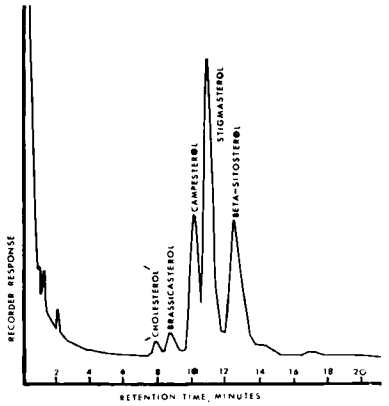

Fig. 3—Chromatogram of sterols from hexane extract.

GLC of the sterol fraction (Table III and Fig. 3) indicated the presence of at least seven components, which were identified by comparison with retention times of standards. Stigmasterol was the most prevalent sterol; Mandiratta and Dutt (1) reported only the presence of sitosterol.

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Keyphrases

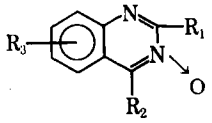
Abrus precatorius seeds—lipids
Lipids, *A. precatorius*—components identified
Column chromatography—separation
GLC—identity

Reaction of 2-Chlorobenzoxazole with Anthranilic Acids and 2-Amino-1-naphthalenesulfonic Acid

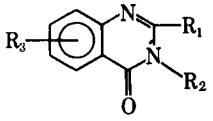
By JOSEPH SAM, J. L. VALENTINE, and C. W. RICHMOND

The preparation of 1-carboxy-12-benzo(*d*)quinazo(2,3-*b*)oxazole-12-one and 2-chloro-12-benzo(*d*)quinazo(2,3-*b*)oxazole-12-one are reported. The reaction of 2-chlorobenzoxazole with 2-amino-1-naphthalenesulfonic acid gave 2-(2-naphthylamino)benzoxazole. Preliminary pharmacological screening indicates mild sedative properties for the described compounds.

NUMEROUS CNS properties have been attributed to quinazoline-3-oxides (I) (1) and quinazolones (II) (2). The incorporation of the latter moiety into a tetraheterocyclic system (*e.g.*, VII)



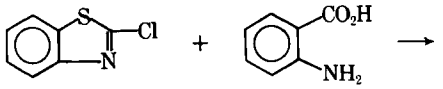
I



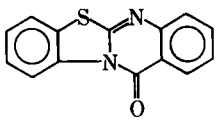
II

which also includes the benzoxazole nucleus was undertaken in order to obtain compounds which might exhibit interesting CNS properties. Benzoxazole derivatives are known to exhibit CNS effects (3).

The preparation of 12-benzo(*d*)quinazo(2,3-*b*)-thiazole-12-one (V) from 2-chlorobenzothiazole (III) and anthranilic acid (IV) has been described by Katz (4); Katz also mentioned 12-benzo(*d*)quinazo-(2,3-*b*)-oxazole-12-one (VII*a*) but did not describe

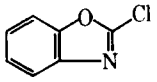


III IV

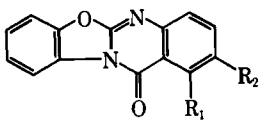


V

its preparation. More recently Sam and Plampin (5) described the preparation of VII*a* *via* condensation of 2-chlorobenzoxazole (VI) and IV. Two related substances, *viz.*, VII*b* and VII*c*, have been



VI

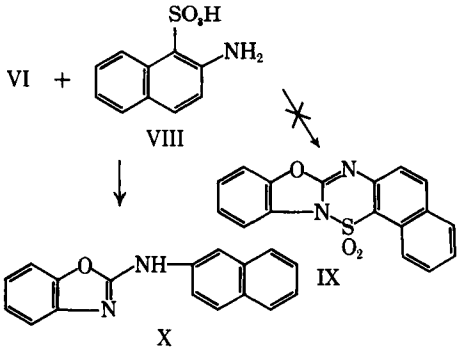


VII

a, $R_1 = R_2 = H$
b, $R_1 = CO_2H, R_2 = H$
c, $R_1 = H, R_2 = Cl$

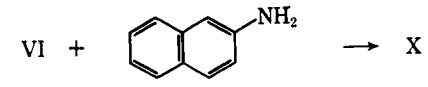
prepared from VI and an appropriately substituted anthranilic acid.

The above results prompted the investigation of the preparation of 12-benzo(*d*)naphtha(*a*)thiadiazo-(2,3-*b*)oxazole-12,12-dioxide (IX) by the condensation of VI with 2-amino-1-naphthalenesulfonic acid (VIII). However, the condensation of VI and VIII



VIII IX X

in either acidic, basic, or neutral medium resulted in either starting materials or intractable residues. The fusion of VI and VIII resulted in 2-(2-amino-naphthyl)benzoxazole (X) which was identified on the basis of physical data and unequivocal synthesis from VI and 2-naphthalamine (XI). The desulfonation inherent in this fusion reaction has not been



XI

previously observed for VIII although the related *1*-naphthalenesulfonic acid is known to undergo desulfonation under acid conditions at 140° (6).

Pharmacological Results[1]—Fasted male albino mice (25–50 g.) were used. The animals were observed closely for signs of pharmacological and toxic effects during the first 2 hr. following intraperitoneal injection. Posttreatment observations were made at 4, 6, 18, 24, and 72 hr. The vehicle for all compounds was a 10% suspension of the respective compound in a 5% acacia solution.

Compound VII*b* exhibited sedation and analgesia at 500 mg./kg. The same effects were noted at 1,000 mg./kg. and 2,000 mg./kg. in addition to ataxia, hypothermia, and opaque clouding of the eyes.

Received April 29, 1968, from the Department of Pharmaceutical Chemistry, The University of Mississippi, University, MS 38677

Accepted for publication May 20, 1968.

[1] The authors are grateful to Dr. W. M. Davis, Department of Pharmacology, School of Pharmacy, The University of Mississippi for pharmacological data.

Compound VII*c* produced writhing, sedation, and diarrhea at 1,000 mg./kg. Compound X exhibited sedation at 500 mg./kg. and 1,000 mg./kg. Sedation was apparent at 2,000 mg./kg. along with writhing and ataxia.

EXPERIMENTAL[2]

1 - Carboxy - 12 - benzo(d)quinazo(2,3 - b)oxazole-12-one (VIIb)—The procedure described by Sam and Plampin (5) for the preparation of VII*a* was followed using 21.7 g. (0.1 mole) of 3-aminophthalic acid and 15.4 g. (0.1 mole) of 2-chlorobenzoxazole. Recrystallization from acetone gave 23.8 g. (85%) of product, m.p. 293–294°; $\nu_{max.}^{KBr}$ 1725 cm.$^{-1}$ (C=O), 1650 (CO_2H); NMR (in d_2-DMSO), 7 proton multiplet at 7.43–8.43 (aromatic), 1 proton broad singlet at 13.0–13.3 (carboxyl hydrogen); upon D_2O exchange the absorption at 13.0–13.3 was absent.

Anal.—Calcd. for $C_{15}H_8N_2O_4$: C, 64.29; H, 2.88; N, 10.00. Found: C, 64.41; H, 2.96; N, 9.82.

2 - Chloro - 12 - benzo(d)quinazo(2,3-b)oxazole-12-one (VIIc)—The procedure described above was followed using 17.1 g. (0.1 mole) of 5-chloroanthranilic acid and 15.4 g. (0.1 mole) of 2-chlorobenzoxazole. Recrystallization from isoamyl alcohol gave 21.6 g. (80%) of product, m.p. 228–229°; $\nu_{max.}^{KBr}$ 1700 cm.$^{-1}$ (C=O); NMR (in d_2-DMSO) 7 proton multiplet at 7.43–8.43 (aromatic).

Anal.—Calcd. for $C_{14}H_7ClN_2O_2$: C, 62.12; H, 2.61; N, 10.35. Found: C, 62.18; H, 2.80; N, 10.38.

2-(2-Naphthylamino)benzoxazole (X)—*Method A*—A mixture of 15.4 g. (0.1 mole) of 2-chlorobenzoxazole and 23.1 g. (0.1 mole) of 2-amino-1-naphthalene-sulfonic acid was fused in a preheated oil bath at 180–190° for 3 hr. The crude product was washed with 5% aqueous NaOH, then recrystallized from cellosolve–water and subsequently from benzene to give 9.5 g. (62%) of product, m.p. 245–245.5°; $\nu_{max.}^{KBr}$ 3300 cm.$^{-1}$ (NH); NMR (in d_2-DMSO), 11 proton multiplet at 7.08–7.3 (aromatic), one proton singlet at 12.5 (NH); upon D_2O exchange the absorption at 12.5 was absent.

Anal.—Calcd. for $C_{17}H_{12}N_2O$: C, 78.44; H, 4.65; N, 10.76. Found: C, 78.45; H, 4.57; N, 10.66.

Method B—A mixture of 15.4 g. (0.1 mole) of 2-chlorobenzoxazole and 14.3 g. (0.1 mole) of 2-naphthylamine was fused in a preheated oil bath at 130–140° for 4 hr. The crude product was treated as in Method A to give 9.5 g. (62%) of product, m.p. 245–245.5°. A mixture melting point with the product from Method A showed no depression. The infrared as well as the NMR spectra were identical.

[2] Melting points were determined on a Fisher-Johns melting apparatus and are corrected. Infrared spectra were obtained on a Perkin-Elmer 137B Infracord spectrophotometer using KBr pellets. The NMR spectra were determined on a Varian A-60A spectrometer using Me_4Si as internal standard; chemical shifts are recorded as δ values.

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Keyphrases

1-Carboxy-12-benzo(*d*)quinazo(2,3-*b*)oxazole-12-one—synthesis
2-Chloro-12-benzo(*d*)quinazo(2,3-*b*)oxazole-12-one—synthesis
2-(2-Naphthylamino)benzoxazole—synthesis
Pharmacological screening
IR spectrophotometry—structure
NMR spectroscopy—structure

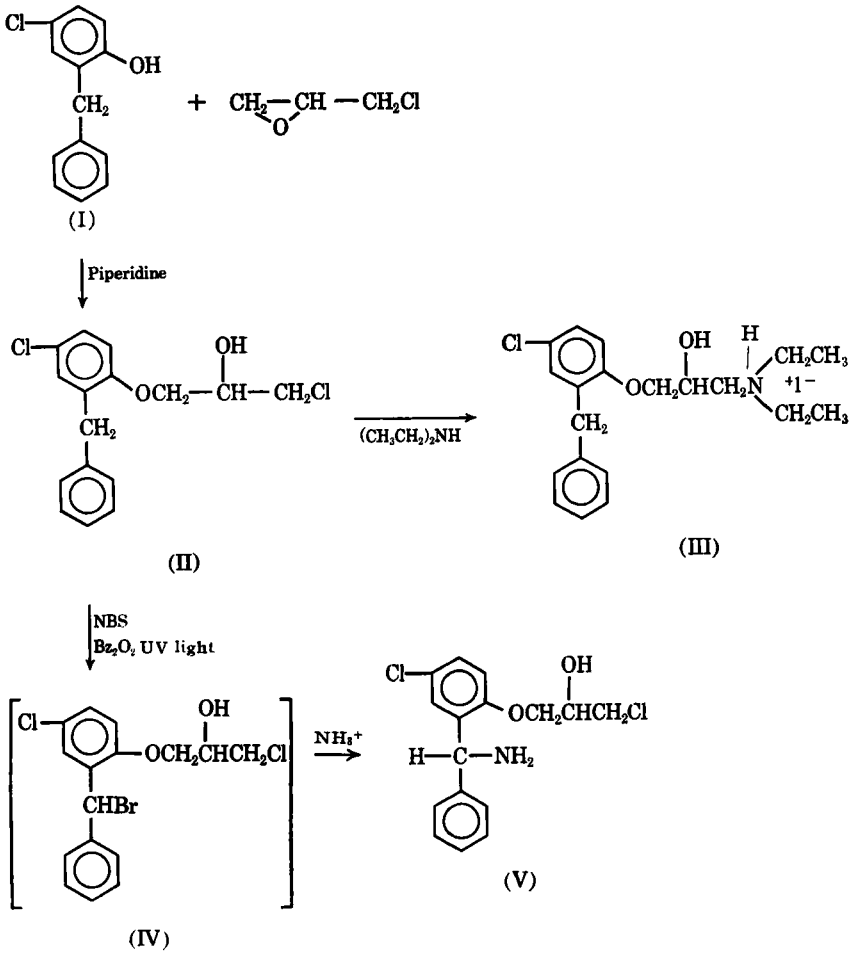
New Compounds: 5-Chloro-2-(3-chloro-2-hydroxypropoxy)-benzhydrylamine and Related Compounds

By HEINO A. LUTS*, and D. YAMASHIRO†

In connection with the chemical-medicinal work 5-chloro-2-(3-chloro-2-hydroxypropoxy)-benzhydrylamine was prepared by a three-step synthesis. Compound I was reacted with epichlorohydrin and the formed 3-(2-benzyl 4-chlorophenoxy)-2-hydroxypropylchloride (II) was then converted to III by free-radical bromination, which was not isolated, but converted to compound V by amination.

EXPERIMENTAL[1]—SCHEME I

3-(2-Benzyl-4-chlorophenoxy)-2-hydroxypropyl chloride (II)—A solution of 21.65 g. (0.100 mole) of 2-benzyl-4-chlorophenol (I) (Dow Santophen), 24.8 g. (0.269 mole) of epichlorohydrin, and 2.0 ml. of piperidine was heated at 90–95° for 4.5 hr. with stirring. Occasional cooling of the exothermic reaction was required during the initial stages. The excess epichlorohydrin was removed by distillation of all material boiling to 40°/0.7 mm. The residual oil was dissolved in an equal volume of chloroform, then shaken for 10 min. with 35 ml. of concentrated hydrochloric acid. The lower chloroform phase was separated and washed with four 30-ml. portions of water. The chloroform solution was steam distilled. The oily, nondistillable residue was extracted with 50 ml. of benzene. The benzene solution was washed with two 30-ml. portions of water, dried over anhydrous calcium sulfate, and evaporated *in vacuo* to remove the solvent. The crude oil was distilled under vacuum twice to yield 8.5 g. (27% yield) of pure 3-(2-benzyl-4-chlorophenoxy)-2-hydroxypropylchloride, b.p. 188–190°/0.6 mm.



Scheme I

Received February 28, 1968, from Horizons Inc., Cleveland, Ohio, and Bristol Laboratories Inc., Syracuse, NY 13201
Accepted for publication April 10, 1968.

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† Stanford Research Institute, Menlo Park, Calif.

[1] All melting points are uncorrected. Microanalyses were performed by G. Robertson, Jr., Florham Park, N. J.

Anal.—Calcd. for $C_{16}H_{16}Cl_2O_2$: C, 61.75; H, 5.18; Cl, 22.79. Found: C, 61.49; H, 5.13; Cl, 22.71.

5-Chloro-2-(3-chloro-2-hydroxypropoxy)benzhydrylamine Hydrochloride (V)—A solution of 44 g. (0.142 mole) of 3-(2-benzyl-4-chlorophenoxy)-2-hydroxypropyl chloride in 150 ml. of carbon tetrachloride was brought to reflux. The solution was exposed to 275-w., 110–125 v., 60-cycle AC General Electric sunlamp held at a distance of 8 in. While stirring the refluxing solution there was added 5.7 g. (0.032 mole) of *N*-bromosuccinimide and 0.31 g. (0.00128 mole) of benzoyl peroxide. The initial dark red solution disappeared in 10 min. The process was repeated three more times until a total of 22.8 g. (0.128 mole) of *N*-bromosuccinimide and 1.24 g. (0.0051 mole) of benzoyl peroxide had been reacted. An additional 1.5 hr. of refluxing under irradiation was permitted. The mixture was filtered to remove the succinimide, and the filtrate was evaporated *in vacuo* to an oil. The crude oil (IV) was dissolved in a mixture of 300 ml. of dimethylformamide and 175 ml. of concentrated ammonium hydroxide with vigorous stirring at room temperature. The solution was allowed to stand overnight and then diluted with 1,600 ml. of water. The crude mixture was extracted with two 300-ml. portions of chloroform. The combined chloroform solutions were washed with two 200-ml. portions of water, dried over anhydrous calcium sulfate, and evaporated *in vacuo* to an oil. The oil was shaken with 800 ml. of ether and 500 ml. of water. The aqueous phase was separated, made basic with 80 ml. of 10% sodium hydroxide, and extracted with two 200-ml. portions of ether. The three ether solutions were combined, washed with two 100-ml. portions of water, dried over anhydrous calcium sulfate, and saturated with dry hydrogen chloride. A total of 7.5 g. (15% yield) of crude salt was obtained. Trituration with chloroform–petroleum ether (75:100 ml.) did not improve the purity of the product, which is somewhat hygroscopic. It was dissolved in 50 ml. of chloroform, treated with charcoal, and the solution evaporated *in vacuo* to an oil. Trituration with anhydrous ether gave 4.87 g. of 5-chloro-2-(3-chloro-2-hydroxypropoxy)-benzhydrylamine hydrochloride, m.p. 97–100°.

Anal.—Calcd. for $C_{16}H_{13}Cl_3NO_2$: C, 52.98; H, 5.00; Cl, 29.33; N, 3.86. Found: C, 53.21; H, 5.24; Cl, 29.21; N, 3.71.

N,N-Diethyl-3-(2-benzyl-4-chlorophenoxy)-2-hydroxypropylamine Hydrochloride (III)—A solution of 31.1 g. (0.100 mole) of 3-(2-benzyl-4-chlorophenoxy)2-hydroxypropyl chloride in 14.6 g. (0.200 mole) of diethylamine was refluxed for 5.5 hr. with stirring. The excess of amine was removed by distillation. The oily residue was dissolved in 250 ml. of ether and extracted with two 100-ml. portions of 10% hydrochloric acid. The combined aqueous acid solutions were washed with 100 ml. of ether and then made basic with 200 ml. of 10% sodium hydroxide. The mixture was extracted with three 100-ml. portions of ether. The combined ether solutions were washed with two 50-ml. portions of water, dried over anhydrous calcium sulfate, and saturated with dry hydrogen chloride. The crude salt was collected and triturated with absolute ethanol–absolute ether (50:250 ml.) to give 16.3 g. (42% yield) of product, m.p. 128–131°. Recrystallization from methanol–ether (75:300 ml.) gave 14.4 g. of pure *N,N*-diethyl-3-(2-benzyl-4-chlorophenoxy)-2-hydroxypropylamine hydrochloride, m.p. 131–133°.

Anal.—Calcd. for $C_{20}H_{27}Cl_2NO_2$: C, 62.50; H, 7.08; Cl, 18.45; N, 3.65. Found: C, 62.53; H, 7.08; Cl, 18.48; N, 3.84.

Keyphrases

5-Chloro-2-(3-chloro-2-hydroxypropoxy)-benzhydrylamine, related compounds—syntheses

UV light—reaction irradiation

Hydrolysis of Aspirin by Rat Small Intestine

Sir:

A recent study of the effect of certain antirheumatic drugs on active amino acid transport across the small intestine revealed that the hamster small intestine is able to hydrolyze aspirin at an appreciable rate (1). This was considered to be due to the presence of esterase(s) in the intestinal epithelium which are known to occur also in all regions of the human gastrointestinal tract (2). It was suggested on this basis that appreciable hydrolysis of aspirin may take place during the absorption of this drug from the gastrointestinal tract (1). A subsequent report by Rowland and co-workers on the kinetics of aspirin disposition in man provided further evidence suggesting aspirin hydrolysis in the gut wall (3). In view of the apparent enzymic nature of the hydrolytic process it was of interest to determine its dependence on aspirin concentration.

Five-centimeter segments of everted small intestine were obtained from male Sprague-Dawley rats as described previously (1). Individual segments were placed in 50-ml. capacity conical flasks containing 15 ml. of 0–120 m*M* aspirin in Krebs-Ringer bicarbonate solution (final pH 7.4). Other segments of small intestine were first immersed in boiling water for 5 min. and then placed in aspirin solutions (controls). Flasks with drug solution but without an intestine segment were also prepared. All flasks were flushed with 95% oxygen–5% carbon dioxide mixture, sealed, and agitated mildly at 37° in a water bath. An aliquot of solution was removed after 1-hr., placed in a chilled test tube containing 1 *N* hydrochloric acid, diluted if necessary, and then extracted immediately with carbon tetrachloride and assayed colorimetrically for salicylic acid by the method of Brodie and co-workers (4).

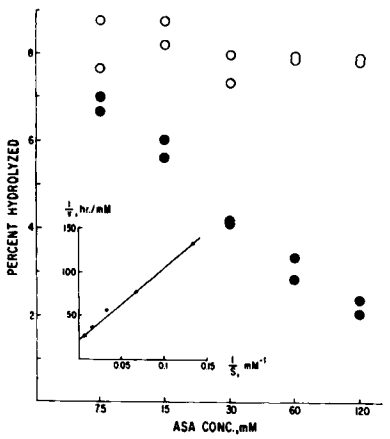

Fig. 1—Hydrolysis of aspirin by rat small intestine as a function of aspirin concentration. Key: ○, *percent hydrolyzed in 1 hr. in the presence of intestine segments inactivated by 5-min. immersion in boiling water (controls);* ●, *percent hydrolyzed in excess of control values by active intestine segments. Inset: Lineweaver-Burk-type plot of the average data.*

The hydrolysis of aspirin by everted intestine segments of rats as a function of aspirin concentration is shown in Fig. 1. The percent hydrolysis decreased markedly with increasing aspirin concentration. A double reciprocal plot of the Lineweaver-Burk-type yielded a linear relationship between the reciprocal of hydrolysis rate and the reciprocal of the initial aspirin concentration (inset of Fig. 1). The percent hydrolysis of aspirin in solutions containing boiled intestine segments was concentration independent and did not differ measurably from that in solutions without intestine.[1] These properties (saturability and heat inactivation) are characteristic of enzymic processes and suggest that aspirin hydrolysis by intestinal tissue is due to esterase(s). The presence of esterases in the human gastrointestinal tract is well established (2) and it is likely that the activity of nonspecific esterase differs in different regions of the gastrointestinal tract. The saturability of aspirin-hydrolyzing activity in the rat intestine is readily apparent at reasonable concentrations of aspirin under the conditions of the present study. It can be expected that this limited capacity of esterase activity will be reflected by a concentration and dose dependence in the extent of aspirin hydrolysis during absorption. Rapid absorption, with high concentrations of aspirin at sites of hydrolysis in the intestinal wall, is likely to result in less extensive hydrolysis of the drug than would be encountered when the same amount of drug is absorbed more slowly. Dosage-form characteristics, inasmuch as they affect the site and rate of release of aspirin in the gastrointestinal tract, may therefore be expected to influence the extent of hydrolysis of aspirin during absorption. The

[1] These control data reflect the extent of hydrolysis of aspirin during the preparation and incubation of the solutions.

magnitude of this effect in man must remain speculative pending appropriately designed clinical trials.

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Keyphrases

Aspirin hydrolysis—rat small intestine
Everted intestine—experimental technique
Hydrolysis, aspirin—concentration effect
Enzymes, intestinal—aspirin hydrolysis effect

A Cardioactive Steroidal Iodoacetate

Sir:

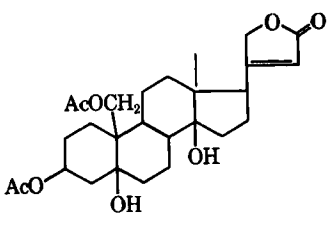
Empirical relationships between the chemical constitution and biological activity of cardiac glycosides and aglycones, *e.g.*, I, have been tabulated (1), and the unsaturated lactone at C-17 appears essential for activity. Moreover, it seems likely that these cardiotonic substances act through inhibition of enzyme systems involved in ion transport (2).

The chemical basis for the essentiality of the lactone is unknown. Portius and Repke (3) have proposed that the α,β-unsaturated carbonyl *Wirkguppierung* is a proton acceptor in hydrogen bonding, whereas Glynn (4) has postulated that addition of essential sulfhydryl groups to the unsaturated center may be involved. We have evaluated the last suggestion by the preparation of analogs.

The reaction of II with chloroacetic anhydride at 55° produced the corresponding 21-chloroacetate ester m.p. 109–111°, $[\alpha]_D$ + 51°[1] which on treatment with sodium iodide in acetone solution gave the iodoacetate III, m.p. 150–155°, $[\alpha]^{20}$ + 49°.

Biological evaluation was performed in the usual way (6) in cats under chloralose-urethan anesthesia. A volume of 1 ml. of 47.5% alcohol containing 0.4 mg. of drug was injected at 3-min. intervals into the femoral vein. The effect of the drug was observed by EKG and blood-pressure readings recorded on a Grass polygraph. The following lethal doses were determined (LD ± SE in mg./kg.): III (1.37 ± 0.26), IV (7) (15.59 ± 2.28). The high activity of III indicates that the lactone ring is not an essential feature for a cardioactive compound.

Iodoacetates are known to react with nucleophiles, such as sulfhydryl groups. Thus a reasonable explanation for the great difference in potency of the iodoacetate III relative to the acetate IV is that alkylation of an essential nucleophilic



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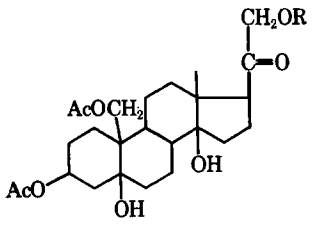

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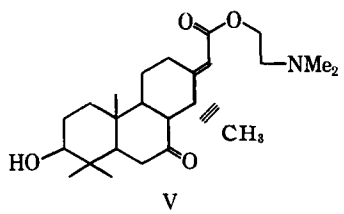

V

Strophanthidiol 3, 19-diacetate (I) (5) in ethyl acetate solution was allowed to react with ozone at −70°, and the resulting ozonide was decomposed with zinc dust in acetic acid. Isolation of the product afforded 3β, 5β, 14β, 19, 21-pentahydroxypregnan-20-one 3,19-diacetate (II).

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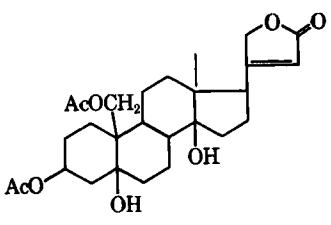

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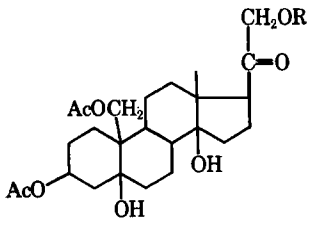

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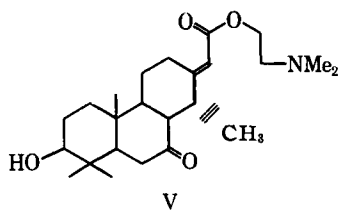

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The cardiotonic activity of cassaine V (9) (LD 1.15 mg./kg.), may be explained in a similar manner, as the α,β-unsaturated ester in this material is also a potential electrophilic reagent. In both cases, the function of the polycyclic portion of the molecule is presumably to induce an inhibitory specific conformational perturbation (10) in the enzyme. This is indicated by the known biological consequences of structural variations in this part of the molecule (1), as well as by the fact that simple unsaturated lactones (11) and simple sulfhydryl reagents (12) are dissimilar from cardiac glycosides in their effects.

The extension of these studies to other electrophilic analogs is in progress.

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Keyphrases

Steroidal iodoacetate
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Lactone rine—cardioactive compounds

Books

REVIEWS

Treatise on Analytical Chemistry. Part III: *Analytical Chemistry in Industry.* Vol. 1. Edited by I. M. KOLTHOFF, P. J. ELVING, and F. H. STROSS. Interscience Publishers, Inc., 605 Third Ave., New York, NY 10016, 1967. xxiv + 455 pp. 17 × 24.5 cm. Price $17.50.

The well-known and respected "Treatise on Analytical Chemistry" series edited by Kolthoff and Elving by now has reached encyclopedic proportions. However, when it came to Part III "Analytical Chemistry in Industry" the editors, as they state in the Preface, deferred for industrially important analytical methods and procedures to Snell and Hilton's even larger "Encyclopedia of Industrial Chemical Analysis" by the same publishers. Therefore, the first volume of Part III with F. Stross as co-editor addresses selected topics to the organization builder, the laboratory designer, the production controller, and the safety conscious among others. The pharmaceutical analyst should approach this volume forewarned that there are few specific references to the pharmaceutical industry. The student eager to explore the attractiveness of industrial employment should be told that the treatise is a "comprehensive account" but comprehensiveness is not apt to stimulate the imagination or inspire enthusiasm. Where is the analytical Watson to write "The Double Focus?"

About individual contributions the following should be briefly reported: In "Analytical Chemistry and the Analytical Chemist in Industry" Royer and Maricle are of the opinion that "analytical chemistry, without doubt, entered industry through the Control Laboratory." In "Methodology of Industrial Analysis" Spauschus makes the excellent point that "the contributions of the analytical chemist to a research program will be most effective if he is given the opportunity to participate while the program is being planned." In "Organization of Analytical Chemistry in the Industrial Research Laboratory" Kirklin makes very cogent comments on the controversial question whether to submit samples for reanalysis "openly" or "blindly." Kirklin also presents a plethora of organization charts but wisely states that "changes in details of organization are made frequently in accordance with the demands of the work." He should have added "and of problems of personality." In "Organization for Analytical Chemistry in Production Control" the same

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"Development of Raw Material and Product Specifications" should be required reading for all purchasing agents. Patek makes the memorable point that "it is almost impossible to develop a good purchase specification without the assistance of an analytical chemist." In the final chapter on "Testing of Consumer Products" by Schwartz and Gaffney the reader not only is treated to the cigarette puffing machine but also will learn that there is a gadget called the Handle-O-Meter to measure the fluffiness of towels.

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New Brunswick, N. J.

Fractional Solidification. Vol. I. Edited by M. ZIEF and W. R. WILCOX. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10019 1967. xvi + 714 pp. 16 × 23.5 cm. Price $28.75.

Fractional Solidification is the first of two volumes. Twenty-two authors from England and the United States have contributed to the text. The contributions of Paul Jannke are of special interest to the pharmaceutical scientist. Each chapter is well documented with references—a majority being recent.

The book is divided into six parts. Part I is devoted to basic principles with contributions dealing with phase diagrams, mass transfer in fractional solidification, constitutional supercooling and microsegregation, polyphase solidification, and heat transfer in fractional solidification. Part II deals with laboratory scale apparatus used in fractional solidification with specifics that deal with batch zone and continuous zone melting, progressive freezing and column crystallization, and zone precipitation and allied techniques. Part III deals with industrial scale equipment. Different authors discuss the Proabd Refiner, Newton Chambers' Process, Rotary-Drum Techniques, Phillips Fractional-Solidification Process, and desalination by freezing. Part IV deals with applications of fractional solidification. Ultrapurification and its relation to pharmaceuticals are discussed by Jannke *et al.* Chapters are devoted to ultrapurity in crystal growth and to bulk purification. Part V is devoted to the economics of fractional solidification, and Part VI contains tables listing the purification and operating parameters for zone melting of inorganic and organic compounds. A great many of the organic compounds are important pharmaceuticals.

This book is of value because the basic knowledge and methods used to produce chemicals of ultrapurity are presented. The processes required to produce ultrapurity are examined in detail. The material should be valuable to those in pharmaceutical and other industries in order to develop and maintain strict specifications on raw materials. This book should prove to be valuable to the researcher in producing ultra-pure crystals in either very small or large scale batches.

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Take as Directed. Edited by F. E. SHIDEMAN and written by JOHN P. RUSSO. The Chemical Rubber Company Press, 18901 Cranwood Parkway, Cleveland, OH 44128, 1967. xiv + 457 pp. 16 × 23.5 cm. Price $14.75.

The editor has fulfilled the objective given on the book cover, "Our modern medicines explained for the layman." Diseases and physiological conditions have been described clearly in terms the layman will understand. The fundamental information given about the drugs is presented in a sound, reasonable way that is a pleasant contrast to the dramatic presentations that frequently are offered to the lay public. This book is an excellent, simplified discussion of drugs and their use in medical treatment today. Pharmacists and pharmaceutical scientists can confidently suggest this book in response to requests from laymen to recommend a simplified—yet authoritative—text discussing drugs in current use.

Staff review

Aromatic Amine Oxides. By EIJI OCHIAI. Translated by Dorothy U. Mizoguchi. Elsevier Publishing Company, 52 Vanderbilt Ave., New York, NY 10017, 1967. ix + 456 pp. 15 × 23 cm. Price $30.00.

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Reviewed by K. Florey
Squibb Institute for Medical Research
New Brunswick, N. J.

Fractional Solidification. Vol. I. Edited by M. ZIEF and W. R. WILCOX. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10019 1967. xvi + 714 pp. 16 × 23.5 cm. Price $28.75.

Fractional Solidification is the first of two volumes. Twenty-two authors from England and the United States have contributed to the text. The contributions of Paul Jannke are of special interest to the pharmaceutical scientist. Each chapter is well documented with references—a majority being recent.

The book is divided into six parts. Part I is devoted to basic principles with contributions dealing with phase diagrams, mass transfer in fractional solidification, constitutional supercooling and microsegregation, polyphase solidification, and heat transfer in fractional solidification. Part II deals with laboratory scale apparatus used in fractional solidification with specifics that deal with batch zone and continuous zone melting, progressive freezing and column crystallization, and zone precipitation and allied techniques. Part III deals with industrial scale equipment. Different authors discuss the Proabd Refiner, Newton Chambers' Process, Rotary-Drum Techniques, Phillips Fractional-Solidification Process, and desalination by freezing. Part IV deals with applications of fractional solidification. Ultrapurification and its relation to pharmaceuticals are discussed by Jannke *et al.* Chapters are devoted to ultrapurity in crystal growth and to bulk purification. Part V is devoted to the economics of fractional solidification, and Part VI contains tables listing the purification and operating parameters for zone melting of inorganic and organic compounds. A great many of the organic compounds are important pharmaceuticals.

This book is of value because the basic knowledge and methods used to produce chemicals of ultrapurity are presented. The processes required to produce ultrapurity are examined in detail. The material should be valuable to those in pharmaceutical and other industries in order to develop and maintain strict specifications on raw materials. This book should prove to be valuable to the researcher in producing ultra-pure crystals in either very small or large scale batches.

The book is printed on paper which provides easy reading, and the print is of adequate size. The authors have used many figures to illustrate their concepts and discussions.

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Reviewed by Frederick C. Bach
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Fort Worth, Texas

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Emulsion Science is divided into five sections or chapters as follows: (1) Principles of Emulsion Formation, by E. S. R. Gopal; (2) The Theory of Stability of Emulsions, by J. A. Kitchener and P. R. Mussellwhite; (3) General Properties of Emulsions and their Constituents, by Philip Sherman; (4) Rheology of Emulsions, by Philip Sherman; (5) Electrical Properties of Emulsions, by Tetsuya Hanai.

The author-editor has done an excellent job in correlating and unifying the contributions of the other authors. The text is well organized and comprehensive with good continuity and a minimum of overlap. The first three sections are primarily descriptive, but sufficiently quantitative to give adequate depth to the subjects covered. While some differences in style and organization may be expected, only a minimum is actually encountered. For example, demulsification is discussed in Section 1 while Section 2 is devoted entirely to emulsion stability. Section 1 includes a discussion on choice of emulsifying agents while Section 3 includes a detailed discussion of the HLB system. Careful attention has been given to minimizing repetition in such instances.

The first three sections cover about 217 pages of the text, while the latter two sections are more theoretical and quantitative, occupying about 260 pages of the text. The section on rheology is rather unique in its approach to rheological properties. Much of the discussion is based on structural effects within the emulsion, not merely on measurement data.

Each section of the text is well documented and contains sufficient breadth and depth to maintain the interest of a broad cross-section of readers.

Reviewed by Robert V. Petersen
College of Pharmacy
The University of Utah
Salt Lake City, Utah

Drugs Affecting the Central Nervous System. Vol. 2 of the *Medicinal Research Series.* Edited by ALFRED BURGER. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1968. xv + 437 pp. 16 × 24 cm. Price $19.75.

This second volume of a series continues the structural approach to drugs affecting the central nervous system. Eight chapters cover the general topics of narcosis, morphine-like analgesics, antidepressants, psychotomimetic agents, 1,4-benzodiazepine derivatives, centrally acting muscle relaxants, and substituted phenothiazines. The chapter on psychotomimetic agents by Albert Hofmann is of considerable interest. It covers not only his personal discovery of the effects of LSD, but is an excellent review of the naturally occurring psychomimetic drugs. As would be expected, the relationship between chemical structure and clinical action, value, or use is not a major contribution of this volume. There is evidence that the familiar nonagreements as to the meaning of words such as tranquilizer, relaxant, antidepressant are present between the various authors. This, however, should not detract from the main value of the series: the presentation in one place of large numbers of chemically related compounds with many of their comparative pharmacologic effects. The chapter on morphine-like analgesics is outstanding. The chemical anatomy of the many compounds is clearly described. There is a precise definition of the meaning of "morphine-like action." There is no attempt made to describe the morphine-like compounds clinically. This volume, like the first, is recommended for all serious students of pharmacology and pharmaceutical chemistry.

Reviewed by R. P. Ahlquist
Medical College of Georgia
Augusta, Ga.

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Despite these shortcomings, this book does provide an assemblage of information which should be useful to teachers and other workers in this field.

Reviewed by Manford J. Robinson
Research and Development Division
Smith Kline & French Laboratories
Philadelphia, Pa.

York, NY 10003, 1968. 16 × 23.5 cm. x + 496 pp. Price 115s $21.00.

Emulsion Science is divided into five sections or chapters as follows: (1) Principles of Emulsion Formation, by E. S. R. Gopal; (2) The Theory of Stability of Emulsions, by J. A. Kitchener and P. R. Mussellwhite; (3) General Properties of Emulsions and their Constituents, by Philip Sherman; (4) Rheology of Emulsions, by Philip Sherman; (5) Electrical Properties of Emulsions, by Tetsuya Hanai.

The author-editor has done an excellent job in correlating and unifying the contributions of the other authors. The text is well organized and comprehensive with good continuity and a minimum of overlap. The first three sections are primarily descriptive, but sufficiently quantitative to give adequate depth to the subjects covered. While some differences in style and organization may be expected, only a minimum is actually encountered. For example, demulsification is discussed in Section 1 while Section 2 is devoted entirely to emulsion stability. Section 1 includes a discussion on choice of emulsifying agents while Section 3 includes a detailed discussion of the HLB system. Careful attention has been given to minimizing repetition in such instances.

The first three sections cover about 217 pages of the text, while the latter two sections are more theoretical and quantitative, occupying about 260 pages of the text. The section on rheology is rather unique in its approach to rheological properties. Much of the discussion is based on structural effects within the emulsion, not merely on measurement data.

Each section of the text is well documented and contains sufficient breadth and depth to maintain the interest of a broad cross-section of readers.

Reviewed by Robert V. Petersen
College of Pharmacy
The University of Utah
Salt Lake City, Utah

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JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Council

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JANUARY TO DECEMBER, 1968

JOURNAL OF Pharmaceutical Sciences

September 1968 volume 57, number 9

Review Article

Sulfonamides, Structure-Activity Relationship, and Mode of Action

Structural Problems of the Antibacterial Action of 4-Aminobenzoic Acid (PABA) Antagonists

By JOACHIM K. SEYDEL

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Structure Specificity of the Activity of PABA and PAS 1457
Structure Specificity of the Activity of SNS 1457
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THIS ARTICLE attempts to review the current knowledge of sulfonamides (SNS),[1] their structure-activity relations, and the mode of action involved. The problem of the mode of action and the relationship between structure and biological and antibacterial action of SNS, which was detected in 1933, are still not completely elucidated. Therefore, it would appear necessary to discuss all important experimental results concerning these problems. However, this is impossible considering that Northey (1) (review, 1948) required 200 pages to discuss the results that were available. Hundreds of publications about the synthesis of new compounds, their chemotherapeutic activity, their biological properties, and their kinetic and enzymatic approaches have been published since then to give an insight into the mode of action. This demonstrates the importance of SNS, which have revolutionized the treatment of bacterial infections. Only some of the important facts can be discussed in this paper, which includes a subjective selection.

There are several statements of many authors concerning the problem of structure-activity rela-

Received from the Borstel Research Institute, Institute for Experimental Biology and Medicine, 2061 Borstel, Hamburg, West Germany.

[1] In this review SNS is used as abbreviation for N^1-substituted sulfanilamides.

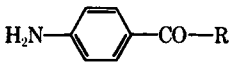
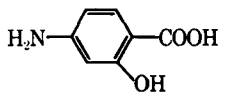
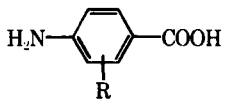
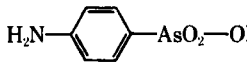
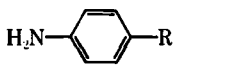
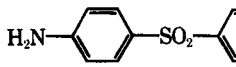
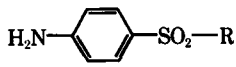
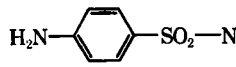
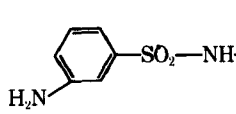
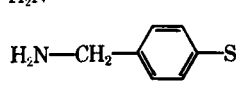
tionships of different chemotherapeutics, but among these statements, none has general validity. Definite results and answers to this question can be expected only if one restricts his attention to a very closely related group of chemical substances. SNS belong to such a group and have been studied in great detail. Setting up an extensive theory of the mode of action of chemotherapeutic agents is not only an academic problem, it should also give quantitative meaning to the chemical, physicochemical, and biological properties of the compound for the therapeutic effects. This can serve not only as a basis for the development of new drugs, but also for the evaluation of different drugs. The increasing difficulties in clinical screening tests (2) necessitate exact experimental standards.

The mode of action of chemotherapeutics, including all chemical, physicochemical, and biochemical reactions involved, can be divided into the pharmacodynamic, toxicological, and the antibacterial mode of action. The antibacterial mode of action consists of (*a*) the transport mechanism to the place of bacterial infection (pharmacokinetic) and through the bacterial membrane and (*b*) the chemical reaction which causes bacterial death or inhibition of growth. The reaction may induce sequential biochemical reactions in bacterial metabolism.

The classification of chemotherapeutics by their mode of action can be based only on the chemistry of the chemical reactions involved in the antibacterial action. As long as the chemistry of the responsible reaction is not clarified, the classification of the chemotherapeutic is doubtful. What are the criteria which decide if an antibacterial compound belongs to the group of SNS? There are substituted SNS, the substituents of which have an antibacterial activity by themselves, for instance sulfanilthiocarbamide (3), some sulfathiazoles, sulfathiadiazoles, and sulfapyridine (4–7), sulfa-3,5-dibromobenzene (8), and some sulfapyrimidine derivatives (9, 10).

If the antibacterial activity of the substituents gets the upper hand, the compound does not belong to the group of SNS with respect to the mode of action. Two criteria which are not identical can be used for classification: the

TABLE I—CLASSIFICATION OF 4-AMINOBENZOIC ACID ANTAGONISTS AND OTHER SULFONAMIDES WITH ANTIBACTERIAL ACTIVITY

4-Aminobenzoic acid antagonists (4-substituted aniline derivatives)		4-Aminobenzoyl[a] derivatives
		4-Aminosalicylic acid[a]
		Other ring substituted[a] 4-aminobenzoic acid derivatives
		4-Aminophenylarsonic acid
		Other 4-substituted aniline derivatives, not sulfone derivatives
		Sulfanilic anilines ("sulfones," DDS derivatives)[b]
		Other sulfanilic acid derivatives (not sulfonamides)[b]
		*p*-Sulfanilamides[b,c]
Other sulfonamides with antibacterial action, which are not 4-aminobenzoic acid antagonists		*m*-Sulfanilamides[c]
		4-(Amino-methyl)[c] benzol sulfonamide (homosulfanilamide)

[a] 4-Aminobenzoic acid derivatives. [b] 4-Sulfanilic acid derivatives. [c] Benzolsulfonamides. [Reproduced with permission from *Jahresber. Borstel*, **5**, 651(1961).]

antagonism of PABA against the antibacterial activity of SNS (11, 12) and the cross-resistance against sulfanilamide (13). By testing the cross-resistance of sulfanilamide and PAS resistant strains, it is possible to obtain a differentiation of the group of PABA antagonists (Table I).

CHEMICAL STRUCTURE AS REASON FOR BIOLOGICAL ACTION

Structure Specificity of the Activity of PABA and PAS—If the antagonistic action of PABA is used as a criterion for the activity of one group of compounds, knowledge is needed of the structure specificity of the action. In the following the rules of Johnson, Green, and Pauli (14), Zetterberg (15), Hossack (16), and others—completed with some additional facts—are given.

1. The antagonistic action against SNS is afforded by the amino group in the 4 position of the carboxyl group (17, 18).

2. Exchange of the amino group for other substituents with the exception of the nitro or acetyl amino group results in ineffective compounds (18).

3. Exchange of the benzene ring for other ring systems causes loss of activity (19).

4. Monosubstitution in the 3 or 2 position of the PABA molecule by halogen, alkyl, and alkoxy substituents results either in bacteriostatic compounds (20–22) or compounds with antagonistic action against SNS. There are completely different statements about the activity of these compounds in the literature; PAS belongs in this group of compounds.

5. Variation in the carboxyl group by exchange or substitution results in compounds which may possess either antagonistic properties (local anesthetics) (23–25), antibacterial, or no activity (26–30).

6. Simultaneous variation at the amino group and substitution at the benzene ring results in inactive compounds.

As previously stated, among the PABA derivatives are compounds which may act as antagonists against SNS, others which have antibacterial properties, and others which are inactive. Some of these compounds are judged differently by different authors, using different concentrations of the drug or different strains. These results are explainable according to Ariens *et al.* (31, 32) by two independent qualities of PABA, PABA derivatives, and SNS, the so-called "affinity" (reciprocal of the dissociation constant of the compound-receptor complex), and the "intrinsic" activity (metabolic activity).

Structure Specificity of the Activity of SNS—Northey (1) developed the following rules about the structure specificity of SNS. These rules are based on a tremendous mass of literature available from several large chemical companies and have been completed by the addition of some new results. The sequence used is the same which is used for PABA and its derivatives.

1. SNS antagonized by PABA possess an amino group in the 4 position of the sulfone group.

2. Exchange of the amino group by H—, HO—, RO—, HOOC—, $H_2N—SO_2—$, alkyl, or halogen substituents causes loss of activity or results in compounds which are not antagonized by PABA (homosulfanilamide). N^4-derivatives are active if the free amino group is obtainable either hydrolytically or enzymatically.

3. Exchange of the benzene ring for other ring systems decreases the activity of SNS.

4. SNS substituted in the benzene ring are mostly inactive; however, there are exceptions (33, 34).

5. Exchange of the SO_2NH group for NH_2—, —CN, $—SO_3H$ (see, however, 35), $—AsO_3H_2$, —NH—CO—CH_3, or $—NO_2$ results in inactive compounds. The activity maintains—in some cases, however, it decreases—if the sulfonamide group is exchanged for $—SO_2H$, $—SO_2C_6H_4—R$, $—SO—C_6H_4—NH_2$, —S—S—$C_6H_4—NH_2$, —SO —SO—$C_6H_4—NH_2$, $—S—(CH_3)_2$, —SO—CH_2 —$C_6H_4—NH_2$, —CO—NH_2, $—CO—C_6H_4—R$, or $—PO(OH)_2$ (28, 36–38).

Monosubstitution in the N^1 position results in essentially more active compounds as compared to the action of sulfanilamide. The most active compounds were obtained with substituents belonging to a heterocyclic system. Acylderivatives at the N^1-nitrogen atom are active in most cases (39). The rule that disubstitution at the N^1-nitrogen decreases the activity (40, 41) corresponds to the hypothesis of Bell and Roblin (41, 42). However there are some exceptions (41, 43–45). One of the most active SNS *in vitro* is sulfanil-(3,4-dimethyl-isoxazole-5-)-acetamide (see Table II).

6. Simultaneous variation at the amino and sulfonamido group performing Assumptions 2 and 5 results in *in vivo* active compounds.

Table I shows some chemotherapeutics and their classification using the given rules. The structure specificity of the antibacterial action of SNS is not easily seen; the same holds true for the structure specificity of the antagonistic action of PABA and its derivatives. The only rule that can be accepted with certainty is that the benzene

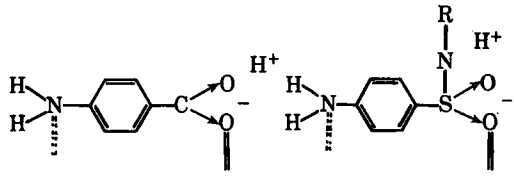
TABLE II—MINIMUM INHIBITION CONCENTRATION (MIC) OF SOME SULFONAMIDES AND THEIR N^1-ACETYL DERIVATIVES

Substance	pKa₂′	MHK *E. coli* μmole/l.
2-Sulfanilamido-pyridine	8.4	8
N^1-Acetyl-2-sulfanilamido-pyridine	—	4
2-Sulfanilamido-5-methyl-pyrimidine	6.8	1
N^1-Acetyl-2-sulfanilamido-5-methyl-pyrimidine	—	1
2-Sulfanilamido-5-ethyl-pyrimidine	6.7	1
N^1-Acetyl-2-sulfanilamido-5-ethyl-pyrimidine	—	1
2-Sulfanilamido-5-isopropyl-pyrimidine	6.7	1
N^1-Acetyl-2-sulfanilamido-5-isopropyl-pyrimidine	—	1
2-Sulfanilamido-5-methoxy-pyrimidine	6.5	2
N^1-Acetyl-2-sulfanilamido-5-methoxy-pyrimidine	—	1
2-Sulfanilamido-3-methoxy-pyrazine	6.1	2
N^1-Acetyl-2-sulfanilamido-3-methoxy-pyrazine	—	0.5
4-Sulfanilamido-2,6-dimethoxy-pyrimidine	5.9	1
N^1-Acetyl-4-sulfanilamido-2,6-dimethoxy-pyrimidine	—	1
4-Sulfanilamido-5,6-dimethoxy-pyrimidine	5.83	1
N^1-Acetyl-4-sulfanilamido-5,6-dimethoxy-pyrimidine	—	1
3-Sulfanilamido-5-methyl-isoxazole	5.7	1
N^1-Acetyl-3-sulfanilamido-5-methyl-isoxazole	—	1
5-Sulfanilamido-3,4-dimethyl-isoxazole	4.9	2
N^1-Acetyl-5-sulfanilamido-3,4-dimethyl-isoxazole	—	0.5
5-Sulfanilamido-1-phenyl-pyrazole	5.8	1
N^1-Acetyl-5-sulfanilamido-1-phenyl-pyrazole	—	0.5

[Reproduced with permission from *Arzneimittel-Forsch.*, 16, 1447(1966).]

ring and the primary aromatic amino group are essential for the biological action. The same is valid for some other PABA antagonists as for instance 4-aminobenzene-arsenic-acid (27, 46), diaminobenzil (36). Obviously the aromatic amino group in the 4 position in SNS is an important or decisive factor for the antibacterial action. Other conclusions are uncertain, for instance, the necessity of an electronegatively charged substituent (but not ionized) in the *para*-position of the amino group, and the distance of the charge from the amino group must be the same as that of the amino group in PABA (41).

There is no doubt that the aromatic amino group of PABA antagonists is involved in a chemical reaction at the site of action (35). Until now the assumption was made that sulfanilamide and PABA have the same partner in reaction. It has been reported that a twofold binding according to Scheme I takes place:



Scheme I

This picture of the geometrical similarity of PABA and sulfonamide molecules obviously was influenced by the ideas of Bell and Roblin (41), see however Alléaume (47, 48).

There is no experimental proof for such a twofold binding. It is better not to call the amino group "haptophore" according to Ehrlich (49) or "toxophile" according to Albert (50), but preferably it should be called the reactive group of the molecule. An explanation will be attempted for the different antibacterial activities of SNS by the different reactivities of their primary aromatic amino groups. A criterion for the reactivity is the "affinity constant" according to Ariens (31). The affinity constant of the amino group in a certain chemical reaction is influenced by intramolecular forces of the molecule. Therefore, it is not necessary to look for a special name for the rest of the molecule as long as the mode of action is discussed. Another question is the meaning of the rest of the molecule for transport mechanism (see protein binding, lipid partition, and ionization).

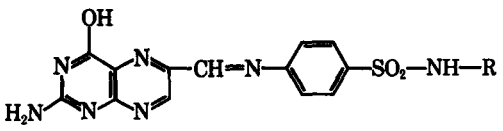
If the structure specificity of SNS is considered from this point of view it is unlikely that structural formulas written in the normal one-dimensional form can contribute to the solution of the question. Even a qualitative grouping of substituents in electropositive and electronegative properties is not sufficient (50). Physical and chemical parameters are needed which describe the condition of the amino group under the influence of certain substituents, for instance, σ-functions according to Hammett (51), rate constants according to Hirsch (52), Kohn and Harris (53), Seydel (43, 54), Brown *et al.* (55), Garrett (56), or optical data for electronic distribution from UV spectra (57–59), IR and NMR spectra (43, 44, 60–62), crystal optical measurements (47), and LCAO calculations (63–67). In considering this question, only a few of Northey's (1) critically discussed mode of action "theories" are of interest. A hypothesis may keep its value if the above-mentioned criteria about the membership of a compound to the group of SNS are considered and correct assumptions are made about the reaction mechanism.

Independent from the metabolic reactions in which SNS may be involved, the question of whether there are any physical or chemical properties of SNS which correlate with the observed antibacterial activity *in vitro* will be discussed. The problem is answered if the minimum inhibitory concentration (MIC) can be calculated by physical or chemical parameters without exception. Bell and Roblin (41) have tried to solve this problem.

CHEMICAL REACTIVITY OF THE PABA ANTAGONISTS

First, some hypotheses and experimental results concerning the reactivity of the primary aromatic amino group will be discussed. In contradiction to the statement of Bell and Roblin (41), there exists a different chemical reactivity of the primary aromatic amino group of different SNS. This can be expressed in reaction rates, pKa values, or electron-density measurements.

Tschesche (68) has developed a hypothesis that SNS are competing with PABA by forming a Schiff base with a pteridinealdehyde. The degree of displacement is a function of concentration; the involved reaction was not considered to be enzymatic but chemical, delivering the following compound:



This compound was considered to be insoluble, thus crystallizing and accumulating within the bacterial cells. In 1950 Tschesche (69) was able to show, that 2-amino-6,9-dioxy-pteridinealdehyde (8) and other pteridines can antagonize the inhibition caused by sulfathiazole. The hydrolysis of the Schiff base is very slow according to Tschesche. From experiments of Wacker, Trebst, and Simon (70) with radioactive SNS, it was not possible to make certain that a Schiff base of that type was formed. This does not mean, however, that the reaction postulated by Tschesche is not possible. It is very likely that under the conditions of the wash process used by Wacker *et al.* (70) a Schiff base could be hydrolyzed. That is even more likely because the action of SNS is considered to be of the bacteriostatic type, which means the bacteria can be liberated from the drug by washing the cells. If the hypothesis is correct, then the sequence of the reactivity of SNS with the pteridinealdehyde should be the same as the sequence of their minimum inhibitory concentration *in vitro*.

A very similar hypothesis was formulated by O'Meara, McNally, and Nelson (71), Bell, Cocker, and O'Meara (72, 73), and Forrest and Walker (74, 75) based on the assumption that SNS was reacting with reductone—an assumed precursor in pteridinealdehyde synthesis—to form a Schiff base. Forrest and Walker (75) were able to support the assumption that the Schiff base from reductone and PABA is a precursor of folic acid by synthesizing pteridine carboxylic acid from 2,4,5-triamino-6-hydroxypyrimidine and reductone. Bell (72) suggested that this reaction was not only blocking folic acid synthesis but also several other reactions where reductone was involved as metabolic compound.

According to O'Meara *et al.* (71) the chemotherapeutic activity of SNS and its susceptibility to the antagonistic action of PABA runs parallel with the stability of the Schiff base formed with glucoreductone. Only qualitative data about the stability are given including a statement that the Schiff base of sulfathiazole is about two times more stable than the Schiff base of PABA.

Bell *et al.* (73), however, obtained the opposite result. They heated a mixture of the Schiff base of sulfathiazole and reductone in sodium acetate with PABA. After 5 min. they obtained crystals which were identified as the Schiff base of PABA and reductone. This result was taken as evidence that SNS can be displaced in Schiff base by PABA in the physiological pH range and therefore would be a chemical explanation of the antagonism. However, there are some objections against this experiment. Reductones were easily oxidized, no exclusion of nitrogen was reported, and the reaction was carried out in heterogeneous mixture. Therefore, the results obtained not only include stability but also solubility constants. The solubility of the Schiff base is very low and differs with different SNS components.

Schiff base formation proceeds in a step reaction, mainly catalyzed by hydroxonium and other oxonium ions (76–78). It is a complex reaction mechanism strongly dependent on pH and changing type of mechanism as a function of pH (79). Therefore, it is more convenient to follow the rate of hydrolysis. Rate constants from such kinetic studies of the hydrolysis of Schiff bases from PABA and SNS with 3-methylreductone were obtained by Seydel *et al.* (44, 54). The sequence of rate constants is similar to the semiquantitative data of O'Meara *et al.* (71); an example is given in Fig. 1. For all studied compounds a minimum of hydrolysis was observed

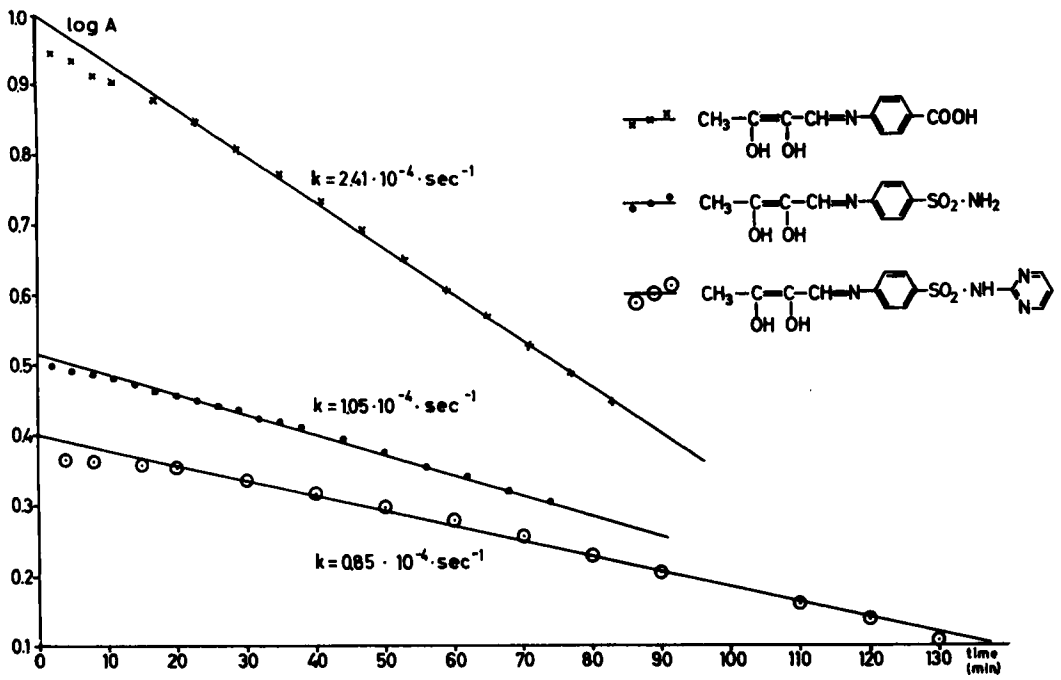


Fig. 1—Apparent first-order plots of Schiff base solvolysis of some sulfonamides, 40°, pH 6.0 phosphate buffer.

between pH 5 and 7. Most of the Schiff bases of SNS studied were more stable than the Schiff base of PABA, but this does not mean that a chemical antagonism of PABA and SNS is not possible. The PABA-reductone compound may be metabolized under *in vivo* conditions thus changing the equilibrium of hydrolysis in favor of the formation of the Schiff base. The antagonism of PABA and SNS may have a metabolic kinetic character in contradiction to the assumption of a more static equilibrium (31, 80, 81). It was not possible, however, to isolate reductone from bacteria (82), and it is known from studies of Brown (83) and Jaenicke and Chan (84) that the biosynthesis of folic acid does not include Schiff base formation, followed by hydration of the double bond. Even *in vivo*, it was not possible to hydrate the double bond of the Schiff base (85). Whatever the reliability of these results may be—with respect to the mode of action—they show that the amino groups of SNS are the reactive groups and that there is a differentiation of the reactivity by the structure of the rest of the molecule. The inductive or conjugative effect of different substituents on the N^1-nitrogen atom of SNS on the primary amino group is shown by some other parameters. Jacobson (86) has studied the effect of substrate structure on the activity of pigeon liver acetyl transferase and compared the effectiveness of several aniline derivatives as acetyl acceptors and donators. The rate of acetyl transfer was seen to decrease as the electronegativity of the *para* substituent decreased; the relative rate of the acceptors was vice versa. Among the compounds studied were sulfanilamide and PABA, with a rate of 6:1 (see Table III).

IONIZATION AND DIPOLE

Ionization of the Amino Group—Although not all of the bacteriostatic acting PABA antagonists possess an ionizing group with a negative charge (—SO_2—NH—), all of them have a primary amino group with a basic character. Tolstoouhov (87) reported that the basic pK_a' values of all active sulfonamides are in the range

Table III—Comparison of Various Derivatives as Acetyl Acceptors[a]

Acetyl Acceptor	Relative Rate
Aniline	0.70
p-Methylaniline	1.00
p-Chloroaniline	1.09
p-Bromoaniline	1.12
p-Nitroaniline	0.34
p-Aminohippuric acid	0.25
Sulfanilamide	0.18
p-Aminosalicylic acid	0.15
p-Aminobenzoic acid	0.03
Sulfanilic acid	0.01

[a] Reaction conditions were as follows: 0.1 *M* Tris pH 8.1; 0.01 *M* EDTA pH 8; 0.03 *M* cysteine HCl(neutralized); 5×10^{-5} *M* acetyl-AABS, A-60 enzyme; and 1×10^{-4} *M* acetyl acceptor amine and temperature 29°. Reaction was followed at 460 mμ. [Reproduced with permission from *J. Biol. Chem.*, **236**, 343 (1961).]

of 1.64–2.34, the most active compounds having a basic pKa_1' in the narrow range of 1.91–2.34. Most emphasis was put on the different pKa_1' values of *p*-, *m*-, and *o*-sulfanilamide (pKa_1' 2.16, 2.89, 1.80), which would explain quite simply the differences in therapeutic activity. The best correlation to activity was obtained by $pH_i = (pKa_1 + pKa_2)/2$.

The earlier results of Bell and Roblin (41), obtained with a larger number of SNS, do not confirm the results of Tolstoouhov (87). This however may be due to insufficient data. In an excellent paper Yoshioka (88) reported the basic pKa_1' values of a homologous series of N^1-arylsulfanilamides. The same compounds were used by Seydel (45, 54, 62) for structure-activity studies. Thus, it was possible to correlate the basic pKa_1' values reported by Yoshioka (88) with the minimum inhibition concentrations reported by Seydel. Figure 2 gives the relationship between those parameters. There is an approximate linear relationship between the MIC and the basic pKa_1' of the N^1-arylsulfonamides. The pKa_1' values of the homologous series of compounds were found to have a linear relationship to the σ-Hammett values of the substituents:

$$\log K_{NH_3}{}^{+} = 0.212\ (\sigma + 0.48\Delta\sigma^{-}R) - 1.815$$

The transmission of the additional resonance effect was considered to be of the cross-conjugation type rather than by a function of —NH—SO_2— like —C=C— (66, 88).

Ionization of the Sulfonamido Group—Bell and Roblin (41, 42) were the first to look for an explanation for the different activity of SNS. The theory of Bell and Roblin published in 1942 is the most important and stimulating one for investigations of structure-activity correlations of SNS. The theory was modified and differentiated by Cowles (89), Fox and Rose (90), Schmelkes *et al.* (91), Brueckner (92), and Klotz (93). The authors stated a functional connection between the acid dissociation constant (pKa_2') and the MIC of SNS. Since then this connection has not been questioned (Fig. 3).

Different hypotheses were developed to explain the observed relation between the acid pKa_2' values and the *in vitro* MIC of SNS. Attention was paid to a possible connection between pKa_2' and the MIC of SNS by the observation of the pH dependency of the inhibitory activity (94) and the antagonizing effect of PABA.

Fox and Rose (90) discovered that the ratio of the MIC of sulfanilamide, sulfathiazole, and sulfapyridine to the antagonistic concentration of PABA varied in the range of 5,000:1–8:1.

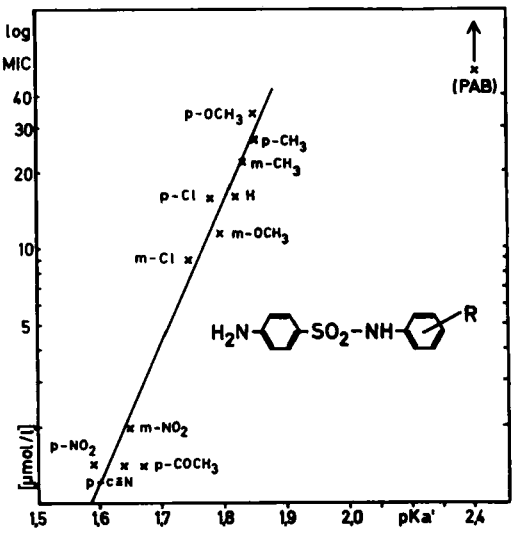


Fig. 2—Correlation between the basic dissociation constant (pKa_1) (88) of some N^1-benzenesulfonamides and their minimum inhibition concentration (MIC) against E. coli.

However, if only the ionized fraction at pH 7 was considered instead of the total concentration, the scattering of the ratio was limited to 1.4:1 up to 6.4:1. The conclusion was derived that only the ionized fraction of the MIC is responsible for the antibacterial action. Schmelkes *et al.* (91) reported that the MIC of sulfanilamide decreased with increasing pH of the culture medium, whereas the activity of sulfadiazine decreased with increasing pH. Cowles (89) considered only the negatively charged form of SNS as active and only the neutral form as able to penetrate the bacterial cell wall. Brueckner (92) differentiated this hypothesis by the assumption of different intra- and extracellular pH values. The results of his experiments only indicate that the molecular fraction of a sulfonamide solution may play an important role in determining the intracellular concentration of the active fraction and that the influence of pH must have a more complex basis than a simple change in the concentration of SNS ions in culture media. To interpret these results, knowledge is needed of the intracellular pH of the bacterial cell and its change as a function of extracellular pH.

Bell and Roblin (41, 42) explained the dependency between pKa_2' and the MIC by the assumption that "the more negative the SO_2 group of N^1-substituted sulfonamide derivatives the more bacteriostatic the compound will be." The acid dissociation constants were shown to furnish an indirect measure of the negative character of the SO_2 group. The hypothesis of Bell and Roblin stated that the unionized molecules

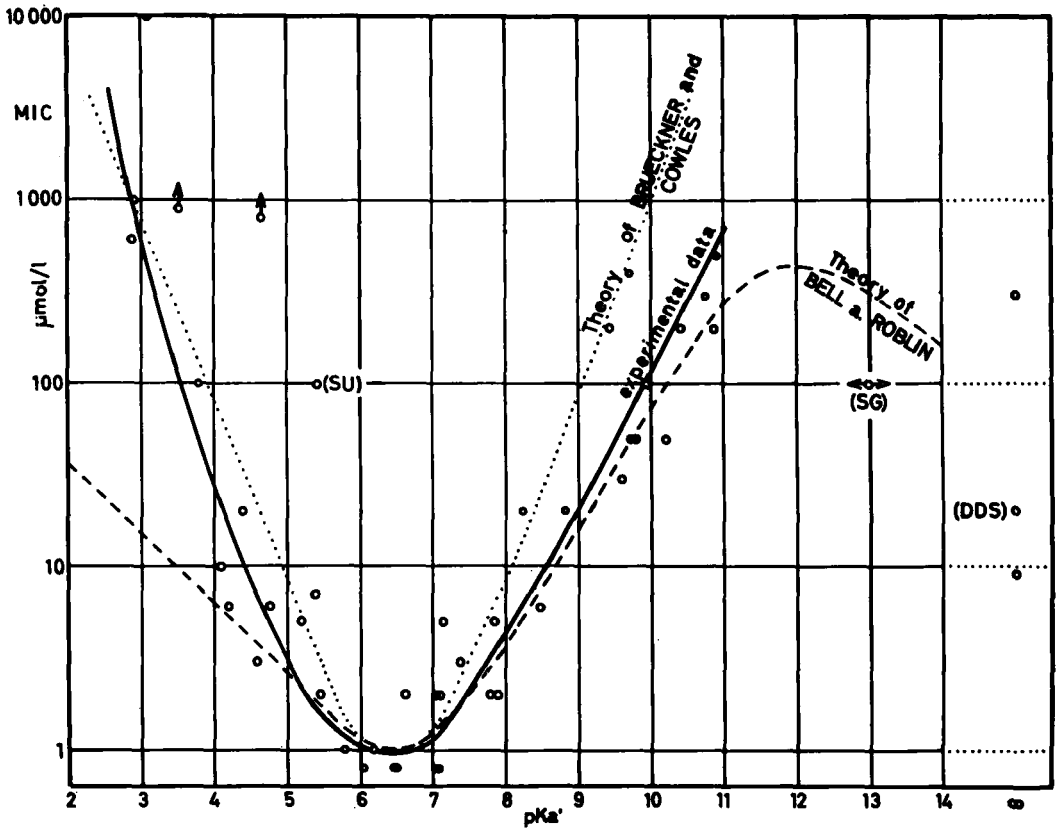


Fig. 3—Plot of the minimum inhibition concentration (MIC, µmole/l.) against the acid dissociation constant (pKa_2) of sulfonamides compared with the theoretical curves of Cowles (89) and Brueckner (92) (···), and of Bell and Roblin (41) (- - -). [Reproduced with permission from Z. Naturforsch., 15b, *628(1960).]*

had a bacteriostatic activity too, however essentially weaker than the ionized form. Further it was supposed that increasing acidity of a compound decreased the negativity of the sulfone group, thus decreasing the bacteriostatic activity of the charged and uncharged molecules. Plotting the MIC against the pKa_2' values of several sulfonamides, Bell and Roblin found a bell-shaped curve with a minimum for the MIC at pKa_2' 6.7. This relationship was used to predict the MIC of a sulfonamide by measuring its pKa_2' value (Fig. 3). There are, however, several exceptions to this hypothesis in addition to the exceptions, sulfaguanidine and sulfanilurea, already stated by Bell and Roblin.

1. There are SNS with pKa_2' values as low as 5.0 or more and still as active as those with pKa values of 6–7. There are SNS with the same pKa_2' but different MIC (44) (see Fig. 4). A striking example is N^1-(3,4-dimethylbenzoyl)-sulfanilamide which has a MIC of 2–4 µmoles/1., the pKa_2' being 4.52 (4.86), and the 2,5- and 2,4-isomers which have a MIC of 22.5 µmoles/1., the pKa_2' being 4.7 [5.1] and 4.6 [5.05], respectively (1, 61). The pKa_2' values in brackets are determined according to Bell and Roblin (41),

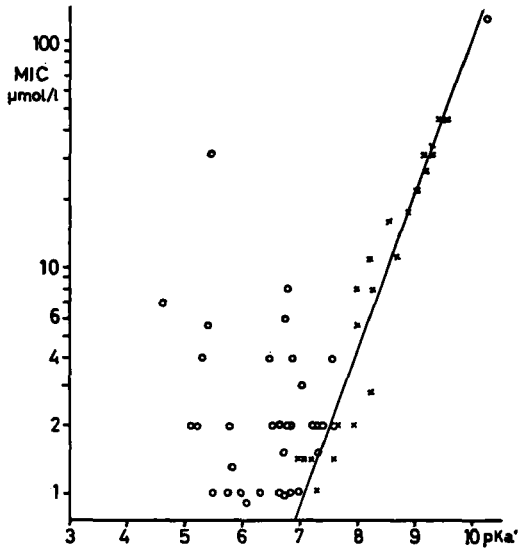


Fig. 4—Relation between the acid dissocation (pKa_2') and the in vitro activity against E. coli *of different sulfonamides* (○) *and of benzenesulfonamides* (×).

for the others a photometric titration method was applied.

It is possible to show in a very homologous series of SNS that there is a *linear* relationship of the pKa_2' and the MIC (45, 54) (see Fig. 4). Using pKa_2' values and MIC data obtained by Bell and Roblin (41), Seydel *et al.* (43), and Seydel and Wempe (61), the bell-shaped curve for the pKa_2'-MIC relation can be separated into linear functions with different slopes (positive and negative) for certain series of substituents (95) (Fig. 5). This provides little evidence in support of a definite pKa_2' which a sulfonamide must possess in order to exhibit maximum activity.

2. N^1-dimethylsulfanilamide is as active as sulfanilamide.

3. N^1-acetylated SNS are as active or even more active than the unacetylated derivatives (Table II) (45). This type of compound has no acidic dissociation constant. The polarization of the SO_2 group should be less in the case of the N^1-acetylated derivatives.

4. There are compounds with a sulfonamide mode of action which do not possess a sulfonamido group, *i.e.*, aminophenylsulfones (96), sulfanilic acid (35), and amino benzil (36), or aminophenyl ketones (37, 28).

5. Furthermore there is no experimental proof that there is a correlation between the degree of polarization of the SO_2 group and the antibacterial activity of SNS (see 32, 34, 35). Therefore it is suggested that the hypothesis of Bell and Roblin fulfills only a minimum condition for the MIC. It is possible to predict from the pKa_2' the MIC of SNS within certain limits of accuracy, but there is no proof for the mode of action theory involved.

Although the relation of ionization to the antibacterial activity and to the mode of action of SNS is very complex, there is no question about the importance of ionization to the physicochemical properties of SNS. The pKa_2' of SNS is directly related to the solubility, the distribution, the partition coefficient and the reabsorption in the kidneys, the permeability of membranes, and the protein binding, to mention only some of the important factors. The solubility is of interest with respect to the toxicologic (crystalluria) (97–100) and the pharmaceutical (absorption) properties of SNS. Therefore the solubilities of SNS have been studied very often (97–104); the functional dependency of pH, pKa_2', the solubility(s), and the base solubility(s_o) is given by the following equation:

$$s = s_o(1 + 10^{pH - pK_a})$$

The distribution has an important influence on the therapeutic effect of SNS; the time needed to reach equilibrium is a function of pH. Sulfathiazole, with pKa_2' of 7, established its equilibrium between blood plasma and erythrocytes *in vitro* in about 4 min., whereas sulfanilacetamide with a pKa_2' of 5.3 needs about 1 hr. (105, 106).

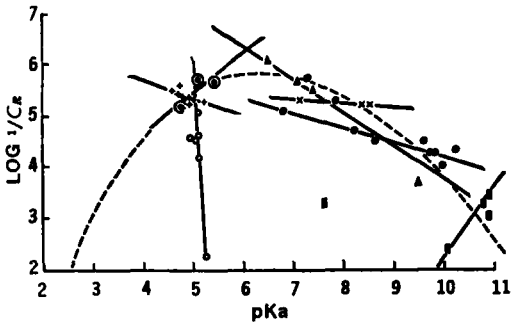


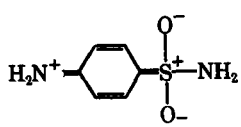
Fig. 5—Correlation between the acid dissociation (pKa_2') and the minimum inhibition concentration (MIC), Key: ⊙, sulfanilamido (substituted) thiazoles, +meta and/or parasubstituted N^1-benzoylsulfanilamides, ○, orthosubstituted N^1-benzoylsulfanilamides; ▲, 2-sulfanilamido (substituted) pyrimidines; ×, 2-sulfanilamido (substituted) pyridines; ●, N^1-(substituted) phenylsulfanilamides; ■ N^1-(substituted) methylsulfanilamides [Reproduced with permission from J. Pharm. Sci., **56**, *640(1967).]*

The partition coefficient plays an important role in the reabsorption of SNS in the kidneys (107). Dependency of the partition coefficient (Q) on the pKa_2' is given by the following equation:

$$Q = \frac{m_o - m_1}{m_1}(1 + 10^{pH - pK_a})$$

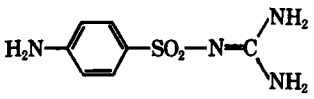
In a detailed study Dettli *et al.* (108) and Portnoff *et al.* (109) were able to demonstrate the change of the excretion rate of SNS by acidifying or alkalizing the urine. The importance of the dissociation constant for the transport of chemotherapeutic agents through membranes is known from several studies (92, 110) and is discussed for SNS by Brueckner (92), and Brown (35), and many other authors.

Dipole Moment of SNS—Kumler and Halverstadt (111) published a paper about the dipole moment of sulfanilamide and related compounds. They stated that there is a contribution of about 3% of the resonance structure in sulfanilamide. Later, Kumler and Daniels (112) and Kumler and Strait (113) proposed a theory that a coplanar amino group resulting from a resonance form

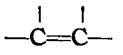


is the main factor effecting activity (see also 114,

115). According to this hypothesis 3-aminobenzene-sulfonamide is inactive because of the inability to build a resonating form; 2-aminobenzene-sulfonamide is inactive in spite of the ability to form a resonance structure, but a hydrogen bond is established between the amino group and the SO_2 group with the consequence of reduction of the active resonance form. Kumler and Daniels (112) explained the low activity of sulfanilurea and the very high activity of sulfaguanidine, which are exceptions of the theory of Bell and Roblin (41) by a resonance form of the ion in which the charge goes mainly to the oxygen rather than to the SO_2 group in case of the sulfanilurea, and by a negative charge appearing on the amide nitrogen in case of the sulfaguanidine. This increases the negativity of the SO_2 group which in turn enhances the coplanar amino resonance form of sulfaguanidine. Since publication of these papers, the resonance hypothesis has been disproved by the papers of Bell, Bone and Roblin (116), Quan *et al.* (33), Quan *et al.* (34), and by NMR measurements, showing that in the case of sulfaguanidine the following form exists (117):



From the paper of Yoshioka *et al.* (88), however, it appears very likely that there is a resonance effect of the R group through the entire molecule and that substituents with a large effect increase the activity of SNS (45). The resonance however is not performed by a SO_2 group acting like



but by cross-conjugation (88).

SPECTRAL DATA AND LCAO PARAMETERS OF SULFONAMIDES AND THEIR PRECURSOR SUBSTANCES IN SYNTHESIS

UV Optical Properties and Structure of SNS—The reactivity of a functional group in a molecule depends on the total structure of the molecule which determines the properties of the compound, for instance, the absorption spectra. UV, IR, and NMR spectra are of special interest because of the relation to the properties of the nucleus and electron density; this again is related to the reactivity of a functional group. Kimmig (57) was the first to study the UV spectra of SNS. He noted that the absorption maxima of active SNS were in the range of 33,000–40,000 cm.$^{-1}$ (300–250 mμ); with increasing activity the maxima shifted toward 33,000 cm.$^{-1}$. The UV absorption spectra of SNS have been studied by several authors (34, 58, 59, 118–123). It was not possible, however, to deduce a general rule for the relation between the position of maximum absorbance and the activity. This is based partly on the fact that the absorbance of the heterocyclic substituents is stronger and shifted toward longer wavelengths (124) in contrast to the absorbance of the important sulfanilamide moiety which is shifted to 38,000 or 40,000 cm.$^{-1}$ and is weak in most cases. The group of interest, the amino group, has no absorbance in the UV range; there is only a certain influence of the primary amino group on the benzene ring absorbance.

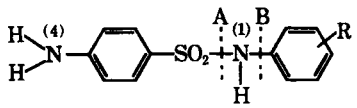
IR Data of the Sulfone Group and the Aromatic Amino Group—Therefore it is much more promising to study the IR spectra of SNS. Vibration frequencies in the infrared characterize the relation between two or three adjacent nuclei in a molecule influenced by other nuclei of the molecule. It is known from several IR-relationship studies that the stretching frequencies arising from vibrations localized in functional groups attached to an aromatic ring can be related to the electronic nature of the ring substituent constant (125).

Many IR measurements of SNS were done for assay methods of drug analysis (126, 127). Other IR measurements were performed to study the imido–amido tautomerism (128) or to see if there is any change of the electronegativity of the SO_2 group (43, 44, 60), which may be correlated to the *in vitro* activity of SNS according to the hypothesis of Bell and Roblin. IR vibrations are suitable for the study of the physical properties of the SO_2 group because of the absence of mass and coupling effects (129). There are two IR absorption frequencies of the SO_2 group, the symmetrical stretching frequency (130) which is little affected by changes in the molecule (1,128–1,665 cm.$^{-1}$ in studied SNS), and the asymmetrical frequency (131), which is in the range of 1,360–1,260 cm.$^{-1}$ in SNS. The exact position of these two frequencies is determined by the electrical character of the substituents adjacent to the sulfur atom. From the position of the IR frequencies it is possible to calculate two parameters with a descriptive physical meaning, the force constant k and the bond angle θ. For both parameters no correlation with the *in vitro* activity of the corresponding sulfonamide was obtained (43, 44, 60, 61). Some of the most active sulfonamides have force constants for the S=O bond of $9.8 \cdot 10^5$ dyne/cm., *i.e.*, the degree of polarization is small.

These data are limited by the fact that these measurements must be performed principally in

solid state. Therefore, the data include association effects. The same is true for the IR data obtained from the vibration frequencies of the primary aromatic amino group, which show some correlation to the activity of SNS (43, 44). However, data obtained from an additional sequence of SNS (61) and data obtained by Brandmüller (60) did not support these findings.

IR and NMR Data of the Aromatic Amino Group of the Precursor Substances in Synthesis (Amines)—To avoid the difficulties arising from the insolubility of SNS in nonpolar solvents, it seems better to study the substituents rather than the complete sulfa drug (45, 62, 132). For this purpose one may split the sulfonamide molecule according to the dotted line B, studying for example substituted chlorobenzene, which may be used for the synthesis of sulfa drugs. Since it is known for sulfathiazole and sulfapyridines (40, 124) and for some 4-sulfapyrimidines and sulfaphenazole (133, 134) that the substituents may be bound to the N^1-nitrogen atom by a double bond, it seems to be more appropriate to split the sulfa drug molecule according to the dotted line A, thus studying the substituted anilines.



To keep the model as simple as possible, anilines were chosen which were substituted in the *ortho*, *meta*, and *para* positions. As shown by Fig. 6 there is an approximately linear relationship between the σ-Hammett values of the substituents in the anilines and the MIC of the corresponding sulfonamides. Similar approaches for other systems have been made by Hansch (135, 136), Zahradnick (137), and Bocek *et al.* (138). It can be seen from Fig. 6 that the more positive the σ-Hammett constant of the amine the more active the corresponding sulfonamide. The IR data obtained from the stretching frequencies of the amino group of the anilines were used to calculate the force constant, the bond angle, and the "s" character of the nitrogen orbitals of the N—H bond expressed as coefficient *b*. The constant *b* is the coefficient of the 2*s* orbital of nitrogen in the hybrid (125, 139).

Plotting the *b* values of the amines against the logarithm of the MIC of the corresponding SNS, an approximately linear relationship was obtained (Fig. 7) (45, 62, 132). The aniline derivatives with *ortho* substituents deviated from the line in most cases. This behavior can be explained by intramolecular hydrogen binding; the hydrogen binding causes a larger bond angle than can be attributed only to the delocalization of the lone electron pair.

More detailed and more sensitive data for all protons of the amines were obtained from NMR measurements (54, 132). The shielding effect of electrons as a function of the substituents were easily followed in these simple molecules. Very small energy differences can be verified; association effects were excluded by dilution technique. Plotting the chemical shift of the amino group protons as a function of substituents against the log MIC of the corresponding SNS, a linear relationship was obtained (Fig. 8) (132). The chemical shift of the amino group was directly related to the pKa of the anilines.

It can be concluded that it is possible to predict the *in vitro* activity of SNS from structural parameters of the basic amines using either NMR or IR data or σ-Hammett constants for calculation;

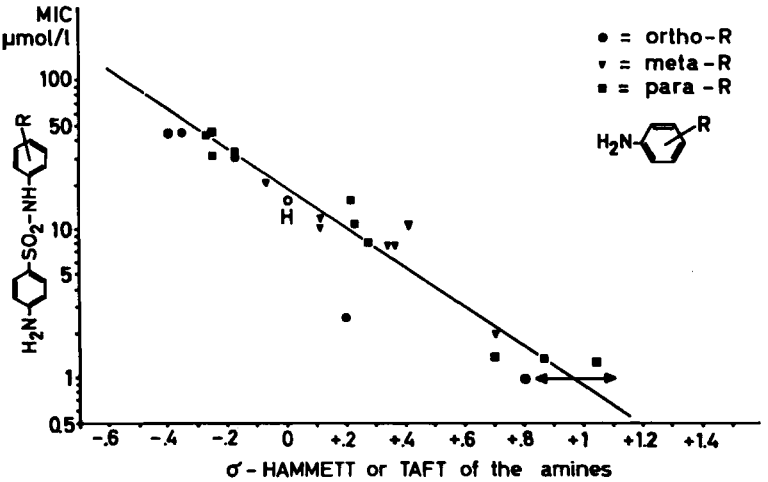


Fig. 6—Relation between the corrected σ-Hammett (210) of the anilines used for the synthesis of sulfonamides and the minimum inhibition concentration of the corresponding sulfonamides against E. coli *(µmole/l.)* [*Reproduced with permission from* Arzneimittel-Forsch., **16**, *1447 (1966).*]

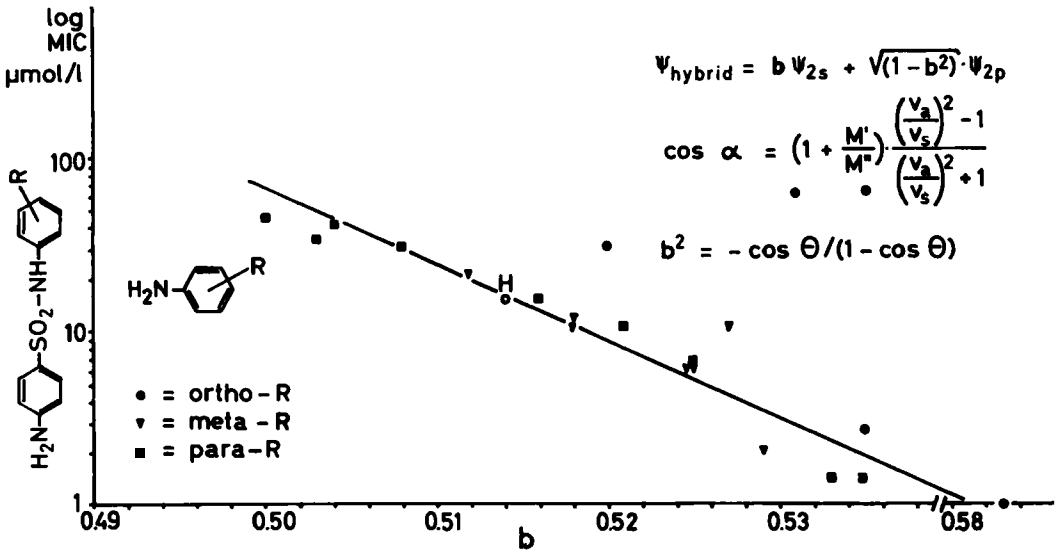


Fig. 7—Correlation between the b *values of the anilines used for the synthesis and the logarithm of the minimum inhibition concentration (MIC) of the corresponding sulfonamides.* [*Reproduced with permission from* Mol. Pharmacol., **2**, *259(1966).*]

this means one can estimate the *in vitro* activity of SNS before synthesis.

Parameters of Crystal Optical Measurements—Alléaume (47) has evaluated from crystal optical measurements the interatomic distances and bond angles of sulfanilamide, 4,4′-diaminodiphenylsulfone, *p*-aminobenzamide, and *p*-aminobenzoic acid. It was concluded by Alléaume that the data support the hypothesis that the different configuration of the primary aromatic amino group may be responsible for the antibacterial activity of SNS. According to Alléaume the configuration of the primary amino group is more of the type sp^3 than sp^2. A hyperconjugation of the substituents at the N^1-nitrogen atom is very likely thus influencing the electron distribution of the primary amino group. More substances have to be measured to come to a final conclusion. It was very clearly shown, however (Fig. 9), that the interatomic distances of the nitrogen atom of the amino group from the benzene carbon are different in PABA on one side and sulfanilamide and diaminodiphenylsulfone on the other side; this is in agreement with the observed different pKa_1' values (87, 88).

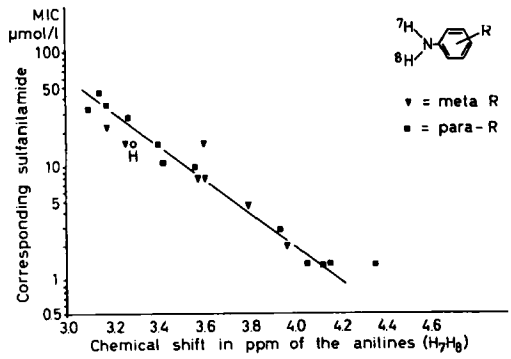


Fig. 8—Relationship between the chemical shift (p.p.m., TMS as internal standard, $CHCl_3$ solution) of the anilines ($^7H^8H$) and the minimum inhibition concentration of the corresponding sulfonamides against E. coli.

Large differences were observed for the bond angle and the interatomic distances of the carboxyl group and the sulfone group. This again makes the proposed similarity (41) of these two functional groups very unlikely.

Parameters from LCAO Calculations—Following the successful application of quantum chemistry to organic chemistry (140–142) and biochemistry (143, 144), greater interest in this method has also been shown in drug research. First calculations have been done for sulfonamide molecules (63–67) and for the sulfone group (66, 145). The data of Schnaare and Martin (63) and Foernzler and Martin (64) show some degree of correlation between the acidic dissociation constant and the electronic charge at the N^1-nitrogen atom. The linear relation which was expected, however, was not obtained. The electronic charge calculated for the oxygen atom of the sulfone group was nearly constant for about 50 different sulfonamides; the oxygen of the sulfone group of the unsubstituted sulfanilamide was more negatively charged than, for instance, the SO_2 of N^1-phenylsulfanilamide. This latter re-

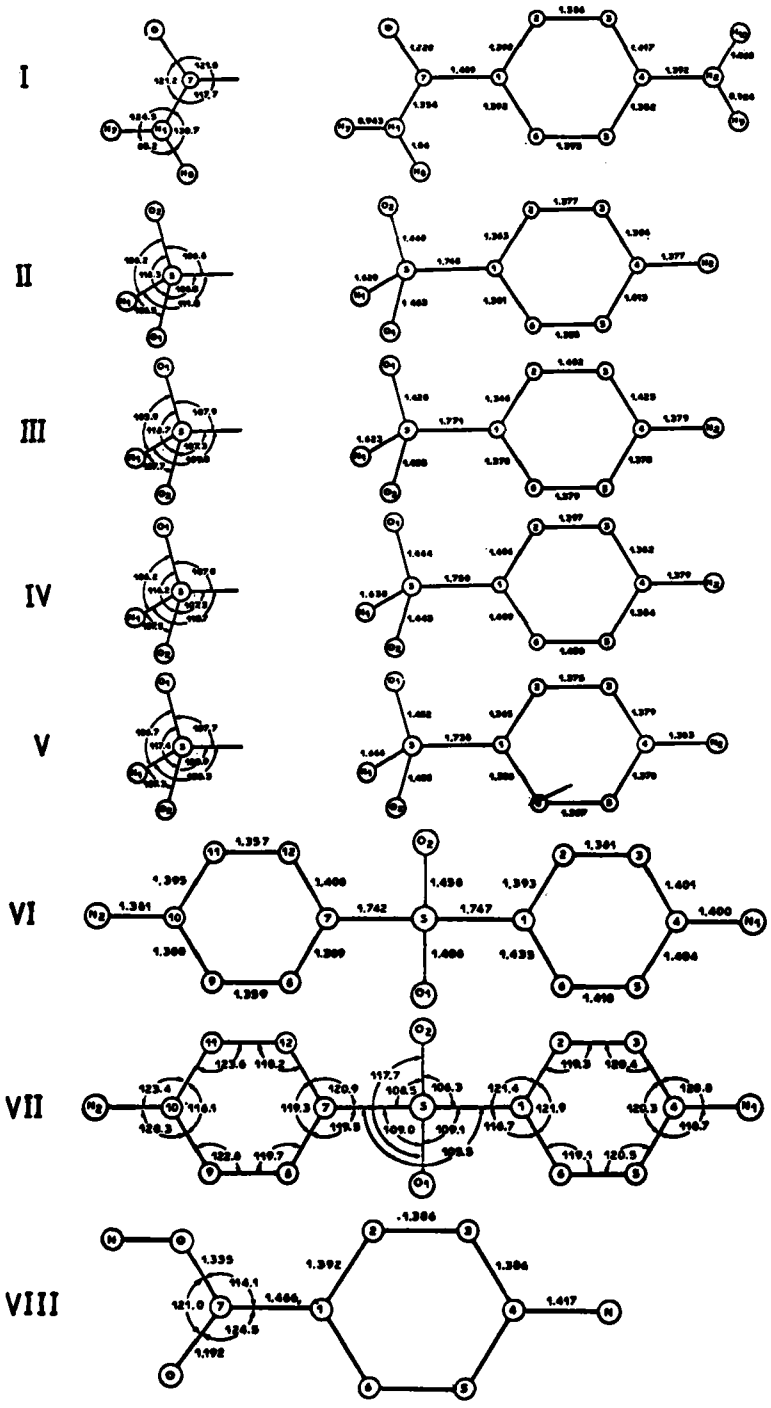


Fig. 9—Atomic distances and valence angles of 4-aminobenzamide, sulfanilamide, diaminodiphenylsulfone, and 4-amino-benzoic acid according to Alléaume (1966, 1967). Key: I, 4-aminobenzamide; II, sulfanilamide monohydrate; III, sulfanilamide α, crystallized from butanol; IV, sulfanilamide β, crystallized from methanol; V, sulfanilamide γ, crystallized from isoamyl alcohol; VI, VII diaminodiphenylsulfone; VIII, 4-aminobenzoic acid [From Alléaume, M., Doctoral dissertation, University of Bordeaux, 1967).]

sult is in contradiction to the hypothesis of Bell and Roblin.

The obtained data for the electronic charge of the primary aromatic amino group, however, show that the parameters used in these calculations are not correct, and the obtained correlation even between pKa_2' and the N^1-nitrogen atom is doubtful. As shown by the pKa_1' values of the aromatic amino group, there are different electronic charges at the N^4-nitrogen atom. If differences of 0.12 pKa units for the acid dissociation (N^1-nitrogen atom) can be described by LCAO method then it should be asked why these differences in electronic charge are not described for the N^4-nitrogen atom. One reason for these difficulties is the treatment of the SO_2 group in LCAO cal-

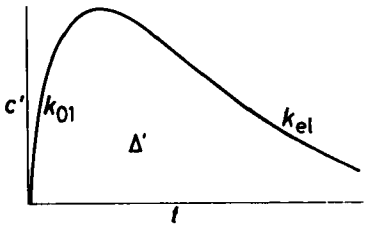
culations. Including the contribution by the sulfur *d*-orbitals in the overall conjugation, Cammarata (65) was able to show in three selected sulfonamide-type compounds that variation in charge at the primary aromatic amino group does occur.

RELATIONSHIP BETWEEN CHEMICAL STRUCTURE OF SNS AND THEIR BIOLOGICAL AND PHARMACOKINETIC PROPERTIES

Besides the *in vitro* activity many other factors are involved in the estimation of the *in vivo* activity of SNS. In spite of the prediction of Bell and Roblin in 1942 (41) that no sulfonamide with a higher activity could be expected (and this is certainly true) and in spite of the evaluation of penicillin which was able to displace SNS from the market for a short time, better SNS were developed in 1950 and 1960 and play an important role in the therapy of infections. This was possible by changing special pharmacokinetic factors, thus reducing the dosage required for therapy (Tables IV and V). These pharmacokinetic factors are mainly absorption, distribution, and elimination coefficients and protein binding, to name some of them. The functional connection of these parameters and their meaning for the dosage regimen of SNS have been studied by Bünger *et al.* (146), Dettli (147), Diller (148), Dost (149, 150), Garrett (151), Krüger-Thiemer (152–154), Nelson (155, 156), Swintosky (157), Wagner (158), and many other authors and is

TABLE IV—DEFINITIONS AND DETERMINATIONS OF THE BIOLOGICAL CONSTANTS NECESSARY FOR THE CALCULATION OF THE DOSAGE SCHEDULES OF SULFA DRUGS

No.	Biological Constants	Symbol	Unit	Experimental Methods of Determination
1	Rate constant of absorption	k'_{01}	h^{-1}	Evaluation of the curve of blood content (po.) in blood plasma of humans
2	Rate constant of elimination; time of half elimination	k'_{el} $t'_{50\%}$	h^{-1} h	
3	Coefficient of distribution relative to the blood plasma	Δ'	ml./g.	
4	Drug concentration in plasma water at half saturation binding	K''	μmole/l.	Evaluation of bound and free drug in human blood plasma by ultrafiltration, dialysis, or ultracentrifugation. Calculation by the law of mass action
5	Maximum specific binding capacity of the plasma proteins	β	μmole/g.	
6	Minimum inhibition concentration	μ	μmole/l.	Evaluation of μ against *E. coli* or other pathogen bacteria in a medium free of antagonists
7	Proportionality constant between μ and $c_{min.}$ for a therapeutical effect of 95%	σ	—	In analogy calculated from the dosage schedules of other clinically effective sulfa drugs



[Reproduced with permission from *Chemotherapia*, **10**, 61(1965).]

TABLE V—CALCULATION OF MAINTENANCE DOSE[a]

Name	Mol. Wt.	μ μmole/ l.	$c_{min.}$ $= \mu\sigma$	K (μmole/ l.)	β (μval/ g.)	A	Δ' (ml./g.)	M 1000· $c_{min.}$	Δ	Φ(τ)	$t'_{50\%}$ (h)	τ (h)	Maint. dose D (mg). calc.	used
Kelfizin	280	1.6	16	366	18.2	4.29	0.21	4.48	0.90	0.32	60	24	90	500
Sinomin, Gantanol	253	0.9	9	536	26.3	4.33	0.22	2.28	0.95	1.24	10	12	190	1000
Sulfadiazine	250	1.0	10	892	16.0	2.19	0.92	2.50	2.01	0.27	17	6	100	1000
										0.61		12	220	
Kynex	280	1.0	10	114	23.2	14.0	0.19	2.80	2.66	0.60	35	24	320	500
Sulfamerazine	264	0.9	9	72	7.4	7.33	0.36	2.38	2.64	0.25	23.5	8	110	1000
										0.92		24	410	
Durenat, Kiron	280	2.0	20	119	18.6	10.3	0.26	5.60	2.68	0.57	37	24	600	500
Sulfuno, Nuprin	267	3.4	34	348	27.0	6.36	0.25	9.08	1.59	1.10	11	12	1110	500
Madribon	310	0.8	8	11	20.5	80.7	0.17	2.48	13.7	0.49	41	24	1170	500
Sulfanilamid	172	128	1280	700	7.0	1.20	0.69	220	0.83	0.76	9	8	9700	1000

[a] $G = 70$ kg.; $w = 0.9337$; $p = 70$ g./l.; $\Delta = \left(w + \frac{\beta \cdot p}{K + \mu \cdot \sigma}\right) \cdot \Delta'$; $D = G \cdot \frac{M}{1000} \cdot c_{min.} \cdot \Delta \cdot \Phi(\tau)$; $\Phi = \left(1 - \frac{k'el}{k'_{01}}\right) \cdot \frac{[1 - \exp(-k'_{01}\tau)] \cdot [1 - \exp(-k'_{el}\tau)]}{\exp(-k'_{el}\tau) - \exp(-k_{01}'\tau)}$. [b] $A = \left(w + \frac{\beta \cdot y}{K + \mu \cdot \sigma}\right)$. [Reproduced with permission from *Chemotherapia*, **10**, 61(1965).]

given by the following equation (152):

$$D = G \times \frac{M}{1000} \times \mu \times \sigma \times \left(w + \frac{\beta \times P}{K' + \mu \times \sigma}\right) \times \Delta' \times \Phi$$

Among these pharmacokinetic factors, the protein binding (binding to albumin) is of great importance. The phenomena of protein binding have been discussed several times in the literature. There is no doubt about the importance of protein binding for the antibacterial action, because of the inactivity of the bound fraction (159–165); in addition there is an influence on the pharmacokinetics (for example, excretion rate and metabolism), demonstrated especially in so-called long-acting SNS (98, 166–169). However, there is no proof for the theory of Klotz (93) that the degree of protein binding is related to the activity and, thus, to the mode of action. As shown by Davis (162), Klotz (170), Goldstein (171), Krüger-Thiemer (98, 152), Nakagaki *et al.* (172), and others, protein binding of drugs may be described with sufficient accuracy by the law of mass action, which is sometimes identified with the adsorbance isotherm of Langmuir (173). The law of mass action for protein binding may be written as follows:

$$c_1' = c_1 \left(w \cdot \frac{\beta \cdot p}{K_1 \cdot c_1}\right)$$

where c_1, drug concentration in plasma water; c_1', drug concn. in water; K_1, apparent dissociation constant of drug–protein complex · β_1, specific binding capacity of plasma proteins (μval./g.); p, protein content of blood plasma (g./l.); w, water content of blood plasma (ml./ml.).

For the achievement of the two parameters of protein binding β and K_1, several methods were developed, for example, ultrafiltration, dialysis, gel filtration (174, 175), and ultracentrifugation techniques (176). The importance of the type of binding involved, electrostatic (ionic type of binding) or hydrophobic, dipole, or van der Waal type of binding is still open to question. Scholtan (177) discussed an important influence of hydrophobic binding in highly bound SNS which possess alkyl or alkoxy groups. According to Scholtan, the reaction energy of the binding reaction is proportional to the molecular refraction of the substance. In a homologous series the protein binding increases from alkyl to alkoxy to halogen substituents (Table VI). On the other hand, papers of Klotz (170), Nakagaki *et al.* (172), Dettli and Spring (178), Krüger-Thiemer *et al.* (152, 179), and Bünger *et al.* (146) show that a stoichiometric type of binding occurs. The constant β, the specific binding capacity of plasma proteins, seems to be the same for all studied sulfonamides; the value corresponds to the ratio of two molecules of drug to one molecule of protein. Moreover, the binding affinity of SNS was estimated to be about 6 Kcal./mole (172). From this number it may be concluded that electrostatic forces are the most important factors involved, which means that the dissociation constant of SNS strongly affects the protein binding.

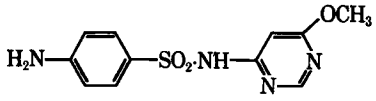
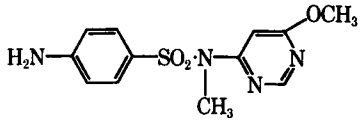
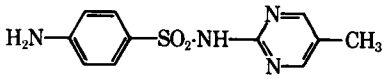
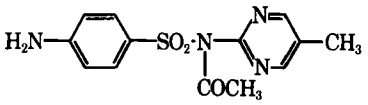
TABLE VI—BINDING CAPACITY OF HUMAN BLOOD SERUM FOR ALKOXY, ALKYL, AND HALOGEN DERIVATIVES OF 2-SULFAPYRIMIDINES AT 37°

No.	Substituent	Position	K	$K\ 10^{-3}$ l./mole	$\Delta F°$ cal./mole
1	H		0.75	1.3	4,430
2	CH_3	5	4.1	7.1	5,480
3	C_2H_5	5	9.5	16.4	6,000
4	C_3H_7 iso	5	28.8	50.0	6,700
5	C_5H_{11}	5	237	445	8,010
6	C_6H_5	5	280	483	8,070
7	OCH_3	5	5.0	8.65	5,640
8	OC_2H_5	5	19.0	33.0	6,400
9	OC_3H_7	5	60.0	104.0	7,100
10	OC_3H_7 iso	5	14.5	25.1	6,240
11	OC_4H_9	5	120	208.0	7,550
12	Cl	5	17	29.4	6,370
13	Br	5	34	59.0	6,770
14	J	5	118	205.0	7,550

[Reproduced with permission from *Arzneimittel-Forsch.*, 14, 348(1964).]

Many studies on structure-protein affinity relations have been conducted (168, 177, 180). The substances used, however, did not belong to a homologous series of compounds or if they did, only alkyl or alkoxy groups with different lengths of the hydrocarbon chain were studied and compared. The influence of the ionization was studied by Seydel (45, 132, 181) using N^1-methylated or N^1-acetylated SNS. In both types of compounds the protein binding was strongly decreased (Table VII). The accuracy of the protein-binding determination in the case of the N^1-acetyl derivative was limited because of the instability of the compound. In a very closely related series of compounds the influence of the type of substituent and of the position of the substituent was studied (45, 132, 181). The influence of different types of substituents was clearly demonstrated (Table VIII). In addition there was a strong influence of the position of the substituent. Obviously the *ortho* substitution resulted in the lowest protein binding among the isomers. This may be explained, at least in part, by a steric hinderance of the N^1-nitrogen atom, which is involved in protein binding by electrostatic forces. The strong overlapping of a methoxy group can be demonstrated by a stereo model. However, this phenomenon is not limited to the benzene sulfonamides. The influence of the position of the substituent on protein binding can be demonstrated also with two pairs of isomeric heterocyclic SNS which are on the market (Table IX). It is difficult to explain these results ac-

TABLE VII—PROTEIN BINDING OF SULFONAMIDES AND THEIR N^1-METHYLATED OR N^1-ACETYLATED DERIVATIVES

Substance	M.p., °C.	MIC [μmole/l.] *E. coli*	Protein Binding Free SNS	
			%	C_o [μmole/l.]
	210–212	0.5	10.5	72
	120–124	≥128	73	106
	274–276	1	11	180
	206	1	~42	56

cording to Scholtan (177) by hydrophobic binding. In addition the data do not support the studies of specific molecular interactions of SNS and protein, shown by NMR relaxation measurements published by Jardetzky (182). Jardetzky was using the broadening of the signals originating from the *p*-aminophenyl moiety in his studies, whereas the lines originating from the substituent ring hydrogens are not involved. The influence of this moiety on protein binding, however, is clearly shown by the data given above. Moreover the N^1-hydrogen is very quickly exchanged and is not observable in NMR and the stated binding energy of 6 Kcal./mole (172) is not coincident with a van der Waal binding type.

Another parameter which may be important for pharmacokinetic properties of the molecule is the partition coefficient, the lipid solubility. The importance of the lipid partition was shown by Koizumi *et al.* (107) and used by Krüger-Thiemer (183) in a new clearance model. Higher lipid solubility means a higher rate of reabsorption in the kidneys, thus increasing the half-life of the drug and decreasing the dosage required. A large number of experimental results obtained for different SNS were published by Rieder (184). Because of the very different substituents used in this study, it is difficult to evaluate structure relationships. In a homologous series the influence of the length of the hydrocarbon chain can be demonstrated (181) (Table X) and again there seems to be a certain influence of the position of the substituent. In a comparison of the partition coefficients of isomers, the *ortho*-substituted compound was found to have an increased lipid solubility. This might be due to steric hinderance of ionization thus increasing lipid solubility. Further studies are necessary to come to a final conclusion.

MODE OF ACTION OF SNS

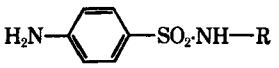
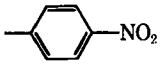
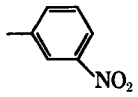
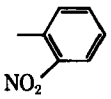
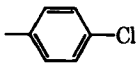
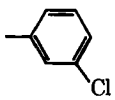
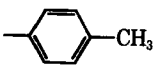
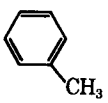
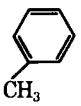
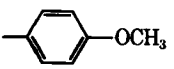
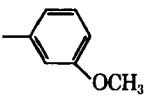
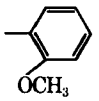
The data discussed above do not give an answer to the mode of action. The developed structure-activity correlations do not include, for instance, that the N^1-nitrogen atom is the reactive center of SNS. From the data it may be derived that there are correlations between the physicochemical properties of the N^1-nitrogen atom and the reactive center, which is very likely the primary aromatic amino group.

In the following the discussion will be restricted to the action of SNS on bacterial strains which are inhibited by SNS and the inhibition of which can be antagonized by PABA or by *p*-aminobenzoyl glutamic acid (82).

What is known about the antibacterial action of SNS besides the structural properties necessary for action?

1. The inhibition by SNS is bacteriostatic, which is not the case for several other drugs like penicillin or isoniazid. This was shown by experiments of Muir *et al.* (185) and Hirsch (52) using a Warburg equipment. Using concentrations in the range of the minimum inhibitory concentration, it was shown in a kinetic approach that there is no death of bacterial cells. It was also shown that the antagonistic effect of PABA is reached in the range of the same molarity as the concentration of sulfathiazole applied for inhibition. The bacteriostatic effect of sulfa drugs can only be obtained in growing cultures. Other studies of a kinetic nature to obtain growth

TABLE VIII—CORRELATION BETWEEN TYPE AND POSITION OF THE SUBSTITUENT AND THE pKa_2', R_f VALUE, AND PROTEIN BINDING OF SOME SUBSTITUTED BENZENE SULFANILAMIDES

	pKa'	R_f Value $CHCl_3/CH_3OH$ 80:15 Silica Gel GF 254	% Unbound[a] $(c.100)/c'$
	6.97	0.56	14.5
	7.67	0.52	20.4
	7.20	0.67	18.3
	8.56	0.58	8.1
	8.28	0.55	12.5
	8.29	0.71	18.6
	9.25	0.54	15.6
	9.05	0.52	20.4
	9.34	0.62	25.2
	9.34	0.61	22.9
	8.72	0.58	25.7
	9.43	0.65	32.3

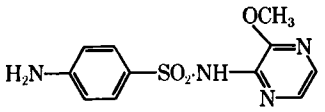
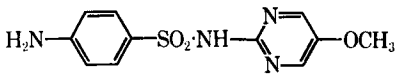
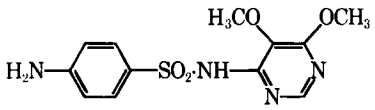
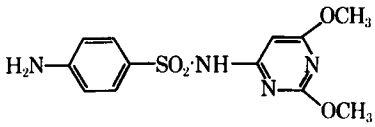
[a] Range of concentration used was 100–400 μmoles/l. Data given are averaged values from 4 runs.

rates and to establish the influence of sulfa drugs on the growth rate were done by Kohn and Harris (53, 186). A more sophisticated approach is that of Garrett *et al.* (56), using the Coulter-counting technique for studying the change of growth rates by changing sulfa drug concentration and reversibility of drug action by dilution technique or addition of PABA.

2. Inhibition of growth by sulfa drugs does not occur immediately, contrary to the activity of many other drugs. Evidence was obtained by respiration curves (52, 53, 186, 187), and a lag phase of about 1–2 hr. was observed. More accurate data were obtained by Garrett (56), who was able to show that this lag phase is 3.7 ± 0.3 generation times. The same lag time was obtained for the different SNS and was interpreted as being the result of the depletion of stored PABA metabolites (56). After a sequential addition of SNS, however, there is again a lag phase until the new steady state is established.

3. The antagonism between sulfa drugs and PABA has been shown by several authors. The antagonistic action of PABA took place imme-

TABLE IX—CORRELATION BETWEEN POSITION OF THE SUBSTITUENT AND THE pKa′ AND PROTEIN BINDING OF SOME SULFONAMIDES USED IN THERAPY

Substance		pKa	% Unbound[a] $(c.100)/c'$
	(Kelfizin)	6.1	54.5
	(Durenat)	6.54	12.7
	(Fanasil)	5.75	14.5
	(Madribon)	5.95	6.2

[a] Range of concentration used was 200–400 μmoles/l. Data given are averaged values from several runs.

diately, whereas the action of sulfa drug needed a lag phase of about 4 mitosic divisions.

In accordance with the well-established bacteriostatic nature of the mode of action of sulfa drugs, Wacker *et al.* (70) could not detect any metabolic transformation or fixation of sulfanilamide using the isotope tracer technique. Since the discovery of the competitive antagonism of PABA (11) against the antibacterial activity of the sulfa drugs, this antagonism has been interpreted by all authors in such a way that SNS displaces the PABA from its site of action at an unknown enzyme system (188) which would be necessary for the biosynthesis of the tetrahydrofolic acid (THFA). As already pointed out, Bell and Roblin (41) have taken a theoretical approach to relate the chemical structure and the strength of the antibacterial activity of SNS. The theory is based on the assumption that the sulfonamide molecule with a more polarized SO_2 group has more structural similarity to the bacterial growth factor PABA, thus fitting in a receptor on an enzyme surface. There is no proof, however, for a structure similarity of the SO_2 group and the carboxy group (see 32, 34, 35). The physicochemical part of the hypothesis of Bell and Roblin (41) was based on analogies rather than on actual measurements of properties of the SO_2 group.

Other review articles concerning the mode of action problems of SNS have been published by

TABLE X—PARTITION COEFFICIENT $[Q = (c_0 + c_1)/c_1]\ (1 + 10^{pH - pKa})$ OF SOME HOMOLOGOUS AND ISOMERIC SULFONAMIDES IN THE SYSTEM OCTANOL/WATER

Substance	C_0 μmole/l.	C_1 μmole/l.	pH − pKa	$1 + 10^{pH-pKa}$	$\frac{K}{\frac{c_0 - c_1}{c_1}}$	$\frac{Q}{K(1 + 10^{pH-pKa})}$
2-Sulfapyrimidine	190	85.5	5.1–6.4	1.05	1.22	1.28
	190	100	6.56–6.4	2.44	0.9	2.20
	357.6	170.8	5.1–6.4	1.05	1.09	1.14
	357.6	220.5	6.56–6.4	2.44	0.62	1.52
2-Sulfa-5-methyl-pyrimidine	50.7	19.2	5.1–6.8	1.02	1.64	1.67
	50.7	27.6	6.56–6.8	1.57	0.84	1.32
2-Sulfa-5-ethyl-pyrimidine	364.8	43.0	5.1–6.7	1.02	7.49	7.68
	364.8	78.0	6.56–6.7	1.72	3.68	6.34
	404.8	58.79	5.1–6.7	1.02	5.90	6.05
	404.8	87.97	6.56–6.7	1.72	3.60	6.20
	372.9	62.2	6.50–6.7	1.63	4.99	8.14
2-Sulfa-5-isopropyl-pyrimidine	484	29.4	5.1–6.6	1.03	15.46	15.9
	484	47.4	6.56–6.6	1.91	9.21	17.8
2-Sulfa-5-methoxy-pyrimidine	217.0	65.6	5.1–6.9	1.02	2.3	2.34
	217.0	100.4	6.56–6.9	1.46	1.16	1.64
2-Sulfa-3-methoxy-pyrazine	6,615	4,110	7.3–6.1	16.85	0.366	6.16
4-Sulfa-2,6-dimethoxy-pyrimidine	3,630	1,725	7.4–6.1	20.95	0.52	10.89
4-Sulfa-5,6-dimethoxy-pyrimidine	16,600	12,800	7.4–5.75	45.67	0.296	13.5

Work and Work (189), Tschesche (82), Mietzsch and Behnisch (115), Kallinich (81), Sexton (190), Wacker (191), and Albert (192). All studies in this field have been done without the knowledge of the reaction chain of the biosynthesis of THFA. Jaenicke and Chan (84) and Brown *et al.* (55, 83, 193) published experimental data which gave proof for the biosynthesis in *E. coli* along the following reaction sequence (Fig. 10). Starting from a pyrimidine derivative, probable guanosine (I) (194, 195), 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine (II) was synthesized. In a cell-free enzyme extract of *E. coli*, to which adenosintriphosphoric acid (ATP) and Mg^{2+} ions were added, the alcohol reacted in a two-step reaction with ATP and ADP to form the corresponding monophosphoric ester (IV) and pyrophosphoric ester (V) according to Jaenicke and Chan (84) or the pyrophosphoric ester was formed enzymatically by the transfer of a pyrophosphate group from ATP to hydroxymethyldihydropteridine according to Weisman and Brown (196). The pyrophosphoric ester then combined with *p*-aminobenzoyl glutamic acid(PAG)or PABA to form dihydrofolic acid (DHFA) or dihydropteroic acid, respectively.

The same reaction took place if formylpteridine, formyltetrahydropteridine (III), or hydroxymethylpteridine was used as the substrate (55, 84); formylpteridine was many times more active

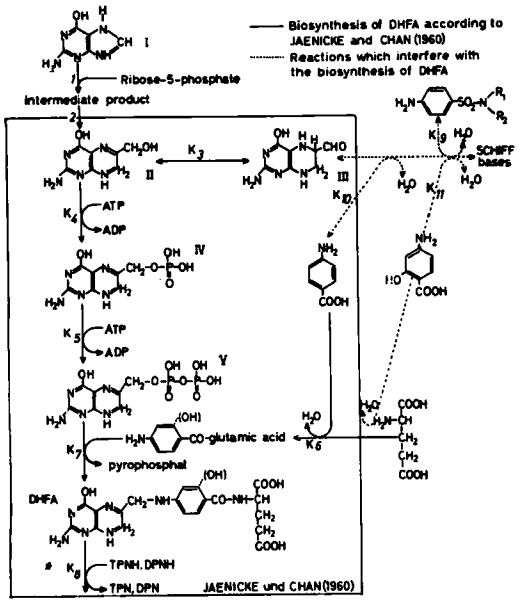


Fig. 10—Pathway for the biosynthesis of DHFA according to Jaenicke and Chan (1960) (——) and reactions which might interfere with the biosynthesis of DHFA (···). [From Jahresber. Borstel, **5,** *651 (1961).]*

than hydroxymethylpteridine (55). It may be correct to assume that a tautomeric equilibrium or a disproportion of the aldehyde and the alcohol exists in watery solutions (74, 75, 84, 193, 197). Dihydro- or tetrahydrofolic acid may act in a similar way as a biological redox system (198).

Brown (35) has used a cell-free enzyme extract in a detailed study of the inhibition of "folate synthesis" by different SNS and of the antagonistic action of PABA. The main results were:

1. The enzymatic inhibition indexes (SNS/PABA) were calculated to be 18 and 0.71 for sulfanilamide and sulfathiazole, respectively, whereas the index published in other papers from studies with bacterial cultures stated 1:1000.

2. In general the sequence of effective inhibitors of enzymatic synthesis of "folate" was the same as in growth inhibition. The exception was sulfanilic acid, which was quite effective in inhibiting folate synthesis but had no effect on growth.

3. A procedure was developed to prove whether or not the inhibition was of the competitive type. From the results it can be seen that the degree of inhibition was different depending on the addition of PABA at the same time as sulfonamide or after a preliminary incubation time. PABA (0.067 mmole) did not reverse the inhibition of folate synthesis by sulfathiazole (0.017 mmole) in the reaction mixture from which PABA was omitted in the first incubation time (2 hr.). On the other hand when PABA (0.067 mmole) and sulfathiazole (0.017 mmole) were added to the reaction mixture at the same time, little inhibition occurred. According to Brown, "the experiment described above indicated that the sulfonamide inhibition could not be described solely as a competitive type" (Table XI). The results were interpreted by Brown in such a way that the irreversible effect might be due merely to depletion of pteridine substrate.

4. Almost complete enzymatic activity was recovered by dialyzing the enzyme system which had been exposed to sulfathiazole during the first incubation period and after new addition of hydroxymethyltetrahydropteridine in the second incubation period. "The fact indicates that the enzyme was not inactivated irreversibly by reaction with sulfathiazole." (Table XI.)

These results indicate that there is not only competition on the receptor site, as suggested by Wood (12) and Bell and Roblin (41). In this case there should be no dependency on the sequence of incubation of sulfonamide and PABA.

5. In another experiment the relation of incubation time to the irreversible inhibition of

TABLE XI—IRREVERSIBLE INHIBITION BY SULFATHIAZOLE OF "FOLATE" SYNTHESIS[a]

Reaction Mixture No.	Component Omitted in First Incubation Period	Component Added Between First and Second Incubation Periods	Folate Equivalents Produced, mcg.
1	Sulfathiazole	None	5.8
2	None	None	5.2
3	Sulfathiazole, *p*-aminobenzoate	*p*-Aminobenzoate	6.4
4	*p*-Aminobenzoate	*p*-Aminobenzoate	1.1
5[a]	Sulfathiazole, *p*-aminobenzoate	All components, except enzyme and sulfathiazole	6.5
6[b]	*p*-Aminobenzoate	All components, except enzyme and sulfathiazole	5.5

[a] The complete reaction mixture contained *p*-aminobenzoate, 0.067 m*M*; hydroxymethyltetrahydropteridine, 0.033 m*M*; ATP, 3.3 m*M*, TPN, 0.067 m*M*; $MgCl_2$, 6.7 m*M*; sulfathiazole, 0.017 m*M*; phosphate buffer, 0.07 *M*; mercaptoethanol, 67 m*M*; and enzyme preparation equivalent to 6 mg. of protein; the total volume of each reaction mixture was 1.5 ml. The first incubation was for 2 hr. anaerobically at 37° in special tubes. Missing components were then added to the reaction mixtures (except for Reaction Mixtures 5 and 6; see footnote *b*) by means of a syringe equipped with a hypodermic needle in order not to disturb anaerobic conditions, and the reaction mixtures were reincubated for 2 hr. at 37°. [b] After the first incubation period, these reaction mixtures were dialyzed for 16 hr. against 2000 vol. of 0.1 *M* phosphate buffer. New substrates and cofactors were then added to the dialyzed solutions, and the reaction mixtures were incubated a second time. [Reproduced with permission from *J. Biol. Chem.*, **237**, 536(1962).]

folate synthesis by sulfathiazole was studied. It was shown that sulfathiazole needs about 2 hr. for full irreversible inhibition, which means the reaction between the sulfonamide and the pteridine derivative is relatively slow. (The concentration used is 0.017 m*M* sulfathiazole and 0.033 m*M* hydroxymethyltetrahydropteridine.) Marked inhibition occurred after approximately 30–60 min. It is interesting to note that about the same time was observed in kinetic studies of growth inhibition by SNS (52, 53, 56), whereas the antagonistic action of PABA occurred immediately.

6. The irreversible inhibition can be overcome, at least to a certain degree, by increasing the concentration of the hydroxymethyltetrahydropteridine. In the author's opinion this cannot be interpreted as a competition on the enzyme surface between PABA and sulfonamide. The antagonistic effect of pteridine substrates was also observed in growing cultures (69, 197).

7. In an isotope tracer experiment in a cell-free enzyme extract where hydroxymethyltetrahydropteridine (0.17 m*M* and sulfanilic-^{14}C acid (0.67 m*M*) were incubated, one or two radioactive compounds were obtained with different R_f values. The nature of these compounds is unknown, but suggested to be a reaction product between sulfanilic acid and the pteridine alcohol. The reaction requires Mg^{2+} ions (Table XII).

TABLE XII—PTERIDINE AND Mg^{++} REQUIREMENTS FOR IRREVERSIBLE INHIBITION BY SULFATHIAZOLE OF "FOLATE" SYNTHESIS

(Reaction mixtures were prepared and incubation procedures were followed as described in Table XI.)

Component Omitted from First and Added Before Second Incubation	Folate Equivalents Produced, mcg.
None	5.6
p-Aminobenzoate	1.2
p-Aminobenzoate, hydroxymethyltetrahydropteridine	6.0
Hydroxymethyltetrahydropteridine	5.0
p-Aminobenzoate, $MgCl_2$	5.1
$MgCl_2$	5.1

[Reproduced with permission from *J. Biol. Chem.*, **237**, 536 (1962).]

It should be mentioned that Wacker *et al.* (70) did not succeed in isolating such a product from bacterial cultures after incubation of sulfanilamide-C^{14}, whereas they were able to isolate a folic acid analog after incubation of *p*-aminosalicylic acid (199).

Under the assumption that the pathway for the biosynthesis of tetrahydrofolic acid and coenzyme F (84) is correct and that an equilibrium between the alcohol and the aldehyde of the pteridine component exists, a hypothesis about the mode of action was developed (44), which may explain the observed phenomena of the antibacterial action of SNS and even 4-aminosalicylic acid without the assumption that PABA and SNS compete in an antagonistic reaction at the same receptor (Fig. 10). Both of the models are in agreement with the kinetically derived equations for the apparent first-order steady-state growth rate (56).

1. Sulfanilamide and other aromatic amines inhibit the biosynthesis of coenzyme F by forming Schiff bases (Fig. 10, reaction sequence *9, 10, 11*) with formyltetrahydropteridine, which is part of a direct reaction sequence (*2, 4, 5, 7*) or in equilibrium with the corresponding alcohol. The relative strength of the inhibitor compounds depends on their affinity for the aldehyde component, according to the different reactivity of the primary amino group and their possible ability to permeate into the bacterial membrane (35, 56, 89, 92). Different heats of activation obtained by Garrett (56), in a kinetic study of growth inhibition by sulfathiazole (ΔH_a = 4.3 Kcal./mole) and by sulfisoxazole (ΔH_a = 15.9 Kcal./mole) were interpreted in such a way "that the receptor site–drug interaction or active transport process have greater significance in the sulfisoxazole than in the sulfathiazole." The ΔH_a value for sulfathiazole justifies the assumption that the passive diffusion process into the bacterial cell is

rate determining. This is not confirmed, however, by the pKa_2' values being 4.9 for sulfisoxazole and 6.8 for sulfathiazole. Later there was no difference in lag phase of 3.7 generation times observed.

The rate of formation of Schiff base depends on the law of mass action. If there is sufficient concentration of sulfonamide, the equilibrium is changed in the direction of the Schiff base. Under these conditions the concentration of aldehyde component decreases and the formation of folic acid also, but it is not completely blocked. This would explain why there is no fixed minimum inhibitory concentration *in vitro*. It would also explain the fact that inhibition does not occur immediately (52, 53, 56, 187). The antagonistic action of PABA, however, does occur immediately.

2. 4-Amino benzoic acid exerts its physiological function and the antagonistic function against SNS in the reaction step *6* or *7* for 4-aminobenzoyl glutamic acid, respectively.

3. All substituted *p*-aminobenzoic acids, which do not inhibit the reaction *6* and *7* and which are incorporated in an active coenzyme F analog may act like PABA as antagonists of SNS [see *p*-aminosalicylic acid (PAS) in enterococci (199)] and the antagonistic action of PAS (200).

4. If a substituted PABA derivative is incorporated in a precursor of coenzyme F, like PABA, however, the further synthesis of the enzyme is blocked or the activity of the coenzyme F is decreased or completely absent, then this PABA derivative acts like an inhibitor of bacterial growth. The relative strength of the inhibitory effects depends on two parameters, the affinity of the compound to the other compound involved in the rate-determining step and on the metabolic activity of the coenzyme F analog (32). These compounds may act like SNS and form Schiff bases (see Statement 1).

5. The relation between PABA and the effect of compounds which act as in Statement 4 depend on the concentration used. In the range of low concentrations PABA acts as an antagonist if the affinity and the metabolic activity is stronger than that of the inhibitors. At high concentrations PABA acts like an inhibitor (see Statement 1).

6. A certain limit of concentration of coenzyme F is necessary for a normal metabolism. Concentrations which are too high or too low disturb bacterial growth.

The new assumption of this hypothesis is that SNS and PABA do not compete in the same reaction. The competitive antagonism between PABA and SNS occurs along the reaction chain *9*, *3*, *4*, *5*, *7*, and *6*, and is not a direct one. Some experimental results shall be discussed concerning this hypothesis.

The PABA antagonists can be divided into two groups, those which react like sulfanilamide, *etc.* (see Statement 1) and compounds which react like compounds mentioned under Statement 4 [*e.g.*, 4-aminosalicylic acid which can be incorporated in a coenzyme F analog (199) and salicylic acid which can be aminated in the 4 position by some bacterial strains (199)]. The division of the compounds into these two groups is supported by some bacteriological experiments (43, 44). According to these results SNS is much more active against a strain of *Mycobacterium smegmatis* than against some strains of *Mycobacterium tuberculosis* and *Mycobacterium bovis*, and the activity of 4-aminosalicylic acid is the reverse. This is in contradiction to the relatively close relation of the MIC of different sulfonamides on different bacterial strains. The relatively strong inhibitory effect of PABA against *Mycobacterium smegmatis* is striking, whereas this compound has nearly no inhibitory effect against *Mycobacterium tuberculosis*. Therefore the inhibitory effect of PABA, which was already noted by Woods (11), belongs to the sulfonamide type (see Statement 1 and Reaction *10* in Fig. 10). The inhibitory effect of PABA occurs only at much higher concentrations than those which afford antagonistic action against SNS. Therefore it could be assumed that this inhibition is caused by Schiff base formation. The experimental results support the assumption that the folic acid metabolism is different in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*.

The 4-aminosalicylic acid (PAS) holds a special position (see Statement 4). The experimental results may be explained by the assumption that the metabolic activity of the PAS analog of coenzyme F is nearly the same as that of the natural coenzyme F, therefore, PAS may act in these bacterial strains (*E. coli*, enterococci) according to Statement 3 of the hypothesis. This explains that the antibacterial activity of SNS against *Staphylococcus* can be antagonized either by PABA or by PAS (200).

With Statement 6 of the hypothesis it may be possible to explain the results of Emmerson and Cushing (201) and Emmerson (202, 203) and Zalokar (204) about mutants of *Neurospora crassa* which either need SNS or SNS and PABA for growth. The results were interpreted as showing that the mutant was producing too much PABA, and the excess PABA was producing a

toxic compound. This process should be more easily inhibited by sulfonamide than the normal function of PABA. According to the new hypothesis it is very likely that the toxic compound is an excess of coenzyme F. It is well known that sulfonamide-resistant strains produce much more PABA (205, 206) and that other sulfonamide-resistant strains need folic acid, which is not the case in normal strains (207, 208). The antagonistic action of PABA against PAS is essentially weaker (209) and can be taken as support of the hypothesis.

The given examples, however, are not sufficient to prove the hypothesis which was developed before the data of Brown (35) were available. Nevertheless most of these data can be interpreted by this hypothesis. It should be noted that the experiments done by Brown do not correspond to the steady-state condition in growing cultures. Only if the reactions are operating in the proximity of their equilibrium is there possible validity to the assumption of an indirect competitive antagonism along the given reaction chain (see Fig. 10).

$$\frac{SB}{DHF} = \frac{SNS \cdot K_3 \cdot K_4 \cdot K_5 \cdot K_6 \cdot K_7 \cdot DHF}{PAG \cdot K_9}$$

where SB = Schiff-base; DHF = dihydrofolic acid; SNS = sulfonamide; PAG = 4-aminobenzoylglutamic acid. The final decision of whether PABA and SNS do compete in an enzymatic reaction for the same compound or whether the competitive antagonism between PABA and SNS is not direct but occurs along a reaction sequence is still open. The final conclusion will be the identification of the compound which was found by Brown (35) in his isotope tracer experiment but could not be detected by Wacker (70). This contradiction may be interpreted in favor of the second assumption. This means that the difference of the experimental findings of Wacker (70), who used growing cultures, and Brown (35), who used cell-free enzyme extracts, might be explained in such a way that the product which is formed in both experiments, was hydrolyzed by the necessary washing process used by Wacker (70). This is possible only if the metabolic compound is a Schiff base type, but not if a covalent bond exists. Under the experimental conditions used by Brown (35), a washing procedure was not necessary and the Schiff base could be detected. On the other hand it should be mentioned that the need for Mg^{2+} ions is in favor of an enzymatic reaction. The hypothesis of the mere receptor-blocking action of SNS and the importance of the polarity of the SO_2 group according to Bell and Roblin (41) is not supported by the experimental findings of the last few years. The results support the assumption that the primary aromatic amino group is a decisive factor.

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Keyphrases

Sulfonamides—structure, activity relationship
Biological action, sulfonamides—chemical structure
PAB antagonists—chemical reactivity
Dipole moment—sulfonamides
Spectral properties—sulfonamides, precursors
Mechanism of action—sulfonamides

Research Articles

Solvolysis of 5-Halouridines and Related Nucleosides

By EDWARD R. GARRETT and GERALD J. YAKATAN*

The neutral and alkaline solvolyses of uridine (UD) derivatives substituted in the 5 position with I (IUD), Br (BUD), Cl (CUD), and CH_3 (MUD) were followed spectrophotometrically. The order of reactivity was IUD > BUD >CUD > UD >>> MUD (essentially stable). Ribosylbarbituric acid (RBA) was identified as a product of solvolysis of IUD and BUD in strong alkali and is itself unstable. The halouridines also degrade to nonchromophoric compounds. The rate-pH profiles for all the halouridines are similar and can be explained by hydroxyl ion attack on both the undissociated and dissociated halouridine. Hydroxyl ion attack on the undissociated species leads to the formation of nonchromophoric products. Hydroxyl ion attack on the dissociated species forms RBA. 5-Hydroxyuridine (OHUD) and dihydropyrimidines are postulated as intermediates. The lability of the halogen substituent enhances the ease of solvolysis. The rate of alkaline solvolysis of BUD is the same as the rate of bromide ion production and shows that the reaction intermediates are highly unstable. The arabino and lyxo derivatives of 5-fluorouracil are solvolyzed faster than all of the halouridines. The Arrhenius parameters for all compounds were determined.

SEVERAL NUCLEOSIDES have been shown to be active chemotherapeutic agents in the treatment of viral diseases (1, 2). Quantitative studies on the stability of nucleosides and the effects of structure and substituents should provide insight into their possible metabolic transformations, and they are of pharmaceutical importance for the estimation of maximum stability and inhibition of the formation of toxic side products (3,

Received April 15, 1968, from the College of Pharmacy, University of Florida, Gainesville, FL 32601
Accepted for publication May 31, 1968.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
This work was supported in part by grants GM-09864-05 and CA 10738-06 from the National Cancer Institute, and the National Institutes of Health, U. S. Public Health Service.
The authors gratefully acknowledge preliminary experiments performed on parts of this work by Drs. Joachim K. Seydel and Alan J. Sharpen.

* NIH Predoctoral Fellow (1965), Fellowship No. 5-F1-GM-29, 944.

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Keyphrases

Sulfonamides—structure, activity relationship
Biological action, sulfonamides—chemical structure
PAB antagonists—chemical reactivity
Dipole moment—sulfonamides
Spectral properties—sulfonamides, precursors
Mechanism of action—sulfonamides

Research Articles

Solvolysis of 5-Halouridines and Related Nucleosides

By EDWARD R. GARRETT and GERALD J. YAKATAN*

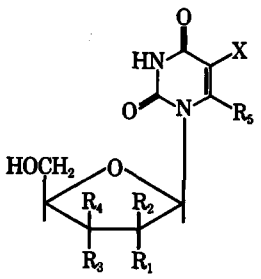
The neutral and alkaline solvolyses of uridine (UD) derivatives substituted in the 5 position with I (IUD), Br (BUD), Cl (CUD), and CH_3 (MUD) were followed spectrophotometrically. The order of reactivity was IUD > BUD >CUD > UD >>> MUD (essentially stable). Ribosylbarbituric acid (RBA) was identified as a product of solvolysis of IUD and BUD in strong alkali and is itself unstable. The halouridines also degrade to nonchromophoric compounds. The rate-pH profiles for all the halouridines are similar and can be explained by hydroxyl ion attack on both the undissociated and dissociated halouridine. Hydroxyl ion attack on the undissociated species leads to the formation of nonchromophoric products. Hydroxyl ion attack on the dissociated species forms RBA. 5-Hydroxyuridine (OHUD) and dihydropyrimidines are postulated as intermediates. The lability of the halogen substituent enhances the ease of solvolysis. The rate of alkaline solvolysis of BUD is the same as the rate of bromide ion production and shows that the reaction intermediates are highly unstable. The arabino and lyxo derivatives of 5-fluorouracil are solvolyzed faster than all of the halouridines. The Arrhenius parameters for all compounds were determined.

SEVERAL NUCLEOSIDES have been shown to be active chemotherapeutic agents in the treatment of viral diseases (1, 2). Quantitative studies on the stability of nucleosides and the effects of structure and substituents should provide insight into their possible metabolic transformations, and they are of pharmaceutical importance for the estimation of maximum stability and inhibition of the formation of toxic side products (3,

Received April 15, 1968, from the College of Pharmacy, University of Florida, Gainesville, FL 32601
Accepted for publication May 31, 1968.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
This work was supported in part by grants GM-09864-05 and CA 10738-06 from the National Cancer Institute, and the National Institutes of Health, U. S. Public Health Service.
The authors gratefully acknowledge preliminary experiments performed on parts of this work by Drs. Joachim K. Seydel and Alan J. Sharpen.

* NIH Predoctoral Fellow (1965), Fellowship No. 5-F1-GM-29, 944.

TABLE I—COMPOUNDS USED IN THIS STUDY



Compd.	X	R_1	R_2	R_3	R_4	R_5
Uridine (UD)	H	OH	H	OH	H	H
5-Iodouridine (IUD)	I	OH	H	OH	H	H
5-Bromouridine (BUD)	Br	OH	H	OH	H	H
5-Chlorouridine (CUD)	Cl	OH	H	OH	H	H
5-Methyluridine (MUD)	CH_3	OH	H	OH	H	H
5-Hydroxyuridine (OHUD)	OH	OH	H	OH	H	H
1-β-D-Ribofuranosylbarbituric acid (RBA)	H	OH	H	OH	H	OH
1-β-D-Lyxofuranosyl-5-fluorouracil (FUL)	F	H	OH	H	OH	H
1-β-D-Arabinofuranosyl-5-fluorouracil (FUA)	F	H	OH	OH	H	H

4, 6, 7). These studies may also provide information regarding new synthetic routes for derived compounds.

This quantitative investigation of the chemical transformations of the 5-halouridines is one aspect of the authors' continuing study of the solvolytic reactions of pyrimidine and purine nucleosides and their derivatives (4–10). The purpose of this paper is to report on the kinetics and possible mechanisms of solvolysis of the 5-substituted halouridines and the determination of the pertinent thermodynamic parameters. The major emphasis was on derivatives of uridine (UD) substituted in the 5 position with I (IUD), Br (BUD), Cl (CUD), and CH_3 (MUD). The structures of some of the pyrimidine nucleosides discussed are given in Table I.

EXPERIMENTAL

Materials—IUD, BUD, CUD, MUD, and 5-hydroxyuridine (OHUD) were purchased from Calbiochem and were all certified as chromatographically homogeneous by the company. UD was purchased from Nutritional Biochemicals Corporation, an elementary analysis, C, H, and N was provided, and the compound was used without further purification. Dr. T. Ukita of the University of Tokyo kindly provided a sample of the sodium salt of 1-β-D ribofuranosylbarbituric acid which was used in the paper chromatography studies. Dr. J. J. Fox of the Sloan-Kettering Research Institute supplied 1-β-D-ribofuranosylbarbituric acid monoalcoholate (SKI No. 30,057) for use in the kinetic studies on this compound. Dr. Fox also provided samples of 1-(β-D-lyxofuranosyl)-5-fluorouracil(FUL) and 1-(β-D-arabinofuranosyl)-5-fluorouracil(FUA). All other materials employed in this study were of analytical reagent grade.

Kinetic Procedures—Appropriate quantities of the nucleosides to produce final concentrations of 10^{-2} *M* were weighed into volumetric flasks and diluted to volume with nitrogen-purged distilled water. From these stock solutions, aliquots were taken and diluted with alkali of appropriate concentration or with an appropriate buffer solution to produce, in general, a final nucleoside concentration of 10^{-4} *M*. The solutions of alkali and buffer were previously equilibrated at the temperature of the study.

The reactions were maintained in constant-temperature baths controlled within 0.1° at selected temperatures between 60 and 80° and samples withdrawn at suitable time intervals. Spectra were recorded on the Cary model 15 recording spectrophotometer or spectrophotometric readings were taken on the Beckman model DU-2 spectrophotometer. Matched spectrophotometric cells (1.00 cm.) were employed for all measurements and a slit width of 0.10 mm. was used. The principal ultraviolet spectral data for the 5-halouridines have been reported previously (8, 11). Alkali solutions were prepared by dilution of a standardized NaOH stock solution with nitrogen-purged distilled water. The NaOH solution was standardized by titration with a standard perchloric acid solution on the Sargent model D recording titrator. All reactions run in buffer solutions were maintained at constant ionic strength of 0.15.

pH and pK_a Measurements—The pH values of the NaOH solutions at the temperatures of study were calculated from activity coefficient data available in the literature (12). The pH values of the buffer solutions were measured directly on a Beckman Zeromatic pH meter using Beckman high temperature electrodes and a thermostated cell to contain the buffer solution at the temperature of study. The pH meter was standardized with standard buffers at the same temperature. The pK_a's of the 5-halouridines were determined by titration of 0.05 meq. of the nucleoside with a standard NaOH solution on the Sargent model D recording titrator. The measurements were carried out in a thermostated cell at 80°.

Paper Chromatography—Paper chromatograms were prepared from Whatman No. 1 chromatography paper. The chromatograms were usually spotted with 25 μl. of sample and developed for more than a 12-cm. travel of solvent front in a *t*-butyl alcohol–methyl ethyl ketone–water–ammonium hydroxide (40:30:20:10) solvent which had previously been reported as useful for the separation of nucleosides and related compounds (13). They were then dried, and the resulting separation was viewed under a short wavelength ultraviolet light. The chromatograms were also placed in an iodine chamber to visualize spots which did not possess an ultraviolet chromophore. The nucleosides were identified by comparison of R_f values of the unknown with known standards spotted on the same chromatogram. Freshly prepared Ehrlich's reagent (14) was used to identify 1-β-D-ribofuranosylbarbituric acid (15).

Bromide Ion Measurement—An Orion bromide ion activity electrode, model 94–35, was used with a calomel reference electrode and a Beckman Zeromatic pH meter to follow bromide ion production from solvolysis of 10^{-2} *M* BUD in 0.40 *N* NaOH at

80°. Aliquots of the degrading solution were taken as a function of time and diluted with 0.5 *N* $HClO_4$ to give a pH of about 1. The resultant potential was used to determine bromide ion concentration. The calibration curve was constructed from the measured potentials of known amounts of NaBr prepared in a similar manner with the same ionic strength and pH values.

RESULTS

Rate Constants—The kinetic parameters describing the solvolysis of the 5-halouridines in alkali and the alkaline buffer region were determined by following the loss of absorbance, A, of the ultraviolet chromophore as a function of time. The spectra of IUD and BUD showed a decided hypsochromic shift in $\lambda_{max.}$ as solvolysis proceeded in strong alkali. A small residual absorbance, A_∞, was noted in some cases at the completion of the reaction. Typical spectral changes as a function of time are given for IUD, BUD, and CUD in 0.40 *N* NaOH in Figs. 1–3. Plots of ln $(A - A_\infty)$ at the $\lambda_{max.}$ *versus* time were reasonably linear for all studies according to the first-order expression:

$$\ln (A - A_\infty) = \ln (A_0 - A_\infty) - kt \quad \text{(Eq. 1)}$$

where A_0 is the absorbance at zero time and k is the apparent first-order rate constant. However, similar plots at lower wavelengths for IUD and BUD indicated the possibility of a sequential process where the intermediate possessed an ultraviolet chromophore.

Subsequent experiments, to be discussed later in this paper, revealed the identity of this intermediate and permitted the determination of the spectrum of this compound. Once this was known, it was possible to choose a wavelength to obtain the apparent first-order loss of the original nucleoside without spectral interference from the intermediate. The rate constants obtained by this method and the conditions under which they were obtained are listed in Tables II and III.

Several typical first-order plots at various alkali concentrations according to Eq. 1 at a wavelength where only IUD absorbs, *i.e.*, 290 mμ, are shown in Fig. 4. Reactions run with a fourfold increase in substrate concentration, in the absence of oxygen, and protected from light showed no significant changes from the apparent first-order rate constants obtained when these parameters were not controlled (see footnotes to Table II). No significant ionic strength effects or buffer catalysis were observed in any of the buffer regions for the solvolysis of the halouridines (see footnotes to Table II).

UD, FUL, and FUA degraded in alkali with no apparent perturbation of the $\lambda_{max.}$ values 263, 268, and 272 mμ, respectively. These compounds solvolyze more rapidly than the halouridines and plots according to Eq. 1 where $A_\infty \sim 0$ are linear and parallel for data obtained at several wavelengths. The obtained rate constants are given in Table III.

The alkaline solvolysis of 5-hydroxyuridine was complex. The $\lambda_{max.}$ of 303 mμ in alkali (or the $\lambda_{max.}$ of 278.5 mμ of the acidified samples) decreased with time with complete destruction of the chromophore. First-order plots of the data showed good linearity at lower temperatures, but apparent sequential rates were observed at higher temperatures (Table III).

Rate-pH Profiles—The rate-pH profiles for all of the 5-halouridines were similar. The profiles were constructed from the apparent first-order rate constants, k, and the pH values at 80° (Table II) and are shown in Fig. 5. The rate-pH profiles indicate specific hydroxyl-ion catalysis as well as a pH independent region of solvolysis. The apparent first-order rate constant at a given pH can be defined as in Eq. 2.

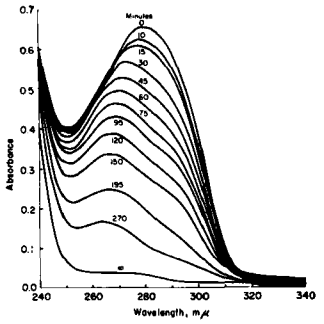


Fig. 1—Typical spectral changes for the solvolysis 10^{-4} M 5-iodouridine in 0.40 N NaOH at 80°. The curves are labeled as to minutes after the start of the reaction.

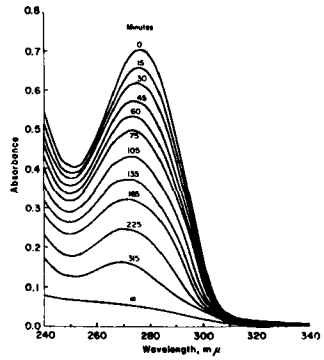


Fig. 2—Typical spectral changes for the solvolysis of 10^{-4} M 5-bromouridine in 0.40 N NaOH at 80°. The curves are labeled as to minutes after the start of the reaction.

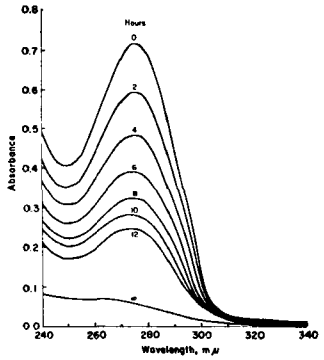


Fig. 3—Typical spectral changes for the solvolysis of 10^{-4} M 5-chlorouridine in 0.40 N NaOH at 80°. The curves are labeled as to hours after the start of the reaction.

TABLE II—APPARENT FIRST-ORDER RATE CONSTANTS, 10^5k IN SEC.$^{-1}$, FOR THE SOLVOLYSIS OF SOME PYRIMIDINE RIBOSIDES AT 80.0°

pH[a]	Medium	IUD Calcd.	IUD Exptl.	BUD Calcd.	BUD Exptl.	CUD Calcd.	CUD Exptl.	RBA Calcd.	RBA Exptl.	UD[f] Exptl.	UD[f] Calcd.
6.00	Phosphate buffer	—	—	0.080	0.125	0.052	0.086	—	—	—	—
6.87[b]	Phosphate buffer	0.539	0.570	0.478	0.546	0.299	0.311	1.40	1.41	—	—
7.09	Phosphate buffer	0.730	0.823	0.688	0.757	0.426	0.443	1.40	1.47	—	—
7.69	Phosphate buffer	1.83	1.82	1.39	1.34	0.825	0.779	1.40	1.40	—	—
7.91	Phosphate buffer	2.30	2.19	1.60	1.69	0.924	0.859	1.41	1.36	—	—
8.28	Phosphate buffer	2.94	2.69	1.86	1.96	—	—	—	—	—	—
9.08	Bicarbonate buffer[g]	3.42	3.28	2.07	2.01	1.18	1.16	1.51	1.57	—	—
9.40	Bicarbonate buffer[g]	3.66	3.50	2.09	2.12	1.19	1.19	1.63	2.13	—	—
9.65[c]	Bicarbonate buffer[g]	3.67	3.60	2.13	2.17	1.21	1.23	1.81	3.45	—	—
9.90	Bicarbonate buffer[g]	3.74	3.80	2.14	2.32	1.22	1.28	2.13	3.70	—	—
10.54	0.01*N* NaOH	3.95	3.72	2.36	2.94	1.28	1.52	4.48	4.11	—	—
11.20	0.05*N* NaOH	4.76	5.01	3.59	4.18	1.56	1.94	13.5	15.8	0.189	0.156
11.49[d]	0.10*N* NaOH	6.50	6.00	4.51	4.81	1.89	2.49	21.4	24.1	0.326	0.270
11.75	0.20*N* NaOH	7.89	8.00	7.28	7.18	2.46	2.70	30.7	31.6	0.491	0.421
12.05[e]	0.40*N* NaOH	13.1	11.0	11.9	9.49	3.71	3.51	41.9	37.2	0.601	0.630
12.23	0.60*N* NaOH	16.2	16.1	14.2	14.3	—	—	—	—	—	—
12.35	0.79*N* NaOH	—	—	—	—	—	—	—	—	0.701	0.840

[a] The pH values of the phosphate and bicarbonate buffer solutions were read at 80° on a pH meter standardized at that temperature. The other pH values were calculated from $pH = pK_w - p\text{OH}$ where $p\text{OH} = -\log\gamma\,[\text{NaOH}]$ where the activity coefficient, γ, and the pK_w values were obtained from the literature (12). [b] Additional values at this pH were: BUD, 0.20 *M* buffer, $\mu = 0.36$, $10^5k = 0.566$; 0.30 *M* buffer, $\mu = 0.36$, $10^5k = 0.530$. [c] Additional values at this pH were: BUD, $\mu = 0.10$, $10^5k = 2.12$; 0.10 *M* buffer, $\mu = 0.21$, $10^5k = 2.21$: IUD, $\mu = 0.21$, 0.10 *M* buffer, $10^5k = 2.21$: IUD, $\mu = 0.21$, 0.10 *M* buffer, $10^5k = 3.70$: CUD, $\mu = 0.10$, $10^5k = 1.28$; $\mu = 0.21$, 0.10 *M* buffer, $10^5k = 1.28$: RBA, $\mu = 0.21$, 0.10 *M* buffer, $10^5k = 3.70$; $\mu = 0.32$, 0.15 *M* buffer, $10^5k = 4.50$. [d] IUD protected from light, $10^5k = 6.10$; BUD with solution purged with nitrogen for 3 min. after each sample was removed, $10^5k = 4.71$. [e] Fourfold increase in initial concentration (4×10^{-4} *M*) gave $10^5k = 9.91$ for BUD and 11.5 for IUD. [f] The experimental data are subject to some error resulting from silica formation in reaction vessels which prevented the accurate determination of any A_∞ values. [g] All bicarbonate–carbonate buffers in rate-pH profile were 0.05 *M*. $[CO_3^=]$ at the various pH values were: 9.08, 0.004 *M*; 9.40, 0.016 *M*; 9.65, 0.028 *M*; 9.90, 0.039 *M*.

TABLE III—APPARENT FIRST-ORDER RATE CONSTANTS, 10^5k IN SEC.$^{-1}$, AND THERMODYNAMIC PARAMETERS FOR THE SOLVOLYSIS OF SOME PYRIMIDINE NUCLEOSIDES AT VARIOUS TEMPERATURES

°C.	IUD 0.4 *N* NaOH	IUD pH 9.81[c]	BUD 0.4 *N* NaOH	BUD pH 9.81[c]	CUD 0.4 *N* NaOH	CUD pH 9.81[c]	RBA 0.4 *N* NaOH	UD 0.4 *N* NaOH	FUL[a] 0.4 *N* NaOH	FUA[b] 0.4 *N* NaOH	OHUD 0.4 *N* NaOH[f]
80	11.0	3.70	9.49	2.30	3.51	1.28	37.2	0.601	—	—	101 14.8
75	7.90	2.26	5.76	1.45	1.92	0.789	—	0.382	—	—	—
70	4.61	1.44	3.47	0.865	1.14[d]	0.484	17.4[e]	0.256	27.8	77.7	67.6 6.92
60	1.94	—	—	—	0.414	—	6.37	—	14.0	38.0	24.7 4.98
50	—	—	—	—	—	—	—	—	5.54	16.1	1.97
40	—	—	—	—	—	—	—	—	2.30	6.12	—
ΔH_a (kcal./mole)	20.4	22.7	24.4	23.7	24.3	23.7	20.5	21.2	18.8	18.8	15.2
Log *P*	8.67	9.61	11.1	10.0	10.6	9.77	9.26	7.90	8.48	8.91	5.52

[a] Additional values were: 0.20 *N* NaOH, 25°, 0.303; borate buffer, pH 9.45, 80°, 15.2. [b] Borate buffer, pH 9.55, 80°, 89.0. [c] Bicarbonate–carbonate buffer. [d] 69.4°. [e] 71.0°. [f] OHUD is susceptible to oxidative degradation and oxygen was not excluded in these experiments. Higher temperatures gave apparent sequential rates and estimates of the rate constants obtained by polyexponential analysis are given. ΔH_a and log *P* values are for the slower reaction.

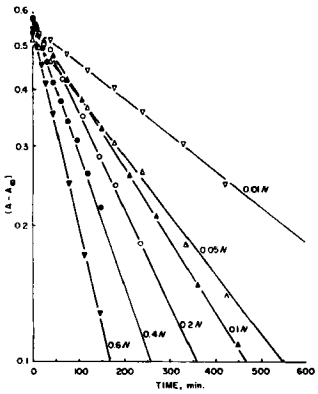


Fig. 4—Typical apparent first-order plots for the solvolysis of 5-iodouridine in various NaOH concentrations at 80°. The absorbance values, A, were measured at 290 mμ and A_∞ is the final asymptotic absorbance.

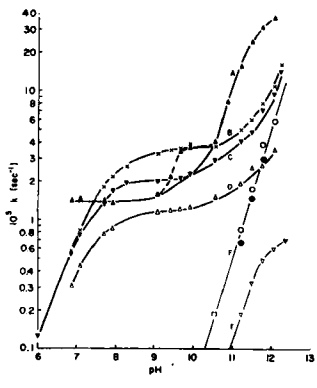


Fig. 5—Rate-pH profiles for the solvolysis of some pyrimidine nucleosides at 80°. Key: A, RBA; B, IUD; C, BUD; D, CUD; E, UD. The open and closed circles for line F show the dependence on pH of the apparent first-order rate constants for the production of RBA from IUD and BUD, respectively.

$$k = \{k_0 + k_{OH}[OH^-]\} f_u + \{k''_0 + k'_{OH}[OH^-]\} f_{u^-} \quad \text{(Eq. 2)}$$

where k_{OH} and k'_{OH} are the bimolecular rate constants for hydroxyl ion attack on the undissociated (f_u) and dissociated (f_{u^-}) halouridine, respectively, and k_0 and k''_0 are the apparent first-order rate constants for water attack on the undissociated and dissociated species, respectively. Hydroxyl ion attack on the undissociated species is kinetically equivalent to water attack on the monoanion (16), and water attack on the undissociated species is either nonexistent or negligible, so that Eq. 2 can be written:

$$k = k_{OH}[OH^-] f_u + k'_{OH}[OH^-] f_{u^-} \quad \text{(Eq. 3)}$$

or as its kinetic equivalent:

$$k = \{k'_0 + k'_{OH}[OH^-]\} f_{u^-} \quad \text{(Eq. 4)}$$

The Eq. 3 can be rewritten by substituting for f_u and f_{u^-} in terms of the equilibrium constant, K_a, for the dissociation of the halouridine to the monoanion (14):

$$k = \{k_{OH}[OH^-]\} \frac{1}{1 + K_a/[H^+]} + \{k'_{OH}[OH^-]\} \frac{1}{1 + [H^+]/K_a} \quad \text{(Eq. 5)}$$

at pH values <9.5, the profiles can be fit by the first term of Eq. 5, *i.e.*,

$$k = \{k_{OH}[OH^-]\} \frac{[H^+]}{[H^+] + K_a} \quad \text{(Eq. 6)}$$

At pH values >9.5, the second term of Eq. 5 contributes to the observed first-order rate constant. The bimolecular rate constants which produce the best fits of the rate-pH profiles and the kinetic pKa values determined from the profiles are given in Table IV. The rate constants calculated from the values of Table IV and Eq. 3 are shown in Table I. The calculated fits obtained for the 5-halouridines are consistent with the experimental data.

The rate-pH profile for uridine (Fig. 5) is different from those for the 5-halouridines. The profile definitely bends over at high pH values and indicates that another pKa′ (attributable to the sugar moiety) influences the rate of reaction. An expression that fits the uridine data is

$$k = k'_{OH}[OH^-] f_{u^-} \quad \text{(Eq. 7)}$$

or its kinetic equivalent

$$k = k''_0 f_{u^-} \quad \text{(Eq. 8)}$$

Table IV—Catalytic Rate Constants for the Solvolysis of Some Pyrimidine Nucleosides at 80°

	IUD	BUD	CUD	RBA	UD
pKa′[a]	7.75	7.40	7.35	11.85[b]	12.1[c]
k_{OH}	3.16	3.56	2.29	—	—
$10^4 k'_{OH}$	2.82	3.02	0.955	39.8	0.479
$10^5 k'_0$	4.15	2.09	1.20	1.40	—

[a] Values reported are determined from best fit of rate-pH profile. pKa′ values determined by titration without control of ionic strength at 80° were: IUD, 7.98; BUD, 7.65; CUD, 7.63. [b] Value reported is for second pKa′ at 80°. Literature value at 25° is 12.4 (15). [c] Value is attributed to sugar pKa′ at 80°.

The catalytic constant and an estimate of the sugar pKa′ of uridine are given in Table IV.

The rate-pH profile for RBA is also different from those of the halouridines. In this case, the second pKa′ is implicated in the rate-pH profile since the second pKa′ for this compound has been reported to be 12.4 at 25° (15) and would be even lower at 80°. A definite bending of the profile in strong alkali can be observed. One of the kinetically equivalent expressions that fit the RBA data for pH values >6.8 >pKa′$_1$ = 3.6 at 25° is

$$k = \{k'_0 + k'_{OH}[OH^-]\} f_{u^-} \quad \text{(Eq. 9)}$$

The Eq. 9 can be rewritten in terms of the second pKa of RBA as:

$$k = \left\{k'_0 + k'_{OH}[OH^-]\right\} \frac{1}{1 + K_{a_2}/[H^+]} \quad \text{(Eq. 10)}$$

Values for the best fit of this profile and the appropriate constants are also found in Tables IV and II, respectively. The discrepancies observed in the bicarbonate buffer region could only be due to catalysis by buffer species since RBA has no pKa in this pH region. The observed buffer catalysis is denoted by a dashed line in the rate-pH profile for RBA (Fig. 5). Buffer catalysis was confirmed by observing an increase in the apparent first-order rate constant with increasing buffer concentration at constant pH (footnote to Table II). The intercept of a plot of $k_{app.}$ *versus* buffer concentration is the apparent first-order rate constant in the absence of buffer effects according to:

$$k_{app.} = k_{hyd.} + k_{buff.}[\text{BUFFER}] \quad \text{(Eq. 11)}$$

The value obtained for $k_{app.}$ was consistent with the calculated value for RBA solvolysis at this pH obtained from Eq. 9. The smooth curve in the rate-pH profile in the bicarbonate buffer region is drawn in agreement with this information.

Dependence of Rate on Temperature—Estimates of the Arrhenius parameters for the solvolysis of several pyrimidine nucleosides were obtained from the slopes and intercepts of plots of the logarithm of the apparent first-order rate constants, k, *versus* the reciprocal of the absolute temperature, T, in accordance with the expression:

$$\log k = \log P - \Delta H_a/2.303\,RT \quad \text{(Eq. 12)}$$

where R is 1.987 cal. deg.$^{-1}$ mole^{-1} and the various k, ΔHa, and log P values at several pH values are given in Table III. Typical Arrhenius plots are shown in Fig. 6.

Identification of Reaction Intermediate—The possibility existed that an intermediate from the alkaline solvolysis of halouridines might be a barbituric acid derivative such as 1-β-D-ribofuranosylbarbituric acid which had been recently synthesized (15). Halouracils do form barbituric acids on attack by alkali (17, 9). Evidence that this compound was an intermediate in the alkaline solvolysis of the halouridines was obtained in the following manner:

(*a*) Ukita and co-workers (15) reported that RBA had pKa's of 3.6 and 12.4 and a $\lambda_{max.}$ in alkali at 263 mμ. By performing *in situ* spectrophotometric pKa measurements on samples of IUD and BUD degraded in 0.40 *N* NaOH at 80° for 1 hr., the authors observed a pKa of 3.7 and another pKa′

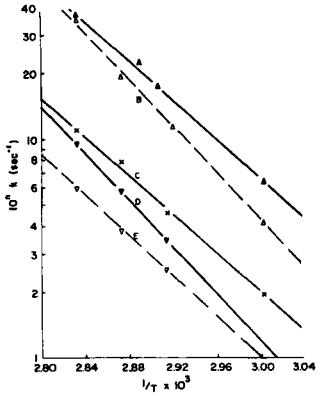


Fig. 6—Typical Arrhenius plots for several pyrimidine nucleosides in 0.40 N NaOH. Key: A, RBA; B, CUD; C, IUD; D, BUD; E, UD; n = 5 for lines A, C, and D; n = 6 for B and E.

attributable to the undegraded halouridine. The degraded mixture also showed a $\lambda_{max.}$ at 264 mμ in strong alkali.

(*b*) The concentration of IUD at any time could be determined from its absorptivity value, ϵ, at a wavelength where the products of degradation did not absorb. The calculated absorbance due to IUD at any time can be subtracted from the total absorbance (Fig. 2) at each wavelength to permit the plotting of these differences against wavelength. The plots for several time intervals at every 5 mμ from 330 to 230 mμ showed the presence of a chromophore with a sharp maximum at 264 mμ consistent with the RBA and different from the spectrum of barbituric acid. The ratios of the absorbances of these difference spectra at any two wavelengths gave constant values which were consistent with the spectra of RBA obtained with the same solvent conditions.

(*c*) The molar absorptivity of RBA in acid solution is negligible (18) but is high in alkaline solution (15,470 in *N* NaOH) (18). This was consistent with the experimental observation that acidified alkaline-degraded BUD showed only the spectrum of BUD with no perturbation of the $\lambda_{max.}$ of BUD in acid solution. Conversion of the acid solution back to alkaline pH values regained the original spectra shown in Fig. 3 with definitive indications of the RBA chromophore. The low ϵ value of RBA in acid solution does not permit its spectral observance in low concentrations.

(*d*) Paper chromatography of alkaline degraded IUD and BUD demonstrated two spots having ultraviolet absorption. One spot was identified as the undegraded halouridine by comparison with a standard. The other spot immediately turned orange on spraying with Ehrlich's reagent, a characteristic reaction for barbituric acid derivatives (15). If left untreated overnight, the RBA spot turned yellow on the chromatogram, another characteristic reaction of barbituric acid derivatives (19). The spot attributed to the undegraded nucleoside neither turned yellow on standing nor was colored orange by Ehrlich's reagent. The R_f value for RBA in the developing system used was 0.40 and a standard spot of pure RBA had exactly the same value. The R_f value for barbituric acid under the same conditions was 0.30. No chromatographic evidence for the formation of the corresponding halouracils was obtained and indicated that lysis of the sugar moiety did not occur.

(*e*) Fox and his co-workers have reported that they have recently succeeded in preparing RBA from the alkaline treatment of BUD (18). The sample of RBA he provided was used in the authors' kinetic studies on this compound.

(*f*) The ultraviolet spectra of degrading IUD and BUD indicate only a hypsochromic shift which never reaches the $\lambda_{max.}$ reported for RBA. This is entirely consistent with an RBA intermediate since it has been shown that RBA degrades in alkali faster than the halouridines and there can never be a very large amount of RBA present at any time. Only the hypersensitivity of the spectrum to RBA caused by the large ϵ value of RBA in relation to those of the halouridines allows the intermediate to be seen at all.

(*g*) The halouracils degrade under similar conditions (9) at rates only one-fifth of those for the halouridines. There was no spectral evidence of halouracil appearance and thus indicated that lysis of the sugar moiety did not occur, at least until after the pyrimidine ring had been cleaved.

Estimation of Rate Constants for Parallel Reactions—As was described previously, plots of ln $(A - A_\infty)$ *versus* time at 265 mμ indicated a sequential process was taking place during solvolysis of the halouridines. This suggested that a model describing the kinetics of solvolysis of the halouridines, XUD, might be:

$$\text{XUD} \xrightarrow{k_1} \text{RBA} \xrightarrow{k_2} \text{Nonchromophoric or rapidly degrading products (NC)} \qquad \text{(Eq. 13)}$$

The apparent first-order rate constants, k_2, were obtained by following the loss of the chromophore due to RBA under conditions equivalent to those used to study the halouridine solvolysis. These values appear in Table II. If the only hydrolysis product of the halouridines is RBA, then the concentration of RBA at any time can be predicted from the obtained values of the rate constants, k_1, for the loss of XUD; k_2 for the solvolysis of RBA, and the initial concentration of the halouridine, $[\text{XUD}]_0$, by (9):

$$\text{RBA}_{\text{PRED}} = [\text{XUD}]_0 \frac{k_1}{k_2 - k_1} (e^{-k_1 t} - e^{-k_2 t}) \qquad \text{(Eq. 14)}$$

When these calculations were carried out over a wide range of sample times and the calculated concentration of RBA converted to absorbance due to RBA, it was observed that the predicted value for the absorbance of RBA at any time was *greater* than the actual absorbance due to RBA as calculated from spectral observations by:

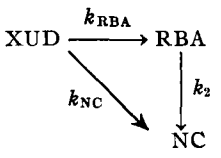
$$A_{264}^{\text{RBA}} = A_{264}^{T} - A_{264}^{\text{IUD}} \qquad \text{(Eq. 15)}$$

where A_{264}^{T} is the total absorbance at 264 mμ at any time and A_{264}^{IUD} is the absorbance at 264 mμ due to IUD at any time. A_{264}^{IUD} can be obtained from:

$$A_{264}^{\text{IUD}} = A_{290}^{T} \times \frac{\epsilon_{264}^{\text{IUD}}}{\epsilon_{290}^{\text{IUD}}} \qquad \text{(Eq. 16)}$$

This information indicated that not all of the

solvolyzed halouridine was going through the RBA intermediate. Since RBA was the only chromophoric product observed, it has been postulated that parallel reactions exist according to:



(Eq. 17)

For this case, the actual concentration of RBA present at any time, RBA_{ACT}, can be formulated (9):

$$RBA_{ACT} = [XUD]_0 \frac{k_{RBA}}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}) \quad \text{(Eq. 18)}$$

where $k_1 = k_{RBA} + k_{NC}$. The ratio of the values RBA_{ACT}/RBA_{PRED} at any time from Eqs. 18 and 14 is

$$\frac{RBA_{ACT}}{RBA_{PRED}} = \frac{k_{RBA}}{k_1} \quad \text{(Eq. 19)}$$

The actual absorbance, $A_{264}^{RBA} = \epsilon_{RBA} RBA_{ACT}$, can be calculated from Eq. 15 and the predicted absorbance, ϵ_{RBA} RBA_{PRED}, can be calculated from Eq. 12 since the ϵ value for RBA is known. Thus the ratio of Eq. 19 can be determined. From this ratio and the known apparent first-order rate constant, k_1, for the loss of XUD, k_{RBA} can be calculated. The apparent first-order rate constant to nonchromophoric products, $k_{NC} = k_1 - k_{RBA}$ can also be estimated.

The values of these ratios were obtained over wide intervals of *A versus* time plots. The resultant rate constants and the yields of RBA at different pH values are presented in Table V. The apparent first-order rate constants for RBA solvolysis were more than 10 times greater than the apparent first-order rate constants for CUD solvolysis in strong alkali. This explains why no RBA was observed and why there were no spectral shifts during CUD solvolysis.

It is interesting to note that the rate constants for the production of RBA from both IUD and BUD follow the same kinetic dependency,

$$k_{RBA} = k'_{OH}[OH^-] f_{u^-} \quad \text{(Eq. 20)}$$

TABLE V—RATE CONSTANTS AND YIELDS IN THE SOLVOLYSIS OF 5-IODO AND 5-BROMO URIDINE TO RIBOSYLBARBITURIC ACID AND NONCHROMOPHORIC PRODUCTS AT 80.0°

pH[a]	$10^5 k_1$[b]	$10^5 k_2$[c]	% RBA	%NC	$10^5 k_{RBA}$	$10^5 k_N$[c]
IUD						
10.54	3.72	4.11	5	95	0.19	3.53
11.20	5.01	15.8	17	83	0.85	4.16
11.49	6.00	24.1	29	71	1.74	4.26
11.75	8.00	31.6	49	51	3.92	4.08
12.05	11.0	37.2	53	47	5.83	5.17
BUD						
10.54	2.94	4.11	0	100	—	—
11.20	4.18	15.8	14	86	0.67	4.14
11.49	4.81	24.1	31	69	1.49	3.32
11.75	7.18	31.6	42	58	3.02	4.16
12.05	9.49	37.2	28	72	2.66	6.83

[a] pH values are calculated from $pH = pKw - pOH$ where $pOH = -\log \gamma NaOH[NaOH]$ using appropriate values at 80°. [b] Overall apparent first-order rate constants for solvolysis of IUD and BUD. [c] Apparent first-order constants for the solvolysis of RBA to nonchromophoric products.

The value obtained for k'_{OH} was 2.47×10^{-4} l./mole-sec. at 80.0°.

Preliminary Studies on Buffer Catalysis of RBA Degradation—Preliminary investigations on the buffer catalysis of RBA observed in the bicarbonate buffer region (Table II, Fig. 5) indicated that carbonate ion is the catalytic species while bicarbonate ion has an inhibitory effect on catalysis. If the difference between the experimental rate constant and the rate constant calculated on the basis of Eqs. 3 or 5 (Table II) is due only to bicarbonate and/or carbonate catalysis,

$$k_{exptl.} - k_{calcd.} = k_{diff.} = k_{CO_3^-}[CO_3^-] + k_{HCO_3^-}[HCO_3^-] \quad \text{(Eq. 21)}$$

Since,

$$[HCO_3^-] = [CO_3^-][H^+]/K_a \quad \text{(Eq. 22)}$$

then,

$$k_{diff.} = \{k_{CO_3^-} + k_{HCO_3^-}[H^+]/K_a\}[CO_3^-] \quad \text{(Eq. 23)}$$

and,

$$\frac{k_{diff.}}{[CO_3^-]} = k_{CO_3^-} + k_{HCO_3^-}[H^+]/K_a \quad \text{(Eq. 24)}$$

Thus, a plot of $k_{diff.}/[CO_3^-]$ *versus* $[H^+]$ should be a straight line with a *positive* slope equal to $k_{HCO_3^-}/K_a$ and an intercept of $k_{CO_3^-}$.

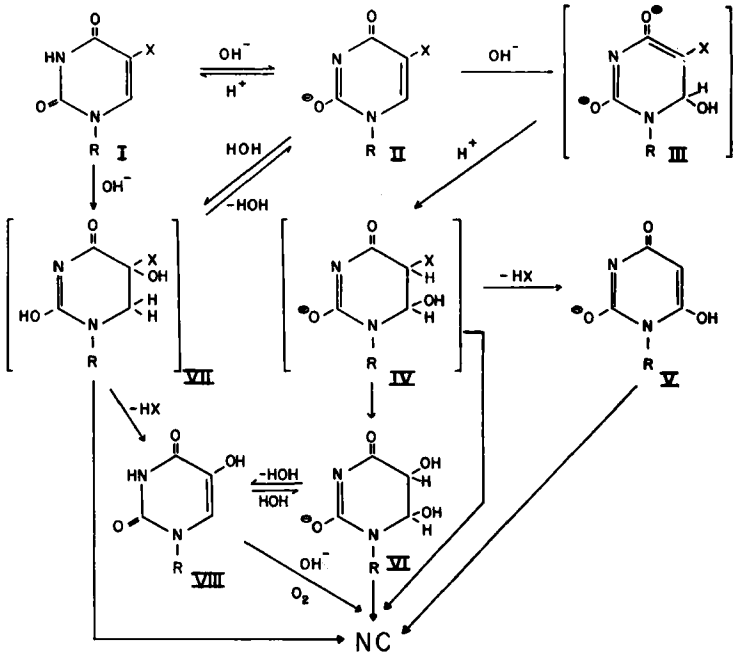
Such a plot was constructed from the six bicarbonate buffer studies of Table II and its footnotes. Five of these studies lay on a straight line of negative slope. This was indicative of the fact that CO_3^- was a catalytic species and that the presence of the undissociated acid, HCO_3^-, inhibited rather than catalyzed the reaction. Estimates at 80° of $k_{CO_3^-}$ and $k_{HCO_3^-}/Ka$ were 45.3×10^{-5} and -3.64×10^5, respectively. The phenomenon of inhibition of solvolysis by weak acids has been observed previously in such cases as acetic acid inhibition of anhydride solvolysis (20).

DISCUSSION

The order of reactivity of the halouridines to alkaline solvolysis is IUD > BUD > CUD. Uridine (UD) is solvolyzed about 5–6 times more slowly than CUD in strong alkali while MUD is stable for 5 days in 0.40 *N* NaOH at 80.0°. These observations would indicate that the lability of the halogen substituent in the 5-position of the pyrimidine ring enhances the ease of the solvolysis. The ability of the halogen to act as a leaving group under these conditions rather than its electronegative effect on making the 6-position susceptibile to hydroxyl ion attack is supported by the fact that the rate constants for the formation of RBA are of the same magnitude for BUD as for IUD (Table IV and Fig. 5). This was also observed for barbituric acid formation from bromo and iodouracil (9).

The fact that barbituric acid and halouracil formation is not observed definitely indicates that the lysis of the sugar moiety from halouridine does not occur, at least until after degradation or cleavage of the pyrimidine ring.

The possible routes of solvolysis of the halouridines consistent with the kinetic and spectra data are given in Scheme I. The observed production (only in strong alkali) of RBA (V) from IUD and BUD must be formulated in agreement with

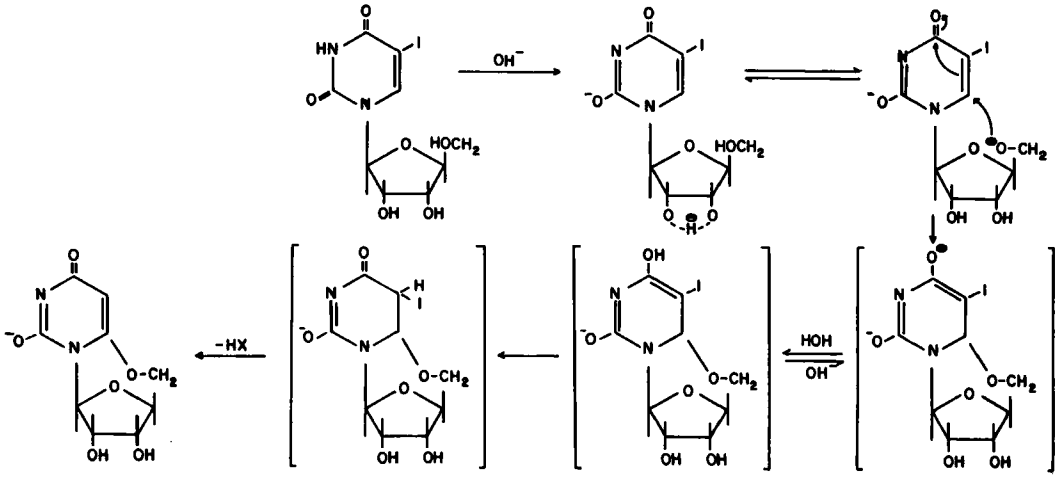


Scheme I

the derived kinetic dependency (Eq. 20) as a direct hydroxyl ion attack at C_6 of the monoanion and subsequent dehydrohalogenation (II → III → IV → V).

The kinetic dependency for RBA formation (Eq. 20) denies the rate-determining hydration of the 5,6 double bond on the monoanion. It would not, however, deny water attack on the dianion which would be kinetically indistinguishable from hydroxyl ion attack on the monoanion, if the halouridine may exist as a dianion at high pH values (*i.e.*, at pH values where a sugar hydroxyl can be ionized) (21). The fact that the rate-pH profiles for IUD and BUD do not bend over at high pH values indicates that an ionized sugar hydroxyl is not involved in an intramolecular nucleophilic attack at C_6 of the pyrimidine base. However, Lipkin and co-workers (22) have reported the formation of a 6,5′-anhydro-ribosylbarbituric acid by "alkaline hydrolysis of 5-iodouridines" which may be a consequence of a secondary dissociation of the sugar moiety (Scheme II).

Fox (17) has reported ~40% yield of a 6,5′-anhydro-2′,3′-*O*-isopropylidene-5-iodouridine with *N* alkali at 55°. It has been hypothesized that the isopropylidene group forces the sugar ring into a conformation that favors cyclization to form 6,5′-anhydronucleosides (23, 24, 17). When Fox treated BUD and IUD with alkali under the same conditions employed for their isopropylidenated derivatives, the reaction was much slower, but "UV data indicated that some 6,5′-anhydro-ribosylbarbituric acid had formed" (17). The authors have no information on the spectral characteristics or alkaline



Scheme II

stability of 6,5′-anhydro-ribosylbarbituric acid. Thus, it cannot be denied that there is the possibility that RBA may be formed by solvolysis of the 6,5′ anhydro intermediate whose formation results on intramolecular condensation of the dianion in the rate-determining step. This reaction rate constant for this process would have to be very large since the second pKa′ of the halouridine is not kinetically observed.

Hydration of the 5,6 double bond of the halouridine monoanion and subsequent dehydrohalogenation could produce compound VIII, 5-hydroxyuridine (OHUD). This compound is extremely unstable in the presence of alkali and is susceptible to oxidative degradation. OHUD degrades rapidly in 0.40 *N* NaOH at 80° showing a nonlinear semilogarithmic plot of absorbance *versus* time which may be interpreted as a sequential reaction, A → B → C, where the estimated rate constants are given in Table III. There is no apparent shift in the $\lambda_{max.}$ of the OHUD chromophore with time. The halouridines degrade much more slowly under equivalent conditions and any OHUD produced could not be observed spectrally. It is not unreasonable to assume that some OHUD is formed in this system, since 5-hydroxydeoxyuridine has been observed as a product of the alkaline solvolysis of iododeoxyuridine (7) and the isopropylidenated derivatives of IUD and BUD (17). Wang (25) also states that OHUD could be formed from BUD.

The formation of unstable dihydropyrimidine compounds may also be responsible for the fraction of the reaction that does not go through RBA. Cleavages of 5,6 dihydropyrimidine nucleosides in alkali at the 3,4 position are well-documented (26) and may lead to nonchromophoric products similar to one isolated from the alkaline treatment of arabinopyrimidine nucleosides (27). The dihydropyrimidines degrade so rapidly in alkali that it would be impossible to observe these intermediates spectrally. Sander has shown, for example, that dihydrouracil degrades about 500 times faster at 25° than IUD does at 80° (28), whereas uracil has high stability (9). It would also be possible for VI to yield VIII by dehydration (25). The postulated intermediates III, IV, and VII must be highly reactive since the rate constant for the loss of BUD as obtained from the production of bromide ion as a function of time is essentially the same as that obtained spectrally for the loss of BUD. Lozeron and co-workers (29) have shown that 5-fluoro-6-hydroxyuracil, a product isolated from the photochemical degradation of 5-fluouracil, is extremely unstable in strong alkali at low temperatures and opens at the 3,4 position.

The possible formation of RBA from the solvolysis of CUD in strong alkali could not be monitored since RBA degrades so much faster than CUD (Fig. 5). However, the shape of the rate-pH profile for CUD is the same as that for IUD and BUD although the rate constants are of a lower order of magnitude.

If one looks at the rate-pH profiles in terms of the formation of RBA and nonchromophoric products, an interesting phenomenon can be observed. The profile for the formation of nonchromophoric products seems to follow the values predicted by Eq. 6, *i.e.*, hydroxyl ion attack on the undissociated molecule or its kinetic equivalent, water attack on the monoanion. The profile describing the formation of RBA (Fig. 5) calls for hydroxyl ion attack on the halouridine monoanion. Thus, the rate-pH profile of the halouridines seems to represent the sum of two different reactions, one of which occurs throughout the entire pH range studied and the other (formation of RBA) occurring only in strong alkali.

RBA is not formed in the bicarbonate or phosphate buffer regions. It was not observed spectrally even though it is solvolyzed in these regions at the same or slower rates than the parent halouridine (Fig. 5), and it has a much higher absorptivity coefficient than the halouridines.

The formation of OHUD and/or unstable dihydropyrimidine derivatives probably accounts for the solvolysis of the halouridines in the buffer regions. Isobarbituric acid has been prepared from bromouracil by treatment with sodium bicarbonate (25, 9). Wang (25) also reported that OHUD was prepared from BUD in over 60% of the theoretical yield using lead oxide and postulated VII as an intermediate. OHUD degrades very rapidly in the bicarbonate buffer region at 80° and would not be observed spectrally.

The fact that UD and MUD are highly stable to alkaline solvolysis does not deny the possibility of a reversible hydration of the 5,6 double bond but further emphasizes the need for an electronegative 5-substituent that can take part in an elimination or substitution reaction, and aid in facilitating the ring opening at the 3,4 position.

The authors have also presented some data on the alkaline solvolysis of FUA and FUL, the 5-fluoro arabino and lyxo analogs of uridine. These compounds solvolyze much more quickly than all the halouridines which can be explained on the basis of Fox's mechanism (27) where intramolecular nucleophilic attack by the 2′-hydroxyl anion is facilitated sterically to give the 6,2′-anhydronucleoside. Subsequent tautomerization and ring cleavage at the 3,4-position of the pyrimidine base (27) produces a compound with no ultraviolet absorption spectrum. This compound can be converted by a much slower process to 1-(β-D-arabinofuranosyl)-2-oxo-4-imidazoline-4-carboxylic acid which does have a chromophore (30). FUA degrades faster than FUL even though FUL has both the 2′ and 3′ sugar hydroxyls in the "up" position and lyxose has a lower pK than arabinose (21). It may be argued that the lyxose anion, where the 2′ and 3′ hydroxyls are vicinal, may be stabilized by a hydrogen-bonded 5-membered ring (31). Thus, the lyxo analog may have a less concentrated negative charge on a specific alkoxide ion and not react as rapidly as the arabino analog in which the negative charge may be more localized at the 2′ position.

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Keyphrases

5-Halouridines, related nucleosides—solvolysis
Solvolysis—halogen-substituted uridines
UV spectrophotometry—solvolysis monitoring
Paper chromatography—separation
UV light—nucleoside visualization
Bromide ion measurement—bromide ion activity electrode

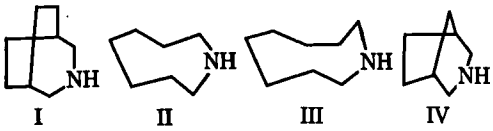
Use of 3-Azabicyclo[3.2.1]octane in the Mannich Reaction

By N. D. POTTI* and W. LEWIS NOBLES

The preparation of a group of ketonic Mannich bases utilizing 3-azabicyclo(3.2.1)-octane as the amine component is described. Also reported are the syntheses of several γ-amino secondary and tertiary alcohols obtained from these Mannich bases by sodium borohydride reduction and the Grignard reaction, respectively. These compounds are being screened for possible pharmacodynamic and chemotherapeutic activity.

AZABICYCLIC RING SYSTEMS are often found in alkaloids, many of which are medicinally useful, *e.g.*, morphine, atropine, *etc.* During the past two decades numerous 3-azabicyclic compounds were synthesized and tested for useful therapeutic activities (1–12). Many of them possessed hypotensive and antibacterial activities.

Nobles and his associates found interesting pharmacological activities associated with derivatives of complex amines like 3-azabicyclo[3.2.2]nonane I (6–8, 12); heptamethyleneimine II (13); and octamethyleneimine III (14).



I II III IV

Certain ketonic Mannich bases derived from such complex amines possessed an unexpected high order of antibacterial activity (15). The object of the present investigation was to extend the study with the complex amine, 3-azabicyclo-[3.2.1]octane (IV). A convenient laboratory method of preparation of this amine is reported elsewhere (16).

Promising pharmacological properties of β-aminoketones prompted several groups of workers to continue their studies with several of the derivatives of β-aminoketones (7–8, 13, 14, 17–19). Among these derivatives were included several γ-amino secondary and tertiary alcohols. In general, these alcohols were reported to be more

Received March 5, 1968, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Mississippi, University, MS 38677
Accepted for publication May 27, 1968.
Abstracted in part from a dissertation presented by N. D. Potti to the Graduate School of the University of Mississippi in partial fulfillment of the Doctor of Philosophy degree requirements.
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(31) Izatt, R. M., Christensen, J. J., Hansen, L. D., and Watt, G. D., *ibid.*, **87**, 2760(1965).

Keyphrases

5-Halouridines, related nucleosides—solvolysis
Solvolysis—halogen-substituted uridines
UV spectrophotometry—solvolysis monitoring
Paper chromatography—separation
UV light—nucleoside visualization
Bromide ion measurement—bromide ion activity electrode

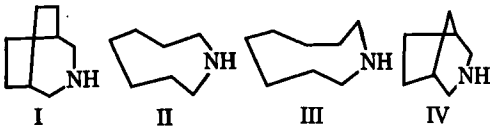
Use of 3-Azabicyclo[3.2.1]octane in the Mannich Reaction

By N. D. POTTI* and W. LEWIS NOBLES

The preparation of a group of ketonic Mannich bases utilizing 3-azabicyclo(3.2.1)-octane as the amine component is described. Also reported are the syntheses of several γ-amino secondary and tertiary alcohols obtained from these Mannich bases by sodium borohydride reduction and the Grignard reaction, respectively. These compounds are being screened for possible pharmacodynamic and chemotherapeutic activity.

AZABICYCLIC RING SYSTEMS are often found in alkaloids, many of which are medicinally useful, *e.g.*, morphine, atropine, *etc.* During the past two decades numerous 3-azabicyclic compounds were synthesized and tested for useful therapeutic activities (1–12). Many of them possessed hypotensive and antibacterial activities.

Nobles and his associates found interesting pharmacological activities associated with derivatives of complex amines like 3-azabicyclo[3.2.2]nonane I (6–8, 12); heptamethyleneimine II (13); and octamethyleneimine III (14).



I II III IV

Certain ketonic Mannich bases derived from such complex amines possessed an unexpected high order of antibacterial activity (15). The object of the present investigation was to extend the study with the complex amine, 3-azabicyclo-[3.2.1]octane (IV). A convenient laboratory method of preparation of this amine is reported elsewhere (16).

Promising pharmacological properties of β-aminoketones prompted several groups of workers to continue their studies with several of the derivatives of β-aminoketones (7–8, 13, 14, 17–19). Among these derivatives were included several γ-amino secondary and tertiary alcohols. In general, these alcohols were reported to be more

Received March 5, 1968, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Mississippi, University, MS 38677
Accepted for publication May 27, 1968.
Abstracted in part from a dissertation presented by N. D. Potti to the Graduate School of the University of Mississippi in partial fulfillment of the Doctor of Philosophy degree requirements.
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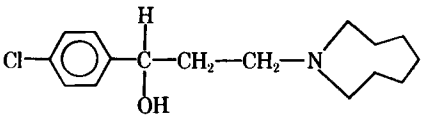
stable than the corresponding ketones and some of them more active, exhibiting additional pharmacological properties. Luts and Nobles (13) reported remarkable analgesic activity for 1-(*p*-chlorophenyl)-3-(1-heptamethyleneimino)-1-propanol (V), the corresponding ketone having no such activity.



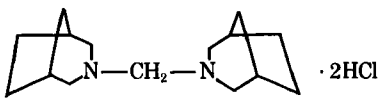
V

In this paper the preparation of a number of β-aminoketones and several γ-aminoalcohols is reported.

DISCUSSION

The β-aminoketones synthesized in this study are listed in Table I. These compounds have been prepared by the well-known Mannich reaction (20). The appropriately substituted aromatic ketone, paraformaldehyde, and 3-azabicyclo[3.2.1]octane hydrochloride reacted to give very good yields of the desired product.

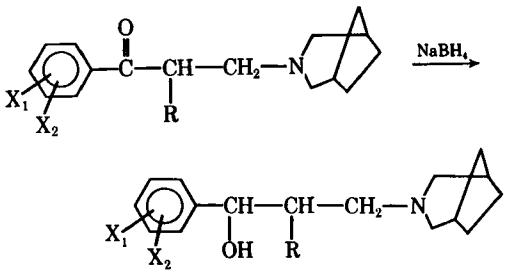
Under the general conditions described in the experimental section, 4-methylpropiophenone did not give the expected β-aminoketone, but resulted in the formation of the methylenebisamine dihydrochloride (VI). Similar observation was reported by Varma and Nobles (21) while attempting the Mannich reaction of indan-1,3-dione using 3-azabicyclo[3.2.2]nonane.



VI

Despite the extensive studies, the mechanism of this reaction is not conclusive. The mechanistic aspect has been dealt with in a recent report (22).

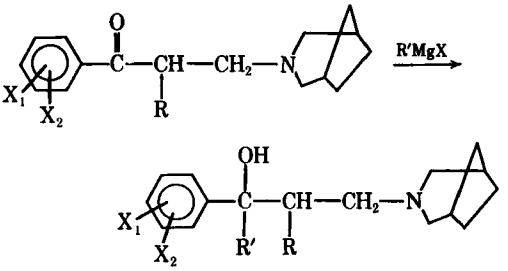
A selected group of β-aminoketones in Table I was reduced to the corresponding γ-amino secondary alcohol. The reduction was effected by treating a methanolic solution of the β-aminoketone with a 6% solution of sodium borohydride in methanol (Scheme I).



Scheme I

These compounds were characterized as hydrochlorides and are listed in Table II.

The third group of compounds described in this paper are the γ-amino tertiary alcohols (Table III). These alcohols were synthesized by the Grignard reaction on selected β-aminoketones, using three different Grignard reagents. (Scheme II.)



Scheme II

The reaction has been carried out in anhydrous ether. The compounds were purified as hydrochlorides. In general the yields are fairly good.

EXPERIMENTAL[1]

The β-aminoketones (Table I)—These compounds were prepared by following the method of Nobles and associates (6–8, 13, 14). All of the ketones in this investigation are commercially available.

A mixture of 0.02 mole of the appropriate ketone, 2.95 g. (0.02 mole) of 3-azabicyclo[3.2.1]octane hydrochloride, 0.9 g. (0.03 mole) of paraformaldehyde, and 5 ml. of ethanol (95%) containing a drop of concentrated hydrochloric acid was refluxed for 3 hr. After refluxing, the volume of the mixture was reduced to one half, using the water aspirator. The residue was cooled and stirred with 30 ml. of acetone. The solid thus separated was filtered and recrystallized from ethanol–acetone mixture. The properties are described in Table I.

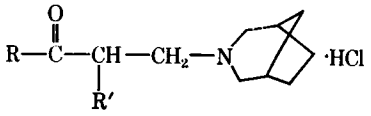
γ-Amino Secondary Alcohol Hydrochlorides (Table II)—The γ-amino secondary alcohols were prepared by the following general method. The appropriate β-aminoketone (0.02 mole) was dissolved in 40 ml. of methanol in a 200-ml. three-necked flask fitted with a condenser, thermometer, dropping funnel, and magnetic stirrer. A solution of 1.52 g. (0.04 mole) of sodium borohydride in 20 ml. of methanol was added to the above with stirring. The temperature of the reaction mixture was maintained between 24–40°, while the stirring was continued for 2 hr. The methanol was removed using water vacuum, and the residue, after the addition of 30 ml. of distilled water, was extracted with three 50-ml. portions of ether. The combined ether extract was washed with 10 ml. of distilled water and dried over anhydrous sodium sulfate. Anhydrous hydrogen chloride was passed through the ether solution to precipitate the aminoalcohol hydrochloride.[2] The precipitate was filtered and recrystallized for ethanol–acetone mixture.

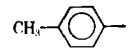
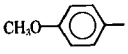
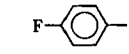
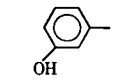
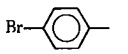
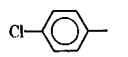
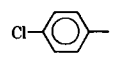
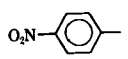
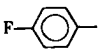
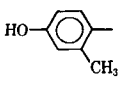
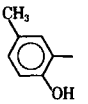
γ-Amino Tertiary Alcohol Hydrochlorides (Table III)—Following the method of Pohland and Sullivan (23), these alcohols were prepared. The com-

[1] All melting points are uncorrected. Melting points were determined on a Thomas-Hoover capillary melting-point apparatus. Infrared spectra were determined on a Perkin-Elmer Infracord spectrophotometer.

[2] Sometimes the precipitate might form a sticky mass, but could be recrystallized from ethanol–acetone mixture.

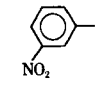
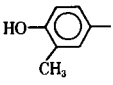
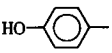
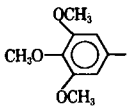
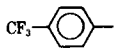
TABLE I—MANNICH BASES (β-AMINO KETONE HYDROCHLORIDES)



No.	R	R′	Yield, %	M.p., °C.[b]	Formula	Anal.[a] Calcd.	Found
1		H	40	198–199	$C_{17}H_{23}NO \cdot HCl$[c]	C, 69.49 H, 8.23 N, 4.77	C, 69.36 H, 8.42 N, 4.66
2		CH_3	77	187–188	$C_{18}H_{25}NO_2 \cdot HCl$[c]	C, 66.75 H, 8.09 N, 4.33	C, 66.74 H, 8.21 N, 4.10
3		CH_3	56	178–179	$C_{17}H_{22}FNO \cdot HCl$[c]	C, 65.48 H, 7.43 N, 4.49	C, 65.56 H, 7.56 N, 4.46
4		H	63	213–214	$C_{16}H_{21}NO_2 \cdot HCl$[c]	C, 64.96 H, 7.50 N, 4.79	C, 64.83 H, 7.49 N, 4.63
5		H	70	198–199	$C_{16}H_{20}BrNO \cdot HCl$[d]	C, 53.50 H, 5.88 N, 3.90	C, 53.58 H, 5.94 N, 3.84
6		CH_3	35	180–181	$C_{17}H_{22}ClNO \cdot HCl$[e]	C, 62.15 H, 7.06 N, 4.27	C, 62.33 H, 7.09 N, 4.08
7		H	48	202–203	$C_{16}H_{20}ClNO \cdot HCl$[c]	C, 61.22 H, 6.74 N, 4.46	C, 61.21 H, 6.70 N, 4.31
8		H	49	177–178	$C_{16}H_{21}NO_2 \cdot HCl$[c]	C, 64.96 H, 7.50 N, 4.74	C, 63.49 H, 7.42 N, 4.50
9		H	51.5	171–172	$C_{17}H_{23}NO_2 \cdot HCl$[e]	C, 65.90 H, 7.81 N, 4.52	C, 66.08 H, 7.79 N, 4.46
10		H	50	200–201	$C_{16}H_{20}BrNO \cdot HCl$[e]	C, 53.57 H, 5.90 N, 3.90	C, 53.78 H, 5.56 N, 3.75
11		H	53	195–196	$C_{16}H_{20}N_2O_3 \cdot HCl$[c]	C, 59.16 H, 6.52 N, 8.63	C, 59.14 H, 6.52 N, 8.39
12		H	52	195–196	$C_{16}H_{20}FNO \cdot HCl$[c]	C, 64.51 H, 7.11 N, 4.71	C, 63.54 H, 7.14 N, 4.81
13		H	53	168–169	$C_{17}H_{23}NO \cdot HCl$[e]	C, 69.50 H, 8.25 N, 4.78	C, 69.70 H, 8.21 N, 4.55
14		H	60	212–213	$C_{17}H_{23}NO_2 \cdot HCl$[c]	C, 65.91 H, 7.75 N, 4.52	C, 65.67 H, 7.77 N, 4.77
15		H	58	188–189	$C_{16}H_{20}N_2O_3 \cdot HCl$[e]	C, 59.16 H, 6.52 N, 8.63	C, 58.94 H, 6.49 N, 8.26
16		H	53	173–174	$C_{14}H_{19}NOS \cdot HCl$[c]	C, 58.82 H, 7.13 N, 4.90	C, 58.80 H, 7.18 N, 4.57
17		H	48	201–202	$C_{17}H_{23}NO_2 \cdot HCl$[c]	C, 65.91 H, 7.75 N, 4.52	C, 65.47 H, 7.85 N, 4.69

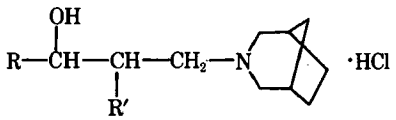
(*Continued on next page.*)

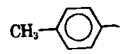
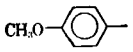
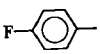
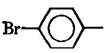
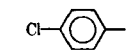
TABLE I (*Continued.*)

No.	R	R′	Yield, %	M.p., °C.[b]	Formula	Anal.[a] Calcd.	Anal.[a] Found
18		H	65	215–216	$C_{16}H_{23}N_2O_3 \cdot HCl$[f]	C, 59.10 H, 6.52 N, 8.62	C, 59.06 H, 6.49 N, 8.55
19		H	55	205–206	$C_{17}H_{23}NO_2 \cdot HCl$[c]	C 65.91 H, 7.75 N, 4.52	C, 65.53 H, 7.88 N, 4.37
20		H	55	195–196	$C_{16}H_{21}NO \cdot HCl$[c]	C, 68.54 H, 7.92 N, 5.0	C, 68.69 H, 8.01 N, 4.88
21		CH_3	48	193–194	$C_{17}H_{23}NO_2 \cdot HCl$[c]	C, 65.91 H, 7.75 N, 4.52	C, 65.93 H, 7.95 N, 4.51
22		H	68	211–212	$C_{19}H_{27}NO_4 \cdot HCl$[d]	C, 61.60 H, 7.63 N, 3.79	C, 60.94 H, 7.44 N, 3.24
23		H	53	204–205	$C_{17}H_{20}F_3NO \cdot HCl$[c]	C, 58.62 H, 6.09 N, 4.03	C, 58.52 H, 6.09 N, 4.31
24		H	50	206–207	$C_{17}H_{20}F_3NO \cdot HCl$[c]	C, 58.62 H, 6.09 N, 4.03	C, 58.45 H, 6.19 N, 3.94
25		H	48	177–178	$C_{17}H_{20}F_3NO \cdot HCl$[e]	C, 58.62 H, 6.09 N, 4.03	C, 58.71 H, 6.07 N, 3.88

[a] Carbon, hydrogen, and nitrogen analyses are through the courtesy of Dr. Paul Craig of Smith Kline & French Laboratories, Philadelphia, Pa. [b] Melting points are uncorrected. [c] Recrystallized from ethanol–acetone mixture. [d] Recrystallized from ethanol. [e] Recrystallized from ethanol–benzene mixture. [f] Recrystallized from ethanol–water mixture.

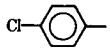
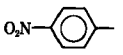
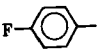
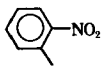
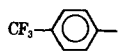
TABLE II—γ-AMINO SECONDARY ALCOHOLS (HYDROCHLORIDE SALTS)[a]



No.[a]	R	R′	Yield, %	M.p., °C.[b]	Formula	Anal.[c] Calcd.	Anal.[c] Found
1		H	68	201–202	$C_{17}H_{25}NO \cdot HCl$	C, 69.02 H, 8.86 N, 4.74	C, 69.08 H, 8.69 N, 4.81
2		CH_3	62	206–207	$C_{18}H_{27}NO_2 \cdot HCl$	C, 66.34 H, 8.66 N, 4.30	C, 66.48 H, 8.63 N, 4.18
3		CH_3	61	227–228	$C_{17}H_{24}FNO \cdot HCl$	C, 65.06 H, 8.03 N, 4.46	C, 65.10 H, 7.91 N, 4.27
4		H	62	209–210	$C_{16}H_{22}BrNO \cdot HCl$	C, 53.27 H, 6.43 N, 3.88	C, 53.17 H, 6.47 N, 3.88
5		H	45	169–170	$C_{16}H_{23}NO_2 \cdot HCl$	C, 64.53 H, 8.12 N, 4.70	C, 64.42 H, 8.02 N, 4.60
6		CH_3	58	254–255	$C_{17}H_{24}ClNO \cdot HCl$	C, 61.51 H, 7.63 N, 4.24	C, 61.52 H, 7.54 N, 6.14

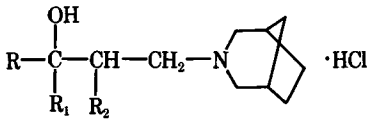
(*Continued on next page.*)

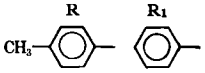
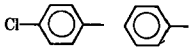
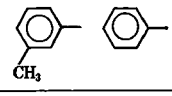
TABLE II (*Continued.*)

No.[a]	R	R′	Yield, %	M.p., °C.[b]	Formula	Anal.[c] Calcd.	Found
7		H	60	195–196	$C_{16}H_{22}ClNO \cdot HCl$	C, 60.76 H, 7.33 N, 4.43	C, 60.75 H, 7.26 N, 4.34
8		H	55	195–196	$C_{17}H_{25}NO_2 \cdot HCl$	C, 65.50 H, 8.41 N, 4.50	C, 65.55 H, 8.33 N, 4.66
9		H	65	177–178	$C_{16}H_{22}BrNO \cdot HCl$	C, 53.27 H, 6.41 N, 3.88	C, 53.39 H, 6.46 N, 4.13
10		H	62	223–224	$C_{16}H_{22}N_2O_3 \cdot HCl$	C, 58.70 H, 7.09 N, 8.56	C, 59.31 H, 7.75 N, 8.47
11		H	58	173–174	$C_{16}H_{22}FNO \cdot HCl$	C, 64.02 H, 7.72 N, 4.67	C, 63.84 H, 7.65 N, 5.05
12		H	69	204–205	$C_{17}H_{25}NO \cdot HCl$	C, 69.02 H, 8.86 N, 4.74	C, 68.91 H, 8.71 N, 4.97
13		H	61	209–210	$C_{16}H_{22}N_2O_3 \cdot HCl$	C, 58.80 H, 7.09 N, 8.56	C, 58.80 H, 6.85 N, 8.46
14		H	70	192–193	$C_{16}H_{23}NO \cdot HCl$	C, 68.19 H, 8.58 N, 4.97	C, 68.07 H, 8.70 N, 5.04
15		H	52	164–165	$C_{17}H_{22}F_3NO \cdot HCl$	C, 58.37 H, 6.62 N, 4.01	C, 58.43 H, 6.60 N, 4.26
16		H	53	221–222	$C_{17}H_{22}F_3NO \cdot HCl$	C, 58.37 H, 6.62 N, 4.01	C, 58.40 H, 6.68 N, 4.07
17		H	48	198–199	$C_{17}H_{22}F_3NO \cdot HCl$	C, 58.37 H, 6.62 N, 4.01	C, 58.12 H, 6.63 N, 4.14

[a] All of these γ-amino secondary alcohol hydrochlorides were crystallized from an alcohol–acetone mixture. [b] Melting points are uncorrected. [c] Carbon, hydrogen, and nitrogen analyses are through the courtesy of Dr. Paul Craig of Smith Kline & French Laboratories, Philadelphia, Pa.

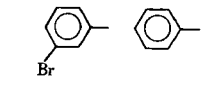
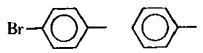
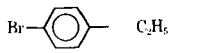
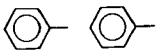
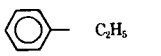
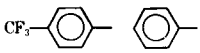
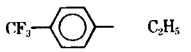
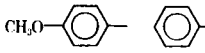
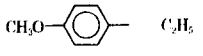
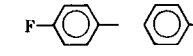
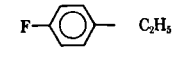
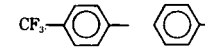
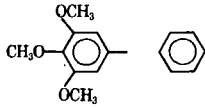
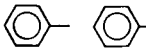
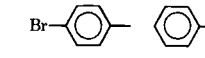
TABLE III—γ-AMINO TERTIARY ALCOHOLS (HYDROCHLORIDE SALTS)[a]



No.[a]	R	R_1	R_2	Yield, %	M.p., °C.[b]	Formula	Anal.[c] Calcd.	Found
1			H	32	247–248	$C_{23}H_{29}NO \cdot HCl$	C, 74.27 H, 8.13 N, 3.76	C, 74.21 H, 8.07 N, 3.75
2			H	43	258–259	$C_{22}H_{26}ClNO \cdot HCl$	C, 67.36 H, 6.94 N, 3.57	C, 66.96 H, 7.16 N, 3.59
3			H	50	229–230	$C_{23}H_{29}NO \cdot HCl$	C, 74.26 H, 8.13 N, 3.76	C, 74.16 H, 8.04 N, 3.78

(*Continued on next page.*)

TABLE III (*Continued.*)

No.[a]	R	R_1	R_2	Yield, %	M.p., °C.[b]	Formula	Anal.[c] Calcd.	Found
4			H	48	241–242	$C_{22}H_{26}BrNO \cdot HCl$	C, 60.48 H, 6.23 N, 3.20	C, 60.30 H, 6.42 N, 3.21
5			H	44	267–268	$C_{22}H_{26}BrNO \cdot HCl$	C, 60.48 H, 6.23 N, 3.20	C, 60.62 H, 6.42 N, 3.04
6			H	43	226–227	$C_{18}H_{26}BrNO \cdot HCl$	C, 55.61 H, 7.00 N, 3.60	C, 55.77 H, 7.20 N, 3.60
7			H	68	247–248	$C_{22}H_{27}NO \cdot HCl$	C, 73.81 H, 7.89 N, 3.91	C, 73.75 H, 8.08 N, 3.93
8			H	72	196–197	$C_{18}H_{27}NO \cdot HCl$	C, 69.78 H, 9.10 N, 4.51	C, 69.65 H, 9.20 N, 4.47
9			H	40	265–266	$C_{23}H_{26}F_3NO \cdot HCl$	C, 64.60 H, 6.39 N, 3.29	C, 64.91 H, 6.59 N, 3.55
10			H	49	225–226	$C_{19}H_{26}F_3NO \cdot HCl$	C, 60.40 H, 7.20 N, 3.70	C, 59.86 H, 7.44 N, 3.46
11			CH_3	51	196–197	$C_{24}H_{31}NO_2 \cdot HCl$	C, 71.70 H, 8.02 N, 3.48	C, 70.12 H, 7.98 N, 3.21
12			CH_3	55	185–186	$C_{20}H_{31}NO_2 \cdot HCl$	C, 67.88 H, 9.11 N, 3.95	C, 68.18 H, 9.20 N, 4.02
13			CH_3	48	228–229	$C_{23}H_{28}FNO \cdot HCl$	C, 70.84 H, 7.49 N, 3.59	C, 70.66 H, 7.56 N, 3.54
14			CH_3	58	207–208	$C_{19}H_{28}FNO \cdot HCl$	C, 66.74 H, 8.55 N, 4.09	C, 66.50 H, 8.56 N, 4.15
15			H	57	240–241	$C_{24}H_{28}F_3NO \cdot HCl$	C, 65.52 H, 6.64 N, 3.18	C, 65.74 H, 6.65 N, 3.30
16			H	28	256–257	$C_{26}H_{35}NO_4 \cdot HCl$	C, 67.60 H, 7.85 N, 3.03	C, 69.52 H, 7.68 N, 2.95
17			H	63	214–215	$C_{23}H_{29}NO \cdot HCl$	C, 74.27 H, 8.13 N, 3.76	C, 74.05 H, 8.04 N, 4.04
18			H	58	247–248	$C_{23}H_{28}BrNO \cdot HCl$	C, 61.26 H, 6.48 N, 3.10	C, 61.08 H, 6.38 N, 2.78

[a] All the aminoalcohol hydrochlorides are crystallized from ethanol–acetone mixture. [b] Melting points are uncorrected. [c] Carbon, hydrogen, nitrogen analyses are through the courtesy of Dr. Paul Craig, Smith Kline & French Laboratories, Philadelphia, Pa.

mercially available "anhydrous" ether was redried by stirring for 5–10 min. with anhydrous magnesium sulfate (one heaping tablespoon per liter) in a stoppered container, using a magnetic stirrer. The anhydrous ether was decanted quickly into the reaction vessel provided with a drying tube containing a mixture of calcium chloride and soda lime.

While phenyl magnesium bromide and ethyl magnesium bromide are available commercially, benzyl magnesium chloride was prepared by following the method of Gilman and Catlin (24). In a 300-ml. dry three-necked flask, fitted with a dropping funnel, a condenser having a drying tube carrying a mixture of calcium chloride and soda lime at the upper end, and a magnetic stirrer, was placed 4.86 g. (0.02 g. atom) of magnesium turnings. To this was added 10 ml. of dry ether. In 100 ml. of dry ether, 25.3 ml. (0.2 mole) of pure benzyl chloride was dissolved.

Five milliliters of this solution was added to the above magnesium turnings-ether mixture. To induce the reaction to begin, a small crystal of iodine was added. The reaction proceeded with development of heat and decolorization of the iodine. The mixture was stirred by means of a magnetic stirrer, and the remainder of the benzyl chloride was added gradually over a period of 30 min., regulating the temperature of the reaction with the aid of an ice water bath. The reagent was used as described in the case of phenyl magnesium bromide.

All of the compounds listed in Table III were prepared by the following general method.

In a 200-ml. round-bottom flask, 0.02 mole of the appropriate aminoketone was dissolved in 75 ml. of anhydrous ether, and the solution was dried with anhydrous magnesium sulfate. Meanwhile, in a dry 300-ml. three-necked flask equipped with dropping funnel, magnetic stirrer, and condenser with drying tube on the upper end, 13.5 ml. of a 3 *M* solution (0.04 mole) of phenyl magnesium bromide and 30 ml. of anhydrous ether was placed. To this, a dry ether solution of the Mannich base as previously prepared, was dropped little by little with vigorous stirring. Stirring was continued for 2 hr., and the mixture was allowed to stand overnight. The reaction flask was cooled in an ice bath. A saturated solution of ammonium chloride was added to decompose the complex. The ether solution was decanted into a separator and washed with 10 ml. of amino-alcohol–ether solution, was filtered, cooled in an ice bath, and dry hydrogen chloride gas was passed through it. The hydrochloride was collected and recrystallized from the alcohol–acetone mixture.

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(2) *Ibid.*, **22**, 1223(1957).
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(7) *Ibid.*, **52**, 46(1963).
(8) *Ibid.*, **53**, 521(1964).
(9) Kreiger, H., *Suomen Kemistilchti*, **36A**, 2(1963).
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(13) Nobles, W. L., and Luts, H. A., *ibid.*, **54**, 67(1965).
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(15) Nobles, W. L., unpublished data, patent application submitted.
(16) Potti, N. D., and Nobles, W. L., *J. Pharm. Sci.*, in press.
(17) Denton, J. J., Turner, R. J., Neier, W. B., Lawson, V. A., and Schedl, H. P., *ibid.*, **71**, 2048(1949).
(18) Katz, L., Karger, L. A., and Cohen, M. S., *J. Org. Chem.*, **19**, 1225(1954).
(19) Lutz, R. E., Allison, R. K., Ashburn, G., Bailey, P. S., Clark, M. T., Codington, J. F., Deinet, A. J., Freek, J. A., Jordan, R. H., Leake, N. H., Martin, T. A., Nicodemus, K. C., Rowlett, R. J., Jr., Shearer, N. H., Smith, J. D., and Wilson, J. W., III, *ibid.*, **12**, 617(1947).
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(21) Varma, R. S., and Nobles, W. L., *J. Pharm. Sci.*, **55**, 1451(1966).
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(23) Pohland, A., and Sullivan, J. R., *J. Am. Chem. Soc.*, **75**, 4458(1953).
(24) Gilman, H., and Catlin, W. E., "Organic Synthesis," Coll. vol. I, Wiley, New York, N. Y., 1951, p. 471.

Keyphrases

Mannich reaction
β-Aminoketones—synthesis
γ-Aminoalcohols—synthesis
3-Azabicyclo[3.2.1]octane—amine component, Mannich reaction

Effect of Sodium Deoxycholate on Gastric Emptying in the Rat

By STUART FELDMAN*, RALPH J. WYNN, and MILO GIBALDI

Sodium deoxycholate delays considerably the gastric emptying of phenol red in rats. Gastric emptying of phenol red in control animals proceeds by apparent first-order kinetics, but a very different kinetic pattern emerges upon administration of the bile salt. Sodium deoxycholate also produces a large net secretion of fluids into the gastric pouch. It is proposed that the resulting increase in gastric volume is the immediate cause for the decreased rate of gastric emptying.

THE EFFECTS of orally administered bile salts on the gastrointestinal absorption of riboflavin (1) and other drugs are currently being investigated in this laboratory. These studies have prompted a consideration of the influence of bile salts on gastric emptying since the rate of passage of drug through the pylorus may have a profound influence on the overall rate and extent of drug absorption (2, 3).

Previous studies (4) have shown that the oral administration of sodium taurodeoxycholate and

Received April 22, 1968, from the Department of Pharmaceutics, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication June 11, 1968.

Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

This work was supported in part by grant AM-11498 from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service, Bethesda, Md.

* Supported by National Institutes of Health training grant fellowship GM 555-06.

Five milliliters of this solution was added to the above magnesium turnings-ether mixture. To induce the reaction to begin, a small crystal of iodine was added. The reaction proceeded with development of heat and decolorization of the iodine. The mixture was stirred by means of a magnetic stirrer, and the remainder of the benzyl chloride was added gradually over a period of 30 min., regulating the temperature of the reaction with the aid of an ice water bath. The reagent was used as described in the case of phenyl magnesium bromide.

All of the compounds listed in Table III were prepared by the following general method.

In a 200-ml. round-bottom flask, 0.02 mole of the appropriate aminoketone was dissolved in 75 ml. of anhydrous ether, and the solution was dried with anhydrous magnesium sulfate. Meanwhile, in a dry 300-ml. three-necked flask equipped with dropping funnel, magnetic stirrer, and condenser with drying tube on the upper end, 13.5 ml. of a 3 *M* solution (0.04 mole) of phenyl magnesium bromide and 30 ml. of anhydrous ether was placed. To this, a dry ether solution of the Mannich base as previously prepared, was dropped little by little with vigorous stirring. Stirring was continued for 2 hr., and the mixture was allowed to stand overnight. The reaction flask was cooled in an ice bath. A saturated solution of ammonium chloride was added to decompose the complex. The ether solution was decanted into a separator and washed with 10 ml. of amino-alcohol–ether solution, was filtered, cooled in an ice bath, and dry hydrogen chloride gas was passed through it. The hydrochloride was collected and recrystallized from the alcohol–acetone mixture.

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Keyphrases

Mannich reaction
β-Aminoketones—synthesis
γ-Aminoalcohols—synthesis
3-Azabicyclo[3.2.1]octane—amine component, Mannich reaction

Effect of Sodium Deoxycholate on Gastric Emptying in the Rat

By STUART FELDMAN*, RALPH J. WYNN, and MILO GIBALDI

Sodium deoxycholate delays considerably the gastric emptying of phenol red in rats. Gastric emptying of phenol red in control animals proceeds by apparent first-order kinetics, but a very different kinetic pattern emerges upon administration of the bile salt. Sodium deoxycholate also produces a large net secretion of fluids into the gastric pouch. It is proposed that the resulting increase in gastric volume is the immediate cause for the decreased rate of gastric emptying.

THE EFFECTS of orally administered bile salts on the gastrointestinal absorption of riboflavin (1) and other drugs are currently being investigated in this laboratory. These studies have prompted a consideration of the influence of bile salts on gastric emptying since the rate of passage of drug through the pylorus may have a profound influence on the overall rate and extent of drug absorption (2, 3).

Previous studies (4) have shown that the oral administration of sodium taurodeoxycholate and

Received April 22, 1968, from the Department of Pharmaceutics, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication June 11, 1968.

Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

This work was supported in part by grant AM-11498 from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service, Bethesda, Md.

* Supported by National Institutes of Health training grant fellowship GM 555-06.

sodium deoxycholate markedly inhibits gastric emptying and proximal intestinal transit after gastric intubation in the rat. The present report concerns the influence of sodium deoxycholate on the pattern of gastric emptying in the rat and the pharmacologic basis of these effects.

EXPERIMENTAL

Male, Sprague-Dawley descent rats (Blue Spruce Farms, Altamont, N. Y.) weighing between 125 and 210 g. were used in all experiments. The animals were fasted for 20–24 hr. prior to gastric intubation. Water was permitted *ad libitum* until 1 hr. before the experiment. One and one-half milliliters of a 70-mg. % phenol red solution containing 0.026 *M* sodium deoxycholate (special enzyme grade, Mann Research Laboratories, New York, N. Y.), was introduced into the rat stomach by intubation. Control animals received 1.5 ml. of 70-mg.% phenol red solution in distilled water.

The rats were sacrificed by decapitation at various times after intubation. An abdominal incision was made immediately to expose the stomach. The stomach was then quickly ligated at the pylorus and cardia, excised, stripped of adhering fat, blotted, and weighed. The stomach and its contents were homogenized in an Eberbach homogenizer and assayed for phenol red as previously described (4). The analytical procedure is a modification of the method of Reynell and Spray (5) and involves alkalinization with 1 *N* NaOH, precipitation of proteins with 30% trichloroacetic acid, realkalinization with NaOH and colorimetric assay at 560 mμ. The stomach weights of a control group of six rats were obtained after sham intubation, immediate sacrifice, and removal of the stomach as described above.

The recovery of known amounts of phenol red added to stomach tissue homogenates is about 93% while recovery of phenol red after incubation in gastric pouches for 1 hr. before homogenization is 87% (6). The presence of bile salt has no effect on the recovery of phenol red added to tissue homogenates (4). In view of the high recovery from gastric preparations, the results are reported on a percent-of-dose basis.

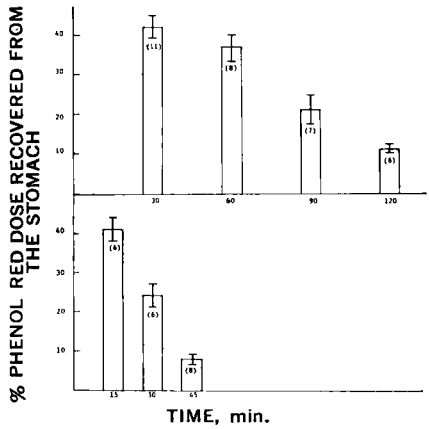

Fig. 1—Recovery of phenol red from rat stomach after gastric intubation. The lower half are data from control animals. The upper half are data from rats co-administered sodium deoxycholate. The vertical lines represent standard error of the mean. The numbers in parentheses indicate the number of animals used for each point.

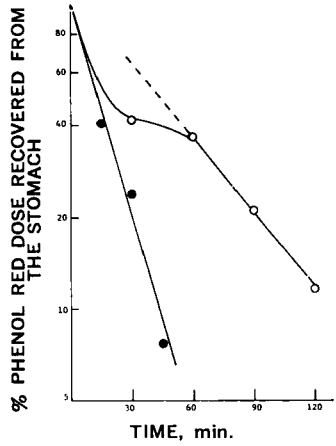

Fig. 2—Semilogarithmic plot of percent of phenol red dose in stomach versus *time. Key: ●, controls; ○, sodium deoxycholate.*

RESULTS

Figure 1 shows the recovery of phenol red from the rat stomach as a function of time after intubation. A considerable delay is observed in the gastric emptying of phenol red in the presence of sodium deoxycholate. This effect is illustrated further in Fig. 2, which shows a semilogarithmic plot of gastric retention *versus* time. The plot indicates that gastric emptying of phenol red in control animals proceeds by apparent first-order kinetics. This finding is consistent with the results of Hunt and MacDonald (7) who showed that the stomach content volume after a liquid test meal decreases in an exponential fashion with time. The "half-life" of gastric emptying in control rats was 13 min.

Figure 2 also shows that sodium deoxycholate modifies the pattern of gastric emptying. In the presence of the bile salt an initial rapid gastric emptying phase followed by a transition region, where little emptying occurs, is noted over the first hour. The second hour after intubation is characterized by an exponential decline in phenol red, with a "half-life" of 36 min.

The average stomach weight (expressed as mg./g. of body weight) of control rats after sham intubation was found to be 8.35 ± 0.23 (*SE*). The percent increases in stomach weight, relative to the control value, of rats receiving phenol red or phenol red and sodium deoxycholate are shown in Figs. 3 and 4, respectively. The greater increase in stomach weights of rats fed sodium deoxycholate is qualitatively consistent with the higher phenol red retention observed in these animals. However, the increase in stomach weight in bile salt-treated rats is significantly greater than predicted from phenol red recovery data.

The stomach weight after intubation reflects a number of factors which are summarized in the fol-

lowing equation:

$$(W_I - W_C) = (W_D - W_E) + (W_S - W_A) \quad \text{(Eq. 1)}$$

where W refers to weight and the subscripts denote stomach weight after intubation (I), stomach weight of controls after sham intubation (C), the weight of the dose (D), the amount of the dose emptied from the stomach (E), the amount of biologic fluids secreted into the gastric pouch (S), and the amount of intubated solution which is absorbed (A).

The term $(W_I - W_C)$ is the observed increase in stomach weight and the term $(W_D - W_E)$ is calculable from the phenol red recovery data. A positive value of the $(W_S - W_A)$ term indicates net secretion of fluids while a negative value indicates net gastric absorption of intubated solution. Values for the $(W_D - W_E)$ term are shown by the cross-hatched area in Fig. 3 at the 15-min. level and in Fig. 4 at the 30-, 60-, and 90-min. levels.

The data in Fig. 4 demonstrate a large net secretion of fluids, induced by the bile salt, which persists for at least 60 min. after intubation. In control rats, receiving only phenol red, there is an indication of net secretion 15 min. after intubation, but at 30 and 45 min. after intubation net absorption occurs (not shown on graph). Hence, sodium deoxycholate significantly enhances gastric secretion.

A semilogarithmic plot of the change in stomach weight after intubation *versus* time is shown in Fig. 5. Similar to the results noted previously with retention data in control animals, the stomach weight declines in an exponential fashion with time, *i.e.*, $(W_I - W_C) = W_D\, e^{-Kt}$. The half-life for this process was 10.5 min. The pattern of the bile salt data in Fig. 5 parallels that shown in Fig. 2. An exponential phase with a half-life of 18 min. is evident between 60 and 120 min. after administration.

DISCUSSION

The effect of sodium deoxycholate on gastric emptying may be explained by considering several

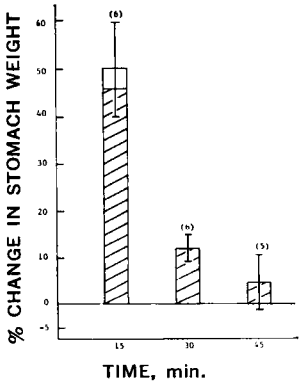

Fig. 3—Change in stomach weight as a function of time after administration of 1.5 ml. of a 70-mg.% phenol red solution. Hatched area at the 15-min. level denotes the value of ($W_D - W_E$). *See text for details. Vertical lines represent standard error of the mean. Numbers in parentheses indicate the number of animals used for each point.*

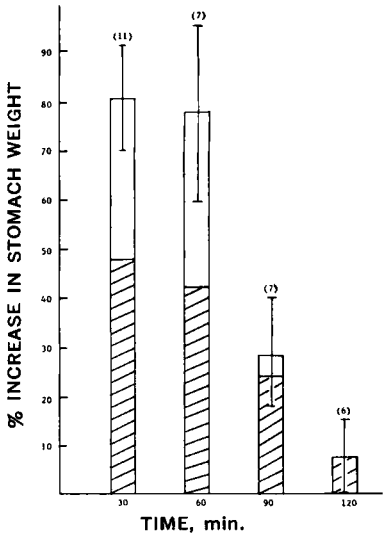

Fig. 4—Change in stomach weight as a function of time after administration of 1.5 ml. of a 70-mg.% phenol red solution in 0.026 M *sodium deoxycholate. Hatched area at 30-, 60-, and 90-min. levels denotes the value of* ($W_D - W_E$). *See text for details. Vertical lines represent standard error of the mean. Numbers in parentheses indicate the number of animals used for each point.*

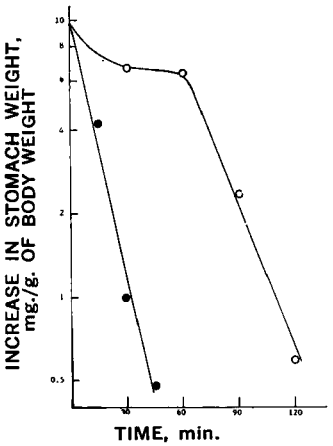

Fig. 5—Semilogarithmic plot of rat stomach weight after intubation minus stomach weight of sham-intubated rats versus *time. Key:* ●, *controls;* ○, *sodium deoxycholate.*

plausible mechanisms. A possibility exists that bile salts exert a direct effect on the musculature of the gastric pouch which results in a decrease in the gastric emptying rate. However, on the basis of literature reports, this possibility is unlikely. Sasaki (8) found that oral administration of bile salts to the rabbit resulted in a slight increase in gastric motility at low doses and in a small decrease in motility at higher levels. Anderson *et al.* (9) recently reported that bile placed in the duodenum had no effect on the contractility of the longitudinal and circular muscles of the canine gastric body and antrum. On the other hand, Davenport (10) ob-

served that natural bile in acid solution when placed in the canine gastric pouch results in an increase in gastric motility. This phenomenon would tend to promote gastric emptying. Hence, it would not appear that bile salts exert an effect on gastric emptying *via* an inhibitory mechanism on gastric motility.

The increase in gastric secretion and lumen volume in the presence of sodium deoxycholate offers an alternate explanation for the decrease in gastric emptying. Hunt and MacDonald (7) have shown that as the volume of a liquid test meal increases the percentage of gastric contents leaving the stomach per unit time decreases. Interestingly, these workers also observed curves similar to those presented in Figs. 2 and 5 for bile salt-treated animals, when large volumes of fluid were administered to the test subjects. The relationship of increased gastric secretions and decreased gastric emptying has also been considered by Sognen (11) who observed a concomitant increase in gastric volume and decrease in gastric emptying upon administration of calcium-binding salts to rats. The present results support the hypothesis that the increase in gastric secretions upon administration of sodium deoxycholate is the immediate cause for the depressed rate of gastric emptying.

The ability of sodium deoxycholate to elicit fluid secretions into the gastric pouch is related to the properties of the bile salts in modifying membrane permeability. Studies in this laboratory suggest that bile salts increase the permeability of the goldfish membrane to various drugs (12) and enhance the rate of translocation of salicylate in everted gut preparations (13). Faust and Wu (14) using the everted gut technique have shown that bile salts, when placed in the mucosal solution, produce a net flux of water from the serosal to mucosal side. Davenport (15) has reported that bile salts are capable of increasing the permeability of the gastric mucosa as judged by H^+ flux.

The alterations in membrane permeability produced by bile salts may or may not be specific. For example, the similar effects on gastric secretion observed with calcium binding substances (11) and sodium deoxycholate suggest a role for calcium depletion from the membrane. This possibility is particularly relevant since Webling *et al.* (16) have shown that bile salts are capable of complexing calcium ions. On the other hand, it appears that injury to the gastric mucosa may also result in changes in membrane permeability and net secretion of fluid. Davenport (17) has noted that the canine gastric mucosa, when damaged by salicylates or fatty acids, produces a large volume of fluid. It has been tentatively suggested that acid itself is the initiating stimulus for fluid production and that histamine liberation by damaging agents is an important mediator.

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Keyphrases

Deoxycholate, sodium—gastric emptying effect, rats
Phenol red—sodium deoxycholate delayed gastric emptying
Stomach weight—sodium deoxycholate effect
Colorimetric analysis—spectrophotometer

Excretion and Distribution of Thiazesim-^{14}C with Its Biotransformation *In Vivo* and *In Vitro*

By J. DREYFUSS, J. R. SWOAP, C. CHINN, and S. M. HESS

Thiazesim-^{14}C [5-(2-dimethylaminoethyl)-2,3-dihydro-2-phenyl-1,5-benzothiazepin-4(5*H*)-one] was demonstrated to have been completely absorbed after administration to rats. After an i.p. dose of 30 mg. of thiazesim-^{14}C per kg. the drug was found in all the tissues that were examined; it was most concentrated in the livers, lungs, and kidneys. The half-life in the brains was about 20 min. The drug was converted in the rat to three major metabolites, two of which were present in unconjugated form and also as sulfate and glucuronide conjugates. Unchanged thiazesim-^{14}C and the three metabolites accounted for 68 and 59 percent of the radioactivity in rat urine and feces, respectively, in the first 24 hr. after administration. More than 90 percent of an oral dose was excreted by rats or by dogs in 3 days; the agent was completely eliminated in 4–6 days. Thiazesim-^{14}C was metabolized by a microsomal enzyme system derived from rat liver. The system required oxygen, NADPH, and nicotinamide. Two major products were obtained by the incubation of the drug with rat liver preparations *in vitro;* they were absent in the excreta of treated rats. Metabolism of thiazesim-^{14}C by liver preparations from the dog, cat, or rabbit was also examined. Although the metabolites appeared to have been similar in all the species examined, the preparations obtained from rat livers metabolized thiazesim-^{14}C twice as fast as microsomes obtained from the other species.

THIAZESIM[1] is an antidepressant agent in humans (1). The compound has been shown to have a specific depressant effect on parts of the amygdalae of various animals; these portions of the brain appear to have been implicated in antidepressant activity (2). Thiazesim depressed the hyperirritability of rats with lesions in the septal area. In addition, the drive by selected aggressive rats to kill mice when they were placed together in a cage was abolished. Thiazesim also blocked the after-discharge produced by stimulation of the amygdaloid nuclei of cats. Afterdischarges due to stimulation to other areas of the limbic system were not affected (3).

This investigation was designed to study the absorption, metabolism, and excretion of thiazesim-^{14}C after administration to animals. The characterizations of the metabolites isolated after treating the animals or after incubation with liver preparations *in vitro* have been described elsewhere in this journal (4).

EXPERIMENTAL

Thiazesim-^{14}C with a specific activity of 0.25 μc./mg. was synthesized by Dr. John Krapcho of The Squibb Institute for Medical Research. The structure of thiazesim-^{14}C is shown in Fig. 1. The purity of the compound was 98% as assayed using infrared spectroscopy and by thin-layer chromatography with the two benzene–ammonia–dioxane systems, as noted in the section on thin-layer chromatography; reference standards of authentic nonradioactive thiazesim were used for comparison. The nonradioactive compound was visualized after chromatography by either the absorption of UV light (254 mμ) against a fluorescent background or by a positive color reaction with Dragendorf's reagent (5).

Extraction Procedure—Except where otherwise noted, male rats of the Holtzman strain weighing 100–250 g. were used. The plasma samples were diluted first with 4 vol. of methanol. The resulting turbid solutions were adjusted to pH 9 with 1 *M* borate buffer, pH 9.4 and 1 *N* NaOH; they were shaken for 15 min. with 2 vol. of benzene and then centrifuged. The benzene was removed and saved. The remaining lower layers and residues were similarly extracted again with a volume of chloroform–methanol (1:1) equal to that of the benzene used above. The combined organic layers were evaporated under reduced pressure at 50° to 5% of the original volume for subsequent use.

Samples of tissues or feces were homogenized with a Potter-Elvehjem or Virtis homogenizer in 4 vol. of 0.1 *N* HCl. Homogenates of tissue and feces or samples of urine were adjusted to pH 9 with 1 *M* borate buffer, pH 9.4, and sufficient 1 *N* NaOH and

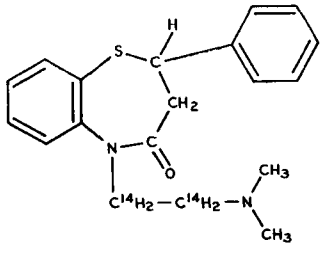

Fig. 1—Structure of thiazesim-^{14}C.

Received March 15, 1968, from the Squibb Institute for Medical Research, New Brunswick, NJ 08903
Accepted for publication June 6, 1968.
During these experiments Mr. Michael Rispoli provided excellent technical assistance, and Mr. Octavian Kocy performed the electrophoretic separations.

[1] Marketed as Altinil, E. R. Squibb & Sons, Inc., also known as SQ 10496

extracted with 2 vol. of benzene. The benzene layer was transferred to a clean glass-stoppered bottle, and the lower layer extracted twice with volumes of chloroform–methanol (1:1) equal to that of the benzene used above. In the case of feces only, a fourth extraction was performed with 2 vol. of methanol.

The extracts were combined, acidified with 1 *N* HCl, and twice the volume of *n*-heptane was added. After shaking for 20 min., the lower layer was removed and concentrated to 5% of the original volume by evaporation under reduced pressure at 50–60°. These procedures extracted 99% of the unchanged thiazesim-^{14}C added to control samples and 75–100% of the radioactivity due to metabolites present in tissues and excreta.

Alumina Column Chromatography—Aluminum oxide was used for the initial separation of metabolites. In order to remove UV-absorbing contaminants, all aluminum oxide was washed with chloroform–methanol (5:1) before use and then dried at 100°. Enough neutral aluminum oxide (Woelm) was added to the evaporated extracts, obtained as described above, to make a thick paste (for 5 ml. of concentrate about 9 g. was added). The paste was taken to complete dryness by warming overnight in a vacuum oven at 50°. The dried sample was placed on top of a column (3 × 15 cm.) of neutral aluminum oxide to which 6% water had been added (6). Fractions of 5 ml. were collected; the column was developed in a stepwise manner with chloroform–methanol (5:1) 80 ml., designated Fraction 1; MeOH–H_2O (1:1) 80 ml., designated Fraction 2; and finally H_2O–NH_3 (20:1) 180 ml., designated Fraction 3 (see Fig. 2). Changes of solvent were made after the first 2 vol. noted above had been collected. Care was taken to maintain a small solvent head during the initial elution procedure until the effluent had appeared. Each of the fractions were assayed for radioactivity by counting a suitable sample in a Packard Tri-Carb liquid scintillation spectrometer. Quenching was determined by the addition of internal standard or with an automatic external standardization system. When nonradioactive thiazesim was used, Fraction 1 was measured by the use of its characteristic absorption at 280 mμ.

Thin-Layer Chromatography—The contents of the tubes in each of the three fractions were pooled and evaporated to about 2% of the original volume.

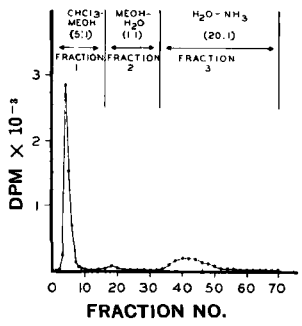

Fig. 2—Alumina column chromatography of a kidney extract containing thiazesim-^{14}C and metabolites. The tissue samples were obtained from rats 30 min. after an i.p. dose of 30 mg. of thiazesim-^{14}C per kg.

Aliquots of the evaporated Fraction 1 were chromatographed on a thin-layer plate with Silica Gel HF (Brinkmann); the plate was developed in benzene–ammonia–dioxane (BAD) (10:10:80 or 60:5:35) (7). Ninety-six percent of the spotted radioactivity obtained from control tissue samples to which thiazesim-^{14}C had been added migrated to the same relative position as authentic thiazesim-^{14}C (R_s = 1). The identity of thiazesim-^{14}C was confirmed by infrared spectroscopy of the eluted sample and by a comparison of the R_s values of the unknown samples with authentic thiazesim-^{14}C in the four solvent systems mentioned below. Other systems that were also used for the chromatography of Fraction 1 or hydrolyzed Fractions 2 and 3 were dioxane–methanol–ammonia–benzene (DMAB) (6:1:2:1) and water–ammonia–dioxane (WAD) (50:1:10).

Enzymatic Hydrolysis—A portion of the evaporated Fraction 2 was hydrolyzed with glucuronidase Mixture A[2] for 18 hr. at 37° to cleave glucuronides. A 0.25-ml. sample was mixed with 0.01 ml. of glucuronidase Mixture A and 0.05 ml. of 1 *M* acetate buffer, pH 5. Alternatively, a 0.25-ml. sample of Fraction 2 was hydrolyzed by mixing it with 0.05 ml. of 1 *M* acetate buffer, pH 5, and 25 units of aryl sulfatase, Type III (Sigma Chemical Co.), followed by incubation for 18 hr. at 37°. The hydrolyzed Fraction 2 was then chromatographed on Silica HF with BAF (10:10:80). A portion of the evaporated Fraction 3 of urine, 0.25 ml., was hydrolyzed by mixing it with 0.025 ml. of glucuronidase Mixture B and 0.05 ml. of 1 *M* acetate buffer, pH 5, followed by incubation for 18 hr. at 37°. The sample of hydrolyzed Fraction 3 was chromatographed on Silica Gel HF in DMAB (6:1:2:1).

Electrophoresis—The evaporated sample of Fraction 3 of feces was spotted on Whatman No. 1 filter paper and subjected to electrophoresis for 6 hr. at 500 v. with a buffer, pH 1.9, which was prepared by mixing 0.6 *M* formic acid with 1 *M* acetic acid.

Combustion Procedure—Tissue or feces samples were combusted in an oxygen atmosphere by the method of Kelly *et al.* (8), as described by Ebert and Hess (9).

Preparation of Subcellular Liver Fractions—All experiments, except those involving enzyme localization and cofactor requirement studies, were performed with the supernatant fraction from liver homogenates that was obtained at 9000×*g*. Livers from male Holtzman rats 250–300 g., male albino rabbits 1.9–2.1 kg., cats of either sex 2–2.2 kg., or male beagle dogs were used. Dogs were sacrificed by a rapid intravenous injection of 5 ml. of saturated KCl; other animals were killed by either stunning or decapitation, or both, and exsanguination. Livers were quickly excised, chilled in ice-cold 1.15% KCl, blotted on filter paper, and weighed. Subsequent operations were performed at 4°. Livers were minced and homogenized in 2 vol. of 1.15% KCl using a Potter-Elvehjem tissue grinder with a Teflon pestle. The homogenate was centrifuged at 9,000×*g* for 15 min. at 4°; the supernatant fraction was used for incubation. Washed microsomes were prepared by centrifuging such a supernatant fraction at 105,000×*g*

[2] Glusulase, Endo Labs, Garden City, N. Y.
[3] Ketodase, Warner-Chilcott, Morris Plains, N. J.

for 60 min.; the pellet was washed twice by resuspending it in a volume of 1.15% KCl equal to that of the supernatant material removed, followed by centrifuging at 105,000×g for 60 min. The pellet obtained at 9,000×g was similarly washed twice; the microsomes or the pellet were finally resuspended in a volume of 1.15% KCl equal to that of the homogenate from which the cell fractions were derived.

Total nitrogen was determined by the micro-Kjeldahl method.

Incubation Conditions In Vitro—Enzyme activity determinations were carried out in either open beakers or conical flasks at 37° on a Dubnoff metabolic shaker. The incubation mixture consisted of 50 μmoles of phosphate buffer, pH 7.4, 2 ml. of supernatant obtained at 9,000×g, 2 μmoles of thiazesim-^{14}C, 5.6 μmoles of glucose-6-phosphate, 0.39 μmole of NADP, 5 μmoles of $MgCl_2$, and 60 μmoles of nicotinamide; the final volume was 4 ml. After 5 min. of incubation at 37°, the substrate was added to initiate the reaction.

Extraction Procedure In Vitro—Samples of the incubation mixtures were removed at suitable intervals and transferred to 45-ml. centrifuge tubes containing 8 ml. of water-saturated butanol and 2 g. of NaCl. The mixture was shaken for 15 min. and centrifuged for 10 min. The butanol layer was transferred to a small round-bottom flask and the residue was extracted once more with 8 ml. of water-saturated butanol. The butanol extracts were combined and evaporated to dryness under reduced pressure; the resulting residue was dissolved in 1 ml. of methanol. After removing the alcohol-insoluble solids by centrifugation, 10 μl. of the supernatant was spotted on plates coated with 250 μ of Silica Gel HF. The plates were developed in BAD (60:5:35 or 10:10:80). The former mixture of solvents provided a means for the rapid separation of unchanged thiazesim; the latter mixture allowed a better resolution of the metabolites. The quantities of unmetabolized thiazesim-^{14}C and its metabolites were determined by measuring the radioactivity found in the sectioned chromatogram. This technique effected recoveries in the range of 75–90% of the radioactive substances in the incubation mixtures.

Excretion Experiments—Male and female rats were placed in cylindrical glass cages that allowed the animals access to condensed milk–water (1:1) but allowed only minimal freedom of movement. To collect feces, a small plastic bottle with the bottom removed and a hole drilled in the cap was used. The rat's tail was threaded through the cup (wide end first) and positioned in such a way that the anus was covered but not the urethra. The bottle cap was affixed and tape was used to immobilize the bottle. With this technique, feces were collected uncontaminated by urine. Urine was collected directly in a vessel cooled over dry ice.

Measurement of Binding to Plasma Proteins—Thiazesim-^{14}C was added to dog plasma. The plasma samples were placed in dialysis bags and tied. The bags were then supported in test tubes lined with wire screens of about 100 squares/cm.[2] The tubes were centrifuged for 45 min. at about 1,000×g in a Sorvall model SS-1 centrifuge or until 0.3 ml. of filtrate had accumulated in the bottom of tubes (10). The radioactivity present in the plasma before centrifugation was measured by dissolving 0.2 ml. of plasma in 1 ml. of NCS solubilizer (Nuclear Chicago), with the subsequent addition of 15 ml. of toluene scintillation fluid. The plasma filtrate was counted directly in Bray's scintillation fluid (11).

Biliary Excretion—Male rats were anesthetized with ether, the common bile duct was exposed and catheterized with a polyethylene tube that passed through the abdominal incision. The incision was sutured and the animal placed in a restraining cage. Bile was collected in tubes for 24 hr.

RESULTS AND DISCUSSION

Absorption and Excretion—Data are given in Table I for the distribution of drug after rats had received oral doses of either 30 or 100 mg. of thiazesim-^{14}C per kg. Peak levels of radioactivity were observed in the blood, brains, and livers within 30 min. These levels of radioactivity declined rapidly thereafter. The amount of radioactivity is expressed in terms of thiazesim-^{14}C equivalents, that is the radioactivity present was assumed to have had the molecular weight and specific activity of thiazesim-^{14}C.

The levels of total radioactivity found in rat brains as a function of time after animals had been dosed i.p. with 30 mg. of thiazesim-^{14}C per kg. are shown in Fig. 3. Radioactivity rapidly disappeared from the brains. Within 10 min. after an i.p. injection, the peak levels in the brains had already been reached; the half-life during the initial linear portion of the curve was about 20 min. No detectable radioactive substances were present in the brains after 2 hr.

The distribution of thiazesim-^{14}C in various organs of the rat was measured during a period of 6 hr. after an i.p. dose of 30 mg. of the drug per kg. (see Table II). The levels of thiazesim-^{14}C equivalents were highest in the livers, lungs, and

TABLE I—DISTRIBUTION OF RADIOACTIVITY AFTER A SINGLE DOSE OF THIAZESIM-^{14}C, EXPRESSED AS THIAZESIM-^{14}C EQUIVALENTS

Time, hr.	Dose, mg./kg.[a]	Blood, mcg./ml.	Brain, mcg./g.	Liver, mcg./g.
0.5	30	6.1 ± 0.9	5.0 ± 1.9	93.5 ± 6.9
1.0	30	3.6 ± 0.4	<1.5	59.6 ± 6.0
3	30	2.0 ± 0.4	<1.5	33.7 ± 3.2
0.5	100	16.8	23.5	178
1	100	12.7	11.6	149
3	100	5.2	4.2	64.0

[a] Rats received p.o. either 30 mg. or 100 mg. of thiazesim-^{14}C per kg.; three rats were used in each experiment at the lower dose level and the values for these are given ± the standard error. Two animals were used at the higher dose; the values represent the mean.

kidneys; intermediate levels were found in the spleens, mesenteric fat, and testes. Relatively low levels were found in the hearts, brains, thigh muscle, and blood. Six hours after the injection the liver contained 17 mcg. of radioactive substances per g., while the other tissues had much lower concentrations. It was interesting to note that the kidney, which might have been expected to have concentrated the drug and its metabolites in the urine, contained only about 0.33 as much as were found in the liver.

Figure 4 summarizes the excretion of radioactivity by male and female rats after single oral doses of 100 mg. of thiazesim-^{14}C per kg. Ninety-three percent of the dose administered to male rats was excreted in 2 days. After 4 days, 99.9 ± 3% of the dose had been excreted; at this time, 1.3% of the dose was left in the whole animal. Thirty-seven percent of the dose was excreted in the urine and 63% of the dose in the feces. Excretion by female rats similarly dosed was also rapid, although 6.7% of the dose still remained in the whole animals after 4 days (see Fig. 4). Male dogs dosed with 5 mg. of thiazesim-^{14}C per kg. excreted the entire dose in 6 days; the excreted dose appeared equally in the urine and feces (see Table III).

The excretion of radioactivity in the bile of rats was more rapid than that in the feces after an i.p. dose of 30 mg. of thiazesim-^{14}C per kg. Within 24 hr. after the injection, 52% of the dose was found in the feces. During the same period other rats, similarly dosed, excreted 70% in the bile (see Fig. 5); the major portion, 66% had been excreted in the bile in the first 6 hr. after the dose. The delay in the excretion of radioactivity in the feces, as compared with the outpouring in the bile, suggested

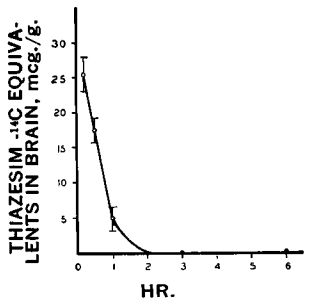

Fig. 3—Thiazesim-^{14}C equivalents present in brains as a function of time. Rats were dosed i.p. with 30 mg. of thiazesim-^{14}C per kg.

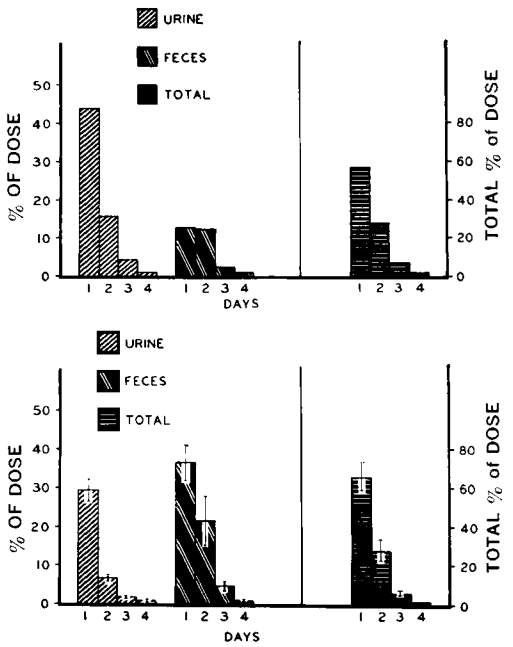

Fig. 4—Excretion of radioactivity measured as percent of dose in male rats (lower) and female rats (upper) dosed p.o. with 100 mg. of thiazesim-^{14}C per kg. Data for male rats are given plus or minus the standard error; n is equal to 3. Data for female rats are the averages of two animals.

TABLE III—EXCRETION OF THIAZESIM-^{14}C BY MALE DOGS[a]

Time, Days	Urine, % of Dose	Feces, % of Dose[b]	Cumulative Excretion, % of Dose
0–1	40.3	13.3	53.6
1–2	7.6	23.3	84.5
2–3	2.2	10.8	97.5
3–4	0.4	2.0	99.9
4–6	—	1.2	101.1
	50.5	50.6	

[a] Dogs received p.o. 5 mg. of thiazesim-^{14}C per kg. All numbers are the averages of values from two beagle dogs. [b] One dog did not excrete any feces during the first day.

that thiazesim and its metabolites may have been absorbed from the intestine.

Separation of Metabolites—In order to isolate and identify metabolites, the total radioactivity

TABLE II—DISTRIBUTION OF THIAZESIM-^{14}C AND METABOLITES IN TISSUES MEASURED AS THIAZESIM-^{14}C EQUIVALENTS, mcg./g.[a]

Tissue	0.5 hr.[b]	1 hr.[b]	3 hr.[b]	6 hr.[b]
Liver	91.4 ± 7.2	44.4 ± 3.7	19.1 ± 2.2	17.0 ± 2.0
Lung	69.2 ± 3.8	23.1 ± 3.3	4.9 ± 0.5	4.1 ± 1.2
Kidney	59.2 ± 5.8	27.7 ± 4.8	6.3 ± 2.1	5.3 ± 0.7
Spleen	46.7 ± 1.5	15.4 ± 1.5	5.3 ± 1.7	2.8 ± 0.9
Mesenteric fat	38.1 ± 2.1	11.0 ± 1.9	4.1 ± 0.5	1.7 ± 0.2
Testes	33.7 ± 2.9	11.3 ± 3.2	3.7 ± 0.5	1.7 ± 0.2
Heart	20.0 ± 2.9	9.3 ± 1.5	2.1 ± 0.8	1.8 ± 0.4
Brain	17.4 ± 1.8	4.8 ± 1.7	<1.5	<1.5
Thigh muscle	16.7 ± 5.2	7.3 ± 1.9	2.0 ± 0.1	<1.5
Blood	8.2 ± 0.5	4.9 ± 0.6	1.5 ± 0.2	1.0 ± 0.3

[a] Rats received i.p. 30 mg. of thiazesim-^{14}C per kg. The values given are ± the standard error; *n* is equal to 3 or 4. [b] Blood levels are in mcg./ml.

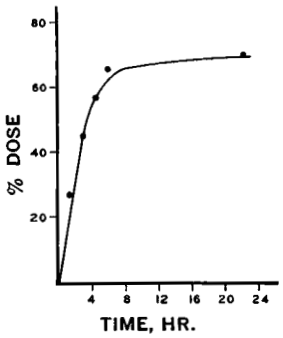

Fig. 5—Excretion of radioactivity in the bile as a function of time. Rats were dosed i.p. with 30 mg. of thiazesim-^{14}C per kg.

extracted from tissues or excreta of the rat and dog was separated grossly into three fractions, as described previously. Unmetabolized thiazesim-^{14}C and metabolites with minor structural alterations were eluted in Fraction 1. Metabolites that were conjugated as sulfate esters were eluted in Fraction 2. Metabolites that were present as glucuronide conjugates, as well as other polar metabolites, were eluted in Fraction 3.

Table IV shows the distribution of conjugated and unconjugated metabolites in various tissues and excreta of the rat and dog in typical experiments. Brains of rats contained primarily unconjugated metabolites and a trace of materials that was isolated in Fraction 2; glucuronide conjugates, Fraction 3, were absent from the brains. Rat plasma contained radioactivity in all three fractions. Livers and kidneys, and the feces of rats, as well as that of dogs, contained primarily unconjugated metabolites. By contrast, rat urine and bile, as well as dog urine, contained primarily conjugated metabolites.

The amounts of thiazesim-^{14}C (expressed as percents of the total radioactivity) present in various tissues and excreta of the rat and dog are shown in Table V. Rat brain contained primarily thiazesim-^{14}C. Rat liver, urine, feces, and bile contained very little unmetabolized thiazesim-^{14}C. Excreta of the dog contained several times more thiazesim^{14}C than was found in the excreta of the rat.

TABLE IV—DISTRIBUTION OF TOTAL EXTRACTED RADIOACTIVITY IN VARIOUS TISSUES AND EXCRETA OF THE RAT AND DOG

Sample[a]	% of Radioactivity		
	Fraction 1	Fraction 2	Fraction 3
Rat brain	93.8	6.2	0.0
Rat plasma	35.5	18.1	46.4
Rat liver	62.0	10.9	27.1
Rat kidney	64.6	4.4	31.0
Rat urine[b]	10.9	11.2	78.0
Rat feces[b]	62.0	8.9	29.1
Rat bile	9.4	18.7	71.8
Dog urine[c]	28.3	42.6	29.9
Dog feces[c]	62.0	14.1	23.9

[a] Male rats were given a dose i.p. of 30 mg. of thiazesim-^{14}C per kg.; organ samples were taken 30 min. after injection of the animals. Excreta samples were taken 24 hr. after dosing the animals. [b] The values cited for rat urine and feces are the results of pooling samples from 2 animals. [c] The values cited for dog urine and feces and for rat bile are the results of averaging values from 2 animals.

TABLE V—FRACTION OF RADIOACTIVITY PRESENT AS THIAZESIM-^{14}C IN VARIOUS TISSUES AND EXCRETA OF THE RAT AND THE DOG

Sample[a]	Amount of Thiazesim Present	
	%	mcg./g.
Rat brain	76.3	13.3
Rat plasma	22.5	3.7
Rat liver	3.9	3.6
Rat kidney	15.0	8.9
Rat urine	1.5	—
Rat feces	5.3	—
Rat bile	1.7	—
Dog urine	9.0	—
Dog feces	15.3	—

[a] Organ samples were taken after 30 min; samples of excreta were collected after 24 hr. Rats received i.p. 30 mg. of thiazesim-^{14}C per kg.; dogs received p.o. 5 mg. of thiazesim^{14}C per kg.

Figure 6 summarizes the results of separating by TLC the radioactive products in Fractions 1 and 2 of excreta and bile of rats and dogs that had received thiazesim-^{14}C. Four metabolites and thiazesim were distinguished. Fraction 2 was hydrolyzed with glucuronidase Mixture A prior to chromatography. The material of Fraction 2 remaining at the origin represents, in part, unhydrolyzed material. After the i.p. administration to rats of 30 mg. of thiazesim per kg., the metabolites in Fraction 1 of the bile were found to have been similar to the metabolites in the urine of rats that had received the same dose either i.p. or p.o. Dogs metabolized thiazesim-^{14}C differently from rats since in the dog greater fractions of the dose in the excreta exhibited R_s values of 0.64 and 1; also the urine of dogs contained a greater proportion of the dose in Fraction 2 than did the urine of rats.

The results of the separation of the components of Fraction 3 of urine after enzymatic hydrolysis are shown in Fig. 7. Five components were separated. With chromatography in BAD (10:10:80), DMAB, and WAD, it was demonstrated that the compounds with R_S values of 0.52 and 0.88 in BAD (10:10:80) (Fig. 6) were identical with the compounds with values of 0.83 and 0.95, respectively, in DMAB (Fig. 7). These components accounted for the bulk of the metabolite radioactivity in the rat. No radioactivity was detected at R_s values of 0.06, 0.28, or 0.45 in the urine of female rats.

The radioactivity present in Fraction 3 of feces and bile was separated into two components by electrophoresis (Fig. 7).

It may be seen from Figs. 6 and 7 that the pattern of excretion was qualitatively and quantitatively similar after i.p. or oral doses of 30 mg. of thiazesim per kg.; this suggested strongly that the drug was completely absorbed after oral administration.

It is noteworthy that the electrophoretic separation of extracts of the feces of rats demonstrated the presence of a much smaller amount of the component at −10.9 cm. than did extracts of bile (Fig. 7). Furthermore, Fraction 3 of bile was hydrolyzed with β-glucuronidase to the extent of 60–70% as opposed to the fraction from feces that was hydrolyzed to the extent of only 30%. An explanation for this quantitative difference between bile and feces, may be that intestinal microorganisms hydrolyzed glucuronide-conjugated metabolites. An alternative explanation may be that glucuronide conjugates were absorbed in the in-

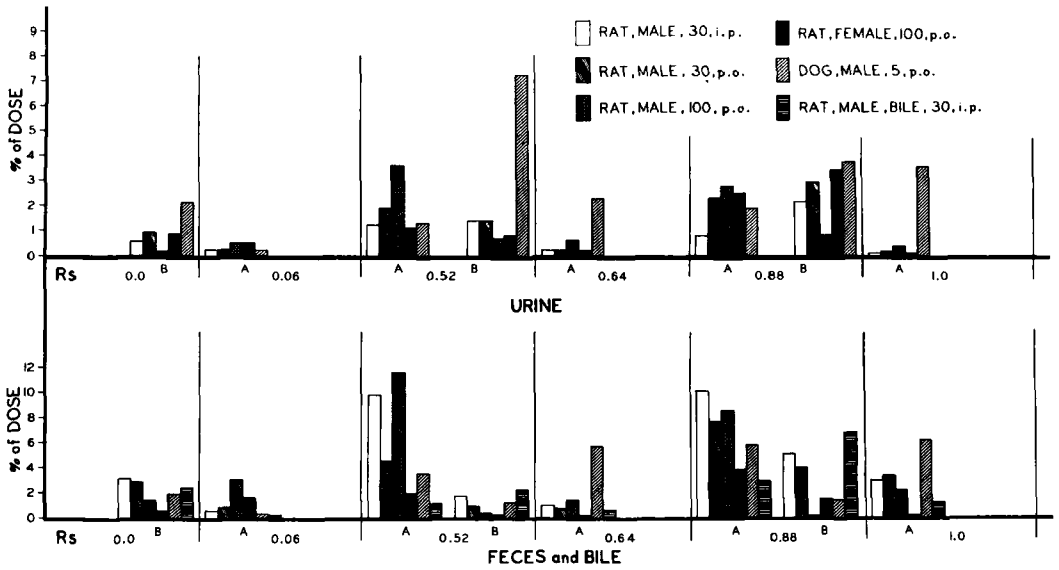

Fig. 6—Distribution of metabolites in Fractions 1 and 2 of rat urine, feces, and bile. Metabolites were located, according to their R_s values relative to thiazesim, on Silica Gel HF plates developed with BAD (10:10:80). Metabolites of Fraction 1 are designated A. *Metabolites of Fraction 2 were chromatographed after enzymatic hydrolysis with glucuronidase mixture A and are designated* B.

testinal tract and subsequently excreted in either the bile or the urine. Williams *et al.* (12) seem to favor the former since they have reported that glucuronide conjugates did not appear to have been absorbed from the intestinal tract, although the hydrolyses of glucuronide conjugates by bacteria have been observed.

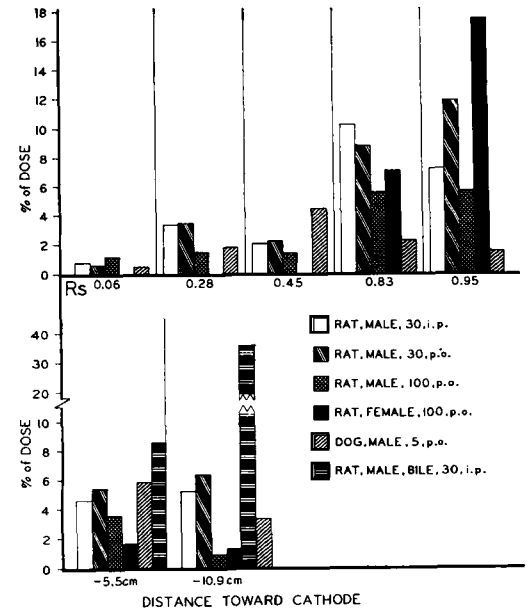

Fig. 7—Distribution of metabolites in Fraction 3 of rat urine, feces, and bile. Components of Fraction 3 of urine (upper) were separated, after enzymatic hydrolysis with glucuronidase mixture B, followed by chromatography on Silica Gel HF plates developed with DMAB (6:1:2:1). Metabolites were located according to their R_s values relative to thiazesim. Components of Fraction 3 of feces or bile (lower) were separated by paper electrophoresis as described in the text.

In order to ascertain whether the metabolites, as isolated from rat feces, had undergone bacterial alterations, bile was collected from rats fitted with indwelling biliary cannulae. The metabolites found in bile in the unconjugated form were the same on the basis of R_s values as those found in feces which suggested that microbial degradation had not produced the metabolites isolated from feces.

The three isolated metabolites, in both their conjugated and unconjugated forms, accounted for 68 and 59% of the amount of radioactivity present in urine and feces, respectively, in 24 hr. A detailed listing of the percent of the dose found in excreta after 24 hr. is shown in Table VI.

The binding of a parent compound to plasma proteins tends to inhibit its metabolism and excretion; in this manner its action in the animal is prolonged. Thiazesim-^{14}C was bound to plasma constituents of the dog. The binding varied between 53–72%, over the range of concentrations (0.33–33 mcg./ml. of plasma) found in a dog that had received i.v. 3 mg. of thiazesim per kg.

Are Microsomal Enzymes Induced By Thiazesim?—Thiazesim did not stimulate the proliferation of drug-metabolizing enzymes in rats. Rats that had been given either 30 mg. of thiazesim per kg. twice daily for 4 days or 98 mg. of thiazesim per kg. per day in the diet for 3 weeks exhibited no decrease in hexobarbital sleep time when compared with a control group that had received no drug. Rats given phenobarbital, a known inducer of drug-metabolizing enzymes (13), demonstrated the expected decrease in the duration of the hexobarbital sleep time.

Metabolism of Thiazesim-^{14}C In Vitro—A microsomal preparation derived from rat liver was capable of catalyzing the metabolism of thiazesim-

TABLE VI—ISOLATED METABOLITES OF THIAZESIM-^{14}C IN RAT EXCRETA IN THE CONJUGATED AND UNCONJUGATED STATES[a]

R_s of Metabolite[b]	% of Dose in 24 hr. Unconjugated Urine	Unconjugated Feces	Sulfate Urine	Sulfate Feces	Glucuronide Urine	Total
0.52	0.8	10.2	1.4	1.7	10.2	24.3
0.64	0.2	1.0	0.0	0.0	0.0	1.2
0.88	1.2	9.8	2.2	5.1	7.1	25.4

[a] Rats received i.p. 30 mg. of thiazesim-^{14}C per kg. [b] R_s values are given relative to that of thiazesim in the solvent system BAD (10:10:80).

^{14}C in the presence of suitable cofactors (Table VII). Specifically, either NADPH or an NADPH-generating system was required. The presence of nicotinamide strongly stimulated the reaction. Incubation in the presence of a nitrogen atmosphere severely inhibited metabolism.

Table VIII shows the results of an experiment that tested various subcellular fractions of rat liver for their ability to metabolize thiazesim-^{14}C. The data showed that either the supernatant fraction obtained at 9,000×g or the microsomal fraction, together with the supernatant fraction collected at 105,000×g, contained the most activity. When the supernatant material obtained at 9,000×g was fractionated at 105,000×g into microsomes and supernatant material, each had greatly diminished activity, however, when the two fractions were combined, activity was equal to that of the parent supernatant fraction. The lack of glucose-6-phosphate dehydrogenase supplementation to the reaction mixture probably accounted for the low activity of the isolated microsomal fraction. The pellet collected at 9,000×g, as well as the whole homogenate, contained very little activity. Furthermore, the supernatant fraction separated at 9,000×g, reconstituted with the pellet derived at 9,000×g, had no activity (data not shown). The lack of activity in the whole homogenate is notable and suggests the presence of inhibitory properties in the material that sediments at 9,000×g. Indeed, Axelrod (14) has shown that the metabolism of drugs by NADPH-dependent microsomal enzymes can be inhibited by substances present in liver homogenates of rats. Gillette *et al.* (15) have found evidence that pyrophosphatases, which hydrolyze NADPH and NADP, can partially account for the inhibitory effects of rat nuclei. An effort was made to obtain increased metabolic activity in the whole homogenate by supplementing the incubation mixture with ATP and DPN, however, the presence of these additional cofactors did not increase the activity. As pointed out by Gillette (16), the binding of substrates to nuclei and mitochondria, components of the whole homogenate, also can result in much slower rates of metabolism in unfractionated homogenates.

Isolation of the metabolic products produced by incubation of the drug *in vitro* with preparations of rat liver revealed that these substances had the same R_s values as those isolated from the excreta of rats dosed with thiazesim-^{14}C (Table IX). It was at once apparent that although the relative concentrations of the metabolites obtained from the *in vitro* system differed from those obtained from the excreta of the rats, the R_s values suggested that the products were the same. The products of

TABLE VII—COFACTOR REQUIREMENTS FOR THIAZESIM-^{14}C METABOLISM IN RAT LIVER *In Vitro*[a]

Additions	Thiazesim-^{14}C Metabolized[b] %	μmoles
None	5.9	0.12
NADPH, 1 μmole	4.1	0.08
NADPH, 4 μmoles	12.0	0.24
NADPH, 4 μmoles + 60 μmoles nicotinamide	27.5	0.55
NADPH-generating system + 60 μmoles nicotinamide	31.0	0.62
NADPH-generating system[c]	2.5	0.05
NADPH-generating system + 60 μmoles nicotinamide, under N_2 atmosphere	9.1	0.18

[a] The data represent the mean of 2 experiments. The basic reaction mixture contained rat liver microsomes (derived from 1 g. of liver), which had been washed twice with 1.15% KCl; 100 μmoles of phosphate buffer, pH 7.4; and 2 μmoles of thiazesim-^{14}C in a final volume of 4 ml.; incubation was for 30 min. at 37°. [b] The figures were calculated after subtracting the amount of thiazesim-^{14}C found in the boiled blank (4.5%). [c] The NADPH-generating system consisted of 5.6 μmoles of glucose-6-phosphate, 0.39 μmole of NADP, and 8 units of glucose-6-phosphate dehydrogenase.

TABLE VIII—RATES OF THIAZESIM-^{14}C METABOLISM IN SUBCELLULAR FRACTIONS OF RAT LIVER

Cell Fraction	Thiazesim-^{14}C Metabolized[a] %	μmoles
Whole homogenate	2.4	0.05
Pellet obtained at 9,000×g	3.7	0.07
9,000×g-Supernatant fraction	25.1	0.50
Microsomes	8.5	0.17
105,000×g-Supernatant fraction	3.3	0.07
Microsomes + 105,000×g-supernatant fraction	26.0	0.52

[a] Incubation was for 30 min. at 37° with subcellular fractions equivalent to 667 mg. of liver. The values given here represent the data of two experiments; the figures were calculated after subtracting the amount of thiazesim-^{14}C found in the boiled blank (4.3%).

TABLE IX—METABOLITES OF THIAZESIM-^{14}C IN EXCRETA AND IN LIVER PREPARATIONS OF THE RAT

R_s[a] of Metabolite	Liver Preparations % Metabolism[b]	Excreta % of Dose[c]
0.05	2.9	0.2
0.11	2.1	0.0
0.52	2.6	25.2
0.64	33.0	1.2
0.88	7.4	24.3

[a] BAD (10:10:80) on Silica Gel HF; R_s is relative to thiazesim. [b] The incubation was carried out using the 9,000×g supernatant fraction, as described in the text. [c] Rats were dosed i.p. with 30 mg. thiazesim-^{14}C per kg.

TABLE X—DISTRIBUTION OF THIAZESIM-^{14}C AND METABOLITES AFTER INCUBATION *In Vitro* WITH LIVER PREPARATIONS FROM THE RAT, DOG, RABBIT, OR CAT[a]

R_s Compd.[b]	Rat, %[c]	Rabbit, %[c]	Dog, %[c]	Cat, %[c]
1.0	51.8	60.8	60.7	69.1
0.88	7.4	9.3	6.0	3.4
0.64	33.0	20.9	10.4	17.4
0.52	2.6	2.8	1.8	0.9
0.11	2.1	2.7	18.2	4.2
0.05	2.9	3.5	2.9	5.1

[a] Incubation was for 90 min. at 37° using the supernatant fraction of livers obtained at 9,000×g and the conditions described in the section on experimental procedures in the text. Samples were extracted and chromatographed on thin-layer plates prepared with Silica Gel HF and developed with BAD (10:10:80) as described in the text. [b] The R_s value is relative to thiazesim. [c] The percentages in the table refer to the distribution of metabolites on the chromatographic plates; they have been corrected to represent the metabolism per g. of nitrogen in the 9,000×g-supernatant; the figures are averages for two experiments with the liver of one animal in each.

incubation with R_s values of 0.64 and 0.88 were isolated, and an effort was made to characterize them (4). It was surprising to learn that the two major products obtained from the incubations *in vitro* were different from the *in vivo* metabolites that had comparable R_s values. No evidence has yet been uncovered to show that the major products formed *in vitro*, those with R_s values of 0.64 and 0.88, were present in any fractions isolated after the administration of thiazesim-^{14}C to animals.

A survey was carried out of the metabolism of thiazesim-^{14}C by several animal species that exhibited somewhat varying sensitivities to the drug. The object of the study was to learn if any correlation existed between the formation of any specific metabolites, or between the overall rate of metabolism and the sensitivity to the drug. The species chosen were the rat, dog, rabbit, and cat. The survey was made by incubating *in vitro* the supernatant fraction obtained by centrifuging at 9,000 × *g*, homogenates of the livers of the animals.

It was observed (Table X) that the metabolites produced *in vitro* exhibited R_s values that corresponded closely to those found *in vivo*. The amounts of each metabolite formed varied somewhat among the species. The only notable difference was found in the incubation mixture prepared with the liver of the dog; the liver of the dog produced about five times more radioactive materials at the R_s of 0.11 than had been produced by the preparations of the other species.

A study of the rates at which thiazesim-^{14}C had been metabolized *in vitro* by the livers of the rat, rabbit, cat, and dog is summarized in Fig. 8. It may be seen that preparations obtained from rats were about twice as active as those obtained from the other species. This difference seemed to be related to the activity of the microsomes, since the quantity of microsomes per mg. of nitrogen in the supernatant fraction obtained at 9,000×*g* was equal in the preparations obtained from the dog and rat. The dog, which was the species most sensitive to the drug,[4] provided incubation mixtures that

[4] Private communication from Dr. J. Poutsiaka of the Toxicology Department of the Squibb Institute for Medical Research.

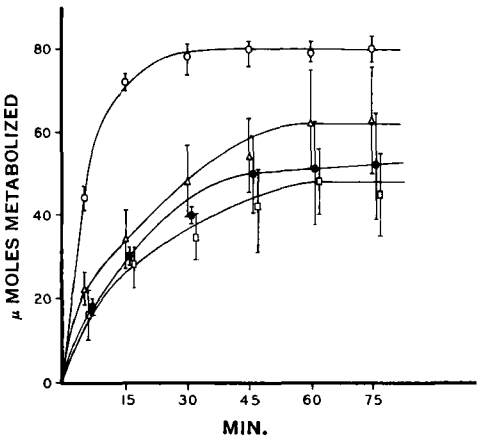

Fig. 8—Rates of thiazesim-^{14}C metabolism by the supernatant fractions (9,000×g) obtained from the liver of various species. Each point represents average values from 2 experiments that had been run in duplicate. Incubation conditions are described in the section on experimental procedure. Metabolism is expressed as μmoles thiazesim-^{14}C metabolized per g. of nitrogen in the 9,000×g-supernatant. Key: ○, rat; △, dog; ●, rabbit; □, cat.

metabolized the drug at a rate that did not differ from the rates exhibited by the rabbit and cat. The LD_{50} values for representatives of the four animal species were determined under fasted conditions. These values for the rat, rabbit, dog, and cat were 560, 220, 70 and 40 mg./kg., respectively. These LD_{50} values corresponded roughly to the rates at which the liver microsomes metabolized thiazesim, however, the correlation was not precise enough to demonstrate whether the rate of thiazesim metabolism was directly related to the degree of toxicity of the compound.

The likelihood that the metabolite isolated at the R_s of 0.11 could account for the varying sensitivities to thiazesim should be considered with caution since the rabbit and cat, which were also more sensitive to the drug than was the rat, produced the same amount of the metabolite as did the rat. In addition, other metabolic alterations, as well as other factors not measured *in vitro*, may have played roles in the varying sensitivities to the drug.

Identification of Metabolites In Vitro and In Vivo—A variety of spectroscopic procedures was used to identify metabolites. The results of these studies are reported in detail in a companion publication (4).

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Keyphrases

Thiazesim-^{14}C—excretion, distribution
Biotransformation, *in vivo*, *in vitro*—thiazesim-^{14}C
Column chromatography—separation
TLC—separation, identity
IR spectrophotometry—identity, purity
Electrophoresis—separation, radioactive metabolites

Metabolism of Thiazesim, 5-(2-Dimethylaminoethyl)-2,3-dihydro-2-phenyl-1,5-benzothiazepin-4(5*H*)-one, in the Rat *In Vivo* and *In Vitro*

By J. DREYFUSS, A. I. COHEN, and S. M. HESS

Three metabolites of thiazesim, 5-(2-dimethylaminoethyl)-2,3-dihydro-2-phenyl-1,5-benzothiazepin-4(5*H*)-one, were isolated from rat feces. Metabolite 1 contained a hydroxyl group in the 2-phenyl ring. Metabolite 2 was dihydroxylated at unknown positions of the 2-phenyl ring. Metabolite 3 was a derivative of Metabolite 1 and, in addition, appears to have had one carbon and two oxygens added to the side chain. Two metabolites of thiazesim (4 and 5) were isolated from rat liver homogenates incubated with NADPH and nicotinamide. Metabolite 4 has been identified as the sulfoxide derivative of thiazesim. Metabolite 5 has not yet been identified, but it was not similar to those isolated *in vivo*. Hydroxylation of either aromatic ring was not an observed metabolic reaction *in vitro*. Sulfoxidation was not an observed metabolic reaction *in vivo*.

A PAPER ELSEWHERE in this journal (1) has described aspects of the metabolism of the antidepressant agent, thiazesim-^{14}C, 5-(2-dimethylaminoethyl)-2,3-dihydro-2-phenyl-1,5-benzothiazepin-4(5*H*)-one, in the rat and dog that relate to its absorption, distribution, excretion, as well as the techniques developed for the separation of both conjugated and unconjugated metabolites. Also discussed were the results obtained by incubating thiazesim-^{14}C with liver preparations from various animal species.

This paper will describe the identification of the metabolic products of thiazesim-^{14}C formed by the rat *in vivo* or by rat liver preparations *in vitro;* these metabolites have not yet been tested for pharmacologic activity. The results showed that the metabolites formed by the rat *in vivo* were not the same as those produced by rat liver preparations *in vitro*.

EXPERIMENTAL

Isolation of Fecal Metabolites—Three metabolites of thiazesim were isolated from feces in an unconjugated form by the following procedure. Rats were repetitively dosed once a day for 4 days with 100 mg. of thiazesim per kg. and the feces samples combined. The feces were homogenized in 4 vol. of methanol that was 0.12 *N* with respect to HCl, and centrifuged. After centrifugation, the supernatant fraction was removed and saved, and the residue was extracted twice more with 4 vol.

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of unacidified methanol. The combined methanol extracts were adjusted to pH 1 to 2 with 1 *N* HCl and 2 vol. of *n*-heptane was added. After shaking, the lower layer was recovered, evaporated, and chromatographed on an alumina column for the isolation of Fraction 1, as described previously (1). The isolated Fraction 1 was evaporated and spotted on 1-mm. Silica Gel HF plates which were developed in benzene–ammonia–dioxane (10:10:80). The appropriate UV positive zones were scraped from the plate, pooled, and packed into columns, 4 cm. diam. × 2 cm. high, which were eluted with chloroform–methanol (5:1). When necessary, each eluted compound was rechromatographed on 1-mm. Silica Gel HF plates in benzene–ammonia–dioxane (60:5:35 or 10:10:80) in order to remove contaminating yellow material.

Preparation and Isolation of In Vitro Metabolites—For the preparation of metabolites *in vitro* in milligram quantities, the following procedure was used. Male Holtzman rats weighing 250–300 g. were killed by decapitation and the livers were quickly removed, chilled in ice-cold 1.15% KCl, blotted on filter paper, and weighed. The livers were homogenized in 2 vol. of 1.15% KCl with a Potter-Elvehjem tissue grinder with a Teflon pestle. The homogenate was centrifuged at 9,000 × *g* for 15 min. at 4°. The incubation mixture consisted of 7.5 mmoles of phosphate buffer, pH 7.4, 300 ml. of the supernatant fraction of rat liver (9,000×*g*), 368 μmoles of thiazesim, and the following amounts of cofactors: $MgCl_2$, 750 μmoles; glucose-6-phosphate, 840 μmoles; NADP, 57.5 μmoles; and nicotinamide, 9 mmoles. Quantities of cofactors equal to those cited above were added after incubation at 37° for 15 and again at 30 min. An additional 322 μmoles of thiazesim was added after 30 min. Incubation was for a total of 60 min. The reaction mixture was extracted with *n*-butanol as previously described (1).

In order to isolate metabolites uncontaminated with nicotinamide, the latter was first removed by chromatography of the methanol extract on a column of Whatman SG 31 silica gel. Sufficient dry silica gel was added to the methanol extract to form a paste which was then dried at 70°. The dry material was placed on top of a column of dry silica gel (5 × 10 cm.) and eluted with water until the effluent no longer absorbed at 260 mμ. At this time, the metabolites and unchanged thiazesim were eluted from the column with methanol–ammonia (95:5). The concentrated eluates were then chromatographed on thin-layer plates, as described above in the section on the isolation of fecal metabolites.

Spectroscopic Analysis—NMR spectra were obtained on either a Varian model A60 spectrometer or on a Varian model A60-A spectrometer equipped with a C1024 time averager.

Mass spectra were obtained on either an Associated Electrical Industries model MS9 spectrometer or on a Consolidated Electrodynamics Corporation model 103C spectrometer. Element maps were obtained by computer analysis from data recorded from the MS9 spectrometer (2).

Metabolite Designations—Three metabolites were isolated from rat feces (1). These metabolites had R_s values of 0.88, 0.64, and 0.52 in benzene–ammonia–dioxane (10:10:80) on Silica Gel HF chromatograms, and were designated as Metabolites 1, 2, and 3, respectively. Two metabolites were isolated after incubation of thiazesim-^{14}C with rat liver preparations. These metabolites had R_s values of 0.88 and 0.64 and were designated as Metabolites 4 and 5, respectively.

Infrared Spectra—Infrared spectra of all compounds were determined as micro-KBr pellets in order to assess the approximate purity of the samples. All samples gave well-defined spectra that were clearly derivatives of thiazesim.

Ultraviolet Spectra—The UV spectra taken at acid pH in methanol solution for the metabolites isolated *in vivo* resembled that found for thiazesim. In each case a prominent peak was seen at 276–278 mμ, as compared with 271–272 mμ for thiazesim. When the spectra for Metabolites 1, 2, and 3, and thiazesim were rerun at pH >10 there was a bathochromic shift to about 300 mμ for the three metabolites, but not for thiazesim. These data suggested the introduction of phenolic hydroxyl groups in the metabolites.

Metabolites 4 and 5, produced from rat liver preparations *in vitro*, exhibited UV spectra at acid pH in methanol solution that consisted of no well-defined peaks, but simply shoulders at 272, 265, 235, and 225 mμ for Metabolite 4 and 265 and 240 mμ for Metabolite 5; these spectral features did not change when the samples were rerun at pH >10. These data suggested that no phenolic hydroxyl groups were present in these metabolites.

Mass Spectra—The mass spectra of thiazesim and Metabolites 1 and 2 gave molecular ions at 326 ($C_{19}H_{22}N_2OS$), 342 and 358, respectively, as shown in Fig. 1. The increase in weight of 16 and 32 mass units of the molecular ions of Metabolites 1 and 2 strongly indicated that the compounds were the mono- and di-oxygenated derivatives of thiazesim. As confirmatory evidence, the mass of the parent ion of Metabolite 1, measured by high resolution mass spectrometry, was 342.1414 which corresponded to the formula $C_{19}H_{22}N_2O_2S$ (calcd. 342.1403).

The origins of the fragmentation peaks of Metabolites 1 and 2 and thiazesim are described below. As an aid in making the assignments, the element maps of Metabolite 1 and thiazesim were prepared. The base peak at *m/e* 58 and the next most intense peak, at *m/e* 71, present in thiazesim and Metabolites 1 and 2, corresponding to the $[CH_3N(CH_3)_2]^+$ and $[CH_2{=}CH{-}N(CH_3)_2]$ ions, respectively, showed the retention of the unaltered side chain in Metabolites 1 and 2, as shown in Scheme I. Furthermore, the presence of the ion at *m/e* 178 (C_9H_8NOS) in the spectra of thiazesim and Metabolites 1 and 2 suggested that the oxygen(s) was not substituted on the heterocyclic ring and must be substituted on the 2-phenyl group.

The assignment of the hydroxyl group(s) to the 2-phenyl ring was established from a comparison of selected peaks of thiazesim and those for Metabolites 1 and 2, which differed by 16 or 32 mass units (see Table I). The structures of the *m/e* 147, 120, and 107 peaks of Metabolite 1 are shown in Scheme II.

Prominent peaks common to all three compounds appeared at *m/e* 109, 136, 151, and 178, which corresponded to the following elemental compositions, as determined from the high resolution mass spectra

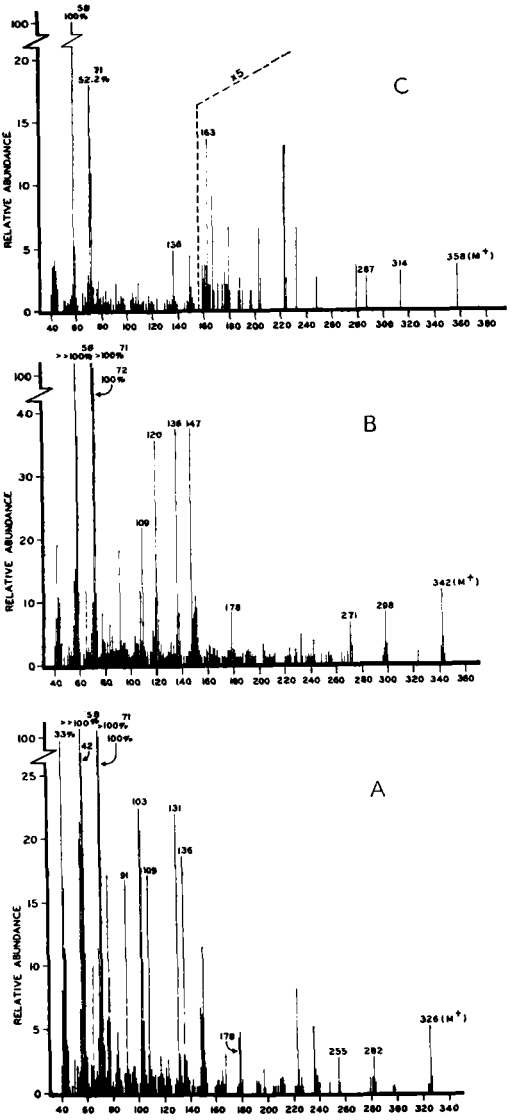
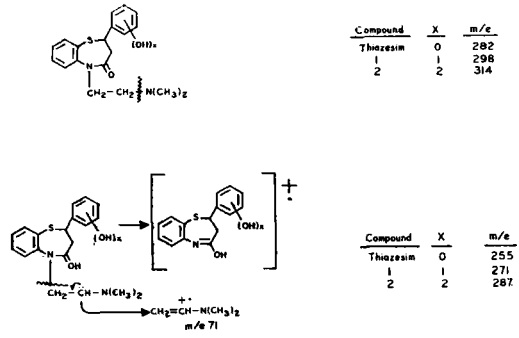
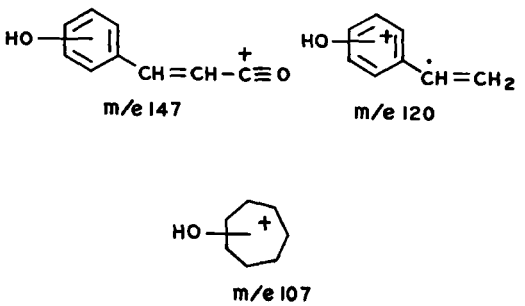


Fig. 1—Mass spectra of thiazesim (A), Metabolite 1 (B), and Metabolite 2 (C).

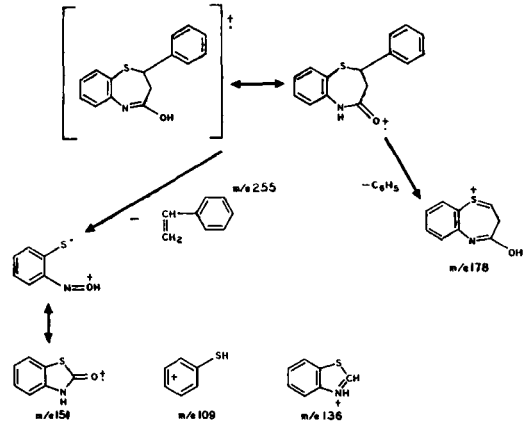


Scheme I—Fragmentation pattern of the side chain of thiazesim and Metabolites 1 and 2



Scheme II—Structures of selected fragments of Metabolite 1

of thiazesim and Metabolite 1, C_6H_5S, C_7H_6NS, C_7H_5NOS, and C_9H_8NOS, respectively. These peaks, corresponding to those aromatic fragments containing *both* nitrogen and sulfur, contained no hydroxyl group(s) either in the parent compound or in Metabolites 1 and 2. The probable structures of these fragments are depicted in Scheme III.



Scheme III—Structures of selected fragments of thiazesim

Metabolite 3 (see Fig. 2) with a molecular ion at *m/e* 386 ($C_{20}H_{22}N_2O_4S$) had fragments common to the nuclear portion of Metabolite 1 at *m/e* 91, 109, 120, 136 (C_7H_6NS), 147 ($C_9H_7O_2$), 242, and 271 ($C_{15}H_{13}NO_2S$). The absence of prominent peaks at *m/e* 58 and 71 demonstrated alterations(s) of the side chain. Further, peaks were present with the loss of oxygen at *m/e* 356 ($C_{19}H_{20}N_2O_3S$), 310 ($C_{18}H_{18}N_2OS$), 217 ($C_{12}H_{13}N_2S$), 203 ($C_{11}H_{11}N_2S$), and with the loss of sulfur at *m/e* 353 ($C_{20}H_{21}N_2O_4$), 323 ($C_{19}H_{20}N_2O_3$), and 277 ($C_{18}H_{17}N_2O$). It is to be noted that the low intensity peak, *m/e* 356, does not appear in the low resolution spectrum of Fig. 2. This peak was, however, detected and its exact mass determined by the use of the Nier peak-matching procedure.

The additional oxygen atoms present in Metabolite 3, compared with Metabolite 1, are not ketonic, aldehydic, acidic, or in an ester grouping, as determined from the infrared spectrum, therefore, the oxygens must be either alcoholic or ethereal. Moreover, the mass-measured formula of the mo-

TABLE I—MASS SPECTRA OF SELECTED FRAGMENTS OF THIAZESIM AND METABOLITES 1 AND 2

Thiazesim		Metabolite 1		Metabolite 2	
m/e	Elemental[a] Composition	m/e	Elemental[a] Composition	m/e	Elemental[b] Composition
282	$C_{17}H_{16}NOS$	298	$C_{17}H_{16}NO_2S$	314	$C_{17}H_{16}NO_3S$
255	$C_{15}H_{13}NOS$	271	$C_{15}H_{13}NO_2S$	287	$C_{15}H_{13}NO_3S$
131	C_9H_7O	147	$C_9H_7O_2$	163	$C_9H_7O_3$
104	C_8H_8	120	C_8H_8O	136	$C_8H_8O_2$
103	C_8H_7	119	C_8H_7O	135	$C_8H_7O_2$
91	C_7H_7	107	C_7H_7O	123	$C_7H_7O_2$

[a] Based on peak matching. [b] Based on low resolution data.

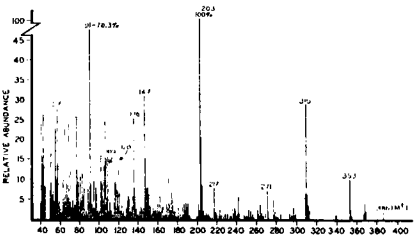
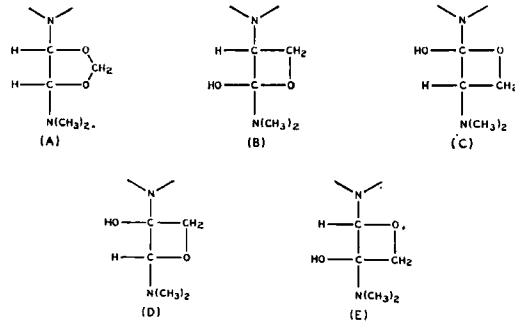


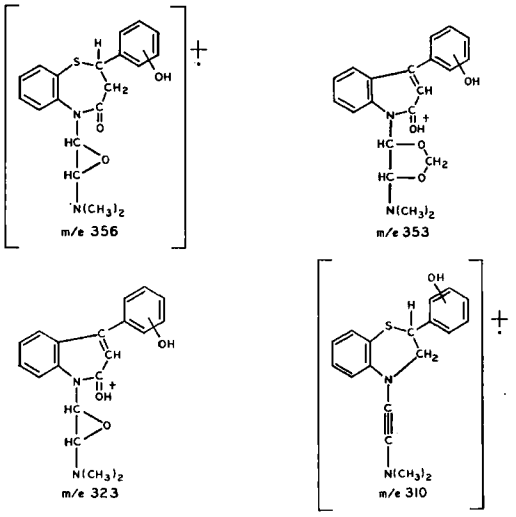
Fig. 2—Mass spectrum of Metabolite 3. This compound was thermally labile and gave variable spectra. Peaks of low intensity at m/e *323, 356, and 368 did not appear in this spectrum. In subsequent spectra, utilizing oscilloscope display of pertinent areas, these weak peaks were recognized and their exact masses determined. The elemental composition found for the mass measured peak at* m/e *368 did not give a reasonable structure based on the elemental composition of the parent ion and was considered to represent an impurity.*

lecular ion (*m/e* 386) corresponded to at least one more degree of unsaturation when compared with Metabolite 1. It can be concluded that at least one of the two incorporated oxygens must be a part of a cyclic ether. Reasonable structures for Metabolite 3 are the methylene dioxy group A or the cyclic ethers B, C, D, and E shown in Scheme IV.



Scheme IV—Possible structures for the side chain of Metabolite 3

It seems reasonable to eliminate from consideration substitution on the *N*-methyl carbon atoms, since atoms at this remote location would have had little effect on the fragmentation process of the remainder of the molecule. In those metabolites with a dimethylaminoethyl side chain, the fragmentation process appeared to be initiated at the side chain (*β*-cleavage). With changes in the side chain, the other fragmentation modes become apparent, particularly the loss of the sulfhydryl group. Since the fragmentation pattern of Metabolite 3 in the high mass region of the spectrum differed markedly from those obtained with thiazesim or Metabolites 1 or 2, it was suspected that modification of the side chain might alter the fragmentation mode of Metabolite 3. In support of this supposition an analog of thiazesim having a dimethylaminopropyl side chain was analyzed by the use of a mass spectrometer. This sample having a molecular ion at *m/e* 340 also gave a prominent *m/e* 307 peak (loss of SH). This result is similar to the loss of an SH group in the case of Metabolite 3. The side chain of thiazesim cleaves very readily since the carbon-carbon bond is *β* to two nitrogens. In a molecule which has an altered side chain, fragmentation modes, such as those originating from the nuclear portion of the molecule, may become more favored. The probable structure of the peaks of highest mass unique to Metabolite 3 are shown in Scheme V.



Scheme V—Structures of selected high-mass fragments of Metabolite 3

Of the five possible structures that accommodate the added elements of one carbon and three oxygens, compared with the parent drug (see Scheme IV), Structure A appears to be the most reasonable on the basis of NMR and mass spectral data. The aromatic proton resonances of Metabolites 1 and 3 were identical, therefore, Metabolite 3 was presumed also to have had the aryl substitution in the 2-phenyl ring. The NMR spectrum of Metabolite 3 had a prominent peak near 4.4 τ which was absent in the spectrum of Metabolite 1. The loca-

tion of this peak was reasonable for the assignment of the methylene dioxy protons. Although the proton count corresponded to only three protons, the region around 4.4 τ also may have included the other two protons on carbons bearing the two methylene dioxy oxygens. The relevant proton resonances of the protons of Structures B–E would have tended to have occurred at τ values higher than those found for the methylene dioxy protons, and certainly only one proton in Structures D and E would be expected to have appeared below 5 τ. In addition, Structures B, C, D, and E are 4-membered cyclic hemiacetals, and would not have been expected to exhibit the stability of the 5-membered cyclic methylene dioxy Structure A.

Although the data obtained with Metabolite 3 tend to support Structure A (Scheme IV), it should be stressed that this tentative proposal is made in the absence of the synthesized compound with which authentic spectra could be prepared.

The mass spectrum of Metabolite 4 gave a molecular ion at *m/e* 342, the same as that found for Metabolite 1, however, the fragmentation patterns for the two compounds were quite different (see Fig. 3). Metabolite 4 showed fragments of high intensity at *m/e* 325 (M-17, loss of OH), and at *m/e* 298 [M-44, loss of $-N(CH_3)_2$]. These results suggested that the additional oxygen incorporated into Metabolite 4 was not present on either of the substituted benzene rings. The strong peaks at *m/e* 58 and 71 indicated that no alteration had occurred to the side chain in Metabolite 4. Sulfoxidation seemed a likely possibility. Accordingly, the mass spectrum was run of the sulfoxide derivative of a thiazesim analog (SQ 10502) that contained an *N*-diethyl amino ethyl instead of an *N*-dimethyl amino ethyl side chain. The M^+ for this compound was found at *m/e* 370. Prominent peaks were found at *m/e* 353 (M-17) and *m/e* 298. The strong peaks found for Metabolite 4 at *m/e* 58 and *m/e* 71 were now displaced by 28 mass units to *m/e* 86 and *m/e* 99 due to the added C_2H_4 of the *N*-diethyl group. Other features of the mass spectra of Metabolite 4 and SQ 10502 were essentially identical. Similarly, the ultraviolet and infrared spectra of Metabolite 4 and SQ 10502 were identical.

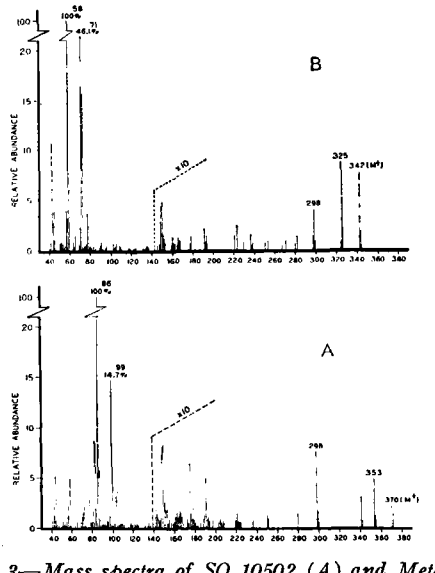


Fig. 3—Mass spectra of SQ 10502 (A) and Metabolite 4 (B). SQ 10502 is the sulfoxide derivative of thiazesim, which also contains an N*-dimethylaminoethyl side chain.*

The complete interpretation of the mass spectrum of Metabolite 5 has been complicated by the absence of a discernible peak for the parent ion. The presence of the peak at *m/e* 255 ($C_{15}H_{13}NOS$), as well as the peaks at *m/e* 136, 131, 109, 104, 103, and 91, demonstrated that the nuclear portion of thiazesim had remained intact. The presence of a stronger *m/e* 57 (C_3H_8N) peak rather than an *m/e* 58 peak, as well as the absence of both the *m/e* 71 peak and a strong *m/e* 44 peak, demonstrated a change in the fragmentation pattern from that observed for thiazesim. These data suggested that changes had occurred in the side chain of Metabolite 5.

Location of Hydroxyl Substituents—When strongly electron-donating groups, such as hydroxyl, are substituted in benzene the protons substituted *ortho* and *para* to the hydroxyl group are preferentially shielded (3). This is to be anticipated, because the increase in electron density will be greatest at the carbons *ortho* and *para* to the functional group and least at the *meta* position. The protons attached to the *ortho* and *para* carbons have an electron density that results in the observed chemical shifts of these protons occurring at a higher field than the *meta* proton.

The three isomeric cresols were used as model compounds for the determination of the position of substitution of the hydroxyl group in Metabolite 1. The three isomers exhibited unique aromatic proton resonances due to the presence of the hydroxyl group and the spin-spin coupling of the aromatic protons. *Para* cresol exhibited the typical A_2B_2 pattern of symmetrically disubstituted aromatic compounds with closely spaced proton resonances at 2.95 and 3.25 τ. The spectrum of the nonsymmetrically substituted *o*-cresol was less well defined, but resonances for the two protons *ortho* and *para* to the hydroxyl group fell in the region between 3.2 and 3.4 τ, while the *meta*-substituted protons resonated at lower fields between 2.8 and 3.17 τ as a complex multiplet. *Meta*-cresol, having three protons either *ortho* or *para* to the hydroxyl group, had these protons resonating near 3.3 τ as a complex multiplet, while the remaining *meta* protons resonated near 2.9 τ.

Figure 4 gives the proton resonances in the aromatic region of the NMR spectrum of the *ortho* and *para* methoxy derivatives on the 2-phenyl ring of thiazesim and of Metabolite 1. The time-averaged NMR spectrum of the aromatic portion of Metabolite 1 showed resonance for two protons rather than four, between 3.15 and 3.5 τ; these τ values agree with those found for *o*-cresol. The lack of symmetry in the aromatic portion of the spectra of *o*-cresol and Metabolite 1 added further evidence in favor of the *ortho* configuration for the hydroxyl group. The region under discussion provides evidence for only two protons; in addition, there is a noteworthy absence of the coupling pattern of an AB quartet which is present in the *p*-methoxy thiazesim spectrum (see Curve A, Fig. 4).

The *meta* substitution of the hydroxyl group may

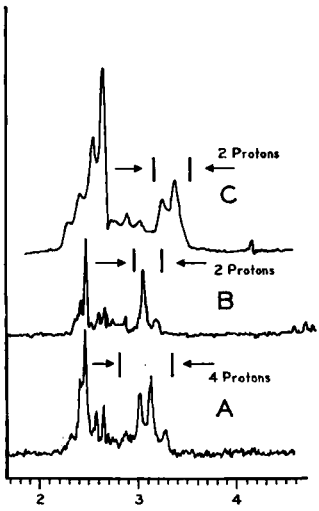


Fig. 4—Proton resonances of the aromatic region of the NMR spectra of p-*methoxy-thiazesim* (*A*), o-*methoxy-thiazesim* (*B*), *and hydroxy-thiazesim, Metabolite 1. The numbers on the abscissa represent τ-values.*

be eliminated *a priori* since the area under the curve between 3.15 and 3.5 τ accounted for only two of four protons. The lack of symmetry in the aromatic proton resonances ruled out the *para*-disubstituted isomer as the probable structure. These data support the view that the hydroxyl group of Metabolite 1 appears to be in the *ortho* position. This interpretation remains tentative, however, until the proposed structure will have been synthesized and the spectra analyzed.

The authors were unable to obtain a satisfactory time-averaged NMR spectrum of Metabolite 2, and, thus, have been unable to locate the hydroxyl groups on the 2-phenyl ring.

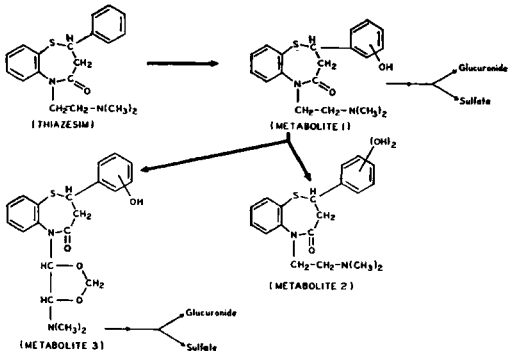
Metabolite 3 exhibited the same pattern for the proton resonances of the 2-phenyl ring as did Metabolite 1, and, therefore, appeared to be a derivative of the latter.

Scheme VI summarizes the probable metabolic events occurring during the biotransformation of thiazesim-^{14}C in the rat *in vivo*.

DISCUSSION

The inability to detect sulfoxidation *in vivo* may be attributed to two possibilities. First, sulfoxidation may actually have occurred, but the equilibrium for the reaction may strongly favor the sulfide form. Second, sulfoxidation could have been a favored reaction, but additional metabolic alterations may occur that result in a very low steady-state level of sulfoxide. A similar line of reasoning would apply if any sulfoxide formed were subsequently converted to the sulfone or sulfate derivatives. Since 30–40% of the dose, has not been identified, such secondary conversions might have been unrecognized.

It is more difficult to understand why hydroxylation, a predominant reaction *in vivo*, was not observed *in vitro*. One could argue that for some reason the conditions of incubation were not suitable for hydroxylation to have occurred. According to Gillette (4), hydroxylation reactions of aromatic



Scheme VI—Summary of biotransformation of thiazesim^{14}*C in the rat* in vivo

compounds require a liver microsomal enzyme system, NADPH, and oxygen. Likewise, Gillette and Kamm (5) have reported that the enzymatic formation of sulfoxides by guinea pig liver microsomes, requires NADPH and oxygen, the same requirements listed above for hydroxylation.

A common metabolic reaction during drug metabolism is *N*-dealkylation and a number of psychoactive drugs undergo this conversion (6). In the metabolism of thiazesim-^{14}C, either *in vivo* or *in vitro*, the authors were unable, so far, to detect the presence of any *N*-demethylated derivatives.

The elucidation of the biotransformation of drugs has been greatly simplified by the introduction of NMR and mass spectrometry. In addition to being able to work with microgram quantities of sample, the advantages of mass spectrometry as an analytical tool are in being able more readily to identify an unexpected structure. The more conventional and more restrictive approach to the identification of metabolites has been to synthesize "expected" metabolites and then to demonstrate their identity by chromatography in several different solvent systems, in comparison with an unknown compound. Schwartz and Bommer (7) have used high-resolution mass spectrometry to identify metabolites of diazepam in the rat.

Additional support for the unusual substitution to the side chain of Metabolite 3 comes from the fragmentation pattern of a known structure containing the methylene dioxy group (8). The compound, 1,3-dioxolane, gives a weak molecular ion (6.0%) at *m/e* 74. Prominent fragments are found at *m/e* 43 and *m/e* 44 which are easily rationalized by the loss of H_2CO, formaldehyde.

The methylene dioxy structure is found in several naturally occurring alkaloids (9). In these alkaloids, the 5-membered methylene dioxy ring is fused to an adjacent benzene ring. Presumably, the methylene dioxy group is formed by the oxidative cyclization of the *o*-methoxyphenol (10). Intermediates establishing the validity of the structure of Metabolite 3 by the latter pathway were not isolated. The transient presence of these compounds may have accounted for the authors' inability to find them; another possibility that existed, of course, is that the metabolic pathway was other than that considered here. To the authors' knowledge, there is no precedent for the natural occurrence of a methylene dioxy group attached to an alkyl side chain in either plants or

animals. In view of the unusual structure, however, this assignment should be considered tentative.

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Keyphrases

Thiazesim metabolism—*in vivo*, *in vitro*
Metabolites, thiazesim—isolated, identified
Liver, rats—*in vitro* metabolism
TLC—separation, identification
Column chromatography—separation
NMR spectroscopy—structure
Mass spectroscopy—structure
UV spectrophotometry—structure

Nonpolar Metabolites of Trifluoperazine in Rats

By C. L. HUANG and K. G. BHANSALI*

Urinary excretion of nonpolar metabolites of trifluoperazine was studied in six albino rats which were administered intraperitoneally with 5 mg. trifluoperazine dihydrochloride daily for a period of 5 days. The 24-hr. urine specimens were collected and analyzed for trifluoperazine sulfoxide and the unchanged drug by means of a spectrophotometric and thin-layer chromatographic techniques. The average urinary excretion of trifluoperazine sulfoxide was found to be 10.77% of the administered dose in the first 24-hr. period. Only a trace or a negligible amount of the unchanged drug was found in urine.

TRIFLUOPERAZINE (TOP) is one of the widely used neuroleptics today. During the past decade TOP has become established as an effective neuroleptic agent. Many clinical reports have appeared attesting to its efficacy in the treatment of certain types of psychotic symptomatology (1–12). TOP, a member of the phenothiazine family, is approximately 10 times more potent than chlorpromazine and is reported to have fewer side reactions. It differs from chlorpromazine by possessing a trifluoromethyl group on position 2 and a piperazinyl function on the propyl side chain. Comparative clinical studies (13–18) have indicated differences in the sedative effect, potency, rapidity of action, and incidence of neurological complications between TOP and other neuroleptics; however, factors responsible for these differences are as yet largely unknown. Despite a large number of clinical investigations reported in the past years, little is known as to its metabolic pathway. Because of the relatively small dosage level, metabolic study of this compound presents a formidable problem especially in man. It would be of interest to study the metabolic pathway of this relatively potent drug and compare it with that of chlorpromazine. Such comparative data might help to explain the question as to why this compound is more potent and has less side effects than chlorpromazine. A survey of the literature revealed that there is little information available regarding the metabolism of TOP. A rapid color test utilizing ferric chloride in mineral acids to detect urinary TOP metabolites was reported (19, 20).

The present investigation is focused on identification and quantitative determination of nonpolar metabolites of TOP in urine of rats. Thin-layer chromatography and paper partition chro-

Received December 21, 1967, from the School of Pharmacy, University of Mississippi, University, MS 38677
Accepted for publication June 5, 1968.
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matography were utilized to elucidate metabolites in this study.

MATERIALS AND METHODS

Six albino rats weighing 150–200 g. were selected. They were administered intraperitoneally with 5 mg. of TOP dihydrochloride in saline solution daily for a period of 5 days. The animals were placed in individual metabolic cages with free access to food and water. Daily 24-hr. urine specimens were collected and analyzed by means of a Beckman DU spectrophotometer.

On the first day of the experiment, all rats were sedated and remained motionless most of the time but were easily aroused by a light touch on their fur. Beginning the second day, animals showed a lighter degree of sedation and were able to feed and to drink water.

Chromatography of Reference TOP—Reference TOP dihydrochloride was dissolved in normal rat urine. An aliquot of 0.1 ml. was placed on a thin-layer plate and the chromatogram was developed in a solvent system, acetone–methanol (1:1) for a period of 20 min. The developed chromatogram was dried and sprayed with uranyl nitrate reagent in concentrated hydrochloric acid (19). A bluish-purple spot with R_f 0.5 was observed. The sensitivity of this reagent to TOP is 5 mcg. in 10-ml. urine specimen.

Synthesis of TOPO—A reference TOPO was synthesized according to the following procedure. TOP dihydrochloride (1 g.) was dissolved in water and hydrogen peroxide (0.3 ml., 30%) was added. The mixture was left overnight at room temperature. Then the mixture was evaporated to dryness *in vacuo* and the residue dissolved in 2 ml. methanol. The methanol solution was chromatographed on Whatman 3 MM paper by a linear chromatographic technique in a solvent system, ethanol–butanol–water (2:5:2) to a height of approximately 30 cm. Then the chromatogram was processed according to the guiding-strip technique below (21). The chromatogram was dried in the air and about 3-cm. strips from both edges were cut and sprayed with 50% sulfuric acid. Three colored bands, namely, light orange (R_f 0.8), orange (R_f 0.7), and pink (R_f 0.6) were observed. The acid-sprayed paper strips were covered with a thin plastic sheet (Saran wrap) and placed back to the original chromatogram. Using these colored strips as a guide, the chromatogram was marked with a lead pencil and cut into three fractions. The accumulated fraction of each band above was extracted several times with methanol in a Waring blender. The combined methanol extracts were evaporated to dryness and the residue recrystallized from methanol to an amorphous powder. From the physical properties, the second band above was identified to be TOPO (m.p. 173–177°, $\lambda_{max.}$, 235, 277, 305 mμ). The first and the third bands were identified to be unchanged TOP and TOP-sulfone, respectively.

Identification of TOP and TOPO in Urine—A urine specimen (50 ml.) from rats administered with TOP dihydrochloride was adjusted to pH 10, and extracted three times with equal volume of methylene dichloride. The combined methylene dichloride extracts were filtered through a layer of anhydrous sodium sulfate and the solvent was removed *in vacuo*. The residue was dissolved in 1 ml. methanol and an aliquot of 0.1 ml. was placed on a thin-layer plate (Chromagram, Eastman). The chromatogram was developed in a solvent system, acetone–methanol (1:1) for 20 min. to a height of about 15 cm. The developed chromatogram was dried in the air and sprayed with uranyl nitrate reagent in concentrated hydrochloric acid. Two spots, with a bluish-purple color (R_f 0.5) and a pink color (R_f 0.4) were observed. They were identified to be the unchanged TOP and TOPO, respectively, from the color reaction, R_f value and cochromatogram with reference TOP and TOPO. The ultraviolet absorption spectra of TOP and TOPO isolated from rat urine are shown in Fig. 1.

Construction of Standard Curves of TOP and TOPO—A series of dilutions of TOP and TOPO in normal rat urine was prepared. An aliquot (10 ml.) of each dilution was adjusted to pH 10 and extracted three times with a 10-ml. portion of methylene dichloride. The combined methylene dichloride extracts were dried over anhydrous sodium sulfate and evaporated to dryness under a reduced pressure. The residue was taken up in 1 ml. methanol and the methanol extract was applied linearly on 10 × 20 cm. thin-layer plate (Chromagram, Eastman). The chromatogram was developed in a solvent system, acetone–methanol (1:1) for 20 min. to a height of about 15 cm. The chromatogram was air dried and scanned under ultraviolet lamps (Mineralight, UVS-11 and UVL-21) to locate the spots. The silica gel support of the chromatogram including the spot was scraped into a container and extracted with methanol (3.5 ml.). The methanol extract was centrifuged and the supernatant liquid was transferred into a cell. The absorbance was recorded in a Beckman DU spectrophotometer at 262 mμ for TOP and 277 mμ for TOPO. The absorbances of both TOP and TOPO followed Beer's law.

Quantitative Determination of TOP and TOPO in Urine—An aliquot (10 ml.) of a 24-hr. urine specimen was measured and analyzed for TOP and

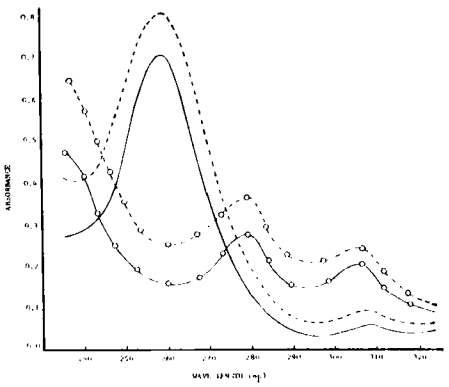

Fig. 1—Ultraviolet absorption spectra of trifluoperazine (TOP) and trifluoperazine sulfoxide (TOPO). Key: ——, reference TOP dehydrochloride; --- TOP isolated from rat urine; O—O, reference TOPO dihydrochloride; O--O, TOPO isolated from rat urine

TABLE I—URINARY EXCRETION OF TRIFLUOPERAZINE SULFOXIDE IN RATS[a]

Rat	Day 1	Day 2	Day 3	Day 4	Day 5
1	0.400	0.206	0.354	0.224	0.213
2	0.832	1.520	0.395	0.266	died
3	0.486	0.304	0.186	0.196	0.187
4	0.364	0.292	0.236	0.248	0.292
5	0.353	0.318	0.218	died	—
6	0.306	0.246	0.227	0.208	0.200
Av. mg. excretion	0.457	0.419	0.269	0.228	0.223
Av. % excretion of the administered dose	10.77	9.89	6.35	5.37	5.25

[a] Five milligrams trifluoperazine dihydrochloride (equivalent to 4.24-mg. base) was administered intraperitoneally for a period of 5 days. Absorbance was recorded in a Beckman DU spectrophotometer at 277 mμ.

TOPO according to the procedure described above for the construction of standard curves. The concentrations (mcg./ml.) were calculated with the factor obtained from the standard curves. The recovery data of the urinary TOPO are shown in Table I.

RESULTS

Six albino rats injected intraperitoneally with 5 mg. (4.24-mg. base) of TOP dihydrochloride were maintained for at least 5 days. Two rats died, one on the fourth day and the other on the fifth day. Average urinary excretion of TOPO on the first day and the second day was found to be 10.77 and 9.89% of the administered dose, respectively. Thereafter, the average daily excretion was almost constant being between 6.35 to 5.25% (22).

In contrast to the relatively high rate of sulfoxide excretion, the unchanged TOP in urine was found to be negligible or only trace which indicated almost complete metabolism of this drug. Preliminary information indicated that the intraperitoneally administered TOP was also excreted in the feces (23–25).

The mother liquor of the urine specimens after the methylene dichloride extraction showed a strong purple color reaction with 50% sulfuric acid. This suggests the presence of polar metabolites such as hydroxides and/or glucuronides of TOP which are not extractable in methylene dichloride.

Thin-layer chromatography coupled with an ultraviolet spectrophotometric technique offers a simple yet sensitive method for a quantitative assay of urinary metabolites of TOP. The sensitivity of this method is 0.7 mcg. in 1-ml. urine specimen. Normal urine components such as urea, creatinine and bile acids did not interfere with the assay of these TOP metabolites because none of these compounds have UV absorption peaks or R_f values in the area where the TOP metabolites are found.

CONCLUSION

TOP dihydrochloride (5 mg.) was injected intraperitoneally to six albino rats daily for a period of 5 days. Twenty-four hour urinary excretion of the nonpolar metabolites, namely, TOPO and the unchanged TOP, were determined spectrophotometrically in a Beckman DU spectrophotometer following linear chromatography on a thin-layer plate.

TOPO was found to be the major nonpolar metabolite in the urine with an average excretion of 10.77 and 9.89% in the first 2 days, respectively. Only a trace or a negligible amount of the unchanged trifluoperazine was found in the urine specimens indicative of almost complete metabolism of this drug in rats at this dosage level (5 mg./rat).

Preliminary study on other metabolites indicated that there is a considerable amount of polar metabolites present in urine after being extracted with methylene dichloride. They are speculated to be hydroxides and/or glucuronides of TOP.

An attempt was also made to isolate the nonpolar metabolites by paper chromatographic technique. However, it did not prove to be a satisfactory one for a spectrophotometric determination of TOP and TOPO as interference of the chromatographic paper in the methanol extract was noticed.

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Keyphrases

Trifluoperazine metabolites—nonpolar
Urinary excretion, rats—trifluoperazine metabolites
TLC—separation, identity
Paper chromatography—separation, identity
UV spectrophotometry—analysis

Further Applications of Pentachlorophenyl Active Esters in Lengthening Peptide Chains from C- and N-Terminal Amino Acids Using Dicyclohexylamine C-Protection

By A. KAPOOR, E. J. DAVIS, and MARY J. GRAETZER

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THE SYSTEMATIC SEARCH for suitable "activated" esters, for the synthesis of peptides *via* the aminolysis of esters started with the historic paper of Wieland and Bernhard, when they reported the synthesis of peptides *via* the phenyl thiolesters (1). For more than a decade various active esters have been used extensively in the synthesis of peptides and polypeptides with known sequence of amino acids (2–5). The pentachlorophenyl active esters, which were first reported in the literature in 1961 (6), afford an excellent method for lengthening the peptide chain. The pentachlorophenyl active esters have the following advantages: (*a*) they are one of the most active esters (7), (*b*) they are generally higher melting compounds than other active esters, which leads to their easy crystallization and purification (8), (*c*) they are conveniently prepared without any significant racemization by *N*,*N*′-dicyclohexylcarbodiimide (DCC) method (9), (*d*) they are stable to controlled hydrogenation conditions and make an excellent combination with *N*-carbobenzoxy and *tert*-butyl protecting groups when the incorporation of trifunctional amino acids in the peptides is desired (8). Previously, the peptide chain was lengthened by coupling pentachlorophenyl active esters of *N*-carbobenzoxy amino acids or peptides with C-methyl protected amino acids or peptides. C-methyl protection at each activation stage and at the end of the synthesis was removed by saponification (8, 10–12). Alkali treatment of peptides is usually associated with a number of problems, *e.g.*, racemization (13), transpeptidation (8, 14), *etc.* In addition, removal of C-methyl protection by alkali becomes more difficult as the number of amino acids increases in

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Accepted for publication May 20, 1968.

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Abstracted in part from a thesis submitted by E. J. Davis to the Graduate Division, College of Pharmacy, St. John's University in partial fulfillment of Master of Science degree requirements.

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the peptide chain (15). MacLaren reported the formation of urea or hydantoin derivatives when *N*-carbobenzoxy peptides were treated with alkali (16).

Avoiding racemization of optically active centers in the synthesis of peptides with biological activity is a major concern and new approaches for the synthesis which would limit the degree of racemization in synthetic peptides are under continual investigation. To overcome racemization and other problems associated with alkali treatment, the ideal approach would be to carry out the coupling of activated N-protected amino acids or peptides with amino acids or peptides C-protected by suitable salt formation, which can easily be removed by mild acid, instead of methyl or ethyl ester protection. Among the active esters, *p*-nitrophenyl esters have been frequently used for coupling reactions with amino acid salts. Various solvent mixtures and pH conditions have been reported; however, the yields were invariably low (17).

As the pentachlorophenyl active esters (OPCP) are among the most active esters, it was considered worthwhile to systematically study their coupling with amino acids or peptides, C-protected by suitable salt formation. The purpose of this paper is to report a general method which conveniently avoids the use of alkali during stepwise lengthening of the peptide chain, both from C- and N-terminal amino acid residues.

RESULTS AND DISCUSSION

C-protection by salt formation with three different bases was investigated as follows: (*a*) Amino acids were converted into their sodium salts by treatment with an equivalent amount of sodium bicarbonate in 5–6 ml. of water. (*b*) Triethylamine salts were prepared by making a slurry of amino acids with calculated amount of triethylamine (TEA) in 10–15 ml. of methylene chloride. (*c*) C-protection by dicyclohexylamine (DCA) was carried out in the same manner as described for TEA.

Z-Ala-OPCP[1] (8), was coupled separately with sodium salts of phenylalanine, alanine, and glycine. A mixture of eights parts of acetonitrile and two parts of water was used as a solvent with 2-hydroxypyridine (18) as a catalyst. After 24 hr. the salt protection from the resulting dipeptides was removed by mild acid (1 *N* hydrochloric acid) treatment and Z-Ala-Phe-OH, Z-Ala-Ala-OH, and Z-Ala-Gly-OH were isolated in 15, 18, and 17% yields, respectively. Increasing the coupling time up to 72 hr. and substituting various other solvents and solvent mixtures, *e.g.*, tetrahydrofuran, ethyl acetate, methylene chloride, dimethylformamide, and different proportions of water in acetone, did not afford any appreciable increase in the yields.

Z-Ala-OPCP was coupled separately in methylene chloride with triethylamine salts of phenylalanine, alanine, and glycine. 2-Hydroxypyridine was used as a catalyst and after 24 hr., removal of triethylamine protection by mild acid, afforded Z-Ala-Phe-OH, Z-Ala-Ala-OH, and Z-Ala-Gly-OH in 40, 45, and 38% yields, respectively; and no increase in yields was observed by increasing the time or changing to different solvents for reaction.

Under identical conditions, DCA salts of phenylalanine, alanine, and glycine were coupled with Z-Ala-OPCP (I) in better yields; Z-Ala-Phe-OH (IV), Z-Ala-Ala-OH (V), and Z-Ala-Gly-OH (VI) were isolated in 62, 61, and 60%, respectively. Z-Gly-OPCP (II) (8) and Z-Phe-OPCP (III) (19) were coupled with a number of other amino acids, C-protected by dicyclohexylamine, and the corresponding N-protected dipeptides (VII–XIII) were isolated in satisfactory yields as indicated in Table I. Since the yields were optimal with methylene chloride, the preceding coupling reactions were carried out in this solvent.

It would seem apparent that the hydrocarbon portion of the base used for C-protection, played an important role in the coupling reactions. This can be justified on the basis of better solubility of DCA salts as compared to sodium or TEA salts of amino acids in the reaction solvents, such as methylene chloride. If this was true, then N-protected OPCP esters of amino acids would be expected to couple better with relatively more soluble di- and tripeptides, C-protected by DCA. In order to establish the effect of solubility of the C-protected component, the coupling of N-protected, C-activated amino acids with di- and tripeptide DCA salts was undertaken. Z-Gly-OPCP(II) coupled with the DCA salt of H-Gly-Phe-OH (XVII) to afford Z-Gly-Gly-Phe-OH (XX) in 68% yield, and Z-Phe-Gly-Phe-OH (XXI) was prepared in 67% yield, by coupling Z-Phe-OPCP (III) with H-Gly-Phe-OH (XVII), C-protected by DCA. The yields were even better when the DCA salts of tripeptides were coupled with activated amino acids. H-Ala-Phe-Gly-OH (XIX) C-protected by DCA, coupled with Z-Gly-OPCP (II) in 75% yield, to afford Z-Gly-Ala-Phe-Gly-OH (XXIII). N-protected dipeptide-OPCP active esters were also coupled in satisfactory yields with amino acids or peptides, C-protected by DCA. In this case, the length of the C-protected components had the similar effects on the yields as noted above. Z-Gly-Ala-OPCP (XIV) coupled with DCA salt of H-Ala-OH to afford Z-Gly-Ala-Ala-OH (XXIV) in 43% yield. However, Z-Gly-Ala-OPCP (XIV) reacted with H-Phe-Gly-OH (XVIII) C-protected by DCA in 54% yield to afford Z-Gly-Ala-Phe-Gly-OH (XXIII). In line with the authors expectations, N-protected tripeptide OPCP active esters coupled in relatively lower yields with DCA salts of amino acids. Z-Gly-Gly-Phe-OPCP (XV) coupled with H-Phe-OH, C-protected by DCA in 38% yield to afford Z-Gly-Gly-Phe-Phe-OH (XXV). It is interesting to note that N-protected OPCP active esters of amino acids could not be coupled with glutamic or aspartic acids with DCA protection on both carboxyl groups. However, the coupling of N-protected-amino acid-OPCP active esters, was found to proceed in satis-

[1] Abbreviations used in this paper for amino acids and peptides are those recommended in "Proceedings of the 5th European Peptide Symposium, Oxford, September 1962." Young, G. T., Ed., Macmillan, New York, N. Y., 1963. Amino acids used in this work were all of L-configuration.

TABLE I—YIELDS AND MELTING POINTS OF N-PROTECTED DIPEPTIDES, MADE THROUGH COUPLING OF N-PROTECTED-OPCP ESTERS OF AMINO ACIDS WITH DCA, C-PROTECTED AMINO ACIDS

Compd.	OPCP Derivative	Amino Acid DCA Salt	Compd.	Dipeptide Formed	Yield, %	M.p., °C.	M.p., °C. (Lit)
I	Z-Ala-OPCP	H-Phe-OH	IV	Z-Ala-Phe-OH	62	121–122	122[a]
I	Z-Ala-OPCP	H-Ala-OH	V	Z-Ala-Ala-OH	61	152	152–153[b]
I	Z-Ala-OPCP	H-Gly-OH	VI	Z-Ala-Gly-OH	60	132–133	132[c]
II	Z-Gly-OPCP	H-Gly-OH	VII	Z-Gly-Gly-OH	55	178	178–179[d]
II	Z-Gly-OPCP	H-lleu-OH	VIII	Z-Gly-lleu-OH	59	115	114–115[e]
II	Z-Gly-OPCP	H-Ala-OH	IX	Z-Gly-Ala-OH	62	120	119.5[f]
II	Z-Gly-OPCP	H-Phe-OH	X	Z-Gly-Phe-OH	62	125–126	126[g]
II	Z-Gly-OPCP	XXVI H-Asp(OBzl)-OH	XI	Z-Gly-Asp(OBzl)-OH	58	99–100	
III	Z-Phe-OPCP	H-Gly-OH	XII	Z-Phe-Gly-OH	61	154–155	154[g]
III	Z-Phe-OPCP	H-Pro-OH	XIII	Z-Phe-Pro-OH	63	110–111	109–110[h]

[a] *Reference 26.* [b] *Reference 27.* [c] *Reference 28.* [d] *Reference 29.* [e] *Reference 30.* [f] *Reference 31.* [g] *Reference 32.* [h] *Reference 33.*

factory yields with monoaminodicarboxylic acids, when one of the carboxyl groups was protected with DCA and the second carboxyl group was protected with a suitable ester, such as benzyl or *t*-butyl, which can be removed without alkali. Z-Gly-OPCP (II) was coupled with β-benzyl aspartate (XXVI) (20), C-protected with DCA, and Z-Gly-Asp(OBzl)-OH (XI) was isolated in 58% yield. From the above results, summarized in Table II, it can be concluded that, when OPCP esters are to be used for coupling with DCA salts of amino acids or peptides, the peptide chain should be lengthened from C-terminal instead of N-terminal amino acid residues.

It was previously reported (21) that a combination of mixed anhydride (isobutyl chloroformate) and pentachlorophenyl active ester methods, provided a suitable approach for stepwise incorporation of amino acids into peptides with an ordered sequence. A complementary reaction sequence to this combination was also reported recently (22) in which the N-protected OPCP active esters of amino acids or peptides were coupled with OPCP active ester hydrochlorides of amino acids. The advantage of the latter approach is significant since all the amino acids or peptides used in the coupling reaction would be in the form of OPCP esters and, therefore, easily crystallized. A limitation of this approach is found in the very poor yields obtained when the coupling of N-protected OPCP active esters of amino acids or peptides is carried out with di- or tripeptide OPCP active ester hydrochlorides. This may be due to the formation of diketopiperazine derivatives in dipeptides and the formation of cyclic peptides or linear polypeptides in the case of tripeptides.

Since the C-activation by mixed anhydrides is stronger than the C-activation by OPCP esters, the yields for coupling with mixed anhydrides are better. However, problems involving cyclization and polymerization are synonymously encountered, though to a somewhat lesser degree when N-protected amino acid or peptides, C-activated by mixed anhydride, are coupled with di- or tripeptides OPCP ester hydrochlorides. It is concluded that, in the case of chain lengthening of peptides from the N-terminal residue, a combination of mixed anhydride and OPCP active ester methods would be most rewarding when single units of amino acid OPCP ester hydrochlorides are incorporated, as outlined in Scheme I. The disadvantage of this approach, as already discussed, is the lower yields at stage *C*.

$$\text{Z-A}_1\text{-OPCP} + \text{H-A}_2\text{-OH} \xrightarrow{\text{DCA}} \text{Z-A}_1\text{-A}_2\text{-OH} \quad (A)$$

$$\text{Z-A}_1\text{-A}_2\text{-OH} + \text{HCl.H-A}_3\text{-OPCP} \xrightarrow[\text{chloroformate}]{\text{isobutyl}} \text{Z-A}_1\text{-A}_2\text{-A}_3\text{-OPCP} \quad (B)$$

$$\text{Z-A}_1\text{-A}_2\text{-A}_3\text{-OPCP} + \text{H-A}_4\text{-OH} \xrightarrow{\text{DCA}} \text{Z-A}_1\text{-A}_2\text{-A}_3\text{-A}_4\text{-OH} \quad (C)$$

A_1, A_2, A_3, and A_4 represent amino acid residues in a peptide sequence

$$\text{Z} = \text{C}_6\text{H}_5\text{—CH}_2\text{—O—}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{—} \qquad \text{—OPCP} = \text{—OC}_6\text{Cl}_5$$

DCA = dicyclohexylamine

Scheme I

With respect to the above discussion, the following approach for extending the peptide chain from the C-terminal residue, as outlined in Scheme II, would be more suitable.

TABLE II—YIELDS AND MELTING POINTS OF N-PROTECTED TRI- AND TETRAPEPTIDES, MADE THROUGH COUPLING OF N-PROTECTED OPCP ESTERS OF AMINO ACIDS AND PEPTIDES, WITH DCA, C-PROTECTED AMINO ACIDS AND PEPTIDES

Compd.	OPCP Derivative	Compd.	DCA Protected Component	Compd.	Peptide Formed	Yield, %	M.p., °C.	M.p., °C. (Lit)
II	Z-Gly-OPCP	XVII	H-Gly-Phe-OH	XX	Z-Gly-Gly-Phe-OH	68	145–146	146[a]
III	Z-Phe-OPCP	XVII	H-Gly-Phe-OH	XXI	Z-Phe-Gly-Phe-OH	67	151–152	150–151[a]
I	Z-Ala-OPCP	XVIII	H-Phe-Gly-OH	XXII	Z-Ala-Phe-Gly-OH	68	171	
II	Z-Gly-OPCP	XIX	H-Ala-Phe-Gly-OH	XXIII	Z-Gly-Ala-Phe-Gly-OH	75	174–175	175[b]
XIV	Z-Gly-Ala-OPCP		H-Ala-OH	XXIV	Z-Gly-Ala-Ala-OH	43	173–174	172[c]
XV	Z-Gly-Gly-Phe-OPCP		H-Phe-OH	XXV	Z-Gly-Gly-Phe-Phe-OH	38	140–142	
XIV	Z-Gly-Ala-OPCP[d]	XVIII	H-Phe-Gly-OH	XXIII	Z-Gly-Ala-Phe-Gly-OH	54	175–176	175[b]
XVI	Z-Gly-Ala-Phe-OPCP		H-Gly-OH	XXIII	Z-Gly-Ala-Phe-Gly-OH	40	175	175[b]

[a] *Reference 34.* [b] *Reference 32.* [c] *Reference 35.* [d] This compound was prepared by coupling through mixed anhydride, *N*-carbobenzoxy glycine with alanine pentachlorophenyl ester hydrochloride (XXVIII).

$$Z\text{-}A_3\text{-}OPCP + H\text{-}A_4\text{-}OH \xrightarrow{DCA} Z\text{-}A_3\text{-}A_4\text{-}OH \quad (a)$$

$$Z\text{-}A_3\text{-}A_4\text{-}OH \xrightarrow[Pd/C]{H_2} H\text{-}A_3\text{-}A_4\text{-}OH \quad (a_1)$$

$$Z\text{-}A_2\ OPCP + H\text{-}A_3\ A_4\text{-}OH \xrightarrow{DCA} Z\text{-}A_2\text{-}A_3\text{-}A_4\text{-}OH \quad (b)$$

$$Z\text{-}A_2\text{-}A_3\text{-}A_4\text{-}OH \xrightarrow[Pd/C]{H_2} H\text{-}A_2\text{-}A_4\text{-}A_4\text{-}OH \quad (b_1)$$

$$Z\text{-}A_1\text{-}OPCP + H\text{-}A_2\text{-}A_3\text{-}A_4\text{-}OH \xrightarrow{DCA} Z\text{-}A_1\text{-}A_2\text{-}A_3\text{-}A_4\text{-}OH \quad (c)$$

Scheme II

In Scheme II, there is a steady increase in the yields from stage $a \rightarrow b \rightarrow c$. The other advantage would be in further limiting the degree of racemization as the C-activated component used would always be a monomer. It has been reported that during coupling of N-protected, C-activated peptides, racemization of C-activated amino acid residue takes place, probably through an oxazolone (23–25). In order to compare the yields, tetrapeptide, Z-Gly-Ala-Phe-Gly-OH (XXIII) was synthesized as described below, both through Schemes I and II. As expected, the overall yield was about 10% more in the case of synthesis through Scheme II.

Synthesis of Z-Gly-Ala-Phe-Gly-OH (XXIII) Through Scheme I—Z-Gly-OPCP (II) was coupled with H-Ala-OH, C-protected by DCA, and after removal of DCA protection by mild acid, Z-Gly-Ala-OH (IX), was isolated in 62% yield. Coupling of N-protected dipeptide IX, with HCl.H-Phe-OPCP (XXVII) through mixed anhydride method, afforded Z-Gly-Ala-Phe-OPCP (XVI) in 74% yield. N-protected tripeptide active ester XVI was coupled with H-Gly-OH, C-protected by DCA, and after removal of DCA protection by mild acid, the desired tetrapeptide, Z-Gly-Ala-Phe-Gly-OH (XXIII) was isolated in 40% yield. Overall yield 18%.

Synthesis of Z-Gly-Ala-Phe-Gly-OH (XXIII) Through Scheme II—Z-Phe-OPCP(III) was coupled with H-Gly-OH, C-protected by DCA, and upon removal of DCA protection by mild acid, Z-Phe-Gly-OH (XII) was isolated in 61% yield. N-protected dipeptide XII, upon hydrogenation, afforded H-Phe-Gly-OH (XVIII) in 93% yield. Z-Ala-OPCP (I) was coupled with dipeptide XVIII, C-protected by DCA. After usual removal of DCA protection, Z-Ala-Phe-Gly-OH (XXII) was isolated in 68% yield. Removal of N-protection from tripeptide XXII by hydrogenation, afforded H-Ala-Phe-Gly-OH (XIX) in 95% yield. Z-Gly-OPCP (II) was coupled with tripeptide XIX, C-protected by DCA, and Z-Gly-Ala-Phe-Gly-OH (XXIII) was isolated in 75% yield. Overall yield 27.5%.

EXPERIMENTAL

All melting points are uncorrected and were taken on a Hoover Uni-Melt apparatus. The microanalyses were carried out by Drs. G. Weiler and F. B. Strauss, Oxford, England. Infrared spectra were determined in KBr pellets with a Beckman IR 8 spectrophotometer. Optical rotations were measured with a Rudolph precision ultraviolet polarimeter, model 200S-340-80Q3. *N*-Carbobenzoxy pentachlorophenyl active esters of amino acid used in this work were prepared by the DCC method, as previously reported (8).

Procedure A

This is the general method for the coupling of N-protected OPCP active ester derivatives with amino acids and peptides C-protected by DCA.

Preparation of N-Carbobenzoxy-alanyl-alanine (V)—To a stirred solution of 2.0 g. (4.24 mmoles) of *N*-carbobenzoxy alanine pentachlorophenyl ester-(I), and 0.1 g. of 2-hydroxypyridine, as a catalyst, in 80 ml. of methylene chloride, was added in 2-ml. portions over the period of 30 min. 10 ml. of a suspension containing 0.377 g. (4.24 mmoles) of alanine and 0.83 ml. (4.24 mmoles) of dicyclohexylamine (DCA). The reaction mixture, after magnetically stirring for 24 hr., at ambient temperature, was spin evaporated *in vacuo*. The residue was dissolved in 100 ml. of ethyl acetate and the resulting solution was treated with 40 ml. of 2 *N* HCl, then filtered to remove the insoluble dicyclohexylamine hydrochloride. The aqueous layer was drawn off and the ethyl acetate layer was washed with two 40-ml. portions of water. The ethyl acetate layer was extracted with three 15-ml. portions of 5% aqueous sodium bicarbonate. The bicarbonate extracts were combined and acidified with 1 *N* HCl; the clear solution became cloudy during the acid addition. The acidified suspension was extracted with two 50-ml. portions of ethyl acetate. The ethyl acetate layer was washed three times with 40-ml. portions of water and dried over anhydrous sodium sulfate. Spin evaporation of ethyl acetate solution *in vacuo* left a brown viscous oil which was crystallized from ethyl acetate–ether–petroleum ether (b.p. 30–60°). The mixture was kept at −15° overnight, then the product, *N*-carbobenzoxy-alanyl-alanine (V) was collected on a filter; 0.76 g., (61%), m.p. 152° [lit. (27) m.p. 152–153°]. *N*-Carbobenzoxy peptides with free C-terminal, listed in Tables I and II were prepared using the above method.

Procedure B

This is a general method for the coupling of N-protected amino acids or peptides through mixed anhydride with pentachlorophenyl active ester hydrochlorides of amino acids.

Preparation of N-Carbobenzoxy Glycyl-alanine Pentachlorophenyl Ester (XIV)–Alanine pentachlorophenyl ester hydrochloride (XXVIII) was prepared by hydrogenation of *N*-carbobenzoxy alanine pentachlorophenyl ester (I) in the presence of anhydrous methanol containing hydrogen chloride, according to the method previously reported (8), in 91% yield, m.p. 216°, $[\alpha]_D^{25}$ +29.2° (c 1.0, methanol).

Anal.—Calcd. for $C_9H_7Cl_5NO_2$: C, 28.87; H, 1.87; N, 3.74. Found: C, 28.91; H, 1.79; N, 3.81.

To a stirred solution of 2.0 g. (9.57 mmoles) of *N*-carbobenzoxy glycine (11) in 50 ml. of tetrahydrofuran at −10° was added 1.33 ml. (9.57 mmoles) of triethylamine and 1.3 ml.(9.57 mmoles) of isobutylchloroformate. After 30 min. 1.33 ml. (9.57 mmoles) of triethylamine was added to the reaction mixture followed by stepwise addition, over a 30-min. period, of a suspension of 3.59 g. (9.57 mmoles) of alanine pentachlorophenyl ester hydrochloride in

10 ml. of tetrahydrofuran. The stirred reaction mixture was left at −10° for 4 hr., then freed from the solvent by spin evaporation *in vacuo*. The residue was triturated with 100 ml. of hot ethyl acetate and filtered free from triethylamine hydrochloride. The filtrate was extracted three times with 30-ml. portions of 1 *N* hydrochloric acid, water, and 5% aqueous sodium bicarbonate and water. The ethyl acetate layer was dried over anhydrous sodium sulfate. The residue obtained after spin evaporation of ethyl acetate was crystallized from ethyl acetate–ether–petroleum ether to yield 4.40 g. (87%) of N-protected dipeptide active ester XIV, m.p. 158–159°, $[\alpha]_D^{19}$ −1.8° (c 1.0, DMF). The infrared spectrum showed peaks at 6.05 μ (amide I), 6.5 μ (amide II), and 5.6 μ (characteristic of pentachlorophenyl esters).

Anal.—Calcd. for $C_{19}H_{15}Cl_5N_2O_5$: C, 43.21; H, 2.84; N, 5.30. Found: C, 43.15; H, 2.91; N, 5.41.

Procedure C

This consisted of removal of *N*-carbobenzoxy protecting group from peptides with free C-terminal residue.

Preparation of Glycyl-phenylalanine (XVII)—A suspension of 0.3 g. palladium-charcoal catalyst (10%) in a 20-ml. mixture of 8 parts of methanol and 2 parts of water, was hydrogenated at atmospheric pressure until no further uptake of hydrogen occurred. A solution of 2.0 g. (5.62 mmoles) of *N*-carbobenzoxy glycyl-phenylalanine(X), in 10 ml. of methanol was added to the reaction mixture and the hydrogenation was carried out until no further uptake of hydrogen occurred (55 ml. of hydrogen in 10 min.). The reaction mixture was filtered free from the catalyst and the filtrate was spin evaporated under reduced pressure. The residue was crystallized from ethanol–ethyl acetate to yield 1.17 g. (94%) of the free dipeptide XVII, m.p. 266–268° dec. [lit. (34) m.p. 264–268° dec.].

Preparation of Phenylalanyl-glycine (XVIII)—*N*-Carbobenzoxy phenylalanyl-glycine (XII) was hydrogenated according to Procedure C, in 93% yield to afford the dipeptide XVIII, m.p. 269–270° dec. [lit. (34) m.p. 270–271° dec.].

Preparation of N-Carbobenzoxy Glycyl-glycyl-phenylalanine (XX)—Using the appropriate amounts, *N*-carbobenzoxy glycine pentachlorophenyl ester (11) was coupled with glycyl-phenylalanine (XVII), C-protected by DCA in the manner described in Procedure A. The N-protected tripeptide XX was obtained in 68% yield, m.p. 145–146° [lit. (34) m.p. 146°].

Preparation of N-carbobenzoxy Phenylalanyl-glycyl-phenylalanine (XXI)—The coupling of N-protected phenylalanine active ester III with the free dipeptide XVII, C-protected by DCA according to Procedure A, afforded the N-protected tripeptide XXI in 67% yield, m.p. 151–152° [lit. (34) m.p. 150–151°].

Preparation of N-Carbobenzoxy Glycyl-alanyl-alanine (XXIV)—N-protected dipeptide active ester XIV was coupled with alanine, C-protected by DCA in the manner described in Procedure A and the desired tripeptide XXIV was isolated in 43% yield, m.p. 173–174° [lit. (35) m.p. 172°].

Preparation of N-Carbobenzoxy Glycyl-glycyl-phenylalanyl-phenylalanine (XXV)—*N*-Carbobenzoxy glycyl-glycyl-phenylalanine pentachlorophenyl ester (XV), was prepared in the manner described (8). N-Protected tripeptide active ester XV was coupled according to Procedure A with phenylalanine C-protected by DCA. The tetrapeptide XXV was isolated in 38% yield, m.p. 140–142°.

Anal.—Calcd. for $C_{30}H_{32}N_4O_7$: C, 64.29; H, 5.72; N, 10.00. Found: C, 64.38; H, 5.81; N, 9.92.

Preparation of N-Carbobenzoxy Glycyl-β-benzyl Aspartic Acid (XI)—β-Benzyl aspartate (XXVI) was prepared according to the method reported by Benoiton (20). From appropriate amounts of N-protected glycine active ester II, and compound XXVI, C-protected by DCA, coupling was carried out following Procedure A. The dipeptide XI was isolated in 58% yield after crystallization from ethyl acetate–ether, m.p. 99–100°, $[\alpha]_D^{20}$ +15.7° (c 1.0, methanol).

Anal.—Calcd. for $C_{21}H_{22}N_2O_7$: C, 60.8; H, 5.32; N, 6.77. Found: C, 60.88; H, 5.13; N, 6.76.

Preparation of N-Carbobenzoxy Glycyl-alanyl-phenylalanyl-glycine (XXIII)—*Method 1*—Coupling of N-protected dipeptide active ester XIV, to free dipeptide XVIII. From 2.0 g. (3.78 mmoles) of *N*-carbobenzoxy glycyl-alanine pentachlorophenyl ester (XIV), and 0.84 g. (3.78 mmoles) of phenylalanyl-glycine (XVIII), C-protected by appropriate amount of DCA, coupling was carried out according to Procedure A. The N-protected tetrapeptide XXIII, 0.99 g. (54%) was isolated after crystallization from methanol–ether, m.p. 175–176° [lit. (32) m.p. 175°].

Method 2—Stepwise chain lengthening of the tetrapeptide XXIII, from N-terminal residue (Scheme I). *N*-Carbobenzoxy glycyl-alanine (IX) was prepared by coupling 2.0 g. (4.38 mmoles) of N-protected glycine active ester II, with 0.39 g. (4.38 mmoles) of alanine C-protected by 0.86 ml. (4.38 mmoles) of DCA. Following the reaction conditions outlined in Procedure A, 0.76 g. (62%) of the N-protected dipeptide IX, was obtained, m.p. 120° [lit. (31) m.p. 119.5°]. Phenylalanine pentachlorophenyl ester hydrochloride (XXVII) was prepared by hydrogenation of *N*-carbobenzoxy phenylalanine pentachlorophenyl ester (III) in the presence of anhydrous methanol containing hydrogen chloride, according to the method previously reported (8), in 93% yield, m.p. 199–200°, $[\alpha]_D^{23}$ −23.4° (c 1.07, methanol).

Anal.—Calcd. for $C_{15}H_{11}Cl_6NO_2$: C, 40.00; H, 2.44; N, 3.11. Found: C, 39.95; H, 2.51; N, 3.08.

N-Carbobenzoxy glycyl-alanyl-phenylalanine pentachlorophenyl ester (XVI) was prepared by mixed anhydride method as outlined in Procedure B. From 1.0 g. (3.57 mmoles) of the N-protected dipeptide IX and 1.6 g. (3.57 mmoles) of the active ester hydrochloride XXVII, 1.78 g. (74%) of N-protected tripeptide active ester XVI was obtained, m.p. 187–189°. Recrystallization from ethyl acetate–ether raised the melting point to 191–192°, $[\alpha]_D^{22}$ −49.4° (c 1.0, DMF). Infrared spectrum showed peaks at 6.04 μ (amide I), 6.5 μ (amide II), and 5.6 μ (pentachlorophenyl ester).

Anal.—Calcd. for $C_{28}H_{24}Cl_6N_3O_6$: C, 49.74; H, 3.56; N, 6.22. Found: C, 50.03; H, 3.48; N, 6.15.

N-Protected tetrapeptide XXIII, was prepared according to Procedure A. From 1.5 g. (2.22 mmoles) of tripeptide active ester XVI, and 0.166 g. (2.22 mmoles) of glycine, C-protected by 0.44 ml.

(2.22 mmoles) of DCA, 0.43 g. (40%) of *N*-carbobenzoxy glycyl-alanyl-phenylalanyl-glycine (XXIII) was obtained, m.p. 175°.

Method 3—Stepwise chain lengthening of the tetrapeptide XXIII, from C-terminal residue (Scheme II). *N*-Carbobenzoxy phenylalanyl-glycine (XII) was prepared according to Procedure A. From 2.0 g. (3.65 mmoles) of N-protected phenylalanine active ester III, and 0.274 g. (3.65 mmoles) of glycine, C-protected by appropriate amount of DCA, 0.79 g. (61%) of the dipeptide XII, was obtained, m.p. 154–155° [lit. (32) m.p. 154°]. Removal of *N*-carbobenzoxy protecting group from XII, as described in Procedure C, afforded the free dipeptide XVIII, in 93% yield.

N-Carbobenzoxy alanyl-phenylalanyl-glycine (XXII), was prepared according to Procedure A, by coupling 2.0 g. (4.24 mmoles) of N-protected alanine active ester I, with 0.94 g. (4.24 mmoles) of the dipeptide XVIII, C-protected by appropriate amount of DCA, and 1.23 g. (68%) of N-protected tripeptide XXII, was obtained, m.p. 168–170°. Recrystallization from methanol–ether raised the melting point to 171°, $[\alpha]_D^{21}$ −46.5° (c 1.02, methanol).

Anal.—Calcd. for $C_{23}H_{25}N_3O_6$: C, 61.82; H, 5.85; N, 9.84. Found: C, 61.78; H, 5.73; N, 9.71.

N-Carbobenzoxy tripeptide XXII was hydrogenated in the manner described in Procedure C, and alanyl-phenylalanyl-glycine (XIX), was obtained in 95% yield upon crystallization from ethanol–ethyl acetate, m.p. 233–234° dec., $[\alpha]_D^{21}$ +17.7° (c 1.02, 1 *N* HCl).

Anal.—Calcd. for $C_{14}H_{19}N_3O_4$: C, 57.34; H, 6.48; N, 14.33. Found: C, 57.45; H, 6.57; N, 14.21.

N-Protected tetrapeptide XXIII, was prepared according to Procedure A. From 1.0 g. (2.18 mmoles) of *N*-carbobenzoxy glycine pentachlorophenyl ester II, and 0.64 g. (2.18 mmoles) of the tripeptide XIX, C-protected by 0.42 ml. (2.18 mmoles) of DCA, 0.79 g. (75%) of *N*-carbobenzoxy-glycyl-alanyl-phenylalanyl-glycine (XXIII) was obtained, m.p. 174–175°.

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Keyphrases

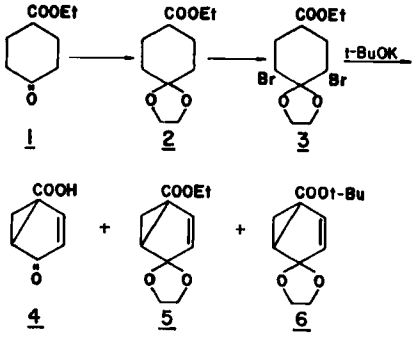
Peptide synthesis
Pentachlorophenyl esters, N-protected—peptide, amino acid coupling
Salt formation effect—peptide chain lengthening
Alkali elimination—peptide chain lengthening
Optical rotation—identity
IR spectrophotometry—structure

Novel Preparation of Bicyclo[3.1.0]hex-2-ene-4-one-1-carboxylic Acid and Related Compounds

By EDWARD E. SMISSMAN, THOMAS L. LEMKE, and MARY WEIR CREESE

An attempt to dehydrohalogenate 4-carbethoxy-2,6-dibromocyclohexanone ethylene ketal afforded bicyclo[3.1.0]hex-2-ene-4-one-1-carboxylic acid, ethyl bicyclo[3.1.0]hex-2-ene-4-one-1-carboxylate ethylene ketal, and *t*-butyl bicyclo[3.1.0]hex-2-ene-4-one-1-carboxylate ethylene ketal. Under milder conditions it was possible to secure 3-bromo-bicyclo[3.1.0]hexane-4-one-1-carboxylic acid ethylene ketal.

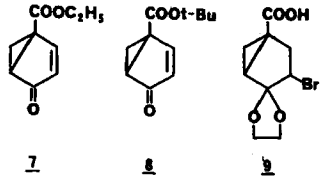
AN ATTEMPT was made to dehydrobrominate 4-carbethoxy-2,6-dibromocyclohexanone ethylene ketal (*3*) in order to prepare ethyl cyclohexa-2,4-dienone-4-carboxylate as a starting material in a synthesis of prephenic acid. Collidine proved unsuccessful in this dehydrohalogenation reaction and therefore potassium *tert*-butoxide in dimethyl sulfoxide (DMSO) was utilized (1) (Scheme I).



Scheme I

The dibromide, *3*, was prepared by first converting 4-carbethoxycyclohexanone (*1*) to the ethylene ketal *2*, which was then brominated by the procedure of Garbisch (1). The dibromide, *3*, appeared to be the *cis* diequatorial dibromide since the NMR spectrum showed a singlet for the methylene protons of the ketal, suggesting that they were located in a symmetrical environment. This would not be the situation if the halogens were *trans*. This assignment is consistent with the findings of Garbisch. A second component was isolated in 8% yield and proved to be 4-carbethoxy-*trans*-2,6-dibromocyclohexanone ethylene ketal. This material exhibited a multiplet for the methylene protons of the ketal, indicating that the methylenes are no longer in a symmetrical environment. A monobromide was isolated in 15% yield.

The 4-carbethoxy-*cis*-2,6-dibromocyclohexanone ethylene ketal (*3*) was dissolved in DMSO and added to a suspension of potassium *tert*-butoxide in DMSO. Acidic and neutral fractions were recovered from the reaction mixture. The acidic material, a low melting solid, proved to be bicyclo [3.1.0]hex-2-ene-4-one-1-carboxylic acid (*4*). The neutral fraction contained two compounds which were identified as ethyl bicyclo[3.1.0]hex-2-ene-4-one-1-carboxylate ethylene ketal (*5*) and *t*-butyl bicyclo[3.1.0]hex-2-ene-4-one-1-carboxylate ethylene ketal (*6*).

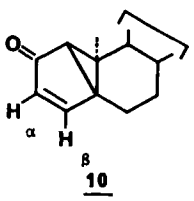


DISCUSSION

Assignment of these structures was based on spectral evidence obtained from the acid, *4*, the ketones, *7* and *8*, which were prepared either by chromatography of the ketals on silica gel or by treatment with hydrochloric acid, and from the ketals, *5* and *6*. The NMR spectrum of ethyl bicyclo[3.1.0]hex-2-ene-4-one-1-carboxylate (*7*) exhibited a doublet at 7.80 δ (J = 6.0 c.p.s.) and a slightly split doublet at 5.60 δ (J = 5.5 and 0.5 c.p.s.), both of which integrated for single protons. These absorptions are similar to those found by Dutler and co-workers (2) for the α and β protons of the steroid systems *10* (partial structure), in which case the α proton appeared at 5.82 δ as a doublet of doublets with J = 5.5 and 0.5–1.0 c.p.s., while the β proton was found as a doublet at 7.20 δ with J = 5.5 c.p.s. The methylene of the ester *7* was found at 4.20 and the methyl at 1.28 δ. The tertiary proton on the cyclopropane ring was found as a doublet of doublets at 2.58 δ with J = 5

Received February 15, 1968, from the Department of Medicinal Chemistry, School of Pharmacy, University of Kansas, Lawrence, KS 66044
Accepted for publication May 14, 1968.
Abstracted in part from a thesis submitted by T. L. Lemke, to the Graduate School, University of Kansas, Lawrence, Kansas, in partial fulfillment of Doctor of Philosophy degree requirements.
This investigation was supported by grant GM-07444 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

and 8 c.p.s., while the methylene protons on the cyclopropane ring appeared as multiplets centered



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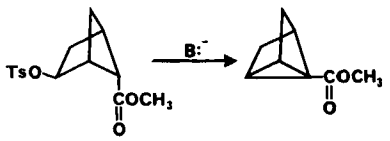
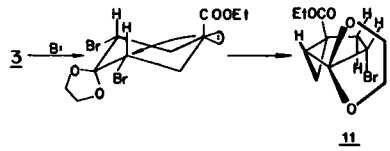
at 2.15 and 1.82 δ. A similar system has recently been reported by Meinwald (Scheme II) (3). A



Scheme II

more detailed analysis of the NMR spectra of these systems is the subject of a subsequent paper (4).

Formation of these bicyclic compounds, *4–6*, probably occurs by base abstraction of the proton α to the carbethoxy group followed by internal nucleophilic displacement of the halogen as shown in Scheme III. A facile dehydrobromination may



11

Scheme III

occur in intermediate *11* due to the nearly axial conformation of the bromine. Since an excess of potassium *t*-butoxide was present, a considerable amount of ester exchange occurred leading to *6*. In addition, it appears that some water was present in either the DMSO or *tert*-butanol causing ester hydrolysis. Under milder conditions it was possible to obtain the bicyclobromo acid, *9*.

EXPERIMENTAL[1]

4-Carbethoxycyclohexanone (*1*)—This material was prepared by two different methods. *Method A*—A mixture of 12.5 g. (0.149 mole) of 2-methoxybutadiene, 11.2 g. (0.112 mole) of ethyl acrylate, and 70 ml. of benzene was placed in an autoclave and heated to 160°. A total time of 1.5 hr. was required to reach this temperature which was maintained for 0.5 hr. The system was then cooled to 25°. Removal of the solvent resulted in recovery of 16.5 g. (80%) of 1-carbethoxy-4-methoxy-3-cyclohexene; IR: 5.78 (*s*) and 6.0 μ (*m*); NMR: 4.50 (1 H, multiplet), 4.07 (2 H, quartet, J = 7 c.p.s.), 3.41 (3 H singlet), 2.00 (7 H, multiplet), and 1.22 (3 H, triplet, J = 7 c.p.s.). Upon treatment with 2,4-dinitrophenylhydrazine a solid formed, which after recrystallization from EtOH had m.p. 115–118°.

To 15 ml. of 1% H_2SO_4 was added 1.70 g. (0.0092 mole) of the enol ether obtained above. The mixture was shaken for 5 min. and then thoroughly extracted with ether. The combined ether layers were dried ($MgSO_4$). Removal of the solvent left a residue of 1.22 g. (84%) of 4-carbethoxycyclohexanone; IR: 5.80 (s); NMR: 4.16 (2 H, quartet, J = 7.0 c.p.s.), 2.71 (1 H, multiplet); 2.20 (8 H, multiplet), 1.28 (3 H, triplet, J = 7.0 c.p.s.). Reaction with 2,4-dinitrophenylhydrazine gave a solid which after recrystallization from 95% ethanol melted at 119–120° [lit. (5, 6) m.p. 125–126°].

Method B—Ethyl *p*-hydroxybenzoate (30 g., 0.18 mole) in 75 ml. of 95% EtOH was hydrogenated in the presence of 3 g. of rhodium-on-alumina catalyst (5%) in a Parr apparatus. The solvent was removed and the remaining oil was distilled under reduced pressure. The product, b.p. 80°/0.1 mm., was obtained in 75% yield. The resulting alcohol, 4-carbethoxycyclohexanol (37 g., 0.22 mole), was dissolved in 200 ml. of acetone and was treated with 27 ml. of Jones reagent. The temperature was maintained at 30–35° throughout the addition. An 89% yield of the desired product was obtained.

4-Carbethoxycyclohexanone Ethylene Ketal (*2*)—To a solution of 1.61 g. (0.0094 mole) of 4-carbethoxycyclohexanone (*1*), 0.73 g. (0.012 mole) of ethylene glycol and 30 ml. of dry benzene was added 53 mg. of *p*-toluenesulfonic acid. The solution was heated under reflux for 4.5 hr., the evolved water being collected in a Dean-Stark trap. The solvent was distilled and the oil, which remained, was taken up in ether and washed with 10 ml. of 10% NaOH. The ether solution was dried ($MgSO_4$). Removal of the solvent left a residue of 1.80 g. (89%) of ethylene ketal, b.p. 85°/0.6 mm.; IR: 5.80 (s), 8.25 (s), 8.50 (s) μ; NMR: 1.22 (3 H, triplet, J = 7.0 c.p.s.), 1.40 to 2.50 (9 H, broad band), 3.87 (4 H, singlet), and 4.10 (2 H, quartet, J = 7.0 c.p.s.). A sample was treated with 2,4-dinitrophenylhydrazine, and in a short time a precipitate resulted. After recrystallization from 95% ethanol the solid melted at 119–121°. The mixed melting point with the phenylhydrazone of 4-carbethoxycyclohexanone showed no depression.

4-Carbethoxy-2,6-cis-dibromocyclohexanone Ethylene Ketal (*3*)—To a solution of 4.76 g. (0.022 mole) of 4-carbethoxycyclohexanone ethylene ketal (*2*) in 50 ml. of diethyl ether was added 7.20 g. (0.045 mole) of Br_2, while stirring. The addition was at such a rate as to cause gentle reflux. Near the end of addition of the bromine a two-phase system developed. A solution containing 1.11 g. (0.048 g. atoms) of sodium in 20 ml. of ethylene glycol was added. Water was added to the reaction mixture and the ether layer was removed. The aqueous layer was extracted with ether and the combined ether layers were dried ($MgSO_4$). Removal of the solvent left a residue of 7.00 g. (85%) of a mixture of products. A portion of the oil,

[1] Melting points were obtained on a Thomas-Hoover Unimelt and are corrected. Infrared data were recorded on Beckman IR-5, IR-8, and IR-10 spectrophotometers, and the ultraviolet data were recorded on a Cary-14 spectrophotometer. NMR data were obtained from a Varian Associates model A-60 spectrometer utilizing CCl_4 as a solvent, unless otherwise stated, with tetramethylsilane as an internal standard, and reported in p.p.m. as δ values. Elemental analyses were performed by Hoffman Microanalytical Laboratories, Wheatridge, Colo., and by Drs. G. Weiler and F. B. Strauss, Oxford, England.

2.14 g., was chromatographed on 50 g. of silica gel (Brinkmann) using benzene as the eluting solvent. The first few fractions, 783 mg., contained the desired dibromide, *3*. The compound was further purified by rechromatographing on silica gel (Brinkmann); IR: 5.78 (s) μ; NMR: 1.32 (3 H, triplet, J = 7.5 c.p.s.) 2.2 to 2.9 (5 H, multiplet), 4.35 (4 H, singlet), 4.25 (4 H, multiplet).

Anal.—Calcd. for $C_{11}H_{16}Br_2O_4$: C, 35.51; H, 4.34. Found: C, 35.59; H, 4.22.

The next oil removed from the column, 460 mg., was 4-carbethoxy-2-bromocyclohexanone ethylene ketal; IR: 5.78 (s) μ; NMR: 1.27 (3 H, triplet, J = 7.5 c.p.s.), 1.5 to 2.9 (7 H, broad multiplet), 4.15 (2 H, quartet, J = 7.5 c.p.s.), 4.08 (5 H, multiplets).

Anal.—Calcd. for $C_{11}H_{17}BrO_4$: C, 45.06; H, 4.84. Found: C, 45.07; H, 5.62.

In a later experiment another oil was isolated during chromatography of the reaction mixture on a silica gel (Brinkmann) column. This material was eluted from the column after the *cis*-dibromide and before the monobromide. It appeared to be 4-carbethoxy-*trans*-2,6-dibromocyclohexanone ethylene ketal; IR: 5.79 (s) μ; NMR: 1.25 (3 H, triplet, J = 7.0 c.p.s.), 2.0–3.1 (5 H, broad multiplet), 4.11 (7 H, multiplet), 4.68 (1 H, doublet of doublets, J = 5.0 and 12.0 c.p.s.); n_D^{25} 1.5221.

Dehydrobromination of 4-Carbethoxy-2,6-cis-dibromocyclohexanone Ethylene Ketal—To a suspension of potassium *tert*-butoxide (prepared by addition of 2.03 g., 0.052 g. atom, of potassium to an excess of dry *tert*-butanol followed by removal of most of the excess alcohol) in 40 ml. of DMSO was added 4.08 g. (0.011 mole) of 4-carbethoxy-*cis*-2,6-dibromocyclohexanone ethylene ketal (*3*) in 10 ml. of DMSO. The reaction mixture was cooled during the addition. After the addition was complete the mixture was stirred at 25° for 3 hr. To the reaction mixture was added 1.80 g. (0.03 mole) of acetic acid in 25 ml. of H_2O. The reaction was made alkaline with $NaHCO_3$ and extracted with ether. The ether extract was dried ($MgSO_4$) (ether extract I). The aqueous layer was acidified with 10% HCl and then extracted with ether. The ether extract was dried ($MgSO_4$) (ether extract II).

Removal of the solvent from ether extract II resulted in recovery of a thick oil, 437 mg., which solidified on standing. The material was bicyclo[3.1.0]hex-2-ene-4-one-1-carboxylic acid (*4*); IR ($CHCl_3$): 1700 to 1745 (s), 1580 (w) cm.$^{-1}$; near IR ($CHCl_3$): 1626 (s), 2217 (m) mμ; NMR ($CDCl_3$): 7.89 (1 H, doublet, J = 6.0 c.p.s.), 5.77 (1 H, broad doublet, J = 5.5 c.p.s.), 11.10 (1 H, singlet), 2.90 (1 H, doublet of doublets, J = 5.0 and 9.0 c.p.s.), 1.8 to 2.4 (2 H, multiplets); λ_{max}^{EtOH} 205 and 249 mμ.

Treatment of *4* with 2,4-dinitrophenylhydrazine resulted in recovery of a dark-red phenylhydrazone which was recrystallized from 95% EtOH, m.p. 202° dec.

Anal.—Calcd. for $C_{13}H_{10}N_4O_6$: C, 49.06; H, 3.18; N, 17.61. Found: C, 49.35; H, 3.32; N, 17.55.

Removal of the solvent from ether extract I resulted in recovery of 2.20 g. of an oil which contained acetic acid, DMSO, and two unknowns as shown by GLC. An NMR spectrum of the crude reaction mixture showed the presence of vinyl and alkoxy protons suggesting that the mixture contained *5* and *6*.

A second sample of the crude oil was chromatographed on silica gel (Brinkmann) using $CHCl_3$ as the eluting solvent. The first few fractions contained a mixture of products. Later fractions contained two oils which were different from those placed on the column. These two oils possessed similar infrared spectra. One of the oils was identified as ethyl bicyclo[3.1.0]hex-2-ene-4-one-1-carboxylate (*7*) by its IR and NMR spectra; infrared: 1720 (s), 1580 (s) cm.$^{-1}$; NMR: 7.80 (1 H, doublet, J = 6.0 c.p.s.), 5.60 (1 H, broad doublet, J = 5.5 and 0.5 c.p.s.), 4.20 (2 H, quartet, J = 7.0 c.p.s.), 1.47 to 2.73 (3 H, multiplets), 1.28 (3 H, triplet, J = 6.5 c.p.s.); λ_{max}^{EtOH} 199 and 250 mμ. It would appear that the ketal group was removed on the column. To verify this a sample of starting oil was shaken with 10% HCl. The aqueous layer was extracted with ether. GLC analysis showed that the material had undergone conversion to the same mixture as was formed during chromatography. The second unknown was identified as *tert*-butyl bicyclo[3.1.0]hex-2-ene-4-one-1-carboxylate (*8*); the NMR was essentially the same as that of *5* with the exception of a singlet at 1.46 and the loss of peaks at 4.20 and 1.28; near IR ($CHCl_3$): 1628 and 22119 mμ with peak height ratio of 4.16 (2219:1628).

Another sample of the crude oil, 652 mg., was heated under reflux for 2 hr. with 25 ml. of 10% NaOH. After cooling the aqueous layer was extracted with ether and the ether was discarded. The aqueous phase was acidified with 10% HCl and extracted with ether. The combined ether extracts were dried ($MgSO_4$). Removal of the solvent resulted in recovery of 200 mg. of a thick oil which solidified on standing and which by spectral data was shown to be the carboxylic acid (*4*).

A final sample was treated with ozone in methylene chloride. The methylene chloride was then replaced by H_2O and hydrogen peroxide, and the mixture was digested on a steam bath. Removal of the solvent left a thick oil which was taken up in ether and dried. After filtration, diazomethane was added and the resulting ester was examined; NMR ($CDCl_3$): 3.80 (6 H, singlet), 3.73 (3 H, singlet), 1.0 to 2.0 (broad bands); near IR ($CHCl_3$):1622 (w), 2209 (m) mμ, indicating this material to be methyl 1,1,2-cyclopropane tricarboxylate.

When the above reaction was performed in a similar manner but the stirring was discontinued after 1 hr., a product containing bromine was obtained. On work-up the acidic material recovered was recrystallized from $CHCl_3$, m.p. 141.5–142.5°.

Anal.—Calcd. for $C_9H_{11}BrO_4$: C, 41.06; H, 4.18. Found: C, 40.99; H, 4.28.[9]

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[9] A detailed discussion of the structure proof of this compound (*9*) by NMR is to be found in *Reference 4*.

Keyphrases

Bicyclo [3.1.0]hex-2-ene-4-one-1-carboxylic acid, related compounds—syntheses
Column chromatography—separation
GLC—analysis
NMR spectroscopy—structure
IR spectrophotometry—structure

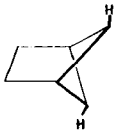
Long-Range Spin-Spin Coupling in Bicyclo[3.1.0]hexane Systems

By ROGER C. BRIDEN, EDWARD E. SMISSMAN*, and MARY WEIR CREESE

The NMR parameters of 3-bromo-bicyclo[3.1.0]hexan-4-one-1-carboxylic acid ethylene ketal and ethyl bicyclo[3.1.0]hex-2-ene-4-one-1-carboxylate have been recorded and the chemical shifts of all protons assigned. Both of these compounds show interesting cases of long-range spin-spin coupling.

LONG-RANGE ^1H-^1H spin-spin coupling in general (1, 2), and coupling through 4-σ bonds in particular (3–6), are receiving increasing attention. Two features are apparent in compounds exhibiting 4-σ bond coupling: first, the compounds have well-defined stereochemistry resulting from the rigidity of the molecule due to a fixed or preferred conformation; second, the H—C—C—C—H skeleton through which the protons are coupled exists in a ⟋⟍⟋ configuration.

The magnitudes of the coupling constants reported for 4-σ bond coupling range from less than 1 to about 7 c.p.s., with values of 1–2 c.p.s. being most common. Some examples of compounds in which long-range 4-σ bond coupling has been observed are shown by *1*, *2*, and *3* (4–6).



J=6.7 Hz

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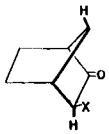

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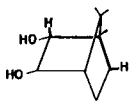
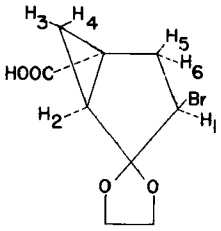
2



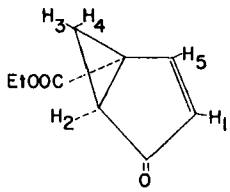
J=1.4 Hz

3

The authors have observed long-range 4-σ bond coupling in 3-bromo-bicyclo[3.1.0]hexan-4-one-1-carboxylic acid ethylene ketal (*4*) and in ethyl bicyclo[3.1.0]hex-2-ene-4-one-1-carboxylate (*5*).



4



5

The 100-Mc.p.s. spectrum of *4* is shown in Fig. 1. The coupling constants and chemical shift values determined from this spectrum are listed in Table I.

RESULTS AND DISCUSSION

The ethylene ketal resonance is assigned to the 3.88-δ absorption on the basis of its four-proton

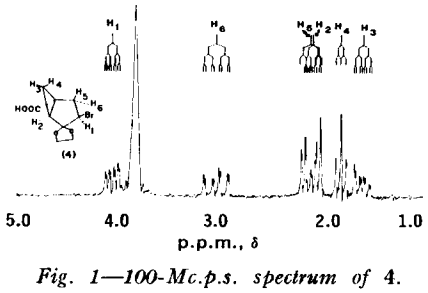


Fig. 1—100-Mc.p.s. spectrum of 4.

Received February 15, 1968, from the Department of Medicinal Chemistry, School of Pharmacy, University of Kansas, Lawrence, KS 66044
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This investigation was supported by grant GM-07444 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
* To whom inquiries concerning this paper should be addressed.

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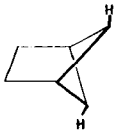

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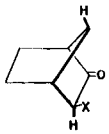
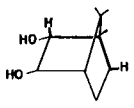
1



J=3-4 Hz

2



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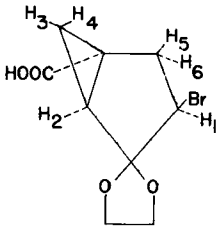
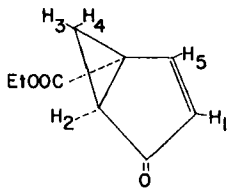


4



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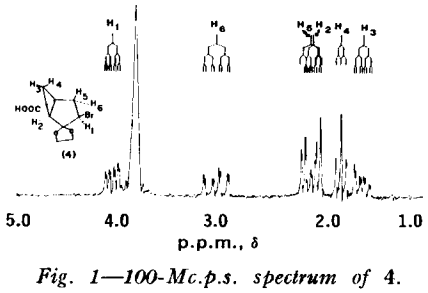

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TABLE I—NMR PARAMETERS

Compd.	Proton Chemical Shifts, δ					
	H-1	H-2	H-3	H-4	H-5	H-6
4	4.08	2.10	1.57	1.79	2.15	3.07
5	5.67	2.76	2.17	1.82	7.87	
	Coupling Constants, c.p.s.					
	Geminal		Vicinal		Long-Range	
4	*J*-34 = 5.5 *J*-56 = 15.0		*J*-15 = 4.0 *J*-16 = 8.5 *J*-23 = 9.5 *J*-24 = 5.5		*J*-12 = 1.5 *J*-36 = 1.5	
5	*J*-43 = 3.0		*J*-15 = 5.5 *J*-24 = 4.5 *J*-23 = 8.5		*J*-12 = 1.0	

integration and its field position. The 4.08-δ absorption is assigned to H-1 as this proton is on a bromine-bearing carbon and thus will absorb furthest downfield. H-1 is coupled to three other protons with coupling constants of 8.5, 4.0, and 1.5 c.p.s. The dihedral angle between H-1 and H-6 is 0°, while that between H-1 and H-5 is 120°. The modified Karplus plot allows the predication that *J*-16 should be about 10 c.p.s. and *J*-15 about 4 c.p.s. (7). Therefore, the observed coupling constants 8.5 and 4.0 c.p.s. have been assigned as *J*-16 and *J*-15, respectively. The 1.5-c.p.s. splitting is assigned to long-range coupling between H-1 and H-2 based on the magnitude of the coupling, the observed ⋁⋁ configuration of the H_1—C—C—C—H_2 system, and the absence of another proton in the molecule to which this coupling can reasonably be assigned. Double resonance experiments (Figs. 2*b* and 3*b*) indicate that the proton (H-2) which is responsible for this 1.5-c.p.s. splitting lies in the upfield portion of the 2.1-δ multiplet. The lack of clean decoupling for protons H-1 and H-6 when irradiating H2 (Fig. 2*b*) may be attributed to the proximity of the H-5 resonance to the point of irradiation and possibly to virtual spin-spin decoupling of H-6 through H-1.

The proton resonance at 3.07 δ is coupled to three other protons with *J* values of 15.0, 8.5, and 1.5 c.p.s. Proton H-6 is assigned to this absorption because of the observed 8.5-c.p.s. coupling between H-1 and H-6. The 15.0-c.p.s. coupling is reasonable for geminal coupling (8) and is probably negative in sign. This coupling is assigned as *J*-65. The remaining 1.5 c.p.s. coupling can be assigned to long-range coupling between H-6 and either H-2 or H-3 as both 5-atom systems exist in a ⋁⋁ configuration. Double-resonance experiments (Figs. 2*a* and 3*a*) show that this 1.5-c.p.s. splitting results from coupling to the proton absorbing at 1.6 δ. As double resonance techniques have indicated that H-2 absorbs in the 2.1-δ region, this 1.5-c.p.s. coupling and the 1.6-δ absorption are assigned to H-3.

Proton H-3 is coupled to three other protons with coupling constants of 9.5, 5.5, and 1.5 c.p.s. The 1.5-c.p.s. splitting has been assigned as *J*-36. The *J* values of 9.5 c.p.s. and 5.5 c.p.s. agree with those reported for *cis* and geminal coupling on cyclopropane rings (8). These couplings are assigned as *J*-32 = 9.5 c.p.s. and *J*-34 = 5.5 c.p.s.

The 2.1-δ resonance can be divided into two parts: one part shows coupling to two other protons with *J* values of 15.0 c.p.s. and 4.0 c.p.s. Proton H-5 is assigned to this part because of its 15.0-c.p.s. coupling to H-6. The coupling constants are assigned as *J*-56 = 15.0 c.p.s. and *J*-51 = 4.0 c.p.s.

Because of its 9.5-c.p.s. coupling constant and double resonance experiments, the second part of the 2.1-δ multiplet is assigned to H-2. The observed coupling constants of 9.5, 5.5, and 1.5 c.p.s. are assigned as *J*-23 = 9.5 c.p.s., *J*-24 = 5.5 c.p.s., and *J*-21 = 1.5 c.p.s.

The final multiplet in the spectrum at 1.79 δ is assigned to H-4. The coupling constants are assigned as *J*-43 = 5.5 c.p.s. and *J*-42 = 5.5 c.p.s.

The 60-Mc.p.s. spectrum of *5* is shown in Fig. 4, and the chemical shifts and coupling constants are listed in Table I. The one-proton doublet at 7.87 δ is assigned to H-5 because the β-olefinic proton of an α,β-unsaturated ketone, cyclic or acyclic, usually lies further downfield than the α-olefinic proton (9).

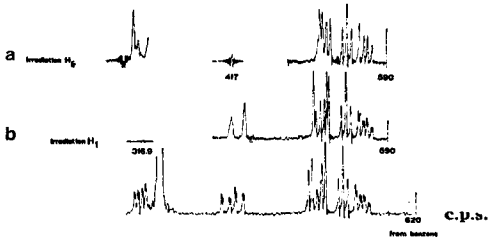

Fig. 3—100-Mc.p.s. spectrum of 4.

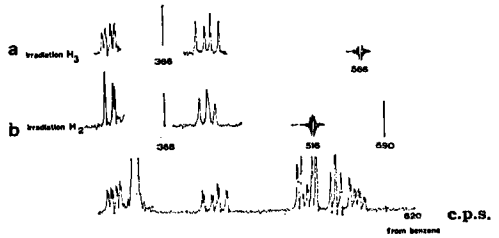

Fig. 2—100-Mc.p.s. spectrum of 4.

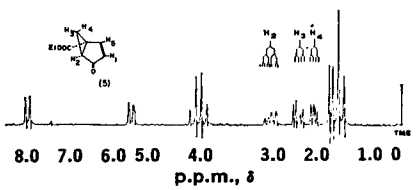

Fig. 4—60-Mc.p.s. spectrum of 5.

The 5.5-c.p.s. splitting is due to vicinal coupling of H-5 with H-1. Proton H-1 is assigned to the 5.67-δ doublet which is further split. The 5.5-c.p.s. coupling of this doublet of doublets is due to H-5, H-1 coupling while the 1.0-c.p.s. splitting is assigned to the long-range coupling of H-1 with H-2. The broad doublet of doublets at 2.76 is assigned to H-2, the 8.5-c.p.s. splitting being due to coupling between H-2 and H-3 which are *cis* to one another. The coupling between the *trans* protons H-4 and H-2 is assigned to the 4.5-c.p.s. splitting and the broadening of each peak to the long-range coupling of H-2 to H-1. The two remaining one-proton multiplets at 2.17 δ and 1.82 δ are assigned to H-3 and H-4, respectively. H-3 is coupled to H-2 by 8.5 c.p.s. and to H-4 by 3.0 c.p.s. H-4 is in like manner coupled to H-2 by 4.5 c.p.s. and to H-3 by 3.0 c.p.s. The triplet at 1.30 δ and the quartet at 4.18 δ are obviously due to the ethyl group of the ester. The 1.49-δ singlet is due to a *tert*-butyl group resonance, the *tert*-butyl ester having been formed by transesterification during the synthesis of *5* (10).

The magnitudes of the vicinal coupling constants observed in these systems are in agreement with values reported previously for some substituted cyclopropanes by Graham and Rogers (8). In these systems they found that *J-cis* ranged from 7.90 to 9.33 c.p.s. and averaged 8.44 c.p.s. and that *J-trans* ranged from 5.28 to 6.55 c.p.s and averaged 5.68 c.p.s. The geminal coupling constant varied over a large range but the sum of *J-cis* + *J-trans* + *J-gem* was virtually constant at 20.68 to 21.61 c.p.s. The coupling constants observed for Compound *4* are *J-cis* = 9.5, *J-trans* = 5.5, and *J-gem* = 5.5 c.p.s., giving a sum of 20.5 c.p.s., which is near the reported range, thus suggesting that the cyclopropane system in this bicyclo compound is near normal. In Compound *5* the analogous coupling constants are 8.5, 4.5, and 3.0 c.p.s., respectively, giving a sum of 16 c.p.s. which is significantly below the reported range. The low value of the geminal coupling constant (*J*-34) is of particular interest and probably results from a combination of effects among which should be included the sensitivity of geminal coupling to the H—C—H bond angle and the substituent effects of the carbonyl and the double bond.

Long-range coupling in Compound *4* occurs between protons H-1 and H-2, and between H-6 and H-3. No coupling is observed between H-6 and H-2. The $\diagup\!\diagdown\!\diagup$ between H-1 and H-2 is similar but more planar than that formed between H-2 and H-6. This, plus substituent effects (3, 11) may account for the absence of coupling between H-2 and H-6. Compound *5* shows only one case of long-range coupling, that between H-1 and H-2. Although the lack of long-range coupling between H-5 and H-3 presumably results from a combination of a substituent and a steric effect caused by the introduction of the double bond, other effects are not ruled out. The absence of long-range coupling between H-5 and H-3 is clear from the half-band width of the H-3 resonance which is, within experimental error, the same as that of the TMS resonance and the methylene quartet of the ethyl ester group.

This bicyclo[3.1.0]hexane system (*4*), which provides an example of relatively rare long-range coupling of cyclopropane hydrogens through 4-σ bonds, also illustrates the utility of the commonly observed $\diagup\!\diagdown\!\diagup$ configuration in long-range spin-spin coupling in assigning chemical shifts of closely related protons in such systems.

EXPERIMENTAL

The proton resonance spectra were obtained on Varian A-60 and HA-100 high-resolution NMR spectrometers. Chemical shifts are reported in δ. The 100-Mc.p.s. spectra were observed using benzene as the lock signal. Each sample was run as a 10–15% solution in deuterochloroform containing TMS as the internal reference.

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Keyphrases

Bicyclo[3.1.0]hexane systems
Spin-spin coupling, long-range—bicyclo[3.1.0]hexane systems
Coupling constants—bicyclo[3.1.0]hexane systems
NMR spectroscopy—chemical shifts

Serum Turbidity Responses of Anti-Inflammatory Drugs in Endocrine-Deficient Rats

By C. COLOMBO, S. J. PILIERO, and L. GROSSO

The turbidimetric responses of sera to heat from cold stress and endocrine-deficient rats and the effects of phenylbutazone, paramethasone, and adrenocorticotropic hormone (ACTH) have been compared. Sera from cold-stress animals (24 hr.) reveal decreases in the turbidimetric response to heat followed by a return to pre-exposure levels 5 days after cold stress. These responses appear to be operative through the pituitary-adrenocortical axis. Adrenalectomized animals show an inhibition in their serum-turbidity responses to heat which can be prevented by the administration of paramethasone. A further inhibition of protein coagulation by heat occurs in phenylbutazone-treated adrenalectomized rats. This decrease in serum-turbidity readings is similar to that observed in phenylbutazone-treated sham-operated animals. Hypophysectomy or pleuriglandular deficiency causes no protein-stabilization effect to heat which is not attributable to a lack of ACTH. The protein-stabilization effect of phenylbutazone as revealed by the decreases in serum-turbidity responses to heat appears to require an intact pituitary.

RECENTLY, Piliero and Colombo (1, 2) developed an *in vivo* assay for screening drugs for potential anti-inflammatory activity. The method, as well as those procedures described by Mizushima *et al.* (3–5) and Glenn and Kooyers (6), is based on the ability of compounds to inhibit heat denaturation of proteins.

Regarding the underlying mechanism of action of the *in vivo* assay as conducted in normal animals, evidence indicates that the activity of many drugs (nonsteroidal) in the normal animal is through a stabilizing effect on serum-protein coagulation which appears to be operative through a protein-binding phenomenon involving lysyl epsilon-amino groups (2). This appears to be in agreement with the hypothesis of Whitehouse and Skidmore (7) that an important property of nonsteroid anti-inflammatory agents is their ability to uncouple oxidative phosphorylation. In view of the lack of turbidity responses of glucocorticoids in normal animals (2), the present study was undertaken to investigate the role of the pituitary-adrenal axis on turbidity changes in cold-stressed rats and in endocrine-deficient animals treated with adrenocorticotropic hormone (ACTH), paramethasone, and phenylbutazone.

MATERIALS AND METHODS

Four-hundred and thirty-three young adult male rats of the Charles River (CD) strain, weighing approximately 200–225 g. were employed in all experiments. All animals were fed a standard laboratory ration with water *ad libitum* and were kept at temperatures of 70–80° F.

Adrenalectomized animals were maintained on 1% sodium chloride given as drinking water while hypophysectomized animals received supplements of 5% glucose solution. In addition, one group in which adrenalectomy was employed as part of a pleuriglandular procedure (hypophysectomy) received a single maintenance dose of 2.5 mg. of cortisone acetate injected intramuscularly. No accessory adrenal tissue was found to be present in any of these animals at the time of sacrifice.

Serum turbidity (1, 2) was assayed in the following manner: (*a*) To 0.1 ml. of nonhemolyzed serum, 2.9 ml. of 0.066 *M* Sørensen phosphate buffer in saline at pH 5.2 was added and gently agitated. This mixture was allowed to stabilize at room temperature for 15 min. (*b*) The mixture was heated in a Precision constant-temperature shaking water bath set at 69° for 30 min. (*c*) Then, the samples were immediately cooled in an ice water bath and read in a spectrophotometer at a wavelength of 645 μ. Changes in serum turbidity are based on the absorbance readings. Plasma fibrinogen was determined by the method of Goodwin (8). There were four experimental groups.

Intact Animals Following Cold-Stress Exposure (Expt. 1)—Thirty-six animals were subjected to cold stress in a cold room set at 5°. At various intervals, 3, 6, 24, and 120 hr. of continuous exposure, six animals were removed from this stress and immediately sacrificed and exsanguinated for serum-turbidity and plasma-fibrinogen levels in comparison to a group of 12 nonexposed animals. In addition, one group of six rats exposed for 24 hr. was allowed an additional exposure of 3 hr. at room temperature before sacrificing and testing of blood samples.

Hypophysectomized and Control Animals (Expt. 2)—Serum-turbidity studies in three different groups of rats were conducted utilizing 120 hypophysectomized and 135 sham-hypophysectomized

Received January 24, 1968, from the Department of Pharmacology, Geigy Research, Division of Geigy Chemical Corporation, Ardsley, NY 10502, and Graduate Department of Biology, Long Island University, Brooklyn, NY 11201

Accepted for publication May 15, 1968.

Taken in part from a thesis presented to the Graduate Department of Biology, Long Island University, by C. Colombo in partial fulfillment of the Master of Science degree requirements, 1967.

TABLE I—PLAN OF EXPERIMENT 2

Group	No. of Rats	Agents Used, *Per Os* Unless Indicated	Dose, 3 Hr. Before Sacrifice
Hypox.	20	3% Corn starch	10 ml./kg.
Sham-hypox.	30	3% Corn starch	10 ml./kg.
Hypox. + ACTH	20	ACTH (i.m.)	3 units
Sham-hypox. + ACTH	10	ACTH (i.m.)	3 units
Hypox. + phenylbutazone	20	Phenylbutazone	100 mg./kg.
Sham-hypox. + phenylbutazone	5	Phenylbutazone	100 mg./kg.
Hypox. + ACTH + phenylbutazone	20	ACTH (i.m.); phenylbutazone	3 units; 100 mg./kg.
Sham-hypox. + ACTH + phenylbutazone	10	ACTH (i.m.); phenylbutazone	3 units; 100 mg./kg.
Sham-hypox.	10	3% Corn starch	10 ml /kg.
Sham-hypox. + ACTH	10	ACTH (i.m.)	3 units
Sham-hypox. + phenylbutazone	10	Phenylbutazone	100 mg./kg.
Sham-hypox. + ACTH + phenylbutazone	10	ACTH (i.m.); phenylbutazone	3 units
Hypox.	10	3% Corn starch	10 ml./kg
Sham-hypox.	10	3% Corn starch	10 ml./kg.
Hypox. + paramethasone	10	Paramethasone	1 mg./kg.
Sham-hypox. + paramethasone	10	Paramethasone	1 mg./kg.
Hypox. + phenylbutazone	10	Phenylbutazone	100 mg./kg.
Sham-hypox. + phenylbutazone	10	Phenylbutazone	100 mg./kg.
Hypox. + paramethasone + phenylbutazone	10	Paramethasone; phenylbutazone	1 mg./kg.; 100 mg./kg.
Sham-hypox. + paramethasone + phenylbutazone	10	Paramethasone; phenylbutazone	1 mg./kg.; 100 mg./kg.

animals, operated 6 weeks previously and experimentally treated as indicated in Table I.

Adrenalectomized and Control Animals (Expt. 3)—Serum-turbidity studies in two different groups of rats were conducted utilizing 50 adrenalectomized and 40 sham-operated animals, operated 2 weeks previously and experimentally treated as indicated in Table II.

Hypophysectomized Adrenalectomized and Control Animals (Expt. 4)—Twenty hypophysectomized animals, operated 6 weeks previously were adrenalectomized. After 14 days of adrenalectomy, 100 mg./kg. body weight of phenylbutazone was administered *per os* to 10 of the pleuriglandular deficient rats. Ten other pleuriglandular deficient rats were given 3% corn starch (10 ml./kg.) as controls. Also included were 20 sham-hypophysectomized-adrenalectomized control rats 10 of which were given 3% corn starch (10 ml./kg.) and the remaining 10 animals given phenylbutazone (100 mg./kg. body weight). Serum-turbidity studies were conducted 3 hr. following the administration of the drug.

RESULTS[1] AND DISCUSSION

Changes in protein structure, such as those brought about by denaturation, as a cause of nonspecific inflammation (9, 10) suggests a basic physicochemical action of anti-inflammatory drugs on proteins (2–6). Previous studies employing nonsteroidal and steroidal agents *in vitro* on the turbidimetric response to heat of Cohn fraction IV-4 (3–5) as well as similar *in vivo* studies employing plasma (6) or serum (1, 2) from adjuvant arthritic rats appear to be in accord with this concept. In the authors' serum-turbidity model employing normal animals only the nonsteroidal agents and not the glucocorticoids have been found to be effective in inhibiting protein coagulation by heat as reflected by decreases in absorbance (2).

The present study (Fig. 1) reveals that cold stress (24 hr.) brings about an inhibition of protein coagulation by heat, accompanied by an increase in fibrinogen levels. However, as reflected by the turbidity response a degree of adaptation appears to occur in the 5-day cold-exposed group in that there is at this time a return in values to pre-exposure levels. Apparently, the fibrinogen levels do not reflect to the same degree this adaptation since, although somewhat lower, the high fibrinogen values are still maintained. The data presented appear to be in agreement with the fact that cold stress involves the pituitary-adrenocortical axis (11, 12).

The serum-turbidity levels of the hypophysectomized rats (Fig. 2) reveal significant increases in comparison to the values obtained for sham-operated animals. This seems to imply that the lack of inhibition of protein coagulation by heat in the hypophysectomized animals may be due to the absence of ACTH. However, that ACTH deficiency is not responsible for the lack of protein-coagulation inhibition by heat in the hypophysectomized animal appears to be attested by the fact that the administration of ACTH was without effect in such animals.

Phenylbutazone has been shown to cause a stabilization of the coagulation of serum proteins by heat (1, 2), an effect which is most likely due to its ability to uncouple phosphorylation (7). The effects of phenylbutazone and paramethasone, administered either singly or in combination, in hypophysectomized and sham-operated rats on the levels of serum turbidity are shown in Fig. 3. Of importance is the observation that phenylbutazone does not cause a significant lowering effect in the hypophysectomized animal in comparison

[1] All statements made in this section regarding differences among the means of the different groups of animals compared are based on statistical analyses for probability values derived from the distribution of Fisher's t. Values of $p < 0.05$ have been considered significant.

TABLE II—PLAN OF EXPERIMENT 3

Group	No. of Rats	Agents Used (*Per Os*)	Dose (3 Hr. Before Sacrifice)
Adx.	10	3% Corn starch	10 ml./kg.
Sham-Adx.	10	3% Corn starch	10 ml./kg.
Adx. + phenylbutazone	10	Phenylbutazone	100 mg./kg.
Sham-Adx. + phenylbutazone	10	Phenylbutazone	100 mg./kg.
Adx.	10	3% Corn starch	10 ml./kg.
Sham-Adx.	10	3% Corn starch	10 ml./kg.
Adx. + paramethasone	10	Paramethasone	1 mg./kg.
Adx. + paramethasone + phenylbutazone	10	Paramethasone; phenylbutazone	1 mg./kg.; 100 mg./kg.
Sham-Adx. + phenylbutazone	10	Phenylbutazone	100 mg./kg.

to the evident lowering noted in the sham-operated group. Significant increases in the absorbance readings are noted in both groups of animals receiving paramethasone alone. This seems to indicate that adrenal insufficiency is not the reason for the effect seen in the hypophysectomized group (Fig. 2). On the other hand when phenylbutazone is administered in combination with paramethasone, the rises previously noted with paramethasone alone are prevented in both groups. The administration of phenylbutazone causes an inhibition of serum-protein coagulation by heat in ACTH-treated sham-operated animals but did not affect the lack of stabilization seen in the hypophysectomized group (Fig. 2).

Figure 4 indicates the effects of phenylbutazone in adrenalectomized rats on the levels of serum turbidity. A decrease in absorbance values occurs in the untreated adrenalectomized rats. Further, the administration of phenylbutazone in the adrenalectomized group results in a significant decrease of the absorbance reading, beyond the lowering observed in the untreated adrenalectomized groups, and is of the same magnitude as seen for the treated sham-operated animals.

The effects of the combination of phenylbutazone and paramethasone in adrenalectomized rats on the levels of serum turbidity are shown in Fig. 5. It will be noted that paramethasone in the adrenalectomized rat results in a significant increase in absorbance readings, an effect previously mentioned for both hypophysectomized and sham-operated groups (Fig. 3). The paramethasone effect can possibly be explained on the basis that such substances prevent the uncoupling of phosphorylation (7). On the other hand, the combination of phenylbutazone and paramethasone administered to the adrenalectomized rat results in the prevention of the rise seen with paramethasone alone. In addition, the lowering normally seen in the adrenalectomized, phenylbutazone-treated animal (Fig. 4) does not occur.

The serum-turbidity levels of the hypophysectomized-adrenalectomized rats (Fig. 6) reveal significant increases in the absorbance readings. Figure 6 also indicates that phenylbutazone in

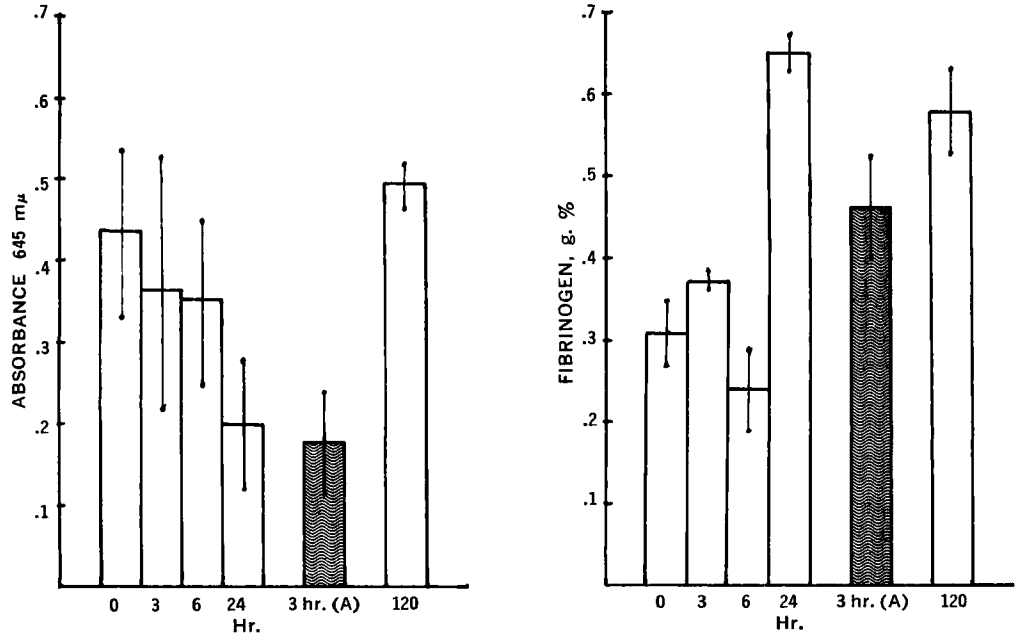

Fig. 1—Effects of cold-stress exposure in rats on the levels of fibrinogen and serum turbidity (absorbance reading, means ± standard errors). Key: A, 3 hr. following 24-hr, cold exposure.

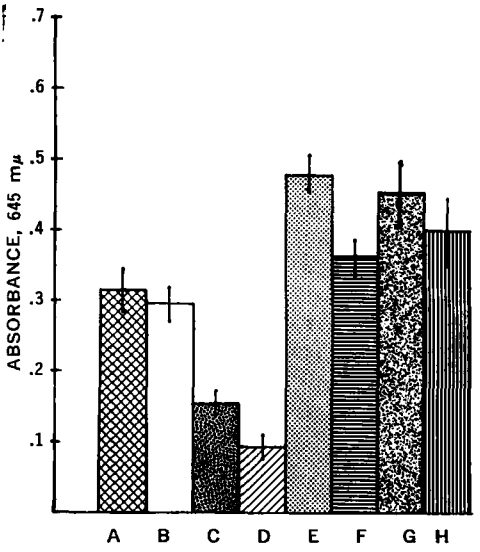

Fig. 2—Effects of phenylbutazone in hypophysectomized and sham-operated rats treated with ACTH (3 units/rat i.m.) on the levels of serum turbidity (absorbance readings, mean ± standard errors). Key: A, sham + ACTH (starch); B, sham control (starch); C, sham + ACTH + phenylbutaz. (100 mg./kg.); D, sham + phenylbutaz. (100 mg./kg.); E, hypox. + ACTH (starch); F, hypox. + ACTH + phenylbutaz. (100 mg./kg.); G, hypox. (starch); H, hypox. + phenylbutaz. (100 mg./kg.).

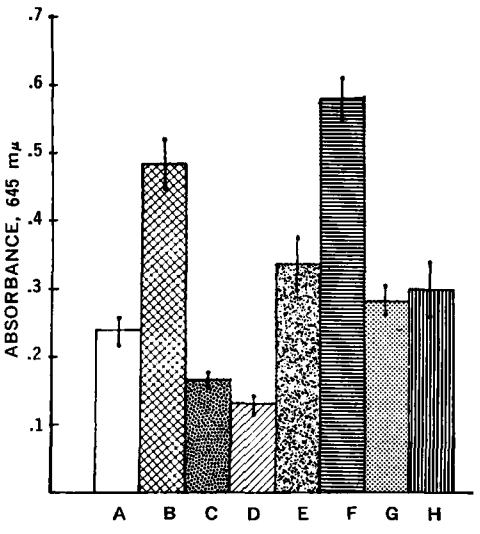

Fig. 3—Effects of phenylbutazone and parametha-sone, administered singly or in combination, in hypophysectomized and sham-operated rats on the levels of serum turbidity (absorbance readings, means ± standard errors). Key: A, sham control (starch); B, sham + parameth. (1 mg./kg.); C, sham + parameth. (1 mg./kg.) + phenylbutaz. (100 mg./kg.); D, sham + phenylbutaz. (100 mg./kg.); E, hypox. (starch); F, hypox. + parameth. (1 mg./kg.); G, hypox. + parameth. (1 mg./kg.) + phenylbutaz. (100 mg./kg.); H, hypox. + phenylbutaz. (100 mg./kg.).

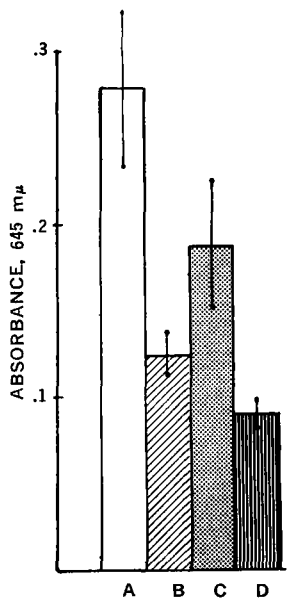

Fig. 4—Effects of phenylbutazone in adrenalectomized rats on the levels of serum turbidity (absorbance readings, means ± standard errors). Key: A, sham control (starch); B, sham + phenylbutaz. (100 mg./kg.); C, adx. (starch); D, adx. + phenylbutaz. (100 mg./kg.).

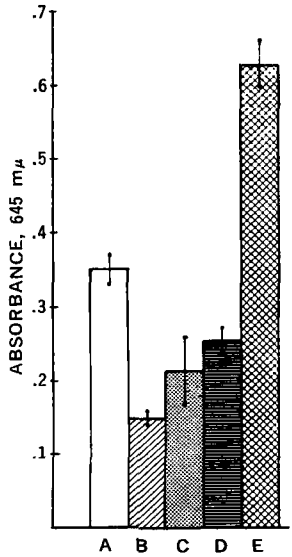

Fig. 5—Effects of phenylbutazone and paramethasone, administered either singly or in combination, in adrenalectomized and sham-operated rats on the levels of serum turbidity (absorbance readings, means ± standard errors). Key: A, sham control (starch); B, sham + phenylbutaz. (100 mg./kg.); C, adx. (starch); D, adx. + parameth. (1 mg./kg.) + phenylbutaz. (100 mg./kg.); E, adx. + parameth. (1 mg./kg.).

such pleuriglandular deficient animals is ineffective in changing the response seen in the phenylbutazone-treated sham-operated group. As previously cited for the hypophysectomized group (Fig. 3), the above results infer that an intact pituitary is

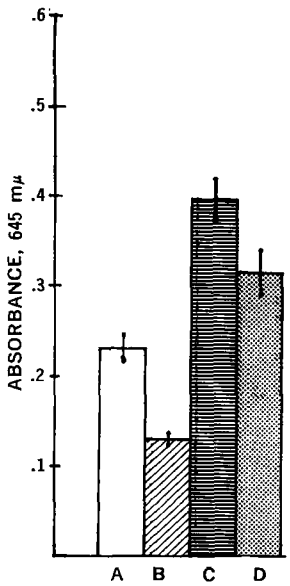

Fig. 6—Effects of phenylbutazone in hypophysectomized-adrenalectomized and sham-operated rats on the levels of serum turbidity (absorbance readings, means ± standard errors). Key: A, sham control (starch); B, sham + phenylbutaz. (100 mg./kg.); C, hypox. + adx. (starch); D, hypox. + adx. + phenylbutaz. (100 mg./kg.).

necessary for the stabilizing action of phenylbutazone.

The results of the present study do not preclude the possibility that endocrines other than the pituitary-adrenal system and/or enzyme systems are involved in the serum-turbidity responses of drugs to heat.

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Keyphrases

Anti-inflammatory activity—screening method
Serum turbidimetric response—heat application
Turbidimetric response, heat—anti-inflammatory agents
Adrenal gland effect—turbidimetric response
Pituitary gland effect—turbidimetric response
Absorbance—analysis, turbidity

Structure-Activity Relationships Among Some Selected Substituted Cyanoacetamides

By HERBERT F. SCHWARTZ, II, ROBERT G. BROWN, EUGENE I. ISAACSON, and JAIME N. DELGADO

Selected *N*-alkyl- and *N,N*-dialkyl-2,2-dialkylcyanoacetamides, including 2-cyanoacyl-, piperidines, pyrrolidines, and morpholines, were synthesized in order to study structure-activity relationships. These were administered to rats and their anticonvulsant activity evaluated by means of the electroshock test. The median effective dose was determined for seven compounds exhibiting protective action, while seven were inactive or displayed convulsant properties. An estimated LD_{50} was determined for the three most effective and least toxic substances. Preliminary pharmacological data have been tabulated and correlations of bioaction with structure are presented.

THE DEMONSTRATION of anticonvulsant activity within a series of 2,2-dialkylcyanoacetamides (1, 2) and in particular, the significant activity of 2-ethyl-2-propylcyanoacetamide and of 2,2-diethylcyanoacetamide against both supramaximal electroshock and subcutaneous pentylenetetrazol led to the consideration of these amides as suitable parent compounds upon which to effect molecular modifications that might serve

Received April 18, 1966, from the Pharmaceutical Chemistry and Pharmacology Laboratories, College of Pharmacy, University of Texas, Austin, TX 78712
Accepted for publication May 15, 1968.

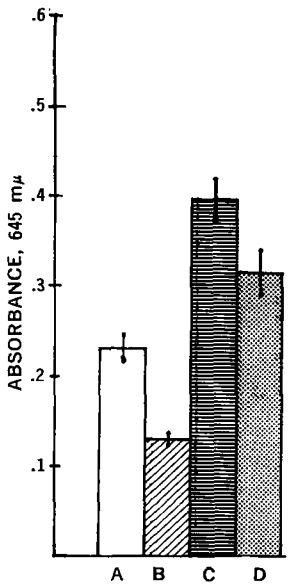

Fig. 6—Effects of phenylbutazone in hypophysectomized-adrenalectomized and sham-operated rats on the levels of serum turbidity (absorbance readings, means ± standard errors). Key: A, sham control (starch); B, sham + phenylbutaz. (100 mg./kg.); C, hypox. + adx. (starch); D, hypox. + adx. + phenylbutaz. (100 mg./kg.).

necessary for the stabilizing action of phenylbutazone.

The results of the present study do not preclude the possibility that endocrines other than the pituitary-adrenal system and/or enzyme systems are involved in the serum-turbidity responses of drugs to heat.

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Keyphrases

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Absorbance—analysis, turbidity

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By HERBERT F. SCHWARTZ, II, ROBERT G. BROWN, EUGENE I. ISAACSON, and JAIME N. DELGADO

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THE DEMONSTRATION of anticonvulsant activity within a series of 2,2-dialkylcyanoacetamides (1, 2) and in particular, the significant activity of 2-ethyl-2-propylcyanoacetamide and of 2,2-diethylcyanoacetamide against both supramaximal electroshock and subcutaneous pentylenetetrazol led to the consideration of these amides as suitable parent compounds upon which to effect molecular modifications that might serve

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as the basis for the study of structure-activity relationships. The compounds investigated are listed in Table I. The synthetic studies which led to the preparation of these compounds have been summarized in a previous report (2).

The present work was undertaken with the hope of delineating structure-activity relationships among these cyanoacetamides. Such structure-activity studies are fundamental to further structural alteration and drug design.

Members of this series include compounds possessing different N-substituents: acyclic or cyclic groups. Such modifications were effected in order to study the influence of N-substitution on anticonvulsant properties. Acyclic *N*-alkyl substituents affect the lipid-water solubility ratio and the electron distribution of the amide function, and these physicochemical factors should influence the bioactivity. The inclusion of heterocyclic compounds provides additional alteration of physicochemical properties. Specifically, the morpholino compound has a greater degree of water solubility than the piperidino analog because of the presence of the ether-oxygen atom. It is useful to speculate that at the molecular level these agents act through a mechanism proposed by Johnson and Eyring (3) to account for the action of certain structurally nonspecific CNS depressants, *e.g.*, urethan and phenobarbital. According to this proposal the depressant reversibly unfolds or conformationally alters a central catalytic protein, probably an enzyme, thus reducing the catalytic ability of the protein, the ultimate manifestation being depression of central activity. A major driving force for the drug–protein interaction and consequent inactivation was demonstrated to be hydrophobic forces. Because hydrogen bonds are important in maintaining native protein structure, Johnson and Eyring theorized that agents that hydrogen bond could also inactivate catalytic proteins through hydrogen bonding (3).

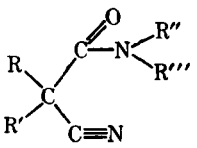
It is interesting to note that many anticonvulsants, *e.g.*, phenobarbital, have a nonpolar hydrocarbon moiety attached to a polar moiety. The latter is often characterized by the presence of an imide or amide function. An imide or amide moiety could conceivably hydrogen bond through the media of the high electron density of the carbonyl oxygen with a partial proton donor present in or on bioactive molecules. The amide or imide function, when the nitrogen bears a hydrogen atom could hydrogen bond through the hydrogen atom. Additionally, when the nitrogen atom does not have an available hydrogen atom there exists the possibility at least in the case of imides, that an electrostatic interaction involving the partial positive charge on the nitrogen atom could take place.

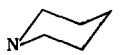
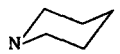
It has been found that *N*-methylated anticonvulsants undergo metabolic demethylation with ease and it has been suggested that the demethylated products are responsible for the action (4–11).

If *N*-dealkylation is important for bioactivity this tends to lead to the conclusion that the presence of the >N—H moiety is important and with respect to the mode of binding of the amide or imide moiety, hydrogen bonding involving the hydrogen of the imide or amide is important.

An objective of this preliminary investigation was to examine the role and possible mode(s) of binding of the polar amide function of a series of anticonvulsant alpha-cyanoacetamides.

Table I—Cyanoacetamides



Compd.[a] No.	R	R′	R″	R‴
1	C_2H_5	C_3H_7	H	H
2	C_2H_5	C_3H_7	H	CH_3
3	C_2H_5	C_3H_7	CH_3	CH_3
4	C_2H_5	C_3H_7	H	C_3H_7
5	C_2H_5	C_3H_7		
6	C_2H_5	C_3H_7		
7	C_3H_7	C_3H_7	H	CH_3
8	C_2H_5	C_2H_5	H	H
9	C_2H_5	C_2H_5	H	CH_3
10	C_2H_5	C_2H_5	CH_3	CH_3
11	C_2H_5	C_2H_5	H	C_3H_7
12	C_2H_5	C_2H_5		
13	C_2H_5	C_2H_5		
14	C_2H_5	C_2H_5		

[a] Compounds 1,8, Schwartz, H. F., and Doerge, R. F., *J. Am. Pharm. Assoc., Sci. Ed.*, **44**, 80(1955); and 9, Conrad, M., and Zart, A., *Ann.*, **340**, 329(1905) have been previously synthesized.

EXPERIMENTAL

Method—Young adult female albino rats, weighing initially 100–120 g.,[1] maintained on commercial laboratory feed[2] and permitted free access to food and water except during actual test periods were administered the drugs. No animal was dosed

[1] Holtzman Co., Madison, Wisc.
[2] Purina laboratory chow.

more frequently than once weekly and use of the animal was discontinued when its weight reached about 250 g. Acute toxicity studies were accomplished on older, heavier animals, thus the LD_{50} values obtained can be considered only approximations.

Pharmacodynamics[3]—Anticonvulsant activity was determined by the supramaximal electroshock seizure test (MES), essentially that described by Toman, Swinyard, and Goodman (12), using ear-clip electrodes and prior application of an acacia-sodium chloride paste. The apparatus used was the Electroshock stimulator model D,[4] adjusted to deliver 150 ma. for 0.2 sec.

Each group of rats was screened for positive response (extensor thrust of the hind limbs) to the electric shock and nonconformers removed from the test group. Checking of the undosed animals was repeated frequently throughout the entire period of evaluation.

The median effective dose (ED_{50}) of each active drug was determined from the dose-response curve obtained by plotting the effect of at least 3 dose levels on log-probability graph paper (13). Groups of at least 10 rats were given various doses until the required points were obtained.

All doses (mg./kg. body weight) were given intraperitoneally, using appropriate concentrations in propylene glycol. Most of the drugs were sufficiently soluble in the solvent to be given in solution, but a few had to be warmed slightly to maintain a homogeneous solution. *N*-Methyl-2,2-dipropylcyanoacetamide and *N*-methyl-2,2-diethylcyanoacetamide were injected as suspensions in propylene glycol only when the concentration of the solution being used was 100 mg./ml. and 200 mg./ml., respectively. Since the volume of solvent was limited to not more than 1.5 ml./kg. of total body weight (14), there exists a wide margin of safety over the toxic dose of propylene glycol as reported by Spiegel and Noseworthy (15).

Initial observations of the action of every compound in intact animals indicated a rather long period of action. These observations were confirmed in a later experiment utilizing ED_{99} doses in animals and checking for effect at 30-min. intervals up to 4 hr. after administration. The time-course studies indicated an optimal effect at the 3-hr. interval. Varying degrees of rapidly developing gross sedation were noted early in the time course of the drug effects. Since it was not desirable to shock the animals more than once, a single 3-hr. time interval was selected between administration and the test for anticonvulsant activity. This is meaningful in terms of a single finite time interval in the intact animal reflecting all processes of distribution and approach to the site of action.

RESULTS

The experimental results are summarized in Tables II and III. Several drugs were active and protected against electroshock. Some were convulsive, whereas others were sedative in action. Only those drugs active against electroshock were quantified. Although convulsive tendencies and/or lack of activity prevented the quantification of others in the series, qualitative observations of drug action were made and are recorded in the tables.

The compounds listed in Table II possess protective action against electroshock. One of the most effective drugs was 2-ethyl-2-propylcyanoacetamide. No toxic symptoms were observed at protective dose levels. *N*-Methyl-2,2-dipropylcyanoacetamide was as active as 2-ethyl-2-propylcyanoacetamide but displayed toxic symptoms at a relatively low dose. 2,2-Diethylcyanoacetamide showed no toxic symptoms in doses required to provide protection. Therapeutic indexes were calculated only for the best compounds (highest activity, least toxic effect).

Dose-effect curves are presented in Figs. 1 and 2 for the active anticonvulsant compounds. Figure 3 shows the comparison of dose-effect curves for anticonvulsant activity and lethality for 2-ethyl-2-propylcyanoacetamide. The Therapeutic Index (TI) was 8.1 and the certain safety factor (LD_1/ED_{99}) 1.7; the latter figure detracting from the favorable TI.

Table III lists those drugs which were inactive or whose activity was considered not useful due to predominantly undesirable actions. Pertinent remarks concerning qualitative observations are also included.

Structure-Activity Relationships—The preliminary pharmacologic studies have provided sufficient information to demonstrate trends in biological activity.

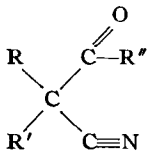
Although the effect of variation of alkyl groups at C2 was not a primary objective of the investigation, experimental results indicate the activity against electroshock to decrease: dipropyl > ethyl, propyl > diethyl for this series of mono-*N*-methyl compounds. The order ethyl, propyl > diethyl is seen also in the unsubstituted amides and in the di-*N*-methyl compounds. This decrease in activity appears to be in accord with current interpretations of distribution phenomena (16) by providing a decreasing lipid solubility in the order given. Among the compounds described, increasing hydrocarbon substitution at C_2 apparently provides a more favorable ratio of lipid-water solubility characteristics, possibly resulting in more effective distribution of drug to the site of action. A comparison of dose levels required to produce a similar degree of sedative effect among compounds differing only in their C2 substituents gave an order of decreasing sedative activity identical to that for decreasing activity against electroshock. The effect of *C*-alkylation on convulsive action could not be evaluated in this series.

N-Alkyl Modifications—(Tables II and III and Fig. 4)—With the exception of compound 8 *versus* 10, which will probably fit the generalization if the confidence limits are lowered to 90%, there is a significant decrease in potency by *N*-monomethylation. There is no further significant decrease in potency with *N*-dimethylation. *N*-Monopropylation and cyclization decreased the potency sufficiently so as to prevent quantification (Table III). The production of convulsions and lack of protection in the cyclic series suggested a complete investigation of dose-response effects would be pointless.

In those compounds having N-atom substitution, simultaneous sedative and convulsive tendencies

[3] The authors acknowledge the technical assistance of Mr. Richard G. Hendrick.
[4] M. L. Wahlquist, Salt Lake City, Utah.

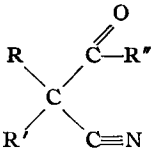
TABLE II—PHARMACOLOGICAL DATA, ACTIVE SUBSTITUTED CYANOACETAMIDES



Designation	R	R′	R″	Dose, mg./kg.	Electroshock, Protection, %	Remarks[a]	ED_{50}, mg./kg.	LD_{50} (Estim.),[b] mg./kg.	TI (Estim.)[c]
7	Propyl	Propyl	NH-Methyl	50	10(1/10)		70(60–82)	450	6.4
				75	60(6/10)				
				85	70(7/10)	[d,e]			
1	Ethyl	Propyl	NH_2	50	25(5/20)		71(60–84)	575	8.1
				75	50(10/20)				
				100	79(15/19)				
2	Ethyl	Propyl	NH-Methyl	75	17(3/18)		98(88–109)		
				100	65(11/17)	[f] (brief)			
				125	61(11/18)	[d,g]			
				150	100(10/10)[h]	[e,d] (brief),[g]			
3	Ethyl	Propyl	*N*-Dimethyl	75	18(2/11)		102(86–120)		
				100	50(5/10)	[f,e]			
				150	82(9/11)	[d,e,i,g]			
				200	100(10/10)[h]	[d,e,i,g]			
8	Ethyl	Ethyl	NH_2	100	0(0/10)[h]		170(152–197)	1000	5.9
				150	40(8/20)				
				190	70(7/10)				
				225	80(8/10)				
				300	90(9/10)				
9	Ethyl	Ethyl	NH-Methyl	190	33(5/15)	[f] (brief)	218(200–238)		
				225	44(7/16)	[f] (brief)			
				250	87(13/15)	[d] (righting ref. greater than 3 hr.)			
10	Ethyl	Ethyl	*N*-Dimethyl	150	6(1/16)	[d,g] (brief)	225(192–263)		
				200	38(6/16)	[d,g]			
				250	60(9/15)	[d,g,e] (brief, one death)			

[a] Qualitative evaluation. [b] Death within 24 hr. [c] LD_{50}/ED_{50} therapeutic index. [d] Depression, loss of righting reflex within 2–5 min. [e] General tenseness or slight muscular tremor, facial muscle twitching, provoked into spasm or convulsion by noise or touch. [f] Mild sedation, no loss of righting reflex, ataxia. [g] Recovery of righting reflex less than 3 hr. [h] Zero and 100% effect corrected according to the method of Litchfield and Wilcoxon (13). [i] Convulsive.

TABLE III—PHARMACOLOGICAL DATA, INACTIVE SUBSTITUTED CYANOACETAMIDES



No.	R	R′	R″	Dose, mg./kg.	Electroshock, % Protection	Remarks[a]
4	Ethyl	Propyl	NH-propyl	25	10(1/10)	
				50	5(1/20)	
				75	14(2/15)	
				100	17(1/6)	
				125	0(0/3)	[b,c]
				150	0(0/1)	[b,c] (72 hr.)
				200	75(3/4)	[b] (3 deaths in 24 hr.)
11	Ethyl	Ethyl	NH-propyl	100	10(1/10)	[b,c]
6	Ethyl	Propyl	Piperidine	100	0(0/9)	[b] (brief)
13	Ethyl	Ethyl	Piperidine	75	15(3/20)	
				150	15(3/20)	
				175	40(2/5)	[b]
				200	0(0/15)	[b]
5	Ethyl	Propyl	Pyrrolidine	200	0(0/10)	[b]
12	Ethyl	Ethyl	Pyrrolidine	200	10(1/10)	[b,d]
14	Ethyl	Ethyl	Morpholine	100	0(0/7)	[c]
				200	0(0/3)	[b] (mild)[c]

[a] Qualitative evaluation. [b] Convulsive. [c] General tenseness or slight muscular tremor, facial muscle twitching, provoked into a spasm or convulsion by noise or touch. [d] Mild sedation, no loss of righting reflex, ataxia.

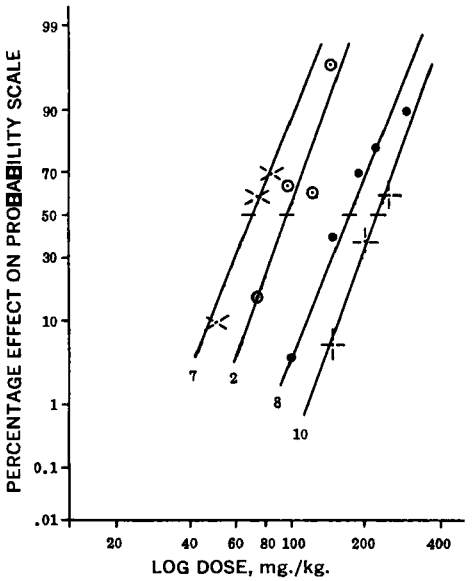


Fig. 1—Dose-response curve of active substituted cyanoacetamides. Key: 7, dipropyl-N-methylcyanoacetamide; 2, ethylpropyl-N-methylcyanoacetamide; 8, diethylcyanoacetamide; 10, diethyl-N-dimethylcyanoacetamide.

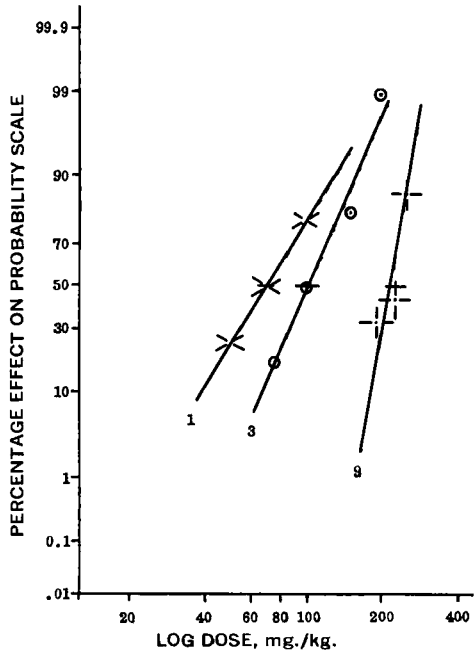


Fig. 2—Dose-response curve of active substituted cyanoacetamides. Key: 1, ethylpropylcyanoacetamide; 3, ethylpropyl-N-dimethylcyanoacetamide; 9, diethyl-N-methylcyanoacetamide.

were present. Both effects were more obvious at higher doses. Although it was not possible to evaluate contributions of *N*-alkylation to sedative effect, it was noted that convulsive tendencies increased with an increase in number of substituents or with an increase in the size or cyclization of

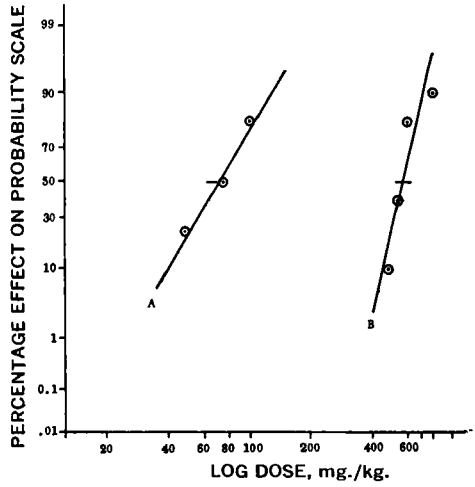


Fig. 3—Comparison of dose effect curves for anticonvulsant activity (A) and lethality (B) compound 1 (ethylpropylcyanoacetamide).

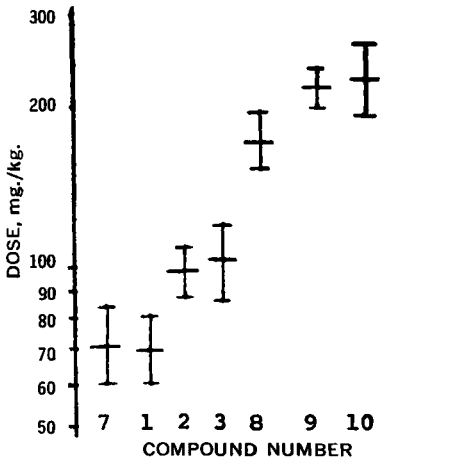


Fig. 4—Comparison of 95% confidence limits for the individual active drugs.

these substituents. The heterocyclic compounds and the *N*-propyl congeners were convulsive by the qualitative evaluation as indicated in Table III.

In this series of amides, mono-*N*-alkylation decreases activity significantly, this may be rationalized in the following ways: (*a*) the amide function is now sterically protected making any type of interaction, including hydrogen bonding *via* the amide N-H moeity, with a bioactive macromolecule less likely, (*b*) the hydrogen atom is now less acidic and consequently will not hydrogen bond as readily, (*c*) a combination of *a* and *b*.

The effect of di-*N*-alkylation now may be considered: there is no significant loss of activity relative to mono-*N*-alkylation. This appears to dispute rationalization *b* above, for this rationalization demands the presence of a hydrogen atom attached to the nitrogen atom. Rationalization *a* is also apparently disputed, for if one alkyl substituent sterically protects the amide function, then two

substituents should protect better and more effectively preclude interaction with a bioactive macromolecule. Rationalization *c*, because it is a combination of *a* and *b*, also is apparently disputed.

The fact that the *N,N*-dialkylamides are as potent as the *N*-mono-alkylamides could be rationalized as follows: (*a*) better distribution to the receptor because of the increased hydrocarbon content, (*b*) a substantial amount of *N*-dealkylation, (*c*) increased hydrophobic interactions. Upon examination however, rationalizations *a* and *c* must be discarded, for on the basis of these, *N*-monoalkylation should not have decreased activity. It thus appears that *N*-dealkylation may be a factor. If this is the case the three rationalizations previously set forth with respect to the effect upon activity of mono-*N*-alkylation may be valid. These rationalizations are consonant with a sterically accessible amide function interacting with the bioactive macromolecule. The interaction possibly involves hydrogen bonding through the media of an amide hydrogen atom.

CONCLUSIONS

The following compounds: 2-ethyl-2-propylcyanoacetamide[5]; *N*-methyl-2,2-dipropylcyanoacetamide; *N*-methyl-2-ethyl-2-propylcyanoacetamide; *N*-dimethyl-2-ethyl-2-propylcyanoacetamide; 2,2-diethylcyanoacetamide[5]; *N*-methyl-2,2-diethylcyanoacetamide[5]; and *N*-dimethyl-2,2-diethylcyanoacetamide have been found active when subjected to testing by an electroshock method using rats. The median effective dose (ED_{50}, mg./kg.) of each active drug was determined and reported. The least toxic and most active compounds were ($ED_{50}LD_{50}$,[6] TI, respectively): *N*-methyl-2,2-dipropylcyanoacetamide, 70, 450, 6.4; 2-ethyl-2-propylcyanoacetamide, 71, 575, 8.1; 2,2-diethylcyanoacetamide, 170, 1,000, 5.9.

[5] See Footnote *a*, Table I.
[6] Approximate.

Convulsive tendencies and/or lack of activity prevented quantification of the seven drugs: *N*-propyl-2-ethyl-2-propylcyanoacetamide; *N*-propyl-2,2-diethylcyanoacetamide; 1-(2-cyano-2-ethylbutyryl)-piperidine; 1-(2-cyano-2-ethylvaleryl)-piperidine; 1-(2-cyano-2-ethylbutyryl)-pyrrolidine; 1-(2-cyanoethylvaleryl)-pyrrolidine; and 4-(2-cyano-2-ethylbutyryl)-morpholine. Qualitative observations were made and recorded.

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ED_{50} values—cyanoacetamides

Development of a Sustained-Release Aspirin Tablet

By EDWARD H. WISEMAN and N. J. FEDERICI*

Several sustained-release aspirin tablets, some of which are capable of attaining and maintaining plasma salicylate concentrations of about 30 mcg./ml. for 8 hr. after ingestion from a total dose of 975 mg. aspirin are described. The design of the final tablet characteristics was guided by analysis of plasma salicylate concentrations *in vivo*, and the rate of aspirin release *in vitro*.

A SUSTAINED-RELEASE product has been defined as "one in which a drug is initially made available to the body in an amount sufficient to cause the desired pharmacological response as rapidly as is consistent with the properties of the drug determining its intrinsic availability for absorption; and one which provides for maintenance

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Accepted for publication July 10, 1968.
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of activity at the initial level for a desirable number of hours in excess of the activity resulting from the usual dose of drug" (1). The advantages of such a dosage form are chiefly the maintenance of therapeutic drug concentrations without incursions into higher concentrations which may lead to undesirable effects, plus the convenience of less frequent dosage. A further possible advantage is the prolonging of drug action throughout sleeping hours without the necessity to ingest medication.

A convenient way to achieve rapidly and then maintain a desired plasma drug concentration is to combine the active ingredient in a multilayer tablet consisting of a sustained-release core with an outer, instantly available coating. This technique was familiar to the authors in connection with products containing triethanolamine trinitrate biphosphate (2), and it was desired to extend the process to sustaining the action of other therapeutic agents, for example aspirin.

In the design of a prolonged action form of a therapeutic agent, it is important that the aims and limitations of the desired formulation be defined. For an aspirin product, the plasma salicylate level required for analgesia in mild acute pain should be maintained for the maximum possible duration, within the limitation of the amount of aspirin which can be conveniently incorporated into a tablet.

A recent careful study was conducted on the relief of post-partum pain with 650 mg. of aspirin (3). In that investigation, pain relief significantly different from placebo control did not appear earlier than 45 min. after drug administration, and did not increase in intensity after 60 min. as determined either by the number of patients obtaining relief or the magnitude of relief of the individual patient. Four hours after aspirin ingestion, pain relief was no longer significantly different from placebo control. Two earlier studies (4, 5), although less rigorously controlled, reached similar conclusions and suggest analogous pain-relief patterns in a variety of clinical states, including headache.

Following the ingestion of 650 mg. of aspirin, by normal men, average plasma salicylate concentrations reached about 30 mcg./ml. at 45 min., with a maximum of about 40 mcg./ml. at 2 hr., and declined to about 30 mcg./ml. at 4 hr. For the purpose of this study, therefore, these results, viewed in conjunction with the clinical studies, were taken to indicate that pain relief occurred during the time when plasma salicylate levels were above 30 mcg./ml.

The desired characteristics and limitations of the sustained-action aspirin tablet were therefore projected as follows. Plasma levels of 30 mcg./ml. should be attained within 1 hr. after dosage and be maintained for 8 hr. Physical limitations on the tablet size dictated that total aspirin content should be not more than 487.5 mg./tablet. The studies undertaken to design a sustained-release formulation with these characteristics, and to investigate the factors which determine these characteristics, are detailed in this report.

METHODS[1]

Preparation of Tablets—All tablets were double layer in composition, with aspirin incorporated into a restraining matrix in one layer, and aspirin and a cornstarch binder in the second layer. The restraining matrix consisted of a vegetable wax, insoluble extenders, a water-soluble binder, and a fatty-acid salt lubricant. The release rate was governed by the ratio of the sustaining base to active ingredient, as well as tablet hardness. Two restraining matrices were investigated.

Matrix A—Calcium phosphate (tribasic, NF; 175 parts), talcum (USP; 25 parts), and carnauba wax (passing No. 30 mesh sieve; 125 parts) were mixed thoroughly, moistened with glucose (USP; 25 parts) in water, and the granulation dried at 60° for 2 hr. The dried granulation was powdered, and finally screened through a No. 30 sieve.

Matrix B—Lactose (USP; 125 parts), sucrose (USP; 125 parts), carnauba wax (passing No. 30 mesh sieve; 75 parts), and polyvinylpyrrolidone (12.5% solution; 4.5 parts) were mixed thoroughly. This granulation was then dried, powdered, and screened as for Matrix A.

For preparation of the restraining matrix granulation, appropriate amounts of restraining matrix (A or B) and acetylsalicylic acid (USP) were mixed with small amounts of magnesium stearate (USP) and cornstarch (USP; dried) and the mixture slugged into 1.9-cm. slugs. The slugs were reduced progressively to pass finally through a No. 20 sieve. This granulation, thoroughly mixed with cornstarch (2%), was used for the sustained layer of the final tablet. The instant release layer consisted of acetylsalicylic acid (USP) and cornstarch (10%). The tablets were made in a Stokes BB2 rotary tableting machine, using a capsule-shaped punch [(1.9 cm. × 0.63 cm.) (0.75 in. × 0.25 in.)], thickness 0.71 cm. (0.28 in.), to the appropriate hardness (8–20 kg., Pfizer tester).

In Vitro Studies—The tablet under investigation was placed in one chamber of the basket of a USP disintegration apparatus, and the basket immersed in 0.1 *N* hydrochloric acid (500 ml.) maintained at 37°. Samples (1 ml.) of the hydrochloric acid were removed at varying times, filtered, and the absorbance at 295 mμ, determined in a Beckman DU spectrophotometer. Acetylsalicylic acid concentrations were then determined by reference to a standard curve of absorbance *versus* concentration of known solutions.

In Vivo Studies[2]—Healthy men who had fasted for 12–14 hr. ingested the tablets under investiga-

[1] The authors wish to thank Miss J. Chiaini, Mr. A. Capozza, and Mr. R. Lebel for expert technical assistance.

[2] Carried out in collaboration with Dr. D. G. Iezzoni.

TABLE I—PLASMA SALICYLATE CONCENTRATIONS (mcg./ml.) IN NORMAL HUMAN MALE SUBJECTS AFTER ORAL ADMINISTRATION OF PROPRIETARY ASPIRIN (650 mg.)

Time After Administration, hr.	C.L.	M.O.	W.O.	C.K.	T.H.	Av.
0	0	0	0	0	0	0
0.5	24	27	13	21	5	18
1	30	36	36	45	20	33
2	35	37	40	41	38	39
3	34	40	37	43	37	37
4	—	—	29	35	31	32
5	20	25	24	27	26	25
6	16	24	18	22	21	20
7	—	22	14	20	—	18
7.75	11	20	12	14	14	14

TABLE II—COMPOSITION AND HARDNESS OF EXPERIMENTAL ASPIRIN TABLETS

Formulation No.	Instant Layer: Aspirin, mg. + 10% Cornstarch	Sustained Layer: Matrix Type	Sustained Layer: Carnauba Wax, mg.	Sustained Layer: Aspirin, mg.	Av. Hardness, kg.
I	162	A	90	324	10.5
II	162	A	140	324	10.5
III	162	A	140	324	11.5
IV	162	A	125	324	11.5
V	162	A	110	324	11.5
VI	162	B	130	324	12.5
VII	162	B	75	324	12.5
VIII	162	B	75	324	11.5

tion (total aspirin, 975 mg.) followed by 200 ml. of water. Blood samples were drawn by venipuncture and transferred immediately to heparinized tubes. Plasma samples were separated by centrifugation and assayed for total salicylate by the method of Brodie *et al.* (6).

RESULTS

The plasma salicylate levels attained in normal man after the ingestion of 650 mg. of a commercial aspirin preparation (Bayer) are shown in Table I. Experimental two-layer tablets were formulated as described in *Methods*. Either the ratio of aspirin: restraining matrix was varied, or, at constant composition, the hardness to which the tablets were pressed was changed. All experimental tablets comprised 162.5 mg. of aspirin in the "instant" layer and 325 mg. aspirin in the "restrained" layer (Table II).

In Vitro Release Studies—The *in vitro* aspirin release from the experimental tablets is shown in

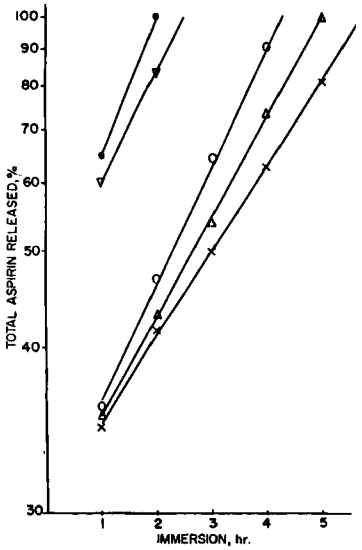

Fig. 1—Rate of aspirin release in vitro *from tablets comprising restraining Matrix A. Each point represents determination on 8–15 tablets. Key: ●, I; ▽, II; ○, V; △, IV; ×, III.*

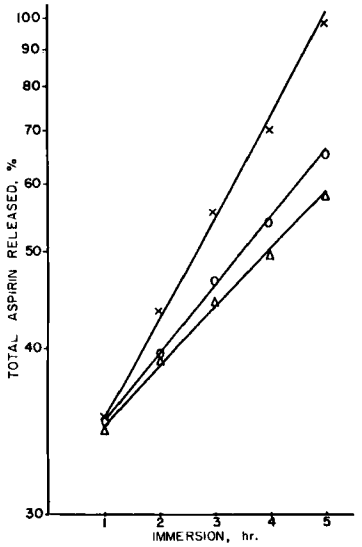

Fig. 2—Rate of aspirin release in vitro *from tablets comprising restraining Matrix B. Each point represents determinations on 8–15 tablets. Key: ×, VIII; ○, VII; △, VI.*

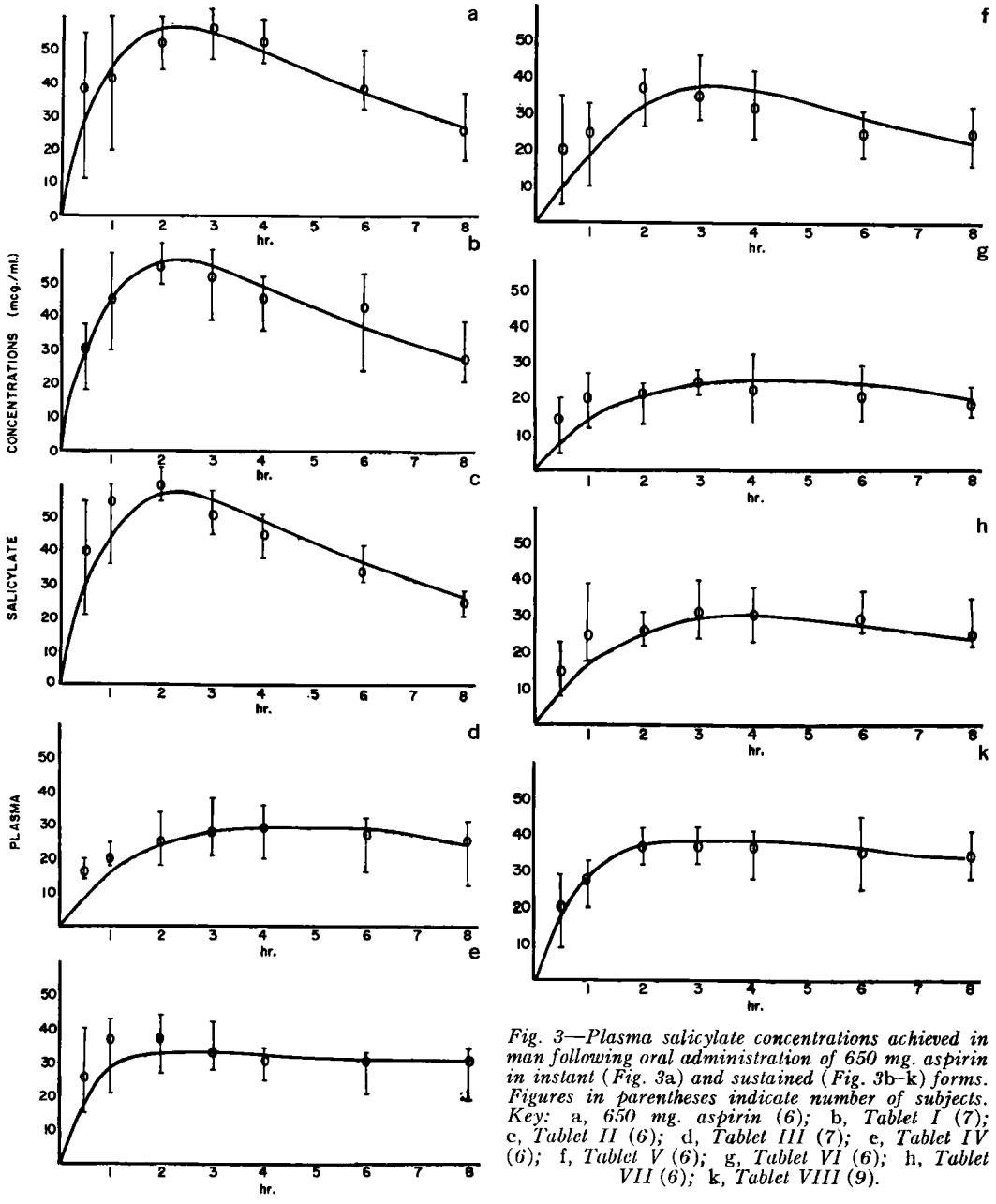

Fig. 3—Plasma salicylate concentrations achieved in man following oral administration of 650 mg. aspirin in instant (Fig. 3a) and sustained (Fig. 3b–k) forms. Figures in parentheses indicate number of subjects. Key: a, 650 mg. aspirin (6); b, Tablet I (7); c, Tablet II (6); d, Tablet III (7); e, Tablet IV (6); f, Tablet V (6); g, Tablet VI (6); h, Tablet VII (6); k, Tablet VIII (9).

Figs. 1 (Matrix A) and 2 (Matrix B). In both series, when tablet hardness was held constant, increasing the ratio of restraining matrix to aspirin content led to a decrease in rate of release of aspirin from the tablet. If the composition of the tablet was held constant, an increase in tablet hardness caused a similar, but less pronounced, decrease in rate of aspirin release.

In Vivo Studies—The plasma salicylate concentrations produced by the experimental tablets are shown in Fig. 3 (*b–k*). Tablets I and II gave no evidence of sustained action ***in vivo***. The time course of plasma salicylate concentrations was indistinguishable from that obtained by oral administration of 975 mg. of nonsustained aspirin (Fig. 3*a*). Tablets III–VIII all exhibited varying degrees of sustained action.

DISCUSSION

For many drugs, it has been shown that the therapeutic effect is proportional to the concentration of drug in the blood (7). The determination of the effective dose of aspirin however, is complicated by the subjective nature of the assay of "pain relief." Nevertheless, the work of investigators skilled in

this type of evaluation parallel in a striking manner the objective plasma salicylate determinations made in this study. Since aspirin has a relatively short plasma half-life (about 4 hr.), "analgesic" plasma salicylate concentrations cannot be maintained much beyond 4 hr. following a single 650-mg. dose. Extended analgesic plasma concentrations may be obtained by giving a second dose 4 hr. after the first, or by giving a larger dose initially. A sustained release form of aspirin would eliminate the frequent dosage required by the first alternative, and eliminate the unnecessarily high plasma drug concentrations in the early hours of the second alternative.

With both of the experimental restraining matrices studies, rate of aspirin release was found to be controlled by the hardness to which the tablet was compressed and the ratio of restraining matrix to aspirin. For example, Tablet II (hardness 10.6 kg.) exhibited no sustained action *in vivo*. Tablet III, of identical composition, but of hardness 11.5 kg., exhibited good sustained properties, but did not exactly conform to the desired specifications. If tablet hardness was kept constant, a reduction in the amount of restraining matrix (Tablets IV and V) led to an increase in both *in vitro* and *in vivo* release rates. Tablet IV had the characteristics desired in a sustained-release preparation, in that it produced plasma salicylate concentrations of about 30 mcg./ml. within 1 hr. of ingestion, and maintained these concentrations for at least 8 hr.

Similar effects were noted with tablets derived from sustaining Matrix B. Tablet VIII performed *in vivo* essentially the same as Tablet IV, while Tablets VI and VII, with higher matrix–aspirin ratios, both displayed slower rates of aspirin release.

The alliance of *in vitro* and *in vivo* techniques proved to be a powerful combination in this investigation. Although *in vitro* studies were unable to provide absolute values for *in vivo* performance, the qualitative picture was maintained. Those tablets which released their aspirin swiftly *in vitro* (I and II) proved, *in vivo*, to be indistinguishable from nonsustained aspirin. As the rate of aspirin release *in vitro* was decreased, either by increasing the amount of restraining matrix or by increasing tablet hardness, the tablets exhibited sustained release over longer periods *in vivo*. Most interestingly, Tablets IV and VIII, although of different restraining matrices, had essentially identical rates of aspirin release *in vitro*, and the time course of plasma salicylate concentrations from these tablets was virtually identical. The entirely artificial *in vitro* rate of release of aspirin from the tablet into dilute hydrochloric acid thus proved to be a reliable guide to tablet performance *in vivo*. *In vitro* release rates were therefore used in the selection of tablets for *in vivo* studies, and finally in the quality control of the production in quantity of Tablets IV and VIII.

Tablets IV and VIII represented the successful attainment of the goal of this study. Both of these tablets, in a single dose of 975 mg., rapidly achieved plasma salicylate concentrations of about 30 mcg./ml., and maintained those concentrations for a period of 8 hr. Either formulation eliminated the need for frequent aspirin ingestion, or of plasma salicylate concentrations above those required for therapeutic effects. The individual plasma salicylate concentrations in subjects receiving Tablet VIII [the tablet in which aspirin showed excellent stability (8)] are shown in Fig. 4. Chronic administration of Tablet VIII (2 tablets = 975 mg. aspirin, every 8 hr. for 56 hr.) resulted in relatively constant plasma salicylate concentrations at all times during treatment. These findings will be reported in detail in a subsequent paper (8).

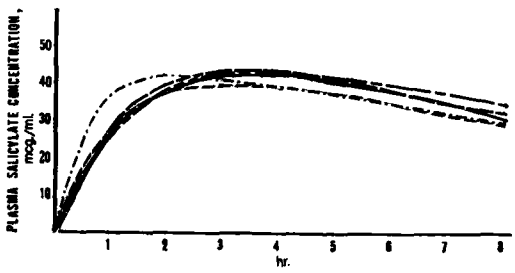

Fig. 4—Individual plasma salicylate concentrations achieved following oral administration of 650 mg. aspirin as sustained Tablet VIII.

SUMMARY

The development of several sustained-release aspirin tablets, which rapidly achieve and maintain constant plasma salicylate concentrations over 8 hr., is described. The tablets are two-layered, consisting of 162.5 mg. aspirin in a rapid-release layer, together with 325 mg. aspirin in a sustained-release layer. Carnauba wax, in combination with soluble extenders, was used as the restraining agent. It was found that the *in vitro* rates of aspirin release into acid media showed excellent correlation with the observed *in vivo* sustained characteristics. One of the experimental sustained-release aspirin tablet gave constant plasma salicylate concentrations on chronic administration.

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Keyphrases

Aspirin—sustained-release tablets
Sustained-release tablets—double layer
Plasma concentration—aspirin, sustained release
Dissolution—aspirin, sustained release
UV spectrophotometry—analysis

Effect of Various Liquids on the Diffusion of Salicylic Acid from Ointment Bases

By CLYDE W. WHITWORTH

Water, alcohol, and dimethyl sulfoxide were each incorporated at 1, 2, and 5 percent concentrations into four ointment bases containing salicylic acid. The bases tested were white petrolatum, a hydrogenated cottonseed oil base, a water-in-oil emulsion base and an oil-in-water base. The diffusion of salicylic acid from five samples of each mixture was determined at 20-min. intervals for a period of 2 hr. All three liquids enhanced diffusion from the white petrolatum base and the water-in-oil base but tended to inhibit diffusion from the cottonseed oil base and the oil-in-water formula. In some instances the concentration of liquid appears to have an effect. Statistical analyses of data point out significance of differences.

THE TRANSFER ability of the skin is probably very strongly influenced by the presence of moisture as suggested by Higuchi (1). He showed that near 100% relative humidity the permeability of glyceryl monostearate was dependent on water activity. Wurster and Kramer (2) showed an increase in absorption of three salicylate esters accompanied increased moisture conditioning. Shelmire (3) has stated that hydration of the stratum corneum appears to increase the rate of passage of all substances which penetrate the skin.

Since moisture greatly influences percutaneous absorption, it was decided that a study of drug release from different ointment bases as influenced by various liquids might yield useful information. It is recognized that *in vitro* drug release measurements may be difficult to correlate with *in vivo* results; however, such studies may be useful in discovering and observing interactions between drugs and vehicles which in turn influence drug absorption. Diffusion techniques have been extensively employed to measure drug release especially from heterogeneous preparations such as ointments (4) and emulsions (5).

EXPERIMENTAL

Three liquids, water, alcohol, and dimethyl sulfoxide, were incorporated at 1, 2, and 5% concentrations into four different ointment bases. The bases consisted of:

(*a*)	White petrolatum USP (Standard Oil Co.)	
(*b*)	Water-in-oil base:	
	White petrolatum USP	64%
	Sorbitan monooleate[1]	6%
	Distilled water	30%
(*c*)	Oil-in-water base:	
	Stearyl alcohol[2]	25%
	White petrolatum USP	25%
	Glycerin[2]	12%
	Polysorbate 80[3]	5%
	Distilled water	33%
(*d*)	Partially hydrogenated cottonseed oil[4]	90%
	Completely hydrogenated cottonseed oil[5]	10%

The bases were prepared by fusion at low temperatures. The salicylic acid was added to the warm base and the mixture stirred until it cooled in order to achieve homogeneity. The liquids were mixed with the ointments just prior to use in order to minimize loss by evaporation.

Five samples of each ointment were packed into hollow plastic stoppers which had a top (open end) diameter of 40 mm., a bottom diameter of 30 mm., and a depth of 25 mm. The open end was then covered by a membrane (supplied by Young Drug Co.) and sealed by a rubber band so that the entire exposed surface of the ointment was brought into contact with the membrane. The containers were then inverted in 60 ml. of distilled water contained in 4-oz. ointment jars and the tops put on the jars to prevent evaporation of the diffusion media. The jars were then placed in a constant-temperature shaker-type water bath at 37.5°. At 20-min. intervals for 2 hr. the diffusion medium of each sample was assayed for salicylic acid content by reading absorbance on a Beckman DU spectrophotometer at 297 mμ. The assay samples were returned to the diffusion media each time in order to maintain volume.

Controls consisted of the base and the salicylic acid without additional liquids. All bases contained 2% by weight of salicylic acid except the oil-in-water base which contained 1% due to the rapid rate of diffusion from this vehicle.

Figures 1–12 are plots of the concentration of salicylic acid in the diffusion media against time. Each point represents the average of five samples.

Received April 15, 1968, from the School of Pharmacy, University of Georgia, Athens, GA 30601
Accepted for publication May 31, 1968.
Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

[1] Trademarked as Arlacel 80, Atlas Chemical Industries, Inc., Wilmington, Del.

[2] Fisher Scientific Co.

[3] Trademarked as Tween 80, Atlas Chemical Industries, Inc., Wilmington, Del.

[4] Trademarked as Cotmar, Procter and Gamble Co., Cincinnati, Ohio.

[5] Trademarked as Cotoflakes, Procter and Gamble Co., Cincinnati, Ohio.

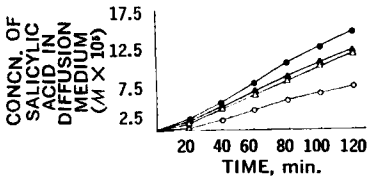

Fig. 1—Diffusion of 2% salicylic acid from white petrolatum (○), and from white petrolatum containing: 1% water (●), 2% water (△), and 5% water (▲).

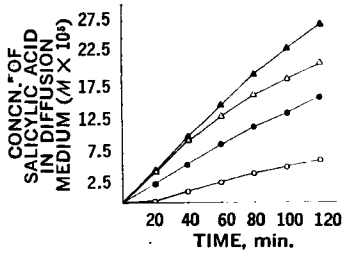

Fig. 2—Diffusion of 2% salicylic acid from white petrolatum (○), and from white petrolatum containing 1% ethanol (●), 2% ethanol (△), and 5% ethanol (▲).

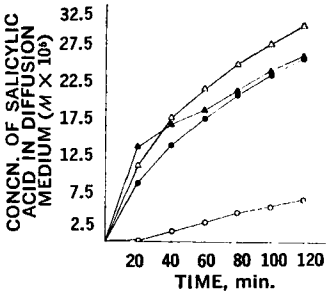

Fig. 3—Diffusion of 2% salicylic acid from white petrolatum (○), and from white petrolatum containing: 1% DMSO (●), 2% DMSO (△), and 5% DMSO (▲).

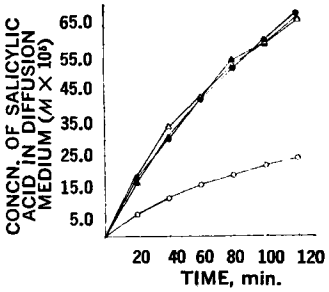

Fig. 4—Diffusion of 2% salicylic acid from a w/o base (○), and from a w/o base containing: 1% water (●), 2% water (△), and 5% water (▲).

RESULTS AND DISCUSSION

White Petrolatum Base—The diffusion of salicylic acid from white petrolatum base proceeded more slowly than from any of the other bases. All concentrations of all three liquids significantly increased the rate of diffusion of the drug, however, as seen in Figs. 1–3. Of the three liquids used water is

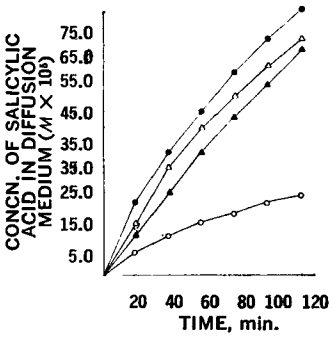

Fig. 5—Diffusion of 2% salicylic acid from a w/o base (○), and from a w/o base containing: 1% ethanol (●), 2% ethanol (△), and 5% ethanol (▲).

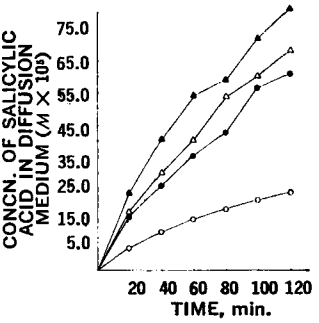

Fig. 6—Diffusion of 2% salicylic acid from a w/o base (○), and from a w/o base containing: 1% DMSO (●), 2% DMSO (△), and 5% DMSO (▲).

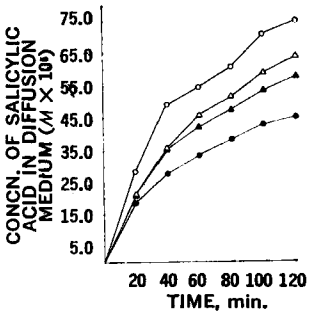

Fig. 7—Diffusion of 1% salicylic acid from an o/w base (○), and from an o/w base containing: 1% water (●), 2% water (△), and 5% water (▲).

seen in Fig. 1 to have the least effect, dimethyl sulfoxide (DMSO) the greatest effect, the results with alcohol falling in between. While it is evident that all three liquids increase diffusion, the influence of the concentration of each liquid on diffusion does not follow a similar pattern for all liquids. In the case of water a significant difference exists only between the results with 1 and 2% water. Other differences may be taken as due to chance. In Fig. 2 each increase in concentration of alcohol shows an increase in rate of diffusion. All differences are significant. As seen in Fig. 3, DMSO has a great effect on the diffusion process with the 2% concentration showing the greatest influence. All differences at 2 hr. are significant except between

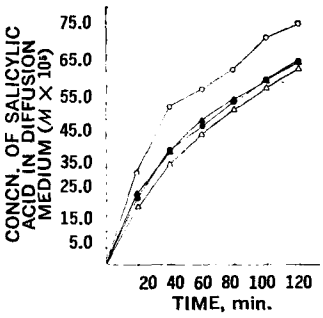

Fig. 8—Diffusion of 1% salicylic acid from an o/w base (○), and from an o/w base containing 1% ethanol (●), 2% ethanol (△), and 5% ethanol (▲).

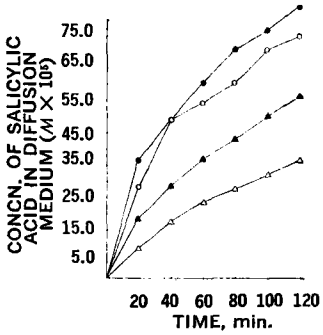

Fig. 9—Diffusion of 1% salicylic acid from an o/w base (○), and from an o/w base containing: 1% DMSO (●), 2% DMSO (△), and 5% DMSO (▲).

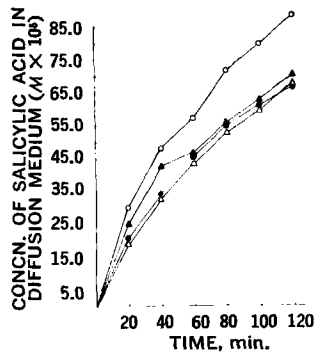

Fig. 10—Diffusion of 2% salicylic acid from a cottonseed oil base (○), and from a cottonseed oil base containing: 1% water (●), 2% water (△), and 5% water (▲).

1 and 5% amounts of DMSO. The crossover seen between the 2 and 5% DMSO is probably due to a lack of homogeneity of the mixture containing 5% DMSO, a condition which tends to diminish as the diffusion process proceeds.

Water-In-Oil Base—Figure 4 shows all three concentrations of water to significantly increase the diffusion of salicylic acid from the water-in-oil emulsion base by about the same amount. Comparisons by statistical analyses show no significant differences in the results from the treated bases at the 2-hr. mark.

Alcohol significantly increases the diffusion of the drug in all concentrations (Fig. 5). The diffusion rate, however, tends to decrease as the concentration of alcohol increases with the 1% concentration giving the greatest rate of passage. All differences are statistically significant.

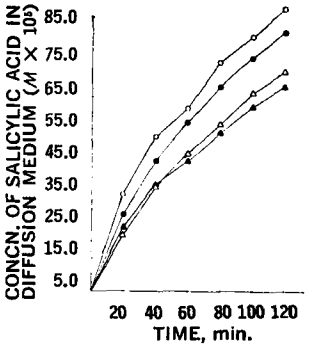

Fig. 11—Diffusion of 2% salicylic acid from a cottonseed oil base (○), and from a cottonseed oil base containing: 1% ethanol (●), 2% ethanol (△), and 5% ethanol (▲).

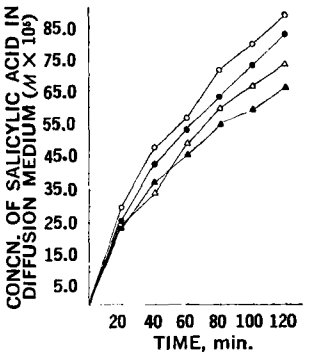

Fig. 12—Diffusion of 2% salicylic acid from a cottonseed oil base (○), and from a cottonseed oil base containing: 1% DMSO (●), 2% DMSO (△), 5% DMSO (▲).

Dimethyl sulfoxide (Fig. 6) significantly increases the diffusion of salicylic acid from this base. The 5% concentration has the greatest effect and all differences in the points of 2 hr. are significant except between 1 and 2%.

Oil-In-Water Base—In the case of the oil-in-water emulsion base it was necessary to reduce the concentration of salicylic acid from 2 to 1% because of the rapid rate of diffusion. In this base all concentrations of all liquids except 1% DMSO were found to be retard diffusion. The 1% concentration of water (Fig. 7) is seen to have the greatest effect in inhibiting the diffusion process. All differences at 2 hr. are significant except between 2 and 5%.

Alcohol in all three concentrations significantly retards diffusion (Fig. 8). However, there is no significant difference among the results from the different amounts of alcohol at the 2-hr. point.

Figure 9 shows 1% dimethyl sulfoxide to significantly increase the diffusion of drug from this base however the 2 and 5% concentrations significantly inhibit the process. The 2% quantity has the greatest effect of inhibition of the three concentrations used.

Cottonseed Oil Base—Results with the cotton-

seed oil base were similar to those with the oil-in-water emulsion in that all three liquids inhibited diffusion. Figure 10 shows the three concentrations of water to significantly retard diffusion. However, the results with water do not differ significantly from each other.

The results with alcohol differ significantly from the control (Fig. 11). As the concentration of alcohol is increased, the retardation of diffusion becomes more pronounced. However, the results with the 2 and 5% do not differ significantly.

The results with DMSO and this base (Fig. 12) are very similar to those obtained with alcohol. All differ significantly from the control. As with the alcohol and this base the difference in results between 2 and 5% DMSO may be taken as due to chance and is not significant. Again as the concentration of liquid is increased inhibition of diffusion becomes more pronounced.

CONCLUSIONS

Undoubtedly the solubility of a drug in an ointment base plays a major part in the diffusion or release of the drug from that base (6). For the most part the solubility of a drug in an intact ointment base is impossible to determine with any degree of confidence due to the fact that one is working with a solid and perhaps a heterogeneous or multiphase system.

Factors which also may influence diffusion in such instances are those of viscosity and the nature of the diffusion membrane. In preliminary experiments alcohol and DMSO in 1, 2, and 5% concentrations did not appreciably influence the passage of salicylic acid from aqueous solutions through the membrane used in this study. From this it is assumed that the liquids used in this study exerted their influence on diffusion by means other than their action on the membrane. Although viscosity measurements were not taken on the ointments, the quantities of liquids added to the various bases were so small that their influence on viscosity would be negligible. If viscosity changes were a great factor in this study, one would expect to get results all going in one direction, probably toward increased diffusion rate, rather than results shown in these experiments, *i.e.*, increased diffusion from two bases and decreased release from the other two.

It is apparent that from this study no general explanation is sufficient to cover all the results. The results indicate that diffusion from ointment bases is greatly influenced by the inclusion of liquids. The characteristics of the base probably determine whether the diffusion process is enhanced or retarded.

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Keyphrases

Salicylic acid diffusion—ointment bases
DMSO, alcohol, water—diffusion effect
Diffusion—ointment base effect
UV spectrophotometry—analysis

Effect of β-Adrenergic Blockade on the Toxicity of Bronchoconstricters in Guinea Pigs

By F. P. LUDUENA and W. B. McKEON, JR.

Propranolol (10 mg./kg.) greatly increased the intravenous toxicity of serotonin, oxotremorine, histamine, and nicotine in guinea pigs. In the case of serotonin, the LD_{50} in the controls was more than 100 times larger than in the propranolol-treated animals. In both groups, bronchoconstriction was the cause of death following the injection of histamine, serotonin, and oxotremorine. In the case of nicotine, lethal bronchoconstriction was produced only in the propranolol-treated animals. The presence or absence of bronchoconstriction was determined by *in vitro* perfusion of the lungs, excised immediately after death.

It has been reported recently that β-adrenergic blockade increases the sensitivity of guinea pigs (1–5) and man (6, 7) to bronchoconstrictors. This potentiation has been attributed to an antagonism of the bronchodilator effect of catecholamines, released as a result of the action of bronchoconstrictors. This suggested that the LD_{50} values of histamine, serotonin, and a cholin-

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Accepted for publication June 11, 1968.
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seed oil base were similar to those with the oil-in-water emulsion in that all three liquids inhibited diffusion. Figure 10 shows the three concentrations of water to significantly retard diffusion. However, the results with water do not differ significantly from each other.

The results with alcohol differ significantly from the control (Fig. 11). As the concentration of alcohol is increased, the retardation of diffusion becomes more pronounced. However, the results with the 2 and 5% do not differ significantly.

The results with DMSO and this base (Fig. 12) are very similar to those obtained with alcohol. All differ significantly from the control. As with the alcohol and this base the difference in results between 2 and 5% DMSO may be taken as due to chance and is not significant. Again as the concentration of liquid is increased inhibition of diffusion becomes more pronounced.

CONCLUSIONS

Undoubtedly the solubility of a drug in an ointment base plays a major part in the diffusion or release of the drug from that base (6). For the most part the solubility of a drug in an intact ointment base is impossible to determine with any degree of confidence due to the fact that one is working with a solid and perhaps a heterogeneous or multiphase system.

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omimetic, such as oxotremorine, represent the effect of doses that surmount the antagonistic effect of released catecholamines on the bronchioles. It may explain the negligible effect of injected catecholamines on the toxicity of histamine in guinea pigs which had been observed in preliminary experiments.

In order to determine the degree of potentiation of the action of the above bronchoconstrictors, experiments were performed on guinea pigs. The toxicity of nicotine was also determined on the assumption that in the presence of propranolol this alkaloid may produce death by bronchoconstriction. It is generally accepted that in untreated animals, the lethal effect of nicotine is due to paralysis of the respiratory muscles (myoneural junction blockage) (8). On the other hand, guinea pig bronchiolar chains (9) and perfused bronchioles (10), are strongly contracted by nicotine. Since nicotine is known to be a releaser of catecholamines from the adrenal gland (11, 12), it was assumed that nicotine bronchoconstriction might become lethal, if the β-agonist effects of endogenous catecholamines were blocked by propranolol.

METHODS

Potentiation of the Lethal Effect of Bronchoconstrictors—Using a special technique for intravenous injection in the ear veins of guinea pigs (13), the LD_{50} of the bronchoconstrictors was determined in control and pretreated albino animals, weighing 225–300 g. In each toxicity determination, three doses, graded at 0.3-log intervals were used. Each dose was tested on 15 guinea pigs. Pretreatment consisted of a dose of 10 mg./kg. of propranolol injected intravenously, approximately 15 min. before the dose of the bronchoconstrictor. Only in the case of nicotine, this drug was injected simultaneously with propranolol.

Lung Perfusion—In order to obtain an indication as to the cause of death, lung perfusion experiments were carried out. In control experiments, the guinea pigs were stunned or killed with a head blow, the chest was opened, and the lungs were excised; one lung was tied off and the other was attached through the trachea to a perfusion apparatus (14) containing Krebs-Henseleit solution at 34–36°. The manometer in the apparatus received approximately 20 ml./min. of perfusion fluid. With no outlet, the fluid level increased in the manometer at an approximate rate of 1.5–1.7 ml./sec. In the case of control animals, an average pressure of 20 ml. was sufficient to force the fluid through the bronchiolar tree, and the lung became rapidly distended. When this happened, the perfusion pressure declined until the lung was maximally distended. The highest perfusion pressure (hereafter called critical perfusion pressure) before the lung was filled with fluid was read off the manometer. This is the value recorded in Table I and could be defined as the minimal perfusion pressure required to force the fluid through the bronchioles. In the experiments where lethal doses of bronchoconstrictor were injected, the lungs were excised immediately after the animal died following the injection. The survival time after lethal doses of serotonin, histamine, and nicotine varied from 3 to 5 min. After oxotremorine the survival time was 7–12 min. Those guinea pigs which received the smaller, nonlethal doses of the bronchoconstrictors, (Table II) were killed approximately 5 min. after injection.

The animals injected with propranolol alone were killed 15 min. after injection. In all cases, the lungs were set up for perfusion as described above for the controls.

The drugs tested were histamine diphosphate, serotonin creatinine sulfate, oxotremorine, nicotine D-bitartrate dihydrate, propranolol hydrochloride, and DCI (dichloroisoproterenol HCl). The doses are expressed in terms of the bases.

RESULTS

The toxicity of the bronchoconstrictor in control and propranolol-pretreated guinea pigs is shown in Table II. Propranolol produced a 7- to 26-fold increase in the toxicity of nicotine, histamine, and oxotremorine. The effect on the toxicity of serotonin was considerably more pronounced; the LD_{50} of serotonin in the control was 105 times larger than in the propranolol-treated animals.

TABLE I—GUINEA PIG LUNG PERFUSION

Bronchoconstrictors	Dose, mg./kg. i.v., as Base	β-Blocker	No. of Lungs Perfused	Mean	Critical Perfusion CPP, cm. H_2O; CPP Reached Max. Value 82 cm. H_2O, Number of Lungs
Controls	—	—	12	20	—
—	—	Propranolol, 10 mg./kg. i.v.	6	52.3	—
Serotonin	54	—	11	>70	(6)
Serotonin	0.4	—	6	20.2	—
Serotonin	0.4	Propranolol[a]	7	>79	(6)
Histamine	0.6	—	13	>77	(11)
Histamine	0.03	—	6	22.8	—
Histamine	0.03	Propranolol[a]	5	>79	(4)
Oxotremorine	1.2	—	6	>60	(1)
Oxotremorine	0.012	—	4	27.5	—
Oxotremorine	0.012	Propranolol[a]	5	>80	(4)
Nicotine	3.0	—	5	23.6	—
Nicotine	1.5	Propranolol[b]	6	>79	(5)

[a] Propranolol HCl, 10 mg./kg. injected i.v. 15 min. before bronchoconstrictor. [b] Propranolol HCl, 10 mg./kg. injected simultaneously with nicotine.

TABLE II—EFFECT OF PROPRANOLOL ON THE TOXICITY OF HISTAMINE OXOTREMORINE, SEROTONIN, AND NICOTINE IN GUINEA PIGS

Bronchoconstrictor, i.v. admin.	Propranolol: Dose, mg./kg.	Propranolol: No. of Doses	Propranolol: Route of Admin.	Propranolol: Interval Between Propanolol and Injections	LD_{50} of Bronchoconstrictor mcg./kg., as Base	Toxicity Increase LD_{50} Ratios
Histamine	0	3	—	—	220 ± 19	22
Histamine	10	3	i.v.	15 min.	9.8 ± 1.2	
Serotonin	0	3	—	—	12,800 ± 290	105
Serotonin	10	3	i.v.	15 min.	122.0 ± 15.6	
Oxotremorine	0	3	—	—	162.0 ± 17.0	26
Oxotremorine	10	3	i.v.	15 min.	6.2 ± 0.4	
Nicotine	0	3	—	—	1,080 ± 150	7
Nicotine	10	3	i.v.	Simultaneous	150 ± 38	

In all cases, with the exception of the lethal effect of nicotine in the animals not pretreated with propranolol, death appears to be due to respiratory obstruction; the pressure required to initiate the perfusion of the lungs (critical perfusion pressure) was much greater than in the unmedicated animal dying from a head blow.

Dichloroisoproterenol (DCI) also increased the toxicity of histamine, confirming that the effect is due to a blockade of β-receptors. The i.v. LD_{50} of histamine was reduced from 220 ± 19 to 54 ± 8.8 mcg./kg. 30 min. after the subcutaneous injection of 10 mg./kg. of DCI.

Perfusion Experiments—The results of the perfusion experiments clearly indicate that lethal bronchoconstriction persists after death, as measured by the increased resistance of the bronchiolar tree to perfusion.

The height of the critical perfusion pressure was much larger in animals dying after lethal doses of known bronchoconstrictors (histamine, serotonin, oxotremorine). The smaller doses (histamine: 0.03 mg./kg., serotonin: 0.4 mg./kg., oxotremorine: 0.012 mg./kg.) which were well-tolerated in the nonpretreated guinea pig, did not result in postmortem bronchoconstriction; the critical perfusion pressure readings after administration of those doses were not much higher than those of the control lungs. On the other hand, the highest critical perfusion pressure readings were obtained with lungs from animals which received the same small doses after propranolol pretreatment, strongly suggesting that bronchoconstriction was the cause of death. After the largest dose of nicotine used (LD_{50} × 3) the critical perfusion pressure was not much larger than that of the controls. A smaller dose injected with propranolol resulted in a pronounced postmortem bronchoconstriction.

DISCUSSION

As suggested by Collier *et al.* (1), Townley *et al.* (2, 3), and others (4, 5), the blockade of β-receptors in the bronchioles would prevent the bronchodialator effect of released epinephrine. The bronchoconstrictors the authors have studied could provoke the release of catecholamines from the adrenal glands by two mechanisms: a direct action on chromaffin cells, and an indirect one provoked by asphyxia and mediated through the splanchnic nerves [Cannon and Hoskins (15), Tournade and Chabrol (16), Houssay and Molinelli (17), Houssay (18), and others]. The output of adrenal catecholamines is increased by histamine [Elliott (19), LaBarre (20), Malmejac, Gross, and Neverre (21), Houssay (18)], serotonin [Poisnier and Douglas (22), Douglas (23)], and muscarinic agents (23, 24). The dose of propranolol was sufficiently large to antagonize the β-receptor effect of released epinephrine.

The experiments suggest that the biogenic substances that produce bronchoconstriction also stimulate the release of endogenous bronchodilators (oxotremorine and nicotine mimic the muscarinic and nicotinic effects of acetylcholine, respectively). That this antagonism is fairly efficient is indicated by the fact that the released catecholamines antagonize many lethal doses of the bronchoconstrictors. In the case of serotonin, the endogenous catecholamines appear to protect against more than 100 LD_{50}'s.

The perfusion experiments indicate that maximal bronchoconstriction is the cause of death in guinea pigs receiving nicotine and propranolol simultaneously. On the other hand, in control animals the intravenous injection of a large dose of nicotine (3 × LD_{50}) kills by a different mechanism.

Propranolol alone, injected i.v. 15 min. before death by a head blow, produced moderate increases in critical perfusion pressure. However, the degree of bronchoconstriction was not sufficiently large to produce visible symptoms of respiratory distress during the intervening 15-min. period.

Another point to be considered is the proportions of receptors occupied by the agonists in the presence and in the absence of propranolol. In the propranolol-treated guinea pigs a dose of 400 mcg./kg. of serotonin killed all the animals injected. It was assumed that the contraction of the bronchioles was maximal. In other words, all or most of the serotonin receptors were occupied. In the absence of propranolol enormous doses of serotonin were needed to produce maximal contraction of the bronchioles. This result could be explained, either by accepting the existence of a very large excess of spare serotonin receptors, or by assuming that endogenous catecholamines, acting on β-receptors, reduce the affinity of serotonin to its specific receptor.

SUMMARY

The intravenous toxicity of histamine, serotonin, oxotremorine, and nicotine in guinea pigs was greatly increased by propranolol (10 mg./kg. i.v.). The LD_{50} values in mg./kg. in control and pre-

treated animals were as follows: histamine, 0.22 ± 0.019 and 0.0098 ± 0.0012; oxotremorine, 0.16 ± 0.017 and 0.0062 ± 0.0004; nicotine, 1.08 ± 0.15 and 0.15 ± 0.038; and serotonin, 12.8 ± 0.29 and 0.12 ± 0.016. The toxicity of histamine was also increased by pretreatment with dichloroisoproterenol.

The resistance of the bronchiolar tree to Krebs-Henseleit perfusion in lungs excised after intravenous injection of lethal doses of bronchoconstrictors was much higher than that of lungs from control animals.

Doses of oxotremorine, serotonin, and histamine which did not change the resistance to perfusion, resulted in pronounced postmortem bronchoconstriction when the animals had received an intravenous dose of 10 mg./kg. of propranolol 15 min. before the bronchoconstrictor. A similar phenomenon was observed when 1.5 mg./kg. of nicotine was injected i.v. with propranolol.

The results indicate that in toxicity tests in control guinea pigs endogenous catecholamines exert a powerful antagonistic action against the bronchoconstrictor effect of histamine, serotonin, and oxotremorine. In the case of these three substances this protective mechanism can be overwhelmed by larger doses; the mechanism of death is bronchoconstriction. Nicotine, which is a bronchoconstrictor in the guinea pig pretreated with propranolol, does not kill untreated guinea pigs by producing bronchoconstriction.

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Critical perfusion pressure—guinea pig lungs

Antimicrobial Activity of Dermatomucosal Agents

By LEO GREENBERG

Dermatomucosal agents act in a local manner on the skin and mucous membranes and are, in general, not regarded as materials which alter the microbial flora of the body. The present study was undertaken to determine whether they are, in fact, microbiologically inert. Nine categories of dermatomucosal agents were established and at least six commonly available products were investigated in each category. Products were evaluated for antimicrobial activity by the "small tube method" and by filter disk zone of inhibition method utilizing 12 organisms representative of the normal aerobic skin flora as substrates. Results indicate a wide range of antimicrobial activity among dermatomucosals with the distinct possibility that such products may, in actual conditions of use, alter normal human skin flora leading either to beneficial or deleterious results.

THERE IS, in modern pharmaceutical terminology, a large group of drugs which can collectively be identified as "dermatomucosal agents." These are compounded and dispensed in a multitude of different ways, but they all possess the property of acting in a local manner on the skin and mucous membranes. Some act purely in a physical or mechanical fashion (*e.g.*, demulcents, protectives) while others have a chemical mode of action (*e.g.*, astringents, anti-

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The technical assistance of Miss June Polak in carrying out this investigation is gratefully acknowledged.

seborrheics). In general, they fall within the scope of cosmetic agents in that they enhance the esthetic properties of the skin in some way, but they are not normally considered as curative of dermatological infection. Thus specific antibacterial or antifungal ointments would fall outside of the scope of this definition and have not been considered in this study.

Antimicrobial activity on the part of a dermatomucosal agent or product arises as the result of (*a*) incorporation of a specific germicide into the formulation as a prophylactic measure, usually for the prevention of possible infection. It may be noted that this procedure has often been attacked as without scientific merit both as ineffective and indiscriminate (1). (*b*) incorporation of a germicide into the formulation for the inhibition or destruction of specific species of microorganisms such as those involved in ammonia dermatitis of infants. (*c*) incorporation into the formulation of a preservative agent. (*d*) antimicrobial activity by a chemical which is largely or entirely incidental to its intended activity.

Since the first three of these are procedures which constitute a routine part of pharmaceutical compounding of cosmetic products (2), it would appear likely that commercial dermatomucosal agents must exert an effect on the microbial flora of man which is either unintentional or exceeds the expected degree and range of response. Verification of this would have more than academic interest; changes in flora by cosmetic agents, whether intentional or unintentional, may conceivably result in serious health problems either from the development of resistant organisms or the accrual of virulent or undesirable species (3). Since some data on this problem are scattered in the literature and some are closely held in company files, the present study was undertaken to report on the antimicrobial spectrum of a large number of representative dermatomucosals.

PROCEDURES

Using standard reference sources (4), nine categories of dermatomucosal agents were identified for study. A tenth category, that of dusting powders was eliminated because of the extreme difficulty in preparing reliable dilutions. At least six common products in each category were purchased locally for evaluation.

Selection of test microorganisms was made on the basis of careful investigation of the resident and transient flora of the skin and mucous membranes. Thus bacteria, which constitute a preponderance of the normal population (5), were obviously of major interest as test substrates. Among the various groups, cocci are of primary importance, with Gram-positive species far outnumbering Gram-negatives. In the case of bacilli, the lipophilic, microaerophilic types are common, as are nonmotile forms of the *Neisseria*, *Mima*, or *Herella* type.

Yeasts are routinely cultured from normal skin and mucous membranes. Particular attention has been paid by clinicians and microbiologists to species of *Candida* because of their potential pathogenicity. However, *Saccharomyces*, *Torulopsis*, and *Pityrosporum* are commonly present. Molds are of course ubiquitous; many are environmental contaminants which are simply in transit from their normal habitat as saprophytes in the soil. Whether transient or resident, they do represent a major segment of the human skin ecology.

From these considerations, 12 microorganisms (seven bacteria, two yeasts, and three molds) representative of human flora were selected as test organisms and typical strains purchased from the American Type Culture Collection.

Tests for inhibitory concentrations of products were carried out by the "small tube method" (6) and by zone inhibition tests utilizing sterile S & S filter paper disks (7). Although these methods are not ideal, depending particularly upon both horizontal and vertical diffusion through the medium (8), they are entirely suitable for routine screening purposes.

Bacterial organisms were maintained on brain heart infusion agar and broth (Difco), while yeasts and molds were cultivated on Sabouraud dextrose agar and broth (Difco). Dilutions were made with sterile distilled water or suspended in sucrose solution if insoluble. Normally, 0.5 ml. of 18–24 hr. broth culture of the test organism was added to each 100 ml. of melted, cooled agar prior to pouring into sterile, plastic Petri dishes or tubes. Incubation in all experiments was at 35° for bacterial and yeast preparations and at 30° for molds. Bacteria and yeast plates and tubes were read after 48 hr. while molds were held for 7 days.

RESULTS AND DISCUSSION

For preliminary general screening, four representative organisms were chosen; these were a Gram-positive coccus, a Gram-negative enteric bacillus, a typical conidiospore-forming mold, and a pathogenic yeast. The activity or lack of activity of 63 commercial preparations against each of these four organisms was determined by filter paper disk method and is recorded qualitatively in Table I. The data indicate antimicrobial activity as follows: 41/63 against *M. citreus;* 31/63 against *E. coli;* 24/63 against *A. niger;* 10/63 against *C. albicans*. The minimum inhibitory concentration for each effective product against each test organism is given in Tables II–V, as well as the highest ineffective concentration tested. It may be noted that the number of effective products in Tables II–V exceeds the totals calculated from Table I. This results from the fact that inhibitory response judged to be "trace" in Table I was recorded as no activity, while being recorded as a minimum effective concentration in later tables.

From the results of these screening procedures, it is evident that antimicrobial activity is the rule among dermatomucosal agents rather than the exception. The sensitivity of micrococci to most

TABLE I—QUANTAL ACTIVITY OF FULL-STRENGTH DERMATOMUCOSAL AGENTS

	Product Active Against			
	Micrococcus citreus	*Escherichia coli*	*Aspergillus niger*	*Candida albicans*
Local anesthetics				
4% Butacaine sulfate	Yes	Yes	No	No
5% Cyclaine hydrochloride	Yes	Yes	No	No
4% Intracaine hydrochloride	Yes	Yes	No	No
4% Metycaine hydrochloride	Yes	Yes	No	No
4% Xylocaine hydrochloride	No	Yes	No	No
4% Procaine hydrochloride	No	No	No	No
Deodorants				
Arrid	Yes	Yes	Yes	No
Right Guard	Yes	Yes	Yes	No
Mennen Spray	Yes	Yes	No	No
Man Power	Yes	No	Yes	No
Ban	Yes	No	Yes	No
Mum Mist	Yes	No	No	No
Secret	Yes	No	No	No
Rubefacients and astringents				
Oil of Wintergreen	Yes	Yes	Yes	Yes
Pronto Gel	Yes	Yes	Yes	Yes
Sloan's Liniment	Yes	Yes	Yes	Yes
St. John's Bay Rum	Yes	Yes	Yes	Yes
Heet	Yes	Yes	Yes	No
Campho-Phenique	Yes	Yes	Yes	No
Omega Oil	Yes	Yes	Yes	No
Banalg	Yes	Yes	Yes	No
Camphorated Oil	No	No	No	No
St. John's After Shave	No	No	No	No
Face washes and cleansing creams				
Paradox Creme Cleanser	Yes	Yes	Yes	Yes
All Clear Face Wash	Yes	Yes	No	No
Du Barry Hand & Body Lotion	No	No	No	No
Nivea Skin Oil	No	No	No	No
Noxema Liquid	No	No	No	No
Water Lily Pore Lotion	No	No	No	No
Hair Tonics				
Trol	Yes	Yes	Yes	Yes
Heads Up	Yes	Yes	No	No
Fitches Ideal	Yes	Yes	No	No
Yardley	Yes	Yes	No	No
Hask	No	Yes	Yes	No
Jeris	Yes	No	No	No
Old Spice	No	No	No	No
St. John's Hair Groom	No	No	No	No
Vaseline Hair Tonic	No	No	No	No
Vitalis	No	No	No	No
Sunscreening agents				
St. John's Sun Tan	Yes	Yes	Yes	No
Tansation Plus	Yes	Yes	No	No
A-Fil	No	No	No	No
Bain de Soleil	No	No	No	No
Coppertone Cream	No	No	No	No
Sun Bath	No	No	No	No
Depilatories				
IMRA	Yes	Yes	Yes	Yes
Nair	Yes	No	Yes	No
Zip	Yes	No	No	No
Neet	No	No	No	No
Sleek	No	No	No	No
Shampoos				
All Clear	Yes	Yes	Yes	Yes
Rinse Away	Yes	Yes	Yes	Yes
Thylox	Yes	Yes	Yes	No
Kreml	Yes	Yes	Yes	No
Sebb	Yes	Yes	No	No
Capsebon	Yes	No	No	No

(Continued on next page.)

TABLE I (*Continued.*)

	Product Active Against			
	Micrococcus citreus	*Escherichia coli*	*Aspergillus niger*	*Candida albicans*
Protectives, demulcents, and emollients				
Paradox Moisturizing Lotion	Yes	No	Yes	No
Trushay	Yes	No	Yes	Yes
Edith Rehnborg Dermojeune Cream	Yes	No	No	No
Edith Rehnborg Moisture Cream	Yes	No	No	No
Jergens Lotion	No	No	No	No
Corn Huskers Lotion	No	No	No	No
Nosekote	No	No	No	No

TABLE II—INHIBITION OF GROWTH OF *Micrococcus Citreus* (BY GENERALLY DECLINING DEGREE OF ACTIVITY)

Item	Class	Effective Growth Inhibiting Concn.	Concn. Ineffectual for Inhibition
Butacaine sulfate	Local anesthetic	1/2500	1/3125
Rinse Away	Shampoo	1/2000	1/3000
Cyclaine hydrochloride	Local anesthetic	1/2000	1/2400
Intracaine hydrochloride	Local anesthetic	1/1875	1/2500
Metycaine hydrochloride	Local anesthetic	1/625	1/1250
Mum Mist	Deodorant	1/400	1/500
Mennen Spray	Deodorant	1/300	1/350
Secret	Deodorant	1/200	1/300
Right Guard	Deodorant	1/150	1/200
Fitches Ideal	Hair tonic	1/100	1/150
Heads Up	Hair tonic	1/100	1/150
Trol	Hair tonic	1/100	1/150
All Clear Face Wash	Cleanser	1/50	1/100
Man Power	Deodorant	1/50	1/100
Oil of Wintergreen	Rubefacient	1/50	1/100
Paradox Creme Cleanser	Cleanser	1/50	1/100
Paradox Moisturizer	Protective	1/50	1/100
Yardley	Hair tonic	1/50	1/100
All Clear	Shampoo	1/80	1/90
Edith Rehnborg Dermojeune Cream	Protective	1/50	1/60
Edith Rehnborg Moisture Cream	Protective	1/50	1/60
Trushay	Protective	1/40	1/50
Sebb	Shampoo	1/40	1/50
Thylox	Shampoo	1/40	1/50
Pronto Gel	Rubefacient	1/30	1/40
Campho-Phenique	Rubefacient	1/20	1/30
Ban	Deodorant	1/20	1/30
Capsebon	Shampoo	1/10	1/20
Kreml	Shampoo	1/10	1/20
Heet	Rubefacient	1/10	1/20
Sloan's Liniment	Rubefacient	1/10	1/20
Zip	Depilatory	1/10	1/20
Nair	Depilatory	1/4	1/10
IMRA	Depilatory	1/4	1/10
Arrid	Deodorant	1/4	1/10
Jeris	Hair tonic	1/4	1/10
St. John's Bay Rum	Astringent	1/4	1/10
Jergen's Lotion	Protective	Full strength	1/4
Banalg	Rubefacient	Full strength	1/4
Omega Oil	Rubefacient	Full strength	1/4
St. John's Sun Tan	Tanning agent	Full strength	1/4
Tansation Plus	Tanning agent	Full strength	1/4

products is not unexpected, but the relative resistance of *Candida albicans* lends support to current fears among some clinicians of an upsurge of yeast overgrowth in areas of the body where the resident bacterial flora has been reduced or eliminated by drug therapy.

To establish a picture of the range of germicidal activity involved, the two most active products in each of the nine categories were tested against eight additional organisms, and the data summarized in Table VI. The reader can determine by inspection the degree of activity of any given

TABLE III—INHIBITION OF GROWTH OF *E. Coli* (BY GENERALLY DECLINING DEGREE OF ACTIVITY)

Item	Class	Effective Growth Inhibiting Concn.	Concn. Ineffectual for Inhibition
Cyclaine hydrochloride	Local anesthetic	1/2500	1/3000
Butacaine sulfate	Local anesthetic	1/1875	1/2500
Xylocaine hydrochloride	Local anesthetic	1/1875	1/2500
Intracaine hydrochloride	Local anesthetic	1/1250	1/1875
Metycaine hydrochloride	Local anesthetic	1/1250	1/1875
Rinse Away	Shampoo	1/250	1/1000
All Clear	Shampoo	1/150	1/200
Oil of Wintergreen	Rubefacient	1/50	1/100
Thylox	Shampoo	1/30	1/40
Heads Up	Hair tonic	1/30	1/40
Yardley	Hair tonic	1/20	1/30
St. John's Bay Rum	Astringent	1/20	1/30
Mennen Spray	Deodorant	1/20	1/30
Arrid	Deodorant	1/10	1/20
Campho-Phenique	Rubefacient	1/10	1/20
Pronto Gel	Rubefacient	1/10	1/20
Sloan's Liniment	Rubefacient	1/10	1/20
Paradox Creme Cleanser	Cleanser	1/10	1/20
All Clear Face Wash	Cleanser	1/10	1/20
Fitches Ideal	Hair tonic	1/10	1/20
Hask	Hair tonic	1/10	1/20
St. John's Sun Tan	Tanning agent	1/10	1/20
IMRA	Depilatory	1/10	1/20
Sebb	Shampoo	1/10	1/20
Tansation Plus	Tanning agent	1/8	1/10
Trol	Hair tonic	1/4	1/6
Right Guard	Deodorant	1/2	1/4
Banalg	Rubefacient	1/2	1/4
Kreml	Shampoo	1/2	1/4
Ban	Deodorant	Full strength	1/10
Mum Mist	Deodorant	Full strength	1/10
Man Power	Deodorant	Full strength	1/10
Nair	Depilatory	Full strength	1/10
Heet	Rubefacient	Full strength	1/2
Omega Oil	Rubefacient	Full strength	1/2

TABLE IV—INHIBITION OF GROWTH OF *A. Niger* (BY GENERALLY DECLINING DEGREE OF ACTIVITY)

Item	Class	Effective Growth Inhibiting Concn.	Concn. Ineffectual for Inhibition
Oil of Wintergreen	Rubefacient	1/50	1/100
All Clear	Shampoo	1/40	1/50
Rinse Away	Shampoo	1/40	1/50
Paradox Creme Cleanser	Cleanser	1/40	1/50
Trol	Hair tonic	1/30	1/40
Paradox Moisturizer	Protective	1/20	1/30
Pronto Gel	Rubefacient	1/20	1/30
Sloan's Liniment	Rubefacient	1/20	1/30
Omega Oil	Rubefacient	1/20	1/30
Thylox	Shampoo	1/20	1/30
St. John's Bay Rum	Astringent	1/10	1/20
Heet	Rubefacient	1/10	1/20
Campho-Phenique	Rubefacient	1/10	1/20
Man Power	Deodorant	1/10	1/20
Right Guard	Deodorant	1/10	1/20
St. John's Sun Tan	Tanning agent	1/10	1/20
Hask	Hair tonic	1/10	1/20
Kreml	Shampoo	1/10	1/20
Trushay	Protective	1/10	1/20
Ban	Deodorant	1/4	1/10
IMRA	Depilatory	1/4	1/20
Nair	Depilatory	1/4	1/10
Capsebon	Shampoo	Full strength	1/10
Jeris	Hair tonic	Full strength	1/10
Mennen Spray	Deodorant	Full strength	1/10
Secret	Deodorant	Full strength	1/10
Old Spice	Hair tonic	Full strength	1/10
Arrid	Deodorant	Full strength	1/4
Banalg	Rubefacient	Full strength	1/4

TABLE V—INHIBITION OF GROWTH OF *Candida Albicans* (BY GENERALLY DECLINING DEGREE OF ACTIVITY)

Item	Class	Effective Growth Inhibiting Concn.	Concn. Ineffectual for Inhibition
Rinse Away	Shampoo	1/200	1/250
Oil of Wintergreen	Rubefacient	1/50	1/100
Paradox Creme Cleanser	Cleanser	1/30	1/40
Trol	Hair tonic	1/30	1/40
All Clear	Shampoo	1/20	1/30
Paradox Moisturizer	Protective	1/10	1/20
Trushay	Protective	1/10	1/20
Sloan's Liniment	Rubefacient	1/10	1/20
Pronto Gel	Rubefacient	1/10	1/20
IMRA	Depilatory	1/10	1/20
Nair	Depilatory	Full strength	1/10
Arrid	Deodorant	Full strength	1/10
Mennen Spray	Deodorant	Full strength	1/10
Heet	Rubefacient	Full strength	1/10
Thylox	Shampoo	Full strength	1/10
St. John's Bay Rum	Astringent	Full strength	1/4

TABLE VI—COMPARATIVE ANTIMICROBIAL ACTIVITY OF FULL-STRENGTH DERMATOMUCOSAL AGENTS[a]

Product	Test Organisms[b] A	B	C	D	E	F	G	H	I	J	K	L
All Clear shampoo	1+	1+	1+	1+	0	1+	1+	0.8	1+	1+	0.9	0
Trol hair tonic	0.9	1+	1+	1+	0.9	1	0.7	0.4	0.9	0.7	0.8	1+
Paradox Creme Cleanser	1+	1+	1+	1+	0	1+	0.8	0.4	0.9	0.8	0.7	1+
Trushay lotion	0.7	1+	1	0.9	1+	0.7	0.6	0	0.6	0.8	0.6	0.4
Rinse Away shampoo	1+	1+	1+	0.9	0	0	0.7	0.7	0.6	0.6	1	0.6
Oil of Wintergreen	0.8	0	1+	1+	0.6	0	0.5	1+	1+	0	1+	0
Mennen Spray deodorant	0.7	1	1+	1+	0.5	0.9	0.5	0.6	T	0	T	0.6
Sloan's Liniment	1	0.6	0.4	0.8	0.8	0.5	0.3	0.5	0.6	0.7	0.5	0.3
St. John's Sun Tan	1+	0.8	0.3	0	1+	0.7	0.5	0.4	0.5	0.6	0	0
Mum Mist deodorant	0.7	0.9	0.5	0.5	0.5	0.8	0.4	T	T	0.9	0	0.6
Paradox Moisturizer	0.3	0.8	0.8	0.7	0.5	0.5	0.3	0	0.7	0.3	0.6	0.7
Heads Up hair tonic	0.5	1	0.9	0.3	1	0.5	0.5	0.5	0	0.5	0	0.3
Butacaine sulfate	0.9	0.7	1	0.7	0	0	0.6	0.8	0	0.3	0	0
All Clear Face Wash	0.7	0.8	0.6	0	0.8	0.6	0.4	0.4	0	0.4	0	0.5
Cyclaine hydrochloride	0.8	0.8	0.6	0	0.3	0	0.7	1	0	0	0	0.3
Tansation Plus	1+	0.3	0.3	0	0.6	0.5	0.4	0.4	0	0	0	0.5
Nair depilatory	0.4	0.3	0.3	0	1+	0.6	0.4	T	0.4	0.3	T	0.3
IMRA depilatory	0.4	0.4	0.3	0	0.3	0.4	0.4	1.4	0.4	0	0.4	0.4

[a] Zone of inhibition of 25-mm. diameter = 1; zone of inhibition greater than 25-mm. diameter = 1+; trace activity = T; and lack of activity = 0. [b] Test organisms: A, *Streptomyces griseus;* B, *Sarcina lutea;* C, *Micrococcus citreus;* D, *Saccharomyces ellipsoides;* E, *Spirillum rubrum;* F, *Neisseria catarrhalis;* G, *Bacillus cereus mycoides;* H, *Escherichia coli;* I, *Aspergillus niger;* J, *Mucor mucedo;* K, *Candida albicans;* L, *Streptococcus faecalis.*

product against any given organism. He can also rank any product in comparison to 17 others and can estimate the general sensitivity of any test organism to chemical agents. From the data, it is clear that all categories participate in the antimicrobial phenomenon (seven different categories are represented among the 10 most active products), and as is to be expected, there is a wide range of activity within each category. It is likewise evident that different species of microorganisms display quite different susceptibilities to chemical agents. However, the marked sensitivity of *Streptomyces griseus* to all types of dermatomucosals was unexpected, and it is noteworthy that the test species of *Streptococcus* and *Neisseria*, genera associated with a high degree of pathogenicity, show a relatively high degree of resistance to chemical agents.

Finally, mention must be made of the fact that in some cases, the response of the test organism to certain concentrations of the dermatomucosal agent was unpredictable. Increase in colony size, enhancement of growth, and induction of sporulation were among the observations recorded, and further studies have been undertaken to determine if these represent consistent, reproducible responses or merely local idiosyncracies.

CONCLUSIONS

1. Evidence is presented that dermatomucosal products, normally intended to serve a cosmetic-type function, possess powerful abilities to affect microbial organisms typical of those normally associated with skin and mucous membranes.

2. Although no specific clinical implications can be drawn from this kind of laboratory investigation, the possibility that dermatomucosal products may significantly alter normal human skin flora leading to either beneficial or deleterious changes (in either case, neither predictable nor expected) cannot be discounted.

3. The data would appear to justify the recommendation that in dermatomucosal formulations, unless specific evidence of beneficial activity has been reported, antimicrobial activity should be kept to the most practical minimum.

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Keyphrases

Dermatomucosal agents—antimicrobial activity

Antimicrobial activity—small tube, disk methods

Cosmetic products—antimicrobial activity

Effects of Heat and Ultraviolet Radiation on the Stability of a Polypropylene–Polyisobutylene Alloy

By ROBERT K. O'LEARY, WALLACE L. GUESS, and JOHN AUTIAN*

Polypropylene and blends of polypropylene are or may be utilized in medical items of equipment, containers, and various devices. Changes in properties of the material may negate its value when used for these purposes. This paper deals with the assessment of the stability of a polypropylene–polyisobutylene alloy when exposed to heat and ultraviolet radiation. Evaluation of stability was based upon (*a*) differential thermal analysis, (*b*) tensile properties, and (*c*) infrared spectroscopy. Results of the study revealed that considerable degradation of the material had occurred when exposed to a combination of heat and radiation, but relatively little degradation when the plastic was exposed to heat only.

PRESENTLY, polypropylene is enjoying widespread use in many applications in pharmacy, dentistry, and medicine as such items as disposable syringes, various types of containers, protective sheets, and a host of other devices. Addition of relatively small concentrations of a second polymer, polyisobutylene, gives added advantages for certain applications, the chief of which is a reduction in water vapor transmission.

Polypropylene is generally less stable to heat than polyethylene, for the most part due to the methyl side groups attached to every other carbon in the polymer (1). These tertiary carbons serve as active sites for oxidative degradations leading to chain scission and embrittlement. Incorporation of small quantities of antioxidants or combinations of antioxidants with other agents into the polymer increases the long-term stability of the final item.

In recent years, the authors have become interested in various man-made polymeric materials as protective barriers against harmful chemicals. It has become clear, however, that depending upon the inherent properties of the polymeric material and the types of stabilizers employed, the shelf-life of the plastic will be a function of time and the environmental conditions imposed upon the item. A protective coating which is initially found to prevent the penetration or permeation of a chemical poison might become, depending upon its storage conditions, sufficiently altered to permit the passage of the contaminating agent.

To gain some knowledge as to the stability of polymeric materials which might be used in medical items, containers, and various devices, a study was initiated to evaluate the properties of a specific material, polypropylene-polyisobutylene alloy, when exposed to heat and ultraviolet radiation.

EXPERIMENTAL

Materials and Apparatus—Polypropylene–polyisobutylene alloy[1] (Pro-Fax N400), 10 mil thick-

Received July 17, 1967, from the *Materials Science Toxicology Laboratory, College of Dentistry and College of Pharmacy, University of Tennessee Medical Units, Memphis, TN 38103, and the Drug Plastic Research and Toxicology Laboratories, University of Texas, Austin, TX

Accepted for publication June 11, 1968.

This study supported in part by contract No. DA18-035-AMC-391 (EA), Research Laboratories, Edgewood Arsenal, Md.

Appreciation is extended to Mr. Glynn R. McDonald for technical assistance during the study.

[1] Five percent polyisobutylene.

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EXPERIMENTAL

Materials and Apparatus—Polypropylene–polyisobutylene alloy[1] (Pro-Fax N400), 10 mil thick-

Received July 17, 1967, from the *Materials Science Toxicology Laboratory, College of Dentistry and College of Pharmacy, University of Tennessee Medical Units, Memphis, TN 38103, and the Drug Plastic Research and Toxicology Laboratories, University of Texas, Austin, TX

Accepted for publication June 11, 1968.

This study supported in part by contract No. DA18-035-AMC-391 (EA), Research Laboratories, Edgewood Arsenal, Md.

Appreciation is extended to Mr. Glynn R. McDonald for technical assistance during the study.

[1] Five percent polyisobutylene.

ness (Hercules Chemical Co., Wilmington, Del.); Differential thermal analysis instrument, model KA-2-HD (Rogert L. Stone, Austin, Tex.); Instron apparatus, model TM (Instron Engineering Corp., Canton, Mass.); Infrared spectrophotometer, model 337 (Perkin-Elmer Corp., Norwalk, Conn.).

Heat and Ultraviolet Radiation—Samples of polypropylene alloy were exposed to heat alone and to a combination of heat and ultraviolet radiation. Figure 1 shows a diagram of the system employed to supply the heat and radiation. A G. E. sunlamp which emits only light rays greater than 2800 Å. was used for both heat and radiation sources. The temperature at the surface of the plastic samples was continually monitored and was found to average 82 ± 2°. In the case of heat treatment alone, a thin cardboard 0.5 mm. thick (0.02 in.) was inserted above the sample, thus effectively shielding the plastic from the radiation without diminishing the heat intensity striking the plastic. The sunlamp was approximately 20 cm. above the samples.

At 24-hr. intervals or at other time periods, depending upon the experiment to be performed, samples were removed from both the heat-treated and heat-ultraviolet treated conditions for analysis, utilizing three techniques: (*a*) differential thermal analysis, (*b*) tensile properties, and (*c*) infrared spectroscopy.

Differential Thermal Analysis (DTA)—The DTA apparatus used in this study has been described in a previous paper (2). In this study, DTA was employed to characterize the thermal behavior of the plastic prior to any type of treatment, and at various time periods under the two types of treatment.

Initial experiments were conducted on 10 samples of plastic in order to establish the temperature at which transitions occurred. Samples were heated in the DTA instrument at a programmed rate of 12.6°/min. until a temperature of 200° was reached. The sample weight was approximately 1.5 mg., while the alumina (Al_2O_3) reference weight was 3.0 mg. To prevent oxidation of the samples during any DTA run, a nitrogen atmosphere was maintained. Figure 2 represents a typical thermogram on an untreated polypropylene–polyisobutylene alloy. In this case, both a heating and a cooling sequence were used, giving rise to an endothermic melt starting at 140° and peaking at 167°. The cooling curve demonstrates an exothermic peak (crystallization) beginning at approximately 119°, and peaking at 107°

At the various time periods and for each of the treatments (heat and radiation), at least three samples of plastic were examined by the procedure described above. Only the DTA heating sequence was employed. From the various thermograms, the onset temperature and peak temperatures were recorded. The areas under the curve representing the melt were converted to standard heats of fusion (ΔH). Table I incorporates the temperature values and constants obtained from the DTA experiments. Figure 3 demonstrates the influence time and treatments have on the peak temperature (melt), while Fig. 4 is a similar relationship except heat of fusion values are plotted against time.

Tensile Properties—Test specimens were prepared from the samples of plastic taken from the hood at the various time periods and stress-strain curves obtained using an Instron instrument, follow-

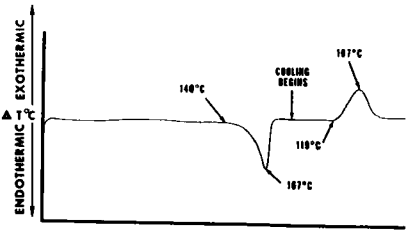


Fig. 2—Typical thermogram of untreated polypropylene–polyisobutylene alloy. A heating and cooling sequence is shown. The curve at the left represents the melt area; the curve at right represents the area associated with crystallization (on cooling.)

TABLE I—SUMMARY OF DTA DATA FROM HEAT-TREATED (82°) POLYPROPYLENE ALLOY AT VARIOUS TIME PERIODS

Hr. Exposed	Melt Onset, °C.	Melt Peak, °C.	ΔH^a cal./g.
0	122.4	165.0	16.1
48	128.1	164.5	16.4
96	130.9	166.7	14.7
120	132.6	165.3	14.6
192	130.9	164.2	16.6
240	131.3	165.3	15.5
264	129.8	165.1	16.5
288	131.8	165.1	16.1

[a] An average of 10 samples used at zero time with a standard deviation of ±1.1 cal./g. All other values based upon an average of three samples with an average deviation falling within ±1.5 cal./g.

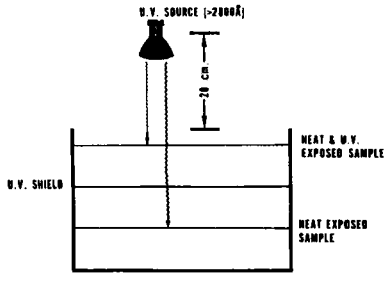


Fig. 1—Diagram showing method of heat and ultraviolet radiation treatment of plastic samples. Distance between samples exposed to radiation and exposed to heat is approximately 20 cm.

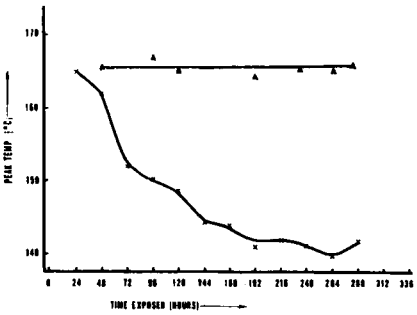


Fig. 3—Comparison of DTA melt peaks of heat-treated and heat-radiation treated samples. Key: ▲, heat treated; ×, heat and UV treated.

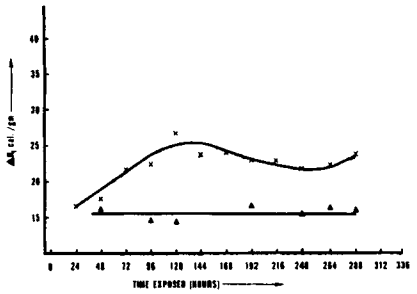


Fig. 4—Comparison of DTA Δ H of heat-treated and heat-radiation treated samples. Key: ▲, heat treated; ×, heat and UV treated.

TABLE II—SUMMARY OF DTA DATA FROM HEAT-RADIATION TREATED POLYPROPYLENE ALLOY AT VARIOUS TIME PERIODS

Hr. Exposed	Melt Onset, °C.	Melt Peak, °C.	ΔH^a, cal./g.
0	122.4	165.0	16.1
24	126.9	164.9	16.9
48	127.0	163.8	17.7
72	104.9	152.0	21.8
96	109.8	150.3	22.5
120	96.9	148.8	26.8
144	99.2	144.4	23.8
168	96.1	144.0	24.1
192	87.5	141.1	23.2
216	94.5	142.0	23.0
240	96.4	141.7	21.9
264	95.8	139.8	22.4
288	91.8	141.6	23.7

[a] An average of 10 samples used at zero time with a standard deviation of ±1.1 cal./g. All other values based upon an average of three samples with an average deviation falling within ±1.5 cal./g.

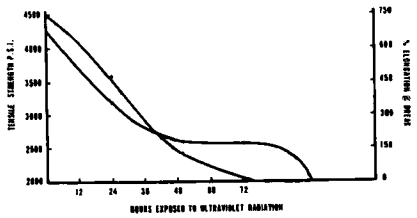


Fig. 5—Effect of heat-radiation treatment on tensile properties of polypropylene–polyisobutylene alloy. Key: ○, tensile strength; △, % elongation.

ing the standard ASTM method.[2] From the various curves, tensile strength and percent elongation were evaluated. Three samples were used for each run for any one period and condition Table II includes the results while Fig. 5 is a plot of the data.

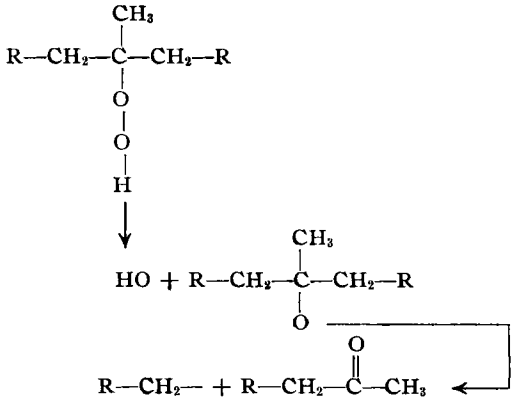
Infrared Spectroscopy—The appearance of carbonyl bands and the intensity of these bands are indications of oxidation of the oleofinic polymers such as polypropylene, and for these reasons infrared spectra were taken of samples of plastic exposed to the two treatments at various time periods. Prior to IR analysis, the films were mounted between KBr salt disks. These spectra were examined and the salient features noted in the 1750 cm.$^{-1}$ wavelength region (corresponding to carbonyl band). Figure 6 includes three spectra representing the carbonyl band of samples removed at several time periods from the heat-radiation treatment.

[2] Die is No. ASTM 1708-59T, determinations made at 25°, 52% RH on a full load of 10 lb., 23 mm. (0.9 in.) gauge length, drive speed of 12.7 mm. (0.5 in.)/min. and a chart speed of 127 cm. (50 in.)/min.

RESULTS AND DISCUSSION

Figure 2 illustrates a typical thermogram for the polypropylene–polyisobutylene alloy and includes the peak area for the melt and the peak area representing the crystallization phase after cooling. The two areas should theoretically correspond, but as is evident from the thermogram, this was not the case. The area under the curve for the crystalline phase was slightly larger than for the melt area. More dramatic, perhaps, is the peak temperature difference for the melt (167°) as compared to the crystallization peak (107°). Heat history plays an extremely important role on transition temperatures, and this, no doubt, was the case with what has been reported here. In one case (melt area), the sample has had a heat history quite different from that of the plastic after it is heated above its melting point and then cooled down in a short time period in the DTA instruments. In the melted state the polymers are all in a random motion with little orientation, but as the sample is cooled the chains start sorting out in a fashion to favor orientation with subsequent crystallization. Even though the degree of crystallization is slightly greater for the sample after the cooling treatment (in DTA instrument), the size of the individual crystallites are much smaller, reflected by the much lower onset and peak temperatures. For the purposes of this study, it was felt more advantageous to examine the area corresponding to the endothermic melt (heating sequence).

Stability studies on polypropylene by several workers have shown that this plastic is quite susceptible to oxidative degradation (3–5). The tertiary carbons in the molecule are active sites for the formation of hydroperoxides which then decompose into alkoxy and hydroxy free radicals. Chain scission takes place when the alkoxy radical at the tertiary carbon atom stabilizes through disproportionation into an alkyl radical and a ketone as shown below (6):



The reaction continues until two radicals combine, terminating chain scission. Other possible reactions are occurring during the degradation, and one or more of these can lead to cross-linking. It is, however, believed that the chain scission is most

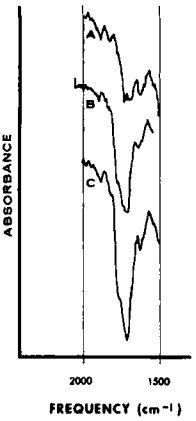


Fig. 6—Effect of heat radiation on IR absorption curve of polypropylene–polyisobutylene alloy (carbonyl formation observed at 1750 cm.$^{-1}$). Key: A, *24 hr.;* B, *48 hr.;* C, *72 hr.*

likely favored over the cross-linking, at least in the initial phases of oxidative degradation.

Table I, Figs. 3 and 4 reveal that heat treatment alone has very little effect on the property of the alloy. Combined heat and radiation, however, has a dramatic effect on the material. As may be noted in Table II and Figs. 3 and 4, there is an appreciable drop in both the melt peak temperature and the heat of fusion (ΔH) during the time the plastic is exposed to the combined heat and radiation condition. A decrease in tensile properties (Table III and Fig. 5) also suggests that polymer alteration is occurring. Infrared studies (Fig. 6), in fact, confirm that oxidative degradation has taken place in the alloy and that this effect increases with time.

Figure 3 shows that the peak temperature (melt) decreases with time for the heat-radiation treated samples of plastic while Fig. 4 shows an increase in the heat of fusion (ΔH) with time. These results may be interpreted in the following manner: with chain scission there is a decrease in molecular weight; these short chains, as well as the possible broad molecular weight distribution of the polymers, create smaller crystallite regions which are more susceptible to melting at lower temperatures.

The increase in the heat of fusion (ΔH), demonstrated in Fig. 4, may be attributed to the formation of more crystallite regions in the previously amorphous zones, due to either rearrangements of the existing polymer chains into a more ordered system or to the degraded polymer chains forming new crystallites. Evidence from Fig. 3 (decrease in melt temperature), Fig. 5 (decrease in tensile properties), and Fig. 6 (decrease in carbonyl function favors the formation of new crystallite regions from the degraded polymers. Oswalt and Turi have also noted that density (crystallinity) increases with oxidative degradation for polypropylene (6).

The decrease in tensile properties (Table III and Fig. 5) with time for the heat-radiated samples of alloy may be interpreted as being due to chain scission occurring in the amorphous regions of the material. The polymers in the amorphous region essentially "tie" the various crystallites into a unified structure having various degrees of toughness and elasticity. Any altering of these ties, such as chain scission, will help collapse the structure with eventual breaking or shattering of the material. It is also possible that a point of degradation may occur where cross-linking in the amorphous zone becomes important and adds to the destruction of the material when some minor force is applied.

Table III—Tensile Properties of Heat-Treated and Heat-Radiation Treated Samples of Polypropylene Alloy at Various Time Periods

Time, hr.	Treatment	Tensile Strength[a]	% Elongation at Break[a]
0	None	4,380 ± 271	746 ± 37
24	Heat	3,830 ± 281	649 ± 35
	Heat and radiation	3,207 ± 291	475 ± 100
50	Heat	3,646 ± 622	654 ± 70
	Heat and radiation	2,578 ± 86	121 ± 101
72	Heat	3,817 ± 194	550 ± 7.8
	Heat and radiation	2,688 ± 36	4 ± 0.3
91.5	Heat	3,718 ± 674	575 ± 96
	Heat and radiation[b]	—	—
117	Heat	3,860 ± 544	592 ± 101
	Heat and radiation[b]	—	—

[a] Tensile properties based upon the average of three samples ± the difference between the lowest and highest values. [b] Tensile properties could not be determined due to brittleness of samples.

In this particular study, it was not possible to assess to what extent the relatively small concentration of polyisobutylene (5%) took part in the degradation. It is felt that this specific polymer would follow the same general chain scission reaction as is the case for polypropylene.

One important aspect of the study reported here is that storage of a protective barrier such as a plastic film may become altered sufficiently to malfunction, causing possible harm or damage to personnel or equipment. It appears that conditions of storage should be established for items to be used as protective devices and that this same concept should extend to all items to be used to protect health and life, as is the case for various devices used in medical and *para*-medical applications.

SUMMARY AND CONCLUSION

Polypropylene and alloys of polypropylene are being used with greater frequency for a number of applications in medical and *para*-medical applications. When the polypropylene alloy is used as a protective film to prevent harm to medical equipment, even slight alterations of the properties of the material due to storage may negate the value of the item. The results reported here for one alloy of polypropylene demonstrate that heat alone (82°) had no significant effect upon the material up to 300 hr. Under similar conditions, except in the presence of ultraviolet rays, the alloy was rapidly destroyed as measured by (*a*) DTA, (*b*) tensile properties, and (*c*) infrared. There appears to be a need for the establishment of storage profiles for certain plastic items if the value of the item is not to be lost with subsequent injury or damage to personnel or equipment.

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Keyphrases

Polypropylene-polyisobutylene alloy—stability
Heat effect—polypropylene alloy
UV radiation effect—polypropylene alloy
Thermal analysis—differential
Tensile properties—polypropylene alloy
IR spectrophotometry—analysis.

Characterization of Complex Formation Between Small Molecules by Membrane Permeation Measurements

By RICHARD H. REUNING* and GERHARD LEVY†

A membrane permeation technique is described which permits characterization of complex formation between small molecules. The method is applicable if the components of the complex diffuse across a nonporous membrane at markedly different rates. The procedure may be used (*a*) to determine the stoichiometry and stability constant of a complex, (*b*) to verify values of stability constants obtained by other methods, (*c*) to check assumptions concerning the stoichiometry of a complex, and (*d*) to determine the degree of complexation of a compound in systems complicated by the existence of two or more simultaneous equilibria, where other methods of determination may fail or be very time consuming. The membrane permeation technique has been applied to the characterization of the complex formation between salicylamide and caffeine at concentrations where appreciable self-association of caffeine occurs.

MANY TECHNIQUES have been developed in recent years for the characterization of drug complexes. Among the methods which have been most commonly used for the analysis of drug interactions are partitioning (1), spectrophotometric (2), equilibrium dialysis (3), dialysis rate (4, 5), and solubility techniques (6). All of the methods available for the study of complex formation are subject to certain limitations peculiar to the particular technique. Some of these limitations which were encountered in the present study are: the partitioning method requires that at least one of the uncomplexed species does not partition into one of the two solvent phases; the spectrophotometric method cannot be applied to systems involving relatively weak molecular interactions and to those which do not manifest a pronounced change in spectral characteristics upon complex formation; the dialysis techniques are limited to the study of interactions between drugs which diffuse across a dialysis membrane and macromolecules which do not; the solubility method does not permit a determination of the stoichiometry of the complex with respect to the less soluble component, if the complex itself is very soluble.[1] Connors and Mollica (8) have recently pointed out that some of the experimental approaches mentioned above may not always yield the same values for an equilibrium constant and that comparative studies with several techniques may yield valuable information concerning the nature of a complex.

Complex formation between salicylamide and caffeine has been detected recently in this laboratory. The salicylamide–caffeine complexing system was used in studies concerning the effect of complex formation on drug absorption, which are

Received April 15, 1968, from the Biopharmaceutics Laboratory, Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication June 4, 1968.

This investigation was supported in part by grant RO1 AM 08753-03 PET from the U. S. Public Health Service, Bethesda, Md., and by Public Health Service Predoctoral Fellowship No. F1-GM-24,005 (for RHR) from the National Institute of General Medical Sciences.

The authors wish to thank Dr. H. B. Kostenbauder for making available the results of his extensive study of the permeation characteristics of synthetic membranes.

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reported elsewhere. Complexation of an appreciable fraction of salicylamide by caffeine requires a relatively high concentration of caffeine at which self-association of the latter occurs (9). While the solubility method could be used to characterize the interaction between salicylamide and caffeine at relatively low concentrations of caffeine, it was felt that an extrapolation of these data to higher concentrations would be unjustified in view of the self-association of caffeine at higher concentrations, and the possible formation of salicylamide–caffeine complexes of stoichiometries other than 1:1. It became desirable, therefore, to develop a technique which permits a determination of the extent of complexation of salicylamide by caffeine at the concentrations and in the buffer system actually employed in the absorption study. Advantage was taken of the fact that salicylamide permeates relatively rapidly across a nylon membrane while caffeine permeates much more slowly. This difference in permeation rates was not sufficient to determine equilibrium concentrations by procedures analogous to equilibrium dialysis. However, it was possible to estimate the extent of salicylamide complexation in a solution of salicylamide and caffeine on one side of the nylon membrane by using varying concentrations of salicylamide on the other side of the membrane, and thereby determining the direction of the concentration gradient of free salicylamide and the concentration at which this gradient is essentially zero.

EXPERIMENTAL

Membrane Permeation Procedures—One hundred and ten milliliters of a solution of 0.384% salicylamide and 2.2% caffeine in pH 5 Krebs-Henseleit Acetate Ringer solution (KHAR)[2] was placed in a 125-ml. conical flask. Ten milliliters of a solution of salicylamide (varying concentrations in KHAR) was placed in a nylon bag.[3] The bag was suspended in the solution contained in the 125-ml. flask. The flasks were agitated at 37° in a water bath shaker and 0.5-ml. samples were withdrawn from each dialysis bag after 1, 4, and 7 hr. The samples were adjusted to pH 1 with HCl and diluted with 0.1 *N* HCl as necessary. One milliliter of diluted sample was added to 5 ml. of Trinder reagent (11) and the absorbance of this solution was measured at 525 mμ using as the blank a solution consisting of 1 ml. 0.1 *N* HCl and 5 ml. Trinder reagent. It was established that caffeine, if present in the solution, does not interfere with this assay for salicylamide.

Similar experiments were carried out with caffeine alone and salicylamide alone and only the solvent inside the nylon bag. Salicylamide was assayed as described above and caffeine was assayed spectrophotometrically at 273 mμ, using KHAR, pH 5.0, as the diluent.

In order to determine the rate of permeation of caffeine in the presence of salicylamide (*i.e.*, to assess a possible difference in the permeation rate of free and complexed caffeine), nylon bags, containing only KHAR were suspended in solutions of either 0.388% caffeine or 0.388% caffeine and 0.543% salicylamide in KHAR. A sample of 0.5 ml. was removed from each nylon bag after 11 hr. of equilibration and assayed for salicylamide and caffeine. This experiment was performed in duplicate. The assays were carried out as described above except that the absorbance at 273 mμ due to salicylamide was subtracted from the total absorbance to obtain that due to caffeine. It was determined that, in the concentration range at which the measurements were carried out, the absorbances of salicylamide and caffeine were additive. Since nylon and many other materials used to make membranes take up salicylamide and other drugs from aqueous solution, it is essential that the membrane is equilibrated with the drug solution before it is used in a permeation experiment. In this study, the nylon bags were immersed in salicylamide solution (same concentration as used in the subsequent experiment) for 24 hr. prior to an experiment. There was no measurable uptake of drug when these pretreated bags were filled with fresh salicylamide solution. The time required for adequate equilibration of a membrane must be determined experimentally for each system. Failure to equilibrate the membrane may result in artifacts and possible misinterpretation of data.

Determination of the Stability Constant of the Salicylamide–Caffeine Complex by the Solubility Method—The solubility method described by Higuchi and Zuck (6) was used to determine the stability constant of the salicylamide–caffeine complex at 37° in pH 5 KHAR.

RESULTS

The results of the membrane permeation study are shown in Fig. 1. There is a pronounced difference between the diffusion rates of salicylamide and caffeine across the nylon membrane, as illustrated in the upper portion of the figure. While salicylamide diffuses across the nylon membrane quite rapidly, there was practically no diffusion of caffeine across the membrane in 7 hr. Even after 11⅔ hr. the concentration ratio (inner–outer solution) of caffeine was less than 0.02 while that of salicylamide had increased to 0.56.

The permeation rate of caffeine in the presence of a concentration of salicylamide sufficient to complex about 50% of the caffeine did not differ measurably from that of caffeine alone. It must be noted, however, that the sensitivity of the measurement was limited due to the extremely slow permeation of caffeine and the interference of salicylamide with the caffeine assay, which necessitated a rather large correction of the absorbance at 273 mμ to account for the considerably higher concentrations of salicylamide present. Thus, minor differences between the relative permeation of caffeine alone and in the presence of salicylamide could not be excluded. These initial findings demonstrated that it is possible

[2] This is similar to Krebs-Henseleit Bicarbonate Ringer solution (10) except that an equimolar amount of acetate buffer was substituted for the bicarbonate.

[3] Tomac Nylon Bags, American Hospital Supply Corp., Evanston, Ill.

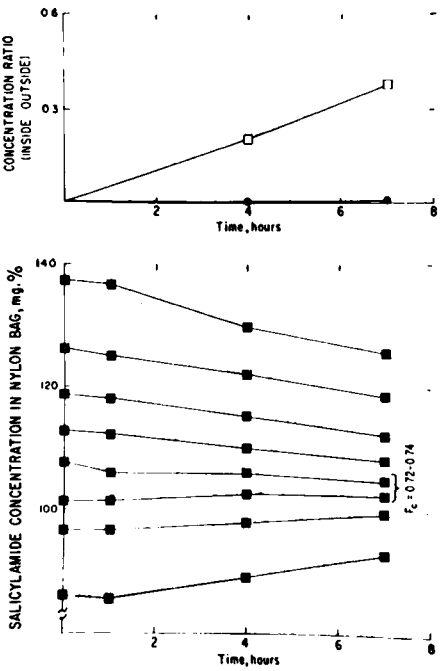

Fig. 1—Determination by the membrane permeation method of the extent of salicylamide complexation by caffeine. Upper part, ratio of drug concentration inside the nylon bag (initially only pH 5 buffer) to that outside (initially, 0.384% salicylamide or 2.2% caffeine in pH 5 buffer) as a function of time. □, salicylamide; ●, caffeine. Lower part, concentration of salicylamide inside the nylon bag as a function of time. Data are from experiments in which initially only salicylamide solution (varying concentrations) was inside the nylon bag and a solution of 0.384% salicylamide and 2.2% caffeine was outside. All data points are averages of two determinations.

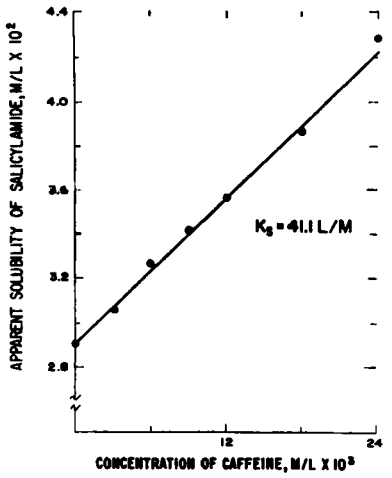

Fig. 2—Total solubility of salicylamide in pH 5 buffer at 37° as a function of total caffeine concentration. Each point represents the average of two determinations.

to maintain an essentially constant concentration of caffeine (both free and complexed) on one side of a nylon membrane for a period of time sufficient to afford considerable transfer of free salicylamide. Consequently, the series of experiments depicted in the lower portion of Fig. 1 was carried out. A solution containing 0.384% salicylamide and 2.2% caffeine was placed into conical flasks and nylon bags containing salicylamide in concentrations ranging from 0.085 to 0.135% were suspended in this solution. The concentrations of salicylamide inside the nylon bag increased or decreased with time, depending on the initial concentration, thereby reflecting the concentration gradient of uncomplexed salicylamide across the membrane. The results show that there was no significant change in the concentration of salicylamide in the solution inside the nylon bag at initial salicylamide concentrations ranging from 0.100 to 0.106%. On the basis of a total salicylamide concentration of 0.384% outside the nylon bag and a concentration of 0.100 to 0.106% of free salicylamide inside the nylon bag, it can be calculated that F_c, the fraction of total salicylamide complexed in the presence of 2.2% caffeine, ranged from 0.72 to 0.74. The value of 0.73 for F_c was used in all subsequent calculations.

The results of the solubility method as applied to the salicylamide–caffeine system are shown in Fig. 2. There was a linear increase in the apparent solubility of salicylamide with increasing concentrations of caffeine. Assuming a 1:1 stoichiometry of the salicylamide–caffeine complex, and neglecting the possibility of some slight dimerization of caffeine at these concentrations, a stability constant of 41.1 l./mole at 37° in pH 5 KHAR was obtained (12).

DISCUSSION

The membrane permeation method may be used to characterize complex formation in systems consisting of two components which exhibit rather pronounced differences in permeation rate across a suitable membrane. A major advantage of the method is that the extent of complexation of a drug or other compound can be determined directly in solutions of any desired composition, without having to determine the stoichiometry of the complex, the stability constant, or the nature of the equilibria involved. It is possible, for example, to determine the extent of drug complexation in systems involving several simultaneous equilibria as long as the drug to be studied diffuses across the membrane appreciably more rapidly than the components with which it interacts. In addition, the membrane permeation method does permit the determination of stoichiometric ratios (when these are simply 1:1, 2:1, *etc.*, and not a mixture) and stability constants in systems characterized by a single complexation equilibrium.

The membrane permeation experiments showed that the fraction of total salicylamide complexed (F_c) in a solution containing 0.384% salicylamide and 2.2% caffeine in pH 5 KHAR at 37° is 0.72–0.74. Using the stability constant obtained by the solubility method (Fig. 2) and neglecting the self-association of caffeine which is known to occur at higher concentrations of this drug (9), it can be calculated (*Reference 6* and *Appendix*) that F_c should be 0.79. A more realistic estimate of F_c can be obtained by taking into account the degree of self-association of caffeine. From the data of

Guttman and Higuchi (9) it was possible to determine a stability constant for caffeine dimerization at 37° in distilled water.[4] This constant, 8 l./mole, was used in the calculations described here since it was found that the stability constant for caffeine dimerization was not measurably different in water and in Ringer's solution, as judged by the partitioning behavior of caffeine (13). Calculations using Method II of the *Appendix*, which incorporates the stability constant of the salicylamide–caffeine complex as determined by the solubility method and the stability constant for caffeine dimerization, yielded a value for F_c of 0.67. Thus, the F_c value of 0.72–0.74 obtained by the membrane permeation method was intermediate between the values calculated from the 1:1 stability constant of the salicylamide–caffeine complex which were uncorrected, and corrected for caffeine dimerization, respectively.[5]

While the results described in the preceding paragraph are consistent with the assumption of a 1:1 stoichiometry of the salicylamide–caffeine complex, a more rigorous test of this assumption is obtained by calculating the stability constant of the complex based on a 2:1 (salicylamide–caffeine) stoichiometric ratio.[6] If the results of the solubility method and the membrane permeation method yield widely different values, a 2:1 stoichiometry can be ruled out. Values for the stability constant of the salicylamide–caffeine complex obtained by both methods assuming either a 1:1 or 2:1 stoichiometry are listed in Table I. The method of calculating the stability constants from the membrane permeation data is described in the *Appendix*. It is evident from the data in Table I that there was no agreement between the stability constants obtained by the two methods assuming a 2:1 stoichiometric ratio, but that there was reasonably good agreement of the stability constants based on a 1:1 ratio. The complexity and multiplicity of the simultaneous equilibria involved are probably responsible for the lack of a very close agreement between the 1:1 stability constant obtained by the solubility method at low caffeine concentration and that obtained by the membrane permeation method at high caffeine concentration.

A more detailed understanding of the applicability of the membrane permeation technique for distinguishing between a 1:1 and a 2:1 stoichiometry of a complex can be obtained by a review of Fig. 3, which is a theoretical plot of the fraction of total salicylamide complexed (F_c) as a function of total salicylamide concentration, with the total caffeine concentration held constant at 0.113 moles/l. (2.2%). The plots were constructed by assuming complete solubility of the complex in the concentration range shown, and a stoichiometric ratio of 2:1 and 1:1, respectively. The curves shown in the figure were calculated by procedures

TABLE I—STABILITY CONSTANTS OF THE SALICYLAMIDE–CAFFEINE COMPLEX FROM MEMBRANE PERMEATION AND SOLUBILITY MEASUREMENTS ASSUMING 1:1 OR 2:1 STOICHIOMETRY

Method	$K_{1:1}$, l./mole	$K_{2:1}$, l.²/mole²
Membrane permeation, uncorrected	29	17×10^2
Membrane permeation, corrected for caffeine dimerization	53	33×10^2
Solubility	41	4.4×10^2

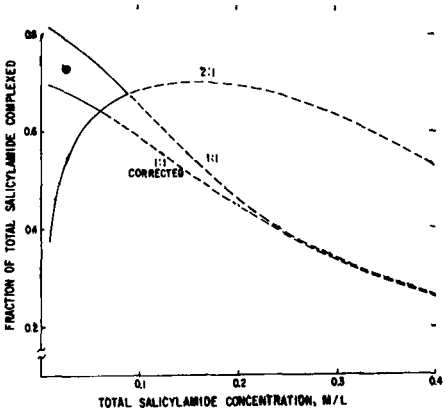

Fig. 3—Theoretical plot of the fraction of total salicylamide complexed (F_c) as a function of total salicylamide concentration. F_c was calculated from the stability constant determined by the solubility method. Numbers in the figure refer to assumed stoichiometric ratios (salicylamide–caffeine). The term "corrected" refers to a correction of the calculation of F_c which takes into account the dimerization of caffeine. Total caffeine concentration was held constant at 0.113 moles/l. The dashed portions of the curves indicate extrapolations of the theoretical calculations beyond the solubility limit of uncomplexed salicylamide. The solid circle represents the experimental value determined directly by the membrane permeation technique.

outlined in the *Appendix* and are based on the stability constants listed in Table I which were obtained by the solubility method. It is apparent that the most pronounced difference in F_c values occurs at the low salicylamide concentrations and that the curves converge as the salicylamide concentration is increased. These curves intersect (*i.e.*, the F_c values are equal) when the solution is saturated with respect to free salicylamide.[7] This is consistent with the fact that the solubility method cannot distinguish between a 1:1 and a 2:1 stoichiometry of an *A-B* complex on the basis of the total solubility of *A* (12). As a matter of theoretical interest, the curves were drawn beyond the solubility limit. The continuing decline of F_c with increasing concentration of total salicylamide reflects the decreasing availability of caffeine for complex formation. Of interest, and perhaps surprising, is the fact that F_c initially increases with total salicylamide concentration in the case of a hypothetical stoichiometric ratio of

[4] The stability constant for caffeine dimerization at 37° was determined by a graphical extrapolation of the constants reported by Guttman and Higuchi (9) at 0, 20, 30, and 35°.

[5] The small difference between the experimental results and those predicted from theoretical calculations based on a 1:1 stoichiometric ratio and corrected for caffeine dimerization cannot be ascribed to the fact that tetramer formation of caffeine was neglected in the calculations. Correction for the latter would actually increase somewhat the difference between the experimental and theoretical results.

[6] The linearity of the solubility phase diagram (Fig. 2) indicates that the stoichiometry with respect to the caffeine component is 1 in this concentration range (12).

[7] Actually, since the curve representing a 2:1 stoichiometric ratio is not corrected for caffeine dimerization, there is a slight difference between the actual solubility limit and the intersection of the "1:1 corrected" curve with the "2:1" curve.

2:1. This reflects, apparently, the fact that the concentration of salicylamide is the limiting factor initially and that caffeine becomes limiting only after an appreciable extent of caffeine complexation is attained. The most important conclusion to be derived from the relationships shown in Fig. 3 is that a distinction between 2:1 and 1:1 stoichiometry can be made most readily at the lower concentrations of salicylamide and that the sensitivity of the membrane permeation method for making such a distinction decreases as the solubility limit of salicylamide is approached. The data point in Fig. 3, which represents the results of the membrane permeation experiments, shows that the determination of F_c for salicylamide was carried out at a concentration at which a clear distinction between a 1:1 and a 2:1 stoichiometric ratio can be made.

APPENDIX

Calculation of the Extent of Complexation of Compound A by Compound B Where B is Subject to Dimerization[8]—The extent of complexation of Compound A by Compound B can be represented by F_c, the fraction of total A which is complexed. The following three methods may be used to determine F_c at relatively low concentrations of B (where dimerization is negligible) and at relatively high concentrations of B (where appreciable dimerization occurs).

Method I—Negligible dimerization of B, 1:1 stoichiometry of the complex.

$$A + B \rightleftharpoons A\text{-}B$$

The designation A-B refers to the complex of A with B. The 1:1 stability constant ($K_{1:1}$) of this equilibrium is defined as:

$$K_{1:1} = \frac{[A\text{-}B]}{[A][B]} \qquad \text{(Eq. 1)}$$

where the brackets refer to molar concentrations. F_c, the fraction of total A in solution which is complexed, is defined as follows:

$$F_c = \frac{[A\text{-}B]}{[A] + [A\text{-}B]} \qquad \text{(Eq. 2)}$$

Equations 1 and 2 can be combined to give the following expression:

$$F_c = \frac{K_{1:1}[B]}{K_{1:1}[B] + 1} \qquad \text{(Eq. 3)}$$

In order to use Eq. 3 to calculate F_c, the concentration of free B must be determined. The total concentrations of A species and B species, A_t and B_t, respectively, are defined as:

$$A_t = [A] + [A\text{-}B] \qquad \text{(Eq. 4)}$$

$$B_t = [B] + [A\text{-}B] \qquad \text{(Eq. 5)}$$

A substitution of Eqs. 4 and 5 into Eq. 1 yields upon rearrangement:

$$K_{1:1}[B]^2 + (1 + K_{1:1}A_t - K_{1:1}B_t)[B] - B_t = 0 \qquad \text{(Eq. 6)}$$

The quadratic formula applied to Eq. 6 then can be used to calculate $[B]$. This in turn permits the calculation of F_c by use of Eq. 3.

[8] Applied to the present study, A denotes salicylamide and B represents caffeine.

Method II—Partial dimerization of B, 1:1 stoichiometry of the complex. Assuming that appreciable dimerization of B occurs, the following simultaneous equilibria are applicable:

$$A + B \rightleftharpoons A\text{-}B$$

$$B + B \rightleftharpoons B\text{-}B$$

where B-B refers to the dimer. The stability constant of the dimer (K_d) is defined as follows:

$$K_d = \frac{[B\text{-}B]}{[B]^2} \qquad \text{(Eq. 7)}$$

As in the case of negligible dimerization of B, F_c can be calculated from Eq. 3. However, $[B]$ must now be determined on the basis of the following relationship:

$$B_t = [B] + 2\,[B\text{-}B] + [A\text{-}B] \qquad \text{(Eq. 8)}$$

This definition of B_t, when combined with Eqs. 1, 4, and 7, yields upon rearrangement:

$$(2K_{1:1}K_d)[B]^3 + (K_{1:1} + 2K_d)[B]^2 + (1 + K_{1:1}A_t - K_{1:1}B_t)[B] - B_t = 0 \qquad \text{(Eq. 9)}$$

Thus, a knowledge of the stability constants of the two simultaneous equilibria ($K_{1:1}$ and K_d) and the total concentrations of A species and B species (A_t and B_t, respectively) allows calculation of $[B]$ from this cubic equation. Methods of solving this type of equation are available[9] (14) and consequently F_c can be calculated by use of Eq. 3.

Method III—Negligible dimerization of B, 2:1 stoichiometry of the complex.

$$2A + B \rightleftharpoons A_2\text{-}B$$

In this equilibrium, the symbol A_2-B represents the 2:1 complex. The 2:1 stability constant is defined as follows:

$$K_{2:1} = \frac{[A_2\text{-}B]}{[A]^2[B]} \qquad \text{(Eq. 10)}$$

In this case the fraction of total A in solution which is complexed (F_c) is defined as:

$$F_c = \frac{2[A_2\text{-}B]}{[A] + 2[A_2\text{-}B]} \qquad \text{(Eq. 11)}$$

Equations 10 and 11 can be combined to give the following expression for F_c:

$$F_c = \frac{2K_{2:1}[A][B]}{1 + 2K_{2:1}[A][B]} \qquad \text{(Eq. 12)}$$

In order to use Eq. 12 it is necessary to determine $[A]$ and $[B]$ from known or experimentally determinable factors. A_t and B_t are defined for this case as follows:

$$A_t = [A] + 2[A_2\text{-}B] \qquad \text{(Eq. 13)}$$

$$B_t = [B] + [A_2\text{-}B] \qquad \text{(Eq. 14)}$$

The following expression for A can be obtained by combining Eq. 10 with Eqs. 13 and 14 and rearranging:

$$0.5K_{2:1}[A]^3 + (K_{2:1}B_t - 0.5K_{2:1}A_t)[A]^2 + 0.5[A] - 0.5A_t = 0 \qquad \text{(Eq. 15)}$$

[9] A digital computer was used in this study to solve the cubic equation.

Solution of this cubic equation yields the value of $[A]$. The value of $[B]$ can be determined from the following two relationships:

$$[A_2\text{-}B] = \frac{A_t\text{-}[A]}{2}$$

$$[B] = B_t - [A_2\text{-}B]$$

With $[A]$ and $[B]$ thus calculated, F_c can now be determined using Eq. 12.

Calculation of Stability Constants from Membrane Permeation Data—The calculation of the stability constants from membrane permeation data is shown here for the case where the calculations are corrected for the dimerization of B. The calculations which are not corrected are merely a simplification of these. The symbols used are the same as those in the previous section.

Complex of 1:1 Stoichiometry—The membrane permeation method provides a value for the concentration of uncomplexed A, $[A]$. The total concentrations A_t and B_t are known. From these values the stability constant ($K_{1:1}$) may be calculated as follows:

$$[A\text{-}B] = A_t - [A]$$

$$[B] = B_t - [A\text{-}B] - 2[B\text{-}B] = B_t - [A\text{-}B] - 2K_d[B]^2$$

$$2K_d[B]^2 + [B] - B_t + [A\text{-}B] = 0$$

The value of $[B]$ can be determined by using the quadratic formula. Then:

$$K_{1:1} = \frac{[A\text{-}B]}{[A][B]}$$

Complex of 2:1 Stoichiometry—Again the membrane permeation experiment provides a value for $[A]$. Then:

$$[A_2\text{-}B] = \frac{A_t\text{-}[A]}{2}$$

$$[B] = B_t - [A_2\text{-}B] - 2[B\text{-}B] = B_t - [A_2\text{-}B] - 2K_d[B]^2$$

$$2K_d[B]^2 + [B] + [A_2\text{-}B] - B_t = 0$$

The determination of $[B]$ then permits calculation of the stability constant:

$$K_{2:1} = \frac{[A_2\text{-}B]}{[A]^2[B]}$$

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Keyphrases

Complex formation—small molecules
Membrane permeation, nylon—complex formation determination
Salicylamine-caffeine—complexing system
Diffusion rates—salicylamide, caffeine
Colorimetric analysis, salicylamide—spectrophotometer
UV spectrophotometry—analysis, caffeine
Stability constants—equations

Imidazoles V

5(or 4)-(3-Alkyl-3-methyl-1-triazeno)imidazole-4(or 5)-carboxamides

By Y. FULMER SHEALY, CHARLES A. KRAUTH*, SARAH J. CLAYTON, ANITA T. SHORTNACY, and W. RUSSELL LASTER, Jr.

Several 5(or 4)-(3-alkyl-3-methyl-1-triazeno)imidazole-4(or 5)-carboxamides (III–X) were prepared from 5-diazoimidazole-4-carboxamide and the appropriate *N*-alkyl methylamine. Most of these derivatives significantly increased the life span of leukemic (L1210) mice. Inhibition of the growth of sarcoma 180, adenocarcinoma 755, and Walker carcinosarcoma 256 was observed, but inhibition of solid tumors was generally accompanied by large, adverse, host-weight changes. Qualitative observations of the sensitivity to light of aqueous alcoholic solutions of the dimethyltriazeno (NSC-45388) and the butylmethyltriazeno derivatives indicate a pronounced variation in stability with the kind of light exposure.

REPRESENTATIVES OF BOTH disubstituted-triazeno and monosubstituted triazeno imidazole-4(or 5)-carboxamides have been previously reported (1, 2), and the modes of dissociation of the simpler members of these two groups have been shown to differ. The substituents on the triazeno group included straight-chain alkyl, cyclic alkyl, aralkyl, aryl, and functionally substituted alkyl and aryl groups, and the number of carbon atoms in the straight-chain groups was doubled in a few representatives in order to perform a preliminary sampling of alkyl groups for antineoplastic effectiveness. Among the disubstituted-triazeno derivatives, 5(or 4)-(3,3-dimethyl-1-triazeno)imidazole-4(or 5)-carboxamide (I, NSC-45388) demonstrated the most interesting activity against mouse neoplasms (3) and is currently undergoing clinical trial (4, 5). Likewise, the monomethyltriazeno derivative (II), despite its instability, was the most effective of the monosubstituted triazeno derivatives in initial evaluations (2). The methyl ester corresponding to I (6) and the *v*-triazole analog of I (7) were also inhibitory to transplanted mouse neoplasms, and among some phenyltriazenes, association of activity with the presence of a methyl group had also been noted (8, 9). For these reasons several 5(or 4)-(3-alkyl-3-methyl-1-triazeno)imidazole-4(or 5)-carboxamides (III–X) were prepared and are recorded in this report. Relevant to the structure-activity question, however, is the fact that the bis(2-chloroethyl)triazeno derivative (10, 11), prepared subsequently to these compounds, is the most active of the triazenoimidazoles *versus* mouse lymphoid leukemia L1210.

The method of preparation, the reaction of 5-diazoimidazole-4-carboxamide with an appropriate amine, was described earlier (1). Subsequently, the yield of the dimethyltriazene (I) was improved considerably by employing ethyl acetate as the solvent, and this modification was used to prepare the new 3-alkyl-3-methyl-1-triazeno derivatives. However, 2-azahypoxanthine (XI), a potential by-product, is less soluble in this solvent and is more likely to contaminate the initially precipitated product. 2-Azahypoxanthine can be removed by washing the crude products with water and by recrystallizing them from methanol. Contamination of the disubstituted triazenes by 2-azahypoxanthine can be detected by thin-layer chromatography (TLC) if proper precautions are taken to prevent the artifactual formation of 2-azahypoxanthine *via* light-catalyzed decomposition of the triazenes during chromatography.

Previously, the light-catalyzed decomposition of several disubstituted triazeno derivatives, as well as the concomitant formation of 2-azahypoxanthine, was demonstrated by ultraviolet spectroscopy (1). The light wavelengths (or other conditions) responsible for this instability have not been defined precisely, but by TLC it has been shown that 2-azahypoxanthine forms quite rapidly when a 50% aqueous alcohol solution of the dimethyltriazene is exposed to sunlight (filtered through glass) or to an ultraviolet lamp emitting principally at 365 mμ. 2-Azahypoxanthine was not detected by TLC in similar solutions during the first 4 days of exposure to incandescent lighting or during the first day of exposure to fluorescent lighting, but a small amount was detectable after longer periods.

Received May 20, 1968, from Southern Research Institute, Birmingham, AL 35205
Accepted for publication June 18, 1968.
This work was supported by contract PH43-64-51 from the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md, and by the C. F. Kettering Foundation.
Previous papers in this series: Part III, *Reference 6*; Part IV, *Reference 2*.
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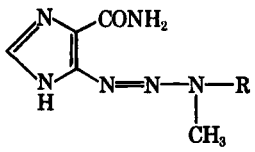
Exposure of solutions of the butylmethyltriazene (IV) to sunlight and to incandescent lighting gave similar results.

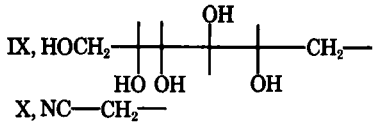


R

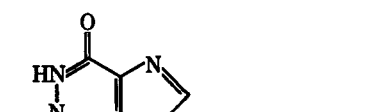
I, CH_3
II, H
III, C_3H_7
IV, *n*-C_4H_9
V, *iso*-C_4H_9

R

VI, *n*-C_5H_{11}
VII,
VIII, $HOCH_2CH_2$—



IX, $HOCH_2$—



X, NC—CH_2—

XI

Antitumor Evaluation—When compounds III–VII were administered daily according to the standard protocol (12), they significantly increased the life span (T/C values of 125–170%) of L1210-bearing mice at doses within the range of 25–75 mg./kg./day (Table I). The hydroxyethyl derivative (VIII) was less effective at 75 mg./kg./day, but it showed significant activity at a considerably higher dose. The optimum dose is presumed to be intermediate (and somewhat higher than the optimum doses of III–VII). Administration of larger single doses of III–VII on the second day after implanting the animals with 10^5 L1210 cells also significantly increased the survival times of the treated animals (T/C values of 125–165%, see Table II). In some of these tests, evidence of toxicity was manifested by a large difference between the treated and control groups in the average host-weight change. When an increase in the survival time of treated mice was accompanied by a large weight loss, it was not possible to decide from these tests whether the enhancing effect on survival time of killing large numbers of leukemic cells was partly cancelled by the depressing effect of toxicity to the host.

The effectiveness of these compounds against solid rodent tumors is not unequivocal because of the adverse host-weight change of treated animals in many of the tests. Most of the previously undescribed compounds (Table III), as well as some of those reported earlier (1), inhibited the growth of sarcoma 180 (S180) and adenocarcinoma 755 (Ca755) to 50% or less of the average weights of untreated tumors without causing host deaths in excess of permissible limits (12). At doses administered in accordance with protocols (12) in effect when S180, Ca755, and L1210 (QD 1–30) constituted the primary screen of the Cancer Chemotherapy National Service Center, the previously described disubstituted triazeno derivatives (1, *cf.* Table IV) lacking a methyl substituent were not active *versus* L1210.[1] Two compounds (V and VII) employed in limited testing *versus* intramuscular Walker carcinosarcoma 256 (W256IM) in rats effectively inhibited tumor growth. However, in most of the solid tumor tests showing inhibition of tumor growth, the treated animals suffered large weight losses, and drug-induced host inanition (13, 14), therefore, may account for much of the observed inhibition of growth of solid tumors. It has been shown (15, 16) that the effectiveness of an anticancer drug may be increased by arriving at an optimum relationship between dose size and frequency of administration, and it is conceivable that further variation in dose size and frequency of administration of these compounds might decrease the chronic toxicity manifested as host weight loss.

EXPERIMENTAL[2]

5(or 4)-(3-Alkyl-3-methyl-1-triazeno)imidazole-4(or 5)-carboxamides—*Preparation*—During all operations involved in the preparation and purification of compounds III–X, they, as well as the starting material, were shielded from light in solution and in the solid state. The following example is typical of the procedures used.

To a solution that was being stirred under a current of nitrogen and that consisted of 60 ml. of *N*-methylpentylamine and 480 ml. of ethyl acetate was added, portionwise during a 1-hr. period, 12 g. of 5-diazoimidazole-4-carboxamide (1, 17). Vigorous stirring was continued for 2 hr.; and the precipitate was then collected by filtration, washed with ethyl acetate, and dried *in vacuo*. The crude product (18.5 g., 88%), shown by TLC to contain some 2-azahypoxanthine (XI), was suspended in 400 ml. of water, the pH was adjusted to 9–10 with 1 *N* NaOH, and the resulting mixture was stirred for 10 min. The solid was collected on a filter, washed well with water, dried *in vacuo;* wt. 16.0 g. (76%). TLC showed that the product was free of XI. Recrystallization of 15.6 g. from methanol with the aid of activated-carbon decolorization afforded 9.9 g. (47% yield) of yellowish white crystals (in two crops) of pure VI.

Compounds III–V and VII–VIII were prepared

[1] The pyrrolidinylazo derivative gave a T/C value of 126% in a single (unconfirmed) test at 250 mg./kg./day.

[2] The authors are indebted to Drs. W. J. Barrett, W. C. Coburn, Jr., and P. D. Sternglanz of the Analytical and Physical Chemistry Division of this Institute for spectral determinations and elemental analyses and to Miss Kathleen Hewson and Mrs. Mary B. Emory for TLC determinations.

TABLE I—DAILY TREATMENT OF L1210 WITH III–X[a]

Compd. No.	Compd. R	Dose, mg./kg./day	Mortality,[a] Deaths/ Total	Av. Wt. Change, g., T/C[a]	Av. Survival Time, Days, T/C	T/C Ratio, %
III	*n*-Propyl	75	0/6	−1.7/+1.1	11.0/8.1	135
		50	0/6	−2.0/+1.1	12.8/8.1	158
		38	0/6	−0.9/+1.1	12.3/8.1	151
IV	*n*-Butyl	120	0/6	−3.1/+1.3	8.8/8.7	101
		80	0/18	−1.6/+1.2	14.0/8.4	167[b]
		40	0/6	−0.9/+1.3	14.3/8.7	164
		20	0/6	−0.1/+1.3	10.8/8.7	124
V	Isobutyl	100	0/6	−3.2/+0.5	9.0/8.3	108
		80[c]	0/6	−2.7/+1.3	10.0/9.1	109
		75	0/12	−1.3/+1.9	13.7/8.7	157[d]
		50	0/12	−0.4/+1.8	12.8/8.6	149[d]
		48[c]	0/6	−0.6/+1.3	12.5/9.1	137
		37	0/6	−0.4/+2.0	15.3/9.0	170
		28[c]	0/6	−1.4/+1.3	11.5/9.1	126
		25	0/6	+0.2/+1.9	12.3/8.5	144
		12.5	0/6	+0.2/+1.9	10.8/8.5	127
VI	*n*-Pentyl	200[c]	0/6	−3.2/+1.3	7.5/9.1	82t[e]
		120[c]	0/6	−3.4/+1.3	8.8/9.1	96
		100	0/6	−2.5/+1.3	13.7/8.6	159
		75	0/6	−2.6/−0.6	10.8/9.2	117
		72[c]	0/6	−2.2/+1.3	11.7/9.1	128
		50	0/6	−3.5/−0.6	11.7/9.2	127
		42[c]	0/6	−2.0/+1.3	11.7/9.1	128
		25	0/6	−1.4/−0.6	12.0/9.2	130
		15	0/6	+0.3/+1.9	10.5/8.8	119
		7.5	0.6	+0.8/+1.9	9.0/8.8	102
VII	Cyclohexyl	153[c]	0/6	−1.9/+1.0	9.2/7.7	83t[e]
		100	0/6	−1.8/+0.7	11.0/8.1	135
		90[c]	0/18	−1.4/+1.2	10.6/8.9	118[b]
		75	0/6	−1.1/+1.3	14.8/8.9	166
		54[c]	2/12	−1.4/+1.4	11.1/8.8	126[b]
		50	0/6	−0.5/+0.7	12.2/8.1	150
		32[c]	0/12	−0.9/+1.4	10.7/8.8	121[b]
		25	0/6	−0.6/+0.7	9.3/8.1	114
VIII	2-Hydroxyethyl	400[c]	0/6	−0.5/+1.2	12.8/9.3	137
		75	0/6	0.0/+1.3	10.7/8.9	120
IX	Pentahydroxyhexyl	200	0/6	+0.4/+1.1	8.2/9.1	90
		100	0/6	+0.9/+1.2	7.8/7.8	100
X	Cyanomethyl	150[f]	0/6	−1.3/+1.3	9.5/9.1	104
		100	0/6	−2.1/+0.5	9.7/8.3	116
		75	0/6	+0.4/+1.5	8.8/8.2	107
		50	0/6	+0.1/+1.5	9.7/8.2	118
		37.5	0/6	+0.5/+1.1	8.5/8.3	102
		25	0/5	+1.8/+1.1	8.4/8.3	101

[a] QD 1–30 days or to death unless otherwise stated. Mice implanted intraperitoneally with 10^6 L1210 cells. T = treated mice, C = control (untreated leukemic) mice. Av. wt. change = average weight change of host animals. Mortality and weight changes determined after the first 4 days of treatment (5 days after implantation). [b] Values in the last 3 columns are averages of 3 experiments. [c] QD 1–15 days or to death. [d] Values in the last 3 columns are averages of 2 experiments. [e] t = toxic. T/C below 85% considered to be caused by toxic dose. [f] QD 1–11 days or to death.

TABLE II—SINGLE-DOSE TREATMENT OF L1210 WITH III–VIII[a]

Compd. No.	Compd. R	Dose mg./kg.	Mortality, Deaths/ Total	Av. Wt. Change, g., T/C	Av. Survival Time, Days, T/C	T/C Ratio, %
III	*n*-Propyl	400	1/4	−2.9/+1.4	7.3/8.7	83t
		300	0/6	−4.1/+1.7	11.0/9.4	117
		200	0/10	−3.4/+1.6	13.0/9.1	143[b]
		133	0/6	−2.4/+1.7	11.7/9.4	124
		100	0/4	−1.1/+1.4	11.0/8.7	126
		89	0/6	−1.3/+1.7	11.0/9.4	117
IV	*n*-Butyl	400	0/6	−4.3/+3.1	11.3/9.2	122
		200	0/6	−2.5/+3.1	13.0/9.2	141
		100	0/6	−0.4/+3.1	11.0/9.2	119
V	Isobutyl	600	6/6			
		400	1/4	−3.9/+4.2	12.7/8.4	151t
		400	3/6	−4.1/+3.1	14.3/9.5	150t
		267	0/6	−2.9/+3.1	12.5/9.5	131
		200	0/4	−0.6/+4.2	10.3/8.4	122
		100	0/4	+1.6/+4.2	9.8/8.4	116

(*Continued on next page.*)

TABLE II (*Continued.*)

Compd. No.	Compd. R	Dose, mg./kg.	Mortality, Deaths/ Total	Av. Wt. Change, g., T/C	Av. Survival Time, Days, T/C	T/C Ratio, %
VI	*n*-Pentyl	600	0/6	−4.1/+3.1	12.2/9.5	128
		400	0/10	−2.8/+3.6	14.9/9.0	165[b]
		267	0/6	−1.9/+3.1	13.3/9.5	140
		200	0/4	−1.0/+4.2	12.8/8.4	152
		178	0/6	−2.1/+3.1	11.2/9.5	117
		100	0/4	+0.9/+4.2	10.0/8.4	119
VII	Cyclohexyl	600	0/12	−1.4/+2.7	12.2/9.4	129[b]
		400	0/16	−1.5/+3.2	11.8/9.1	130[c]
		267	0/12	−1.2/+2.7	11.5/9.4	122[b]
		200	0/4	−0.1/+4.2	10.8/8.4	128
		178	2/12	−1.8/+2.7	11.2/9.4	119[b]
		100	0/4	+0.8/+4.2	10.5/8.4	125
VIII	2-Hydroxyethyl	400	0/4	+1.6/+4.2	9.0/8.4	107
		200	0/4	+2.6/+4.2	9.5/8.4	113
		100	0/4	+3.1/+4.2	9.0/8.4	107

[a] Mice implanted intraperitoneally with 10^5 L1210 cells. T = treated mice, C = control mice. Av. wt. change = average weight change of host animals. Mortality and weight changes determined after the first 4 days of treatment (5 days after implantation). t = toxic (see *Footnote e*, Table II). [b] Values in the last 3 columns are averages of 2 experiments. [c] Values in the last three columns are averages of 3 experiments.

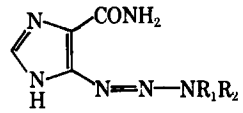
TABLE III—5 (OR 4)-(3-ALKYL-3-METHYL-1-TRIAZENO)IMIDAZOLE-4 (OR 5)-CARBOXAMIDES[a]

Compd. No.	Compd. R	Yield[b]	Formula	Anal. Calcd.	Anal. Found	Ultraviolet Absorption λ_{max}	Ultraviolet Absorption $\epsilon \times 10^{-3}$	Ultraviolet Absorption Solvent[c]
III	C_3H_7	57	$C_8H_{14}N_6O$	C, 45.70	45.63	236	12.4	CH_3OH
				H, 6.71	6.54	327	18.1	
				N, 39.97	40.24	237	11.7	Buffer
						331	18.2	
IV	*n*-C_4H_9	60	$C_9H_{16}N_6O$	C, 48.20	48.44	237	11.6	Buffer
				H, 7.19	7.16	331	18.3	
				N, 37.48	37.73	249	13.4	Base
						345	16.2	
V	*iso*-C_4H_9	54	$C_9H_{16}N_6O$	C, 48.20	48.33	237	11.7	Buffer
				H, 7.19	7.36	331	18.8	
				N, 37.48	37.70	249	13.6	Base
						345	16.8	
VI	*n*-C_5H_{11}	47	$C_{10}H_{18}N_6O$	C, 50.41	50.67	238	11.7	Buffer
				H, 7.61	7.41	331	18.6	
				N, 35.26	35.25	249	13.6	Base
						345	16.5	
VII		43	$C_{11}H_{18}N_6O$	C, 52.78	52.74	238	11.2	Buffer
				H, 7.25	6.98	333	19.4	
				N, 33.58	33.84	249	13.4	Base
						345	17.2	
VIII	$HOCH_2CH_2$—	35	$C_7H_{12}N_6O_2$	C, 39.62	39.56	237	11.7	Buffer
				H, 5.70	5.65	329	17.3	
				N, 39.61	39.45	248	13.3	Base
						344	15.6	
IX	$HOCH_2(CHOH)_4$—CH_2—	25	$C_{11}H_{20}N_6O_6$	C, 39.75	39.84			
				H, 6.07	6.02			
				N, 25.29	24.96			
X	$NCCH_2$—	55	$C_7H_9N_7O$	C, 40.57	40.72			
				H, 4.38	4.25			
				N, 47.32	47.11			
I[d]	CH_3					237	12.2	Buffer
						329	18.0	
						248	13.9	Base
						344	15.9	

[a] When these triazenes are heated in a capillary tube or on a Kofler-Heizbank apparatus, they decompose with intense darkening and evolution of gas. The decomposition temperatures vary markedly with the temperature at which heating is started and with the rate of heating. Decomposition at high temperatures may be explosive. [b] Yield of analytically pure product. [c] Buffer = phosphate buffer, pH 7. Base = 0.1 *N* NaOH. All of these derivatives produced absorption maxima in 0.1 *N* HCl at 223–226, *ca.* 275 (sh), and 323–327 mμ. Solutions for UV spectra were prepared by dissolving the sample in methanol and diluting an aliquot (5 ml. or less) to 50 ml. with the designated solvent. All spectra were recorded with a Cary model 14 recording spectrophotometer. [d] *Reference 1.*

and purified similarly. Compound IX was prepared by adding 5 g. of the diazoimidazolecarboxamide to a solution of 21.4 g. of 1-deoxy-1-(methylamino)-glucitol in 1.5 l. of methanol and stirring the mixture for 24 hr. The precipitate was not purified further. Methylaminoacetonitrile for the preparation of X was extracted with two 250-ml. portions of chloroform from a solution consisting of 50 g. of the amine

TABLE IV—EVALUATION OF III–X AND RELATED COMPOUNDS *versus* SOLID TUMORS[a]



No.	R_1	R_2	Tumor	Dose, mg./kg./day	Mortality, Deaths/Total	Av. Wt. Change, g., T/C	Av. Tumor Wt.,[b] T/C	T/C Ratio, %
III	*n*-C_3H_7	CH_3	S180	91	3/6	−3.7/+1.4	618/1826	t
				62	1.6	−3.4/+1.4	625/1826	34
				46	0/6	−2.2/+1.4	678/1826	37
				31	1/6	−3.3/+1.4	1018/1826	55
			Ca755	50	5/10	−3.4/−1.1	128/1169	t
				40	1/10	−2.5/−1.1	544/1169	37
				30	0/10	−2.7/+1.6	341/1129	30
				20	3/10	−0.9/+1.6	441/1129	39
				15	1/10	−1.3/+1.6	539/1129	47
				10	1/10	+0.2/+1.6	581/1129	51
IV	*n*-C_4H_9	CH_3	S180	187	6/6			t
				125	4/12	−5.8/+1.1	212/1142	19[c]
				62	0/6	−5.0/+2.5	361/1148	31
				31	0/6	−1.3/+2.5	510/1148	44
			Ca755	90	5/10	−4.7/+2.9	94/1392	t
				75	2/10	−3.1/+3.1	77/1476	5
				60	1/10	−4.4/+2.9	112/954	11
				37	0/10	+0.1/+3.1	699/1476	47
				18	0/10	+1.9/+3.1	1264/1476	85
V	*iso*-C_4H_9	CH_3	S180	75	0/7	−6.5/−1.0	509/1774	28
				50	0/7	−3.1/+1.4	544/1323	41
				25	0/7	−3.4/+1.4	1222/1323	92
			W256IM	200	1/12	−13/+16	0.8/8.7	9[c]
VI	*n*-C_5H_{11}	CH_3	S180	150	3/6	−3.7/+0.4	230/1434	t
				75	2/12	−4.7/+0.2	419/1411	30[c]
				50	0/12	−4.2/+0.2	654/1411	46[c]
			Ca755	100	6/10	−3.9/+6.1	173/2499	t
				50	0/10	−2.5/+3.9	1212/2814	43
VII		CH_3	S180	75	0/7	−4.3/+1.2	673/1353	49
			Ca755	200	10/10			t
				100	3/32	−2.9/+4.8	568/2440	24[d]
				50	0/10	+0.3/+3.9	2347/2814	83
			W256IM	400	2/6	−17/+17	0/4.2	0
				200	1/18	−8/+18	0.6/7.1	8[d]
				100	0/6	−4/+17	0.3/4.2	7
				50	0/6	+9/+17	2.2/4.2	52
VIII	$HOCH_2CH_2$—	CH_3	S180	250	1/12	−1.5/+0.1	706/1056	67[c]
				125	0/6	−2.4/0.0	788/1078	73
			Ca755	375	5/10	−4.2/+3.1	10/1523	t
				250	0/10	−1.9/+3.7	72/1146	6
				187	0/10	+0.2/+3.1	396/1523	26
				93	0/10	+2.1/+3.1	871/1523	57
IX	$C_6H_{13}O_5$	CH_3	S180	300	0/6	+1.0/+1.0	1486/1006	147
				200	0/6	+2.4/+2.0	1450/1529	94
X	NC—CH_2—	CH_3	S180	300	3/6	−6.0/−0.3	165/1231	t
				225	1/6	−3.3/−0.3	469/1231	38
				150	1/6	−2.1/+1.1	725/940	77
			Ca755	300	10/10			t
				150	4/10	−1.8/+2.4	578/1502	t
				150	0/10	0.0/+3.0	293/703	41
				75	0/10	+0.9/+0.8	748/1018	73
e	C_6H_5	CH_3	S180	250	1/6	−1.7/−0.3	374/919	40
				125	1/6	−0.7/−0.7	353/753	46
				62	0/6	−1.7/−0.7	749/753	99
			Ca755	200	5/10	−4.1/+1.7	107/779	t
				100	0/10	−2.7/+2.2	406/992	40
e	C_2H_5	C_2H_5	S180	250	4/6	−2.1/−1.1	763/1014	75
				125	0/6	−4.1/−0.1	398/758	52
				62	0/6	−1.2/+2.2	620/603	102
e	C_4H_9	C_4H_9	S180	500	6/6			t
				250	0/7	−2.5/+1.1	525/1227	42
			Ca755	375	6/10	−2.0/+2.5	144/1539	t
				250	0/10	−1.9/+1.2	602/838	71
e	C_8H_{17}	C_8H_{17}	S180	500	0/6	+0.4/−0.1	1115/1066	104

(*Continued on next page.*)

TABLE IV (*Continued.*)

No.	R_1	R_2	Tumor	Dose, mg./kg./day	Mortality, Deaths/Total	Av. Wt. Change, g., T/C	Av. Tumor Wt.,[b] T/C	T/C Ratio, %
e	$C_6H_5CH_2$—	$C_6H_5CH_2$—	S180	250	0/6	−2.5/−0.3	250/879	28
				125	2/6	−1.1/−0.3	565/919	61
				62	1/7	+0.3/+1.1	735/1227	59
				31	1/7	−0.9/+1.1	606/1227	49
			Ca755	500	2/10	+1.6/+1.4	771/614	125
				250	0/10	−0.1/+2.3	896/1253	71
e	R_1R_2N = 2-pyrrolidinyl		S180	250	0/6	+1.0/+1.7	919/857	107
				125	0/6	+2.5/+1.7	632/857	73
			Ca755	500	4/10	−1.0/+0.9	205/654	t
				250	1/10	−1.6/+0.2	611/782	78
e	R_1R_2 = 2-piperidino		S180	500	6/6			t
				250	1/6	+0.2/+1.8	1387/2108	65
			Ca755	375	4/10	−2.4/+0.9	291/654	t
				250	0/10	+3.0/+4.6	991/1838	53
				250	3/10	−0.3/+1.4	885/1055	83
				188	0/10	+1.6/+3.4	743/1660	44

[a] T = treated animals, C = control animals, t = toxic. Avg. wt. change = average weight change of host animals. Mortality and weight changes determined at the end of the tests. [b] Average tumor weights in milligrams for S180 and Ca755 and in grams for W256IM. [c] Values in the last 3 columns are averages of 2 experiments. [d] Values in the last 3 columns are averages of 3 experiments. [e] *Reference 1.*

hydrochloride, 250 ml. of saturated sodium chloride solution, and sufficient 6 *N* NaOH to raise the pH to 8.3. Stirring a mixture of the dried ($MgSO_4$) chloroform solution, 250 ml. of methanol, and 6.0 g. of the diazoimidazolecarboxamide for 48 hr. gave 5 g. of X that was homogeneous according to TLC. Additional data obtained in characterizing compounds III–X are summarized in Table III.

Thin-layer Chromatography—2-Azahypoxanthine was separated from the triazenes by TLC on silica gel in ethyl acetate–methanol (9:1) (Solvent A). In this system, however, the triazenes generally remained near the origin. Chloroform–methanol (3:1) (Solvent B) failed to separate significantly 2-azahypoxanthine from some of the triazenes on silica gel, but this solvent mixture moved the triazenes well and permitted examination of triazene specimens for slower moving impurities, which were sometimes observed in crude samples. The two solvent mixtures were, therefore, used to complement each other in evaluating a triazene sample. Subsequently, a butanol–water–15 *N* ammonia system (86:14:4) (Solvent C) was also employed to examine specimens of I (NSC-45388) for the presence of both XI and 5(or 4)-aminoimidazole-4(or 5)-carboxamide (AIC), a precursor and potential contaminant of I. The order of increasing distance from the origin on TLC plates developed in this solvent system was XI, AIC, I. Hypoxanthine, a potential contaminant of the AIC precursor, was not separated from XI in this solvent system, but in Solvent A it moved more slowly than XI.

Detection was by ultraviolet light (365 mμ), by ultraviolet light (254 mμ) after spraying with an optical whitening agent,[3] and by iodine vapor. The triazenes were protected from light during TLC.

Sensitivity to Light—The solutions of the dimethyltriazene (I) and the butylmethyltriazene (IV) described below were contained in Pyrex volumetric flasks, and, unless otherwise stated, 10-mcg. aliquots were applied to TLC plates and developed in Solvents A and B. Water in the aliquots usually produced weak spots at the origin in these solvent systems. 2-Azahypoxanthine (XI) and AIC (for Solvent C) were spotted as reference compounds at 1 and 0.5 mcg., respectively. Both compounds are easily detectable in these (and smaller) amounts, but no attempt was made to quantitate the results described below by employing minimum amounts of reference compounds and maximum amounts of aliquots.

A 50% aqueous ethanol solution of I protected from light showed no evidence of 2-azahypoxanthine formation during a period of 4 days at room temperature. A similar solution of I exposed [about 21 cm. (6 in.) distant] to an ultraviolet lamp emitting principally at 365 mμ contained a small amount of XI after 1 hr. of exposure, and the conversion of I to XI was near completion after 3 hr.[4] TLC of a 20-mcg. aliquot in Solvent System C did not reveal any AIC after 3 hr. of exposure.

Solutions of I and IV in 50% aqueous methanol (5 mg./ml.) were exposed to light under the conditions described below, and the results determined by TLC of aliquots removed at appropriate time intervals were as follows.

(*a*) 2-Azahypoxanthine (XI) was not detectable within 4 days in the I and IV solutions exposed continuously to incandescent lighting (only). A small amount of XI could be detected in the solution of I after 7 days.

(*b*) 2-Azahypoxanthine was detectable within 0.5 hr. in a solution of I exposed to direct sunlight. Conversion of I to XI was estimated to be near 50% of completion within 1 hr. and to be essentially complete within 3 hr. Aliquots removed from a solution of IV after 1-hr. and 3-hr. periods of exposure in the same way gave similar results. The light reaching these solutions passed through both window glass and Pyrex glass. In order to determine whether AIC might also be formed, a second solution was exposed to direct sunlight passing only through the Pyrex glass of the flask, and 20-mcg. aliquots were chromatographed in Solvent System C. After 3 hr. of exposure, only XI was detectable.

(*c*) A solution of I exposed to fluorescent lighting (15-w., 42-cm. fluorescent lamp, approximately 30 cm. from the solution) showed no XI after 24 hr., a small amount after 48 hr., and an increased amount after 96 hr.

[3] Ultraphor WT by BASF Colors and Chemicals, Inc., Charlotte, N. C.

[4] An assay method based on the photochemical decomposition of I was recently reported, Reference 18.

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Keyphrases

Imidazoles—synthesis
TLC—separation, identity
UV light—identity
Antitumor activity—imidazoles
Degradation, imidazoles—light exposure

Specificity of Fluorometry of 5-Hydroxytryptamine by Means of Products with Ninhydrin

By W. B. QUAY

The specificity and relevant technical factors of the fluorometry of 5-hydroxytryptamine (5-HT) by means of its product with ninhydrin (1,2,3-indantrione) were studied in experiments with 121 test compounds and tissues from rats and cockroaches. Aromatic amines producing fluorescence capable of confusion with that from 5-HT were: a number of carboline derivatives, a few hydroxyindole congenors of 5-HT, 4-(aminomethyl)-piperidine, and 2-(2-aminoethyl)-pyridine. Comparisons of results from two different fluorometric procedures showed close correspondence with most mammalian tissues, but not for stomach, nor for cockroach tissues. More significant in the discrimination between 5-HT and the related compounds were the time and temperature-dependent fluorescence development and decay curves of incubations with ninhydrin.

5-HYDROXYTRYPTAMINE (5-HT) occurs widely and is of special interest in its association with neural and neuroeffector mechanisms (1). Its measurements in tissues has been by both bioassay and chemical means. Of the latter, fluorometric procedures are of paramount utility. One of these depends upon the fluorescence of 5-HT (and other 5-hydroxy and 5-methoxy indoles) in strong acid (fluorescence maximum at 540 mμ, excitation maximum at 295 mμ) (2, 3). Another depends on the fluorescence of a reaction product of 5-HT and ninhydrin (1,2,3-indantrione hydrate) (4, 5). This second technique is the more sensitive and has been thought to be more specific (5). In practice this appears to be true for most mammalian and reptilian tissues studied so far (6). But for some groups of animals and kinds of tissues the results by the two methods are divergent, with the ninhydrin technique giving falsely high values (Quay, unpublished results).

This report is a summary of an evaluation of the specificity of the fluorometric technique for 5-HT with ninhydrin, with special attention to amines most apt to produce a reaction product, and to derivatives and relatives of 5-HT. Several types of limitations in specificity are described, and methods for checking specificity are noted.

EXPERIMENTAL

The 121 compounds tested consisted of 72 indoleamines and their derivatives, 11 catecholamines and

Received March 11, 1968, from the Department of Zoology, University of California, Berkeley, CA 94720
Accepted for publication June 14, 1968.
This work was supported in part by a research grant (NB-06296), U. S. Public Health Service, Bethesda, Md.

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derivatives, and 38 amino acids and other N-containing compounds. Immediately before use small samples of these were weighed to the nearest microgram with a quartz helix balance (Microchemical Specialties Corp.), dissolved, then diluted to a standard concentration. These and all other solutions were made with demineralized distilled water.

The fluorescence of the compounds at 490 mμ with excitation at 380 mμ (uncorrected) was measured with an Aminco-Bowman spectrophotofluorometer after five different producers: (*a*) untreated and dissolved in 0.05 *M* pH 7.0 sodium phosphate buffer; (*b*) dissolved in 0.05 *M* pH 7.0 buffer and incubated with ninhydrin at either 60 or 75°, and in a few cases at 40 and 85°, for a precise time, followed by quick cooling and dilution (1 μmole in 250 μl. with 50 μl. 1.78% ninhydrin + 250 μl. H_2O); (*c*) dissolved in 0.1 *N* HCl (DuPont) 0.5% *l*-ascorbic acid (Eastman), washed with diethyl ether, extracted into *n*-butanol (Eastman), passed into 0.05 *M* pH 7.0 buffer, and reacted with ninhydrin at 60° for 1 hr. [method of Wilhoft and Quay (6)]; (*d*) the same procedure but without the ether washes; (*e*) a scaled-down version of the method of Snyder *et al.* (5) summarized as follows: (*a*) 1 ml. of sample, blank, or standard in 0.4 *N* perchloric acid is placed in a 5-ml. glass-stoppered centrifuge tube (for tissue samples, the supernatant of an ice-cold homogenate in 0.4 *N* perchloric acid is used); (*b*) the pH is brought to 10 by adding NaOH and checking with a glass electrode pH meter; (*c*) 100 μl. 0.5 *M* borate buffer at pH 10, 600 mg. NaCl (Merck reagent), and 3 ml. of *n*-butanol are added; (*d*) the tubes are stoppered, shaken, and centrifuged; (*e*) the butanol supernatant is transferred to a 15-ml. glass-stoppered centrifuge tube containing 250 μl. 0.05 *M* phosphate buffer at pH 7.0 and 8 ml. of heptane (Phillips Petroleum, pure); (*f*) this second tube is stoppered, shaken, centrifuged, and most of the heptane phase is aspirated off and discarded; (*g*) 50 μl. of 1.78% ninhydrin is added to the aqueous phase, which is then incubated for 30 min. in a water bath at 75°; (*h*) immediately 250 μl. H_2O is added, the tube is agitated, and cooled.

RESULTS

Fluorescence of Carbolines With and Without Ninhydrin—It was found that of the compounds studied, a number of the carbolines (Fig. 1) and their derivatives at pH 7.0 fluoresce intensely at 490 mμ when irradiated at 380–385 mμ, without prior incubation with ninhydrin. Carbolines and derivatives could be divided into three groups on the basis of this fluorescence (Table I): (*a*) those with strong, moderate, or weak fluorescence without ninhydrin and some reduction in fluorescence intensity after incubation with ninhydrin for 1 hr. at 60°; (*b*) those with weak fluorescence without ninhydrin and with increased fluorescence or little change after ninhydrin, and (*c*) those lacking notable fluorescence at 490 mμ with and without ninhydrin. In general, carbolines fluorescing most strongly were those with double bonds at positions 2–3 and 4–5 (Fig. 1). Derivatives (tetrahydro) lacking these double bonds include the four with no notable fluorescence at 490 mμ.

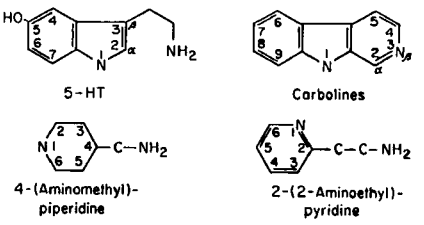

Fig. 1—Compounds giving fluorescence at 490 mμ (excitation at 380 mμ) either in neutral solution alone (carbolines) or after incubation with ninhydrin (others).

TABLE I—RELATIVE FLUORESCENCE OF CARBOLINES AND RELATED COMPOUNDS WITHOUT (0) AND WITH (+) PRIOR INCUBATION WITH NINHYDRIN[a]

Compd.	Relative Fluorescene 0	+
Group *A*		
Harmaline	>1,000	60
Harmalol	>1,000	10
Harmane	450	336
6-Methoxy-harmane	412	303
Harmine	403	278
2-Methylnorharman	318	258
2-Methylharmine	314	192
2-Methyltetrahydroharmol	301	13
2-Methyl-leptafluorine	153	0
6-Methoxy-*l*-tetrahydro-norharmanone	39	31
Tetrahydronorharmane carboxylic acid	36	19
Leptaflorine	25	1
6-Methoxy-tetrahydro-harman	15	5
N-(*py*)-propyltetrahydro-harmine	11	5
Eleagnine-1,3-dicarboxylic acid	10	1
Harminic acid	9	3
Noreleagnine	8	5
Tetrahydroharmane carboxylic acid	5	3
Leptacladine	3	0
Group *B*		
6-Methoxy-harmalan	5	32
6-Hydroxy-tetrahydro-harman	4	24
N-(*py*)-propyltetrahydro-harmane	3	6
Group *C*		
1-Oxo-1,2,3,4-tetrahydro-beta-carboline	0	0
Raunitidine	0	0
Tetrahydroharman	0	0
1-Tetrahydronorharmanone	0	0

[a] Compounds in pH 7.0, 0.05 phosphate buffer either incubated 1 hr. at 60° with ninhydrin (1 μ*M* in 250 μl. with 50 μl. 1.78% ninhydrin) or with H_2O alone, then diluted; fluorescence at 490 mμ; excitation at 380–385 mμ; tabulated values are means of 3 to 4 replicate pairs of tubes after subtraction of blanks (= incubated buffer with and without ninhydrin).

Carbolines with the above fluorescence characteristics were not significantly extracted by *n*-butanol in the three procedures studied, with four exceptions (Table II). The three extraction methods did not differ greatly in their results as far as these four carbolines are concerned. However, a slight reduction in the amount of 2-methylnorharman carried through the procedure was brought about

TABLE II—RELATIVE RECOVERY AND FLUORESCENCE PRODUCTION BY INDOLE DERIVATIVES ASSAYED ACCORDING TO THREE PROCEDURES[a]

Compd.[b]	Methods[c] I	II	III
5-HT	100	100	100
2-Methylnorharman	49	67	60
2-Methylharmine	40	32	38
2-Methyltetrahydroharmol	8	15	2
Harmalol	2	5	≃0
6-HT	7	4	—

[a] Comparisons made with 5-HT set at 100; results are means of two to four replicate series; fluorescence at 490 mμ; excitation at 380–385 mμ. [b] Tested at concentrations of 6–16 mμmoles/ml. [c] I = procedure of (6) with ether washes; II = the same, minus ether washes; III = procedure of (5).

by the ether washes, and possibly a slight reduction in the amounts of the hydroxylated compounds (2-methyltetrahydroharmol and harmalol) occurred in Method III, employing perchloric acid initially and a higher incubation temperature terminally.

Fluorescence of 5-HT and Other Indoles—In neutral 0.05 *M* phosphate buffer, 5-HT, and certain other indoles fluoresced at 490 mμ when irradiated at 380 mμ after having been incubated with ninhydrin. None fluoresced at 490 mμ without incubation with ninhydrin. The number of indoles in addition to 5-HT behaving this way was small, but depended in part on the time, temperature, and other conditions of the incubation with ninhydrin. Thus when equal concentrations (1×10^{-6} *M*/0.25 ml.) of these compounds in 0.05 *M* pH 7.0 buffer were incubated with ninhydrin at 75° simultaneously, the development and decay of the resultant fluorescent products followed different courses for the different compounds (Fig. 2). Fluorescence resulting during incubations of 30 min. or less could be largely attributed to 5-HT. With longer incubation times the fluorescence of products of some other normally occurring indoles increased as the 5-HT fluorescent product gradually decreased. The most intensely fluorescent of these other compounds was that from bufotenin (*N*-dimethyl-5-hydroxytryptamine; NDM-5-HT). Less, but still significantly, fluorescent were those from 5-hydroxyindole-3-acetic acid (5-HIAA) and

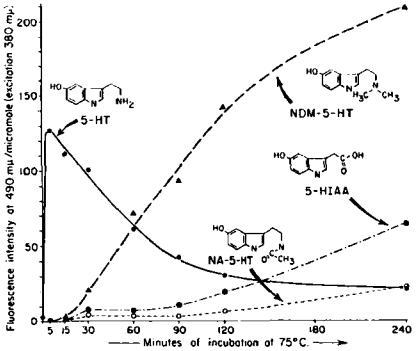

Fig. 2—Development and decay of fluorescent products during incubations of 5-hydroxyindoles with ninhydrin.

N-acetyl-5-hydroxytryptamine (NA-5-HT). Closely related and normally occurring indoles that failed to produce notable fluorescence at 490 mμ at any time during the incubation with ninhydrin at 75° included: tryptophan, 5-hydroxytryptophan, 5-methoxytryptamine, 5-methoxyindole-3-acetic acid, and melatonin (*N*-acetyl-5-methoxytryptamine). This was true if freshly dissolved indole standards were used. If older solutions were used, even after but a few days of refrigeration, some of the latter compounds through the agency of oxidation derivatives fluoresced with ninhydrin. Most significant among these was 5-hydroxytryptophan, several day-old solutions of which exhibited a pattern of fluorescence development intermediate to those of 5-HIAA and NDM-5-HT (Fig. 2).

A set of incubations identical to those represented in Fig. 2 but for the temperature being 60° instead of 75° showed the following differences: (*a*) Near peak fluorescence was attained by 5-HT with ninhydrin after an incubation of about 20 min. and constituted the start of a plateau in fluorescence intensity lasting through incubation times up to about 90 min., and with slow decline thereafter. (*b*) Fluorescence was produced neither by 5-HIAA nor by *N*-acetyl-5-hydroxytryptamine. (*c*) Fluorescence produced from bufotenine was not clearly evident until after an hour of incubation, and then its intensity was only 5% of that produced by 5-HT. Not until after 3 or 4 hr. of incubation did the fluorescence from bufotenine approach that from 5-HT.

Under these conditions besides 5-HT, 6-hydroxytryptamine (6-HT) also produced fluorescence with ninhydrin at 490 mμ with activation at 380 mμ. Although the resultant fluorescence per μmole of 6-HT was much less, 6-HT readily passes along with 5-HT through the extraction procedures (Table II).

Comparison was made of the fluorescence produced from the creatinine·H_2SO_4·H_2O complex of 5-HT with that from the hydrogen oxalate. On a per μmole basis the two were not significantly different and were at least within 5% of each other in level of fluorescence emitted.

Fluorescence of Other Amines and N-containing Compounds—The only other compounds that were both extracted by *n*-butanol and showed as much fluorescence as 5-HT after incubation with ninhydrin were 4-(aminomethyl)-piperidine and 2-(2-aminoethyl)-pyridine (Fig. 1). After certain conditions of incubation with ninhydrin a number of other compounds also produced fluorescence at 490 mμ (activation at 380 mμ), but of very low intensity. Furthermore, none of these amines passed the *n*-butanol extraction. Of these the most productive of fluorescence were lysine and cysteine (Table III).

Results from Tissues—Application of these methods to pooled samples of cockroach cephalic ganglia, nerve cords, and fat bodies demonstrated that the ninhydrin method (5, 6) gave mean "5-HT" values of 13, 10, and 22 mcg./g., respectively (Quay and Herman, unpublished), while the modified Bogdanski method (2, 3) gave trace values, comparable to those published by Welsh and Moorhead (7) for 5-HT in cockroach tissues (<0.02 to 0.05 mcg./g.).

Comparison of the results from the two methods

TABLE IV—ORGAN CONTENTS OF 5-HT ACCORDING TO TWO PROCEDURES

Organ[a]	N	5-HT[b] A: $\bar{x} \pm S_E$	5-HT[b] B: $\bar{x} \pm S_E$
Pineal	5	10.30 ± 1.04	11.27 ± 0.44
Duodenum	3	1.07 ± 0.08	1.06 ± 0.06
Stomach (pyloric)	4	0.77 ± 0.12	6.07 ± 0.41
Spleen	4	0.71 ± 0.24	1.27 ± 0.33
Lung	5	0.49 ± 0.16	0.45 ± 0.24
Liver	5	0.30 ± 0.02	0.22 ± 0.03
Adrenal	4	0.26 ± 0.04	0.25 ± 0.02

[a] Homogenates of adult rat organs provided equal aliquots for simultaneous determinations by two methods. [b] Mean ($\bar{x}$) mg/g. of 5-HT base [± standard error of the mean (S_E)] from N replicate organ samplings assayed terminally by: *A*—fluorescence at 540 mμ [excitation at 295 mμ; method of Quay (3)], *B*—fluorescence at 490 mμ (excitation at 380 mμ) after 1 hr. with ninhydrin at 60° [method of Wilhoft and Quay (6)].

applied to mammalian tissues showed equivalence for most but not all tissues tested (Table IV). Most clearly in the case of the pyloric stomach are the 5-HT values from the ninhydrin technique consistently and markedly greater—approximately eight times greater. The divergence of the spleen's values (Table IV) is not statistically significant, perhaps due to the great individual variation.

Study of the 5-HT content of different brain regions from rats by modified ninhydrin (6) and Bogdanski methods (2, 3) showed close agreement for most regions. But if higher temperatures and longer incubation times were used in the ninhydrin method, falsely high values for 5-HT were obtained. 5-HT either alone or added to brain homogenates had a fluorescent product with ninhydrin which followed the same time course of fluorescence during incubation, with rise, plateau and fall. However, from within homogenates of some brain regions ninhydrin-reactive, fluorescence-producing material was progressively more active at higher temperatures and longer incubation times. Identity of these other brain amines is being investigated.

DISCUSSION AND CONCLUSIONS

The specificity of fluorometric procedures for 5-HT based on a ninhydrin-reaction product is seen to be questionable in the absence of confirmatory results from other methods, and is less than appreciated previously (5). This is likely to be especially true for the tissues and body fluids of various invertebrate animals, which frequently have higher levels of free amines of diverse kinds (8). The same should be at least of potential concern in human and other mammalian tissues from pathologic and experimental situations in which free amines or metabolites may be abnormally high.

Auxiliary methods for the confirmation of the identity and apparent levels of 5-HT are numerous and may be found with the aid of recent reviews (9–11). Certain features of the ninhydrin reaction itself lend themselves also as means for ascertaining the identity of fluorescence-producing tissue constituents. The behavior of these according to temperature, time, and chemical environment will prove instructive in study of certain tissue amines, and will aid in confirming the identification of such

TABLE III—RELATIVE FLUORESCENCE OF INDOLES AND OTHER COMPOUNDS AFTER INCUBATION WITH NINHYDRIN FOR 1 hr. AT 60°[a]

Compd.[b]	Relative Fluorescence
5-Hydroxytryptamine	100
Bufotenine	5
6-Hydroxytryptamine	5
2-(2-Aminoethyl)-pyridine	79
4-(Aminomethyl)-piperidine	29
Lysine	9
4-Aminomethylpyridine	5
Cysteine	2

[a] Procedure and notes as in Table I. [b] The following indoles and derivatives failed to form products fluorescing at 490 mμ after incubation with ninhydrin for 1 hr. at 60°: *N*-acetyl-5-hydroxytryptamine, 5-methoxyindole-3-acetic acid, melatonin, 5-methoxytryptamine, 5-hydroxytryptophan, tryptophan, 5-hydroxyindole-3-acetic acid, bufotenine, tryptamine, α-methyltryptamine, 4-hydroxytryptamine, 3-indolylacetamide, 5-methyltryptophan, 3-methylindole, 3-(dimethylaminomethyl)indole (=gramine), *o*-phosphoryl-4-hydroxy-*N*-dimethyltryptamine (=psilocybin), 4-hydroxy-*N*-dimethyltryptamine (=psilocin), 4-hydroxytryptophan, 6-methyl-tryptophan, tryptophol, *N,N*-dimethyltryptamine, 5-benzyloxygramine, 3-(2-aminobutyl)-6-methoxyindole acetate, 6-methoxyacetyltryptamine, 5-methoxy-gramine, 6-hydroxy-5-methoxytryptamine, 6-hydroxymelatonin, *N*-acetyl-5-hydroxy-6-methoxytryptamine, 7-methyltryptamine, 5-methyltryptamine, kynurenine, dehydrobufotenine, *N*-methyltryptamine, 3-(2-dibutylaminoethyl)-5-hydroxyindole, 5-hydroxy-α methyltryptophan, 5-methoxytryptophan, 5-hydroxy-*N*ω-methyltryptamine, 5-benzyloxytryptamine, carbazole, and 5-aminoindole. Other amines and *N*-containing compounds studied and which produced extremely little or no fluorescence after 1 hr. with ninhydrin at 60° were: tyramine, 3,4-dihydroxyphenylalanine, dopamine, norepinephrine, epinephrine, phenylalanine, tyrosine, normetanephrine, octopamine, 3-methoxy-4-hydroxy-phenylethylamine, 3-methoxy-4-methoxy-phenylethylamine, histamine, 1,4-methylhistamine, 4-amino-6-hydroxypyrazolo(3,4-*d*)pyramidine, glycine, DL-α-alanine, serine, cystine, gamma amino-butyric acid, *p*-aminohippuric acid, threonine (allo-free), valine, norvaline, methionine, leucine, isoleucine, norleucine, citrulline, thyroxin, proline, aspartic acid, glutamic acid, arginine, histidine, ethionine, asparagine, adenine, adenosine, ethyl *p*-aminobenzoate, benzidine, 2,6-lutidine, glutamine, and pyridine.

constituents as 5-HT. Thus kinetic rather than endpoint analyses may be expected to be suitably informative.

In the ninhydrin methods, at least three classes of aromatic amines have been shown in these studies to be able to contribute fluorescence readily confused with that from 5-HT. These are: (*a*) carboline derivatives, fluorescent at 490 mμ without the necessity of reaction with ninhydrin; (*b*) certain hydroxyindoles resembling 5-HT and reacting with ninhydrin to produce similar fluorescence, and (*c*) certain other compounds having suitably reactive and exposed amine groups, such as those in 4-(aminomethyl)-piperidine and 2-(2-aminoethyl)-pyridine. At least many of these are not yet known to occur in animal tissues, and may be of theoretical interest only. On the other hand, hydroxyindole congeners of 5-HT do pose potential problems in determinations of 5-HT in tissues. Most of these can be excluded from 5-HT extracts by the use of suitable solvents and conditions in washing and extraction (3). Three may remain in part, however, to contribute something less than 5% of the fluorescence produced by the same amount of 5-HT. These are 6-HT (Table I), *N*-acetylserotonin and 5-hydroxytryptophan (5-HTP). The last two of these can be removed by suitable washes, such as with diethyl ether (3), a procedure not without other hazards however. 5-HTP is a problem as a fluorescence producer with ninhydrin only after some deterioration in solution has occurred.

The time course of fluorescent product formation from ninhydrin is different for these compounds as compared with 5-HT. Therefore, careful study can discriminate between them.

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Keyphrases

5-Hydroxytryptamine in tissue—analysis
Tissue analysis—5-hydroxytryptamine
Fluorometry, specificity—5-hydroxytryptamine analysis
Ninhydrin reagent
Amine interference—5-hydroxytryptamine analysis

Some Conditions Under Which Pentobarbital Stimulates Spontaneous Motor Activity of Mice

By NATHAN WATZMAN, HERBERT BARRY, III, WILLIAM J. KINNARD, JR., and JOSEPH P. BUCKLEY

The effects of 4 doses of sodium pentobarbital (10, 20, 40, 80 mg./kg., p.o.) on spontaneous motor activity of mice were tested in photocell cages during a 2-hr. period immediately after drug or saline administration. A total of 480 albino mice was divided among several experimental conditions. Increasing doses produced progressively increasing activity in the last 0.5 hr. when normal activity was at a low level. In the first 0.5 hr., the usual intense activity was unchanged by the three lower doses and greatly depressed by the highest dose. Throughout the session, the three lower doses had a stimulant effect on animals tested in a lighted environment in groups of five but not singly, and a small but consistent stimulant effect on both single and grouped animals in a dark environment. Animals fasted for 24 hr. prior to the test were greatly stimulated by the lowest dose and were greatly depressed by the highest dose. The results indicate that the stimulant effect of barbiturates requires both inhibitory influences on normal activity and conditions increasing susceptibility to arousal.

CLINICALLY, subhypnotic doses of barbiturates have been known to produce excitement, euphoria, and confusion rather than central nervous system depression (1). Many investigators have reported a stimulant effect in laboratory animals with low doses and depression with high doses. Kinnard and Carr (2) reported that spontaneous motor activity of mice was increased by low doses and decreased by high doses of sodium amobarbital and sodium secobarbital. A stimulant effect of low doses of barbiturates on spontaneous motor activity of mice has also been demonstrated in several subsequent experiments (3–7).

The stimulant action of barbiturates has been analyzed in situations where the drug enhances a previously rewarded response that was subsequently inhibited by punishment or nonreward (8). This stimulant effect has generally been attributed to a disinhibitory action which at certain doses is stronger than the intrinsic depressant action (9). Dews (10) reported that in a variety of food-rewarded tasks, conditions

Received May 2, 1968, from the Department of Pharmacology, University of Pittsburgh School of Pharmacy, Pittsburgh, PA 15213
Accepted for publication June 17, 1968.
Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
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The time course of fluorescent product formation from ninhydrin is different for these compounds as compared with 5-HT. Therefore, careful study can discriminate between them.

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Keyphrases

5-Hydroxytryptamine in tissue—analysis
Tissue analysis—5-hydroxytryptamine
Fluorometry, specificity—5-hydroxytryptamine analysis
Ninhydrin reagent
Amine interference—5-hydroxytryptamine analysis

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which depressed the normal response rate of pigeons increased the stimulant effect of amobarbital.

Spontaneous motor activity, in common with all behavior, is controlled by opposing excitatory and inhibitory influences. Therefore, it is reasonable to assume that the demonstrated disinhibitory action of barbiturates might account for their stimulant effect on spontaneous motor activity. Such an assumption might be tested by comparing the effects of barbiturates under different conditions which are known to influence the strength of the inhibitory tendency. However, the effects of barbiturates on spontaneous motor activity have generally been tested only under a single set of conditions. An exception to this was a brief report by Wright *et al.* (11) that doses of pentobarbital which depressed spontaneous motor activity of single mice had no effect or increased activity of mice tested in groups of three. Evidence for greater susceptibility of aggregated mice to a stimulant effect is also found in a report by Brown (3) that pentobarbital and phenobarbital greatly increased spontaneous motor activity of mice tested in groups of five, whereas observations of isolated mice indicated a depressant effect of the same doses.

In the present study, effects of pentobarbital on spontaneous motor activity of mice were compared under several experimentally varied conditions: test aggregation, illumination of the test environment, and satiation or fasting for 24 hr. prior to the test. Lower activity levels, which might be attributed to inhibitory influences, were previously found (12) in animals tested singly, or in the light, or after satiation. A prolonged, 2-hr. session permitted measurement of the drug effect throughout a period when normal activity decreases greatly, as previously shown in the same test situation (12, 13).

METHOD

Subjects—The subjects were 480 male Swiss-Webster mice (Taconic Farms, N. Y.) weighing approximately 18–20 g. and housed in groups containing 20 animals per cage.

Apparatus—The experimental work was performed in four circular, 6-beam photocell activity cages (Actophotometers, Metro Industries, Inc., N. Y.), 33 cm. (13 in.) in diameter, operating simultaneously in a sound-attenuated room. The same units have been used in prior studies (12, 13); characteristics of the apparatus were discussed in another prior study (14) and in a review article (15).

Procedures—The 2-hr. test session was initiated immediately after oral administration of sodium pentobarbital (10, 20, 40, or 80 mg./kg.) or placebo (saline, 0.1 ml./10 g. body weight). The groups tested under these five dosage conditions were each subdivided according to three additional experimental conditions: test aggregation (singly or in groups of five mice), illumination (light or dark test environment), and feeding condition (satiated or fasted for 24 hr. prior to the test session). A complete factorial design for the five dosage conditions and the two conditions of each of the other three variables resulted in 40 combinations of conditions ($5 \times 2 \times 2 \times 2$). Each of these was replicated in all four actophotometer units, thus giving a total of 160 conditions, 80 comprising single mice and 80 comprising aggregations of five mice. In order to provide variation in experimental conditions among the four animals or groups tested simultaneously in the different units, each of these animals or groups was under a different dosage condition. The activity counts for each 0.5 hr. and for the total 2 hr. were transformed into square root scores for the reasons discussed previously (13). Statistical significance was tested by an analysis of variance program (BMD 02V, for the IBM 7090 computer), according to the methods described previously (12).

RESULTS

The first 0.5 hr. of the session might not be expected to provide sufficient time for the full effects of the drug because of the oral route of administration and the very brief interval prior to the beginning of the session. Nevertheless, Fig. 1 shows a very strong depressant effect of the highest pentobarbital dose (80 mg./kg.) on activity in the first 0.5 hr. Inspection of the counts recorded for the first 15 min. showed that the effect occurred also in that shorter time period. The test of statistical significance for the first 0.5 hr. revealed a highly reliable difference among the five dosage conditions ($F = 82.1$, $df = 4/117$, $p < 0.001$). Figure 1 shows that the difference was almost entirely attributable to the effect of the highest dose, and a statistical analysis for the same period, omitting the highest dosage condition, shows no significant differences among the four remaining dosage groups.

The activity of the group with the highest drug dosage increased rapidly after the second 0.5 hr., and was the highest among the five groups in the

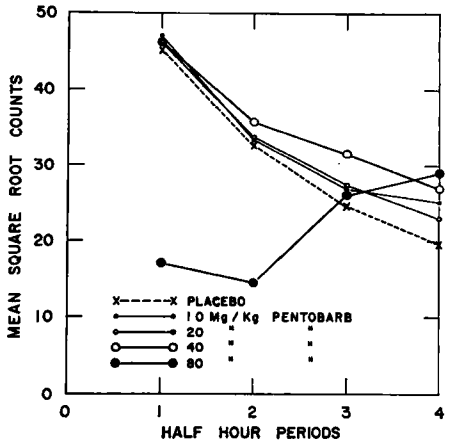

Fig. 1—Spontaneous motor activity of the five dosage groups in each 0.5-hr. period.

fourth 0.5 hr. Meanwhile, the activity of the other four groups decreased progressively. A statistical test of this trend for these four dosage groups during the successive 0.5 hr. periods, with the orthogonal polynomials used previously (12–14), revealed a linear decrease in activity which was highly reliable ($F = 540.0$, $df = 1/288$, $p < 0.001$). The activity of the groups given the three lower doses of pentobarbital decreased more slowly than the activity of the placebo group, so that each of these drug groups was higher than the placebo group at the end of the 2-hr. session. The gradual emergence of the difference among these four dosage conditions, during the successive 0.5-hr. periods, is indicated by a significant interaction between the linear trend for the four periods and the linear trend for the four dosage conditions ($F = 5.86$, $df = 1/288$, $p < 0.05$). A comparison among all five dosage groups in the fourth 0.5 hr. shows an almost uniformly progressive increase in activity with increasing doses. This linear trend was statistically highly reliable ($F = 7.91$, $df = 1/117$, $p < 0.01$).

The effects of two of the other three experimental variables also depended on the portion of the 2-hr. session. The analysis for the four 0.5-hr. periods, excluding the highest pentobarbital dose, showed no difference between the fasted and the satiated animals in activity for the first 0.5 hr., but the subsequent decrease in activity was greater for the satiated animals, resulting in a progressively greater superiority in activity of the fasted animals in successive 0.5-hr. periods. This interaction between fasting condition and the linear function of 0.5-hr. periods was statistically highly reliable ($F = 19.0$, $df = 1/288$, $p < 0.001$). Similarly, the animals tested in the dark showed a superior level of activity which was very slight in the first 0.5 hr. and progressively increased in successive 0.5-hr. periods, with a statistically reliable interaction between illumination and the linear function of 0.5-hr. periods ($F = 8.48$, $df = 1/288$, $p < 0.01$). Activity was much higher for the aggregated than single animals throughout the 2-hr. session ($F = 168.6$, $df = 1/288$, $p < 0.001$), but this difference persisted at approximately the same level throughout the session, as is indicated by the lack of a significant interaction between aggregation and 0.5-hr. periods.

The influence of the experimental conditions on the response to the drug may be measured by the interaction between dosage conditions and the experimental variables. The analysis of the four 0.5-hr. periods showed no significant effect of the periods on these interactions, measured by the three-way interactions for periods, dosages, and any of the other variables. Therefore, the drug effects are compared under the different experimental conditions for the total 2-hr. session.

Figure 2 shows the effect of fasting on the response to pentobarbital. For the fasted animals, the drug had a strong stimulant effect at the lowest dose but a marked depressant effect at the highest dose, whereas the satiated animals were less strongly affected by the various dosage conditions. The superior activity level of the fasted animals at an intermediate dosage condition (10 mg./kg.) but not at the extreme conditions (placebo and the higher doses) is measured by the quadratic orthogonal polynomial. A significantly different drug effect for the satiated and fasted animals is found in the interaction between fasting and the quadratic trend of dosage with all five dosages ($F = 19.1$, $df = 1/117$, $p < 0.001$) and also with the highest dosage omitted ($F = 8.16$, $df = 1/93$, $p < 0.01$). Thus, fasting enhanced sensitivity both to the stimulating effect of a low dose and to the depressant effect of a high dose.

The relationship of the aggregation and illumination conditions with each other influenced their effects on the response to pentobarbital, as indicated by a statistically significant three-way interaction among the four dosage conditions (excluding the highest dose), aggregation and illumination ($F = 3.09$, $df = 3/93$, $p < 0.05$). Figure 3 divides these complex influences on the response to pentobarbital into their simple components by the use of two graphs, both of which compare the single with the aggregated animals under a single illumination condition. In the lighted environment, the three lower pentobarbital doses substantially stimulated the aggregated but not single animals. The interaction between aggregation and the linear function of dosage (omitting the highest dose) was statistically

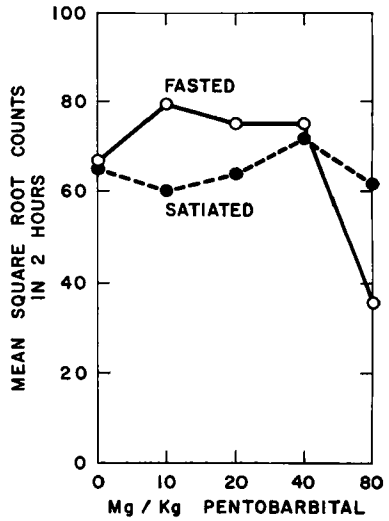

Fig. 2—Comparison of fasted with satiated animals under the different dosage conditions for the total 2 hr.

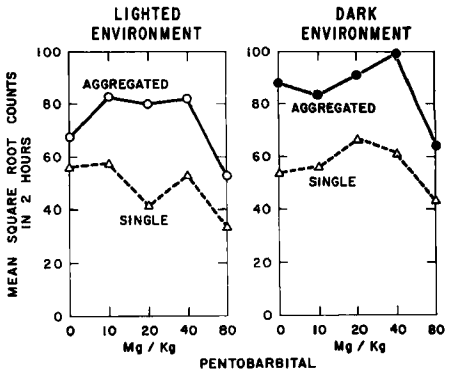

Fig. 3—Effects of test illumination and aggregation under the different dosage conditions for the total 2 hr.

significant ($F = 6.72$, $df = 1/48$, $p < 0.05$). In the dark environment, there was no significant differential effect of aggregation on the response to pentobarbital, but the drug had a stimulant effect on both single and aggregated animals, with a statistically significant linear function of dosage, excluding the highest dose ($F = 6.49$, $df = 1/48$, $p < 0.05$). Thus, in the lighted environment, pentobarbital stimulated only the aggregated animals, whereas in the dark environment the drug had a stimulant effect which was smaller but not dependent on aggregation condition.

DISCUSSION AND CONCLUSIONS

The theory of the disinhibitory effect of barbiturates (8, 9) implies that conditions which strengthen the inhibitory tendencies on behavior, thus reducing the normal activity level, will enhance the stimulant effects of the drug. Some of the present findings clearly support this theory. A stimulant drug effect was not found in the period of intense exploratory activity, during the initial 0.5 hr. in the novel environment. The stimulant drug effect at the end of the session might be attributed to a disinhibitory action on the behavioral mechanisms which cause the activity of the placebo animals to decrease to a low level. This stimulation is not due to compensation for earlier depressed levels of activity, because a depressant effect was seen only with the highest of the four drug doses. The later portions of the session were necessarily associated with longer intervals after drug administration, which enabled recovery from the depressant effect of the highest dose, but none of the prior studies (3–7) has given evidence that the stimulant effect on spontaneous motor activity occurs only at a long interval after drug administration.

Further indication of a disinhibitory effect of pentobarbital is seen in the strongly stimulant drug effect on aggregated animals tested in the light. The lighted environment decreases normal activity in mice, and the aggregation condition may have enhanced this depressant tendency, as indicated by much lower activity scores in the light than in the dark. The three lower doses of pentobarbital increased these scores in the light to the level for the placebo animals tested in the dark. Aggregated animals have been observed to cluster together and become quiescent late in the session; the higher activity counts of the aggregated animals are due to the larger number of opportunities to interrupt the photobeams, and a measure of counts per animal would yield lower scores in the aggregated than in the isolated condition.

Some of the present findings suggest that the stimulant drug effect is enhanced by conditions which increase rather than decrease the normal activity level. The lowest dose of pentobarbital stimulated the fasted but not the satiated animals. The three lower doses had a consistent although small stimulant effect in the dark environment, regardless of aggregation condition. Prior findings have also given evidence that a certain degree of arousal may enhance the stimulant effect of barbiturates. Aggregation was considered to have such an arousing effect in a prior report (3) that barbiturates increased spontaneous motor activity of aggregated but not isolated mice. Quigley *et al.* (16) observed that pentobarbital, amobarbital and barbital evoked excitement in dogs with a nervous temperament, whereas simple depression occurred in quiet, well-behaved animals. The dogs had been trained to lie down quietly for an extended time period in the test situation, which thereby required considerable inhibition of natural activity. In general, a combination of inhibitory influences and susceptibility to arousal appears to be necessary for a stimulant effect of barbiturates. If the animals are already maximally aroused, as when initially introduced into a new environment, the barbiturate cannot induce a further stimulation. If the conditions are predominantly inhibitory, the normal quiescence appears to be enhanced rather than reversed by barbiturates. The stimulant effect of barbiturates appears to be maximized by strongly conflicting behavior tendencies, such as approach and avoidance in an instrumental learning situation (8, 9) or excitatory and inhibitory tendencies after the initial introduction to a novel situation, as in the later portion of the test reported in the present paper.

The present study gives evidence of multiple and complex factors which influence the effect of pentobarbital on spontaneous motor activity in mice. Various excitatory and inhibitory tendencies determine the activity counts registered at a particular time, under particular conditions. The present study has identified only a few of the conditions which influence the drug effect. In a prior study by Harris *et al.* (7) 90 mg./kg. pentobarbital, administered orally, greatly increased spontaneous motor activity of mice in a 30-min. test beginning 30 min. after drug or placebo administration. A somewhat lower dose (80 mg./kg.) had a depressant effect under the most nearly equivalent conditions of the present experiment (satiated animals tested singly during the second 0.5 hr.). Some characteristics of the animals or of the test situation in the present experiment evidently caused either greater resistance to the drug's stimulant effects or greater susceptibility to its depressant effects. Further studies, comparing the drug effects under experimentally varied conditions, are needed to identify more comprehensively the conditions which determine the stimulant and depressant effects of barbiturates.

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Keyphrases

Phenobarbital effects—motor activity, mice
Stimulant effects—phenobarbital
Motor activity—phenobarbital administration
Environment effect—phenobarbital activity
Fasting, satiation effects—phenobarbital activity

Mechanisms of Action of Cryptenamine

By MARIE L. JANDHYALA and JOSEPH P. BUCKLEY

The hypotensive effect of cryptenamine has been reported to be markedly attenuated by adrenalectomy or by treatment with *N,N*-diisopropyl-*N'*-isoamyl-*N'*-diethylaminoethylurea (P-286) in atropinized animals. Cryptenamine also appeared to potentiate the response of isoproterenol on β-adrenergic receptors. This present study was conducted to further investigate these two phenomena. Cryptenamine potentiated the response of epinephrine on the β-adrenergic receptors in the intact dog heart, partially blocked the effect of isoproterenol on the isolated guinea pig auricle, and had no effect on the isoproterenol-induced relaxation of the isolated guinea pig tracheal chain. Cryptenamine produced positive inotropic and chronotropic effects on the isolated guinea pig heart and potentiated the inotropic effect of epinephrine and inhibited its chronotropic effects in this preparation. These data suggest that the effects of cryptenamine on β-adrenergic receptors are variable and that either potentiation or inhibition may be observed depending on the effector organ and on the species of animal studied. Cryptenamine also significantly decreased the epinephrine content of the adrenal venous blood while increasing the norepinephrine content suggesting that the drug may inhibit the methylation of norepinephrine in the adrenal medulla.

CRYPTENAMINE, an alkaloidal mixture, prepared from *Veratrum viride* by a nonaqueous benzene triethylamine extraction procedure, has been reported to have a ratio of emetic to effective hypotensive dose superior to that of other veratrum preparations (1, 2). Finnerty (2) reported that in humans the divergence between the hypotensive and emetic doses of cryptenamine was apparent on intravenous administration. McCall and his colleagues (3) studied the effects of cryptenamine on cerebral circulation and cerebral oxygen consumption in patients with toxemia of pregnancy. They observed that on intravenous administration of cryptenamine cerebral blood flow and cerebral oxygen metabolic rate were increased significantly while the respiration quotient of the brain remained normal. In these experiments, comparison with other veratrum preparations indicated fewer side effects. Although a satisfactory ambulatory treatment of hypertension by the oral administration of cryptenamine has been reported (4), Abreu (5) failed to demonstrate any superiority of cryptenamine over protoveratrine *A* in dogs as to the ratio of emetic to hypotensive doses.

Recently, Jandhyala and Buckley (6) reported that cryptenamine sensitized β-adrenergic receptors and that it might possibly stimulate the release of epinephrine from the adrenal medulla and that these factors might contribute to the overall hypotension. They reported that the hypotensive effects of cryptenamine were inhibited or abolished by pretreatment with *N,N*-diisopropyl-*N'*-isoamyl-*N'*-diethylaminoethylurea (P-286), bretylium, pronethalol, reserpine, α-methyl-dopa, and by adrenalectomy in atropinized animals. Cryptenamine also potentiated the isoproterenol-induced relaxation of the vasculature in the denervated perfused hind limb of the dog. Cryptenamine potentiated the

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depressor response induced by minute doses of epinephrine, suggesting a sensitization of β-adrenergic receptors. In addition, these investigators reported that cryptenamine acted on the baro- and chemoreceptors in the carotid sinus complex inducing a reflexogenic hypotensive response. Most of the evidence for the increased release of epinephrine from the adrenal medulla, as reported by Jandhyala and Buckley (6) was indirect.

The purpose of this investigation was to further study the mechanism of action of cryptenamine, especially as related to the release of catecholamines from the adrenal medulla and to the effects of cryptenamine on β-adrenergic receptors in selected tissues in different species.

METHODS

Effects of Cryptenamine and Protoveratrine A on the Epinephrine Content of Adrenal Venous Blood—Mongrel dogs of either sex were anesthetized with sodium pentobarbital, 35 mg./kg., i.v. The left adrenal vein was catheterized with PE 205 tubing, and 500 units/kg. of heparin administered i.v. Blood was permitted to flow freely from the catheter and collected continuously. Blood flow was measured by collection of 1-min. samples into a 10-ml. graduated cylinder from which an 8-ml. sample was withdrawn for catecholamine analysis prior to, and 5, 15, 30, 60, and 120 min. after the administration of cryptenamine, 5 mcg./kg., i.v. or protoveratrine-*A*, 4 mcg./kg., i.v. Samples to be analyzed were collected under ice, centrifuged at 2000×*g* for 10 min., and the plasma frozen and maintained at −10° until analyzed. Blood not used for catecholamine analysis was injected *via* a femoral vein in 20–30 ml. aliquots. Blood volume was maintained by addition of saline containing red blood cells from the centrifuged blood in amounts equal to plasma removed.

After allowing the frozen plasma samples to thaw at room temperature, an equal volume of 1.6 *N* perchloric acid was added and the samples agitated on a Vortex mixer to precipitate the proteins. Assays of catecholamines were performed according to the method of Brodie *et al.* (7), as modified by Bennett *et al.* (8).

Effects of Cryptenamine, Protoveratrine A and Protoveratrine B on Isoproterenol-Induced Relaxation of Vasculature in the Denervated Perfused Hind Limb of the Dog—Mongrel dogs of either sex were anesthetized with sodium pentobarbital, 35 mg./kg., i.v., and a femoral artery was catheterized to record arterial blood pressure. One hind limb was denervated by severing the femoral and sciatic nerve trunks and vascularly isolated by ligating the muscle with a 21-gauge stainless steel wire placed under the femoral artery and vein and tightened with a Schiffrin wire tightener. The distal segment of the femoral artery of the isolated limb was catheterized with PE 205 tubing and perfused with blood drawn from the central segment of the same artery. A constant flow of blood was maintained utilizing a Sigmamotor pump and perfusion pressure determined from a T tube placed between the pump and the isolated limb *via* a Statham pressure transducer.

In order to study the effects of cryptenamine, protoveratrine *A*, and protoveratrine *B* on β-adrenergic receptors, four different doses of isoproterenol, 1, 2, 5, and 10 mcg., at 10-min. intervals, were administered intraarterially into the limb followed by the i.v. administration of one of the following doses: cryptenamine, 5 mcg./kg.; protoveratrine *A*, 1 mcg./kg., 2 mcg./kg., or 5 mcg./kg.; or protoveratrine *B*, 1 mcg./kg. or 2 mcg./kg. After a 30-min. stabilization period, the doses of isoproterenol were repeated.

Effect of Cryptenamine on the Cardiac Responses of Epinephrine in Atropinized Dogs—Mongrel dogs of either sex were anesthetized with sodium pentobarbital, 35 mg./kg., i.v. and a femoral artery cannulated for the recording of blood pressure. The trachea was cannulated and the animal placed on positive pressure respiration (Mine Safety Appliance Co.), utilizing a mixture of 95% oxygen and 5% carbon dioxide. The thoracic cavity was entered at the fourth intercostal space utilizing electrocautery to minimize bleeding, and the pericardium incised and sutured to the chest wall so as to gain easy access to the heart. A Walton-Brodie strain gauge arch was sutured to the right ventricle to determine the force of contraction and the physiological responses recorded on a Grass polygraph. The animals were pretreated with atropine sulfate, 1 mg./kg., i.v., to block the parasympathetic receptors involved in the reflexogenic mechanism, thus making it possible to study the nature of the efferent pathways.

Effect of Cryptenamine on the Epinephrine Response in Isolated Guinea Pig Heart—Guinea pigs were sacrificed by cervical dislocation and the lungs, heart, and ascending aorta quickly excised. The pericardium was removed, the pulmonary vessels ligated, and the lungs separated from the heart and saline perfused into the aorta to clear the coronary arteries of blood. A cannula was inserted into the aorta, the heart suspended in a Langendorf unit, and perfused with oxygenated Locke's solution (NaCl, 9.0 g.; KCl, 0.42 g.; $CaCl_2$, 0.24 g.; $NaHCO_3$, 0.5 g.; dextrose, 2.0 g., glass-distilled water q.s. 1,000 ml., warmed to 38°). The reservoir was adjusted to produce an infusion pressure of 40–50 mm. Hg which closed the semilunar valves thus forcing the fluid through the coronary vessels. Contractions of the heart were recorded on a Grass polygraph utilizing a force-displacement transducer.

Drugs were administered directly into the cannula, *via* a three-way stopcock. Epinephrine HCl (0.5 or 1.0 mcg. of the base) was administered initially and the perfusion continued for approximately 5 min., at which time cardiac activity had returned to basal level. This procedure was repeated until the response was reproducible. Cryptenamine, 10 mcg., was then injected; and after cardiac activity reached a constant level, the same dose of epinephrine was repeated.

Effect of Cryptenamine on Isoproterenol Response in Isolated Guinea Pig Atria—Guinea pigs were sacrificed by cervical dislocation, the thoracic cavity opened, and the pericardium removed. The ventricles were removed by cutting through the atria-ventricular junction, and the remaining parts of the aorta and pulmonary artery freed and gently drawn apart, producing an atrial strip approxi-

mately 3 cm. in length. Silk thread was tied to each end, and the preparation suspended in oxygenated Locke's solution which was maintained at a constant temperature at 29–30° in a 50-ml. muscle bath. The contractions were recorded on a Beckman dynograph utilizing a Grass force-displacement transducer.

Drugs were administered into the isolated organ bath in volumes less than 5% of the total bath volume after the preparation had been permitted to stabilize for 15–30 min. Between each administration of isoproterenol (2–8 ng./ml.), the preparation was washed at least 3 times at 5-min. intervals. When the response was reproducible, cryptenamine was added (50 ng./ml.), and 30 min. later, the same dose of isoproterenol repeated. Preliminary data indicated that a minimum period of 30 min. was necessary to alter the responses of isoproterenol.

Effect of Cryptenamine on Isoproterenol Responses in Guinea Pig Tracheal Smooth Muscle—Guinea pigs were sacrificed by cervical dislocation, the trachea removed, and sectioned into 12 equal rings. These were connected in series by short loops of silk thread so that the dorsal smooth muscle bands were vertical. The chain was set up in a 10-ml. organ bath containing oxygenated (95% O_2 + 5% CO_2) Krebs-Henseleit solution (NaCl, 6.90 g.; KCl, 0.35 g.; $CaCl_2$, 0.28 g.; $NaHCO_3$, 2.10 g.; KH_2PO_4, 0.16 g.; $MgSO_4$, $7H_2O$, 0.29 g.; dextrose, 1.0 g.; glass-distilled water q.s. 1,000 ml. maintained at 38°). An isotonic lever was utilized to record the contractions on a slowly moving kymograph. The effect of isoproterenol (0.2–0.8 ng./ml.) prior to and following cryptenamine, 50 ng./10 ml. bath were investigated. Isoproterenol (0.2–0.8 ng./ml.) was administered, the maximal effect recorded, and the preparation washed five times at 5-min. intervals. This was repeated until reproducible responses were obtained. Cryptenamine was added to the bath following the last injection and isoproterenol (concentration equal to that administered prior to cryptenamine) administered 5 min. later.

Statistical significance was determined using Student's *t* test throughout this study.

RESULTS

Effects of Cryptenamine and Protoveratrine A on the Epinephrine Content of Adrenal Venous Blood—Cryptenamine and protoveratrine *A* decreased the rate of secretion of epinephrine from the adrenal medulla and increased the rate of norepinephrine secretion (Tables I–IV and Figs. 1 and 2). These results suggest that cryptenamine and protoveratrine *A* may block the enzymes which methylate norepinephrine into epinephrine. It is also probable that the increase in the pressor response that Krayer (9) and Krayer and Mendez (10) obtained when they administered blood plasma from dogs which received veratridine or protoveratrine to pithed rats or cats may not have been due to an increase in epinephrine content but in the amount of norepinephrine.

Effects of Cryptenamine, Protoveratrine A and Protoveratrine B on Isoproterenol-Induced Relaxation of Vasculature in the Denervated Perfused Hind Limb of the Dog—The results are summarized in Tables V–X. Neither protoveratrine *A* nor protoveratrine *B* potentiated the effects of iso-

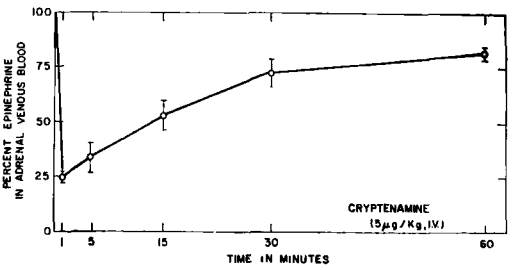

Fig. 1—Percent of control epinephrine content in adrenal venous blood.

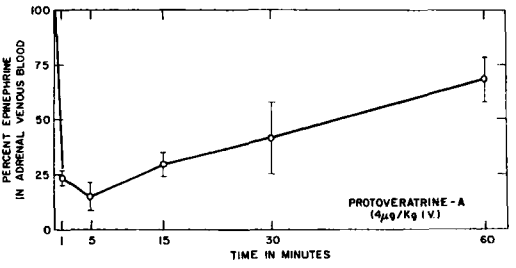

Fig. 2—Percent of control epinephrine content in adrenal venous blood.

TABLE I—EFFECT OF CRYPTENAMINE (5 MCG./KG., I.V.) ON THE ADRENAL VENOUS BLOOD EPINEPHRINE CONTENT (MCG./MIN.)

Dog	Control	*I*[a]	*II*[b]	*III*[c]	*IV*[d]	*V*[e]	*VI*[f]
1	0.899	0.203	0.298	0.487	0.756	0.813	—
2	0.894	0.204	0.596	0.659	0.840	—	0.831
3	2.467	0.941	1.150	1.277	1.468	2.058	2.139
4	0.510	0.160	0.192	0.403	0.438	0.456	0.593
5	0.334	0.104	0.031	0.096	0.229	0.289	0.310
6	2.701	0.608	1.094	1.154	1.849	2.181	2.139
7	1.602	0.139	0.192	0.468	0.486	1.082	1.165
8	1.105	0.236	0.182	0.711	0.885	0.953	1.033
Mean	1.314	0.324	0.466	0.657	0.869	1.109	1.193
± *SE*	0.308	0.104	0.202	0.138	0.192	0.279	0.283
p		<0.01	<0.05	<0.01	<0.05	<0.10	<0.10

[a] Blood collected immediately after administration of cryptenamine. [b] Blood collected 5 min. after administration of cryptenamine. [c] Blood collected 15 min. after administration of cryptenamine. [d] Blood collected 30 min. after administration of cryptenamine. [e] Blood collected 60 min. after administration of cryptenamine. [f] Blood collected 120 min. after administration of cryptenamine.

TABLE II—EFFECT OF CRYPTENAMINE (5 MCG./KG., I.V.) ON THE ADRENAL VENOUS BLOOD NOREPINEPHRINE CONTENT (MCG./MIN.)

Dog	Control	I[a]	II[b]	III[c]	IV[d]	V[e]	VI[f]
1	0.053	0.185	0.195	0.469	0.402	0.116	—
2	0.053	0.127	0.187	0.235	0.141	—	0.138
3	0.035	0.153	0.215	0.211	0.079	0.076	0.073
4	0.015	0.047	0.084	0.206	0.200	0.184	0.129
5	0.021	0.037	0.043	0.034	0.021	0.021	0.022
6	0.176	0.239	0.166	0.159	0.122	0.117	0.117
7	0.238	0.495	0.546	0.699	0.562	0.449	0.335
8	0.248	0.236	0.286	0.318	0.220	0.295	0.295
Mean	0.090	0.190	0.215	0.278	0.217	0.180	0.157
± *SE*	0.034	0.051	0.062	0.069	0.064	0.055	0.043
p		<0.05	<0.05	<0.05	<0.10	<0.10	<0.10

[a] Blood collected immediately after administration of cryptenamine. [b] Blood collected 5 min. after administration of cryptenamine. [c] Blood collected 15 min. after administration of cryptenamine. [d] Blood collected 30 min. after administration of cryptenamine. [e] Blood collected 60 min. after administration of cryptenamine. [f] Blood collected 120 min. after administration of cryptenamine.

TABLE III—EFFECT OF PROTOVERATRINE *A* (4 MCG./KG., I.V.) ON THE ADRENAL VENOUS BLOOD EPINEPHRINE CONTENT (MCG./MIN.)

Dog	Control	I[a]	II[b]	III[c]	IV[d]	V[e]	VI[f]
1	0.462	0.097	0.028	0.154	0.152	0.358	0.615
2	1.806	0.343	0.226	0.343	0.349	0.874	1.658
3	0.265	0.079	0.068	0.097	0.194	0.217	0.247
Mean	0.844	0.173	0.107	0.198	0.232	0.483	0.840
± *SE*	0.484	0.042	0.086	0.057	0.060	0.199	0.422

[a] Blood collected immediately after administration of protoveratrine *A*. [b] Blood collected 5 min. after administration of protoveratrine *A*. [c] Blood collected 15 min. after administration of protoveratrine. *A* [d] Blood collected 30 min. after administration of protoveratrine *A*. [e] Blood collected 60 min. after administration of protoveratrine *A*. [f] Blood collected 120 min. after administration of protoveratrine *A*.

TABLE IV—EFFECT OF PROTOVERATRINE *A* (4 MCG./KG. I.V.) ON THE ADRENAL VENOUS BLOOD NOREPINEPHRINE CONTENT (MCG./MIN.)

Dog	Control	I[a]	II[b]	III[c]	IV[d]	V[e]	VI[f]
1	0.015	0.117	0.151	0.220	0.225	0.196	0.048
2	0.349	0.428	0.443	0.528	0.526	0.481	0.300
3	0.011	0.142	0.158	0.274	0.273	0.091	0.016
Mean	0.125	0.229	0.251	0.341	0.341	0.256	0.122
± *SE*	0.112	0.099	0.096	0.095	0.093	0.138	0.089

[a] Blood collected immediately after administration of protoveratrine *A*. [b] Blood collected 5 min. after administration of protoveratrine *A*. [c] Blood collected 15 min. after administration of protoveratrine *A*. [d] Blood collected 30 min. after administration of protoveratrine *A*. [e] Blood collected 60 min. after administration of protoveratrine *A*. [f] Blood collected 120 min. after administration of protoveratrine *A*.

proterenol on the β-adrenergic receptors, but a slight blocking effect was observed with some of the doses, 90–120 min. after administration of the alkaloids. Cryptenamine did potentiate the response to isoproterenol in the denervated perfused hind limb (Table V).

Effects of Cryptenamine on the Cardiac Responses of Epinephrine in Atropinized Dogs—Pretreatment of the animals with atropine sulfate partially blocked the hypotension and bradycardia, and slightly decreased the duration of activity of cryptenamine as has previously been reported (6). Three doses of epinephrine, 50 ng./kg., 100 ng./kg., and 200 ng./kg. were administered i.v. to the animals before and after administration of cryptenamine, 5 mcg./kg., i.v. Cryptenamine potentiated the effect of epinephrine on the myocardial contractile force (Table XI) but did not alter the effect of epinephrine on the blood pressure.

Effect of Cryptenamine on the Epinephrine Response in Isolated Guinea Pig Heart—Cryptenamine, 10 mcg., produced positive inotropic effects on the heart and also appeared to potentiate the inotropic effect of epinephrine; however, the chronotropic activity was inhibited (Table XII).

Effect of Cryptenamine on Isoproterenol Response in Isolated Guinea Pig Atria—Cryptenamine, 50 ng./ml., markedly reduced the positive chronotropic and inotropic effects of isoproterenol (Table XIII). If cryptenamine remained in contact with the isolated atria for more than 1 hr., it produced a negative chronotropic effect, which as reported by Hawkins (11) was irreversible and did not disappear when the bathing fluid was replaced with fresh Locke's solution.

Effect of Cryptenamine on Isoproterenol Response in Guinea Pig Tracheal Chain—The data, summarized in Table XIV, indicate that cryptenamine, 5 ng./ml., did not affect the isoproterenol-induced relaxation of the guinea pig tracheal chain.

DISCUSSION

It has been reported that cryptenamine appeared to sensitize β-adrenergic receptors in the vasculature

TABLE V—EFFECT OF CRYPTENAMINE (5 MCG./KG., I.V.) ON THE ISOPROTERENOL-INDUCED RELAXATION IN THE DENERVATED PERFUSED HIND LIMB OF THE DOG

Sex and Weight, kg.	Before Cryptenamine: Decrease in Perfusion Pressure to Isoproterenol, mm. Hg				After Cryptenamine: Decrease in Perfusion Pressure to Isoproterenol, mm. Hg			
	1 mcg.	2 mcg.	5 mcg.	10 mcg.	1 mcg.	2 mcg.	5 mcg.	10 mcg.
F 10.4	16	18	23	28	20	26	30	39
M 12.0	25	35	40	45	30	45	47	53
M 10.8	20	25	33	35	25	33	40	42
F 9.4	26	30	38	40	32	38	45	48
Mean	21.8	27.0	33.5	37.0	26.8	33.5	40.5	45.5
± *SE*	2.32	3.63	3.80	3.63	2.69	4.01	3.80	3.12
p					<0.01	<0.01	<0.01	<0.01

TABLE VI—EFFECT OF PROTOVERATRINE *A* (1 MCG./KG., I.V.) ON THE ISOPROTERENOL-INDUCED RELAXATION IN THE DENERVATED PERFUSED HIND LIMB OF THE DOG

Sex and Weight, kg.	Before Protoveratrine: Decrease in Perfusion Pressure after Isoproterenol, mm. Hg				After Protoveratrine: Decrease in Perfusion Pressure after Isoproterenol, mm. Hg			
	1 mcg.	2 mcg.	5 mcg.	10 mcg.	1 mcg.	2 mcg.	5 mcg.	10 mcg.
F 11.8	42	47	62	65	37	46	60	62
M 9.5	10	16	24	32	10	17	25	33
F 11.0	20	25	33	35	20	25	30	30
M 12.0	23	32	35	38	25	33	38	40
Mean	23.8	30.0	38.5	42.5	23.0	30.3	38.3	41.3
± *SE*	6.69	6.54	8.19	7.60	5.61	6.18	7.73	7.23
p					>0.95	>0.95	>0.95	>0.95

TABLE VII—EFFECT OF PROTOVERATRINE *A* (2 MCG./KG., I.V.) ON THE ISOPROTERENOL-INDUCED RELAXATION IN THE DENERVATED PERFUSED HIND LIMB OF THE DOG

Sex and Weight, kg.	Before Protoveratrine: Decrease in Perfusion Pressure to Isoproterenol, mm. Hg				After Protoveratrine: Decrease in Perfusion Pressure to Isoproterenol, mm. Hg			
	1 mcg.	2 mcg.	5 mcg.	10 mcg.	1 mcg.	2 mcg.	5 mcg.	10 mcg.
M 10.7	23	43	46	51	23	25	38	49
M 12.9	35	38	40	44	15	20	25	34
M 10.4	23	25	35	42	22	20	25	30
M 12.0	25	32	35	38	20	27	35	38
Mean	26.5	34.5	29.0	43.8	20.0	23.0	30.8	37.8
± *SE*	2.87	3.88	2.16	2.71	1.78	1.80	3.38	4.09
p		—			>0.3	>0.1	>0.1	>0.2

TABLE VIII—EFFECT OF PROTOVERATRINE *A* (5 MCG./KG., I.V.) ON THE ISOPROTERENOL-INDUCED RELAXATION IN THE DENERVATED HIND LIMB OF THE DOG

Sex and Weight, kg.	Before Protoveratrine: Decrease in Perfusion Pressure to Isoproterenol, mm. Hg				After Protoveratrine: Decrease in Perfusion Pressure to Isoproterenol, mm. Hg			
	1 mcg.	2 mcg.	5 mcg.	10 mcg.	1 mcg.	2 mcg.	5 mcg.	10 mcg.
F 10.0	17	20	34	36	13	15	20	30
F 7.6	25	32	35	37	35	33	40	43
F 8.5	25	28	40	45	25	25	36	40
M 8.5	18	20	24	29	15	12	14	20
Mean	21.5	25.0	33.3	36.8	22.0	21.2	27.5	33.3
± *SE*	2.09	3.0	3.35	3.28	5.07	4.80	6.24	5.21
p					>0.9	>0.2	>0.3	>0.4

of dogs and nictitating membrane of cats (6). Cryptenamine and several other veratrum alkaloids were investigated for their effect on β-adrenergic receptors in various organs. The data indicated that cryptenamine did not affect isoproterenol-induced relaxation of guinea pig tracheal chain, potentiated the inotropic effect and inhibited the chronotropic action of epinephrine on the isolated guinea pig heart, potentiated the positive chronotropic effect of epinephrine in the intact heart of atropinized dogs, and inhibited the characteristic isoproterenol response on the isolated guinea pig atria. These results are consistent with the published work by Lands *et al.* (12) which indicated that there was a certain degree of variation in affinity and intrinsic activity of β-adrenergic receptors in various organs. This and other published work (13) suggest that the epinephrine-sensitive β-adrenergic receptors may be activated to a greater or lesser degree in various organs and species. Although the emphasis in this discussion has been placed on the action of cryptenamine on receptor sites, it is

TABLE IX—EFFECT OF PROTOVERATRINE *B* (1 MCG./KG., I.V.) ON THE ISOPROTERENOL-INDUCED RELAXATION IN THE DENERVATED PERFUSED HIND LIMB OF THE DOG

Sex and Weight, kg.	Before Protoveratrine: Decrease in Perfusion Pressure to Isoproterenol, mm. Hg				After Protoveratrine: Decrease in Perfusion Pressure to Isoproterenol, mm. Hg			
	1 mcg.	2 mcg.	5 mcg.	10 mcg.	1 mcg.	2 mcg.	5 mcg.	10 mcg.
M 13.6	30	45	48	50	25	37	50	50
M 11.4	40	45	50	55	40	50	55	60
F 9.7	8	15	20	25	10	15	20	25
M 10.7	40	45	48	52	32	32	42	50
Mean	29.5	37.5	41.5	45.5	26.8	33.5	41.8	46.3
± *SE*	7.54	7.50	7.18	6.91	6.37	7.24	7.73	7.47
p					>0.4	>0.4	>0.4	>0.7

TABLE X—EFFECT OF PROTOVERATRINE *B* (2 MCG./KG., I.V.) ON THE ISOPROTERENOL-INDUCED RELAXATION IN THE DENERVATED PERFUSED HIND LIMB OF THE DOG

Sex and Weight, kg.	Before Protoveratrine: Decrease in Perfusion Pressure to Isoproterenol, mm. Hg				After Protoveratrine: Decrease in Perfusion Pressure to Isoproterenol, mm. Hg			
	1 mcg.	2 mcg.	5 mcg.	10 mcg.	1 mcg.	2 mcg.	5 mcg.	10 mcg.
M 12.3	30	45	47	50	30	37	40	50
F 8.4	40	45	55	55	35	35	50	55
F 13.1	25	35	43	48	25	30	35	38
M 8.4	23	25	28	30	20	22	25	27
Mean	29.5	37.5	43.3	45.8	27.5	31.0	37.5	42.5
± *SE*	3.80	4.79	5.66	5.45	3.23	3.34	5.20	6.28
p					>0.1	>0.05	>0.05	>0.3

possible that these alkaloids are affecting one or more enzyme systems within the cell (possibly adenylcyclase or phosphordiesterase) or acting directly on the vascular smooth muscle contractile elements. Since protoveratrine *A* and *B* failed to enhance the vasodilator effect of isoproterenol in the denervated dog hind limb, this activity must be attributed to other constitutents in cryptenamine.

Krayer (14) and others (15) have reported that at the peak blood pressure rise following intravenous injection of 1 mg. of protoveratrine to vagotomized dogs, a concentration of epinephrine in the order of 1:100,000 may be reached in the suprarenal venous blood suggesting that the release of epinephrine was a direct effect of veratrum alkaloids on the adrenal medulla. Mendez (15) and Krayer and Acheson (16) reported that an increase in pressor responses was obtained when they administered blood plasma from dogs which received protoveratrine or veratrine to pithed cats and rats and suggested that the rise in blood pressure was due to an increase of epinephrine levels. Jandhyala and Buckley (6) reported that the release of epinephrine from the adrenal medulla may contribute to the hypotensive activity of cryptenamine *via* its action on β-adrenergic receptors. This conclusion was made from indirect evidence in adrenalectomized dogs and dogs pretreated with P-286, reserpine, guanethidine, and α-methyldopa, which suggested the possible release of catecholamines from the adrenal medulla by cryptenamine. However, the actual content of catecholamines in the adrenal venous blood was not determined in their studies.

TABLE XI—EFFECT OF CRYPTENAMINE (5 mcg./kg.) ON INOTROPIC RESPONSE TO EPINEPHRINE IN ATROPINIZED DOGS (1 mg./kg.)

Epi Dose, mcg./kg.	*N*	Before Cryptenamine Average % Increase in Contractile Force ± *SE*	After Cryptenamine Average % Increase in Contractile Force ± *SE*
0.05	3	9.33 ± 2.46	15.63 ± 3.66
0.10	4	15.72 ± 3.91	22.0 ± 3.47
0.20	4	20.43 ± 3.53	32.50 ± 8.85

The results obtained in this present investigation did not agree with the above conclusions, in that the total adrenal venous catecholamine content was slightly decreased following the administration of cryptenamine and protoveratrine *A*. The epinephrine content of adrenal venous blood was decreased while the norepinephrine content markedly increased, suggesting that cryptenamine and protoveratrine *A* may be interfering with the conversion of norepinephrine into epinephrine. Epinephrine is synthesized in the adrenal medulla from norepinephrine by the enzyme, phenylethanolamine-*N*-methyl transferase (PNMT) (17), in a reaction in-

TABLE XII—EFFECT OF CRYPTENAMINE ON EPINEPHRINE RESPONSE IN THE ISOLATED GUINEA PIG HEART

Epi Dose, mcg./ml.	*N*	Before Cryptenamine[a]: Average % Increase in Amplitude ± *SE*	Before Cryptenamine[a]: Average % Increase in Rate ± *SE*	After Cryptenamine: Average % Increase in Amplitude ± *SE*	After Cryptenamine: Average % Increase in Rate ± *SE*
0.5	4	32.88 ± 7.03	40.78 ± 10.10	81.83 ± 17.34[b]	17.13 ± 4.55
1.0	6	68.25 ± 8.69	35.40 ± 9.67	116.87 ± 17.44[b]	20.80 ± 3.09

[a] 10 mcg. [b] Significant at $p < 0.05$ when compared to response prior to cryptenamine.

TABLE XIII—EFFECT OF CRYPTENAMINE (50 NG./ML.) ON ISOPROTERENOL-INDUCED RESPONSES ON GUINEA PIG ATRIA

Iso Dose, mcg./ml.	N	Before Cryptenamine: Average % Increase in Amplitude ± SE	Before Cryptenamine: Average % Increase in Rate ± SE	After Cryptenamine: Average % Increase in Amplitude ± SE	After Cryptenamine: Average % Increase in Rate ± SE
2	4	54.76 ± 5.44	22.40 ± 4.76	15.26 ± 6.94[a]	15.82 ± 3.71
4	4	120.18 ± 16.80	39.15 ± 5.06	42.53 ± 4.83[a]	31.88 ± 3.32
8	4	110.31 ± 18.58	27.98 ± 0.32	45.88 ± 4.58[a]	26.05 ± 6.45

[a] $p < 0.05$ when compared to response prior to cryptenamine.

TABLE XIV—EFFECT OF CRYPTENAMINE[a] ON ISOPROTERENOL-INDUCED RELAXATION IN GUINEA PIG TRACHEAL CHAIN

Iso Dose ng./ml.	N	Before Cryptenamine Average (mm.) Relaxation ± SE	After Cryptenamine Average (mm.) Relaxation ± SE
0.2	4	12.5 ± 0.87	12.5 ± 0.87
0.4	4	16.4 ± 1.10	16.4 ± 0.10
0.8	4	22.4 ± 0.93	22.6 ± 0.93

[a] 5 ng./ml.

volving *S*-adenosyl methionine as a methyl donor (18). One control mechanism for the synthesis of catecholamines, which applies to the synthesis of epinephrine in the adrenal medulla has recently been described (19). It was suggested (19) that the synthesis of PNMT, responsible for the formation of epinephrine, is controlled by adrenal glucocorticoids. When adrenal steroid secretion was depressed by hypophysectomy, the activity of the epinephrine-synthesizing enzyme falls, but can be restored by pituitary adrenocorticotropic hormone (ACTH) or adrenal glucocorticoid administration. It is possible that cryptenamine and protoveratrine *A* affect the PNMT enzyme *via* this endocrine system and prevent the formation of epinephrine from norepinephrine and thus increase the norepinephrine concentration in the adrenal venous blood.

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Keyphrases

Cryptenamine
Hypotensive activity, cryptenamine—mechanism
Epinephrine, norepinephrine activity—cryptenamine effect
Protoveratrine *A*, *B* activity—cryptenamine effect
Atropine activity—cryptenamine effect

Improved Method for Diffusion Coefficient Determinations Employing the Silver Membrane Filter

By ARTHUR H. GOLDBERG and WILLIAM I. HIGUCHI

A convenient and rapid method for determining diffusion coefficients has been developed that is based on the quasi-stationary diaphragm cell method. A silver filter membrane is used to separate two compartments of the apparatus that are stirred magnetically with synchronous motors. The advantages are the good precision of the data, running times of the experiments, and the extended repeated usability of a given membrane.

THERE RECENTLY has been much interest shown in drug dissolution rate mechanisms as well as in other diffusionally characterized transport processes. Regardless of the situation, quantification requires diffusion coefficient determination as one parameter. A method for determining the diffusion coefficient has been described by Northrup and Anson (1) and by McBain (2). Desai *et al.* (3) employed a method similar to the Northrup-McBain technique using sintered-glass disks to separate the drug solution from the "sink." Singh (4) found improvement by using organic polymer membrane filters.

Almost all membrane filters lack the physical strength required for repeated use. They would, after soaking in the drug solutions, lose their integrity, causing changes of area available for transport. One membrane filter,[1] impregnated with glass fibers, was found to maintain itself rather well for as long as 75–80 hr. of experimentation but would then slowly slough its surface. Recently, a new membrane filter was marketed, made from silver,[2] and has been used with the apparatus shown in Fig. 1. This filter has tensile strength as an advantage over the other membrane filters, and the advantage of thinness over the sintered-glass disks. This allows for faster, easier wetting of all the pores. This insures having the same porosity and tortuosity in each succeeding trial, and hence, better reproducibility. Also, the thinner the membrane, assuming all other factors are constant, the faster the rate of transport through it. This advantage, however, makes the overall diffusion rate more stirring speed dependent, due to the greater importance of the aqueous diffusion layers next to the membrane. Therefore, single speed (300 r.p.m.) synchronous motors were employed to control the hydrodynamics. To these motors, 5.6 cm. (2.25 in.) magnetic cylinders were attached, which in turn rotated Teflon-coated magnetic stir bars within each side of the cell. The silver filters, once installed in the cell, may be used over and over again, and appear to be "permanent," *i.e.*, after soaking in drug solution for more than 2 months, there were no apparent deleterious

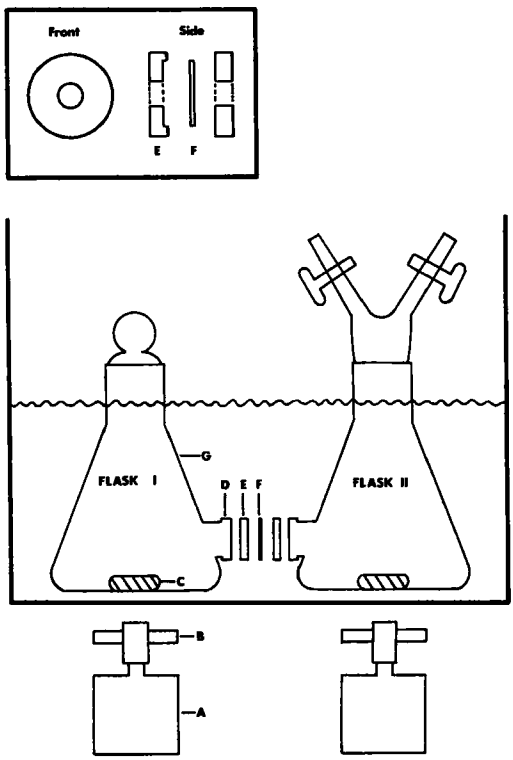

Fig. 1—Schematic diagram of cell used to determine diffusion coefficients. Inset is enlarged schematic of the Teflon washers. Key: A, *synchronous motor, 300 r.p.m.;* B, *5.6-cm. magnetic (cylindrical);* C, *5-cm. Teflon-coated stirring bar;* D, *ground-glass end;* E, *Teflon washer;* F, *silver membrane;* G, *250-ml. conical flask, adapted as shown.*

Received March 14, 1968, from the College of Pharmacy, University of Michigan, Ann Arbor, MI 48104
Accepted for publication June 17, 1968.
This investigation was supported in part by fellowship 5-F1-GM-34,237-02 from The National Institute of General Medical Sciences, and in part by grant GM-13368-01 from the National Institute of General Medical Sciences, U. S. Public Health Service, Bethesda, Md.

[1] Versapor filter, Gelman Industries, Ann Arbor, Mich.
[2] Flotronics Membrane, Selas Flotronics, Spring House, Pa.

effects, despite there being what appears to be a silver oxide film on the filters. Due to its thinness (2 mils), most diffusion coefficients can be determined in about 2 hr. once a cell is calibrated.

The other change is in the cell itself. Desai and workers (3) used a cell of unit construction. The cell used here is similar to that described by Singh (4) and is formed from two 250-ml. conical flasks that have been previously modified to contain side-arm attachments with ground-glass ends, as in Fig. 1. Between these ground-glass ends are placed two Teflon washers, one of which effectively has a rim around its perimeter (see cut in Fig. 1). The 37-mm. diameter silver filter of 1.2 μ pore size is placed between the two Teflon washers. The flasks are then clamped together using a triangular clamp fastened by nuts and bolts. To insure complete wetting of the entire membrane, Flask I is initially filled with a 0.05% polysorbate 80 solution and allowed to flow into Flask II. The cell is then allowed to bathe overnight in the surfactant solution. Then the cell is emptied, filled with enough distilled water to completely cover the membrane, and stirred for 2–3 days. The cell is then emptied and rinsed before use. This procedure is only necessary the first time a cell is constructed. Thereafter, the cell is kept filled with water when not in use. The cell is never dismantled. If the cell is taken apart, new calibrations are required.

Theoretical Considerations—The thickness of the silver membrane is approximately only 50 μ. Since the membranes are purported to have a tortuosity of near unity, and a high porosity, it becomes evident that mass transfer resistance through the aqueous films on either side of the membrane may contribute significantly to the cell constant. Thus it is necessary to explore the question of whether or not mass transport in the aqueous films is essentially molecular diffusion. If, for example, mass transfer mechanisms other than molecular diffusion is important (5), one may expect a nonlinear dependence upon the diffusion coefficient.

To determine this dependency, cell constants can be calculated using several substances of known diffusion coefficients. These diffusion coefficients encompass as large a difference between them as is practically feasible, and in the range of the diffusion coefficients to be measured.

EXPERIMENTAL

For the rate determinations, the flasks are filled and stoppered in a similar manner as previously described (3). The solution containing the drug is put into Flask II, along with a 5-cm. (2-in.) Teflon-coated magnetic stirring bar. The "Y" tube (see Fig. 1) is filled to overflowing by injecting the drug solution through the hole of one stopcock, and then closing both stopcocks. Distilled water (250 ml.) is then added to Flask I, along with an identical stirring bar to the one in Flask II. The entire cell is then immersed in a constant-temperature water bath, under which are placed the synchronous motors. After enough time to allow for steady-state diffusion and temperature equilibration, 10-ml. samples are withdrawn from Flask I, and are replaced by 10 ml. of distilled water. The concentration of drug appearing in Flask I is corrected for the volume replacement factor.

The calculation of the cell constant is given as:

$$L = \frac{G_{(\text{known})}}{D_{(\text{known})}\,(C_2 - C_1)} \qquad \text{(Eq. 1)}$$

where L is the cell constant, G is the rate of transport from Flask II to Flask I of a substance whose diffusion coefficient is known, D is the diffusion coefficient of that substance, C_1 and C_2 are the concentrations in Flasks I and II, respectively. C_1 is zero if no drug is put into Flask I. Once the cell constant, L, has been determined, Eq. 1 is rewritten to:

$$D_{(\text{unknown})} = \frac{G_{(\text{unknown})}}{L_{(\text{cell})}\,(C_2 - C_1)} \qquad \text{(Eq. 2)}$$

The rate, G, is determined as discussed earlier, leaving only the diffusion coefficient, D, to be calculated.

The cell constants were determined at 25 and 30° using KCl solutions and sucrose solutions. Sucrose has diffusion coefficients approximately one-fourth that of potassium chloride. The integral diffusion coefficient for 0.10 M KCl is 1.873×10^{-5} cm.^{2} sec.$^{-1}$ (6) at 25° and 2.093×10^{-5} cm.^{2} sec.$^{-1}$ (7) at 30°. The diffusion coefficients of sucrose, 1% and 0.1% both at 25° are reported as 5.148×10^{-6} cm.^{2} sec.$^{-1}$ and 5.213×10^{-6} cm.^{2} sec.$^{-1}$, respectively (8).

The precision of the cells was then tested by quadruplicate determinations of the diffusion coefficient of benzoic acid, 0.01 M at 30°. Benzoic acid was also used to determine the effect of stirring on the cell constant, by recalibrating the cell using 150 r.p.m. synchronous motors.

DISCUSSION

The calibrations performed in this laboratory indicate that these cells are an improvement over the ones previously employed. The cell constants calculated using Eq. 1, for each trial of two cells using potassium chloride and sucrose are reported in Table I, along with their standard deviations. One

Table I—Cell Constant Determinations Under Varying Conditions

	Cell Constant, cm.	
	Cell B	Cell C
Potassium chloride, 0.1 M, 25°	84.17	87.17
Potassium chloride, 0.1 M, 30°	84.92	87.92
Sucrose, 1.0%, 25°	84.50	89.72
Sucrose, 0.1%, 25°	84.31	87.31
Mean	84.49	88.03
SD	0.275 (±.33%)	1.018 (±1.16%)

cell (B) has a cell constant of 84.49 cm. and the other a cell constant of 88.03 cm. In order to determine whether this standard deviation is due to the changing conditions or to the technique itself, the diffusion coefficient of benzoic acid, 0.01 M was repeatedly determined at 30°. The results are reported in Table II. The diffusion coefficient found (1.113×10^{-5} cm.^{2} sec.$^{-1}$) is in excellent agreement with the reported value of 1.11×10^{-5} cm.^{2} sec.$^{-1}$ (9). Typical data for the benzoic acid rates are shown in Fig. 2.

After noting that the determinations made using benzoic acid showed a slightly larger standard deviation than obtained by the potassium chloride and sucrose calibrations, it was assumed that the small differences found with potassium chloride and sucrose were not due to the changing conditions. This shows that small temperature changes do not affect the cell constant, nor does a 2% change in viscosity. If, however, it is assumed that the standard deviation (from the benzoic acid trials) is attributable to the exponential dependency of the diffusion coefficient, it can be shown that at 300 r.p.m. the maximum dependency is $D^{(1.00 \pm 0.08)}$. For the purposes found to date in the pharmaceutical literature, this leads to only small errors in the determined diffusion coefficient.

If the cell constant is recalculated using 150 r.p.m. rather than 300 r.p.m., a large reduction in cell constant is obtained. The cell constants at 150 r.p.m. for cells B and C were 68.53 and 74.25 cm., respectively. The reproducibility of each cell was excellent. This result indicates the necessity for constant speed stirring. The lower the rates of stirring, the greater the magnification of the differences between the cells. When four more cells were calibrated at 300 r.p.m., the cell constants ranged from 83.33 to 102.22 cm., with each individual cell showing about the same standard deviations as reported for cells B and C. This indicates the necessity of calibrating each cell individually, and using the individual cell constants for calculating the diffusion coefficients obtained from the rate experiments.

TABLE II—REPEATED DETERMINATIONS OF DIFFUSION COEFFICIENT OF BENZOIC ACID, 0.01 M, AT 30.0°

Trial	G (mg./sec.) ($\times 10^3$)	Concn. (mg./ml.)	D ($\times 10^5$)
	Cell B (L = 84.49 cm.)		
1	1.122	1.2212	1.087
2	1.151	1.1813	1.153
3	1.185	1.2188	1.151
4	1.133	1.1813	1.135
	Cell C (L = 88.03 cm.)		
1	1.169	1.2212	1.087
2	1.139	1.1813	1.095
3	1.200	1.2188	1.118
4	1.118	1.1813	1.075
Mean	1.113		
SD	0.029	(± 2.6%)	

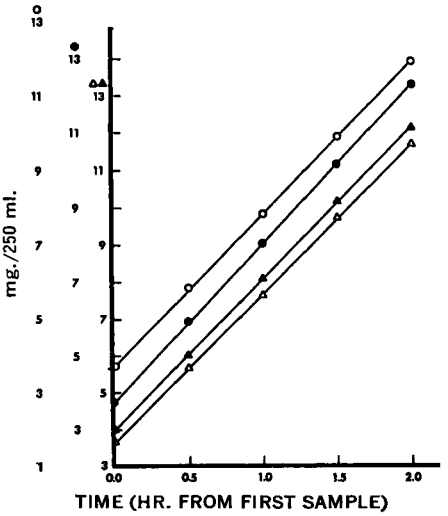

Fig. 2—Typical data from rate experiments. Slope of line is G of Eq. 1. Key: △, ▲, ○, ● are four different rate experiments from cell B.

SUMMARY

An improved method for determining diffusion coefficients is proposed, encompassing the use of a new silver membrane. The advantages are the speed at which diffusion coefficients can be determined, and the good precision of the data. It was also shown that, despite a stirring-rate dependent barrier being present, the diffusion coefficients obtained agree well with the literature and that diffusion through this barrier appears to follow molecular diffusion. It was also shown that small changes in temperature and/or viscosity do not affect the cell constant. These cells offer better precision than the previously reported cells using sintered-glass disks. They combine the physical stability of sintered-glass disks with the advantages of thin membrane filters.

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Keyphrases

Diffusion coefficients—determination
Silver membrane filter—diffusion coefficients
Diagram—diffusion cell

Synthesis of Xanthoxime and Pyridine-3-aldoxime Esters and Ethers as Potential Anticholinergics

By SUSHIL K. GOMER, EUGENE I. ISAACSON, ROBERT G. BROWN, and JAIME N. DELGADO

The synthesis of a series of oxime esters and ethers was accomplished by acylation and *O*-alkylations of 3-pyridinealdoxime and xanthoxime. The esterifications were effected by treating the oximes with benzoyl chloride whereas the *O*-alkylations were effected by treating the oximes with β-dimethylaminoethyl and γ-dimethylaminopropyl chlorides. In the course of this investigation, five compounds were synthesized and evaluated for their anticholinergic activity. This study reveals that the oxime esters and ethers possessing the bulkier xanthene moiety are, in general, more anticholinergic than those derived from 3-pyridinealdoxime. 9-(γ-Dimethylaminopropyloximino)-xanthene methyl bromide was found to be the most potent anticholinergic agent among the new compounds evaluated.

Previous work in these laboratories has led to the synthesis of oxime esters and ethers derived from 1-methyl-4-oximinopiperidine, 3-oximinotropane, benzophenone oxime, and dibenzosuberone oxime (1, 2). Since these studies have demonstrated that certain oxime derivatives possess interesting anticholinergic activity (1), it was decided to extend the series by preparing analogous compounds from pyridine-3-carboxaldehyde and xanthone as potential anticholinergic agents. These carbonyl compounds were selected as starting materials because they provide the basis for comparing the effect of a tricyclic moiety with the pyridine heterocycle on anticholinergic activity. The present work encompasses the synthesis and preliminary pharmacologic evaluation of the oxime derivatives delineated in Tables I and II.

The synthesis of 3-pyridinealdoxime presented no difficulties. For general preparative purposes the method of Ginsburg and Wilson (3) was employed. The utilization of this procedure entailed adjusting an aqueous solution of the aldehyde and hydroxylamine hydrochloride to neutrality by the addition of a 10% sodium hydroxide solution and heating for 20 min.

The synthesis of xanthoxime proceeded with greater difficulty than did the synthesis of 3-pyridinealdoxime. However, application of the method of Campbell *et al.* (4) proved to be successful. Refluxing the ketone together with hydroxylamine hydrochloride in anhydrous pyridine as base and solvent for 24 hr. gave the oxime in yields to 80% of theory.

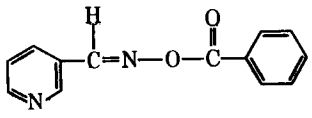
Received February 28, 1968, from The College of Pharmacy, The University of Texas, Austin, TX 78712
Accepted for publication June 11, 1968.

The oximes were converted to the ester derivatives by conventional acylation procedures employing the acyl chloride. 3-(Benzoyloximino)-pyridine was prepared using a number of solvents. The best yields were obtained when the solvent was anhydrous benzene or anhydrous ether. In the preparation of 9-(benzoyloximino)xanthene anhydrous ether was used as solvent.

The preparation of the oxime ethers was accomplished by dissolving the oximes in ethanol, adding sodium hydride, and subsequently adding dropwise a solution of the respective aminoalkylhalides in ethanol. The ethers, all containing the aliphatic amino function, were characterized as the picrate or methyl halide derivatives.

The compounds were synthesized in order to evaluate the influence of certain groups on bioactivity, with a specific point in mind being that the oximino ester and oximino ether linkages could serve as groups favoring an interaction with the cholinergic receptor, provided that other groups are present. Thus, if the oximino linkage could serve as a binding group, then the presence of a cationic center, for example, the quaternary ammonium function, characteristic of many agents acting at cholinergic receptors, should promote affinity for the receptor. The result of interaction with the receptor could be either a cholinergic or anticholinergic action depending upon other molecular features. For example, relatively large nonpolar and semiplanar ring systems are frequently associated with anticholinergic activity. It would be interesting to contrast such a grouping with a smaller and more polar moiety.

TABLE I—PHYSICAL CONSTANTS AND ANALYTICAL DATA OXIME ESTERS



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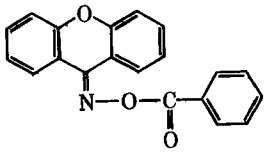
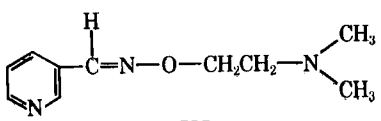


II

Compd. No.	Derivatives	M.p., °C.	% Yield	Empirical Formula	Anal. Calcd.	Anal. Found
I	—	—	—	—	—	—
I-*A*	Hydrochloride salt	124–124.5	62	$C_{13}H_{11}ClN_2O_2$	C, 59.46 H, 4.22	59.59 4.35
II	—	126.5–127	98	$C_{20}H_{13}NO_3$	C, 76.17 H, 4.15	75.98 4.13

TABLE II—PHYSICAL CONSTANTS AND ANALYTICAL DATA OXIME ETHERS



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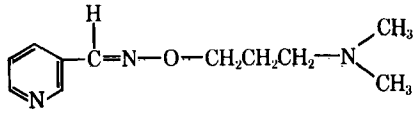

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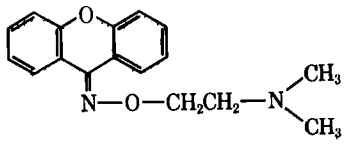
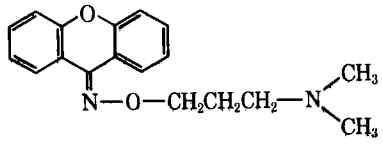


V



VI

Compd. No.	Derivatives	M.p., °C.	% Yield	Empirical Formula	Anal. Calcd.	Anal. Found
III	—	—	67	$C_{10}H_{15}N_3O$	—	—
III-*A*	Picrate	134.5–135	—	$C_{16}H_{18}N_6O_8$	C, 45.50 H, 4.29	45.54 4.50
III-*B*	Dimethyl dibromide	226–226.5	—	$C_{12}H_{21}Br_2N_3O$	C, 37.61 H, 5.52	37.67 5.35
IV	—	—	56.4	$C_{11}H_{17}N_3O$	—	—
IV-*A*	Picrate	130	—	$C_{17}H_{20}N_6O_8$	C, 46.78 H, 4.62	46.87 4.75
IV-*B*	Dimethyl diiodide	166–166.5	—	$C_{13}H_{23}I_2N_3O$	C, 31.78 H, 4.72	32.37 4.78
V	—	—	60	$C_{17}H_{18}N_2O_2$	—	—
V-*A*	Picrate	177–178.5	—	$C_{23}H_{21}N_5O_9$	C, 54.01 H, 4.14	54.26 4.38
VI	—	—	65.8	$C_{18}H_{20}N_2O_2$	—	—
VI-*A*	Picrate	134–135	—	$C_{24}H_{23}N_5O_9$	C, 54.86 H, 4.41	54.84 4.39
VI-*B*	Methyl bromide	240	—	$C_{19}H_{23}BrN_2O_2$	C, 58.32 H, 5.92	58.20 5.88

EXPERIMENTAL[1]

Synthesis of Oximes

3-Pyridinealdoxime—This compound was prepared according to the method of Ginsburg and Wilson (3). The oxime was recrystallized from 95% ethanol. The analytically pure sample melted at 150°; reported m.p. 150–151° (3). The yield was 1.8 g. (85% of theory).

Xanthoxime—The procedure of Campbell *et al.* (4) was adapted to the synthesis of this compound. The oxime was recrystallized from anhydrous benzene. An analytically pure sample of this compound melted at 161°; reported m.p. 161° (4). The yield was 8.5 g. (80% of theory).

[1] Reported melting points are uncorrected. A Thomas-Hoover Unimelt apparatus was used for melting point determinations. Schwarzkopf Microanalytical Laboratory, Woodside, N. Y., conducted the elemental analysis.

Synthesis of Oxime Esters

3-(Benzoyloximino)pyridine (I)—This compound was prepared from 3-pyridinealdoxime (3.05 g., 0.025 mole) and benzoyl chloride (4.21 g., 0.03 mole) in 1,4-dioxane according to the procedure of Gass and Bope (5). The yield was improved when anhydrous benzene was the solvent. However, anhydrous ether was found to be the most suitable reaction solvent. The hydrochloride salt (I-*A*) was prepared and recrystallized from ethanol to give the analytically pure sample, m.p. 124°. The yield was 3.2 g. (62% of theory).

9-(Benzoyloximino)xanthene (II)—Xanthoxime (3.37 g.; 0.016 mole) in anhydrous ether (200 ml.) was placed in a 500-ml., 3-necked round-bottom flask fitted with a stirrer, dropping funnel, and a condenser with a drying tube. Benzoyl chloride (2.24 g., 0.01 mole) dissolved in anhydrous ether (2.5 ml.) was added dropwise with stirring at room

temperature. Anhydrous benzene (100 ml.) was added twice at 15-min. intervals. The reaction mixture was washed with 10% potassium carbonate solution. The nonaqueous layer was dried over anhydrous sodium sulfate and filtered. The solvents were distilled off under reduced pressure. The solid residue was recrystallized from absolute ethanol. The analytically pure sample melted at 126.5–127°; the yield was 4.9 g. (98% of theory).

Synthesis of Oxime Ethers

3-(β-Dimethylaminoethyloximino)pyridine (III)—3-Pyridinealdoxime (6.1 g., 0.05 mole) was placed with ethanol (75 ml.) in a 3-necked round-bottom flask fitted with stirrer, a dropping funnel, and a water condenser with a drying tube. Sodium hydride (4.70 g. of a 51.6% mineral oil dispersion; 0.01 mole) was added slowly in small portions. The reaction mixture was stirred for 30 min. β-Dimethylaminoethylchloride hydrochloride (7.25 g., 0.05 mole) in ethanol (50 ml.) was added dropwise with stirring to the refluxing reaction mixture. The mixture was refluxed and stirred for 4 hr. Strictly anhydrous conditions were observed throughout the period. The reaction mixture was filtered, and the solvent was distilled off under reduced pressure. The product was obtained as an orange, viscous oil. The yield was 6.4 g. (67% of theory). The oxime ether was characterized as the picrate (III-*A*) and dimethyl dibromide (III-*B*) derivatives.

The other oxime ethers, 3-(γ-dimethylaminopropyloximino)pyridine (IV), 9-(β-dimethylaminoethyloximino)xanthene (V), and 9(γ-dimethylaminopropyloximino)xanthene (VI) were synthesized according to the method described for 3-(β-dimethylaminoethyloximino)pyridine with reflux periods of 6, 30, and 48 hr., respectively. Each of these was characterized as the picrate derivative, IV-*A*, V-*A*, and VI-*A*, respectively. 3-(γ-Dimethylaminopropyloximino)pyridine (IV) also was characterized as the methyl iodide derivative (IV-*B*) and 9(γ-dimethylaminopropyloximino)xanthene as the methyl bromide derivative (VI-*B*).

PHARMACOLOGICAL EVALUATION

The pharmacological evaluation of the synthesized compounds was undertaken to assess their spasmolytic activity against the spasm induced by predetermined doses of the spasmogens, acetylcholine bromide and methacholine[2] chloride, on the excised strips of smooth muscle of rat ileum. The method involves a quantitative comparison, *in vitro*, of the spasmolytic activity of each experimental compound in parallel with a reference drug, atropine sulfate, under reproducible conditions.

The smooth muscle employed as test tissue was a strip of 1–2 cm. in length from an anesthetized male or female albino rat which had been recently sacrificed. The strip was suspended in an 80-ml. bath chamber filled to a constant volume (70 ml.) with the Sollmann-Rademaeker solution at a constant temperature of 37°. The muscle contractions were amplified and recorded on an E and M Physiograph through a myograph attachment.

The method was essentially that of Magnus as modified by Ariens (6). This method uses logarithmic dilutions of spasmogens. The Ariens method yields an S-shaped cumulative dose-response curve when percent contraction (response) is plotted against log millimole dose. The potencies of the experimental compounds relative to the reference drug were determined in a series of experiments. The five compounds reported in Table III include one (II) that was markedly inactive against the spasmogens. A second compound (III-*B*) exhibited cholinomimetic activity as reported in Fig. 1. The remaining three compounds showed antispasmodic activity as summarized in Figs. 2, 3, and 4. Relative potencies of the antispasmodics are presented in Table III.

STRUCTURE-ACTIVITY RELATIONSHIPS

The pharmacological evaluation of the experimental compounds reveals that most possess perceptible antispasmodic activity, although the order of acetylcholine antagonism is low as compared with atropine sulfate. Compound VI-*B* is the most active of the experimental compounds. It is $^1/_{62}$ as active as atropine sulfate. It possesses the relatively large xanthene group. Replacement of the large and bulky xanthene group in VI-*B* with a methylpyridinium group yields com-

TABLE III—RELATIVE POTENCIES OF DERIVATIVES OF OXIME ESTERS AND OXIME ETHERS

Compound No.	Activity	Relative Potency Compared with Atropine Sulfate
I-*A*	Anticholinergic	1/40,000
II	Inactive	—
III-*B*	Cholinomimetic	—
IV-*B*	Anticholinergic	1/666
VI-*B*	Anticholinergic	1/62.5

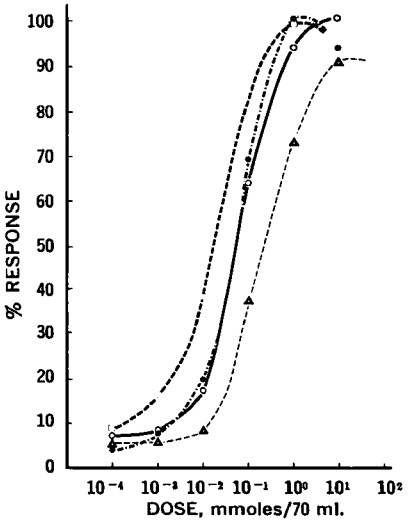


*Fig. 1—Investigation of activity of Compound III-*B *on rat ileum. Key: cumulative dose response curves. ○, acetylcholine bromide (control); ●, acetylcholine bromide (control after pretreatments); △, pretreatment with 1.0 × 10⁻³ mmoles atropine sulfate; □, pretreatment with 1.0 × 10 mmoles III-*B.

[2] Mecholyl chloride, Merck & Co., Rahway, N. J.

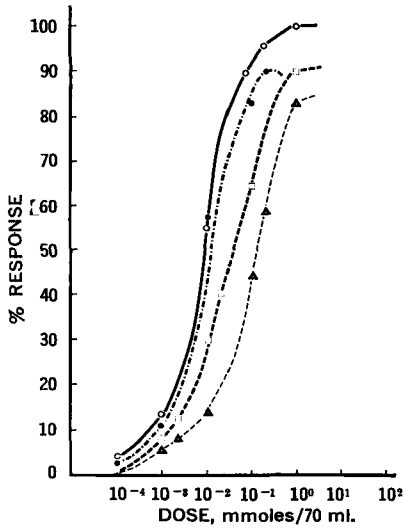


Fig. 2—Investigation of spasmolytic activity *of compound I-*a *on rat ileum. Key: Cumulative dose-response curves.* ○, *methacholine chloride (control);* △, *pretreatment with 1.0 × 10^{-4} mmoles atropine sulfate;* □, *pretreatment with 1.0 × 10^{0} mmoles I-*A*;* ●, *methacholine chloride (control after pretreatments).*

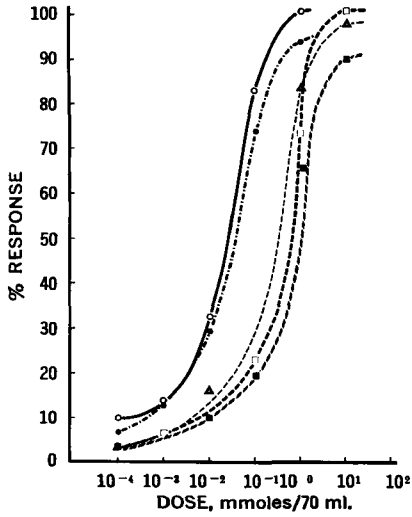


*Fig. 3—Investigation of spasmolytic activity of Compound IV-*B *on rat ileum. Key: cumulative dose-response curves* ○, *acetylcholine bromide (control);* ●, *acetylcholine bromide (control after pretreatments);* △, *pretreatment with 1.0 × 10^{-3} mmoles atropine sulfate;* □, *pretreatment with 1.0 × 10 mmoles IV-*B*;* ■, *pretreatments with 2.0 × 10 mmoles IV-*B.

pound IV-*B* which is about 10 times less active than the former.

Compound III-*B* somewhat resembles acetylcholine in structure. It has a *N*-methyl-3-pyridinealdoximino moiety replacing the acetoxy group. In broad qualitative terms, it thus has structural features related to a second choline moiety, *i.e.*, a quaternary ammonium function, a three carbon chain, and a site of high electron density. This compound shows cholinomimetic activity. It is

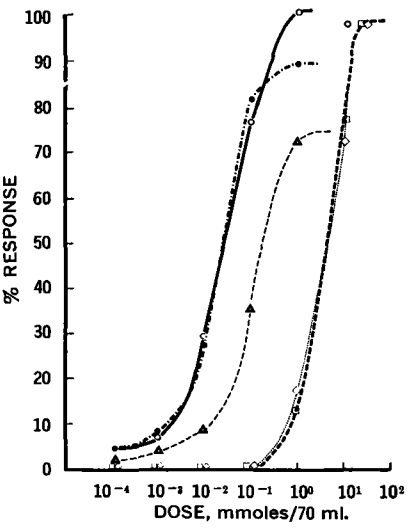
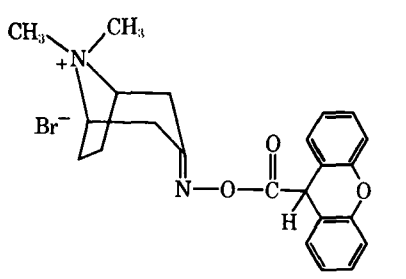


*Fig. 4—Investigation of spasmolytic activity of Compound VI-*B *on rat ileum. Key: cumulative dose-response curves.* ○, *acetylcholine bromide (control);* △, *pretreatment with 1.0 × 10^{-3} mmoles atropine sulfate;* □, ◇, *pretreatment with 1.0 × 10 mmoles, VI-*B*;* ●, *acetylcholine bromide (control after pretreatments).*

interesting to note that IV-*B*, differing from III-*B* by the insertion of a —CH_2— group, possesses discernible anticholinergic activity.

Compound II is poorly soluble in an aqueous medium. Lack of distribution and accessibility to the site of action may partly account for its inactivity, but it should be noted that this compound does not possess a cationic head, one of the pharmacophoric moieties commonly implicated in the drug–receptor interaction, which also should be important. Compound I-*A* would be present to some extent in the protonated form under the test conditions. Compounds III-*B*, IV-*B*, VII have the quaternary ammonium function and are active. These studies indicate that although an ester function is not absolutely essential (7), the presence of a related site of polarity is important. Many potent anticholinergic drugs possess interatomic distances between the cationic nitrogen and a polar and/or polarizable group (*e.g.*, ester) comparable to those of acetylcholine, in addition to large hydrophobic and semirigid moieties. In this connection, it is noteworthy that compound VI-*B* is less active than the oxime ester 3-(xanthene-9-acyloximino)tropane methyl bromide (VII), reported previously (1). The compound VII is six times more active than atropine. This difference



VII

in anticholinergic activity between VII and the experimental compound VI-*B* might be due in part to the difference in steric rigidity and bulkiness of the tropane N-function and the acyclic N-function of VI-*B*.

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Keyphrases

Xanthoxime esters, ethers—synthesis
Pyridine-3-aldoxime esters, ethers—synthesis
Anticholinergic activity—screening
Ileum strips—spasmolytic testing

Morphine-Induced Fetal Malformations I

Exencephaly and Axial Skeletal Fusions

By HOWARD S. HARPEL, JR.* and RONALD F. GAUTIERI

High subcutaneous doses of morphine sulfate, 100–500 mg./kg., administered to CF-1 mice on Day 8 or Day 9 of gestation are teratogenic and result in a large number of fetuses with exencephaly and axial skeletal fusions. Retardation in food consumption is not primarily responsible for these effects even though fasting alone affects embryonic development. Based upon the narrow range between the maternal LD_{50} and the teratogenic doses in this species, the teratogenic potential of morphine sulfate is low.

With the current interest in drug-induced embryopathies, it is surprising that the recent literature is devoid of teratogenic studies involving the narcotic analgesics. In 1931, Myers (1) reported that daily morphine administration beginning 3–5 months before mating and continuing throughout gestation had little effect upon the growth and reproduction of the albino rat. Although the litters were of average size and composed of young that appeared normal, the method of examination was not as extensive as the techniques currently employed in teratogenic studies. Moreover, ample time was available for the female rats to become tolerant to morphine before conception, and consequently, all development occurred in a morphine-adapted environment.

Therefore, it was imperative to determine the consequences of morphine sulfate administration during the critical stages of development in a common laboratory mammal by administering high subcutaneous doses to nontolerant CF-1 mice on the eighth or ninth day of gestation. Moreover, during the investigation it became clear that drug administration produced a temporary decrease in food intake, and for this reason the effect of food deprivation on each of these 2 days also was investigated.

EXPERIMENTAL

CF-1 albino mice weighing between 20–25 g. were obtained from Carworth Farms, Inc., New City, N. Y. Females were caged in groups of 25 to 30 for at least 2 weeks after arrival and were not mated until they weighed at least 25 g. Males and gravid females were caged individually in metal cages measuring 12.5 × 15 × 10 cm. with a wire mesh front and floor. The colony was maintained on Purina laboratory chow and tap water *ad libitum*. Sodium chloride (0.9%) and morphine sulfate[1] (4.0%) prepared weekly in distilled water were

Received January 31, 1968, from the School of Pharmacy, Temple University, Philadelphia PA 19140
Accepted for publication June 20, 1968.
Abstracted in part from a thesis presented by Howard S. Harpel, Jr. to the Graduate School, Temple University, in partial fulfillment of Doctor of Philosophy degree requirements.
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administered subcutaneously in the upper right abdominal area.

The doses of morphine chosen for use in this study were based on the LD_{50} in nongravid mice. The LD_{50} of 525 mg./kg. (500–551 mg./kg., 95% confidence limits) was established by administration of 4 doses (400–700 mg./kg.) to 10 mice at each dose level (2).

To produce timed pregnancies, two females were placed in the cage of a fertile male at 4:00 p.m. The next day at 8:00 a.m., females exhibiting a vaginal plug of coagulated ejaculate were considered gravid. The day of the appearance of the vaginal plug was designated as Day 0. Gravid females were weighed, caged individually, and left undisturbed until the morning of Day 6 when they were weighed again. Pregnancy was confirmed by a weight gain of two or more grams. At this time the gravid animals were randomly assigned to one of 15 experimental categories: an untreated control group; two saline control groups receiving 0.3 ml. of 0.9% saline solution on either Day 8 or Day 9; 10 morphine-treated groups receiving 100, 200, 300, 400 or 500 mg./kg. of morphine sulfate on either Day 8 or Day 9; and two food-deprivation groups that were deprived of access to food on either Day 8 or Day 9. All solutions were injected on either Day 8 or Day 9 between 8:00 and 9:00 a.m.

Every animal was weighed on the morning of Day 6 through Day 13 to standardize the effects of handling. A laparotomy was performed immediately on mice killed by drug treatment in order to reconfirm pregnancy and count implantation sites. A similar procedure was followed with animals whose body weight changes indicated that complete resorption of the litter was occurring.

On Day 18, after sacrifice by dislocation of the cervical vertebrae, the uterine horns were exposed surgically. The number and position of fetuses and resorption sites were counted and recorded. Fetal viability was determined by reflex movement of the limbs in response to mechanical stimuli applied to the uterine wall with a blunt probe.

After transection of the right horn between the ovary and the first fetus, the fetus was removed by blunt dissection, blotted dry with absorbent paper, weighed and measured from the crown to the rump. It was examined for external defects, and the sex determined. Each member of the litter was removed in turn and examined in this manner. Every second fetus was fixed for soft tissue examination, and the remainder were prepared for skeletal examination. To establish the uterine position occupied by the fetus, a straight pin with a colored head was inserted into the base of the tail or axilla before fixation with Bouin's. Specimens were stored in 70% ethanol after 2 weeks in the fixative. Before sectioning, the specimens were reexamined for external defects under a low-power binocular microscope. Free-hand sections were made with a thin, double-edged razor blade according to the method of Wilson (3).

For skeletal examination, specimens were prepared by alkaline maceration, staining with alizarin red S, and clearing according to the method of Staples and Schnell (4).

Food consumption was measured to determine if drug treatment altered food intake in a manner that might correlate with effects upon the conceptus. Feeders were made from 120 g. clear glass ointment jars, 5.5 cm. in height and 7 cm. in diameter, with hard plastic tops. A hole 2 cm. in diameter drilled in the center of the lid allowed free access to food but prevented scattering and loss of uneaten food. These feeders were filled daily with 120 g. of finely ground Purina laboratory chow. They were placed in the cage on the morning the vaginal plug was discovered.

The mice accepted the feeder very readily, but data from Day 0 and Day 1 were often inconsistent with the normal pattern that was established by Day 2. Such inconsistencies for the first 2 days were found with most of the animals tested.

The statistical significance of variations among the experimental groups was estimated by the Student t test for continuous variates and the unadjusted chi-square or Yates' corrected chi-square for binomial proportions (5). Probabilities, p, greater than 0.05 were considered to represent insignificant differences.

RESULTS

Maternal Effects—Regardless of the dose or day of administration, gravid mice given morphine exhibited stimulation followed by depression and a hyperactive period of recovery all of which differed mainly in duration and intensity. After 24 hr., all survivors, irrespective of dosage, appeared normal in spontaneous activity and appearance. Any loss in body weight was regained by the second morning after treatment in conjunction with a return of food consumption to normal levels.

There were no deaths among the gravid mice treated with 100, 200, or 300 mg./kg. of morphine on Day 8 or Day 9. However, deaths did occur with the two higher doses employed; 3 of 14 or 21% were killed by 400 mg./kg. on Day 8, and 2 of 13 or 15% were killed on Day 9. After a dose of 500 mg./kg., 6 of 15 or 40% were killed on either Day 8 or Day 9. These results did not differ significantly from the expected number of deaths in nongravid animals calculated in the LD_{50} determination.

During gestation, 32 control females gained an average of 20.5 g. and the mean maternal weight gain of this and the other experimental groups is tabulated in Table I. It can be seen that the various treatments did not alter significantly the amount of weight gained with the exception of the group which received 500 mg./kg. of morphine on Day 9.

The mean change in body weight 24 hr. after treatment with saline on Day 8 and Day 9 was an increase of 0.2 and 0.7 g., respectively. Among the morphine-treated groups weight loss did not vary significantly with dosage. Irrespective of dosage, mean weight loss in the groups treated with morphine on Day 8 was 1.6 g., and 10 g. in those treated on Day 9. The largest weight losses were seen with the food-deprivation groups; in the Day 8 group the loss was 6.3 g. and in the Day 9 group 4.9 g.

In all mice, with the exception of those which resorbed completely, body weight returned to normal by the second morning after treatment and continued to increase in the normal pattern.

Food Consumption—The absolute amount of food consumed daily varies roughly as a function of body weight, and comparison of absolute consumption was not considered valid. However, net changes

TABLE I—MEAN MATERNAL WEIGHT GAIN, NUMBER OF IMPLANTATION SITES, AND NUMBER OF RESORPTIONS

Treatment[a]	Maternal Weight Gain, g.			Implantation Sites			Fetal Resorptions		
	N[b]	$\bar{x}$[c]	s[d]	N[e]	$\bar{x}$	s	N[e]	$\bar{x}$	s
Control	32	20.5	4.1	43	10.8	1.97	43	1.2	1.22
S-8	8	22.2	4.0	8	11.3	1.06	8	0.6	0.77
S-9	9	18.7	5.0	9	10.3	1.32	9	1.3	1.38
M-1-8	8	23.0	3.8	8	11.4	1.30	8	0.9	0.95
M-2-8	6	20.7	4.2	6	11.7	2.50	6	1.0	1.64
M-3-8	6	19.8	3.1	6	10.2	1.17	6	1.2	1.09
M-4-8	11	20.1	8.1	11	10.8	1.40	11	1.6	1.97
M-5-9	6	23.0	3.0	7	11.6	1.34	7	1.0	1.41
FD-8	6	21.0	6.5	6	11.3	1.21	6	1.7	2.34
M-1-9	5	17.0	4.6	6	11.3	2.14	6	1.3	1.52
M-2-9	9	21.7	4.6	9	10.9	1.27	9	1.3	1.34
M-3-9	10	23.1	4.2	10	11.7	1.70	10	0.7	0.67
M-4-9	9	22.3	5.5	9	11.8	1.64	9	2.6[f]	2.79
M-5-9	5	16.2[f]	4.6	7	11.8	1.68	7	2.6[f]	2.12
FD-9	7	21.3	2.6	7	11.1	2.27	7	1.3	1.79

[a] Treatment: Control, untreated; S, saline-treated; M, morphine-treated; FD, food deprivation; 1,2,3,4, or 5 indicates dose of morphine in hundreds of mg./kg.; 8 or 9 indicates the day of treatment. [b] Number of observations. [c] Arithmetic mean. [d] Standard deviation. [e] Does not include pregnancies in which complete resorption of litter occurred. [f] Statistically significant in comparison with control, $p < 0.05$.

in consumption for each animal are comparable and have been used to calculate the mean net change before and after treatment for each group. The mean net changes in food consumption on the critical days of the test are tabulated in Table II. The effect of treatment on Day 8 is seen by comparison of consumption on Day 7 and Day 8, while the effect of treatment on Day 9 is seen by comparison of Day 8 and Day 9.

In all morphine-treated groups food consumption was decreased significantly; saline injection produced minimal effect; and in the food-deprivation group consumption was nil, because access to food was denied.

For animals treated on Day 8, comparison of consumption on the day following treatment, Day 9, with that on the day before treatment, Day 7, indicated that in all groups food intake returned to normal levels. For animals treated on Day 9, comparison of consumption on the day following treatment, Day 10, with that on the day before treatment, Day 8, indicated that food consumption was still slightly reduced in the groups administered 100, 200, 300, and 500 mg./kg. of morphine; however, consumption returned to normal levels by the next day.

Implantations and Resorptions—The mean number of implantation sites observed at term in each treatment category are tabulated in Table I.

Any area on the uterine wall showing evidence that nidation had occurred was counted as an implantation site. Therefore, the number of implantation sites represented the total number of viable fetuses and resorption sites observed at term.

In 43 control litters the mean number of implantation sites was 10.8. The mean number of implantation sites in mice that did not survive treatment with morphine on either Day 8 or Day 9 was 12.3 and 11.7, respectively. Although the mean value in many of the treatment groups was slightly higher, none of these values differed significantly from the control group.

Complete resorption occurred in only one of the 44 control pregnancies. After administration of 500 mg./kg. of morphine on Day 8, one of nine mice resorbed completely; and with the same dosage on

TABLE II—FOOD CONSUMPTION

Treatment[a]	N	Mean Changes in Food Consumption, g.					
		Between 2 Days Before Treatment[b]		Between Day Before and Day of Treatment[c]		Between Day Before and Day After Treatment[d]	
Nongravid	7	+0.4	−0.2	−0.2	+0.2	+1.0	+0.7
Control	7	+0.8	+0.8	+0.8	+1.1	+0.6	+0.8
S-8	6	−0.9		+0.3		+0.7	
M-1-8	6	+0.5		−3.1		−0.4	
M-2-8	6	+0.5		−2.5		0.0	
M-3-8	5	+0.8		−2.2		0.0	
M-4-8	5	+0.7		−4.4		+0.5	
M-5-8	5	+1.7		−6.1		−0.7	
FD-8	5	−0.2		—		+0.5	
S-9	6		−0.6		−0.1		−0.4
M-1-9	4		+1.6		−4.0		−0.9
M-2-9	5		+0.1		−3.4		−0.4
M-3-9	6		+0.5		−4.1		−0.8
M-4-9	5		+0.6		−4.5		+0.1
M-5-9	5		+1.3		−6.1		−1.4
FD-9	7		+0.6		—		+0.9

[a] See Table I. [b] For mice treated on Day 8, represents Day 6–7. For mice treated on Day 9, represents Day 7–8. [c] For mice treated on Day 8, represents Day 7–8. For mice treated on Day 9, represents Day 8–9. [d] For mice treated on Day 8, represents Day 7–9. For mice treated on Day 9, represents Day 8–10.

Day 9, one of eight mice resorbed completely. After food deprivation on Day 8, three of nine pregnancies terminated in complete resorption. This rate of resorption, 33 %, was significantly higher than the 2% seen in the control group. There were no complete resorptions in any of the other groups.

The number of intrauterine deaths was increased significantly only in the groups administered 400 or 500 mg./kg. of morphine on Day 9.

Intrauterine deaths and subsequent resorption sites were further classified according to their gross appearance. The three classifications were: first, total resorption in which the site was marked by a small green knob with no evidence of any other placental or fetal tissue; second, partial resorption in which the placenta still possessed a discrete disk shape and fetal tissue, if detectable, was an amorphous mass; and third, recent death in which necrosis had begun but the external features of the fetus were still recognizable. The tabulation of fetal resorption by type is given in Table III.

It can be seen in the groups treated with 400 or 500 mg./kg. on Day 9 that the number of total resorptions was not extraordinary, but the number of partial resorptions was very high. This type of resorption was responsible for the significant difference in the resorption rate when compared to the controls.

TABLE III—CLASSIFICATION OF FETAL RESORPTIONS

Treatment[a]	Type of Resorption		
	Total	Partial	Recent Death
Control	47	3	1
S-8	4	1	
S-9	10	1	1
M-1-8	6	1	
M-2-8	5	1	
M-3-8	7		
M-4-8	11	3	
M-5-8	5	1	1
FD-8	8	2	
M-1-9	8		
M-2-9	11	1	1
M-3-9	5	2	
M-4-9	10	16	
M-5-9	6	10	1
FD-9	8	1	

[a] See Table I.

The mean weight and crown-rump length of the fetuses in each treatment group are tabulated in Table IV.

Soft Tissue Malformations—The soft tissue defect with the highest incidence was exencephaly which is characterized by the extensive exteriorization of cerebral tissue due to the absence of the skin and bones of the cranial fault (Fig. 1). Diagnosis of exencephaly could be made upon inspection of the litter before the uterine horn was opened because each uterine swelling associated with this defect was dark red and edematous. This hydramnios was caused by bloody cerebrospinal fluid entering the amnionic sac from the open cerebral ventricles.

All of the exencephalic fetuses were viable, and their respiratory movements were initiated spontaneously as quickly as normal littermates. However, in contrast to the pinkish skin color of the normal fetus, all exencephalic fetuses were blanched and anemic in appearance.

Tissue sections from the malformed fetuses show the neural elements to be markedly disorganized, though in some instances rudimentary organization of the hemispheres had occurred.

The number of exencephalic fetuses and the number of litters containing defective young are tabulated in Table V.

Fetuses with exencephaly were evenly divided between the two uterine horns; irrespective of maternal treatment, 13 were found in the right horn and 14 in the left. Furthermore, within each horn the defective fetuses appeared to be randomly distributed with respect to uterine position. The number of male and female exencephalic fetuses were 12 and 15, respectively.

A number of other defects was found in association with exencephaly induced by morphine. The eyes which usually are closed until 14 days after birth were often open. In two fetuses one or both eyes were closed but misshapen. In one fetus both eyes were absent, and in another the right eye was missing. Two fetuses had cleft palate. In seven of the fetuses the snout was shortened so that the lower jaw and tongue appeared to protrude beyond the nose. In four fetuses the internal ear appeared to be larger than in littermates.

TABLE IV—WEIGHTS AND CROWN-RUMP LENGTHS OF FETUSES

Treatment[a]	Weight, g.			Length, cm.		
	N	$\bar{x}$	s	N	$\bar{x}$	s
Control	320	1.2	0.224	298	2.3	0.100
S-8	80	1.2	0.100	74	2.3	0.141
S-9	84	1.2	0.161	82	2.3	0.173
M-1-8	84	1.2	0.100	84	2.3	0.141
M-2-8	64	1.1[b]	0.100	64	2.3	0.141
M-3-8	54	1.2	0.0	54	2.3	0.173
M-4-8	101	1.1[b]	0.100	101	2.2[b]	0.100
M-5-8	86	1.1[b]	0.141	86	2.2[b]	0.141
FD-8	58	1.1[b]	0.141	57	2.2[b]	0.141
M-1-9	54	1.1[b]	0.469	53	2.1[b]	0.141
M-2-9	85	1.1[b]	0.118	85	2.3	0.173
M-3-9	110	1.1[b]	0.141	109	2.3	0.173
M-4-9	111	1.1[b]	0.141	111	2.2[b]	0.173
M-5-9	66	1.0[b]	0.155	65	2.1[b]	0.100
FD-9	69	1.1[b]	0.141	69	2.2[b]	0.100

[a] See Table I. [b] Statistically significant in comparison with control, $p < 0.05$.

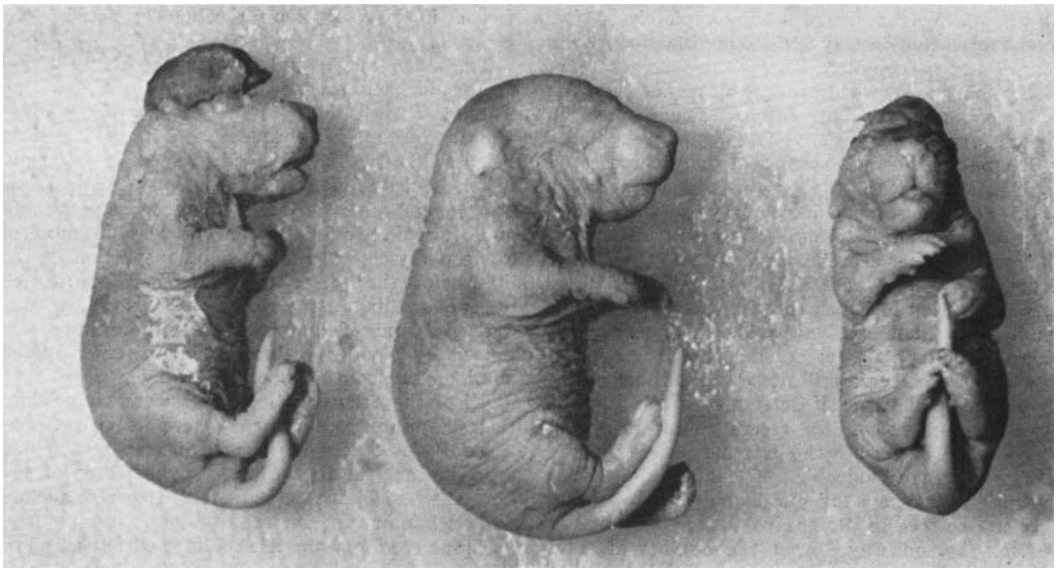

Fig. 1—Near-term mouse fetuses from a female treated with 500 mg./kg. of morphine sulfate on Day 8. In the exencephalic fetuses note the open eye and shortened snout (left) and protrusion of the tongue (right). The middle fetus is a normal littermate.

The weight of the exencephalic fetuses was often but not always less than the mean weight of the normal fetuses. The mean fetal weight of the defective fetuses from the 300 mg./kg. group on Day 8 was equal to the control weight; after 400 mg./kg. it was 0.9 g., $s = 0.08$; and after 500 mg./kg. it was 1 g., $s = 0.07$. The latter two values are significantly lower than the mean fetal weights of all fetuses in their respective groups.

The crown-rump length of exencephalic fetuses was not reduced in comparison to the control fetuses. In fact, the mean crown-rump length was greater in the groups treated with 300 or 400 mg./kg. of morphine on Day 8. Some difficulty was encountered in measuring the total length of fetuses with exencephaly due to the protrusion of neural tissue. This may account for the apparently greater length of these fetuses. Therefore, the fetal weight appears to be a better indication of actual fetal size.

Missing from the skull of the exencephalic fetal skeletons were the frontal, parietal, interparietal, and occipital ossification centers which at this time normally show extensive ossification. In many exencephalic skulls the premaxilla and nasal bones were shortened, but the mandible was of normal size and shape.

Other than exencephaly and defects associated with exencephaly, only a few other soft tissue malformations were found in all of the fetuses examined. In six fetuses testicular descent was retarded; bilaterally in one fetus in the 100 mg./kg. morphine Day 9 group; on the left in a single fetus from the 500 mg./kg. morphine Day 8 group, the 200 mg./kg. morphine Day 9 group and food-deprivation Day 9 group; and on the right in a single fetus from the saline Day 8 group and the food-deprivation Day 9 group. A single fetus with the left pinna misshapen and one fetus with a cleft palate were found in the group given 300 mg./kg. of mor-

TABLE V—THE OCCURRENCE OF EXENCEPHALY AND AXIAL SKELETAL FUSIONS

Treatment[a]	Exencephaly				Axial Skeletal Fusions			
	Litters Absent	Litters Present	Normal Fetuses	Abnormal Fetuses	Litters Absent	Litters Present	Normal Fetuses	Abnormal Fetuses
Control	42	1	386	1	30	0	134	0
S-8	7	0	74	0	7	0	37	0
S-9	10	0	88	0	10	0	45	0
M-1-8	8	0	83	0	8	0	40	0
M-2-8	6	0	64	0	6	0	30	0
M-3-8	3	3[b]	49	5[b]	6	0	26	0
M-4-8	8	3[b]	86	6[b]	6	3[b]	42	4[b]
M-5-8	4	4[b]	75	11[b]	5	3[b]	40	5[b]
FD-8	5	1	57	1	2	4[b]	24	8[b]
M-1-9	4	2[b]	51	2[b]	4	1	17	2[b]
M-2-9	9	0	79	0	3	5[b]	31	10[b]
M-3-9	10	0	108	0	6	4[b]	46	10[b]
M-4-9	10	1	110	1	4	7[b]	40	18[b]
M-5-9	8	0	66	0	3	4[b]	23	9[b]
FD-9	7	0	69	0	7	0	35	0

[a] See Table I. [b] Statistically significant in comparison with control, $p < 0.05$.

phine on Day 9. A fetus from the group treated with 400 mg./kg. of morphine on Day 9 had cleft palate, spina bifida, misshapen external ears, anophthalmia on the right, and microophthalmia on the left.

Skeletal Malformations—The number of fetuses with rib and vertebral fusions as well as the number of litters with these defects are tabulated in Table V. Fetuses with rib and vertebral fusions after treatment with 500 mg./kg. of morphine on Day 9 are illustrated in Fig. 2.

The extent of the rib fusion varied from fusion only at the base where the ribs join the vertebral column to fusion throughout their entire length. The number of ribs involved on any one side varied from a minimum of two to a maximum of eight. Also, fusions involving the same ribs occurred both bilaterally and unilaterally in about equal numbers. After treatment with morphine on Day 8, none of the ribs below the eighth pair were involved in fusions, while the number of fusions involving each of the ribs one through eight was about equal. After food deprivation on Day 8, only ribs four through twelve were involved; a few fusions were seen in ribs four through eight and in the twelfth, but the highest number were in ribs nine through eleven. After treatment with morphine on Day 9, fusions were found involving all ribs, but 90% involved ribs posterior to the sixth pair.

DISCUSSION

The results of this investigation clearly demonstrate that morphine sulfate administered in high doses on Day 8 or Day 9 is teratogenic in CF-1 mice.

The administration of 100 mg./kg. of morphine on Day 8 had no apparent effect upon development. Doses of 200 mg./kg. lowered the mean fetal weight, but no morphological abnormality was detected. With doses of 300 mg./kg. exencephaly was found in one-half of the litters examined even though the mean fetal weight was not decreased significantly. After doses of 400 or 500 mg./kg. exencephaly and axial skeletal fusions were found in significant numbers along with a decrease in the mean fetal weight and crown-rump length.

All doses of morphine employed on Day 9 produced fusions in the axial skeleton and a decrease in the mean fetal weight. Doses of 400 and 500 mg./kg. produced a significant increase in the number of partial fetal resorptions as well as a decrease in the mean crown-rump length. The mean maternal weight gain was significantly decreased after 500 mg./kg. In contrast to Day 8, the number of exencephalic fetuses produced was significant only in the 100 mg./kg. group. However, it does appear that on Day 9 mouse embryos are more susceptible to morphine-induced abnormalities.

Food deprivation on Day 8 was also teratogenic. Axial skeletal fusions were produced in significant numbers as well as one exencephalic fetus. Furthermore, the mean fetal weight and crown-rump length were decreased significantly. However, food deprivation on Day 9 did not result in teratogenesis, although the mean fetal weight and crown-rump length were decreased significantly.

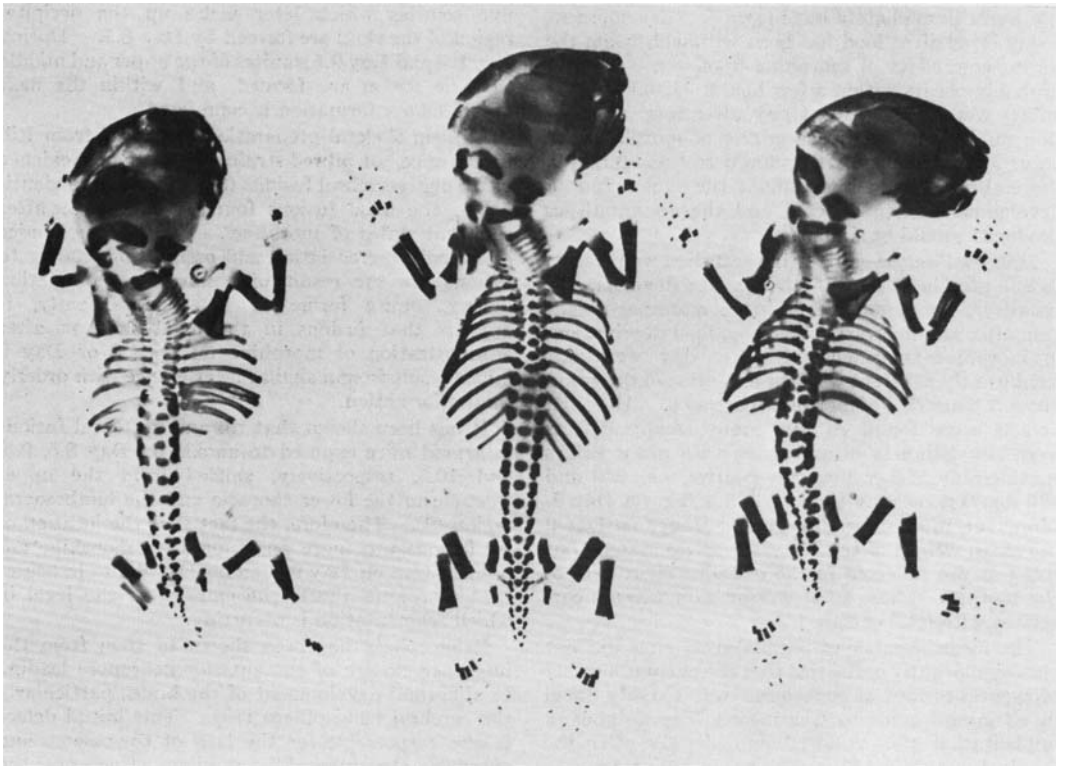

Fig. 2—Alizarin skeletal preparations of near-term mouse fetuses from a female treated with 400 mg./kg. of morphine sulfate on Day 9 showing fusions of the lower ribs and vertebral column. The middle fetus is a normal littermate.

In three of nine mice subjected to food deprivation on Day 8, complete resorption occurred. Death of entire litters may have resulted from an embryocidal effect upon all embryos or maternal effects incompatible with the maintenance of pregnancy. Among the six litters carried to term, the number of resorptions was not increased significantly. After food deprivation on Day 9 all litters were carried to term without an increase in the number of fetal resorptions.

Retardation of food consumption by morphine does not seem to be responsible for the teratogenic effects of the drug even though food deprivation does have definite effects upon embryonic development; for, if the teratogenic effects of morphine were caused by a decrease in food consumption, abnormalities in both the morphine-treated groups and the food-deprivation groups would be similar after treatment on the same day.

Although morphine treatment on Day 8 produced a large number of exencephalic fetuses, only one was found after food deprivation on this day. Moreover, unlike axial skeletal fusions after morphine on Day 8, a large number of fusions were found posterior to the eighth pair of ribs after food deprivation on this day. In fact, the low incidence of exencephaly and the location of axial skeletal fusions after food-deprivation on Day 8 compared quite favorably to the results obtained with morphine treatment 1 day later. Although a large number of axial skeletal fusions were found after morphine treatment on Day 9, the axial skeleton was not affected by food deprivation on this day.

It appears the teratogenic effects of starvation do not occur immediately but may affect development a day or so after food has been withheld, while the teratogenic effect of morphine upon the conceptus probably occurs within a few hours. If teratogenic effects were delayed for 1 day after food deprivation on Day 8 and if the effects of morphine did occur almost at once after administration on Day 9, the embryos would be in about the same stage of development when affected and the abnormalities produced should be similar.

Maternal weight gain during gestation would seem to be a good indication of fetal size and development. However, the differences in mean maternal weight gain after administration of saline, food deprivation, or morphine treatment on either day were not significantly different with the exception of the group given 500 mg./kg. of morphine on Day 9. Although defects were found in this group, many defects were also found in other groups with mean values considerably higher than the control, *i.e.*, 300 and 400 mg./kg. on Day 9 and 500 mg./kg. on Day 8. Moreover, in all morphine-treated groups on Day 9 the mean weight of the fetuses was decreased, but this was not reflected in the overall weight gain of the mother. Thus, total weight gain was not correlated with fetal effects.

The mean number of implantation sites did not vary significantly indicating that the average number of zygotes formed at conception was roughly equal in all groups prior to treatment. The number of implantation sites counted immediately after the death of mice killed by morphine on either day was not significantly different from the control indicating that gestation was proceeding normally up to the time of treatment.

The time of intrauterine death can be estimated by the degree of resorption that has occurred because autolysis, maceration, and disappearance of the products of conception may take several days.

Resorptions classified as recent deaths must have occurred only a few days before sacrifice, because the fetal and placental tissue is necrotic but intact. The number found in the control and treated groups would not suggest any increase as a result of treatment.

The number of total resorptions in each group compared favorably to the number found in the control group indicating no effect referable to treatment. In fact, many of the deaths marked by total resorption sites could have occurred even before treatment on Day 8 or Day 9.

It seems logical to assume that deaths marked by partial resorption occurred after the treatment days. Compared with the control group, the incidence of partial resorptions in the treated groups was not increased markedly with the exception of those administered 400 or 500 mg./kg. of morphine on Day 9. The increase in intrauterine death during this time must have been caused by the embryocidal action of morphine.

The axial skeleton, derived mainly from mesoderm, originates from the primitive streak at the posterior end of the embryo. As the embryo grows, the primitive streak moves in a cranio-caudal direction leaving behind a dense band of undifferentiated mesenchyme. This mesenchyme is divided into segments or somites by intersegmental fissures and intersegmental vessels from the dorsal aorta (6). According to Otis and Brent (7), the first five somites which later make up the occipital region of the skull are formed by Day 8.5. During Day 8.5 and Day 9.5 somites of the upper and middle thoracic region are formed, and within the next 48 hr. somite formation is completed.

Alizarin skeletal preparations prepared from Rib fusion mice, an inbred strain with a high incidence of rib and vertebral fusions (8), appear to be identical to the axial fusions found in CF-1 mice after sufficient doses of morphine. In Rib fusion mice abnormal differentiation and ossification leading to fusions are the result of abnormal segmentation during somite formation (8). Consequently, it appears that fusions in the axial skeleton after administration of morphine on Day 8 or Day 9 might result from a similar interference with orderly somite formation.

It has been shown that rib and vertebral fusions in gravid mice exposed to anoxia on Day 8.5, 9.5, and 10.5, respectively, shifted from the upper thoracic to the lower thoracic and the lumbosacral region (9). Therefore, the fact that the location of rib fusions was more posterior after morphine administration on Day 9 than on Day 8 also indicates that morphine affects the embryo at the level in which segmentation is occurring.

Exencephaly has been shown to stem from the improper closure of the anterior neuropore leading to abnormal development of the brain, particularly the cerebral hemispheres (10). This initial defect is also responsible for the lack of the osseous and cutaneous structures of the cranium. Consequently, the brain is subject to degenerative and hemorrhagic changes as it lies unprotected on the floor of the skull. In man and other mammals with gestation

periods longer than the rodent most of the cerebral tissue may be completely destroyed leading to anencephaly (10).

In mice exencephaly and axial skeletal fusions have been reported after many types of treatment including the administration of insulin (11), galactoflavin (12), vitamins (9) and urethan (13) as well as riboflavin deficiency (12), X-ray (12), and anoxia (14). Although the mechanisms are not completely understood, all of these must interfere with one or more processes necessary for proper closure of the neural tube and segmentation.

The description of exencephaly produced in mice by the administration of urethan on Day 7 (13) is quite similar to the appearance of this defect after morphine administration on Day 8. According to Sinclair (13), the antimitotic effects of urethan did not seem to be the cause of the deformity because the usual number of mitotic figures was seen in the germinal layers of treated embryos. The fact that both agents are central nervous system depressants might indicate a common mechanism of action.

Gautieri and Ciuchta (15) have shown that morphine produces marked and sustained constriction of the fetal vasculature of the perfused human placenta that is characterized by a slow progressive decrease in the volume flow rate of the perfusate ranging from 35 to 92% after infusion of 0.2–0.4 mg. of the sulfate salt; this effect is reversed by the subsequent administration of nalorphine hydrochloride. Moreover, several investigators have shown that mice subjected to anoxia on Day 8 or Day 9 gave birth to a large number of young with exencephaly and axial skeletal fusions (14, 16). Therefore, evaluation of the extent and consequences of maternal and fetal anoxia after morphine administration should provide some insight into the mechanism of the teratogenic action of morphine.

Although congenital abnormalities were produced by morphine, the doses required were extremely high. Even the lowest dose utilized far exceeded the usual effective analgesic dose in this species by at least 10 times. Moreover, doses of 400 and 500 mg./kg. were lethal to 18 and 40% of the gravid animals. These higher doses are quite close to the LD_{50} of 525 mg./kg. in nongravid mice. Therefore, if the criterion for judgment of safety is the ratio of the LD_{50} dose to the teratogenic dose as has been suggested (17), it must be concluded that the teratogenic potential of morphine sulfate is low.

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Keyphrases

Morphine—teratology, mice
Malformations—fetal, mice
Food deprivation—mouse fetus
Gravid mice—morphine effects
Fetal implantations, resorptions, mice—morphine effect

Polymer–Drug Interacted Systems in the Physicochemical Design of Pharmaceutical Dosage Forms I

Drug Salts with PVM/MA and with a PVM/MA Hemi-Ester

By C. R. WILLIS, JR.* and GILBERT S. BANKER

The applicability of a polymer-drug interacted system in the physicochemical design of pharmaceutical dosage forms has been investigated. Salts of poly(methyl vinyl ether/maleic anhydride) and methapyrilene base were prepared. *In vitro* release patterns were determined by an equilibrium dialysis procedure. Dialytic release rates of the polymer-drug salt in solution demonstrated no appreciable difference in drug availability with respect to the free drug or its hydrochloride salt. *In vitro* dissolution rates of the polymer-drug salt in solid form showed similar results. Polymer-drug salts formed with the 1,12-dihydroxyoctadecane hemi-ester of poly(methyl vinyl ether/maleic anhydride) exhibited prolongation of the release of the drug from granular and tableted forms *in vitro*.

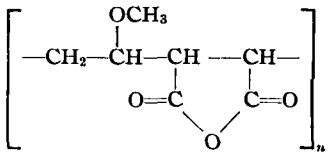
SOLUBILIZATION of various medicinal compounds by interaction with macromolecules has been extensively investigated. Such systems are usually produced by donor-acceptor mechanisms or hydrogen bonding. Unfortunately, few of these investigations have yielded products of practical value in the design of pharmaceutical dosage forms due to the low ratio of active ingredient incorporated in the system. In addition, recovery of these complexes from the reaction media is difficult or impossible in many instances.

Formation of polymer-drug salts provides a practical basis for the investigation of the physicochemical design of pharmaceutical dosage forms and subsequent biopharmaceutic evaluations (1). Such salts have been shown to alter the biologic distribution of the drug moiety as well as prolong release when administered parenterally (2). A recent patent (3), describes a method for preparing sustained-release dosage forms by the formation of polymer-drug salts. The product is based on the interaction of a therapeutically active amine with a crosslinked copolymer containing reactive carboxyl groups. Srinivas (4) studied the reaction between carboxyl-containing polymers and aliphatic and aromatic amines, and amine drug bases. Potentiometric and viscometric data indicated salt formation, with the polymer-drug salts exhibiting controlled release properties. The stability of thiamine is reported to have been enhanced by salt formation of the free base with carbonic and sulfonic acid derivatives of an unsaturated polymerizable material (5).

This investigation was undertaken to further study the application of polymer-drug mono- and di-salts of a dicarboxylic acid copolymer and of drug salts of a hemi-ester of the copolymer, in the physicochemical design of pharmaceutical dosage forms.

EXPERIMENTAL

Materials and Equipment—Poly (methyl vinyl ether/maleic anhydride),[1] (PVM/MA) (I) (6), is a free-flowing polymeric powder whose structural formula may be represented as shown below.



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PVM/MA is a linear polymeric anhydride which is soluble over a wide pH range. Good stability is exhibited in both acid and alkaline solutions. The pH of a 5% solution of the polymer is approximately 2.0. An aqueous solution of this concentration

Received April 15, 1968, from the Industrial and Physical Pharmacy Department, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907

Accepted for publication June 17, 1968.

Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

This investigation was supported in part by a fellowship from the National Institutes of Health (2-F1-MH-20, 190-03).

Abstracted from a thesis submitted to the Graduate School of Purdue University, by C. R. Willis, Jr., in partial fulfillment of the Doctor of Philosophy degree requirements.

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[1] Gantrez AN-169. Supplied through the courtesy of General Aniline & Film Corporation, New York, N. Y.

exhibits a Brookfield viscosity of 200 cps. The polymer is purported to be relatively nontoxic (6).

Methapyrilene, which was prepared from the commercially available hydrochloride salt[2] (USP), was selected as the model amine drug base for use in this investigation. The drug base has a molecular weight of 261.38 and occurs as a viscous oil, slightly yellow in color. The uncharged base has a pKb of 5.15, while the conjugate acid exhibits a pKa of 8.85 (7).

1,12-Dihydroxyoctadecane[3] (DOD) was used as the hydroxyl-containing moiety in the esterification of PVM/MA. This compound occurs as a white waxy solid which melts at 61 to 65°.[4]

Esterification reactions were carried out in the open-bottom portion of a 500-ml. split reaction flask[5] fitted with a heating mantle. The use of this apparatus permitted the easy retrieval of the esterified products which would be difficult to remove from conventional reaction flasks.

A Carver[6] laboratory press was used in the preparation of tablets employed in this investigation. All tablets were prepared with the use of 0.9-cm. (3/8-in.) standard cup punches. The tablets were compressed under a 5,000 lb. load, which was released immediately upon attaining that load.

Assay of Methapyrilene—Methapyrilene and its derivatives were assayed at 292.5 and 323.5 mμ[7] by a differential ultraviolet procedure. This assay employs the summation of absorbance maxima induced by a 3.2 unit pH differential, using buffer solutions of pH 7.4 and 10.6. The use of this assay procedure permitted the determination of unknown quantities of methapyrilene in the presence of other materials which would normally interfere with conventional ultraviolet spectrophotometric determinations.

Reactivity of PVM/MA—The reactivity of PVM/MA was evaluated by potentiometric titration in aqueous solution. Changes in the potential of the solution were monitored with the use of a research pH meter[8] equipped with standard glass indicator and calomel reference electrodes. PVM/MA was titrated with 0.1 *M* and 1 *M* sodium hydroxide and with methapyrilene base. Both the titration curves and their first derivative plots were constructed. End points were determined by evaluation of the first derivative plots.

Esterification of PVM/MA—Hemi-esters of PVM/MA and 1,12-dihydroxyoctadecane were prepared by melting the DOD in the open-bottom portion of the split reaction flask, and dispersing the PVM/MA powder in the melt. The temperature was then increased and the mixture was stirred. Esterification took place within 0.5 hr. as evidenced by solidification of the reaction mixture. The maximum temperature attained during the process was 120°. When the product had solidified, the heat was immediately removed. After cooling at room temperature, the ester was milled in a mortar and classified with the use of nested A.S.T.M. standard screens. All of the hemi-esters were prepared using excess quantities of DOD due to the fact that a stoichiometric amount of the DOD (1.33 g./g. of PVM/MA) proved to be an insufficient quantity in which to disperse the PVM/MA during the esterification reaction. The product was prepared in 2:1, 3:1, and 4:1 ratios.

Formation of Polymer-Drug Salts—Methapyrilene salts of PVM/MA were prepared in aqueous dispersions at 25° with agitation provided by a magnetic stirrer. Methapyrilene oil was weighed in a tared 250-ml. beaker and distilled water (100–150 ml.) added. The mixture was then stirred vigorously to disperse the oil (2–5 min.). PVM/MA was slowly added, by sprinkling it into the vortex created by the agitation. The solution was stirred at a moderate rate for 24–48 hr. to achieve salt formation (this length of time is necessary for complete hydrolysis of PVM/MA at room temperature). The amount of methapyrilene oil and PVM/MA employed for mono- or di-salt formation was calculated on a stoichiometric basis using the unit molecular weight (156) of PVM/MA. Salts remaining in solution (partial and mono-salts) were assayed directly, while the insoluble salts (di-salts) were filtered out and dried under vacuum at 25°. In the case of insoluble salts, the supernatant solution was also collected for assay.

Salts of methapyrilene with the PVM/MA-DOD hemi-esters were prepared as described above. Only mono-salts of PVM/MA could be prepared in the case of the hemi-esters since one of the carboxyl groups of PVM/MA was tied up by the ester group. Due to the low solubility in water of the hemi-esters, the salt formation reaction time of these systems was increased considerably. These systems were stirred in aqueous dispersion for 72–96 hr.

Dialysis Studies—Drug release patterns of methapyrilene in solution were determined by the use of an equilibrium dialysis procedure. Dialysis sacs[9] were prepared to contain 20 ml. of the drug or its salts in aqueous solution. The sacs were securely tied and checked for leaks before placing them in a 3-oz. powder jar containing 70 ml. of artificial gastric or intestinal fluid (without enzymes). The jar was then capped and sealed with a waterproof tape before placing it in a rotating water-bath apparatus maintained at 37 ± 1°. All samples evaluated in this manner were allowed to rotate for preselected time periods of 0.25, 0.5, 1, 2, 3, and 4 hr. The dialytic release patterns of base methapyrilene, methapyrilene hydrochloride, and a partial salt of methapyrilene with PVM/MA were determined by the use of this procedure.

Dissolution Rate Studies—Polymer-drug salts and drug salts of the PVM/MA-DOD hemi-ester which were prepared in solid form were evaluated for their release characteristics by a dissolution procedure. Samples of the salts were placed in previously tared 3-oz. powder jars and accurately weighed. Artificial gastrointestinal fluids were then pipeted into the jars which were subsequently capped and sealed in the manner employed in the dialysis procedure. Methapyrilene release by the dissolution method was studied for periods up to 3 hr. in artificial gastric fluid, and up to 8 hr. in artificial intestinal fluid (without enzymes). The release patterns of particulate materials were determined in

[2] Abbott Laboratories, North Chicago, Ill.
[3] K & K Laboratories, Inc., Plainview, N. Y.
[4] Determined on a hot plate melting point apparatus.
[5] Kontes Glass Company, Vineland, N. J.
[6] Fred S. Carver, Inc., Summit, N. J.
[7] Model DU spectrophotometer, Beckman Instrument Co., Fullerton, Calif.
Model 7403, Leeds and Northrup, Philadelphia, Pa.
[9] NoJax Casing, Size 30, Visking Company, Chicago, Ill.

50-ml. volumes of the gastrointestinal fluids and the release of tableted preparations in 70-ml. volumes. Assays were performed on the filtered fluids to determine drug concentrations following the various intervals in the water bath.

RESULTS AND DISCUSSION

Reactivity of PVM/MA—Titrations of PVM/MA in aqueous solution showed that the dicarboxylic acid hydrolysis product of the polymer exhibits both mono- and di-salt formation with sodium ions. Titrations of 100 and 150 mg. of PVM/MA in 150–200 ml. of distilled water were carried out using 0.1 *M* sodium hydroxide titrant. Analyses of the first derivative titration curves obtained showed that mono-salt formation occurred within 2.3% error relative to the calculated theoretical value based on the unit molecular weight of PVM/MA. Di-salt formation was not indicated with the use of the 0.1 *M* sodium hydroxide titrant, although the titrations were continued well beyond the theoretical di-salt end point. Titration of PVM/MA with 1 *M* sodium hydroxide titrant (Fig. 1) provided both mono- and di-salt formation. Analyses of the first derivative titration curves showed that the mono- and di-salt formation end points occurred within 0.6–4.8% error relative to the theoretical values. The ability of PVM/MA to react stoichiometrically in aqueous solution with sodium ions (Fig. 1), was taken as an indication of possible polymer–drug interaction capabilities with an amine drug base.

Esterification of PVM/MA—PVM/MA was shown to esterify within 0.5 hr. when dispersed in a melt of 1,12-dihydroxyoctadecane (DOD) and heated to 120°. The PVM/MA-DOD hemi-ester was found to be insoluble in artificial gastrointestinal fluids when prepared as described in the *Experimental* section. Swelling of the hemi-ester was observed in artificial intestinal fluid (without enzyme) at 25°, but was found to be negligible in distilled water and artificial gastric fluid (without enzyme). Increased DOD content of the product (*i.e.*, 3:1 and 4:1 ratios) reduced swelling dramatically in all three media. The 2:1 DOD-polymer material underwent a percent increase in volume due to swelling of about 15% in gastric fluid, 80% in intestinal fluid, and 25% in distilled water. The 3:1 DOD-polymer material underwent only a 10% swelling volume increase in water and intestinal fluid, and the 4:1 material did not swell appreciably in any of the three solvent media. The solid hemi-ester was observed to soften at 50–60° and begin to brown at 130°. The formation of the hemi-ester of PVM/MA with DOD was thought to be a method by which the solubility of the reactive PVM/MA could be reduced while retaining an anionic nucleophilic site potentially applicable to the formation of a polymer-drug salt with an amine drug base.

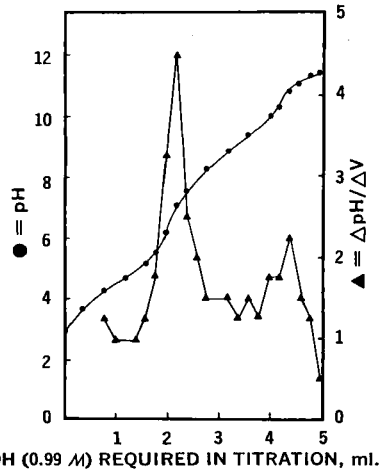

Fig. 1—Potentiometric titration of PVM/MA in aqueous solution.

Formation of Polymer-Drug Salts—The ability of PVM/MA to enter into the formation of a polymer–drug interacted system with an amine drug base was investigated using methapyrilene as the model drug. The formation of a PVM/MA-methapyrilene salt was shown by titrating the polymer in aqueous solution using methapyrilene base as the titrant. Solubilization of the base occurred in the aqueous PVM/MA solution as a result of mono-salt formation as indicated by the titration curve (Fig. 2). The titration of PVM/MA in aqueous solution produced mono-salt formation end points within 2.8% (Table I), of the stoichiometric value, indicating a constant w/w ratio of combination (Fig. 3). Methapyrilene titration of the polymer to the di-salt end point was not practical due to the low solubility of both the titrant and the titrate. While the titration curve (Fig. 2), did indicate a second end point, the first derivative peak was not considered valid in relation to the artifacts observed. A 1:1 mixture of dioxane and water was found to maintain the polymer-drug salt in solution but again the methapyrilene titration curve did not clearly discern the second end point.

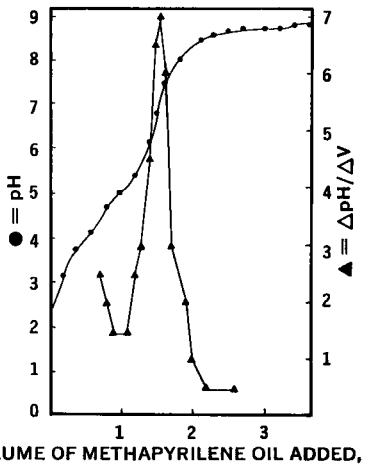

Fig. 2—Titration of PVM/MA in aqueous solution with methapyrilene base.

TABLE I—PVM/MA METHAPYRILENE BASE TITRATION DATA

Weight of Titrate, g.	ml. of Titrant	g. of Titrant	Calcd. Wt., g.	% Error
1.0019	1.530	1.6785	1.6780	0.03
0.7513	1.115	1.2233	1.2585	2.80
0.5010	0.775	0.8500	0.8390	1.31
0.2477	0.370	0.4060	0.4150	2.17

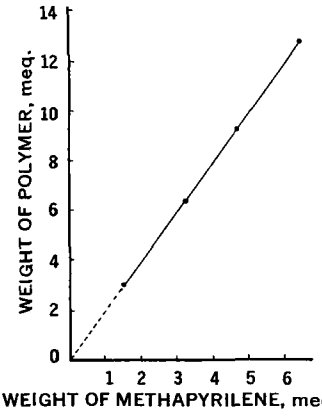

Fig. 3—Weight of PVM/MA versus *weight of methapyrilene base in formation of mono-salt.*

PVM/MA-methapyrilene salt formation was achieved by adding methapyrilene base to an aqueous solution of hydrolyzed PVM/MA in the titration procedure; however, it was found that salt formation was most conveniently accomplished by adding the PVM/MA powder to an aqueous dispersion of methapyrilene "oil." This procedure requires the gradual addition of the powdered polymer to the vortex of a vigorously agitated methapyrilene dispersion. By the use of this technique and with prolonged agitation of the system at 25°, stoichiometric quantities of the polymer were employed to produce partial, mono-, and di-salts of methapyrilene. Salts of methapyrilene and the PVM/MA-DOD hemi-ester were also produced in this manner as described in the *Experimental* section.

Evaluation of Polymer–Drug Interacted Systems—The availability of methapyrilene from the PVM/MA and PVM/MA-DOD hemi-ester salts was studied using the dissolution and dialysis procedures described previously.

A partial salt of PVM/MA and methapyrilene base was prepared by reacting 1 g. of the polymer with an equal weight of methapyrilene base. After stirring at 25° for 72 hr. the solution was filtered and diluted to 250 ml. in a volumetric flask. Assay of the solution indicated that the methapyrilene concentration was 3.9 mg./ml. This solution was dialyzed against the artificial gastrointestinal fluids and the availability of methapyrilene from the system was compared to the release patterns of the hydrochloride salt (Fig. 4) and the free base (Fig. 5), obtained in a like manner. The release pattern of the drug from the polymer salt did not differ substantially from that of the free base or the hydrochloride salt, but the precentage release rate of methapyrilene from the PVM/MA salt in both artificial intestinal and gastric fluids was more rapid than either the hydrochloride salt or the free base.

The di-salt of PVM/MA was prepared by reacting 3.35 g. of methapyrilene base with 1 g. of the polymer. Precipitation of the product from the aqueous system occurred within 6 hr., and after drying, the solid product was found to contain 3.15 g. of drug. Analysis of drug availability from the coarsely subdivided product by the dissolution method indicated that 98% methapyrilene release was attained within 15 min., in the gastrointestinal fluids.

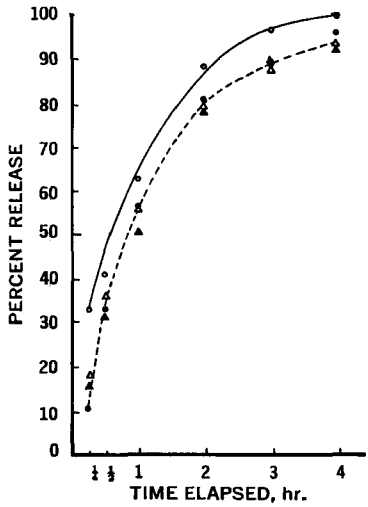

Fig. 4—Equilibrium dialysis release pattern of the PVM/MA-methapyrilene partial salt in comparison to methapyrilene hydrochloride in artificial gastric (●▲) and intestinal (○△) fluids at 37 ± 1°. Key: ▲△, methapyrilene hydrochloride; ●○, methapyrilene-PVM/MA partial salt.

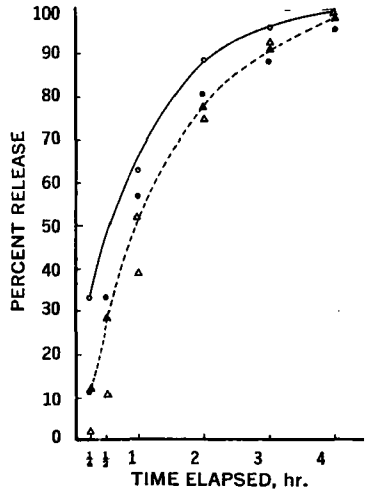

Fig. 5—Equilibrium dialysis release pattern of the PVM/MA-methapyrilene partial salt in comparison to methapyrilene base in artificial gastric (●▲) and intestinal (○△) fluids at 37 ± 1°. Key: ▲△, methapyrilene base; ●○, methapyrilene-PVM/MA partial salt.

Methapyrilene salts of the PVM/MA hemi-esters were prepared by reaction in aqueous dispersion as described in the *Experimental* section. These PVM/MA hemi-ester salts were prepared in granular form and evaluated for methapyrilene release by the dissolution method. The drug content of the hemi-esters ranged from 21 to 33% by weight, and was determined for each product by assaying the supernatant of the reaction dispersion for unreacted drug. The release of methapyrilene from granules of 20/40 mesh and smaller than 40 mesh was determined for each of the three hemi-esters described in the *Experimental* section. The release of metha-

pyrilene from the granules appeared to be dependent upon both the particulate dimensions and the amount of DOD incorporated. As was the case with the partial salt drug of PVM/MA, the release of methapyrilene was initially greater in artificial intestinal fluid (Fig. 6) than in artificial gastric fluid (Fig. 7). In both dissolution test fluids, for each granule particle size fraction studied, the order of drug release was most rapid from the 2:1 DOD-polymer ester, and least rapid from the 4:1 DOD-polymer ester. This finding corresponds with the swelling properties of the DOD-polymer esters, since the 4:1 ratio material evidenced the lowest extent of swelling. The coarser particle size fraction of each product in both test fluids exhibited the slower rate of release.

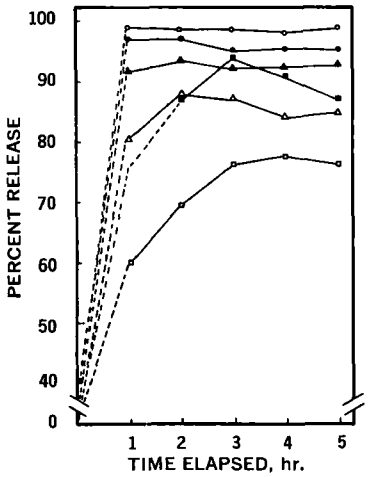

Fig. 6—Release of methapyrilene from granules of the hemi-ester salt in artificial intestinal fluid at 37 ± 1°. Key: ● 2:1 ester (> 40 mesh); ○, 2:1 ester (20/40 mesh); ▲, 3:1 ester (> 40 mesh); △, 3:1 ester (20/40 mesh); ■, 4:1 ester (> 40 mesh); □, 4:1 ester (20/40 mesh).

Samples of the granular hemi-ester salt were compressed into 0.9-cm. ($^3/_8$-in.) tablets weighing approximately 250 mg. The tablets were compressed under a load of 5,000 p.s.i. using a hand-operated hydraulic press as described in the *Experimental* section. The drug content of the tablets was determined on the basis of tablet weight and the drug content of the hemi-esters from which the tablets were compressed. Release-rate analyses of methapyrilene from the tablets prepared using both 20/40 mesh granules and granules smaller than 40 mesh, showed that drug release was substantially retarded by compression of the granules. As was apparently characteristic of the PVM/MA methapyrilene salts, the drug release from the tablets in artificial intestinal fluid (Fig. 8) was more rapid than in artificial gastric fluid (Fig. 9). The release of methapyrilene from the granular particles was dependent upon both particle size and the amount of DOD present in the system.

Drug release from the tablets in gastric fluid also indicated dependence on the amount of DOD in the systems, with the 4:1 hemi-ester salt again exhibiting the lowest rate of release. However, the 3:1 hemi-ester salt now demonstrated the most rapid rate of release (Fig. 9). The tablets prepared from the coarser particle-size fractions of a given ester product exhibited a more rapid release rate (Fig. 9), in the systems in which a particle size effect was noted, suggesting that the tablets prepared from the larger granules were more porous.

Drug release from the tablets in intestinal fluid followed that in gastric fluid, in that the 4:1 hemi-ester salt (>40 mesh) exhibited the slowest rate and the 3:1 hemi-ester salt (20/40 mesh) exhibited the most rapid initial release rate. The rates of release of the other systems were compressed between the rates of these two systems in an indeterminate order. The *in vitro* release pattern of the compressed

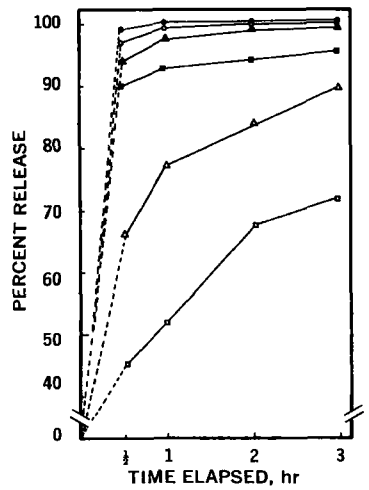

Fig. 7—Release of methapyrilene from granules of the hemi-ester salt in artificial gastric fluid at 37 ± 1°. Key: ●, 2:1 ester (> 40 mesh); ○, 2:1 ester (20/40 mesh); ▲, 3:1 ester (> 40 mesh); △, 3:1 ester (20/40 mesh); ■, 4:1 ester (> 40 mesh); □, 4:1 ester (20/40 mesh).

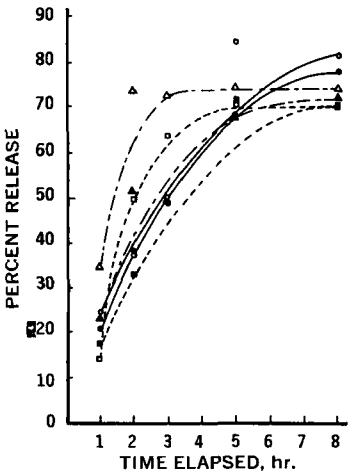

Fig. 8—Release of methapyrilene from tablets of the hemi-ester salt in artificial intestinal fluid at 37 ± 1°. Key: ●, 2:1 ester (> 40 mesh); ○, 2:1 ester (20/40 mesh); ▲, 3:1 ester (> 40 mesh); △, 3:1 ester (20/40 mesh); ■, 4:1 ester (> 40 mesh); □, 4:1 ester (20/40 mesh).

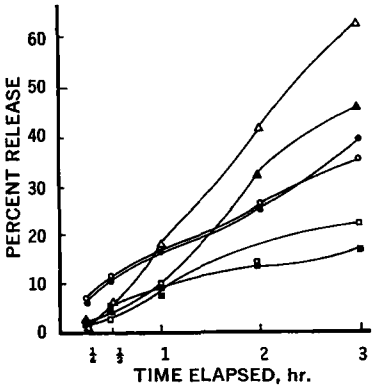

Fig. 9—Release of methapyrilene from tablets of the hemi-ester salt in artificial gastric fluid at 37 ± 1°. Key: ●, 2:1 ester (> 40 mesh); ○, 2:1 ester (20/40 mesh); ▲, 3:1 ester (> 40 mesh); △, 3:1 ester (20/40 mesh); ■, 4:1 ester (> 40 mesh); □, 4:1 ester (20/40 mesh).

tablet DOD-polymer ester drug salts were much more promising that that of the particulate materials. The tablets did not disintegrate under test, but gradually eroded.

SUMMARY

Polymer–drug interacted systems were prepared and evaluated as an approach to the physicochemical design of pharmaceutical dosage forms.

An amine drug base (methapyrilene) was found to form salts with the dicarboxylic acid hydrolysis product of poly (methyl vinyl ether/maleic anhydride) containing as much as 3.15 parts of drug per part of polymer. Release-rate analyses of the polymer drug salts indicated methapyrilene availability comparable to that from the free base or the hydrochloride salt. Increased initial drug release in artificial intestinal fluid was characteristic of the polymer-drug salt.

Hemi-esters of poly (methyl vinyl ether/maleic anhydride) with 1,12-dihydroxyoctadecane were prepared. The hemi-esters, containing excess DOD, were found to react with methapyrilene to form hemi-ester salts of the drug. Methapyrilene release rates from the hemi-ester salts were studied with the use of tablets and granules and found to be substantially reduced in comparison to the polymer salts or the hydrochloride salt and free base forms of the drug.

The potential application of a drug–polymer interacted system as an approach to the preparation of controlled and prolonged-release dosage forms was illustrated.

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Keyphrases

Dosage forms–polymer-drug interacted system
Poly (methyl, vinyl ether/maleic anhydride) and hemi-ester—test polymers
Methapyriline—test drug
Salt formation—methapyriline, polymers
Dissolution rates—polymer-drug system
Dialysis rates—polymer-drug system
UV spectrophotometry—analysis
Potentiometric titration—reactivity analysis

Effect of Adsorbents on Drug Absorption: Importance of Preequilibrating Drug and Adsorbent

By DONALD L. SORBY

Absorption of promazine from the human gastrointestinal tract is unaffected by small amounts of activated attapulgite and activated charcoal when drug and adsorbent are not equilibrated before administration of the test dose. These results are contrasted to previous experiments where the marked inhibition of absorption occurred with the same doses of drug and adsorbent in equilibrated systems.

PREVIOUS PUBLICATIONS (1, 2) have reported the effects of adsorbent materials on the absorption of promazine from the gastrointestinal tract of human subjects. Dosage forms which contained either activated attapulgite, 500 mg. or activated charcoal, 100 mg. equilibrated in an aqueous medium with 50 mg. promazine were studied (1). In such systems, both adsorbents interfered with absorption of promazine. Activated attapulgite slowed the initial rate of appearance of drug in the urine but there was no significant decrease in the total availability of promazine. Activated charcoal decreased both the rate and extent of absorption. The differences between the two adsorbents with respect to their effects on promazine absorption were related to similar differences in the way promazine was desorbed during *in vitro* experiments.

The effect on promazine absorption produced by a commercial antidiarrhea mixture containing activated attapulgite and pectin was studied (2) under conditions where the drug was not equilibrated with adsorbent prior to administering to the test subject. The experiment was designed to test whether previous observations (1) would be applicable under conditions more similar to clinical use. It was observed that the antidiarrhea mixture slowed the rate of absorption and also reduced the availability of promazine. In comparison to previous studies (1) the test subject was dosed with rather large quantities of adsorbent. Thus, the experiment did not specifically determine whether failure to equilibrate drug and adsorbent was important to effect on absorption. Research reported here was intended to determine whether preequilibration of drug and adsorbent is important to effects on drug absorption.

EXPERIMENTAL

Dosage Forms—Dosage forms consisted of promazine hydrochloride 50 mg. dissolved in 45 ml. distilled water. Suspensions were prepared to contain either 500 mg. activated attapulgite or 100 mg. activated charcoal in 50 ml. distilled water. If equilibrated, the activated attapulgite dosage form would contain 33.8 mg. promazine adsorbed. The activated charcoal would contain 23.5 mg. promazine adsorbed. These dosage forms were very nearly identical to those employed previously (1) except that the drug and adsorbent were not at equilibrium or, for that matter, even in contact with one another.

Adsorption Rate, In Vitro—A suspension of the adsorbent in distilled water as described under *Dosage Forms* was placed in a round-bottom flask. The flask was placed in a thermostat at 37.0° and fitted with a stirring apparatus. The rate of stirring was adjusted to 850 r.p.m. which was sufficient to maintain a homogeneous suspension of the adsorbent. The promazine solution described under *Dosage Forms* was then added and a timer started. Aliquots were removed from the flask at appropriate times and were immediately centrifuged. The concentration of promazine in the supernate was measured spectrophotometrically by a procedure described elsewhere (3). The total elapsed time between withdrawal of the aliquot and removal of supernate from the adsorbent was slightly less than 3 min.

In Vivo Tests—At the beginning of each experiment, the test subject first consumed the adsorbent suspension and immediately afterward, the solution of promazine. In all other respects the experimental method was identical to that reported previously (2). The results are summarized in Table I and in Fig. 1.

RESULTS AND DISCUSSION

Statistical comparisons of data in Table I were made by a t test for independent sample means. Two-tailed t values were used in assessing the significance of differences between means. In no case were the differences significant ($p > 0.10$) between mean values of promazine equivalents excreted at the various time intervals. Thus the results of *in vivo* tests show that within the experimental accuracy of the procedure neither adsorbent significantly affected promazine adsorption. This is in direct contrast to results obtained previously (1) where marked inhibition of absorption occurred with similar dosage forms when drug and adsorbent were preequilibrated before administration. Previous experiments with an antidiarrhea mixture (2) also showed an inhibition of promazine absorption.

Figure 2 shows results of *in vitro* studies assessing the rate of adsorption of promazine in a well-stirred system. It is noted that the majority of the interaction has taken place within the first 5 min.; however, some adsorption continues over a

Received April 1, 1968, from the School of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122

Accepted for publication May 10, 1968.

This investigation was supported by research funds from the Academic Senate, University of California, San Francisco, Calif.

TABLE I—CUMULATIVE PROMAZINE EQUIVALENTS PRESENT IN TOTAL URINE SAMPLES AT THE END OF VARIOUS TIME INTERVALS FOLLOWING ADMINISTRATION OF TEST DOSAGE FORMS[a]

Time After Administration of Test Dose, hr.	Mean Solution[b] Dosage Form	—Promazine Equivalents— Excreted Attapulgite[b] Dosage Form	Charcoal[b] Dosage Form
1	121 ± 78[c]	80 ± 23[c]	140 ± 152[c]
2	739 ± 265	773 ± 108	659 ± 164
3	1393 ± 303	1507 ± 279	1283 ± 269
4	1929 ± 411	2184 ± 451	1786 ± 539
5	2233 ± 429	2625 ± 567	2087 ± 547
6	2494 ± 480	2953 ± 416	2307 ± 543
8	2920 ± 496	3391 ± 851	2650 ± 518
10	3238 ± 452	3717 ± 958	2944 ● 554
12	3495 ± 434	3981 ± 1053	3147 ± 577
15	3801 ± 433	4319 ± 1152	3411 ± 636
18	4068 ± 448	4547 ± 1183	3617 ± 606
24	4429 ± 589	4895 ± 1213	3935 ± 503
30	4720 ± 747	4981 ± 1168	4114 ± 490
36	4906 ± 944	5091 ± 1084	4228 ± 528

[a] A promazine equivalent is defined as representing the amount of promazine which, if carried through the assay procedure, would give an identical assay value as the urine sample in question. Units of promazine equivalents are micrograms. [b] Mean of five experiments. [c] Plus-minus values represent the 95% confidence intervals about means.

considerably longer period. In the gastrointestinal tract where stirring is obviously much slower, one might expect a slower rate of interaction between drug and adsorbent. Even though most of the drug appears to be rapidly adsorbed *in vitro*, it also appears that under *in vivo* conditions, this is not of significance. Several other factors may be important in this situation.

In previous experiments with an antidiarrhea mixture (2) involving nonequilibrated conditions, large amounts of adsorbent were employed. In addition, the subject was predosed with adsorbent over a 12-hr. period. Under such conditions one

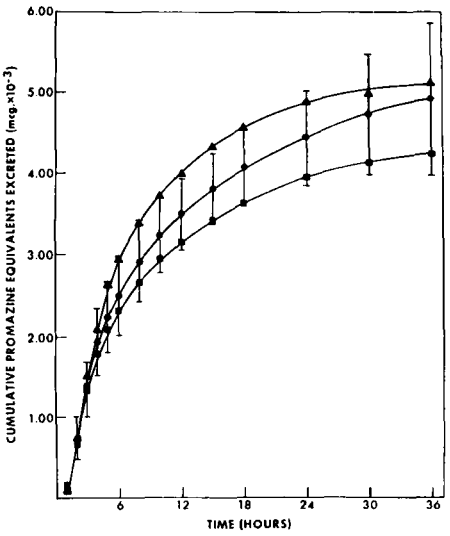

Fig. 1—Cumulative amounts of promazine equivalents excreted in the urine following administration of promazine hydrochloride (50 mg.) administered in simple aqueous solution. Key: ●, promazine administered alone; △, promazine administered immediately following 0.500 g. activated attapulgite in aqueous suspension; □, promazine administered immediately following 0.100 g. activated charcoal in aqueous suspension. Bars denote 95% confidence intervals about means of simple solution data.

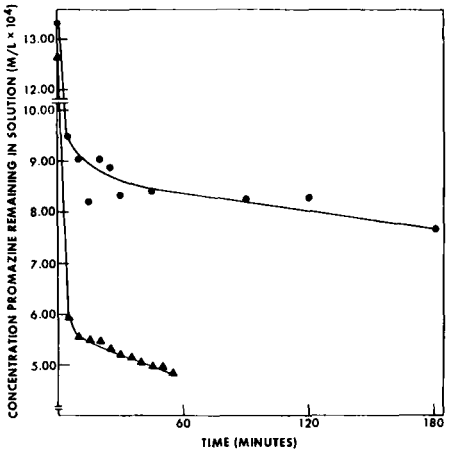

Fig. 2—Rate of adsorption of promazine by activated attapulgite and activated charcoal in a system stirred at 850 r.p.m. Theoretical concentration of promazine at time zero is 16.4 × 10^{-4} M. Key: ●, activated charcoal adsorbent; △, activated attapulgite adsorbent.

would expect adsorbent to be well distributed throughout the gastrointestinal tract. Mixing between drug and adsorbent would be facilitated by virtue of the probable widespread distribution of the relatively large amounts of adsorbent materials. In this experiment, however, a relatively small amount of adsorbent is employed. It is possible that mixing between drug and adsorbent in the gut is quite poor. Promazine appears to be quite rapidly absorbed, see data for drug in solution, and it is probable that significant amounts were absorbed before sufficient mixing could occur between drug and adsorbent. It may also be possible that the presence of high concentrations of hydrogen ion in the stomach retarded adsorption. Hydrogen ion was shown (3) to depress promazine adsorption by kaolin, talc, and activated charcoal.

CONCLUSIONS

It is concluded as a result of the experiments reported here that preequilibration between drug and adsorbent is an important factor with respect to the effect of an adsorbent on uptake of drug from the gastrointestinal tract. Maximal adsorbent effects should occur in the preequilibrated systems. In view of results obtained with the antidiarrhea mixture, it is suggested that the quantity of adsorbent administered may also be important. Predosing of adsorbent may also lead to greater effect on drug absorption in nonequilibrated systems.

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Keyphrases

Promazine absorption—adsorbent effect
Preequilibration—promazine, adsorbent
Absorption, effect—promazine-adsorbent preequilibration

Determination of *p*-Hydroxyphenoxy Methyl Penicillin in Phenoxymethyl Penicillin Fermentation Samples

By JAN BIRNER

A method based on paper chromatography and biological assay has been developed for the determination of *p*-hydroxyphenoxy methyl penicillin in phenoxy methyl penicillin (penicillin V) fermentation samples. The penicillin broth is extracted into methyl isobutyl ketone. The extract is chromatographed in triplicate using Whatman No. 1 paper treated with sodium citrate at pH 5.5 and ethyl ether saturated with 28 percent ammonium sulfate. The penicillins are located by reference to a strip cut from the chromatogram and developed colorimetrically. The appropriate areas are then cut out, eluted separately with 1 percent phosphate buffer, and the eluates are determined by biological assay. As the R_f values for phenoxymethyl penicillin and *p*-hydroxyphenoxy methyl penicillin under the conditions described are, respectively, 0.60 and 0.13, the separation is complete.

PARA-HYDROXYPHENOXY methyl penicillin was first detected in phenoxymethyl penicillin (penicillin V) fermentation broth samples by de Flines *et al.* (1). According to these authors the mold (*Penicillium chrysogenum*, strain WLS 49-133) is able either to oxidize phenoxyacetic acid to *p*-hydroxyphenoxy acetic acid and utilize this compound as a precursor for the synthesis of *p*-hydroxyphenoxy methyl penicillin or else can hydroxylate phenoxymethyl penicillin directly.

The *p*-hydroxyphenoxy methyl penicillin was isolated by the above-mentioned workers in the form of crystalline free acid.

Although its antibacterial spectrum is similar to that of penicillin V, *p*-hydroxyphenoxy methyl penicillin is much less stable in acid solutions. Vanderhaeghe *et al.* (2) found *p*-hydroxyphenoxy methyl penicillin as a metabolite of penicillin V in urine, and they have separated the two compounds by means of paper chromatography.

During the manufacture of penicillin V by fermentation, the assay of samples by one or other of the commonly used methods (hydroxylamine, iodimetric, or biological) usually furnishes values representing the sum of the penicillins contained in them. If they are to be estimated individually it is necessary that they be first separated. The procedure described shows how this may be done.

EXPERIMENTAL

Reagents, Solutions, and Materials—Sulfuric acid 12 *N* A.R.; ammonium sulfate A.R.; sodium sulfate anhydrous A.R.; methyl isobutyl ketone, redistilled sodium citrate A.R.; ethyl ether redistilled; starch, soluble laboratory reagent; 0.1 *N* iodine in 4% potassium iodide A.R.; glacial acetic acid; ammonia 0.880 sp. gr.; phosphate buffer 1% pH = 7.0; Whatman No. 1 paper for chromatography.

Procedure—Pipet 2 ml. of filtered broth into a suitable centrifuge tube fitted with a glass-ground stopper; add approximately 2 g. of solid ammonium sulfate and 0.1 ml of 12 *N* sulfuric acid. Mix by swirling this solution (pH approx. 2.0), add from a pipet 5 ml. of methyl isobutyl ketone (M.I.B.K.), stopper the tube, shake vigorously for 45 sec., and then centrifuge for 5 min. Transfer a portion of the organic layer into another dry tube by means of a Pasteur pipet, dry, by shaking with a small amount of anhydrous sodium sulfate, and finally transfer it by filtration into a third dry test tube.

Treat Whatman No. 1 chromatography paper by passing through a 10% solution of sodium citrate, adjusted to pH 5.5 by hydrochloric acid, followed by drying. Using a microsyringe (capacity 20–50 µl., in 1 µl. divisions), apply to the paper three aliquots, each 20 µl. of the extract. From a freshly prepared standard solution of penicillin V in methanol containing 1 mg. of the antibiotic per ml., apply 10 µl. as a control. The four spots are placed 5 cm. from the bottom of the paper and 5 cm. apart.

Chromatography is performed by the ascending technique. Redistilled ethyl ether to be used as development solvent is equilibrated at room temperature against an ammonium sulfate solution, prepared by dissolving 40 Gm. of the salt in 100 ml. of water. Suspend the chromatogram in a suitable chromatographic tank (or in a jar lined with filter paper) containing equilibrated ethyl ether and a vessel with water.

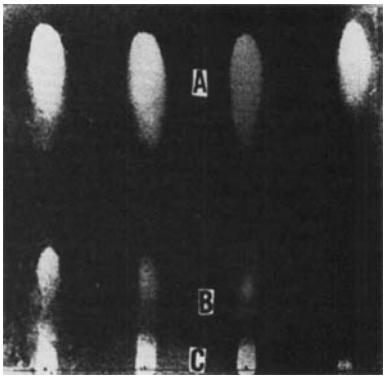

Fig. 1—Chromatogram for methyl isobutyl ketone extract of phenoxymethyl penicillin. Key: A, phenoxymethyl penicillin; B, p-hydroxyphenoxy methyl penicillin; C, biologically inactive.

Received December 11, 1967, from the Commonwealth Serum Laboratories, Parkville, Victoria 3052, Australia.
Accepted for publication February 13, 1968.

TABLE I—RESULTS OF ASSAYS OF FERMENTATION SAMPLES

Fermentation Batch No.	Units of Penicillin Recovered from Chromatogram From 8 μl. of Broth: Pen. V	p-Hydroxy Pen. V	Total	10 mcg., Pen. V[a]	Control Assayed Directly, in Units	p-Hydroxy Pen. V, %
1	58.5	2.20	60.70	16.60	67.20	3.78
2	76.20	3.38	79.58	17.10	87.00	4.24
3	56.00	2.00	58.00	16.50	63.90	3.44
4	66.15	2.76	68.91	16.38	77.40	4.00
5	60.30	2.46	62.76	16.50	66.60	3.90

[a] 1 mcg. pen. V equals 1.695 unit.

After an hour's equilibration, start chromatography from outside by pushing to the bottom of the tank the rod on which the paper is suspended. Following development (up to 16 hr.), cut off the first strip containing 20 μl. of the extract, hold for a few sec. over a jet of steam, then hang for 10 min. in a closed jar containing concentrated ammonia. Spray the strip with starch-iodine solution, prepared by mixing 50 ml. of 1% soluble starch solution, 3 ml. glacial acetic acid, and 1 ml. 0.1 *N* iodine. The white spots on a blue background, which appear after a few min., indicate the position of the corresponding penicillins. A photograph of a chromatogram developed as a whole colorimetrically is shown as Fig. 1. The colored strip is used as a template for locating the penicillins on the remaining part of the chromatogram. Cut out the corresponding areas of the chromatogram (sample in duplicate and standard penicillin V), place in 1.9 cm. × 15.2 cm. test tubes and elute with a measured amount of 1% phosphate buffer pH 7.0. The amount of buffer used for elution per tube is 4 ml. for the *p*-hydroxyphenoxy methyl penicillin and 10 ml. for phenoxymethyl penicillin. Elution is achieved by shaking the stoppered test tubes for 0.5 hr. on a shaking machine. Transfer the same volume of original organic extract as was applied to the chromatogram (20 μl.) directly into a test tube and extract with 10 ml. of buffer to serve as a control. Assay biologically the penicillin content of the solutions as penicillin V and calculate the percentage of *p*-hydroxyphenoxy methyl penicillin.

RESULTS

The data for experiments performed on five samples taken from penicillin fermentation tanks are shown in Table I.

The completeness of extraction of penicillin V (99.82% recovery) under the described conditions was confirmed by biological assay.

The developed method permits the determination of the *p*-hydroxyphenoxy methyl penicillin in the fermented broth during all stages of the fermentation. It was also used in the consequent processes of penicillin extraction and purification and as a qualitative test in the final product.

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Keyphrases

p-Hydroxyphenoxy methyl penicillin—analysis
Phenoxymethyl penicillin fermentations—*p*-hydroxyphenoxymethyl penicillin determination
Paper chromatography—separation
Biological analysis—penicillins

Antifertility Factors from Plants I. Preliminary Extraction and Screening

By CHARLES F. BARFKNECHT and HUNG CHIH PENG

Three plants were examined for possible antifertility effects. *Prunus emarginata* and *Lonicera ciliosa* were found to reduce litter production in mice, while *Lysichitum americanum* was not active. The initial extraction and screening procedures are described.

THE ETHNOBOTANICAL literature contains a report of the use of plant teas to affect the reproductive processes by Indians of the Northwest U. S. Specifically, the roots of *Lysichitum americanum*, the leaves of *Lonicera ciliosa*, and the wood of *Prunus emarginata* were used for antifertility and abortifacient effects (1). Generally the women drank the teas prepared from the ground plants, but some tribes made no distinction on the sex of the person. The report also failed to mention a dosage regimen, but apparently more than one dose was necessary to obtain the antifertility effect. It is the purpose of this study to verify the antifertility activity and determine the constituents responsible.

Cranston (2) studied the effect of *Lithospermum ruderale* extracts on mice. *Lithospermum* was used by Indians of the Southwest U. S. to affect reproduction. She prepared an ethanolic extract

Received August 25, 1967, from the Division of Medicinal Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA 52240 and the College of Pharmacy, Idaho State University, Pocatello, ID 83201

Accepted for publication May 10, 1968.

This investigation was supported by a grant from the National Institute of Child Health and Human Development.

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of the ground plant and fed a 20% extract diet to young adult males and females 10 days prior to and 10 days during cohabitation. The ethanolic extract, which was more potent than a simple aqueous extract, reduced litter production from 70% in the control group to 34% in the treated group. There was no effect on the number of young per litter or on the activity or physical appearance of the adult mice.

Another report of a natural feeding assay of antifertility activity was made by Dewald and co-workers (3). They studied the effectiveness of a synthetic compound in mice by combining it with the food and allowing the pregnancies to go full term. Activity was again expressed as a ratio of the litter production in the treated group *versus* litter production in the control group.

DISCUSSION

For the plants in this study a 50% aqueous ethanolic solution was found to be a more efficient and more effective extraction system than a hot aqueous decoction. The natural feeding method of assay outlined previously was used with the exception that the extract food combination was reformed into pieces resembling the normal food. Since mice are gnawing creatures, it was felt that this technique would encourage them to feed normally. No difference in average food intake was found for any group, thus endorsing the technique.

In the initial screening to determine the validity of the ethnobotanical report, the discovery of antifertility activity was more important than establishing either which sex was affected or what was the mechanism of action. Thus the extracts were fed to both sexes prior to and during cohabitation. The results of the screening are summarized in Table I. Two consecutive runs were made, separated by 1 month, using two different concentrations. The consecutive runs permitted the evaluation of the extracts' effects on virgin and nonvirgin mice, the determination of the relative effectiveness of the dosage levels, and the gathering of the maximum data from the mouse colony. The extracts did not appear to affect the behavior or activity of the mice. Those extracts which caused reduction of litter production did so without decreasing the number of young per litter or without causing visible birth defects.

Present studies are directed toward the isolation of the active antifertility factors from *Lonicera ciliosa* and *Prunus emarginata*. Preliminary work on *Lonicera* leaves indicates the presence of alkaloid and flavonoid components. References to the antifertility effect of both components are found in the literature (4, 5). Two alkaloids have been separated and detected by thin-layer chromatography. One of them has been isolated in sufficient quantities to begin structure elucidation studies. The results of these studies will be the subject of a future communication.

Table I—Antifertility Screening of Plant Extracts

	Run	No. Females	No. Litters	% Litters	Av. Dose Extract/ Day/Mouse, mg.
Control	1st	15	9	60	—
	2nd	18	11	60	—
Lysichitum, roots	1st	15	7	46	10
	2nd	15	8	56	20
Prunus, wood	1st	12	4	33	10
	2nd	12	4	33	20
Lonicera, leaves	1st	15	5	33	10
	2nd	15	4	27	20

EXPERIMENTAL

Collection—The plants were gathered in the vicinity of Corvallis, Ore., in late summer, 1966 and authenticated under the direction of LaRea D. Johnston, Oregon State University Herbarium, Corvallis, to whom the authors are extremely grateful. Voucher specimens are on deposit in the Oregon State University Herbarium and in the College of Pharmacy, University of Iowa.

Extraction—The air-dried plant parts, mentioned in the ethnobotanical report were ground in a Waring blender with 50% v/v aqueous ethanol (20 ml./g. of plant material). The mixture was macerated 3 days with occasional stirring, filtered through paper, and evaporated to dryness under reduced pressure at temperatures not exceeding 50°. By this procedure extracts of 13.0, 1.7, and 17.0% were obtained for *Lysichitum*, *Prunus*, and *Lonicera*, respectively. Preliminary experiments had shown this extraction procedure to be the best method.

Food-Extract Combination—Purina dog chow, the normal food, was ground in a mill. The dried extract was dissolved in a minimum of 50% ethanol, combined with the proper amount of ground dog chow to prepare a 1 or 2% extract diet, and mixed into a mass. The mass was cut into pieces and allowed to air-dry at temperatures not exceeding 40°.

Screening—White mice, 8–10 weeks old (Berkeley Pacific Laboratories, Berkeley, Calif.), were maintained on a diet of Purina dog chow and water for 3 weeks prior to screening. To begin the screening, separated males and females (four to a cage) were fed the dog chow extract diet for 2 weeks. The only exceptions were the mice which would constitute the control group, they received only dog chow throughout the entire screening period. The mice were cohabitated, one male and three females per cage, and fed the test diet an additional 3 weeks. After this period, the males were removed from the cages and the cages examined for litters daily during the next 4 weeks. After the last litter was born, a period of 4 weeks elapsed before the second run. The second run was conducted in an analogous manner. No attempt was made to maintain the mice in the same test group during both runs.

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Keyphrases

Antifertility factors—plant extracts
Prunus emarginata extract—mouse litter reduction
Lonicera ciliosa extract—mouse litter reduction

Structure and Activity in Molluscicides II. Activity Assay of Catalase and Peroxidase in Snails

By I. NABIH, M. T. EL-WASSIMY, and J. METRI

Catalase and peroxidase, protoporphyrin enzymes present in snails and affected by molluscicidal agents, were studied in some species of snails of biological importance (*Biomphalaria* and *Bulinus* types, intermediate hosts for schistosomiasis, intestinal and renal, respectively). The activity values of these enzymes in snails infected with *miracidia* of *Schistosomes* were less than those for the noninfected ones. Also, determinations were run for snails of *Lymnaea* (intermediate host for cattle liver fluke, *Fasciola hepatica*) and *Physa* types.

SCHISTOSOMIASIS is a world health problem, and after malaria, it is the most widespread disease in tropical and subtropical regions. It infects about 200,000,000 (1) of the world inhabitants causing tremendous losses in the areas it prevails. Unfortunately, the disease is still out of control.

One of the major approaches in the combat of the disease is eradication by molluscicides of snails, the intermediate vector of the parasite.

In spite of the extensive work with molluscicides, no approach has yet been made in the establishment of a structure-activity relationship.

In a previous communication (2) the authors reported the relation between the specificity in structure of molluscicides of phenolic type and the activity of both protoporphyrin enzymes, peroxidase and catalase, within the snail's body. The former enzyme is involved in the biological formation of free radicals from substrates of aromatic amines and phenols.

The color developed, due to the formation of trivalent nitrogen-free radicals by the uninfected snails, from *p*-phenylenediamine dehydrochloride and its derivatives led to a new quick method for identification of infected and noninfected snails (the infected snail does not develop the color or only faintly, depending on the intensity and age of infection).

The activity in phenolic molluscicides depends mainly on the nature of substituents on the ring. This may be explained due to the initial formation of a phenoxy radical through the snails' body enzymes. The subsequent transformation of the radical to the snail toxic quinoid structure is similarly dependent on that nature of substituents on the ring.

In the present work, quantitative microassays for the activity of both enzymes, peroxidase and catalase, were run in different species of snails of biological importance.

For these studies, fresh water snails of *Biomphalaria alexandrina* and *Bulinus truncatus* types were selected. Both types are prevalent in this country and act as intermediate hosts for *Schistosoma mansoni* and *Schistosoma hematobium*, respectively.

Also, the studies were run on snails of *Lymnaea caillaudi* (intermediate host for the cattle liver fluke, *Fasciola hepatica*) and the *Physa acuta* types.

The method used for activity assay of peroxidase, present in snails' whole extract, depended on spectrophotometric measurement for the rate of oxidation of *p*-phenylenediamine as a substrate at the wavelength 485 mμ in a buffered solution at pH 7 (3, 4).

For catalase, the activity assay depended on the spectrophotometric measurements for the rate of decomposition of hydrogen peroxide through the enzyme present in the snails' extract at wavelength 240 mμ in a buffered mixture at pH 7 (5–7).

Determinations were run on snails at two different seasons (summer and winter). Under both conditions the snails, after collection from the field, were directly submitted to assay determinations. The temperature ranged between 33° in summer and 15° in winter.

The results reveal that the activity differs from season to season. In summer the activity is higher than in winter. Also, it differs according to the species in the same season.

These findings agree with the observed changes in the rate of the oxygen consumption by fresh water molluscs which depends largely on the temperature, season, and species. Species studied showed increase in the oxygen consumption with increasing temperature, but the precise relationship varies from species to species and at different seasons within a species (8–10).

Also, activity determinations were run on snails of the *Biomphalaria* type infected with the *miracidia* of *Schistosoma mansoni*. It had been observed that the activity for both enzymes is less in the infected snails than the noninfected ones. These findings may be rationalized since the *Schistosomes* live anaerobically (11). They habitate the liver in the snail's body, and may tend to destroy, as a defense mechanism, any enzyme or biological factor that would furnish free oxygen in their surroundings as this would interfere with their anaerobic metabolism.

Also, it can be due to damage in function of organs in the snail's body, due to infection, involved in the biogenesis of both enzymes.

The studies for the end transformation products with their intermediate for each of the important molluscicides *in vivo* and *in vitro* by both enzymes are now under investigation and will be reported later.

EXPERIMENTAL

All spectrophotometric measurements were run on a Unicam SP 500 spectrophotometer using cells with a 1-cm. light path. Snails were freshly collected from the field.

Received March 13, 1968, from the National Research Centre, Dokki, Cairo, U.A.R.
Accepted for publication May 14, 1968.

Extract Preparation

A known weight of snails of the same type was homogenized in a blender with 0.006 *M* phosphate buffer, pH 7 (Solution I diluted 1:10) at 1–4°, then centrifuged (3,500 T/min., for 10 min.). The sediment was stirred with cold phosphate buffer 0.006 *M* and allowed to stand in the cold with occasional shaking. The extraction process was repeated three times. The whole extract was cleaned through centrifugation several times. The final volume of the extract was recorded. The extraction did not take longer than 24 hr.

Activity Measurements

Catalase—*Reagents*—potassium dihydrogen phosphate, A.R., KH_2PO_4; disodium hydrogen phosphate, A.R., $Na_2HPO_4.2H_2O$; hydrogen peroxide, *ca.* 30% (w/v).

Preparation of Solutions—Solution I: phosphate buffer (0.06 *M*, pH 7): 3.522 g. KH_2PO_4 and 7.268 g. $Na_2HPO_4.2H_2O$, were dissolved in doubly distilled water and made up to 1,000 ml. Solution II: hydrogen peroxide–phosphate buffer (0.06 *M* phosphate, *ca.* 1.25×10^{-2} *M* H_2O_2 pH 7). 0.16 ml. H_2O_2 30% (w/v) was diluted to 100 ml. with buffer (Solution I). The absorbance of this solution should be about 0.500 at 240 mμ with a 1-cm. light-path cell. This solution should be freshly prepared.

Procedure—Measurements were carried at wavelength 240 mμ in silica cells of 1-cm. light path, the final volume was about 3 ml. against a control (cell containing H_2O_2-free phosphate buffer, Solution I).

In the experimental cell, 3 ml. hydrogen peroxide–phosphate buffer (Solution II) was placed with about 0.05 ml. extract. The time at (Δ) required for a decrease in the absorbance from 0.450 to 0.400 was recorded; if it was longer than 60 sec. the measurement was repeated with a more concentrated solution of the sample.

Calculations (*cf. Reference 12*)—The activity = $17/\Delta t$ units per assay mixture in the cell, where one unit is defined as "the amount of enzyme which liberates half the peroxide oxygen from a hydrogen proxide solution of any concentration in 100 seconds at 25°C. (7)."

Table I illustrates results obtained for catalase activity[1] in different species of snails.

Peroxidase—*Reagents*—Hydrogen peroxide about 30% (w/v); *p*-phenylenediamine; potassium dihydrogen phosphate, A.R., KH_2PO_4; disodium hydrogen phosphate, A.R., $Na_2HPO_4 \cdot 2H_2O$.

Preparation of Solutions—All solutions were prepared in bidistilled water. (*a*) Hydrogen peroxide (3×10^{-3} *M*). Stock solution: 0.66 ml. of hydrogen peroxide (30% w/v) was diluted to 200 ml. Before use this stock solution was diluted 1:10. (*b*) *p*-Phenylenediamine (1% w/v), 1 g. of *p*-phenylenediamine was dissolved in about 60 ml. hot bidistilled water, then filtered and the filter was washed with about 25 ml. The total filtrate was quickly cooled and diluted to 100 ml. (*c*) Phosphate buffer (0.067 *M* pH 7), 3.522 g. KH_2PO_4 and 7.268 g. $Na_2HPO_4.2H_2O$ were dissolved in water and made up to 100 ml.

Table II illustrates results obtained for catalase activity in different species of snails.

Spectrophotometric Measurements—Wavelength 485 mμ, 1-cm. light path, final volume in the cell *ca.* 3.5 ml. Control cell as for experimental cell but containing bidistilled water instead of hydrogen peroxide. One-milliliter sample, 2 ml. phosphate buffer (III), 0.1 ml. H_2O_2 (I), and 0.1 ml. *p*-phenylenediamine (II) were pipeted successively into the cell and thoroughly mixed. Using a stopwatch, the absorbance after 30 sec., 15 sec., and 3 min. was recorded.

Calculation (*cf. Reference 13*)—*K''* activity per 1-g. snails

$$\frac{\Delta E \times \text{volume of enzyme extract}}{\Delta t \quad \text{weight of snails}}$$

where ΔE = change in absorbance during limited period of time (ΔE) and Δt = measured time reaction.

[1] The activity is in units (7) per g. snails.

TABLE I—CATALASE ACTIVITY IN DIFFERENT SPECIES OF SNAILS[a]

Type	Activity[b]	Temperature, °C.
Biomphalaria alexandrina	25.87	33
	24.207	20
(Infected)	13.26	21.5
	11.53	20
Bulinus truncatus	58.43	33
	36.33	30
	27.67	15
Lymnaea caillaudi	29.27	33
	21.67	27
	19.174	20
Physa acuta	44.35	33
	30.78	30
	28.019	20
	27.58	18

[a] Results are mean values for duplicate determinations of duplicate samples. [b] These units can be converted to Kat. f. if multiplied by 10.8 (12).

TABLE II—PEROXIDASE ACTIVITY IN DIFFERENT TYPES OF SNAILS[a]

Type	Activity[b] per g. Snails	Temperature, °C.
Biomphalaria alexandrina	754×10^{-5}	28
	682×10^{-5}	18
(Infected)	546×10^{-5}	21.5
	462×10^{-5}	18
Bulinus truncatus	513×10^{-5}	20
	452×10^{-5}	15
Lymnaea caillaudi	451×10^{-5}	27
	422×10^{-5}	20
Physa acuta	264×10^{-5}	20
	245×10^{-5}	18

[a] Results are mean values for duplicate determinations of duplicate samples. [b] The peroxidase activity unit is defined as the amount of purpurogallin (mg.) which is formed in 5 min. at 25° from 1.25 g. pyrogallol in 500 ml. solution containing 12.5 mg. H_2O_2 (13).

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Keyphrases

Molluscicides—structure, activity relationship
Snails, *Schistosoma masoni* infection—analysis
Peroxidase activity—*p*-phenylenediamine oxidation
Colorimetric analysis—spectrophotometer
Catalase activity—hydrogen peroxide decomposition
UV spectrophotometry—analysis

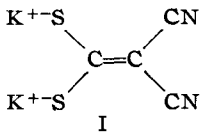
Antiradiation Compounds X. Derivatives of 3,3-Dimercaptoacrylonitrile

By WILLIAM O. FOYE and JOEL M. KAUFFMAN

The dipotassium salt of 2-cyano-3,3-dimercaptoacrylonitrile was found to give fair protection to mice *versus* X-rays. Derivatives prepared for further radioprotective evaluation include dialkyl thioethers, metal complexes, and fused heterocyclic dithioles. A series of 3,3-dimercaptoacrylonitriles bearing aromatic and heterocyclic rings was also obtained from the condensation of arylacetonitriles with carbon disulfide in the presence of alkoxide ion. Of these various derivatives, the zinc complex and the benzoyl derivative showed some radioprotective properties.

Condensations of carbon disulfide with certain amines and mercaptans have given dithiocarbamates (1, 2) and trithiocarbonates (3) with radioprotective activity. In order to determine whether nonbasic compounds, unrelated to the 2-mercaptoethylamine structure, having a dithio acid group may have radioprotective properties, condensations of a number of active methylene compounds with carbon disulfide have been carried out. Some of the products obtained have been examined for radiation protection in mice. Radiation protection of animals by dithio acids has not been previously reported.

Reaction of malononitrile with carbon disulfide is known to give the dipotassium salt of 2-cyano-3,3-dimercaptoacrylonitrile (4) (I). Brown's procedure was repeated, utilizing potassium hydroxide in 95% ethanol,



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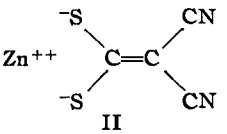

II

to give a product melting at 313–316° (dec) (m.p. previously listed as > 250°) with loss of water below 300°. Analysis indicated a monohydrate, and the infrared absorption spectrum showed the presence of water and C≡N, C=C, and C—S bonds (960 and 872 cm.$^{-1}$) (5). A trihydrate of the sodium salt of this compound has been reported by Soderback (6). Antiradiation tests carried out at the Walter Reed Army Institute of Research[1] showed this compound to give fair protection to mice *versus* 825 r (X-rays) at a dose level of 350–700 mg./kg. Accordingly, derivatives of this compound have been prepared as well as analogs in which one of the cyano groups was replaced, primarily by aromatic rings.

A zinc complex (II) was obtained as the hemihydrate which was much less water soluble than the potassium salt. The infrared spectrum was similar to that of the potassium salt, except that the absorption due to CS_2 appeared as a wide doublet centered at 880 cm.$^{-1}$. A cupric complex was also obtained as the hemihydrate, extremely insoluble in water, but a ferrous complex was too rapidly oxidized to be characterized.

The dimethylthioether and diethylthioether are known compounds (7) and were prepared for testing, but the monomethyl- or monoethyl-thioether could not be isolated. The dibutylthioether could not be crystallized or purified as well. Condensations of I with 2,3-dichloropyrazine and 2,3-dichloroquinoxaline were successful, however, giving pyrazino-[2,3-*d*]-1,3-dithiole-$\Delta^{2,\alpha}$-malononitrile (III) and quinoxalino[2,3-*d*]-1,3-dithiole-$\Delta^{2,\alpha}$-malononitrile.

Carbon disulfide condensation with cyanoacetamide to give IV*a* has been reported (4), using the hydroxide–ethanol method. An attempt to repeat

Received February 26, 1968, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston, MA 02115
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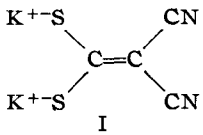

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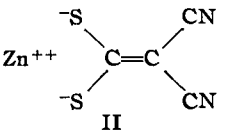

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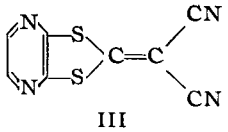
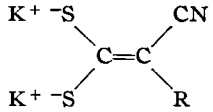


III

this gave only potassium ethyl xanthate. Condensation with ethyl cyanoacetate (4, 8, 9) took place readily using hydroxide–ethanol, and the product (IV*b*) was obtained unsolvated, unlike the other members of this series. The carbon analyses were low and erratic, as was the case with nearly all the compounds described below. This was probably due to retention of carbon as cyanide in the inorganic residue formed on the combustion catalyst. The weight of the residue was found to correspond to the amount the carbon analysis was low, calculated as cyanide, in one case investigated. Infrared absorption, however, showed the presence of C≡N, C=C, and C—S in this and the following compounds, which with generally satisfactory analyses for H, N, and S leaves no question as to the structures.

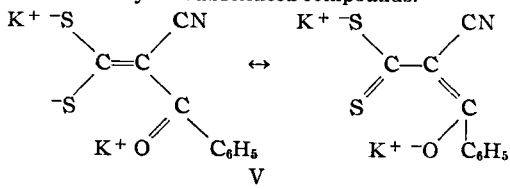


IV*a* R = $CONH_2$ *d* R = 2-furyl
b R = $CO_2C_2H_5$ *e* R = 2-thienyl
c R = C_6H_5 *f* R = 2-pyridyl
g R = 2-quinolyl

Condensation of carbon disulfide with phenylacetonitrile has been reported to occur in ether with sodamide but the product was not isolated (8). It has also been claimed to take place in methanol with potassium hydroxide, but no physical constants were reported for this compound (9). Repetition of this synthesis using potassium *n*-propoxide gave only xanthate. Use of *t*-butoxide, however, which is 100 times more basic than *n*-propoxide (10), in *t*-butanol–ether gave a good yield of condensation product (IV*c*), isolated as the hemi-*t*-butanolate. The reaction of carbanions with carbon disulfide appears to be instantaneous, but difficulties occur when too weak a base is used. A low concentration of carbanion apparently allows the competing reaction of alkoxide with carbon disulfide to predominate, leading to xanthates as major products.

This was illustrated in the case of condensation with furan-2-acetonitrile. Hydroxide in 95% ethanol gave xanthate, *n*-propoxide gave a mixture of xanthate and dithio acid (IV*d*), and *t*-butoxide gave dithio acid in good yield as the hemi-*t*-butanolate. The corresponding thiophene, pyridine, and quinoline compounds were also isolated as the hemialcoholates. Acetonitriles bearing electron-deficient rings such as pyridine and quinoline were sufficiently acidic to allow proton removal by *n*-propoxide. All of these aromatic-substituted compounds were extremely hygroscopic, liquefying after exposure to moist air for a few seconds to a few minutes. Handling in a glove bag under dry nitrogen was necessary. However, aqueous solutions of the compounds appeared to be stable at room temperature. None of these compounds could be recrystallized without decomposition.

A condensation product (V) from benzoylacetonitrile and carbon disulfide was obtained unsolvated and in poor yield. Infrared absorption was similar to that observed for the other compounds in the series, but no absorption attributable to a carbonyl group was evident. Instead, bands were observed at 1580, 1530, 1440, and 1270 $cm.^{-1}$ which are expected for metal chelates or salts of enols of 1,3-diketones (5), so the resonating structure V appears to be the most logical structure. Soderback (6) has pointed out the existence of similar resonating forms of dicyanodimercaptoethylene. The higher degree of resonance in this compound accounts for its greater stability and ease of handling, in comparison to the heterocyclic-substituted compounds.



V

Antiradiation Properties—Antiradiation tests were carried out in mice as previously described (11). Data received show that the dimethylthioether and diethylthioether of I were inactive *versus* 825 r (X-rays), whereas the zinc complex of I showed slight activity *versus* 1000 r (γ-rays) at a dose level of 25 mg./kg. The condensation product of carbon disulfide with ethyl cyanoacetate (IV*b*), however, gave 50% protection to mice *versus* 600 r (γ-rays) at at a dose of 100 mg./kg. The phenyl, furyl, and thienyl dimercaptoacrylonitriles, (IV*c*,*d*,*e*) gave no protection *versus* 825 r (X-rays), but the benzoyl derivative (V) gave slight protection (17% survival) with a dose of 120 mg./kg.

EXPERIMENTAL

Methods—Melting points were determined in capillaries with a Mel-Temp block and are corrected. Infrared spectra were obtained with a Perkin-Elmer Infracord 137B spectrometer in KBr pellets. Carbon-hydrogen analyses were done by Weiler and Strauss, Oxford, England, or by Carol K. Fitz, Needham, Mass. Nitrogen analyses were done by the Kjeldahl method and sulfur analyses by peroxide fusion.

2-Cyano-3,3-dimercaptoacrylonitrile, Dipotassium, Salt, Monohydrate—The procedure of Brown (4) gave an 83% yield of yellow crystals, m.p. 313–316° (dec); $\nu_{min.}^{KBr}$ 3400 (doublet) (OH) 2190 (C≡N), 1610 (C=C), 1310 (C—C), 1235 (C—C), 960 (C—S), 872 (C—S) $cm.^{-1}$.

Anal.—Calcd. for $C_4H_2K_2N_2OS_2$: C, 20.3; H, 0.9; K, 33.0; N, 11.9; S, 27.2. Found: C, 20.3; H, 1.1; K, 32.8; N, 11.8; S, 27.0.

2-Cyano-3,3-dimercaptoacrylonitrile, Zinc Complex—A clear solution of the potassium salt of 2-cyano-3,3-dimercaptoacrylonitrile (2.36 g., 0.01 mole) in 5 ml. of water was treated dropwise at 25° with a solution of zinc nitrate hexahydrate (2.97 g., 0.01 mole) in 2 ml. of water. The resulting mixture was cooled at 5°, filtered, and the light yellow solid was washed with a few ml. of ice water and dried, giving 1.8 g. (84% yield) of product which was unmelted at 360°: $\nu_{min.}^{KBr}$ 3550 (doublet), 2200, 1610, 1400, 1380, 1335, 945, 885, 875 $cm.^{-1}$.

Anal.—Calcd. for $C_4N_2S_2Zn \cdot {}^1/_2\ H_2O$: C, 22.4; H, 0.47; N, 13.1. Found: C, 22.6; H, 0.43; N, 13.4.

2-Cyano-3,3-dimercaptoacrylonitrile, Cupric Com-

plex—The potassium salt of 2-cyano-3,3-dimercaptoacrylonitrile (2.36 g., 0.01 mole) in 14 ml. of water was treated with a solution of cupric nitrate trihydrate (2.41 g., 0.01 mole) in 2 ml. of water. Bulky, red-brown solid appeared at once, and heat was evolved. The product was filtered, washed with water and propanol, and dried to give 2.2 g. (100%) of material unmelted at 360° (darkens above 250°).

Anal.—Calcd. for $C_4CuN_2S_2 \cdot {}^1/_2 H_2O$: C, 22.6; H, 0.47; N, 13.2. Found: C, 23.6; H, 0.42; N, 13.0.

Pyrazino[2,3-*d*]-1,3-dithiole-$\Delta^{2,\alpha}$-malononitrile—The potassium salt of 2-cyano-3,3-dimercaptoacrylonitrile (7.08 g., 0.03 mole) was dissolved in 50 ml. of dimethylformamide, and 2,3-dichloropyrazine (4.44 g., 0.03 mole) (gift of American Cyanamid Co.) was added. The solution was heated at 85° for 16 hr. during which time it became black. It was diluted with 100 ml. of water, cooled to 5°, and filtered. The dried, brown powder (2.0 g.) was sublimed at 0.5 mm. using a free flame to give 1.1 g. (17%) of yellow powder, m.p. 175–205° and 0.25 g. (4%) of yellow needles, m.p. 212–213° (analytical sample); $\nu_{min.}^{Kbr}$ 2210, 1480, 1340, 1155, 1075, 850 cm.$^{-1}$.

Anal.—Calcd. for $C_8H_2N_4S_2$: C, 44.0; H, 0.93; N, 25.7; S, 29.3. Found: C, 43.9; H, 1.16; N, 25.4; S, 29.0.

Quinoxalino [2,3-*d*]-1,3-dithiole-$\Delta^{2,\alpha}$-malononitrile—The potassium salt of 2-cyano-3,3-dimercaptoacrylonitrile (7.08 g., 0.03 mole), 2,3-dichloroquinoxaline (6.0 g., 0.03 mole) (Eastman Organic Chemicals), methanol (200 ml.), and water (20 ml.) were refluxed 2 hr., diluted with 150 ml. of water, and filtered. Recrystallization of the solid from 250 ml. of dimethylformamide and drying at 140°/0.2 mm. for 2 hr. gave bright yellow crystals, 3.2 g. (25%), m.p. 298–320° (dec.); $\nu_{min.}^{KBr}$ 2210, 1505, 1250, 1180, 1120, 780, 770 cm.$^{-1}$.

Anal.—Calcd. for $C_{12}H_4N_4S_2$: N, 20.8; S, 23.9. Found: N, 20.5; S, 23.6.

All manipulations in this and the following procedures were carried out under nitrogen.

2-Phenyl-3,3-dimercaptoacrylonitrile, Dipotassium Salt—Trimmed potassium metal (3.9 g., 0.1 mole) was allowed to dissolve in 100 ml. of *t*-butanol under nitrogen with stirring. Phenylacetonitrile (5.8 ml., 0.05 mole) in 25 ml. of dry ether was added in one portion at 30°, and carbon disulfide (3.8 g., 0.05 mole) in 25 ml. of dry ether was added dropwise at 10° during 10 min. The yellow product was filtered at 5°, washed with ether, and dried to give 10.2 g. (67%) of extremely deliquescent material, m.p. 45–60°; $\nu_{min.}^{KBr}$ 3400 (OH), 2180 (C≡N), 1620 (C=C), 1600, 1500, 1390, 910, 760, 700 cm.$^{-1}$.

Anal.—Calcd. for $C_9H_5K_2NS_2 \cdot {}^1/_2 C_4H_9OH$: N, 4.6; S, 20.9. Found: N, 4.7; S, 21.2.

2-(2-Furyl)-3,3-dimercaptoacrylonitrile, Dipotassium Salt—The previous procedure was applied to furan-2-acetonitrile (Aldrich Chemical Co.) to give an 87% yield of deliquescent tan powder, m.p. 110° (dec.); $\nu_{min.}^{KBr}$ 3400, 2180, 1625, 1570, 1500, 1400, 1350, 920, 765, 730 cm.$^{-1}$.

Anal.—Calcd. for $C_7H_3K_2NOS_2 \cdot {}^1/_2 C_4H_9OH$: N, 4.7; S, 21.6. Found: N, 4.5; S, 20.9.

2-(2-Thienyl)-3,3-dimercaptoacrylonitrile, Dipotassium Salt—The previous procedure was applied to thiophene-2-acetonitrile (12) to give a 76% yield of deliquescent yellow powder, m.p. 160–170° (dec.); $\nu_{min.}^{KBr}$ 3400, 2180, 1625, 1610, 1500, 1480, 1440, 1380, 1325, 1240, 920, 775, 690 cm.$^{-1}$.

Anal.—Calcd. for $C_7H_3K_2NS_2 \cdot {}^1/_2 C_4H_9OH$: N, 4.5; S, 30.8. Found: N, 3.9; S, 30.9.

2-(2-Pyridyl)-3,3-dimercaptoacrylonitrile, Dipotassium Salt—The previous procedure was employed with pyridine-2-acetonitrile (13) except that propanol was used in place of *t*-butanol. Bright yellow solid was obtained in 59% yield, after washing with propanol, m.p. 290–310° (dec.), and was extremely hygroscopic. $\nu_{min.}^{KBr}$ 3300, 2180, 1630, 1590, 1460, 1425, 1380, 1295, 1260, 920, 825, 775, 745, 670 cm.$^{-1}$.

Anal.—Calcd. for $C_8H_4K_2N_2S_2 \cdot {}^1/_2 C_3H_7OH$: N, 9.3; S, 21.3. Found: N, 9.3; S, 21.1.

2-(2-Quinolyl)-3,3-dimercaptoacrylonitrile, Dipotassium Salt—The previous procedure was employed with quinoline-2-acetonitrile (14) to give an 81% yield of bright orange, granular solid, m.p. 225–235° (dec.) (loses liquid at 200°) which was very hygroscopic. $\nu_{min.}^{KBr}$ 3300, 2180, 1640, 1610, 1600, 1550, 1500, 1450, 1420, 1350, 1310, 1270, 1240, 1050, 920, 895, 825, 780, 760, 750 cm.$^{-1}$.

Anal.—Calcd. for $C_{12}H_6K_2N_2S_2 \cdot {}^1/_2 C_3H_7OH$: N, 8.0; S, 18.3. Found: N, 8.0; S, 18.0.

2-Benzoyl-3,3-dimercaptoacrylonitrile, Dipotassium Salt—Potassium hydroxide (4.8 g., 0.072 mole) dissolved in 50 ml. of methanol was treated with a solution of carbon disulfide (2.2 ml., 0.036 mole) and benzoylacetonitrile (5.24 g., 0.036 mole) in 100 ml. of 50% ether–methanol. The solid that separated after 4 days at 20° was washed with 100 ml. of 50% ether–methanol and dried to give 0.8 g. (7.5%) of light-colored solid, m.p. 247–248° (dec.); $\nu_{min.}^{KBr}$ 2200, 1580, 1530, 1440, 1350, 1270, 910, 795, 775, 720, 690 cm.$^{-1}$.

Anal.—Calcd. for $C_{10}H_5K_2NOS_2$: C, 40.3; H, 1.7; N, 4.7. Found: C, 40.2. H, 2.1; N, 4.9.

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Keyphrases

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3,3-Dimercaptoacrylonitrile derivatives—synthesis
IR spectrophotometry—identity
Radioprotective activity—3,3-dimercaptoacrylonitrile derivatives

Antiradiation Compounds XI. Sulfur-Containing Derivatives of Nitriles and Aminonitriles

By WILLIAM O. FOYE and JOEL M. KAUFFMAN

Attempts to obtain iminothio esters and dithio esters from α-aminoacetonitrile gave stable products only with the amino group acylated by the carbobenzoxy function. An iminothio ester derived from β-aminopropionitrile was found to be relatively stable as the dihydrochloride, however. Examples of both α- and β-cyanoalkylthiosulfates were found to be stable as the sodium salts. Antiradiation testing of the β-amino iminothio ester dihydrochloride and the α-cyanothiosulfate showed no protective properties.

INORGANIC CYANIDES have shown radiation-protective properties in animals (1), but relatively few organic nitriles have been tested for this property. Malononitrile (2), hydroxyacetonitrile (3), 2-hydroxybutyronitrile (4), and α-benzyloxyiminopropionitrile (5) have all shown some degree of radiation protection, and several examples of cyano-containing dithio acid dianions (6) are radiation protective. The *N*-cyano derivative of 2-mercaptoethylguanidine (MEG) has also been found protective in animals, but less so than MEG itself (7). An attempt has been made, therefore, to obtain sulfur-containing derivatives of organic nitriles which might have protective ability, and in particular those which might lead to sulfur-containing derivatives of amino acids *in vivo*. Glycine itself is radioprotective for some enzymes (8), and its thioamide has shown radiation-protective properties in plants (9).

In regard to the synthesis of amino dithio acids, no method of liberating an amine or amine salt from an N-blocked derivative in the presence of a dithiocarboxylic acid or ester is known. It appeared possible to obtain dithio esters or iminothio esters with free amino groups, however. Accordingly, benzyl cyanomethylcarbamate (I) was prepared

$$C_6H_5CH_2OCONHCH_2CN \xrightarrow[HCl]{C_2H_5SH}$$

I

$$C_6H_5CH_2OCONHCH_2\overset{\overset{NH_2^+Cl^-}{\|}}{C}SC_2H_5 \xrightarrow[Na_2CO_3]{H_2S}$$

II

$$C_6H_5CH_2OCONHCH_2CS_2C_2H_5$$

III

from carbobenzoxy chloride, aminoacetonitrile bisulfate, and sodium carbonate. Although the preparation of I from aminoacetonitrile hydrochloride with gradual addition of sodium hydroxide has been reported (10), the method described here gives a cleaner product with less trouble, since carbonate provides the optimum pH for the reaction. The product can also be recrystallized rather than reprecipitated. Reaction with ethanethiol and hydrogen chloride gave the iminothio ester (II), which was converted to the dithio ester (III) with hydrogen sulfide and sodium carbonate. The iminothio ester (II) remained stable only long enough for chlorine and nitrogen analyses, which could be run immediately after isolation of the product. Characteristic absorption for C=S in (III) was shown in the infrared at 1050 cm.$^{-1}$ and in the ultraviolet at 270 and 365 mμ.

Attempts to remove the carbobenzoxy group from the α-aminodithio ester were made with hydrogen bromide in acetic acid, hydrogen chloride in acetic acid, trifluoroacetic acid, and phosphonium iodide. All of these methods, as well as aqueous procedures, failed. Attempts to thiohydrolyze the dithio ester (III) to the dithio acid with hydrogen sulfide and triethylamine also failed.

Benzyl cyanoethylcarbamate (IV) was prepared from aminopropionitrile fumarate and sodium carbonate rather than from the free amine and hydroxide as previously reported (11). Reaction with methanethiol and hydrogen chloride gave the iminothio ester (V), which surprisingly had suffered loss of the carbobenzoxy group. Apparently, a distance of two carbons is required between an unblocked amino and iminothio ester functions for stability.

$$C_6H_5CH_2OCONHCH_2CH_2CN \xrightarrow[HCl]{CH_3SH}$$

IV

$$Cl^-{}^+NH_3CH_2CH_2\overset{\overset{NH_2^+Cl^-}{\|}}{C}SCH_3$$

V

To obtain compounds containing both cyano and reactive sulfur functions, the preparation of sodium cyanomethylthiosulfate was attempted using chloroacetonitrile and sodium thiosulfate. Analysis indicated the product (VI) to be a monohydrate, but the infrared spectrum showed only weak absorption for a cyano group and much stronger absorption at 1,640 cm.$^{-1}$. Suspecting that the product might be the amide rather than the nitrile, the amide (VII) was prepared from chloroacetamide and sodium thiosulfate. The amide differed in crystalline form, solubility, infrared absorption, melting behavior, and R_f value on thin-layer chromatography, thus confirming that the nitrile had been obtained. The homologous cyanoethylthiosulfate was also prepared, and again obtained as the monohydrate.

$Na^+{}^-O_3SSCH_2CN \cdot H_2O$ VI

$Na^+{}^-O_3SSCH_2CONH_2$ VII

Received February 26, 1968, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston MA 02115

Accepted for publication June 17, 1968.

This project was supported by U. S. Public Health Service research grant RH00297, National Center for Radiological Health, for which the authors are grateful.

Antiradiation Screening—Results obtained from the Walter Reed Army Institute of Research, through the courtesy of Drs. D. P. Jacobus and T. R. Sweeney, showed that Compounds V and VI provided no protection in mice. Compound V was tested *versus* 1,000r (γ-rays) and Compound VI was tested *versus* 825r (X-rays), both compounds administered at dose levels of less than 50 mg./kg.

EXPERIMENTAL

Melting points were determined in capillaries on a Mel-Temp block (Laboratory Devices Co.) and are corrected. Elemental analyses were determined by Dr. Carol K. Fitz, Needham, Mass. Infrared absorption spectra were obtained with a Perkin-Elmer model 137B spectrometer with sodium chloride optics. Evaporations were done using a rotary evaporator at 40 mm.

Benzyl Cyanomethylcarbamate (I)—Aminoacetonitrile bisulfate (89 g., 0.58 mole) (Aldrich Chemicals), water (500 ml.) and ice (500 g.) were stirred while sodium carbonate monohydrate (220 g., 1.77 moles) was added. Additional ice (1 kg.) and ether (1 l.) were added, the temperature of the ether layer dropped to −2°, and carbobenzoxy chloride (94 g., 0.55 mole) (Nutritional Biochemicals) was poured in at once. Stirring was continued overnight as the mixture warmed to room temperature, and the ether layer was dried ($MgSO_4$) and evaporated. The residue was recrystallized from carbon tetrachloride–methanol, giving 73.7 g. (70%) of white needles, m.p. 61–63° [lit. (10) m.p. 62°]; $\nu_{min.}^{KBr}$ 3320 (NH), 2260 (very weak) (C≡N), 1700 (C=0) cm.$^{-1}$.

Benzyl 2-Ethylthio-2-iminoethylcarbamate Hydrochloride (II)—Benzyl cyanomethylcarbamate (1.9 g., 0.01 mole), ethanethiol (1.5 ml., 0.02 mole) (Eastman Organic Chemicals), and dry ether (20 ml.) were treated with hydrogen chloride at 4° for 30 min. The solution became cloudy and deposited 3.2 g. (100%) of white product after 3 days storage at 4°; m.p. 138–142° (dec.). The analytical sample was obtained by recrystallization from chloroform–ligroin (1:1); m.p. 135–141° (dec.); $\nu_{min.}^{CHCl_3}$ 2860 (NH^+), 1720 (C=O) cm.$^{-1}$.

Anal.—Calcd. for $C_{12}H_{17}ClN_2O_2S$: Cl, 12.28; N, 9.69. Found: Cl, 12.71; N, 9.88.

Ethyl N-Carbobenzoxydithioglycinate (III)—The iminothio ester (II) (1.9 g., 0.007 mole) and sodium carbonate (3.2 g., 0.03 mole) were covered with 30 ml. of dry ether and treated with dry hydrogen sulfide ($CaCl_2$) at a rate sufficient to stir the mixture for 4.5 hr. Filtration and removal of solvent from the filtrate in a dry nitrogen stream left 0.4 g. (22%) of bright yellow crystals, m.p. 60–64°, 150° (dec.). The compound was insoluble in cold alkaline solution, but soluble in sodium hydroxide solution after warming for 5 min. The analytical sample was obtained by recrystallization from ether–ligroin, m.p. 64–65°; $\nu_{min.}^{CCl_4}$ 1730 (C=O), 1050 (C=S) cm.$^{-1}$; $\lambda_{max.}$ (CH_3OH) 270 (ϵ 14), 365 (ϵ 16) mμ.

Anal.—Calcd. for $C_{12}H_{15}NO_2S_2$: C, 53.60; H, 5.62; N, 5.21. Found: C, 53.64; H, 5.56; N, 5.22.

Benzyl 2-Cyanoethylcarbamate (IV)—2-Aminopropionitrile fumarate (15.4 g., 0.06 mole) (Aldrich Chemicals) was carbobenzoxylated by the method above. In the purification of the product, propanol was added to the ether solution until all organic solid was dissolved. Dilution of the dried solution with ligroin and cooling gave glistening white plates in two crops totaling 17.6 g. (72%); m.p. 69–71° [lit. (11) m.p. 74°]; $\nu_{min.}^{KBr}$ 3310 (NH), 2250° (weak) (C≡N), 1695 (C=O) cm.$^{-1}$.

3-Imino-3-methylthiopropylamine Dihydrochloride (V)—This reaction, and all handling of the product, was carried out under dry nitrogen. The previous product (10.2 g., 0.05 mole) was dissolved in 300 ml. of dry 1,2-dimethoxyethane (distilled over sodium hydride), cooled to 5°, treated with 6 ml. of methanethiol (Eastman Organic Chemicals), and saturated with hydrogen chloride. Crystal formation appeared complete in 1 day. Solvent was decanted after 1 week, and the white, crystalline residue was washed with 70 ml. of dry 1,2-dimethoxyethane and vacuum dried, giving 9.1 g. (95%), m.p. 203–208° (dec.). It was hygroscopic and soluble in cold water. Analyses were obtained for chlorine and nitrogen immediately after isolation, but the product suffered partial decomposition before carbon-hydrogen analyses could be obtained.

Anal.—Calcd. for $C_4H_{12}Cl_2N_2S$: Cl, 37.1; N, 14.7. Found: Cl, 37.0; N, 14.6.

Sodium Cyanomethylthiosulfate, Monohydrate (VI)—Sodium thiosulfate pentahydrate (59.6 g., 0.2 mole) was dissolved in 50 ml. of distilled water and treated with 50 ml. of 95% ethanol and then chloroacetonitrile (15.1 g., 0.2 mole) (Aldrich Chemicals). The mixture was boiled under reflux for 20 min., cooled, and freed of solvent. The residue was leached with 100 ml. of boiling 95% ethanol, then with another 20-ml. portion of the same, and the extracts were cooled at −5° to give 32.0 g. (86%) of white prisms. Recrystallization from 95% ethanol gave the analytical sample; m.p. 150° (dec.); $\nu_{min.}^{KBr}$ 3490 (OH), 2250 (C≡N), 1640 (CH), 1380 (SO_2), 1220 (SO_2), 1040 cm.$^{-1}$.

Anal.—Calcd. for $C_2H_4NNaO_4S_2$: C, 12.43; H, 2.10; N, 7.25; Na, 11.9. Found: C, 12.5; H, 1.9; N, 7.35; Na, 11.9.

The Feigl spot test (12) for an aliphatic nitrile was strongly positive. On Eastman Chromagram sheets, using propanol–water, and iodine vapor as developer, this nitrile had an R_f = 0.44, whereas the amide (VII) had an R_f = 0.25.

Sodium Carboxamidomethylthiosulfate (VII)—Chloroacetamide (13) (2.4 g., 0.026 mole) in 12 ml. of warm ethanol solution and sodium thiosulfate pentahydrate (6.4 g., 0.026 mole) in 12 ml. of warm water were mixed and refluxed for 1 hr. The solvent was evaporated, and the residue was taken up in 15 ml. of ethanol with additional water for complete solution, and white needles precipitated at 5° (2.6 g., 55%); m.p. 180–203° (dec.); $\nu_{min.}^{KBr}$ 1670 (C=O), 1260 (SO_2), 1200 (SO_2), 1040 cm.$^{-1}$.

Anal.—Calcd. for $C_2H_4NNaO_4S_2$: C, 12.43; H, 2.10; N, 7.25. Found: C, 12.56; H, 2.44; N, 7.24.

Sodium Cyanoethylthiosulfate Monohydrate—The same procedure as used for VI was employed with 2-bromopropionitrile (26.8 g., 0.2 mole) and sodium thiosulfate pentahydrate (59.6 g., 0.2 mole). After 1 hr. of refluxing, 33.4 g. (81%) of white solid was extracted and washed with cold 95% ethanol; infrared peaks were similar to those of VI; m.p. 250° (dec.).

Anal.—Calcd. for $C_3H_6NNaO_4S_2$: C, 17.40; H, 2.92; N, 6.76. Found: C, 17.58; H, 3.60; N, 6.10.

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Antiradiation compounds
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Paper chromatography—identity
IR spectrophotometry—structure
UV spectrophotometry—structure

Pyrrolo [2,3-*d*]Pyrimidines

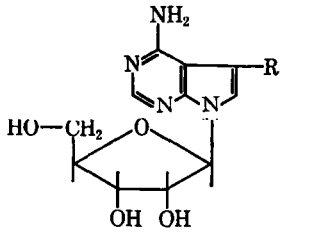
By RICHARD H. HAMMER

The synthesis of a series of 4-alkylamino-7-methyl analogs of tubercidin from 4-chloro-7-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidine is described.

TUBERCIDIN (4-amino-7-β-D-ribofuranosyl-7*H*-pyrrolo[2,3-*d*]pyrimidine) (I) and two other closely related naturally occurring nucleosides containing the pyrrolo[2,3-*d*]pyrimidine structure, toyocamycin (II), and sangivamycin (III), show pronounced cytotoxic activity (1–3). As a result of this activity and the unique stability of the base-sugar bond to enzymatic (4) and *in vitro* acid cleavage,[1] considerable interest in the synthesis and evaluation of tubercidin analogs has developed.

Recently, Gerster *et al.* (5) synthesized a series of 4-substituted derivatives of tubercidin with the ribofuranosyl group intact on the 7 position. Montgomery and Hewson (6) have observed a decrease in cytotoxic activity, compared to tubercidin, when substituting aliphatic and cycloaliphatic groups at the 7 position with a free amino group in the 4 position. Cell-culture cytotoxity studies on Compounds IV and V (Table III), synthesized in this laboratory (7), indicate an increase in activity when the bulky, lipophilic benzyl group is substituted on the mercapto group of IV. These observations prompted the synthesis of a series of 4-alkylamino-7-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidines (VIII*a*-VIII*f*) where the series extends from the simpler alkyls, such as ethyl, to heterocyclic rings such as pyrrolidyl and piperidyl. In order to simulate the pKa of tubercidin, the described compounds all have a methyl group substituted on the 7-nitrogen.

Synthesis of these compounds has been accomplished through methylation of 4-chloro-7*H*-pyrrolo-[2,3-*d*]pyrimidine (VI) with methyl iodide (8) and reaction of this 7-methyl product (VII) with the appropriate amine in a Parr bomb at elevated temperatures to give the respective 4-alkylamino-7-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidine compound (VIII*a*–VIII*f*).



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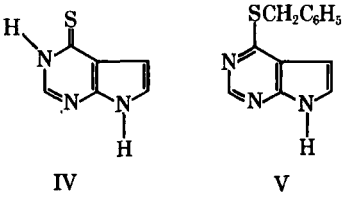

IV V

EXPERIMENTAL

Melting points were obtained on a Thomas-Hoover capillary melting-point apparatus and are uncorrected. Ultraviolet analysis was recorded on a Beckman model DB spectrophotometer. Titrimetric pKa values were obtained on a Sargent titrator, model D at 25°. NMR spectra were obtained on a Varian A-60 instrument at a field strength of 60 Mc./sec. Microanalysis was conducted by Galbraith Laboratories, Inc., Knoxville, Tenn.

Method A—To 0.175–0.20 g. of VII (8) was

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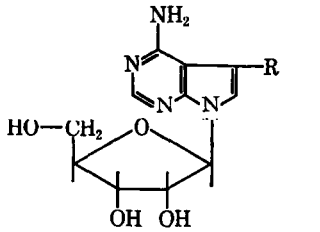

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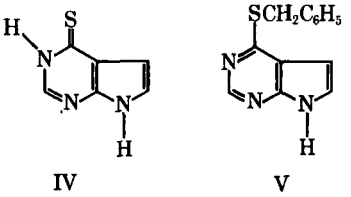

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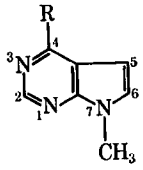
added a 1-mole excess of alkylamine and 15–25 ml. of 95% ethanol. The solution was heated with stirring in a Parr bomb at 125–130° for 6–6.5 hr. The reaction mixture was acidified to approximately pH 2 with 20% HCl, filtered, and the filtrate adjusted to pH 10 with 20% NaOH. The mixture was evaporated to dryness and the resultant oily syrup was taken up in water–chloroform and subsequently extracted with chloroform. Removal of the chloroform left a syrup which was purified using preparative thin-layer chromatography to yield crystalline, analytically pure samples (1-mm. Silica Gel GF_{254}; solvent system, ethyl acetate–methanol, 9:1). Including fluorescent zones on the starting line and solvent front, four bands were observed under short wavelength UV light. Extraction of the second band from the solvent front with 95% ethanol and evaporation of the solvent yielded the products which were characterized as the free base and/or the HCl salt. See Tables I and II. In the case of VIII*b*, after extraction from the plate and removal of the solvent, it was necessary to triturate with petroleum ether to obtain the product.

Method B—After heating the reactants in a Parr bomb at 125–130° for 6–6.5 hr. as outlined under Method A, the solution was concentrated to a viscous oily syrup and purified *via* two consecutive preparative thin-layer chromatography steps (Method A) to give the final product which was analyzed as the free base and/or the HCl salt. See Tables I and II.

RESULTS AND DISCUSSION

The five-step procedure of Davall's (9) led to the 4-chloro-7*H*-pyrrolo[2,3-*d*]pyrimidine intermediate (VI) which was converted to the 7-methyl analog (VII) by methylation with methyl iodide (8). Nucleophilic displacement of the 4-chloro group of VII and other 4-chloro-7-alkyl-7*H*-pyrrolo[2,3-*d*]-pyrimidines with methanolic ammonia to give the

TABLE I—PHYSICAL PROPERTIES OF



Compd.[a]	R	Method	% Yield	M.p., °C. Base	M.p., °C. HCl	Molecular Formula	Anal. Calcd	Anal. Found	pKa[b]
VIII*a*	4-Ethylamino	A	52	156–58[c]	233–34 dec.	$C_9H_{12}N_4$	C 61.37 H 6.81 N 31.82	C 61.25 H 6.95 N 31.67	5.64
VIII*b*	4-*n*-Propylamino	A B	32 50	118–20	203–04 dec.	$C_{10}H_{14}N_4$	C 63.16 H 7.36 N 29.47	C 63.00 H 7.48 N 29.24	5.52
VIII*c*	4-*n*-Pentylamino	B	59	72–3[d]	67–9	$C_{12}H_{18}N$	C 66.04 H 8.33 N 25.68	C 65.85 H 8.08 H 25.53	5.66
VIII*d*	4-*n*-Hexylamino[e]	B	57	71–2	158–60	$C_{13}H_{20}N_4$	C 67.06 H 8.69 N 24.14	C 65.78 H 8.63 N 23.71	—
VIII*e*	4-(1-Pyrrolidyl)[e]	B	80	99–100	[f]	$C_{11}H_{14}N_4 \cdot HCl$	C 55.37 H 6.45 N 23.48	C 54.34 H 6.36 N 23.00	—
VIII*f*	4-(1-Piperidyl)	B	59[g]	—	195–96 dec.	$C_{12}H_{18}N_4 \cdot HCl$	C 57.04 H 7.59 N 22.17	C 56.81 H 7.28 N 21.95	5.28

[a] The 4-*n*-butyl compound was also prepared. M.p. (free base) 112–113°. Calcd. for $C_{11}H_{16}N_4$: C, 64.71; H, 7.87; N, 27.44. Found: C, 64.57; H, 7.90; N, 27.20. [b] Dissociation constants were determined on the HCl salts in distilled water at 25°. [c] Reported: 159° (10). [d] Reported 125–127° (10). See *Results and Discussion*. [e] Compounds VIII*d* and VIII*e* were not within acceptable limits for carbon analysis. Insufficient amounts of both samples prohibited further analysis. [f] Begins darkening around 240° and melts with decomposition at 260°. [g] Calculated as the HCl salt from starting material VII.

TABLE II—UV AND NMR ANALYSIS

Compd.	λmax. mμ (ε) pH 1.0[a]	Ethanol	pH 13.0	Compd.	NMR (τ, p.p.m.) Protons NHCN	=CH	NCH_3	NCH_2	CH_2 & CH_3	$J_{5,6}$ c.p.s.
VIII*a*	232 (19,370) 274 (12,360)	273 (12,150)	273 (11,970)	VIII*a*[b]	1.93	2.82 3.38	6.28	6.52	8.71 (CH_3)	3.5
VIII*b*	232 (19,020) 274 (13,120)	276 (13,310)	275 (12,930)							
VIII*c*	233 (20,140) 274 (12,940)	276 (12,730)	276 (12,940)							
VIII*d*	232 (18,580) 274 (11,840)	278 (9,750)	275 (12,770)	VIII*c*[c]	1.83	2.88 3.41	6.28	6.51	8.56 9.09 } complex, overlapping	3.5
VIII*e*[d]	237 (12,600) 280 (9,700)	281 (9,450)	282 (9,390)	VIII*d*[e]	1.65	3.12 3.66	6.20	6.43	8.08 8.62 9.12 9.94	3.5
VIII*f*[d]	238 (13,640) 284 (11,620)	288 (16,170)	290 (12,880)							

[a] pH 1.0 (0.1 *N* HCl); ethanol, 95%; pH 13.0 (0.1 *N* NaOH). [b] HCl salt in water-*d* with tetramethylammoniumtetrafluoroborate as the internal standard. [c] Determined in d_6-dimethylsulfoxide. [d] HCl salt. [e] Determined in chloroform-*d* with tetramethylsilane as the internal standard.

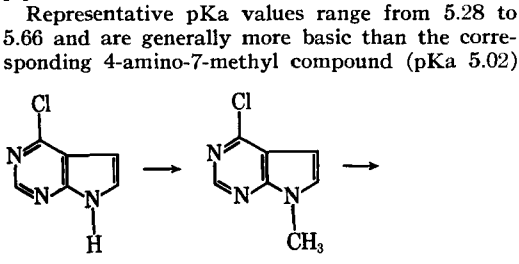
4-amino derivatives proceeds smoothly although requiring higher temperatures than the corresponding purines (6, 8). Hitchings *et al.* (10) reported the synthesis of various 4-alkylamino-7*H*-pyrrolo-[2,3-*d*]pyrimidines by heating the 4-chloro starting material at elevated temperatures with an alkylamine. The facility by which the 4-chloro group of pyrrolo[2,3-*d*]pyrimidines serves as a leaving group for a variety of nucleophilic substitutions is therefore well established (8).

Two methods have been employed for the synthesis of VIII*a*–VIII*f*. The first method (Method A), which is similar to the method employed by Hitchings (10), produced low yields of product. The second method (Method B) whereby the volume of solvent was reduced and the acid-base steps were eliminated increased the yield of VIII*b* from 32 to 50%. Method B was therefore adopted for the synthesis of VIII*c*–VIII*f*. See Table I.

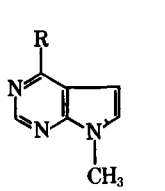
The ultraviolet spectra of the secondary amines (VIII*a*–VIII*d*) have maximums at 274 mμ (ε 11,840–13,120) and a more intense maximum at 232–233 mμ (ε 18,580–20,140) at pH 1, while in ethanol or at pH 13, only a broad absorption band at 273–278 mμ (ε 9,750–13,310) is observed. The spectra of the two tertiary amines, VIII*e* and VIII*f*, undergo a bathochromic shift to 280–284 mμ and 237–238 mμ at pH 1, and a shift to 281–290 mμ in ethanol or at pH 13 with the lower maximum having the more intense absorption. These spectra are characteristic of 4-amino and 4-propylamino-7*H*-pyrrolo[2,3-*d*]pyrimidines (6, 8, 10) as well as the corresponding purines (11). See Table II.

Representative proton magnetic resonance spectra were obtained on two of the free bases (VIII*c* and VIII*d*) and a HCl salt (VIII*a*). The pyrrole ring protons (C_5-H, C_6-H) both have coupling constants ($J_{5,6}$) = 3.5 c.p.s. The chemical shift (τ) of the 7-NCH_3 is 6.2–6.28 p.p.m. The chemical shift of the C_5-H is 2.82–3.12 p.p.m. and for the C_6-H, 3.38–3.66 p.p.m. The chemical shift of the pyrimidine ring C_2-H is further downfield at 1.65–1.93 p.p.m.

Representative pKa values range from 5.28 to 5.66 and are generally more basic than the corresponding 4-amino-7-methyl compound (pKa 5.02)



VI VII



VIII*a*, R = NHC_2H_5
VIII*b*, R = NHC_3H_7-*n*
VIII*c*, R = NHC_5H_{11}-*n*
VIII*d*, R = NHC_6H_{13}-*n*
VIII*e*, R = NC_4H_8
VIII*f*, R = NC_5H_{10}

TABLE III—CELL CULTURE CYTOTOXICITY

Compound	ED_{50} (mcg./ml.)[a]
VIII*a*[b]	25
VIII*b*[b]	27
VIII*c*[b]	47
VIII*d*[c]	56
VIII*e*[b]	42
VIII*f*[b]	100
VII[c]	>100
4-Amino-7-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (8)[c]	>100
4-Mercapto-7-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (8)[c]	>100
4-Chloro-7*H*-pyrrolo[2,3-*d*] pyrimidine (7) (VI)[c]	26
4-Mercapto-7*H*-pyrrolo[2,3-*d*] pyrimidine (7) (IV)[c]	>100
4-Benzylthio-7*H*-pyrrolo[2,3-*d*] pyrimidine (7) (V)[c]	13
4-Methoxy-7*H*-pyrrolo[2,3-*d*] pyrimidine (7)[c]	28

[a] The dose (mcg./ml.) that inhibits growth of cells to 50% of control growth. [b] HCl salt. [c] Free base.

(8), and tubercidin (pKa 5.3) (12). These pKa's are all favorable for producing high ratios of unionized to ionized forms of the drug at the pH of body fluids (7.4), and one would predict a high intracellular level of these drugs provided that they have high partition coefficients.

Synthesis of compounds VIII*a* and VIII*c* have previously been reported (10). The m.p. reported in the literature for VIII*a* agrees with the m.p. of the compound prepared in this laboratory. However, the m.p. previously reported for VIII*c* is in disagreement with that of the compound prepared in this laboratory. The structural assignments for VI and VII, intermediates in the synthesis of VIII*c*, are well established (8, 9). Reaction of VII with *n*-pentylamine to form VIII*c* as reported here is supported by comparison of the UV spectrum of VII (8) and Compounds VIII*a*, VIII*b*, and VIII*d* to the spectrum of VIII*c*. The bathochromic shift from 229 mμ (ε 23,050) and 272 mμ (ε 4,550) of VII (8) to 233 mμ (ε 20,140) and 274 mμ (ε 12,940) at pH 1, similar shifts at other pH's, plus similarity of the UV spectrum of VIII*c* to VIII*a*, VIII*b*, and VIII*d* indicate 4-alkylamino substitution. In addition, the C, H, N analysis and NMR of the free base (VIII*c*) and pKa data further support this. See Tables I and II. Based on this data, VIII*c* is considered to be the 4-*n*-pentylamino-7-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidine structure with physical properties as reported in Tables I and II.

Cytotoxicity studies have been conducted by the Cancer Chemotherapy National Service Center. The results obtained from several pyrrolo[2,3-*d*]-pyrimidine derivatives, synthesized and reported in previous papers (7, 8) and compounds VIII*a*-VIII*f* are given in Table III.

The compounds were all inactive although derivatives substituted with 4-alkylamino substituents (VIII*a*-VIII*e*) and 4-benzylthiopyrrolo[2,3-*d*]pyrimidine (7) (V) were all more active than the unsubstituted 4-amino-7-methyl- (8) and 4-mercaptopyrrolo-[2,3-*d*]pyrimidines (7) (IV), respectively. Conceivably, a bulky group on the 7 position, such as a cyclopentyl or cyclohexyl group, in addition to a 4-alkylamino- or 4-benzylthio- group, would increase activity.

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Two alkaloids, in addition to the six previously described, have been isolated from the leaves of *Peumus boldus* Molina. They were characterized by chromatographic and spectral data and shown to be the noraporphines, laurotetanine and laurolitsine.

DURING A chromatographic study of the alkaloids of the leaves of the South American shrub *Peumus boldus* Molina (*Monimiaceae*) (boldo leaves), the authors detected the presence of at least 17 alkaloids in a nonquaternary fraction obtained by chloroform extraction of the plant material after treatment with ammonia (1). The alkaloids were numbered 1–17 according to increasing R_f on thin-layer chromatography (TLC), and two were isolated and characterized as reticuline and the aporphine, isoboldine. Earlier, the aporphines boldine (I) (2), *N*-methyllaurotetanine (II), and isocorydine and the noraporphine, norisocorydine had been isolated (3). On the basis of paper chromatographic evidence the presence of sparteine has also been suggested (4).

This paper describes the isolation from boldo leaves and the characterization of two closely related noraporphines, laurotetanine (Alkaloid 6) (III) and laurolitsine (norboldine; Alkaloid 2) (IV). TLC examination of the crude alkaloid extract indicates that the leaves contain 0.005–0.007% laurotetanine and approximately 0.001% laurolitsine.

These alkaloids have been isolated from a number of species, particularly in the family *Lauraceae* (5, 6), sometimes together with boldine (5). A recent report describes the isolation of laurotetanine and *N*-methyllaurotetanine from another *Monimiaceae*: *Palmeria fengeriana* Perk (7).

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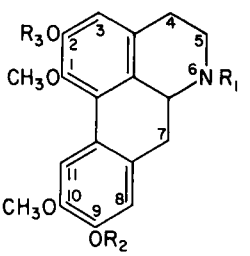

I, $R_1 = CH_3$; $R_2 = R_3 = H$
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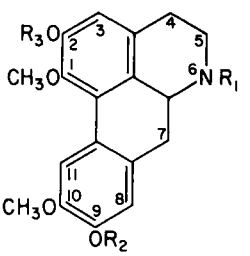

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Keyphrases

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Laurolitsine—isolation, identification
Column chromatography—separation
TLC—separation, identity
UV spectrophotometry—identity
IR spectrophotometry—identity
NMR, mass spectroscopy—structure

Polyamide Layer Chromatography of Organophosphorus Pesticides

By J. T. HUANG*, H. C. HSIU, T. B. SHIH, U. T. CHOU, K. T. WANG, and C. T. CHENG

Thin-layer chromatographic system and spray reagents are described for rapid differentiation of 11 organophosphorus pesticides.

THE USE of paper or thin-layer chromatography to determine and identify the organophosphorus pesticides had been widely studied and reviewed by Zweig (1). Recently, several workers used the formamide impregnated paper (2) and thin-layer (3) to separate organophosphorus pesticides. In previous reports (4, 5) better separation of opium alkaloids was obtained with polyamide layer, therefore, this method was applied to separate the organophosphorus pesticides. This note describes the results obtained.

EXPERIMENTAL

Material—Eleven organophosphorus pesticides, FDA standard were used. The solvents and chemicals are the reagent grade of Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Thin-Layer Plates—All plates used were 15 × 15 cm. of polyamide layer sheet described by Wang (6).

Chromatographic Procedure—The standard techniques of ascending thin-layer chromatography (7) were employed.

Visualization—Four methods were employed: (*a*) iodine vapor exposing, (*b*) 5% alcoholic potassium hydroxide solution spraying, (*c*) iodoplatinate reagent spraying (8), (*d*) first spraying 5% alcoholic potassium hydroxide solution on the plate, allowing the plate to dry, then exposing the plate with iodine vapor for 5 min., followed by exposing ammonia vapor.

Received March 22, 1968, from the Department of Pharmacy, Taipei Medical College, Taipei, China; Department of Chemistry, National Taiwan University, Taipei, China (K. T. Wang); and Taiwan Provincial Health Department, Tai-Chang, Taiwan, China (C. T. Cheng).
Accepted for publication June 11, 1968.
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Keyphrases

Alkaloids—*Peumus boldus*
Laurotetanine—isolation, identification
Laurolitsine—isolation, identification
Column chromatography—separation
TLC—separation, identity
UV spectrophotometry—identity
IR spectrophotometry—identity
NMR, mass spectroscopy—structure

Polyamide Layer Chromatography of Organophosphorus Pesticides

By J. T. HUANG*, H. C. HSIU, T. B. SHIH, U. T. CHOU, K. T. WANG, and C. T. CHENG

Thin-layer chromatographic system and spray reagents are described for rapid differentiation of 11 organophosphorus pesticides.

THE USE of paper or thin-layer chromatography to determine and identify the organophosphorus pesticides had been widely studied and reviewed by Zweig (1). Recently, several workers used the formamide impregnated paper (2) and thin-layer (3) to separate organophosphorus pesticides. In previous reports (4, 5) better separation of opium alkaloids was obtained with polyamide layer, therefore, this method was applied to separate the organophosphorus pesticides. This note describes the results obtained.

EXPERIMENTAL

Material—Eleven organophosphorus pesticides, FDA standard were used. The solvents and chemicals are the reagent grade of Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Thin-Layer Plates—All plates used were 15 × 15 cm. of polyamide layer sheet described by Wang (6).

Chromatographic Procedure—The standard techniques of ascending thin-layer chromatography (7) were employed.

Visualization—Four methods were employed: (*a*) iodine vapor exposing, (*b*) 5% alcoholic potassium hydroxide solution spraying, (*c*) iodoplatinate reagent spraying (8), (*d*) first spraying 5% alcoholic potassium hydroxide solution on the plate, allowing the plate to dry, then exposing the plate with iodine vapor for 5 min., followed by exposing ammonia vapor.

Received March 22, 1968, from the Department of Pharmacy, Taipei Medical College, Taipei, China; Department of Chemistry, National Taiwan University, Taipei, China (K. T. Wang); and Taiwan Provincial Health Department, Tai-Chang, Taiwan, China (C. T. Cheng).
Accepted for publication June 11, 1968.
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TABLE I—CHROMATOGRAPHIC DATA

Pesticides	R_f Value Solvent Systems[a] I	II	III	IV	Color of Spot Visualization[b] 1	2	3	4
2-Iso-propoxyphenyl-*N*-methylcarbamate	0.80	0.77	0.70	0.82	+	Y	—	W
Isopropylmethylpyrimidil diethylthiophosphate	0.87	0.75	0.45	0.59	+	Y	—	—
O,O-Diethyl-*S*-2 (ethylthio) ethylphosphorodithioate	0.62	0.25	0.30	0.36	+	Y	—	—
O-Ethyl-*p*-nitrophenyl-thiobenzene phosphate	0.40	0.12	0.10	0.20	+	—	Y	B
O,O-Dimethyl-*S*-4-oxo-1,2,3-benzotriazin-3-(4*H*)-ylmethyl phosphosphorodithioate	0.65	0.48	0.35	0.54	+	Y	—	B
Phthalimido-methyl-*O,O*-dimethyl phosphorodithioate	0.65	0.45	0.85	0.54	+	Y	—	W
Dimethyl-paranitrophenylthiophosphate	0.58	0.38	0.25	0.50	+	—	Y	Y
S-1,2-Bis-(ethoxycarbonyl)-ethyl-*O,O*-dimethylphosphorodithioate	0.77	0.57	0.73	0.68	+	—	—	W
O,O-Dimethyl-(*O*-2,4,5-trichloro-phenyl) phosphorothioate	0.48	0.14	0.11	0.03	+	—	—	—
S-(*p*-Chlorophenythiomethyl)- *O,O*-diethyl phosphorodithioate	0.35	0.09[c]	0.10[c]	0.11[c]	+	Y	—	—
Diethyl-paranitrophenylthiophosphate	0.55	0.28	0.16	0.37	+	—	Y	B

[a] I, isopropanol–water–formic acid (2:3:1); II, formaldehyde–formic acid (10:1); III, water–methanol–ammonia (4:5:2); IV, acetone–water–formamide (1:2:1); Loading, 1.0 mcg. in 0.01 ml. methanol. [b] Y, yellow; W, white; (+) positive action to detection reagent; (—) negative action to detection reagent; B, brown spot on yellowish background. [c] Tailing.

RESULTS AND DISCUSSION

R_f values for the organophosphorus pesticides are in Table I. The detection limit of each compound is approximately 0.1 mcg. with iodine vapor. It is possible to differentiate each compound by using the different response to the detection reagents. For example, 2-iso-propoxyphenyl-*N*-methylcarbamate failed to give a color spot when sprayed with alcoholic potassium hydroxide solution, but it gave a yellow spot with the iodoplatinate reagent. Also, 2-iso-propoxyphenyl-*N*-methylcarbamate was detected by using visualization method number four described above. This method produced a chromatogram characterized by a white spot on a brown-yellow background.

The experiment indicates that this method is very useful for identification of organophosphorus pesticides. It is convenient in handling the chromatograms and is highly reproducible. Another important feature of this polyamide layer chromatogram is a possible direct filing. The development of this method as official to detect pesticides residues in vegetables is currently under investigation.

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Keyphrases

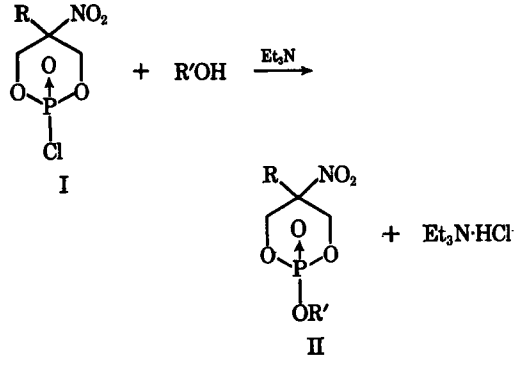
Organophosphorus pesticides—analysis
TLC, polyamide—analysis

1,3,2-Dioxaphosphorinane 2-Oxides I. Preparation of Some 2-Alkoxy, 2-Acyl, and 2-Hydroxy-5-alkyl-5-nitro-1,3,2-dioxaphosphorinane 2-Oxides as Potential Antitumor Agents

By JOHN H. BILLMAN, RALPH F. MAY, and JANE E. HEARD

A series of compounds consisting of 2-alkoxy, 2-acyl, and 2-hydroxy-5-alkyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxides has been prepared. Several amine salts of the 2-hydroxy compounds have been isolated. All of the compounds prepared were submitted for antitumor evaluation.

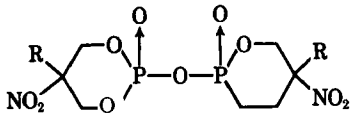
In an earlier publication (1) a report was made from this laboratory concerning the synthesis of phosphorochloridic acids I as possible intermediates leading to some potential antitumor agents.



Scheme I

Recently, several different derivatives of Compound I were prepared in order to determine what type of moiety in the 2 position might lend antitumor activity to the compound.

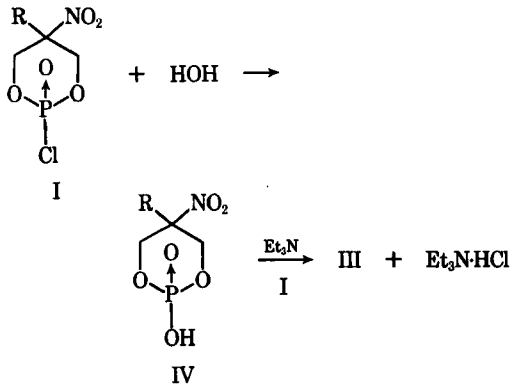
Three 2-alkoxy substituted compounds of Type II were prepared according to Scheme I. Although this synthesis appears to be straightforward, some



III

of the phosphorochloridic acid I failed to react as expected. Instead, a fairly large amount of the pyrophosphate III was produced thereby reducing the yield of the desired ester II.

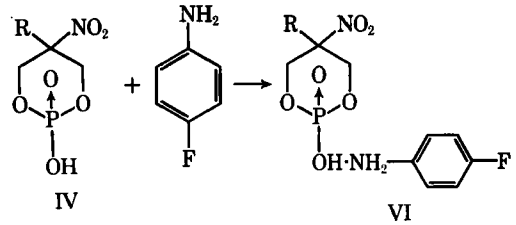
Since the 2-hydroxy Compound IV is readily prepared by refluxing the corresponding chloridic acid I in a 1 : 1 mixture of acetone–water, it was decided that Compound III was the result of traces of moisture in the reaction system. Once formed, Compound IV could then react with the phosphorochloridic acid I as is shown in Scheme II to yield the pyrophosphate III.



Scheme II

In order to prove that Scheme II is a possible pathway to the pyrophosphates, it has now been shown that a phosphorochloridic acid I and a 2-hydroxy compound of Type IV yield the expected anhydride in the presence of triethylamine.

Several amine salts of the 2-hydroxy-5-alkyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxide IV were prepared, not only to improve the hydrolytic properties of the acid but to introduce some amines into the molecule which reportedly have antitumor activity. These salts were prepared as in Scheme III.



Scheme III

Three mixed anhydrides of Type VII were prepared as illustrated in Scheme IV. In view of the fact that these compounds are anhydrides and therefore, should be very active, it is expected that

Received April 8, 1968, from the Department of Chemistry, Indiana University, Bloomington, IN 47401

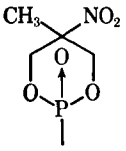
Accepted for publication June 14, 1968.

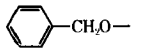
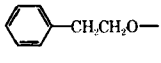
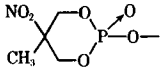
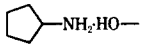
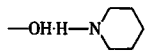
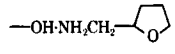
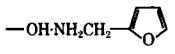
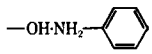
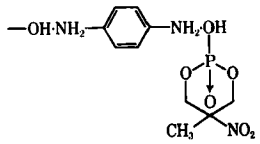
This investigation was supported by a Public Health Service grant (CA-06448-04) from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

This is part of a Ph.D. thesis submitted to the Graduate School, Indiana University, 1967, and part of a M.A. thesis submitted to the Graduate School, Indiana University, 1968.

The authors wish to thank the Commercial Solvent Corporation for samples of the nitroalcohols they furnished for this project.

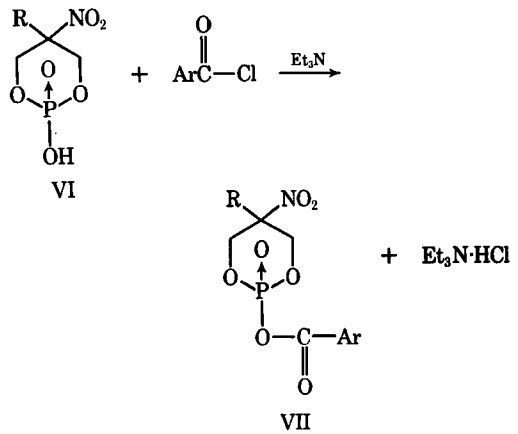
TABLE I—2-ACYL AND 2-HYDROXY-5-METHYL-5-NITRO-1,3,2-DIOXAPHOSPHORINANE 2-OXIDES



Compd. No.	R	Formula	Yield (pure), %	M.p., °C.	% N Calcd.	% N Found	Infrared Assignment, μ	
1	HO—	$C_4H_8NO_6P$	77.6	169–171	7.12	7.13	8.20	9.80
2	CH_3CH_2O—	$C_6H_{12}NO_6P$	86.5	99–100	6.22	6.27	7.82	9.50
3		$C_{11}H_{14}NO_6P$	15.0	152–154	4.89	4.84	7.80	9.62
4		$C_{12}H_{16}NO_6P$	64.5	105–107	4.66	4.65	7.80	9.40
5		$C_8H_{14}N_2O_{10}P_2$	64.5	250–252	7.30	7.44	7.52	10.00
6		$C_9H_{19}N_2O_6P$	99.2	248–250	9.94	9.92	8.12	9.45
7		$C_9H_{19}N_2O_6P$	83.8	204.5–206.5	9.93	9.93	8.2	9.4, 10.05
8		$C_9H_{19}N_2O_7P$	73.3	213–215	9.40	9.30	8.15	9.4, 10.05
9		$C_9H_{15}N_2O_7P$	75.5	204–206	9.52	9.67	8.2	9.3, 10
10		$C_{10}H_{15}N_2O_6P$	68.0	225–227	9.65	9.45	8.3	9.5
11		$C_{14}H_{24}N_4O_{12}P_2$	90.5	243–245	11.16	10.88	8.15	9.3, 9.98

they might act as phosphorylating and/or acylating agents. If this turns out to be the case, such compounds might very well interfere with biological systems involving the chemistry of RNA, DNA, *etc.* Compounds are given in Tables I and II.

Biological Results—To date, no biological data are available on any of the above compounds. All testing is being carried out by the Cancer Chemotherapy National Service Center (CCNSC), Bethesda, Md.



Scheme IV

EXPERIMENTAL[1]

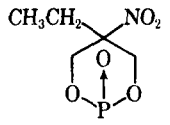
2-Alkoxy-5-alkyl-5-nitro-1,3,2-dioxaphosphorinane 2-Oxide—The synthesis of these compounds is typified by the procedure for 2-benzoxy-5-methyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxide. The benzyl alcohol 2.5 g. (0.0232 mole) and triethylamine 4.7 g. (0.0464 mole) mixture in acetone was added dropwise to a 200-ml., stirring, acetone solution of the phosphorochloridic acid 5 g. (0.0232 mole). The solution was allowed to reflux overnight resulting in a precipitate of amine hydrochloride. The solid was removed, and the acetone was evaporated *in vacuo* to afford an oil. The expected product was extracted from this oil with ethyl acetate and collected as a solid when the solvent was removed under reduced pressure. The material was recrystallized from 2-propanol to give 1 g. (15.0% yield) of pure product (m.p. 152–154°).

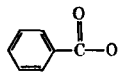
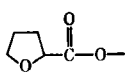
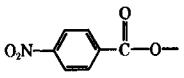
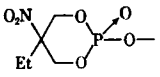
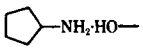
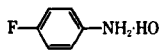
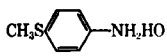
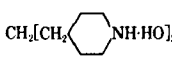
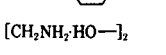
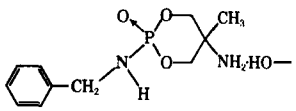
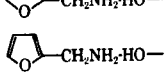
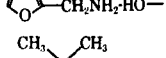
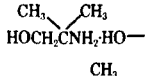
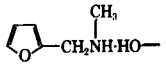
Anal.—Calcd. for $C_{11}H_{14}NO_6P$: N, 4.89. Found: N, 4.84.

2-Hydroxy-5-methyl-5-nitro-1,3,2-dioxaphos-

[1] All melting points were taken on a Thomas-Hoover device and are corrected. The analyses were performed by Midwest Microlab, Inc., Indianapolis, Ind.

TABLE II—2-ALKOXY-5-ALKYL-5-NITRO-1,3,2-DIOXAPHOSPHORINANE 2-OXIDES



Compd. No.	R	Formula	Yield (pure), %	M.p., °C.	% N Calcd.	% N Found	Infrared Assignment, μ	
1	HO—	$C_5H_{10}NO_6P$	63.0	117–119	6.64	6.73	8.30	9.90
2		$C_{12}H_{14}NO_7P$	80.5	134–136	4.45	4.47	8.00	9.80
3		$C_{10}H_{12}NO_8P$	56.8	119–121	4.60	4.63	7.90	9.80
4		$C_{12}H_{13}N_2O_9P$	70.0	214–216	7.78	7.88	8.60	9.60
5		$C_{10}H_{18}N_2O_{11}P$	73.8	255–257	6.95	6.97	7.60	9.25
6	Na^+O^-—	$C_5H_9NNaO_6P$	90.6	300–302	6.01	6.10	7.98	9.22
7		$C_{10}H_{21}N_2O_6P$	98.1	222–224	9.45	9.48	8.15	9.30
8		$C_{11}H_{16}FN_2O_6P$	80.3	222–224	8.75	8.75	8.20	9.32
9		$C_{12}H_{19}N_2O_6PS$	85.5	188–190	8.00	8.01	8.28	9.31
10		$C_{22}H_{46}N_4O_{12}P_2$	87.0	227–229	8.81	8.74	8.20	9.33
11		$C_{12}H_{28}N_4O_{12}P_2$	79.0	257–258	11.62	11.80	8.15	9.25
12		$C_{16}H_{27}N_3O_9P_2$	78.5	206–208	9.00	9.12	8.20	9.30
13		$C_{10}H_{21}N_2O_7P$	69.0	183–185	9.00	8.99	8.12	9.35
14		$C_{10}H_{17}N_2O_7P$	85.0	185–187	9.08	8.91	8.12	9.22
15		$C_9H_{21}N_2O_7P$	85.8	211–213	9.34	9.12	8.00	9.35
16		$C_{11}H_{19}N_2O_7P$	95.6	124–126	8.70	8.59	8.20	9.20

phorinane 2-Oxide—This compound and the 5-ethyl product as well were prepared by a procedure similar to that of Edmundson (2).

Anal.—Calcd. for $C_4H_8NO_6P$: N, 7.12. Found: N, 7.13.

2-Hydroxy-5-methyl-5-nitro-1,3,2-dioxaphosphorinane 2-Oxide, Furfurylamine Salt—The following synthesis is typical of the procedure used for preparing salts. Five grams (0.025 mole) of 2-hydroxy-5-methyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxide was dissolved in 100 ml. of hot acetone. A mixture of 2.42 g. (0.025 mole) of furfurylamine and 10 ml. acetone was added slowly to the hot solution with constant stirring. The salt formed rapidly and was collected by filtration after cooling. Recrystallization in 2-propanol and water gave 5.6 g. (75.5% yield) of white crystalline material, m.p. 204–206°.

Anal.—Calcd. for $C_9H_{15}N_2O_7P$: N, 9.52. Found: N, 9.67.

2-Acyl-5-alkyl-5-nitro-1,3,2-dioxaphosphorinane 2-Oxide—The synthesis of this series of compounds is illustrated by the synthesis of 2-benzoyl-5-ethyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxide. The acetone solutions of the 2-hydroxy-5-ethyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxide 5 g. (0.0237 mole) and the benzoyl chloride 3.5 g. (0.0237 mole) were combined with no noticeable reaction. The triethylamine 4.8 g. (0.0474 mole) was added dropwise with stirring to give an immediate precipitate of

triethylamine hydrochloride. The solid was removed by filtration, and the filtrate was evaporated to dryness *in vacuo* to give a white solid. The material was recrystallized from acetonitrile to give 6 g. (80.5% yield) of pure product (m.p. 134–136°).

Anal.—Calcd. for $C_{12}H_{14}NO_7P$: N, 4.45. Found: N, 4.47.

Bis(5-alkyl-5-nitro-2-oxo-1,3,2-dioxaphosphorinanyl) Oxide—The synthesis of this type of compound is typified by the synthesis of the methyl derivative. The phosphorochloridic acid 20 g. (0.0928 mole) was dissolved in undried acetone, and the triethylamine 9.4 g. (0.0928 mole) was added. Within 10 min. the solid product began to form, and the reaction was complete in 2 hr. The solid was removed by filtration and dried, yielding 11.5 g. (64.5% yield) of pure product (m.p. 250–252°).

Anal.—Calcd. for $C_8H_{14}N_2O_{11}P_2$: N, 7.46. Found: N, 7.44.

REFERENCES

(1) Billman, J. H., Meisenheimer, J. L., and May, R. F., *J. Med. Chem.*, **9**, 772 (1966).
(2) Edmundson, R. S., *Tetrahedron*, **21**, 2379(1965).

Keyphrases

2-Alkoxy-5-alkyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxides—synthesis
2-Acyl-5-alkyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxides—synthesis
2-Hydroxy-5-alkyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxides—synthesis
IR spectrophotometry—structure

New Compounds: Synthesis of Some Phosphorus-Nitrogen Compounds for Pharmacological Study III

By A. ABOU-MOUSTAFA and M. KHALIFA

Phosphorus-nitrogen compounds containing moieties of the so-called long-acting sulfonamides were prepared by reacting the sulfonamides with PCl_3 or $POCl_3$ in a 2:1 ratio. In the case of PCl_3 the condensation took place according to the above ratio with sulfaphenazole, sulfamethoxypyridazine, and sulfamethoxydiazine. With sulfadimethoxine two products were obtained: a derivative of phosphorus triamide and a derivative of diamidophosphorus acid; this corresponds to the condensation of 1 mole of the acid chloride with 3 moles and with 2 moles of the sulfonamide, respectively. In the $POCl_3$ condensations the ratio mentioned earlier was obeyed only with sulfamethoxydiazine while with the rest, 3 moles of the sulfonamide condensed with 1 mole of the oxychloride.

In continuation of the work which has been started in this laboratory on the condensation of a number of sulfa drugs with phosphorus trichloride and phosphorus oxychloride (1, 2), the so-called long-acting sulfonamides were condensed with the same acid chlorides for two reasons: to complete the picture required for the pharmacological study and to compare once more the reactivity of the two acid chlorides in these condensation reactions. This latter objective was dealt with in a previous publication (2) and from a consideration of the reaction time, the yields, and the mode in which the sulfa drug had condensed with the acid chloride, it was concluded that phosphorus oxychloride is more reactive than the trichloride. The results obtained from the present investigation conform with those obtained earlier and confirm the finding that phosphorus oxychloride is more reactive than the trichloride.

With the trichloride the condensation was complete after 24 hr. while with the oxychloride 12 hr. was sufficient. Moreover, higher yields were obtained with the oxychloride (70–88%) than with the trichloride (58–80%). Finally the manner in which the sulfa compound condensed with the acid chloride was significant. In three cases the sulfonamide–oxychloride condensation ratio was 3:1 while in the fourth only two molecules of the sulfonamide condensed with one molecule of the acid chloride. This latter mode was the rule in the phosphorus trichloride condensations except in the case of sulfadimethoxine where a mixture of the tri- and diamide derivatives was obtained with the former predominating.

The superior reactivity of phosphorus oxychloride to that of the trichloride is probably due to the fact that the phosphorus atom in the oxychloride is more electrophilic than that in the trichloride—a property which expectedly would render it more vulnerable to attack by nucleophilic reagents and this is in keeping with what has been reported earlier (3).

That the phosphorus atom in the synthesized compounds is linked to the N^4 of the sulfa drug was shown qualitatively by the failure of all the condensation products to diazotize, their solubility in dilute alkali, and their insolubility in dilute mineral acids.

Received March 5, 1968, from the Pharmaceutical Chemistry Department, Faculty of Pharmacy, Riyad University, Riyad, Saudi Arabia.
Accepted for publication April 17, 1968.
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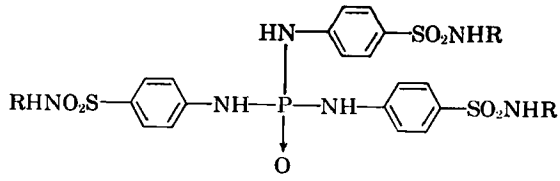
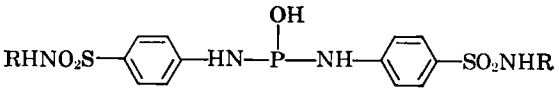
TABLE I—*N*-SUBSTITUTED DERIVATIVES OF PHOSPHORIC TRIAMIDE



Compd.	*R*	% Yield	M.p., °C.[a]	Formula	Anal.,[b] Calcd.	Found
I	6-Methoxy-3-pyridazinyl	76	219–222 265 dec.	$C_{33}H_{33}N_{12}O_{10}PS_3$	N, 18.99	19.49
II	2-Phenyl-3-pyrazolyl	88	170–174 242 dec.	$C_{45}H_{39}N_{12}O_7PS_3$	N, 17.02 C, 54.75 H, 3.98	16.67 54.18 4.06
III	2,4-Dimethoxy-6-pyrimidinyl	70	245–248 270 dec.	$C_{36}H_{39}N_{12}O_{13}PS_3$	N, 17.24	17.85

[a] Liquid crystal. Melting points were performed by the capillary tube method and are uncorrected. [b] Analyses performed by Janssen Pharmaceutica, Beerse, Belgium.

TABLE II—*N*-SUBSTITUTED DERIVATIVES OF PHOSPHORODIAMIDOUS ACID



Compd.	R	% Yield	M.p., °C.[a]	Formula	Anal.,[b] Calcd.	Found
IV	6-Methoxy-3-pyridazinyl	58	187–190 248 dec.	$C_{22}H_{23}N_8O_7PS_2$	N, 18.47	18.42
V	2-Phenyl-3-pyrazolyl	80	178–182 255 dec.	$C_{30}H_{27}N_8O_5PS_2$	N, 16.60	16.49
VI	5-Methoxy-2-pyrimidinyl	67	155–158 232 dec.	$C_{22}H_{23}N_8O_7PS_2$	N, 18.48	18.16

[a] Liquid crystal. Melting points were performed by the capillary tube method and are uncorrected. [b] Analyses performed by Janssen Pharmaceutica, Beerse, Belgium.

EXPERIMENTAL

***N*-Substituted Derivatives of Phosphoric Triamide and *N*-Substituted Derivatives of Phosphorodiamidous Acid**—*General procedure*—Compounds I–VI were prepared by the gradual addition of the acid chloride (0.01 mole) in pyridine–chloroform (20 ml.) to the sulfa compound (0.02 mole) dissolved in the same solvent (120 ml.). Reactions were completed after reflux periods of 12 (I–III) or 24 (IV–VI) hr. Then the solvent was distilled *in vacuo*, the residue suspended in dilute hydrochloric acid, filtered, and washed with water until the washings gave a negative chloride test with silver nitrate T.S. The dried products were crystallized from aqueous ethanol. (See Tables I and II.)

***N,N'*-di[*N*-(5-Methoxy-2-pyrimidinyl)-*p*-sulfamoyl Phenyl] Phosphorodiamidic Acid**—This compound was prepared by condensing sulfamethoxydiazine (8.5 g.) dissolved in pyridine–chloroform (160 ml.) with phosphorus oxychloride (2.33 g.) dissolved in 40 ml. of the same solvent. The reaction mixture was refluxed for 12 hr., the solvent distilled *in vacuo*, and the residue worked up in the usual manner. The product which was obtained in 76% yield melted at 225–227°, 260° dec. (liquid crystal) after being crystallized from aqueous ethanol.

Anal.—Calcd. for $C_{22}H_{23}N_8O_8PS_2$: N, 17.94. Found: N, 17.97.

***N,N',N''*-tri[*N*-(2,4-Dimethoxy-6-pyrimidinyl)-*p*-sulfamoyl Phenyl] Phosphorus Triamide and *N, N'*-Di [*N*-(2,4-Dimethoxy-6-pyrimidinyl)-*p*-sulfamoyl Phenyl] Phosphorodiamidous Acid**—These were prepared by condensing sulfadimethoxine (8.8 g.) dissolved in pyridine-chloroform (150 ml.) with phosphorus trichloride (1.94 g.) dissolved in 30 ml. of the same solvent. The reaction mixture was refluxed for 24 hr., then the solvent was distilled *in vacuo* and the residue was worked up as usual. Crystallization of the product thus obtained from (*ca.* 300 ml.) 70% aqueous ethanol yielded (5.85 g.) of a compound which on recrystallization from the same solvent melted at 290° dec. Concentration of the mother liquor afforded (1.87 g.) of a second compound which melted at 190–193°, 245–250° dec. (liquid crystal) after being recrystallized from 50% aqueous ethanol.

Former compound: *Anal.*—Calcd. for $C_{36}H_{39}N_{12}O_{12}PS_3$: N, 17.51. Found: N, 18.16.

Latter compound: *Anal.*—Calcd. for $C_{24}H_{27}N_8O_9PS_2$: N, 16.86. Found: N, 17.14.

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(1) Abou-Moustafa, A., Khalifa, M., and El Mangowri, H., *J. Pharm. Sci.*, **56**, 1357(1967).
(2) Abou-Moustafa, A., and Khalifa, M., *ibid.*, **57**, 532 (1968).
(3) Hudson, R. F., "Structure and Mechanism in Organophosphorus Chemistry," Academic Press, Inc., New York, N. Y., 1965, p. 90.

Keyphrases

Phosphorus-nitrogen compounds—synthesis
Sulfonamides-phosphorus tri- and oxychloride—condensation

Novel Method for Studying Protein Binding

Sir:

The binding of drugs to proteins has been the subject of numerous investigations and many experimental techniques have been described for determining the fraction of total drug concentration which is unbound and/or bound in a protein-small molecule system (1, 2). Equilibrium dialysis and ultrafiltration are widely used methods and have general applicability in protein binding studies. Both methods are, however, somewhat tedious, require a considerable number of individual measurements to define binding behavior, and are rather inconvenient to use over a wide range of temperatures. In addition, equilibrium dialytic studies require a rather long equilibration time and the ultrafiltration approach can suffer from the approximations necessary to assess the changing protein concentration during the course of filtration.

We wish to report details of a rather simple method for studying the protein binding of small molecules which is based on the determination of the rate of dialysis of a small molecule from a protein-containing compartment. The method differs from other dynamic dialytic approaches suggested, for example, by Stein (3) and Agren and Elofsson (4) in that the time course of disappearance of small molecule from a protein compartment is followed for extended periods of time. In our system, the relative concentrations of bound and unbound small molecule change continuously and, as a result, data which are obtained from a single kinetic run permit characterization of binding behavior over a wide range of small molecule concentrations. The method is based on the fact that nondiffusible protein-small molecule complexes are reversibly formed in the protein compartment and that the rate of loss of small molecule from that compartment is directly proportional to the concentration of unbound small molecule, provided that care is taken to insure that sink conditions are maintained for the diffusing species, *i.e.* that back diffusion into the protein compartment is insignificant.

The experimental system consisted of a jacketed beaker (internal height = 9 cm., internal diameter = 6.5 cm.) in combination with a constant-temperature water bath and circulator which provided excellent temperature stability. A large three-hole rubber stopper was obtained to fit the beaker. One hole accommodated a short piece of glass tubing (i.d. = 1 cm.). Two-hundred milliliters of buffer solution was placed in the beaker. Hydrated cellophane dialysis tubing [width = 2.47 cm. (0.984 in.)] was knotted at one end to form a bag (length = 7 cm.) and was attached with a rubber band to the glass tubing of the stopper. Seven milliliters of small molecule or small molecule-protein solution was placed in the bag. The stopper with the attached bag was fitted on the beaker. The solution external to the bag was stirred with a magnetic stirrer. Care was taken to insure that there was sufficient clearance between the bag and the bottom of the beaker to allow free motion of the stirring bar. The solution within the bag was stirred with a twisted glass rod which passed through the glass tubing and was connected to a variable speed stirring motor. All experiments were conducted at constant temperature, pH, liquid volume, bag size, and stirring rate. One-hundred milliliters of the solution external to the bag was periodically removed through one hole of the stopper and immediately replaced with 100 ml. of fresh buffer which was introduced through the other hole. In this manner the concentration of small molecule in the external solution was always maintained at a level sufficiently lower than that in the protein compartment to approximate sink conditions. The concentration of small molecule in the removed sample was determined spectrophotometrically and the concentration of small molecule in the protein compartment was calculated from a knowledge of the initial concentration and the total amount of small molecule which had appeared in the external solution.

Typical results are shown in the form of semi-log plots in Fig. 1 and depict experiments in which phenol red was employed as the small molecule and bovine serum albumin (BSA) was the macromolecule. In the absence of protein, the dialytic behavior obeyed the expected rate law:

$$\frac{-d(Dt)}{dT} = K(Df) \qquad \text{(Eq. 1)}$$

where $[-d(Dt)/dT]$ = rate of loss of small molecule from the dialysis sac. K = first-order rate constant which characterizes the diffusion

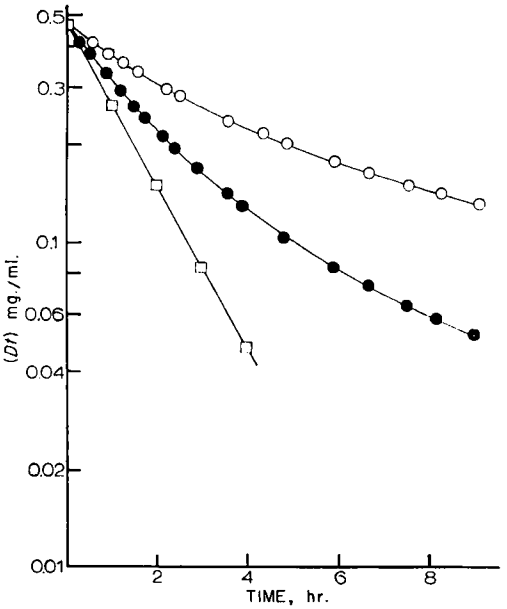

Fig. 1—The loss of phenol red from inside a dialysis bag, in the presence and absence of BSA. The studies were run at pH 7.3 and 25°. Key: ○, 1.442% BSA; ●, 0.618% BSA; □, 0% BSA.

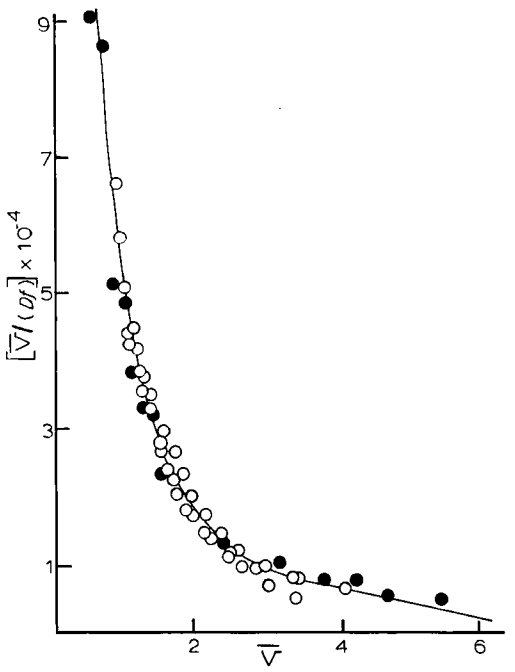

Fig. 2—The binding of phenol red by BSA at pH 7.3 and 25°. Key: ●, ultrafiltration data; ○, kinetic data.

process and which incorporates the area and thickness of the membrane. (Df) = concentration of unbound small molecule in the dialysis sac.

In the presence of protein, it is seen that the semilog plots were not linear but exhibited marked curvature. The curvature reflects the fact that as the concentration of small molecule in the protein compartment decreased, the fraction of small molecule which was bound increased. The concentration of unbound small molecule (Df) in the protein compartment at any total drug concentration (Dt) can be calculated from Eq. 1 with a knowledge of K and the instantaneous rate $[-d(Dt)/dT]$ at that particular (Dt). The value for K was obtained from the slope of the semilog plot of (Dt) *versus* time in the absence of macromolecule. The instantaneous rate at a value of (Dt) in the presence of macromolecule can be estimated graphically from a plot of (Dt) *versus* time. We, however, found it more convenient and accurate to fit data from plots of (Dt) *versus* time to an empirical equation with the aid of a digital computer (a six-parameter triexponential equation has been found to yield excellent fits with all systems studied thus far). The differential of the empirical equation was then calculated to obtain instantaneous rates at various values of (Dt) from which (Df) values were obtained. These two values permitted computation of the concentration of bound small molecule. The binding data obtained in this manner were used to construct the Scatchard plot of Fig. 2 where $(\bar{v}/Df)$ *versus* $\bar{v}$ was plotted ($\bar{v}$ = moles of bound small molecule per mole of protein). The open circles represent data points obtained from the dynamic dialysis experiments and the closed circles those obtained from ultrafiltration studies.

Table I—Summary of Binding Constants

Method	n_1[a]	n_2	k_1[b]	k_2
		Phenol Red		
Experimental				
Ultrafiltration and kinetic	1	6	1.74×10^5	1.97×10^3
Literature (5)				
Ultrafiltration	1	6	1.1×10^5	1.2×10^3
		Methyl Orange		
Experimental				
Kinetic	22	—	4.8×10^4	—
Literature (6-7)				
Equilibrium dialysis	22	—	4.4×10^4	—

[a] n_i = number of binding sites in the i'th class; [b] k_i = association constant for the i'th class.

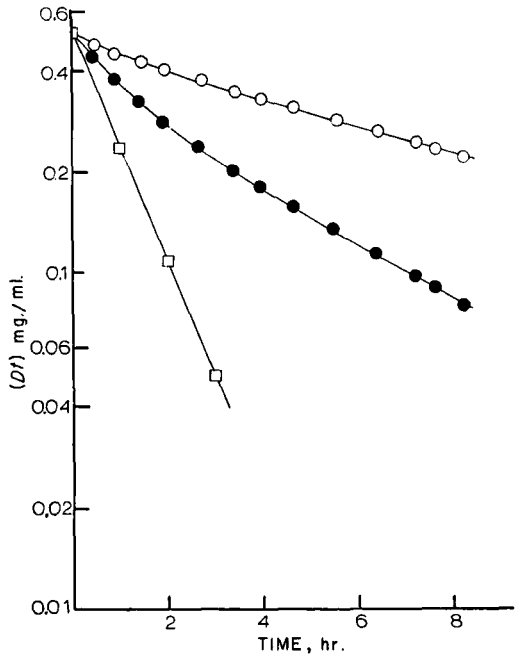

Fig. 3—The loss of methyl orange from inside a dialysis bag, in the presence and absence of BSA. The studies were run at pH 7.3 and 25°. Key: ○, 1.235% BSA; ●, 0.620% BSA; □, 0% BSA.

There is close agreement between data obtained by the two different techniques. The Scatchard plot was mathematically analyzed in terms of the fundamental binding parameters (n = number of sites in a class, k = intrinsic association constant characterizing the binding to a site) and was found to be consistent with an interaction involving two classes of sites on the protein molecule. Values for n and k are given in Table I where literature values are also shown. Figure 3 shows the dialytic behavior of methyl orange in the absence and presence of BSA. These data were analyzed in a similar manner. The binding parameters are shown in Table I and are seen to be in excellent agreement with values previously reported.

These preliminary results show the promise of this approach for studying protein binding. The relative ease and rapidity of the method, the minimum amount of sample preparation required, the convenient means of temperature control, and the economical utilization of protein are definite advantages of this approach. These factors become especially important when the supply of protein is limited or when a variety of experimental conditions such as pH or temperature are to be studied.

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Dialysis—protein binding determination
Diffusion—small molecules from protein containing compartment

Apparent Discrepancy Between Theory and Experimental Data for Dissolution from the Rotating Disk Under Stirred and Unstirred Conditions

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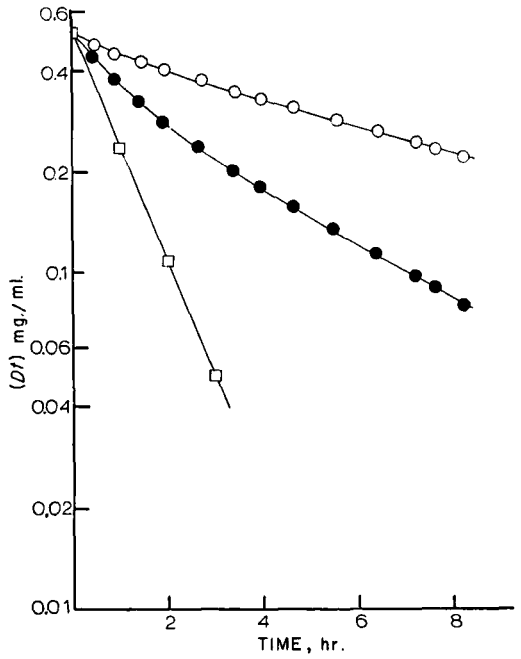

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We believe that the excellent fit of their data to the diffusion layer model was intrinsic to the method used to calculate theoretical values for the mean micellar diffusion coefficient. It can be shown that the choice of models was arbitrary and that the diffusion layer model can be fitted equally well to static dissolution data. Figure 1 shows the result of using their approach to calculate a mean micellar diffusion coefficient (5.86×10^{-6} cm.^{2}/sec.) from their static rate data and assuming that the diffusion layer model is applicable. Equation 1 was used for this calculation along with their solubility data, and diffusion coefficient in water (1.2×10^{-5} cm.^{2}/sec.).

$$R = \frac{DC_s + D'C_s'}{DC_s} \qquad \text{(Eq. 1)}$$

D' is the diffusion coefficient of the micelle solubilized drug that must be calculated from the chosen model, and C_s' is the solubility increase due to micellar solubilization. D is the aqueous diffusion coefficient of the drug and C_s is the aqueous solubility of the drug. R is the ratio of the dissolution rate in surfactant solution to that in water. As expected, the diffusion layer model fits the static conditions, while the stirred data do not correspond to any of the represented models.

It has been shown (3), however, that the rotating disk dissolution rate follows the Levich equation and hence should be applied to the stirred rate data. Using their same approach, the Levich equation was used to calculate a mean micellar diffusion coefficient (0.828×10^{-6} cm.^{2}/sec.) from their stirred data. The Levich equation can be represented by the following expression:

$$R = \frac{D_{\text{eff.}}^{2/3}C_t}{D^{2/3}C_s} = \frac{(D'C_s' + DC_s)^{2/3}\,(C_s + C_s')^{1/3}}{D^{2/3}C_s} \qquad \text{(Eq. 2)}$$

In this equation C_t is the total solubility of drug, which is $C_s + C_s'$, and $D_{\text{eff.}}$ is the effective diffusion coefficient which can be written as

$$D_{\text{eff.}} = \frac{DC_s + D'C_s'}{C_s + C_s'} \qquad \text{(Eq. 3)}$$

Using the calculated mean micellar diffusion coefficient, theoretical relative dissolution rates were again determined for the Danckwerts', rotating disk, and diffusion layer models. These theoretical plots are shown in Fig. 2 and shows that the Danckwerts' theory appears to fit the static data, while the rotating disk theory fits the stirred data, as expected. The diffusion layer theory predicts considerably lower relative rates than the experimental values.

An alternate approach was also utilized, whereby the Danckwerts' model was fitted to the static rate data in order to calculate another mean micellar diffusion coefficient (1.27×10^{-6} cm./sec.). The Danckwerts' model can be given as

$$R = \frac{D_{\text{eff.}}^{1/2}C_t}{D^{1/2}C_s} = \frac{(D'C_s' + DC_s)^{1/2}\,(C_s' + C_s)^{1/2}}{D^{1/2}C_s} \qquad \text{(Eq. 4)}$$

The theoretical plots for each model can be seen in Fig. 3 and yielded essentially the same agree-

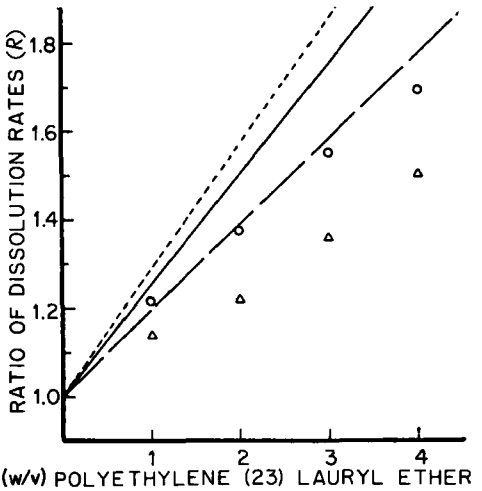


Fig. 1—Comparison of theoretical and experimental dissolution rate ratios assuming $D' = 5.86 \times 10^{-6}$ *cm.^{2}/sec., calculated from Eq. 1. Key: static, ○; 100 r.p.m., △; theoretical ratios predicted by Danckwerts' theory, ---; rotating disk theory, ——; diffusion layer theory, – – –.*

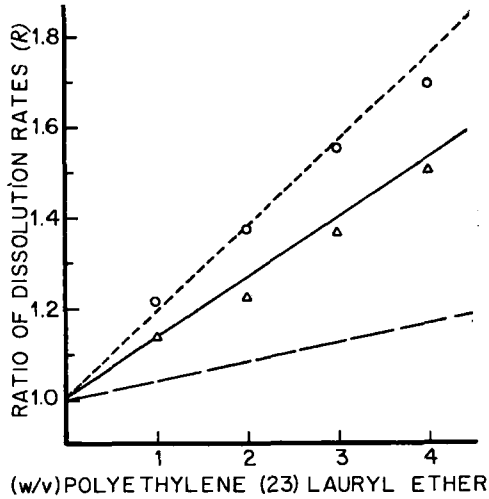
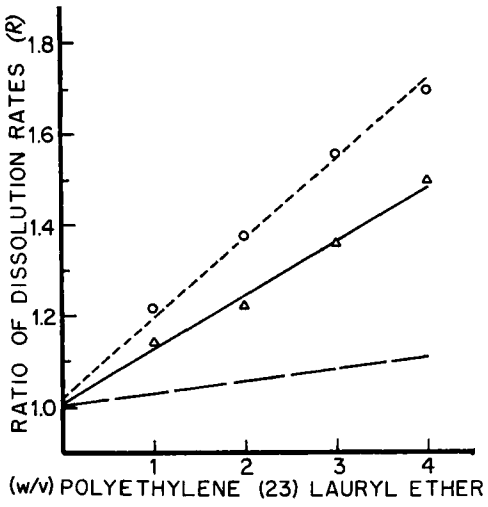


Fig. 2—Comparison of theoretical and experimental dissolution rate ratios assuming $D' = 0.828 \times 10^{-6}$ *cm.^{2}/sec., calculated from Eq. 2. Key: static, ○; 100 r.p.m., △; theoretical ratios predicted by Danckwerts' theory, ---; rotating disk theory, ——; diffusion layer theory, – – –.*



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ment between theory and experimental data as Fig. 2.

Despite this good agreement, one must be cautious when using this approach because of the assumption that the micellar diffusion coefficient remains constant with increasing surfactant concentration. Changes in viscosity, micelle interactions, micelle type, *etc.*, could significantly alter the micelle diffusion coefficient as surfactant concentration is changed. For these reasons, we feel that the approach utilized in our study should be used to clearly elucidate the specific dissolution mechanism operative under a given set of conditions, *i.e.*, the correlation of the relative dissolution rates with the corresponding effective diffusion coefficients, which are independently determined.

It also should be noted that the hydrodynamics associated with a rotating disk is such that the diffusion layer model *per se* is not applicable and the the Levich equation must be used. The rotating disk model represents one of the few instances of an exact mathematical solution to a classical hydrodynamic problem, while the diffusion layer model assumes a uniform one-dimensional diffusion layer. Therefore, this diffusion layer model cannot be applied to the diffusional flux from a rotating disk, which is influenced by both a centrifugal force and a concentration gradient. In view of this and the reasonable agreement of the Danckwerts' prediction to their static data, this analysis confirms our conclusions. Since they used a completely different drug-surfactant system from that used in our study, it appears that our conclusions are generally applicable to micellar transport systems.

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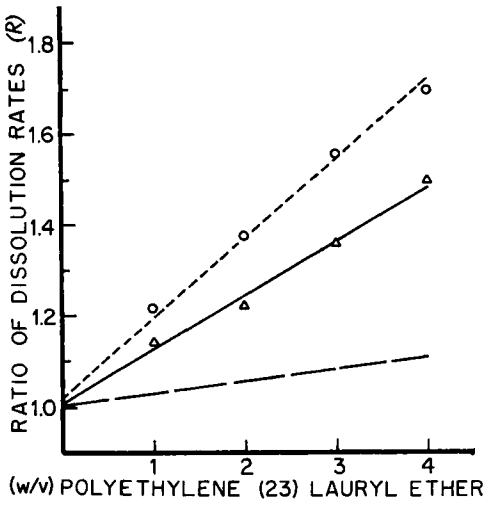

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A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Council

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JANUARY TO DECEMBER, 1968

JOURNAL OF Pharmaceutical Sciences

October 1968 volume 57, number 10

Review Article

Biosynthesis of the Tetracycline Antibiotics

By L. A. MITSCHER

INVESTIGATION of the metabolic products of biological systems, accelerated in recent years by the discovery and application of chromatographic and spectroscopic methods, has resulted in the structural elucidation of an enormous number of natural substances. The endeavor to bring classifying order into this accumulation of data has resulted over the years in various unifying structural theories (1–5). These have often been put forth with the stated or implied assumption that the structures of the molecules reflect the underlying biological processes by which they are formed. Classic examples of such systems would include the isoprene rule, the polyketide theory, and the biogenesis of alkaloids from amino acids. Among the most exciting discoveries of recent years has been the finding of experimental evidence indicating that many of these systems do indeed have a real biological basis. As a result we now recognize great families of natural products derived from the ubiquitous primary metabolites: acetate-malonate, shikimate-prephenate, and acetate-mevalonate. An accelerating effort in recent years has resulted in the gathering of numerous examples of members of each of these great classes, along with a number of examples of natural products containing structural features derived from more than one class of precursor. Even while this great unifying effort has gone forth, examples have begun to appear of studies involving the next logical phase of study, elucidation of the detailed biosynthetic pathways linking the primary metabolites with their derived natural products. The morphine alkaloids (6, 7), the steroids (8–10), and the tetracycline antibiotics (11–14) constitute the systems best studied to date. These systems are of outstanding medical and scientific importance and, interestingly, each belongs to a different precursor-product class and they may well serve as prototypes for the many studies to come. It is the purpose of this review to describe the present state of our knowledge of the biosynthesis of the tetracycline antibiotics and their relationship to the mainstream of acetate–malonate-derived microbial products. A number of practical applications has been accomplished in this area making this study especially instructive for those interested in the preparation of new antibiotics.

The tetracyclines are highly oxygenated hydronaphthacene derivatives produced by fermentation of various *Streptomyces* species, by structural modification of fermentation tetracyclines, and, in certain cases, by total synthesis. The formulas of those tetracyclines which are produced wholly or primarily by fermentative processes are given in Table I, which also contains the numbering and lettering system used for these substances.

Many of the compounds that comprise the domain of the natural products chemist are classified as secondary metabolites. A secondary metabolite may be defined as a substance with no presently apparent utility (such as structure, energy, reproduction, growth, *etc.*) to the producing organism and which is elaborated, usually, when the primary growth and replication

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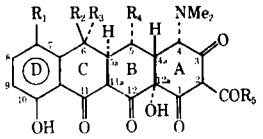
TABLE I—TETRACYCLINES PRODUCED BY FERMENTATION



No.	Compd.	R_1	R_2	R_3	R_4	R_5
I	Chlortetracycline	Cl	OH	Me	H	NH_2
II	Oxytetracycline	H	OH	Me	OH	NH_2
III	Tetracycline	H	OH	Me	H	NH_2
IV	6-Demethylchlortetracycline	Cl	OH	H	H	NH_2
V	Oxychlortetracycline	Cl	OH	Me	OH	NH_2
VI	2-Acetyldecarboxamidooxytetracycline	H	OH	Me	OH	CH_3
VII	2-Acetyldecarboxamidotetracycline	H	OH	Me	H	CH_3
VIII	2-Acetyldecarboxamidochlortetracycline	Cl	OH	Me	H	CH_3

phase of colony or plant development has ceased. These substances are often produced in relatively large quantites, are frequently of complex structure, and occasionally have useful physiological properties in human and veterinary practice. The tetracycline antibiotics are excellent examples of such metabolites.

An excellent review of the chemistry and general history of the tetracyclines up to about 1962 has been published (15), and the synthesis of tetracycline analogs has been reviewed (16). A number of reviews of the biosynthesis of the tetracyclines from various viewpoints has also appeared in recent years (11–14).

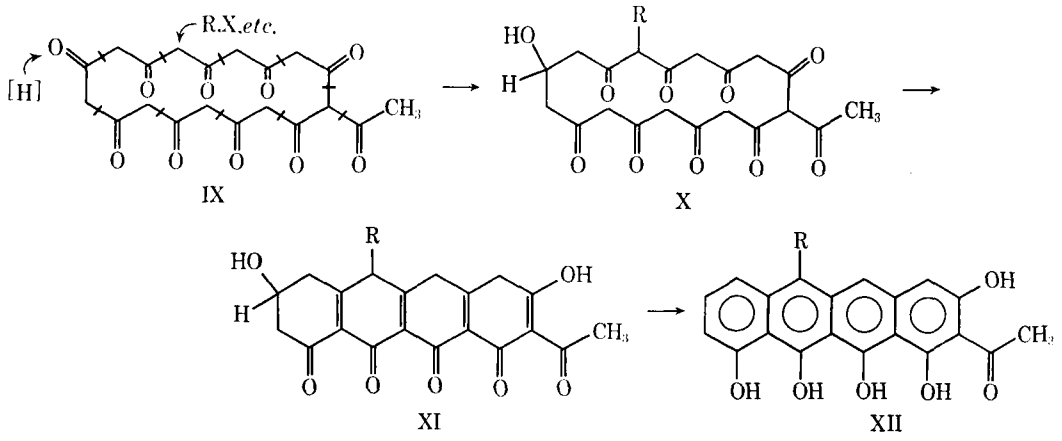
FUNDAMENTAL UNITS

Before any experimental evidence was available, Birch (17), Robinson (3), and Woodward (18) predicted on theoretical grounds that the carbon skeleton of the tetracyclines is constructed from "acetate" units linked together head-to-tail. At about the same time Snell (19, 20) was able to show that sodium acetate-2-^{14}C was incorporated into oxytetracycline (II) to the extent of 5–10%. The same result was obtained independently by Miller *et al.* (21). Birch, Snell, and Thompson (22, 23) and Gatenbeck (24) subsequently were able to show by degradation studies that the carbon atoms were indeed derived from acetate carboxyl and methyl in an alternating sequence although, because of experimental difficulties, complete degradation of the tetracyclines to isolate every carbon atom for absolutely rigorous proof has still not been accomplished. The C- and N-methyl groups are derived from methionine (21, 22, 25) as expected, and the nitrogen atom at C_4 likely comes from glutamic acid. Certain tetracyclines (I, IV, V, VIII) have a chlorine atom at C_7. In chlortetracycline (I) biosynthesis, this atom comes from chloride ion in the medium and is not a necessary feature for overall tetracycline formation as the final concentration of tetracyclines produced by a given mutant appears to be independent of the chloride ion concentration in the medium (27). When care is taken to exclude chloride ion from the medium, normally halogenating strains produce unhalogenated tetracyclines in undiminished quantity. Some strains will accept bromide ion as a substitute and thus produce bromotetracycline ($I, R_1 = Br$) (26–29). This substance is quite active biologically though it has not found clinical application. A number of compounds, some of which are more readily recognized as antithyroid drugs, inhibit chlorination (30, 31).

POLYKETIDE THEORY AND TETRACYCLINE BIOSYNTHESIS

The manner in which these fundamental building blocks are assembled can be predicted using the general tenets of the polyketide theory as elaborated in a masterful series of experiments by Birch and his school (32–34).

According to this theory the majority of the phenolic secondary metabolites produced by microorganisms (particularly the fungi) can be considered to be polymers derived from various metabolic acids, with "acetate" forming the most common component. For simplicity of exposition the basic tenets of polyketide theory can be illustrated through the hypothetical tetracene derivative (XII) which can be supposed to have been formed from 10 "acetate-equivalents" linked head-to-tail in a regular manner to form hypothetical intermediate IX, which is called a polyketide because of the alternation of carbonyl and methylene groups in a large ring. This substance can be likened to a zipper which is ready to close. The carbon skeleton of the aromatic rings is formed by a series of transannular carbonyl-methylene condensations followed by enolizations. A large open-ring system such as X is capable of assuming many shapes by simple folding. The shape assumed during cyclization will determine the aromatic ring system formed. Control of the specificity of the process is considered to be one of the functions of the enzymes involved. The majority of those oxygens not involved in ring closures are retained in the product as, indeed, it was this circumstance that



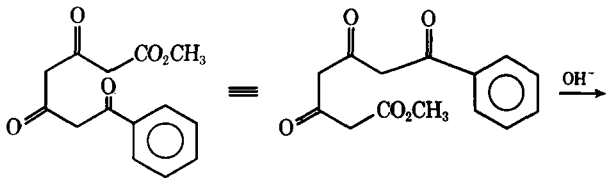
Scheme I

led Birch to propose polyketide involvement in natural processes in the first place. A number of secondary reactions are also frequently encountered. The different oxygenation patterns of the two terminal rings in XII are particularly illustrative of one of the proposed ways in which the basic oxygenation pattern can be altered. Direct removal of a phenolic hydroxyl group as such, in the laboratory, is a high-energy process. A process involving much lower energy requirements and with ample precedent in fatty acid biosynthesis is simple reduction to a β-hydroxyketone followed by dehydration. One presumes a biological parallel. These reactions presumably occur before cyclization to hypothetical XII and are illustrated by formulas X and XI (Scheme I). It is interesting to note that oxygen atoms are frequently "missing" in analogous positions from a variety of polyketide-derived aromatic products and it is tempting to suggest that this oxygen has no essential function in the cyclization or activation process as it has been formulated. This observation has recently been emphasized in the elaboration of an ingenious theory which attempts to explain the steric control of cyclization on the basis of chelation with metal ions on an enzyme surface. Examination of this hypothesis is highly recommended to those with a special interest in this topic (35). Additional functions, such as halogens, alkyl groups, isoprene units, extra oxygen functions, *etc.*, can readily be visualized as being introduced into the activated methylene groups of the polyketides by electrophilic-type reactions. In fact, in microbial products thought to be elaborated from polyketides, such "extra" functions are almost invariably found on methyl-derived carbons—almost never occurring on carboxyl-derived carbons. It must be emphasized, however, that these "extra" functions may also be introduced after the fully aromatic stage is achieved. This will be clearly apparent later when the sequential aspects of tetracycline biosynthesis are discussed. These few general considerations, as simple as they are, are sufficient to rationalize the formation of the vast majority of polyketide-derived substances. The enormous utility of polyketide theory, not only in rationalizing biogenesis but also in predicting structures when a series of otherwise equivalent expressions is available, has led to its virtually general acceptance despite the fact that no one has yet succeeded in isolating a polyketide as such from either chemical or biological sources. Some interesting beginnings have been made in mimicking these processes by syntheses of poly-β-carbonyl derivatives and examining their rearrangement products (36–41) and some suggestive structures have been isolated from unnatural, or blocked, biological systems (42–48).

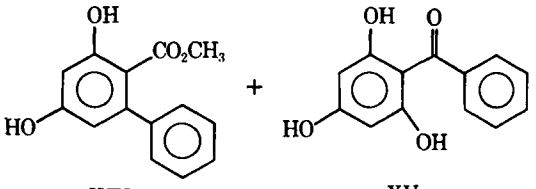
For example, synthetic triketoester (XIII) undergoes cyclization in alkaline solution to give a β-resorcylic ester (XIV) and/or an acylphloroglucinol (XV) (39) (Scheme II). The product composition varied widely depending upon the reaction conditions. These products are precisely those predicted by the original polyketide theory (17) and naturally occurring examples of both types of products are numerous (the phenyl substituent, however, is not usual).

Synthetic pyrones (XVI) behave in a similar manner and presumably do so by the intermediacy of poly-β-ketones (XVII) (36). The product in this case (XVIII) has been isolated from natural sources as well (Scheme III).

When tropolone (XIX) biosynthesis is interrupted by the use of enzymatic blocking agents, "tetraacetic lactone" (XXI) can be isolated (48) (Scheme IV). This material clearly looks like a rejected or incomplete polyketide (48).

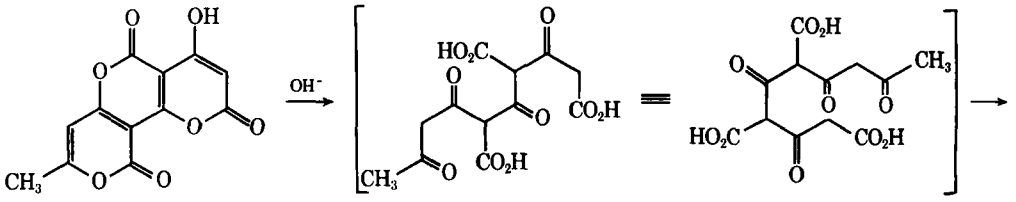


XIII

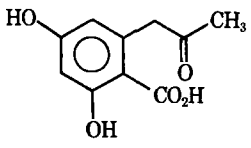


XIV XV

Scheme II



XVI XVII

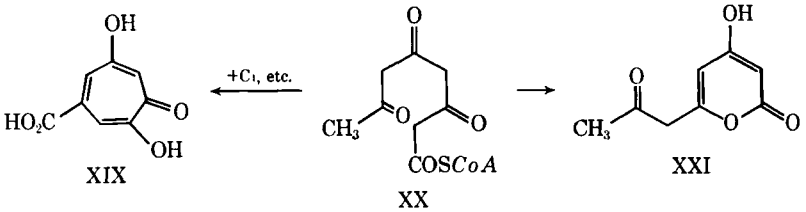


XVIII

Scheme III

Many other examples could be quoted (36–48) but these are sufficient to illustrate the point that materials resembling polyketides can be isolated from natural systems and that these materials have the general properties required of them by polyketide theory. Ultimate success in this difficult area will undoubtedly depend on development of suitable means of stabilizing the highly reactive polyketides once they are freed from their enzymatic supports.

This exposition is, however, like most appealingly simple approximations, now known to be an understatement. Polyketide formation may be viewed in a practical sense as an aberration in fatty acid biosynthesis in which most of the same apparatus is utilized but to a different end. The growing chain is not reduced, dehydrated, and reduced, successively, as in typical fatty acid biosynthesis. The polyketocarbonyl structure persists in the growing chain. The polyene macrolides would appear on this basis to be intermediate forms biosynthetically between the fatty acids and the aromatic metabolites. It has now become apparent that acetyl coenzyme A and malonyl coezyme A are the main fatty acid and polyketide precursors (thus the term "acetate-



XIX XX XXI

Scheme IV

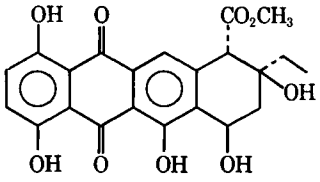
$$CH_3CO{-}S\mathit{CoA} + CO_2 + ATP \rightarrow HO_2C{-}CH_2COS\mathit{CoA} + ADP + Pi \quad (a)$$

$$H{-}O{-}\overset{\overset{O}{\|}}{C}{-}CH_2{-}CO{-}S\mathit{CoA} \rightarrow H^+ + CO_2 + \bar{C}H_2{-}CO{-}S{-}\mathit{CoA} \quad (b)$$

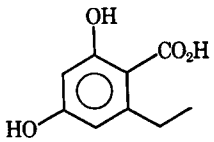
$$\underset{\text{primer}}{CH_3CO{-}S\mathit{CoA}} + \underset{\text{extender}}{\bar{C}H_2CO{-}S\mathit{CoA}} \rightarrow CH_3{-}CO{-}CH_2{-}CO{-}S\mathit{CoA} \quad (c)$$

Scheme V

equivalent" used in early work is amply justified!) (24, 49–51). In polymer terms acetyl *CoA* is thought to serve as the primer with the thiol ester grouping being easily displaced (a good



XXII



XXIII

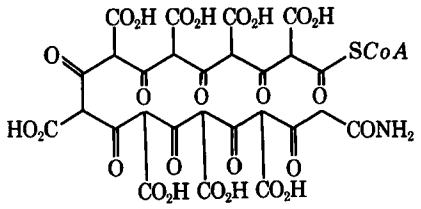
"leaving group") facilitating coupling through nucleophilic reaction. Carboxylation of acetyl *CoA* to form malonyl *CoA* provides the extender, facilitating the reaction by increasing the ease of development of a negative charge on the acetate

It must be emphasized that these expressions are expository and do not exclude the possibility that the sequence involved is concerted and, therefore, that the carbon dioxide may be lost subsequent to or during the coupling rather than, as illustrated above, before. As the role of carbon dioxide is essentially catalytic, the involvement of malonate was missed in early work and the fairly recent appreciation of its central role in these reactions is of outstanding importance. Reduction of the growing chain would lead to fatty acid biosynthesis, whereas persistance would lead to aromatic products. This suggests that one factor controlling the amount of metabolism down these two branches may be the amount of reducing capacity (NADPH) available to the organism at the time.

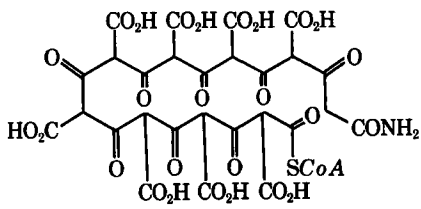
A number of other common metabolic acids, particularly propionate [either naturally as in the case of ϵ-pyrromycinone (XXII) (52) or by forcing as with homoorsellenic acid (XXIII) (53)], are known to serve as primers, and chain extension can occur *via* other acids, such as methylmalonate, as is the case in most nonpolyene macrolide antibiotics (54).

TETRACYCLINES AS POLYKETIDES

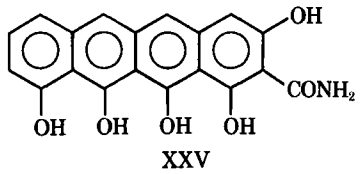
In the case of the tetracyclines specifically, it is now generally, though not entirely without reservation (14, 55), accepted that malonamyl *CoA* serves as the primer and that extension occurs *via* eight malonate units so that more sophisticated versions of polyketide IX can be put forth X(XIV and XXVI) (Scheme VI). As there is no



XXIV



XXVI



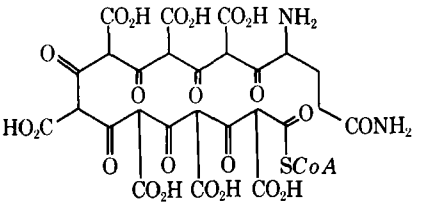
XXV

Scheme VI

methyl carbon. These ideas are illustrated in Scheme V, *a*, *b*, and *c*.

convenient terminal methyl group to act as a marker, the exact folding is not obvious and both

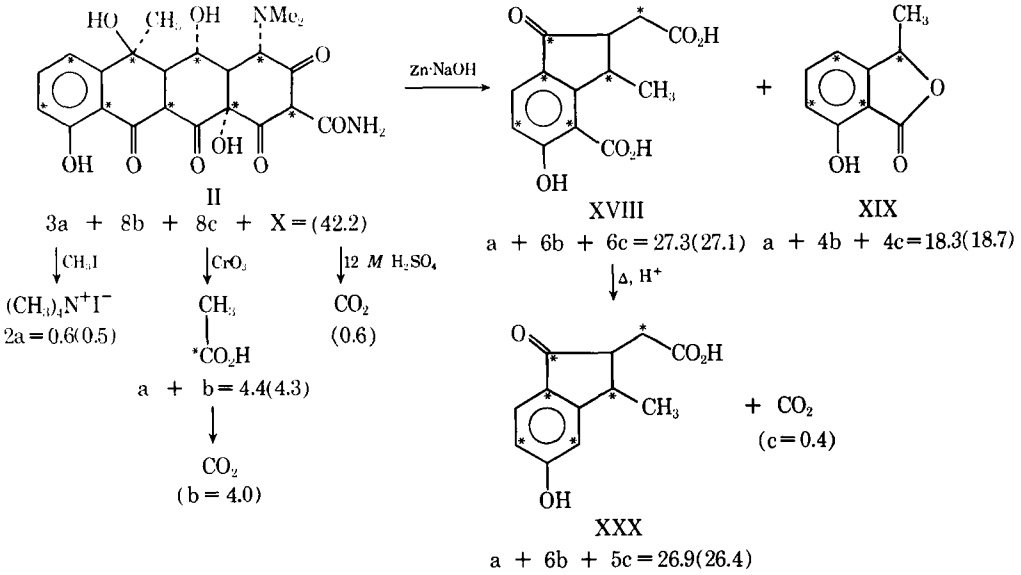
XXIV and XXVI equally satisfy the available evidence. There is no direct evidence for the involvement of malonamyl *CoA*. An interesting theory which has not been entirely abandoned (14) suggested that the majority of Ring A of oxytetracycline was derived from a glutamine unit. This ingenious idea would require the involvement of hypothetical polyketide XXVII, and was advanced prior to the recognition of the role of malonate in polyketide biosynthesis (22, 23). Having noticed that the amount of



XXVII

radioactivity incorporated into Ring A was often lower than anticipated when radioactive acetate was fed, an alternate primer unit was sought. It was found that glutamic acid-2-^{14}C did lead to oxytetracycline with most of the radioactivity in Ring A. Unfortunately Ring A is very difficult to degrade cleanly and in good yield to well-defined products, so that most of the work on the primer unit still depends on obtaining the extent of labeling by difference and the definitive experiment involving intact incorporation of a doubly labeled starter has yet to be reported.

After observing that the extent of labeling of the carboxamide carbon was higher than seemed likely from metabolic scrambling of acetate, an effect which Snell and Birch themselves questioned (23), Gatenbeck obtained results (24) which suggest strongly that malonate, thought to be in the form of malonamyl *CoA*, is the starter unit and that the higher than expected labeling of the carboxamide function resulted from metabolic oxidation of acetate to carbon dioxide followed by fixation into malonate *via* the tricarboxylic acid cycle. The earlier result with glutamate could be explained in the same way—that is, that glutamate acts as a metabolic source of carbon dioxide. The timing of the fixation and the time of incorporation of the starting unit could explain the variable radioactivity in the primer unit. It will be noted from inspection of formulas XXIV and XXVI that all of the carbon dioxide molecules fixed into the hypothetical polyketide are subsequently lost *with the single exception of the one in the starter unit.* Thus this theory predicts a specific enrichment of Ring A by carbon dioxide, an effect Gatenbeck was able to achieve using radioactive sodium bicarbonate. Glutamate had no specific promoting effect over the other Krebs cycle acids tested. Using the data of Birch and Snell (22, 23) and Gatenbeck's hypothesis (24), it is possible to achieve an excellent fit for the experimental data (Scheme VII).



Scheme VII—Incorporation of radioactivity into oxytetracycline from $^{14}CH_3CO_2H$. Calculated figures are followed by normalized experimental values in parentheses.

Radioactivity in Scheme VII is expressed as relative molar activities (R.M.A.) $\times 10^{-3}$. R.M.A. is defined as the number of counts/

100 sec. for an infinitely thick sample of 1 cm.^{2} cross-sectional area. Thus it is directly proportional to the molar specific activity. Using methyl-labeled acetate the molecule as a whole had R.M.A. = 42.2×10^3 in this particular experiment and the parameters involved are fourfold:

a = contribution due to the one-carbon pool derived *via* oxidation of the acetic acid and subsequent general metabolic incorporation of the labeled carbon dioxide. This could be measured directly by isolation of the amino methyls, which are methionine derived, and from the activity of the C_6 methyl determined by Kuhn-Roth oxidation and subsequent degradation. a = 0.3.

b = contribution of the methyl of acetic acid. This is measurable from the intensity of the activity of the C_6 carbon, isolated as the carboxyl end of the acetic acid from the Kuhn-Roth oxidation. b = 4.1.

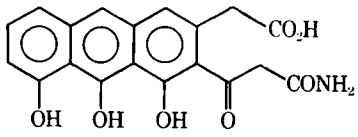
c = contribution of the carboxyl of acetic acid. The radioactivity of this carbon is due to metabolic scrambling and experience has indicated that a value of 5–10% is appropriate. This could, however, be measured directly as the activity of the carboxyl in terracinoic acid (XVIII). c = 0.4.

x = activity in the primer unit can be calculated using the values for a, b, and c. If the molecule is wholly acetate derived, then its makeup will be 3a + 8b + 8c + x = 42.2 and therefore x = 5.3. This would give an incorporation level of about 12.5% into the primer unit which is equivalent to that which Gatenbeck found using radio-labeled malonate (10–20%). Birch determined the level in the carboxamide carbon to be 0.6. Thus the starter would be b + c + 0.6 = 5.1 which is reasonably close to the expected value (b + c = 4.5). As the acetate involved in the starter unit must undergo at least one additional reaction compared with those used in extension, the agreement should not be expected to be exact. The variability observed in this unit in various experiments (14, 24) might reflect the relative efficiencies of these two processes and the extent to which the organism uses these building blocks for other purposes between the time when they are assembled and when they are incorporated into tetracyclines.

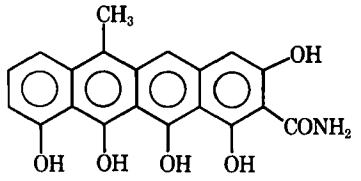
While it is true that specific degradation of Ring A is still to be achieved and double-labeling studies with malonic acid semiamide have either failed (55) or have not yet been reported, the balance of evidence currently favors Gatenbeck's modification of Birch and Snell's hypothesis. One additional bit of circumstantial evidence favors this theory. The production of 2-acetyl-2-decarboxamidotetracycline derivatives (VI,VII, VIII) by certain mutant strains (56–58) is most readily rationalized by presuming a block in malonamyl *CoA* formation and the subsequent utilization of acetoacetyl *CoA* in its place. This would implicate polyketides XXIV or XXVI rather than XXVII. No experimental confirmation of this inference by labeling studies has yet appeared. Another circumstantial bit of supporting evidence derives from experiments indicating (*vide infra*) that the C_4 nitrogen is introduced at a later stage and does not therefore come as part of the primer unit. Thus the evidence available at present is in favor of the whole carbon skeleton being acetate-malonate derived and so the tetracyclines appear to be typical, although unusually complex, polyketide-derived molecules. No other known polyketide product has malonic acid semiamide as the presumed starter unit.

PRETETRAMIDS

Having concluded that hypothetical polyketides XXIV and XXVI are satisfactory rationalizations for the experimental evidence, it is immensely gratifying to find that certain aromatic degradation products of the tetracyclines which closely resemble XXV are in fact excellent biological precursors of the tetracyclines. The simplest of these, pretetramid (XXV) is very close indeed (59, 60). The biotransformation yields of XXV to finished antibiotic ranged from 3–75% depending upon the substrate and the mutant used. Pretetramid is the earliest recognizable and well-characterized specific tetracycline precursor known at present. Protetrone (XXXI), isolated from a blocked mutant culture, appears to be a shunt product resulting from an imperfection in the cyclization process (11). Synthesis of aromatic pretetramid derivatives and subsequent bioconversion is a tool that should allow the preparation of novel tetracyclines. This approach depends upon the subsequent enzymes being relatively insensitive to the presence of unusual structural features and it is disappointing that, so far, no really unusual tetracyclines have reportedly been made in this way. Still, work with the various pretetramids has given considerable insight into the biological reactions involved. Pretetramids lacking a C_6 methyl group do not give rise to 6-methylated tetracyclines, so methylation may be presumed to be a very early step. Synthetic 6-methyl pretetramid (XXXII) is readily bioconverted to 6-methylated tetracyclines as theory would demand. Obviously then, the subsequent en-



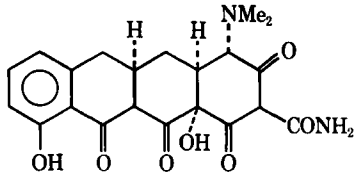
XXXI



XXXII

zymes in the two series are insensitive to the presence or absence of the C_6 methyl and the biosynthesis follows a parallel course from here on.

These inferences are quite consistent with the observation that a number of mutant cultures produce 6-demethyltetracycline derivatives (61, 62) and, under certain conditions, normal strains can be induced to form 6-demethyl derivatives by the use of enzymatic-blocking agents such as various sulfonamides, aminopterin, and ethionine (63–66). These appear to operate by inhibiting one-carbon transfer reactions and methionine is usually able to overcome these inhibitions. Ethionine dramatically reduces the antibiotic-producing potential of some *S. aureofaciens* strains. More will be made of this observation later. Under certain conditions, ethionine can substitute for methionine, so ethionine can to a certain extent both repress methionine utilization-formation or substitute for it in biosynthesis in some strains, and ethylmethylaminotetracycline was prepared in this way (67). The practical importance of the 6-demethyltetracyclines lies in their greater acid stability as compared with the normal tetracyclines which contain a tertiary-benzylic hydroxyl at C_6 which is readily lost to form the anhydrotetracyclines (XLII). These later substances are strongly bound to serum proteins and are not clinically useful as antibiotics. The tendency to form anhydrotetracyclines is diminished by removal of the C_6 methyl. A chemically derived product of the 6-demethyltetracyclines, 6-demethyl-6-deoxytetracycline (XXXIII), is the simplest known tetracycline possessing essentially full antibiotic activity and has been used to prepare a variety of new tetracyclines by electrophilic reactions under conditions which normal tetracyclines would not survive (68). 7-Chloro-6-demethyltetracycline (IV) was produced from blocked mutants and is an article of commerce, representing the first economic fruit of these fundamental

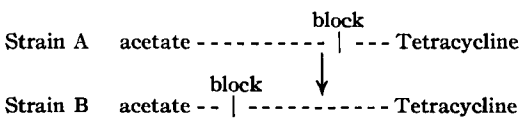


XXXIII

studies. In contrast to the C_6 methylation, C_7 chlorination of various pretetramids does take place using chlorinating *S. aureofaciens* strains so this is a later step (53).

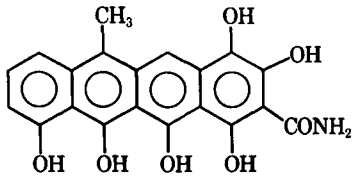
With the presumptive role of these partially and wholly synthetic derivatives established, the isolation of 4-hydroxy-6-methylpretetramid (XXXIV) from a blocked mutant was a very significant advance indeed (69, 70). The methodology involved should be of general utility in similar sequence problems and bears some comment. In contrast to mutations involving pathways in primary metabolism which are relatively easy to detect for they often lead to death or nutritional dependence on media factors, mutations deleting enzymes in secondary metabolism are apparently not usually lethal. The cell can survive quite nicely whether it produces tetracyline or not. To make the detection of productive mutants easier, the phenomenon of cosynthesis was used in a brilliant series of investigations by J. R. D. McCormick and his group at Lederle. This approach has recently been described in detail (11, 71) and can be synopsized figuratively as shown in Scheme VIII.

Cosynthesis depends upon the use of two nutritionally-intact blocked mutants, neither of which is able alone to synthesize active antibiotic. If certain conditions are met, however, growth of the two strains in mixed culture can produce tetracyclines. Thus consider Mutants A and B—one blocked (lacking at least one important enzyme or cofactor for a given step in the sequence) early and the other blocked late in the sequence (Scheme VIII). If a stable intermediate is

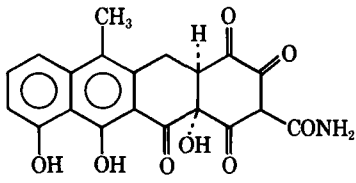


Scheme VIII—A simplified illustration of cosynthesis.

assembled by Mutant A, is excreted into the medium, and is taken up by Mutant B, Mutant B has the necessary enzymes to finish the synthesis. This ingenious method allows one not only to detect the presence of a stable intermediate but also to demonstrate its biological capabilities. Barring coincidence, positive results in



XXXIV



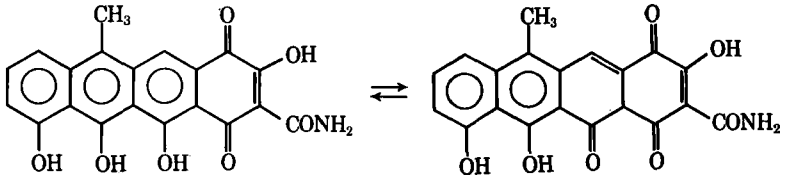
XXXV

this whole cell system are quite meaningful whereas negative results may not be (due to permeability problems, instability, *etc.*). Given enough mutants, the position of the break in the biosynthetic path can be worked out with some certainty as has been done with at least 16 mutant strains (11). The use of these strains obviates the necessity of using radio-labeled precursors as net synthesis is detected by biological activity (antibiosis).

Individually nonproductive strains V655 and and ED1369, in mixed fermentation, produced 7-chlortetracycline (60). Strain ED1369 was also able to form chlortetracycline when various fractions from V655 mash were added. Using this as an assay method, McCormick was able to isolate and characterize the elaborated agent as 4-hydroxy-6-methyl pretetramid (XXXIV) and to show that the pure product was bioconvertable to chlortetracycline in up to 75% yield. These findings strongly support the polyketide-pretetramid hypothesis and suggest that 4-hydroxylation is the next step in the sequence and that chlorination occurs subsequently. Transformation of 6-methylpretetramid to 4-hydroxy-6-methylpretetramid has been accomplished recently by purely chemical means though under fairly drastic conditions (72). As the 4-hydroxypretetramids are now fairly readily prepared by partial synthesis, and are at least one enzymatic stage further down the pathway, once again one can hope that new tetracyclines will result from mixed chemical-biological studies. The experiments reported to date, however, have not achieved this goal (12, 73, 74).

STEPWISE DEAROMATIZATION—THE 4-KETOANHYDROTETRACYCLINES

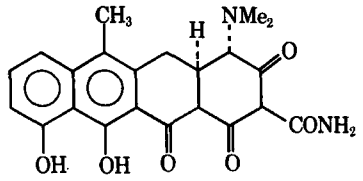
The next derivatives for which precursor activity has been demonstrated are the 4-ketoanhydrotetracyclines (XXXV). These substances have been prepared synthetically in a variety of ways (75, 76) and preliminary accounts of successful bioconversion have appeared using both whole cell (12) and cell-free systems (13). The details of the path between XXXIV and XXXV are obscure but one can propose, for the sake of argument, oxidation to quinone XXXVI which formally accepts a mole of water stereospecifically or (when represented by its enolic form XXXVII) (Scheme IX), more likely, by analogy with later steps, undergoes oxygenation of the β-diketo-system followed by reduction of the 4*a*, 5-double bond. Once again whole cell systems are able to halogenate these substances. Cell-free systems have not exhibited chlorinating ability here, perhaps due to the lack of necessary cofactors or instability of the responsible enzymes (13).



XXXVI XXXVII

Scheme IX

The biological 12*a*-hydroxylation of certain synthetic anhydrotetracycline (XXXVIII) derivatives has been accomplished but not by tetracycline-producing strains, and these reactions

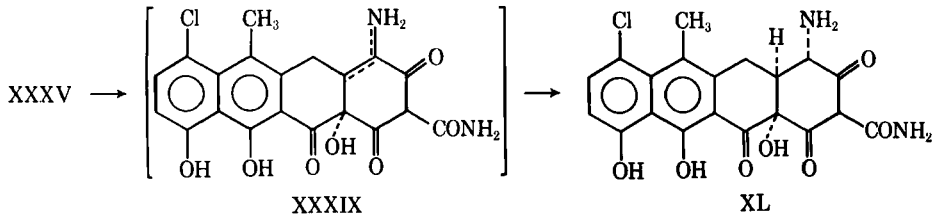


XXXVIII

may be taken, for our purposes, as being outside the normal pathway (77). Chemical hydroxylation of the same type of derivative has also been carried out (78, 79).

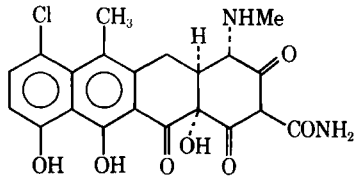
4-AMINOANHYDROTETRACYCLINES

The next piece of evidence bearing on the proposed sequence was provided by experiments involving genetically complete strains. Addition

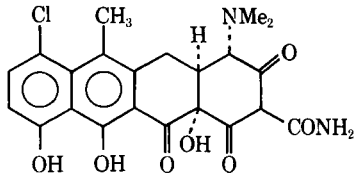


Scheme X

of ethionine at a suitable stage apparently represses methionine synthesis and, in the presence of an insufficient supply of methionine, tetracycline biosynthesis is shut off at an intermediate stage. The presence of precursors could be detected conveniently and quantitatively by assaying cellular extracts in the presence of methionine-$^{14}CH_3$ in a cell-free enzyme preparation made by sonification of suitable *Streptomycete* strains. The preparation of cell-free enzyme systems capable of elaborating secondary metabolites has usually been unsuccessful, thus it was most gratifying to find conditions whereby this could be accomplished with tetracycline-producing strains and, further, to find that they were capable of transforming even fairly early precursors (all the way back to the 4-ketoderivatives) (13). The competence of these soluble enzyme systems is an especially powerful tool because diffusion and cellular permeability are no longer factors of concern. Thus, providing the appropriate cofactors are supplied, the results obtainable take on a different order of reliability when using this sort of system. With this experimental approach it was possible to find conditions whereby treatment of whole cell cultures of *S. aureofaciens* and *S. rimosus* with ethionine resulted in high yields of these blocked intermediates and the products were isolated and characterized (Scheme X). These materials were shown to be 4-aminoanhydrotetracyclines (represented by XL) and, further, it was shown that they were past the point of halogenation. This narrowly restricts the position in the sequence where this step occurs and from this point onward, a parallel biosynthesis is utilized for halo and nonhalo-tetracyclines. Various degrees of methylation are present in these substances providing additional evidence that the three carbons of the tetracyclines which are methionine derived are sensitive to different extents to the action of ethionine. It could be shown by addition of limiting amounts of *S*-adenosylmethionine that the amino derivatives were stepwise alkylated to the monomethylamino (XLI) and then the dimethylaminoanhydrotetracyclines (XLII). The 4-β-epimers are not biologically convertable by these systems indicating something of the steric requirements of the enzymes involved (80). Discovery of the introduction of



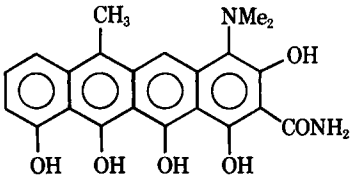
XLI



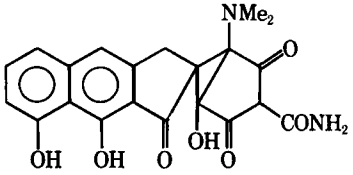
XLII

nitrogen at this stage is added circumstantial evidence against glutamate as a direct precursor of substantial portions of Ring A.

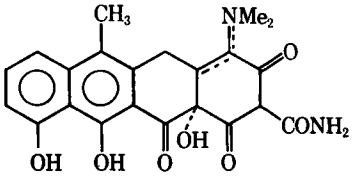
One problem of concern in all sequence work involving mutants or chemical blocking agents is the possibility that some of the substances discovered may have good precursor activity but not be in fact actual intermediates. Such stabilized shunt products or extraneous materials could be converted to mainstream intermediates by enzymes not ordinarily involved in the normal biosynthetic sequence. A wonderfully instructive example of this pitfall comes from work with 4-dimethylaminopretetramids (XLIII) which are available by degradation of tetracyclines (77). These compounds serve as reasonably efficient precursors in whole-cell systems (59). However, in the presence of methionine-$^{14}CH_3$ the dimethylamino group of the finished tetracyclines became radioactive and one is forced to conclude that the cell must remove the dimethylamino function, perhaps producing a 4-hydroxypretetramid or its corresponding quinone, and then reintroduce this function later in its normal time. The alternate possibility, stepwise dealkylation followed by realkylation, seems less likely although must still be allowed as a possibility. This serves as a warning in drawing hasty conclusions.



XLIII



XLIV

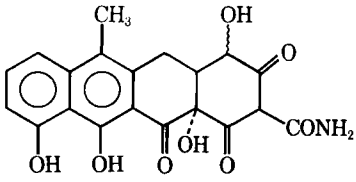


XLV

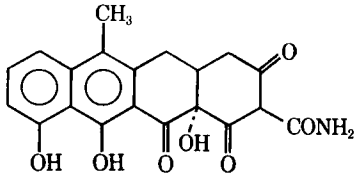
In this context the aminoanhydrotetracyclines, although highly active as precursors, were at first thought by some investigators to be stabilized shunt products (12). Thus at least two alternate sequences may be put forth between the 4-ketoanhydrotetracyclines and the anhydrotetracyclines. The evidence for the published sequence (12) was based, in part, on the structure of metatetrene, which shunt product has been isolated from mutant strain T219 and reported to have novel structure XLIV. This structure is now considered to be erroneous and the intermediacy of XLV is now doubted although not disproven (82, 104).[1] Such evidence as is now available favors pathway XXXV → XXXIX → XL → XLII and this sequence is generally accepted. Thus the C_4 nitrogen appears to be introduced into the sequence by reductive amination at the 4-ketoanhydrotetracycline stage and the products are stepwise methylated to the anhydrotetracyclines. Interestingly, the closely related 4-hydroxy (XLVI) and 4-desoxyanhydrotetracyclines (XLVII) are inert (12).

It has been demonstrated for one group of mutants (Class I) that the rate of chloride utilization for tetracycline biosynthesis is independent of the chloride concentration (26) so the overall rate-limiting (slow) step must be prior to the aminoanhydro stage for halogenation precedes the formation of XL and probably XXXIX.

[1] Subsequent to the submission of this review, an article appeared describing isolation of 4-aminoanhydrodemethylchlortetracycline (the 6-demethyl analog of XL) from a blocked mutant (104). This discovery is strong confirmatory evidence for the sequence related herein.



XLVI



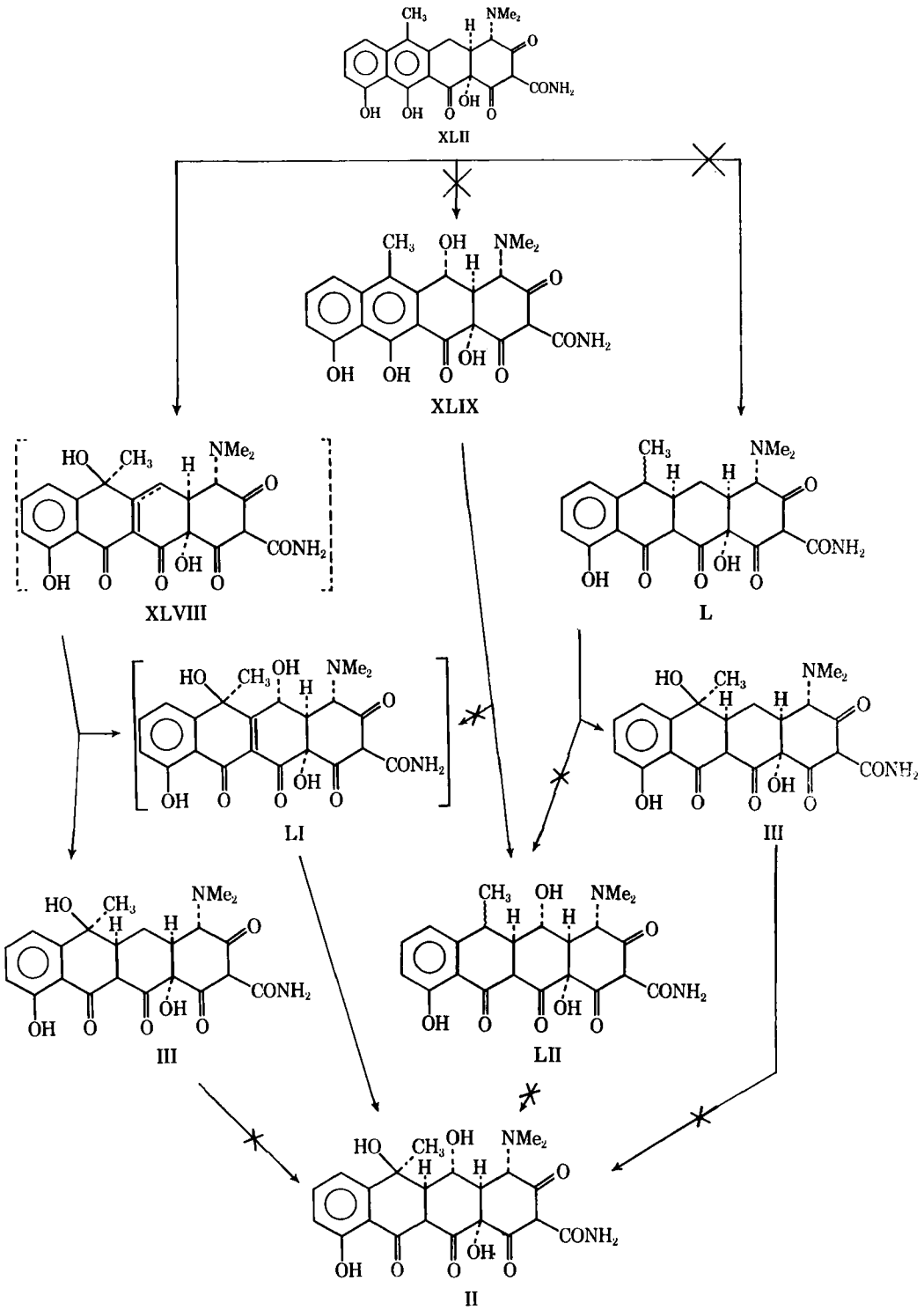
XLVII

ANHYDROTETRACYCLINES

The products of the methylation, the anhydrotetracyclines, are very readily formed by dehydration of tetracyclines carrying an oxygen at C_6 and have been available by partial synthesis for a long time. They are readily transformed to finished tetracyclines by a number of whole-cell and cell-free systems (11, 14), but have not yet been reported as having been isolated from normal, blocked, or mutant cultures. This is surprising for they are stable chemically and their *in vitro* antibiotic properties should make them readily detectable. It has been surmised that they may be toxic to the producing organism so that inability to carry out the next step may cause them to accumulate to a toxic level and such mutations might then become self-limiting (12).

TERMINAL REACTIONS

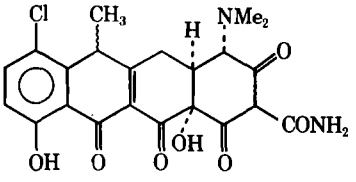
The remaining reactions in the sequence are clear. Because the number of possible intermediates in the sequence between anhydrotetracycline (XXXIII) and oxytetracycline (II) are limited, the method of approach used was to obtain as many as possible of the plausible intermediates and test them one after another in a cell-free system (83). The results are given in Scheme XI. Those reactions which could not be demonstrated in cell-free enzyme preparations have been blocked with an X. Note that there is only one contiguous sequence between anhydrotetracycline (XLII) and oxytetracycline (II). Compound XLVIII is encased in dotted brackets as it has been prepared in microgram quantities only. Compound LI is encased in solid brackets as it has only been detected on paper chromatograms and has not been isolated as a single chemical entity. The other substances have been fully characterized.



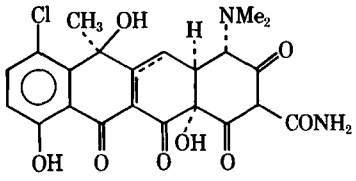
Scheme XI—Terminal reactions in tetracycline biosynthesis.

Certain strains utilize air as an oxidizing agent and transform anhydrotetracycline (XLII) to 5*a*(11*a*)-dehydrotetracycline (XLVIII). The 7-chloro analog of XLVIII (LV) was one of the first tetracycline intermediates to be isolated from a blocked mutant and to be converted biologically to a finished antibiotic (71, 83–85). It is interesting to note that the latter transforma-

tion has also been achieved in the laboratory by photochemical oxygenation of anhydrotetracyclines (86, 87) but also that no evidence is available for a free hydroperoxide intermediate in cell-free studies (13). The anhydrotetracyclines can be drawn in their ketonic tautomeric state (LIV) in which the C_6 hydrogen is both benzylic and allylic and should be very susceptible to free-radical oxygenation processes. Interest-



LIV



LV

ingly, the *in vitro* photooxygenation appears to fail unless a C_7 chlorine atom is present (86, 87). A biological feature of interest here is that *S. rimosus* strains are not able to carry out a C_6 oxygenation reaction on anhydrotetracyclines bearing a C_7 chlorine (84). This presents an interesting contrast in structural requirements between the biological and photochemical process. As the aminoanhydrotetracyclines do not serve as substrates for the C_6 oxygenating system in either *S. aureofaciens* or *S. rimosus* cell-free systems, it is easy to see why they accumulate under ethionine blockade and to explain the otherwise puzzling observation that no amino or methylaminotetracyclines have been isolated from biological systems, particularly those leading to 6-demethylchlortetracycline under ethionine blockade (13).

5*a*(11*a*)-Dehydrotetracycline has proven to be too unstable for successful isolation in more than microgram amounts; however, sufficient material was isolated by elution from paper chromatograms that its biological role could be evaluated in whole-cell systems. This substance is a key intermediate, serving as the branch point at which the biosynthesis of oxytetracycline (II) and tetracycline (III) diverge. Once again a point is reached where parallel pathways develop after the introduction of one of the "extra" features of the tetracyclines. As had been predicted (84, 88), reduction with NaDPH under aerobic conditions with *S. aureofaciens* enzymes leads to tetracycline whereas 5-oxygenation under aerobic conditions (with *S. rimosus* enzymes) leads to oxytetracycline.[2] When 5*a*(11*a*)-dehydrochlortetracycline is used, biological reduction produces chlortetracycline. It is possible to convert 6-deoxytetracycline to tetracycline in cell-free systems, but the anticedent reaction, conversion of anhydrotetracycline to 6-deoxytetracycline, has not been observed despite careful attempts. If this failure is fundamental rather than tactical, then the oxygenation of L is an incidental reaction and does not represent a valid *in vivo* pathway. Thus all the available evidence at present supports paths XLII → XLVIII → LI → II and XLII → XLVIII → III for the terminal sequences.

It is interesting to note that cultures producing mixtures of either oxytetracycline and tetracycline or chlortetracycline and tetracycline are not uncommon. However, no reports have appeared of cultures producing mixtures of chlortetracycline and oxytetracycline. This interesting fact bore on a problem of longstanding interest, namely: why was oxychlortetracycline (V) not known in nature, and how could this "hybrid" be prepared? It is possible to hypothesize that each sequence of reactions leading to the formation of a secondary metabolite has as its ultimate aim the elaboration of a "target substance" (89). This material may be considered to be the most highly elaborated (in terms of the extra functions added to the basic polyketide chain) metabolite that the culture was able to produce in its evolutionary history. In these terms oxychlortetracycline (V) might serve as the target substance that all tetracycline-producing *Streptomycetes* once made. In the course of time, mutations, in the light of this theory, conceivably caused the deletion of one or more enzymes and all strains eventually lost the ability to produce this material. This is entertaining, though purely speculative, but it heightens interest in the recent successful preparation of oxychlortetracycline through use of biosynthetic theory and methodology (90–92).

Knowing that *S. rimosus* was unable to handle anhydrochlortetracycline (84), and having the terminal sequence for tetracycline and oxytetracycline in hand, examination of the chlorinated analogs of XLVIII and LI in *S. rimosus* systems seemed called for in the hope that the nonconversion of anhydrochlortetracycline was due to the C_7-chlorofunction being inhibitory for the

[2] J. R. D. McCormick, (87) *Reference 12*, p. 82, reports that 5*a*(11*a*)-dehydrotetracycline undergoes a facile allylic rearrangement to 5*β*-hydroxyanhydrooxytetracycline. Analogous reactions in a similar series have been reported (88).

early enzymes and that subsequent enzymes would be less specific in their structural requirements.

It was indeed found to be possible to transform 5*a*(11*a*)-dehydrochlortetracycline (LV) to oxychlortetracycline (V) in excellent yield using either cell-free or whole-cell *S. rimosus* preparations. It is apparent that *S. rimosus* lacks the enzymes or cofactors necessary for halogenation of tetracycline precursors and, even if it did not, it would be unable to carry the precursor past the anhydrochlortetracycline stage, for the 7-halogen apparently inhibits the C_6-oxygenase of *S. rimosus*. This, then, explains on an enzymatic level why oxychlortetracycline remained so elusive for so long. It also suggests that natural strains producing LV may yet be found and, indeed, the pronounced instability of LV may be a partial explanation for this lack (in addition to the enzymatic factors discussed above).

SUMMARY

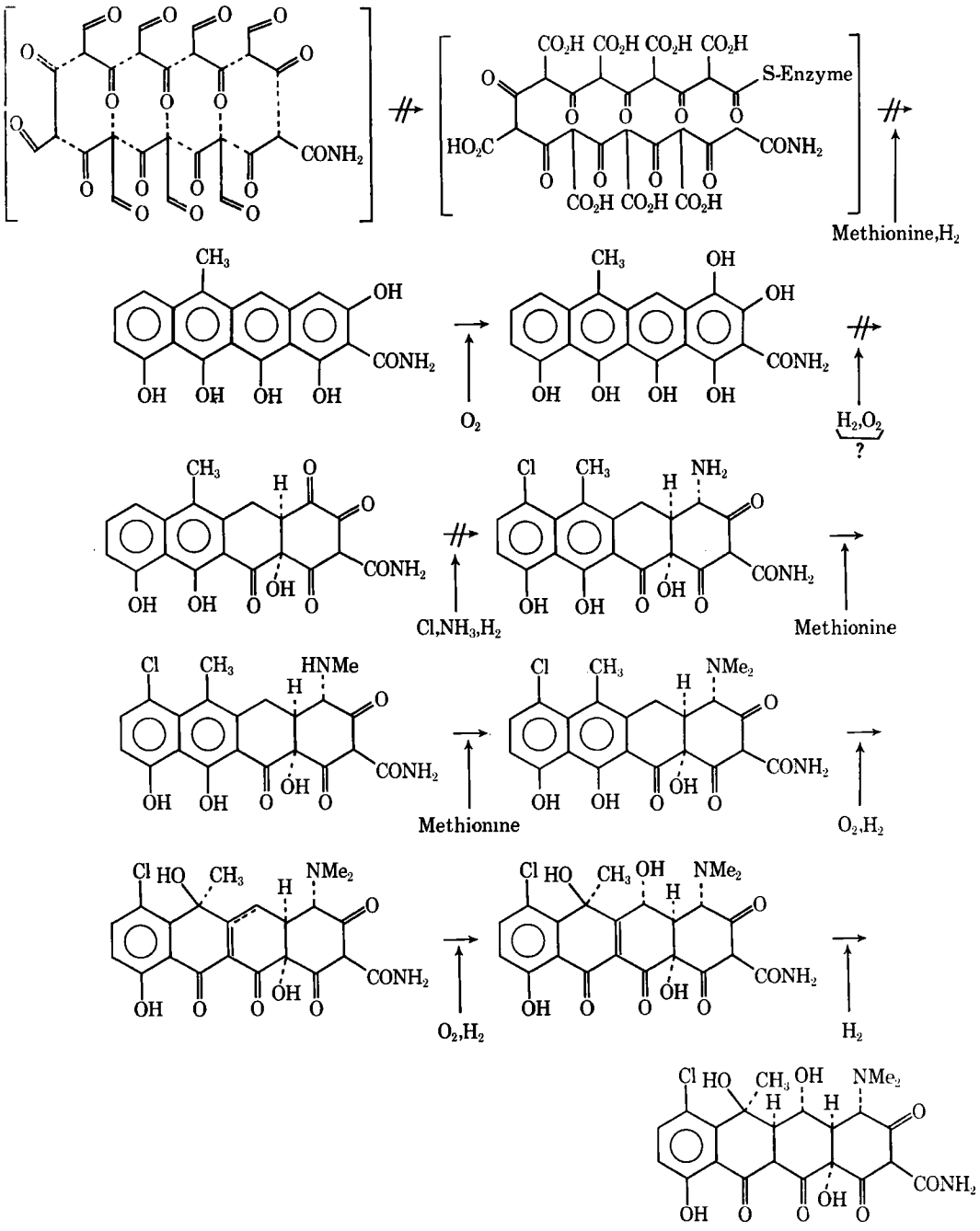
Our current understanding of the biosynthetic scheme by which the tetracycline antibiotics are synthesized in nature is set forth in Scheme XII. Oxychlortetracycline is the most highly elaborated tetracycline presently known, embodying the structural elements of chlortetracycline, tetracycline, and oxytetracycline, so it was chosen to exemplify the sequence. Lack of enzymes or cofactors causes the deletion of one or more steps, several of which serve as branch points. Omission of halogen, for example, leads to oxytetracycline and omission of the final hydroxylation step leads to chlortetracycline, and so on.

FUTURE GOALS

It is possible, indeed one of the hopes of this review, that successful preparation of new antibiotics making use of biosynthetic theory either to suggest new structures or new means of preparation, as in several cases mentioned herein, will stimulate an increasing number of other workers to take a similar approach. This is particularly important at a time when empirical screening methods for new antibiotics have clearly reached the point of diminishing returns considering the effort expended. Even in fields as thoroughly studied as the tetracyclines there are goals left to achieve. By no means the only remaining lack, but illustrative of the point, is the preparation of the still missing 8-hydroxytetracyclines. Polyketide theory suggests that they should exist, and experience with oxychlortetracycline suggests that enzymatic factors may in part be at fault. Miller (13) has discussed the theoretical parameters involved in preparing 6-demethyloxytetracycline. There appears at present to be no theoretical reason why this compound should not be prepared by biochemical manipulation of a suitable *S. rimosus* system. On the other hand the reaction sequences in Scheme XI suggest that a 6-deoxytetracycline antibiotic would be unlikely as a natural fermentation product (13), just as V was not a natural product in a strict sense. Very little work has been done on the enzymes themselves involved in secondary metabolism and many fascinating questions remain to be solved here.

Certainly the conclusion is warranted that a great deal of sequential information, even though some of the pieces are still largely circumstantial, is now available dealing with the intimate steps by which various *Streptomyces* species elaborate the tetracycline antibiotics starting with very simple primary metabolites. It is also apparent that there are no real surprises in terms of the types of reactions involved. The major unique characteristic of secondary metabolism is the substrates themselves. It is fascinating to consider that, using a relatively limited number of reactions over and over again in various sequences, microbes are capable of elaborating such a multitude of compounds. The complexity of these substances, including the tetracyclines, has taxed the ingenuity and been the joy of structural, synthetic, and biological chemists for many years. Despite their central role in mankind's attack against disease, it is curious that there is little real evidence that secondary metabolites are of much importance to the organism itself. Darwinian arguments can be advanced in the case of the tetracyclines and other antibiotics but for the vast majority of secondary metabolites such arguments are not supportable. Either we have not yet detected a function for the majority of these materials or they are themselves metabolic "flotsam."

Still, it is possible to rationalize the value of secondary metabolism to the organism (bearing in mind that a more direct, but currently obscure, primary function may someday be discovered). The important factor may not be the compounds themselves but rather the process whereby they are formed. Secondary metabolism is often considered to begin when some essential media constituent has become exhausted thus subjecting the culture to metabolic stress. The organism has the choice of death or adaptation to the new circumstances of its environment. A whole sequence of enzymes may be induced (89) pro-



Scheme XII—A summary of the biosynthetic pathway of the tetracycline antibiotics as currently understood. These reactions which appear to involve more than one step and intermediates which have not yet been well characterized are signified by hatched arrows (⫽→).

ducing substances not normally present during primary metabolism. It is alternatively possible that shutting off the normal reactions of primary metabolism may permit the diversion of newly excess raw materials into pathways which are present in primary metabolism but not operating except to a minor extent. It is also possible that a few bridging enzymes are induced by the sudden piling up of a metabolite normally rapidly turning over and not present in large amount. This would lead down new paths of metabolism involving enzymes which normally have some other catalytic function, or perhaps only a structural function. The answer as to which if any of these possible interpretations is correct must await the next logical phase of inquiry—study of

the enzymes themselves; a few pioneering papers have appeared in this area (93–102). This would bear on the question of whether the function of secondary metabolism in the microorganism is to maintain intact for as long as possible the largest number of enzymes and cofactors. This would have survival value by permitting continued operation, even at the expense of a great deal of cellular energy, on the chance that environmental conditions would change and allow the cells to resume primary metabolism (103). The future promises to provide answers to this and many other intriguing questions raised by the phenomenon of secondary metabolism.

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Keyphrases

Tetracycline antibiotics—biosynthesis review
Biosynthetic processes—tetracyclines
Polyketide theory—tetracycline biosynthesis
Structure, basic—tetracyclines
Pretetramids—tetracycline precursors
4-Ketoanhydrotetracyclines—dearomatization
4-Aminoanhydrotetracyclines—tetracycline intermediates
Oxidation, tetracyclines—final biosynthetic step

Research Articles

Nuclear *In Vitro* Method of Continuously Evaluating Release Rates of Solid Dosage Forms

By WILLIAM J. McCLINTOCK*, DANE O. KILDSIG, WAYNE V. KESSLER, and GILBERT S. BANKER

A nuclear *in vitro* continuous release rate measuring method has been developed and evaluated which permits determination of the release rates of ^{14}C-labeled materials from solid dosage forms. The nuclear apparatus consisted of a scintillation flow cell and a liquid scintillation spectrometer which recorded count rates automatically. The nuclear method was compared with a spectral method in measuring the release rates of ^{14}C-labeled caffeine and carrier caffeine from three different tablet systems. The two methods were equally precise and there was no statistical difference in the release rates determined by the two methods of analysis for any of the tablet systems studied. The nuclear method may be more convenient and less sensitive to the presence of interfering substances than a spectral method. The nuclear method can be employed as a continuous and automated methodology to reflect the effect of formulation or physical changes on the release rate of dosage forms.

CONCERN WITH maximum drug availability and effectiveness has resulted in the establishment of various dissolution tests as *in vitro* indicators of availability. Often the comparison of the apparatus used in one laboratory and that used in another yields significant differences in the results of dissolution tests on the same dosage forms and drugs. The rotating-bottle method (1–3), the modified USP disintegration testing basket attached to a Gershberg-Stoll apparatus (4, 5), and the rotating disk (6–8), involve the periodic removal of samples followed by some form of quantitative analysis. Automated methods of evaluating *in vitro* release rates have been developed which employ continuous spectrophotometric analysis (9, 10). However, the use of radionuclides as labels on drug molecules affords a direct method of determining dissolution rates regardless of the drug's spectral characteristics.

Several investigators have substituted radionuclide detection for chemical analysis in the mea-

Received May 22, 1968, from the Industrial and Physical Pharmacy Department, School of Pharmacy and Pharmacal Science, Purdue University, Lafayette, IN 47907
Accepted for publication July 12, 1968.
This investigation was supported by fellowship 1-F1-GM-21,146 from the Institute of General Medical Sciences, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
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sure of drug release, but have retained the sampling methodology (11–13). Other workers, employing nondrug labeled systems, have investigated continuous flow methods and various flow cells. Schram and Lombaert (14) used a spiral of polyethylene tubing filled with anthracene powder, inserted into a flat plastic vial, and placed on the window of a multiplier phototube to measure the radionuclide concentration of a continuously flowing eluate. Rapkin and Packard (15) used a cell packed with crystals of anthracene and placed between two multiplier phototubes. The counting efficiencies for ^{14}C using anthracene crystals were similar to those found in counting vials, according to Rapkin and Packard (15), which indicated that continuous flow measurements appeared to be a practical method of determining concentrations of ^{14}C-labeled materials in solution.

In a previous investigation (16), a method of continuously measuring the release characteristics of γ-labeled drugs was developed. The purpose of this research was to develop a system which would continuously and quantitatively measure the concentration of ^{14}C-labeled drugs in solution. Such a method would be independent of the physical and chemical properties of the other components of the dosage form, the spectral properties of the drug, and eliminate interference problems often found in spectrophotometric analyses.

EXPERIMENTAL

Apparatus and Methods—The nuclear system was composed of a Packard scintillation flow cell,[1] a Packard model 3314 liquid scintillation spectrometer,[1] and a flow system designed to maintain the dissolution medium at 37 ± 1°. The Packard flow cell was a plastic cylinder containing a U-shaped channel for anthracene packing and an aluminum cap. To provide greater flow rates and to ensure leak-free operation, the cap was modified (Fig. 1) from the original manufacturer's model by boring two threaded holes, 0.8 cm. in diameter in place of the approximately 0.8 mm. ($^1/_{32}$ in.) inlet-outlet openings of the manufacturer's model. Two cylindrical brass fittings were constructed (Fig. 1) with a 0.625 cm. i.d. for threading into the top of the cell. The fittings were securely tightened into the cell with the aid of rubber O-rings to assure a water-tight fit. The cap had a groove cut around the circumference to allow the use of another O-ring which sealed out light when the cell was in the spectrometer.

The cell was firmly packed with 1.8 g. of flaked anthracene crystals[1] and each tube opening covered with a plug of glass wool and an 80-mesh monel metal screen. The flow cell, which had a 4-ml. volume when empty, had a 1.2-ml. liquid volume when filled with the flaked anthracene crystals.

[1] Packard Instruments Co., Inc., Downers Grove, Ill.

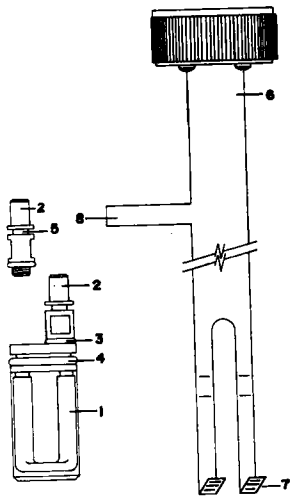

Fig. 1—The scintillation flow cell. Key: 1, flow cell; 2, brass-threaded fittings for inlet and outlet hose connections; 3, rubber O-ring (leak seal); 4, rubber O-ring (light seal); 5, flange for positioning handle prongs; 6, positioning handle; 7, positioning handle prongs; 8, location bar.

After packing the cell the brass fittings were securely tightened in place and the inlet and outlet tubing was connected.

A steel handle was fabricated (Fig. 1) with prongs which could slide into flanges provided in the brass fittings of the flow cell. The handle was employed to accurately position the flow cell in the scintillation counter insuring reproducible counting geometry and to remove the cell from the counter. A location bar on the handle further facilitated positioning of the flow cell in the scintillation well.

The liquid scintillation spectrometer was operated in the repeat mode and with a preset time of 5 min. and a preset count of 900,000 counts. An optimum gain for ^{14}C was used. A background count was established by pumping the solvent, 400 ml. of water, through the cell during a 15-min. period prior to a release study.

Preparation of Dosage Form—Three tablet formulations were prepared as shown in Table I. The

TABLE I—TABLET FORMULATIONS OF THE THREE TABLET GROUPS

Group	Percent Composition by Weight—Caffeine[a]	Magnesium Stearate	Starch
A	40	60	
B	40	40	20
C	40	20	40

[a] Labeled caffeine, approximately 1 μc./200 mg., was used.

caffeine-^{14}C was solvent distributed in the carrier caffeine using isopropanol and the resulting labeled caffeine had a specific activity of approximately 1 μc./200 mg. Accurately weighed samples of the formulations were compressed on a Carver laboratory press[2] under 2,000 lb. of force to yield tablets weighing approximately 500 mg.

[2] Fred S. Carver, Inc., Summit, N. J.

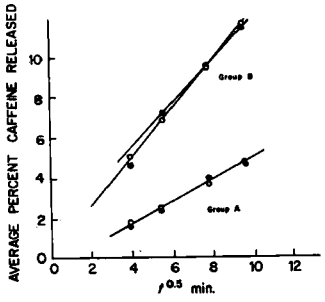

Fig. 2—Average percent caffeine released from Group A and Group B tablets with time as measured by the scintillation and spectral methods. Key: O, spectral; ●, nuclear.

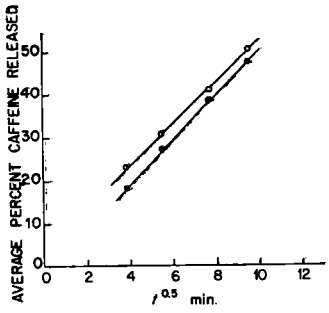

Fig. 3—Average percent caffeine released by tablet Group C as measured by scintillation counting and spectrophotometry. Key: O, spectral; ●, nuclear.

Method of Operation—The tablet was placed in a weighted 60-mesh monel metal screen enclosure which was pyramidal in shape. The enclosure was attached to the arm of a Vanderkamp[3] tablet disintegration apparatus and suspended in the dissolution vessel. The spectrometer was set to print cumulative counts at 5-min. intervals. During the 10-sec. time period required for printing the cumulative count, the pump was stopped and 10-ml. samples were taken for spectrophotometric analysis. After appropriate dilution and filtering, the caffeine was assayed at 273 mμ using a Beckman DU-2 spectrophotometer.[4]

both methods of analysis tablet Group C released caffeine nearly 10 times faster than Group A and more than four times faster than Group B. The release rates for a particular tablet group, as determined by the nuclear and spectral methods of analysis, were compared by two special t tests described by Brownlee (17) to determine whether parallelism existed and to determine whether the two lines represented a single coincident line.

The rates of release as determined using the two methods of analysis, within each tablet group, were first compared for parallelism using a two-tailed

TABLE II—MEAN PERCENT CAFFEINE RELEASED AND STANDARD DEVIATION VALUES FOR TABLET GROUPS A, B, AND C AT GIVEN TIME INTERVALS

	Scintillation				Spectrophotometry		
Group	n[a]	Minutes	Y %[b]	SD[c]	n	$\bar{Y}$ %	SD
A	6	15	1.68	0.23	6	1.79	0.19
	4	30	2.54	0.067	5	2.57	0.23
	6	60	3.93	0.73	5	3.68	0.17
	6	90	4.73	0.65	5	4.72	0.32
B	5	15	4.56	0.63	6	5.02	0.76
	4	30	7.21	0.46	6	6.79	0.83
	6	60	9.63	1.24	5	9.40	0.39
	4	90	11.44	0.42	4	11.59	0.34
C	4	15	18.42	3.36	4	23.16	3.70
	4	30	27.36	4.05	4	30.76	5.18
	4	60	38.67	4.84	4	40.94	4.63
	4	90	47.54	4.71	4	50.44	5.64

[a] n = number of data points used to calculate mean values. [b] $\bar{Y}$% = the mean percent caffeine released. [c] SD = the standard deviation of the mean.

RESULTS AND DISCUSSION

Release Rates—The comparison between the nuclear and spectrophotometric methods of measuring the release of caffeine from three different tablet formulations is shown in Figs. 2 and 3. The release of caffeine from the tablets was linear with square root of time as predicted by Higuchi (18) for a leaching process and as shown in a previous investigation (16) of similar formulations. The mean percent caffeine released at any time period was calculated from at least four determinations, and standard deviation is shown in Table II, for each tablet group.

The release rates were calculated from their regression line slopes (Table III). According to t test and the hypothesis $b_1 - b_2 = 0$ with the critical regions of rejection being set at 0.25. The observed t values (Table IV) were less in every case than the table values for t. The rates of release within each group of tablets for each method of analysis may therefore be regarded as parallel or equal.

TABLE III—REGRESSION LINE SLOPES OF RELEASE PERCENTAGES *versus* SQUARE ROOT OF TIME (AVERAGE PERCENT CAFFEINE RELEASED/MIN.$^{1/2}$)

Group	Scintillation	Spectrophotometry
A	0.553	0.518
B	1.223	1.147
C	5.204	4.844

[3] Van-Kel Industries, Inc., Livingston, N. J.
[4] Beckman Instruments, Inc., Fullerton, Calif.

TABLE IV—RESULTS OF *t* TESTS FOR PARALLEL AND IDENTICAL REGRESSION SLOPES

Group	Parallel	Identical
A	t (a = 0.25) = 0.6038 table t = 0.681	t (a = 0.10) = 1.266 table t = 1.303
B	t (a = 0.25) = 0.6649 table t = 0.681	t (a = 0.05) = 1.445 table t = 1.684
C	t (a = 0.25) = 0.4427 table t = 0.683	t (a = 0.025) = 1.994 table t = 2.045

The rates of release within each tablet group were next compared statistically to determine if they were identical as well as parallel. Brownlee (17) describes the lines as being identical when the two lines can be regarded as a single coincident line. A null hypothesis that the two lines within each tablet group were identical could be accepted (could not be rejected) at a critical rejection region of 0.10 or less (Table IV). The rates of release of each tablet group, as measured by either analytical method, could therefore be regarded as producing a single coincident line.

The variances of different sources of error with each method of analysis were also analyzed statistically. The scintillation method of measurement tended to yield slightly higher variance values than the spectrophotometric method. However, in no case were these differences significant. In general, when the variance of one analytical method was high, the variance of the other method for the same tablet group was also high.

SUMMARY

Many new drug substances are being routinely ^{14}C-labeled early in the drug product development sequence to facilitate illucidation of metabolites and metabolic pathways. It is suggested that the availability of ^{14}C-labeled drug might facilitate drug property characterization, and dosage form design, especially according to the preformulation research protocols recently implemented in many pharmaceutical laboratories. The use of ^{14}C-labeled drug, when available, could expedite preformulation research and drug design related to dissolution rate and other determinations. The necessity of extracting drug from dissolution media containing enzymes of other interfering components of the media, or containing polymeric materials or other interfering dosage form excipients, may be avoided using a nuclear method. The necessity, sometimes encountered, of developing special assay methods or assay modifications during product design can be avoided. In addition, continuous and automated analyses may readily be established for static determinations or for dynamic tests such as continuous dissolution rate analysis.

A method has been described in this investigation by which these objectives could be accomplished, employing ^{14}C-labeled materials and utilizing a scintillation flow cell and scintillation spectrometer. The nuclear method was found to be equally precise and much more convenient than a sampling technique and a spectral analytical method.

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Keyphrases

Release rates—solid dosage forms
Nuclear *in vitro* method—continuous evaluation, release rates
Apparatus—nuclear *in vitro* release rates
UV spectrophotometry—analysis
Scintillation counting—analysis
Caffeine-^{14}C—test compound

Iron-Adjuvant Interactions Observed with Diffuse Reflectance Spectroscopy

By MICHAEL BORNSTEIN*, JOHN L. LACH†, and BILL J. MUNDEN

Numerous adjuvants have been screened for the existence of chemical and physical interactions with iron compounds. Results indicate that calcium hydroxide, magnesium hydroxide, magnesium oxide, magnesium carbonate, magnesium citrate, zinc oxide, and potassium iodide do undergo large spectral and visual color changes on equilibration with ferrous sulfate in aqueous and nonaqueous systems.

ALTHOUGH chemical reactions of iron in solution have been previously investigated, very little information is available in the literature concerning the possible interactions of highly insoluble excipient materials with iron. In this communication, ferrous sulfate reactions have been studied with various organic and inorganic materials, using diffuse reflectance techniques.

Lach and Bornstein have recently shown that many of these insoluble adjuvants do undergo strong chemical interactions with a number of therapeutic agents (1–3) as well as certified dyes (4). Clarke (5) found that the addition of citric acid to a ferrous sulfate elixir increased its stability. Freedman (6), Uprety (7), and associates investigated the formation and stability of iron ascorbate complexes. Wagner (8) synthesized iron-gentisate complexes to be used for colorimetric assay. Lange and associates (9) studied a number of commercial preparations with respect to their ability to liberate iron from metallo-organic iron chelates and concluded that chelated iron is carried through the GI tract with less loss and lower toxicity than is ionic iron.

In view of the fact that the literature dealing with inorganic salt-iron complexes is limited and since many inorganic salts are not only integral parts of dosage forms, but may in themselves serve as the active therapeutic agent, it was felt that information concerning their solid-solid interactions would be of pharmaceutical importance.

EXPERIMENTAL

Reagents—Dried ferrous sulfate USP (Mallinckrodt Chem. Works), ferric chloride ACS (J. T. Baker Co.), sodium hydroxide pellets ACS (J. T. Baker Co.), acacia, aluminum hydroxide, aluminum stearate, calcium carbonate (dense), calcium hydroxide, cetyl alcohol, citric acid, kaolin, lactose, magnesium carbonate (light), magnesium hydroxide (heavy), magnesium citrate, magnesium stearate, Pharmasorb [colloidal (Minerals and Chemicals Philipp)], Pharmasorb [regular (Minerals and Chemicals Philipp)], polyethylene glycol 6,000, potassium iodide, sodium chloride, sodium phosphate (dibasic), sodium phosphate (tribasic), starch USP, stearic acid (flakes), stearyl alcohol (granular), Sterotex [edible peanut, cottonseed, and soybean oils (Capital City Products Co.)], tannic acid, tragacanth, distilled water, chloroform AR, and dehydrated alcohol.

Preparation of Ferric Hydroxide—Carefully weigh 50.0 g. triturated ferric chloride and 120.0 g. sodium hydroxide pellets. Place 1 l. distilled water into a 2-l. beaker and add the sodium hydroxide slowly. Stir until dissolved and add the ferric chloride. Mix for 15 min., vacuum filter, and wash the precipitate. Freeze the precipitate with the aid of a dry ice–methanol mixture, lyophilize for 24 hr., and triturate the resulting dark brown powder with a glass mortar and pestle.

Apparatus—The apparatus and technique of measurement have been reported (1–4).

Equilibration Technique—The procedure for preparing the samples and their corresponding controls has been reported (4). Thirty milliliters of aqueous or nonaqueous dispersion media has been employed in the equilibrations reported in this communication. A lyophilization technique (4) was used to remove the aqueous dispersion medium. The other nonaqueous solvents employed, including chloroform and dehydrated alcohol, were removed with the aid of ambient temperature vacuum evaporation.

RESULTS AND DISCUSSION

In view of the numerous difficulties encountered in pharmaceutical dosage forms containing iron, ferrous sulfate was selected as the inorganic material for this study of salt-adjuvant interaction. Based on DRS data obtained with respect to spectral intensity and visual color changes, it was evident that many of the interactions taking place were of a complex nature dealing with chemical and physical changes. These observations are summarized in Tables I and II.

For the purpose of illustration and discussion,

Received August 21, 1967, from Pitman-Moore Division of The Dow Chemical Co., Research Center, Zionsville, IN 46077

Accepted for publication July 8, 1968.

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The authors wish to thank Mr. John P. Walsh for his laboratory assistance.

TABLE I—IRON-CALCIUM CARBONATE REACTIONS USING AQUEOUS AND NONAQUEOUS DISPERSION MEDIA

Iron	Concentration Iron:$CaCO_3$, g.	Dispersion Medium	DRS Change[a]	Visual Color Change[a]
Ferric hydroxide	0.100:2.00	Water	0	No change[b]
Ferric hydroxide	0.400:2.00	Water	0-S	No change
Ferric hydroxide	1.00 :2.00	Water	0-S	No change
Ferric hydroxide	0.100:2.00	Ethanol	0-S	No change
Ferric hydroxide	0.400:2.00	Ethanol	0-S	No change
Ferric hydroxide	1.00 :2.00	Ethanol	S	No change
Ferric hydroxide	0.100:2.00	Chloroform	0-S	No change
Ferric hydroxide	0.400:2.00	Chloroform	S	No change
Ferric hydroxide	1.00 :2.00	Chloroform	0	No change
Ferrous sulfate	0.700:2.00	Water	L	Off-white to brownish-green
Ferrous sulfate	0.700:2.00	Ethanol	0	No change
Ferrous sulfate	0.700:2.00	Chloroform	M-L	Off-white to golden-brown

[a] Effects observed in comparing physically mixed controls to equilibrated samples. [b] No significant visual color change apparent. 0, No significant spectral change observed (≃DRS change of less than 5 reflectance units [RU] at $\lambda_{max.}$). S, Small spectral change (≃DRS change of 5–20 RU at $\lambda_{max.}$). M, Medium spectral change (≃DRS change of 20–50 RU at $\lambda_{max.}$). L = Large spectral change (≃DRS change of 50 or more RU at $\lambda_{max.}$).

several systems are presented in greater detail since space limitation does not permit a discussion of all the systems involved.

The diffuse reflectance data of an equilibrated $FeSO_4$-$CaCO_3$ sample and control are presented in Table I and illustrated in Fig. 1. An examination of these results indicates that some change has taken place as seen by the spectral differences reported for the control and the equilibrated and lyophilized sample. These observed changes are due not only to a surface adsorption phenomenon, but also to the existence of chemical reactions. The latter is true since although the solubility of $CaCO_3$ ($K_{sp.} = 0.87 \times 10^{-8}$) is limited, it is nevertheless sufficient to result in chemical reaction of small amounts of ferrous-ferric hydroxide. Although it is recognized that ferrous salts undergo oxidation, the addition of the oxidizing agent ascorbic

TABLE II—FERROUS SULFATE-ADJUVANT REACTIONS USING AQUEOUS DISPERSION MEDIA

Adjuvant	Concentration $FeSO_4$:Adjuvant, g.	DRS Change[a]	Visual Color Change[a]
Acacia	0.700:2.00	M	Beige to deep tan
Aluminum hydroxide	0.700:6.00	M-L	Tan to brown
Aluminum stearate	0.700:2.00	S-M	Off-white to yellow
Calcium carbonate	0.700:2.00	L	Off-white to dark gold
Calcium hydroxide	0.700:2.00	M-L	Tan to brown
Cetyl alcohol	0.700:2.00	0-S	No change[b]
Citric acid	0.700:2.00	S-M	No change
Kaolin	0.700:2.00	S-M	Off-white to dull gold
Lactose	0.700:2.00	S-M	Off-white to gray
Magnesium carbonate	0.700:2.00	L	Off-white to light brown
Magnesium citrate	0.700:2.00	L	White to olive green
Magnesium hydroxide	2.00 :2.00	L	Tan to olive green
Magnesium oxide	0.700:2.00	L	Off-white to olive green
Magnesium stearate	0.700:2.00	L	Light pink to golden brown
Pharmasorb (colloidal)	0.700:2.00	S-M	Tan to dull gold
Pharmasorb (regular)	0.700:2.00	S-M	No change
Polyethylene glycol 6,000	0.700:2.00	0	No change
Potassium iodide	0.700:2.00	L	Gold to dark brown
Sodium chloride	0.700:2.00	0	No change
Sodium phosphate (dibasic)	0.700:2.00	S-M	Gray to bluish-gray
Sodium phosphate (tribasic)	0.700:2.00	S	Tan to medium brown
Starch, USP	0.700:2.00	M	Off-white to yellow
Stearic acid	0.700:2.00	0-S	No change
Stearyl alcohol	0.700:2.00	S	No change
Sterotex	0.700:2.00	S	No change
Tannic acid	0.200:4.00	0-S	Tan to gray
Tragacanth	0.700:2.00	S-M	Beige to yellow
Zinc oxide	0.700:2.00	L	Beige to olive green

[a] Effects observed in comparing physically mixed controls to equilibrated samples. [b] No significant visual color change apparent. 0, No significant spectral change observed (≃DRS change of less than 5 reflectance units [RU] at $\lambda_{max.}$). S, Small spectral change (≃DRS change of 5–20 RU at $\lambda_{max.}$). M, Medium spectral change (≃DRS change of 20–50 RU at $\lambda_{max.}$). L, Large spectral change (≃DRS change of 50 or more RU at $\lambda_{max.}$).

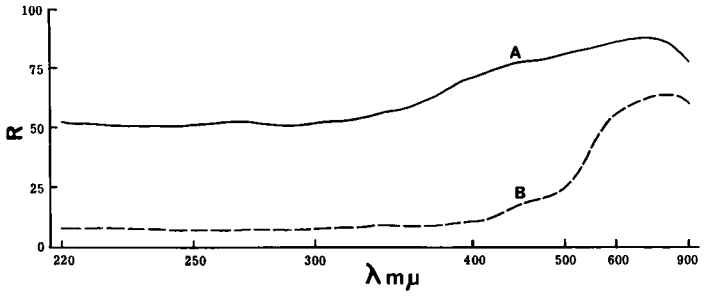

Fig. 1—DRS of dried ferrous sulfate (700 mg.) and calcium carbonate (2.00 g.). Key: A, control; B, water-equilibrated sample.

acid to the $FeSO_4$-$CaCO_3$ system did not alter the spectral characteristics to any appreciable extent.

The possibility that the spectral changes observed are due primarily to iron hydroxide formation was investigated and the results are presented in Table I and Fig. 2. The equilibration of a freshly prepared sample of $Fe(OH)_3$ with $CaCO_3$ at various iron concentrations, as shown in Fig. 2, indicates that the spectral changes observed here do not totally account for differences seen in the previous system. An examination of this $Fe(OH)_3$-$CaCO_3$ system indicates only minor spectral changes in comparing the control with the equilibrated sample. Only an intensity change, due to greater amounts of iron, is observed. The latter is due to a concentration effect (1, 3). Comparable spectra, Table I, were also obtained for this $Fe(OH)_3$-$CaCO_3$ equilibration when using chloroform or dehydrated alcohol as the dispersion medium. These results suggest that surface adsorption effects were minor in this $Fe(OH)_3$-$CaCO_3$ system since there was little spectral change between the controls and equilibrated samples in both aqueous and nonaqueous dispersion media.

Spectral changes observed in the $FeSO_4$-$CaCO_3$ aqueous system not only represent changes resulting from iron hydroxide formation, but also possibly due to the presence of iron oxides as well as spectral changes brought about by chemisorption of the various ionic species onto the insoluble calcium carbonate. It is significant to point out here that although the chances of iron hydroxide formation from ferrous sulfate in a chloroform medium are highly remote, the spectral changes obtained on equilibrating $FeSO_4$ wih $CaCO_3$ in this nonaqueous system did nevertheless produce significant spectral changes. Not only were these changes large, but they were also accompanied by important visual color changes. For example, the control, represented by Curve 3A (Fig. 3), is off-white in color as compared to a golden-brown chloroform equilibrated sample observed in Curve 3C. This is interesting in that the equilibration of ferrous sulfate with calcium carbon-

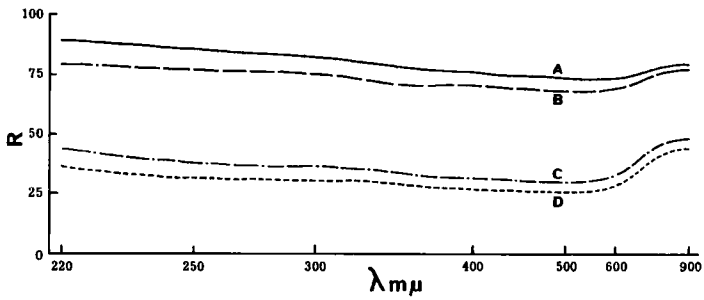

Fig. 2—DRS showing the effects of varying iron hydroxide concentrations in water-equilibrated samples, using 2.00 g. calcium carbonate as the adsorbent. Key: A, control, 100 mg. ferric hydroxide; B, sample, 100 mg. ferric hydroxide; C, control, 1.00 g. ferric hydroxide; D, sample, 1.00 g. ferric hydroxide.

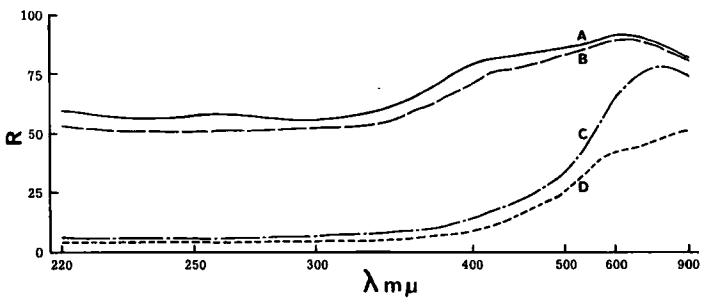

Fig. 3—DRS showing the effects of various solvents on equilibrating dried ferrous sulfate (700 mg.) with calcium carbonate (2.00 g.). Key: A, control; B, sample, equilibrated in 30 ml. dehydrated ethanol; C, sample, equilibrated in 30 ml. chloroform; D, sample, equilibrated in 30 ml. distilled water.

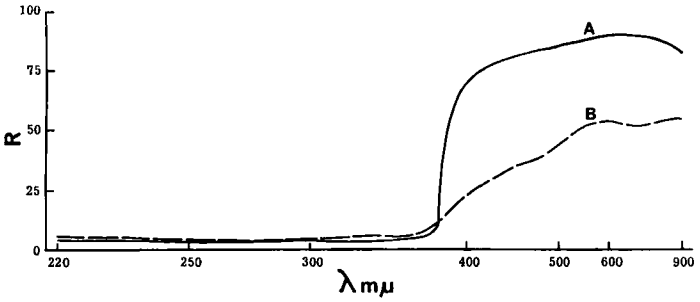

Fig. 4—DRS of dried ferrous sulfate (700 mg.) and zinc oxide (2.00 g.). Key: A, control; B, water-equilibrated sample.

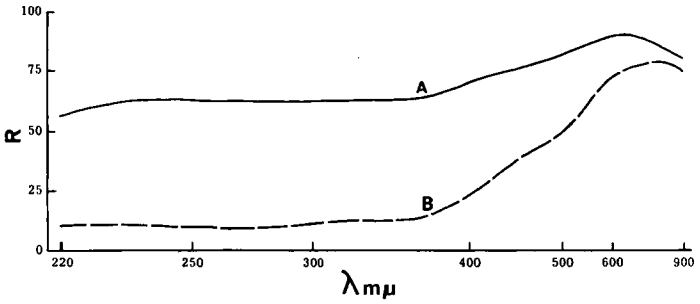

Fig. 5—DRS of dried ferrous sulfate (700 mg.) and magnesium stearate (2.00 g.). Key: A, control; B, water-equilibrated sample.

ate in a chloroform medium was expected to produce no significant changes since chemical reaction of these two inorganic salts in this medium is unlikely. Curve 3D, representing an aqueous equilibrated sample, which was brownish-green in color, indicates the presence of more interaction than the latter chloroform system. This greater reaction difference in the pressence of water is attributed to ionic reaction species. The $FeSO_4$-MgO, -$MgCO_3$, and ZnO systems presented in Table II exhibit spectral changes comparable to those observed for the $FeSO_4$-$CaCO_3$ equilibration, indicative of strong chemical and physical interaction. Another sample of this interaction is further illustrated in Fig. 4.

Although there is no evident reaction of ferrous sulfate with stearic acid, equilibration of ferrous sulfate with magnesium stearate does, however, produce a significant change as seen in Fig. 5. It is interesting to note the spectral changes brought about by the reaction of ferrous sulfate with citric acid and magnesium citrate presented in Table II as well as Figs. 6 and 7. Interaction of citric acid and its magnesium salt with ferrous sulfate was expected to result in spectral changes, in that iron-citrate complexes are known (1). It is of further interest to point out that the larger spectral changes observed with the magnesium citrate system were to be expected in view of the greater availability of the free citrate ion in solution.

Furthermore, although the spectral changes

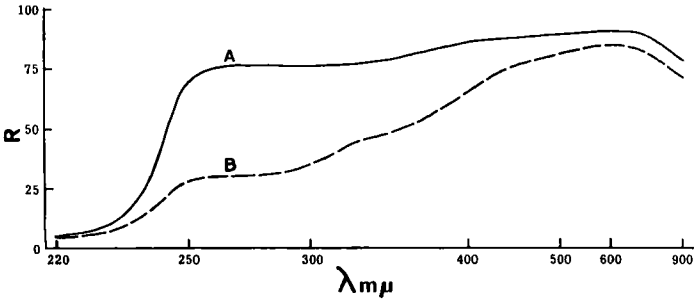

Fig. 6—DRS of dried ferrous sulfate (700 mg.) and citric acid (2.00 g.). Key: A, control; B, water-equilibrated sample.

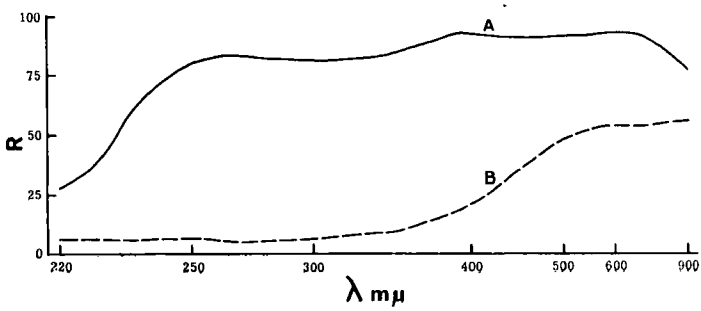

Fig. 7—DRS of dried ferrous sulfate (700 mg.) and magnesium citrate (2.00 g.). Key: A, control; B, water-equilibrated sample.

observed with other excipients studied in this investigation exhibit smaller changes, the spectral and color differences observed were indicative of some interaction. The diversity of the excipient or other type materials found in Table II also indicates that this ferrous sulfate interaction is widespread.

It should therefore be recognized that although analytical data with respect to a dosage form may substantiate the presence of the labeled amount of metallic ion, the form in which it exists in this product may certainly be one that is not available for a therapeutic response (11). Based on this ferrous sulfate-excipient interaction data, it does not seem unreasonable that other iron salts as well as salts used for other therapeutic purposes would also undergo such a phenomenon. A consideration of this possibility is therefore necessary in the development of pharmaceutical products.

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Keyphrases

Iron—adjuvant interactions
Ferrous sulfate-adjuvant reactions—aqueous dispersion
Diffuse reflectance spectroscopy—analysis

Tumor Localizing Agents VI

Radioiodinated Analogs of Dichlorodiphenyldichloroethane (DDD)

By R. E. COUNSELL, V. V. RANADE, P. POCHA, R. E. WILLETTE, and W. DiGUILIO

The preparation of 1,1-dichloro-2-(*p*-chlorophenyl)-2-(*o*-iodophenyl-^{125}I)ethane and 1,1-dichloro-2-(*p*-chlorophenyl)-2-(*m*-iodophenyl-^{125}I)ethane is reported. These compounds were prepared by chlorination of the appropriate iodoacetophenone, reduction to the corresponding carbinol, followed by acid-catalyzed condensation with chlorobenzene. Radioiodination was accomplished by isotope exchange with iodide-125. Preliminary studies in rats indicate that the position of the radioiodine has little influence on the predilection of these agents for adrenal tissue.

Wolf and Tubis (1) recently reviewed the rapidly expanding field of radiopharmaceuticals. As noted by these workers, various gamma-emitting radionuclides are now employed to externally scan many organs and major parts of the body.

Scanning has become a valuable diagnostic technique and permits the visualization of an internal organ by outlining the distribution of a radionuclide concentrating within an organ or tumor. With this technique, iodine-131 has been used to localize tumors of the thyroid, and chlormerodrin labeled with mercury-197 has served to delineate brain and kidney tumors. To date, however, no radiopharmaceutical is available for photoscanning the adrenal gland, although several laboratories are currently in the process of attempting to develop such an agent.

Nagai *et al.* (2) have reported that ^{131}I-labeled stigmasterol was useful in some cases for adrenal scanning. The agent was not regularly successful, however, because of the uptake in the liver and the excretion of free ^{131}I in the stomach. Similarly, because human adrenal glands con-

Received May 22, 1968, from the Laboratory of Medicinal Chemistry, College of Pharmacy, The University of Michigan and Veterans Administration Hospital, Ann Arbor, MI 48104

Accepted for publication June 28, 1968.

Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

This work was supported by grants CA-08349-01 and 02 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md., and PRA-18 from the American Cancer Society, New York, N. Y.

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Nagai *et al.* (2) have reported that ^{131}I-labeled stigmasterol was useful in some cases for adrenal scanning. The agent was not regularly successful, however, because of the uptake in the liver and the excretion of free ^{131}I in the stomach. Similarly, because human adrenal glands con-

Received May 22, 1968, from the Laboratory of Medicinal Chemistry, College of Pharmacy, The University of Michigan and Veterans Administration Hospital, Ann Arbor, MI 48104

Accepted for publication June 28, 1968.

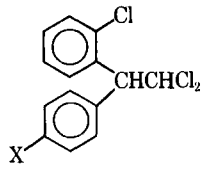
Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

This work was supported by grants CA-08349-01 and 02 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md., and PRA-18 from the American Cancer Society, New York, N. Y.

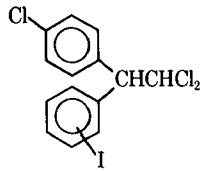
Part V, Counsell, R. E., Hong, B. H., Willette, R. E., and V. V. Ranade, *Steroids*, *11*, 817(1968).

tain a high concentration of ascorbic acid, Mishkin and Castronovo (3) evaluated ^{14}C-labeled ascorbic acid as a possible lead to an adrenal scanning agent. Unfortunately, their studies revealed that the liver interfered once again and concentrated approximately 35 times more ascorbic acid than did the adrenal.

Previous reports from this laboratory (4) have indicated that certain radioiodinated analogs of 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane (*o,p'*-DDD, I*a*) may offer some promise for adrenal photoscanning in humans. For example, preliminary studies with 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-iodophenyl)-ethane-^{125}I (I*b*) showed that the general order of concentration 4 hr. following administration to rats was adrenal > fat > liver and other tissues. Additional preclinical studies in dogs are currently in progress with this agent.



I*a*, X = Cl
b, X = I

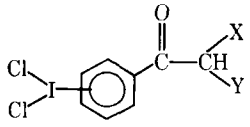


II*a*, *o*-iodo
b, *m*-iodo
c, *p*-iodo

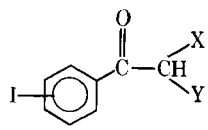
The author's studies with radioiodinated DDD analogs were prompted by the fact that *o,p'*-DDD causes adrenal atrophy in dogs (5) and is currently the treatment of choice for adrenocortical carcinoma (6). It apparently exerts its antitumor action by concentrating in adrenal tissue (7). Interestingly, isomeric *p,p'*-DDD was found to be essentially devoid of adrenolytic activity in dogs (5).

In an effort to obtain further information regarding structure and adrenal localization, this paper describes the synthesis and compares the tissue distribution of additional radioiodinated DDD analogs in which the position of the aromatic iodine has been varied (II).

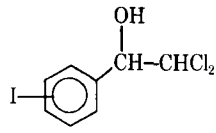
The preparation of the *p,p'*-isomer, II*c*, was reported previously (4) and involved the acid-catalyzed condensation of iodobenzene with 2,2-dichloro-1-(*p*-chlorophenyl)ethanol. For the preparation of II*a* and *b*, the *ortho* and *meta* isomers of 2,2-dichloro-1-(iodophenyl)ethanol (VIII) were required. These compounds were prepared by side chain chlorination of the appropriate iodoacetophenone and subsequent reduction to the carbinol with lithium tri-*t*-butoxyaluminum hydride.



III X = Y = H
IV X = Cl, Y = H
V X = Y = Cl



VI X = Cl, Y = H
VII X = Y = Cl



VIII

a, *o*-iodo; *b*, *m*-iodo

The chlorination of the iodoacetophenones by methods previously employed for the chloroacetophenones was complicated by the ability of aryl iodides to readily add 1 mole of chlorine to form dichlorides (8). For example, when chlorine gas was passed into an acetic acid solution of *m*-iodoacetophenone, the internal temperature quickly rose to 50° and a yellow precipitate of the dichloride (III*b*) formed immediately. An examination of the filtrate afforded a low yield of the chloroketone, VI*b*. When chloroform was used as the solvent, III*b* was obtained exclusively. If chlorination was continued at 50–60°, the yellow dichloride gradually dissolved and quenching the reaction at this stage furnished the chloroketone VI*b* in good yield. If chlorination was conducted above 70°, a second yellow precipitate formed which was the dichloride of the dichloroacetophenone, V*b*. The dichlorides are unstable and liberate chlorine on heating to give the appropriately substituted iodoacetophenones. Thus, the dichloroketone VII*b* was readily obtained by heating V*b* to 150°. No nuclear chlorination was observed to occur.

Chlorination of *o*-iodoacetophenone at 70°, on the other hand, gave rise to the dichloride of the trichloroketone. This product rapidly lost chlorine upon heating to afford 2,2,2-trichloro-2'-iodoacetophenone. This structure was established on the basis of elemental analysis, the presence of only aromatic protons in the NMR, and its quantitative conversion to *o*-iodobenzoic acid upon treatment with aqueous sodium hydroxide. The desired dichloroketone VII*a* was obtained in excellent yield by a slight modification of the chlorination conditions.

The carbinols (VIII*a* and VIII*b*) obtained after reduction of the halogenated acetophenones with lithium tri-*t*-butoxyaluminum hydride were condensed with chlorobenzene. The resulting

TABLE I—TISSUE/LIVER RATIOS OF RADIOACTIVITY AT 4 hr. IN RATS

Compd.-^{125}I	Adrenal	Perirenal Fat	Serum	Muscle	Kidney
I*b*	1.66 (0.12)[a]	2.11 (1.38)	0.66 (0.47)	0.48 (0.41)	0.55 (0.22)
II*a*	0.74 (0.09)	0.99 (0.20)	0.42 (0.24)	0.28 (0.06)	0.24 (0.01)
II*b*	1.47 (0.57)	1.33 (0.40)	0.41 (0.15)	0.39 (0.14)	0.44 (0.10)
II*c*	2.22 (0.48)	2.01 (1.05)	0.32 (0.04)	0.34 (0.16)	0.42 (0.07)

[a] Standard deviation in parentheses.

iodinated analogs of DDD were then subjected to isotope exchange with iodide-125 under conditions similar to those previously reported from this laboratory (4).

Tissue Distribution Studies—Preliminary tissue distribution data in male rats are summarized in Table I. The values are expressed as a ratio of the concentration of radioactivity found in a particular tissue to that for liver. Since the liver is the nearest interfering organ in adrenal scanning, it is essential that a compound exhibit a high adrenal/liver ratio in order to be considered for follow-up studies.

In contrast with results in the DDD series (6, 7), the radioiodinated analogs did not differ greatly in their capacity to concentrate in adrenal tissue. In fact, these preliminary results would tend to indicate that the *p,p'*-isomer (II*c*) has the greatest specificity for adrenal tissue. In all cases, the concentration of radioactivity in fat increased progressively and was three to four times higher than the adrenal content at 24 hr. More detailed studies in rats and additional studies in dogs are now in progress and will be reported elsewhere.

EXPERIMENTAL[1]

Chlorination of o-Iodoacetophenone—*Method A*—A solution of *o*-iodoacetophenone[2] (5 g.) in acetic acid (30 ml.) was treated with Cl_2 gas at 50–55°. Nitrogen was then passed through the reaction mixture to remove excess chlorine and hydrogen chloride. The temperature was raised to 60–65° and Cl_2 passed into the solution for 3.5 hr. Excess Cl_2 was swept out with N_2 as before and the solution allowed to cool. The solution was then poured into water (300 ml.) containing sodium sulfate (2 g.) and sodium acetate (6 g.). The mixture was extracted with ether and the ether extract was washed well with water and dried (Na_2SO_4). The solvent was removed and the residual oil distilled *in vacuo*. This gave pure VII*a* (6 g., 87%) as a yellow mobile liquid, b.p. 112–114° (0.2 mm.), $\nu_{max.}$ 3010, 2950, 1280, and 750 cm.$^{-1}$. The NMR spectrum showed a single peak at 6.64 δ (—$CHCl_2$).

Anal.—Calcd. for $C_8H_5Cl_2IO$: C, 30.52; H, 1.60. Found: C, 30.66; H, 1.57.

Method B—Chlorine gas was passed into a solution of *o*-iodoacetophenone (2 g.) in acetic acid (15 ml.). A yellow precipitate formed immediately and the temperature of the reaction mixture increased to 55°. The gas flow was stopped and the reaction mixture heated until the solid dissolved (65–70°). The gas flow was started and continued for 30 min. The mixture was refrigerated and the yellow precipitate collected (2.7 g.), m.p. 73–90° with evolution of Cl_2. This product was heated in an oil bath (130°) until gas evolution stopped and the residual oil distilled. This gave 2 g. of a pale yellow oil, b.p. 108–110° (0.25 mm.). The NMR spectrum displayed only aromatic protons. Elemental analysis of this product and its quantitative conversion to *o*-iodobenzoic acid upon treatment with aqueous NaOH established the product as 2,2,2-trichloro-2'-iodoacetophenone.

Anal.—Calcd. for $C_8H_4Cl_3IO$: C, 27.50; H, 1.15. Found: C, 27.66; H, 1.34.

Chlorination of m-Iodoacetophenone — *Method A*—Chlorine gas was bubbled through a solution of *m*-iodoacetophenone[2] (5 g.) in acetic acid (15 ml.). The temperature of the reaction rose to 50° and a yellow precipitate began to form immediately. Filtration and washing with water gave the dichloride of starting material (III*b*, 7 g.), m.p. 100° (from $CHCl_3$) with evolution of Cl_2 and $\nu_{max.}$ 1680 cm.$^{-1}$ (C=O).

Anal.—Calcd. for $C_8H_7Cl_2IO$: C, 30.31; H, 2.23; Cl, 22.37; I, 40.03. Found: C, 30.49; H, 2.28; Cl, 22.09; I, 40.21.

The compound was unstable to heat and light and lost Cl_2 on standing at room temperature, to yield *m*-iodoacetophenone. The mother liquors from the reaction were poured into ice water and extracted with ether, dried (Na_2SO_4), and evaporated to give the monochloroacetophenone, VI*b*. Recrystallization from CH_2Cl_2-hexane gave pure VI*b* (0.1 g.), m.p. 69–70°, $\nu_{max.}$ 1710 cm.$^{-1}$ (C=O). The NMR spectrum showed a sharp singlet at 4.64 δ integrating for two protons.

Anal.—Calcd. for C_8H_6ClIO: C, 34.25; H, 2.16. Found: C, 34.07; H, 2.08.

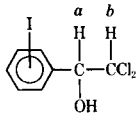
When the reaction was repeated using dry chloroform as solvent instead of acetic acid, the dichloride (III*b*) was formed exclusively.

Method B—Chlorine gas was passed through a solution of *m*-iodoacetophenone (5 g.) in acetic acid (50 ml.). A yellow precipitate formed immediately but dissolved upon heating the reaction mixture to 70°. Chlorination was continued at this temperature until yellow needles began to

[1] Melting points were taken on a Fisher-Johns melting point apparatus and are corrected. Elemental analyses were performed by Spang Microanalytical Laboratories, Ann Arbor, Mich. Infrared spectra were taken in KBr disks on a Perkin-Elmer 337 spectrophotometer. The NMR spectra were obtained with a Varian A-60 spectrometer in $CDCl_3$ at a concentration of 10%, with tetramethylsilane as internal reference. Thin-layer chromatograms (TLC) were run with 1 in. wide Eastman Chromagrams, type K301R with fluorescence indicator, developed with benzene, and spots detected with UV light and iodine vapor. Chromagrams were scanned with an Atomic Associates RCS-363 Radiochromatogram scanner. The specific activities were determined with a Beckman scintillation spectrometer model 530.

[2] Prepared according to Bowman, R. E., *J. Chem. Soc.*, 1950, 322.

TABLE II—CHEMICAL DATA FOR



Compd.	B.p., °C.(mm.)	Yield, %	Anal.[a] C	Anal.[a] H	Chemical Shifts (δ) H(a)	Chemical Shifts (δ) H(b)	J_{AX}, c.p.s.
VIII*a*	115–117 (0.2)	62	30.47	2.37	5.31	6.06	3.5
b	123–124 (0.12)	35	30.40	2.35	4.90	5.80	5

[a] *Anal.*—Calcd. for $C_8H_7Cl_2IO$: C, 30.29; H, 2.21.

separate (20 min.). The reaction mixture was allowed to cool and the product was collected by filtration. The product was washed with dry ether to give the dichloride of the α-dichloroketone (V*b*, 5.5 g.), m.p. 97–115°. Recrystallization of a sample from benzene gave pure V*b*, m.p. 125–127°, which was extremely unstable and promptly lost Cl_2. The remaining crude V*b* was heated to 150° until the evolution of gas ceased. The residual oil was distilled *in vacuo* to afford the dichloroacetophenone, VII*b* (2.7 g.), b.p. 108° (0.1 mm.), $\nu_{max.}$ 1725 cm.$^{-1}$ (C=O). The NMR showed a singlet at 5.04 δ (—COCH—) integrating for one proton.

Anal.—Calcd. for $C_8H_5Cl_2IO$: C, 30.51; H, 1.60. Found: C, 30.50; H, 1.77.

Method C—A solution of *m*-iodoacetophenone (10 g.) in acetic acid (35 ml.) was warmed to 50° and Cl_2 gas passed through. The yellow precipitate that formed initially was dissolved by warming to 60° and Cl_2 gas passed into the reaction mixture for an additional 2 hr. The warm reaction mixture was then poured onto ice whereupon a white solid formed. This was extracted with benzene, dried ($MgSO_4$), and evaporated to give a colorless oil which crystallized on refrigeration. The solid was collected and washed with benzene–pentane to give 2-chloro-3′-iodoacetophenone (VI*b*) as white needles (6.8 g.), m.p. 53–58°. Recrystallization of 0.5 g. from CH_2Cl_2–hexane afforded 0.2 g. as silky needles, m.p. 69–70°. In addition to aromatic protons, the NMR showed a singlet at 4.63 δ (—CH_2Cl).

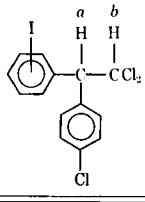
Anal.—Calcd. for C_8H_6ClIO: C, 34.25; H, 2.16. Found: C, 34.07; H, 2.08.

Reduction of Chlorinated Iodoacetophenones—*General Method*—A solution of the dichloroketones (VII, 20 m*M*) in tetrahydrofuran (20 ml.) was added dropwise with stirring to a solution of lithium tri-*t*-butoxyaluminum hydride (30 m*M*) in tetrahydrofuran (40 ml.). The mixture was stirred at room temperature for 2–5 hr. and poured into ice water (500 ml.) containing acetic acid (25 ml.). The mixture was extracted with chloroform, the extract dried ($MgSO_4$), and the solvent removed *in vacuo*. The residual pale yellow oils were fractionally distilled to give the corresponding carbinols, VIII (see Table II).

Condensation of VIII with Chlorobenzene—*General Method*—To a solution of VIII (1 g.) in chlorobenzene (3 ml.) was added dropwise with stirring a saturated solution of boron trifluoride in sulfuric acid. The addition required 30 min. whereupon the mixture was heated at 40–45° for 4 hr. The mixture was extracted with benzene, and the extract washed with water and dried ($MgSO_4$). The solvent was removed *in vacuo* and the oily residue triturated with petroleum ether (30–40°). The resulting solid was collected by filtration and recrystallized from 95% EtOH (see Table III).

Isotope Exchange—*General Method*—A solution containing 3 mc. of $Na^{125}I$ was placed in a 10-ml. round-bottom flask and evaporated to dryness at 100° under a gentle stream of N_2. Ethylene glycol (1 ml.) was added, the external temperature raised to 175–180°, and the iodinated compound (II) added. The solution was magnetically stirred for 5 hr., cooled, and diluted with water. The mixture was extracted with benzene and the extract washed with water, dried ($MgSO_4$, Darco), and concentrated to dryness. The products were purified by recrystallization from 95% ethanol and the purity

TABLE III—CHEMICAL DATA FOR



Compd.	M.p., °C.	Yield, %	Anal.[a] C	Anal.[a] H	Chemical Shifts (δ) H(a)	Chemical Shifts (δ) H(b)	J_{AX}, c.p.s.
II*a*	104–105	62	40.70	2.52	5.05[b]	6.29	8
b	87.5–88.5	31	40.95	2.50	4.48	6.27	8

[a] *Anal.*—Calcd. for $C_{14}H_{10}Cl_2I$: C, 40.87; H, 2.45. [b] The deshielding effect of *ortho*-substituted halogens on benzylic protons has been noted previously (9).

TABLE IV—ISOTOPE EXCHANGE FOR COMPOUNDS II

Compd.	Recovery, %	Exchange, %	Spec. Act., mc./mg.
IIa	10	15	5.22
b	70	62.8	17.58

established by (*a*) melting point and (*b*) TLC and a radiochromatogram of the strip (see Table IV).

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(2) Nagai, T., Solis, B. A., Koh, C., Hasegawa, M., and Goto, R., *J. Nucl. Med.*, **8**, 352(1967).
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Keyphrases

Tumor localizing agents—synthesis
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Radioiodination—iodide-125
Tissue distribution—radioiodinated dichlorodiphenyldichloroethane analogs

Interrelationship of Phosphate Nutrition, Nitrogen Metabolism, and Accumulation of Key Secondary Metabolites in Saprophytic Cultures of *Psilocybe cubensis, Psilocybe cyanescens,* and *Panaeolus campanulatus*

By J. M. NEAL, R. G. BENEDICT, and L. R. BRADY

The three basidiomycetes were grown on rotary shakers in four nutrient media containing various amounts of phosphate to determine the relative effect of this nutrient on the trichloroacetic acid-soluble and -insoluble nitrogen metabolites and to detect possible correlation between the fungal free amino acid pool (soluble nitrogen) and accumulation of characteristic hydroxytryptamine derivatives. The species were selected to represent different patterns of metabolism in fruiting bodies and vegetative mycelia. Vegetative mycelium of *P. cubensis* was characterized by a relatively high soluble nitrogen component and by the capacity to accumulate psilocybin and psilocin under selected conditions. The closely related *P. cyanescens* was less responsive to variations in phosphate nutrient and lacked the capacity to accumulate appreciable quantities of soluble nitrogenous compounds or detectable quantities of key tryptamines. *P. campanulatus* grown in phosphate-rich media appeared to have an adequate free amino acid pool and to excrete some exocellular nitrogen metabolites during longer incubation periods; no 5-hydroxytryptamine derivatives were detected in cultures of this fungus.

OBSERVATIONS on the occurrence of various secondary metabolites in basidiomycetes have demonstrated the medicinal interest in certain of these fungi (1). However, extensive investigation or utilization of many of the empiric observations has been delayed by the lack of basic biologic knowledge which would permit manipulation of the basidiomycetes in the manner of antibiotic-producing actinomycetes or various commercially useful ascomycetes. Information on the occurrence of many basidiomycete metabolites suggests that fungal fruiting bodies and

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Accepted for publication July 11, 1968.
Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
Abstracted from a thesis submitted by John M. Neal to the Graduate School, University of Washington, Seattle, in partial fulfillment of Master of Science degree requirements.
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saprophytic mycelia frequently exhibit different metabolic capabilities. Such distinctive metabolism is reminiscent of the situation with *Claviceps* species where lysergic acid alkaloids were known to occur in the sclerotium or resting stage of the fungus as early as 1875, but successful production of such derivatives in saprophytic culture was not achieved until 1953 (2).

Mushrooms belonging to the related genera *Panaeolus* and *Psilocybe* are known to contain 5- and 4-hydroxyindole derivatives, respectively. Patterns of accumulation of these metabolites during different growth phases of these fungi reveal a good potential for comparative studies which may contribute to an understanding of basic metabolic interrelationships. Serotonin and 5-hydroxytryptophan accumulate in the fruiting bodies of various species of *Panaeolus* (3–6), but these 5-hydroxyindoles have never been detected in mycelial cultures (7). Psychedelic 4-hydroxytryptamines, usually psilocybin and psilocin, occur in the carpophores of at least 13 *Psilocybe* species (8–10). However, production of 4-hydroxyindoles in submerged culture has been reported for only *Psilocybe cubensis* (Earle) Sing. (11) and *P. baeocystis* Sing. and Smith (12), and no detectable quantities of these compounds were observed in mycelial cultures of other species such as *P. cyanescens* Wakefield (11) which have been investigated.

Presumably, genetic factors contribute either directly or indirectly to the observed differences in patterns of accumulation of indolylic amines in vegetative mycelium and carpophore tissues, but very little is known about the influence of various nutrients or other environmental factors on the accumulation of these compounds or about other biologic processes in these fungi. The relationship of phosphate nutrition to nitrogen metabolism and accumulation of secondary metabolites was selected as a logical focus for initial biologic studies. A reduced availability of phosphate in culture media has been associated with an increased accumulation of such secondary metabolites as clavine alkaloids by *Claviceps* strain 47A (13), chlortetracycline by *Streptomyces aureofaciens* Duggar (14), and volucrisporin by *Volucrispora aurantiaca* Haskins (15). Precursor moieties for clavine alkaloids, chlortetracycline, and volucrisporin are known to be derived, at least in part, from amino acids. The response pattern of phosphate deficiency on nitrogen metabolism in fungi is not well established, but phosphate deficiencies in angiosperms are associated generally with a large increase in free amino acid nitrogen in the cells and a corresponding decrease in protein nitrogen.

Panaeolus campanulatus (Fr.) Quél., *Psilocybe cubensis*, and *P. cyanescens* represented different metabolic capabilities, and isolates of these fungi were selected for this study. The influence of phosphate was studied by growing the fungi in nutrient solutions differing essentially in phosphate content and by noting the distribution pattern of nitrogen between soluble and insoluble cellular components. The presence or absence of detectable quantities of key hydroxyindole derivatives was determined, and the various observations were reviewed for correlating metabolic patterns.

EXPERIMENTAL

Cultures and Nutrient Media—The isolates of *P. campanulatus* and *P. cyanescens* were prepared in 1961 from carpophores collected in western Washington. The culture of *P. cubensis* was obtained from the U.S.D.A Northern Regional Research Laboratories, Peoria, Ill., under the designation NRRL A-9109.

Catalfomo and Tyler (11) reported a medium which permitted psilocybin production when *P. cubensis* was grown in submerged cultures. Preliminary experiments showed that *P. campanulatus* and *P. cyanescens* also developed abundant growth in this medium. Thus, it was designated Medium A (Table I) and selected as a basis for modifications to accommodate the objectives of the proposed study. Determination of phosphate in the yeast extract used in test Media B, C, and D showed approximately 28.6 mg. of phosphate ion per g., and since it was considered desirable to test a

Table I—Composition of Media

Ingredient	g./l. A	B	C	D	E
Glucose	10	10	10	10	10
Ammonium succinate	1	1	1	1	1
Thiamine HCl	0.003	0.003	0.003	0.003	0.003
Difco Yeast Extract	0.5	1	1	1	0.0
KH_2PO_4	0.1	0.250	0.075	0.0	0.005
K_2SO_4	0.0	0.322	0.432	0.482	0.478
Trace minerals[a]					

Add distilled water to make 1 l. adjust, pH to 5.5 with HCl, and autoclave at 15 p.s.i. for 15 min.

[a] All media contained the following trace minerals/l.: $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 0.05 mg.; $ZnSO_4\cdot 7H_2O$, 0.3 mg.; $MnCl_2\cdot 4H_2O$, 0.35 mg.; $FeSO_4\cdot 7H_2O$, 2.5 mg.; $CuSO_4\cdot 5H_2O$, 0.5 mg.; $MgSO_4\cdot 7H_2O$, 500 mg.

medium with very little available phosphate, this component was omitted from Medium E. Since potassium deficiency in some higher plants is associated with a reduced incorporation of amino acids into proteins (16), various amounts of K_2SO_4 were added to the test media to provide a uniform potassium ion concentration.

Growth of Mycelia—Uniform inoculum for each fungus was considered critical. Since the experimental design projected the use of four media and the harvesting of triplicate samples after each of four incubation periods, it was necessary to have sufficient inoculum for 48 cultures. Starter cultures grown in Medium A provided the uniform inoculum. Mycelial mats were cut from three agar-slant stock cultures of each fungus and blended in a Waring micro-blender with 30 ml. of medium. Mycelia of the different fungi varied in friability and required different blending periods to obtain comparable comminution; preliminary observations resulted in the selection of 10-, 15-, and 20-sec. blending periods for *P. campanulatus*, *P. cubensis*, and *P. cyanescens*, respectively. Two-milliliter aliquots of the resulting mycelial suspension were transferred aseptically to six 500-ml. conical flasks each containing 100 ml. of medium. The culture flasks were agitated at 200 r.p.m. on a rotary Gump shaker at 25 ± 2°; after growing for 7 days, the mycelia were separated from the nutrient broths by filtering through a pad of four layers of sterile gauze with the aid of suction. The combined mycelial pellets from the six starter cultures were washed thoroughly with sterile distilled water, transferred to a Waring micro-blender, suspended in 80 ml. of sterile distilled water, and blended for the same period of time utilized for the mycelium from the agar-slant cultures. A 2-ml. volume of this suspension was pipeted aseptically to each 500-ml. conical test flask containing 100 ml. of the appropriate medium. The cultures were placed on the rotary shaker and allowed to grow at 25 ± 2° until harvested.

Approximately 9 days had been reported to permit maximum growth of *P. cubensis* (11). Incubation periods of 5, 7, 9, and 11 days were arbitrarily selected for this study. The mycelial pellets were separated by suction filtration using Whatman No. 1 filter paper. Four milliliters of nutrient broth from each culture was placed in a 4-dr. vial and retained for phosphate determination and thin-layer chromatographic evaluation. The mycelial pellets were washed thoroughly with distilled water and placed on Petri dishes left in a forced draft oven overnight at 47°. The dried material was weighed to provide a proximate indication of growth, ground to a fine powder in a mortar and pestle, and subjected to analyses for phosphate content, nitrogen distribution, and key-metabolite accumulation.

Phosphate Determination—The procedure of Weil-Malherbe and Green (17) was employed with minor modification to determine the total free and organically bound phosphate in media, culture filtrates, and dried mycelia. Accurately measured volumes (varying from 0.1 to 4.0 ml., as needed) of the media and nutrient broths were transferred to 2 × 17-cm. test tubes. The tubes were placed in a boiling water bath and an air stream directed on the solutions until the samples were evaporated to dryness. Each dried residue was digested by adding 1.2 ml. of 60% $HClO_4$ and one drop of fuming HNO_3 and heating over a microburner until the solution became clear and colorless (usually 6 to 10 min.). After cooling, 4 ml. of distilled water was added, and the solution was transferred to a 12-ml. glass-stoppered centrifuge tube. Six milliliters of isobutyl alcohol and 1 ml. of aqueous 5% $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ solution were added to the centrifuge tube, and the mixture was shaken vigorously for 15 sec. After centrifugation, the clear isobutyl alcohol layer was transferred with a pipet to another 12-ml. centrifuge tube, approximately 2 g. of anhydrous Na_2SO_4 was added, and the mixture was agitated for 45 sec. followed by centrifugation. A 5-ml. aliquot of the isobutyl alcohol fraction was placed in a 1.6 × 15-cm. test tube with subsequent addition of 5 ml. of an acidic ethanol solution (prepared by adding 10 ml. of concentrated H_2SO_4 to 490 ml. of absolute ethanol) and 0.2 ml. of a freshly prepared, dilute solution of $SnCl_2\cdot 2H_2O$.[1] After thorough mixing, the solution was centrifuged, and the color intensity of the blue complex was measured in a Bausch & Lomb Spectronic 20 colorimeter. The maximum absorption of the blue complex was determined to be 639 mμ with a Beckman DB recording spectrophotometer, and this wavelength was employed for the colorimetric measurements. The quantity of phosphate in the sample was calculated after comparing the observed absorbance with a standard curve. The standard curve was prepared by subjecting known quantities of KH_2PO_4 to the digestion treatment and other standard manipulations.

Quantitation of phosphate in mycelia was accomplished by digesting an accurately weighed sample of the dried mycelial pellets (usually 5 to 10 mg.) with $HClO_4$ and fuming HNO_3 as described for the broth residue and processing the resultant solution in the prescribed manner. The relatively high concentrations of phosphate in the mycelia necessitated dilution of the final solution with isobutyl alcohol to permit colorimetric evaluation; tests with solutions containing known quantities of phosphate revealed that the accuracy of the assay was unaffected by the use of this alcohol as a diluent.

Nitrogen Determination—A method distinguishing free and bound amino acids or nitrogen was necessary to obtain an indication of the amino acids in fungal cells and their incorporation into proteins. Trichloroacetic acid is commonly used as a protein-precipitating agent (18), and treatment of microbial material with 10% trichloroacetic acid has been effective in extracting free amino acids while precipitating the cellular proteins (19). Separation of the cellular nitrogenous constituents into trichloroacetic acid-soluble and -insoluble fractions does not provide an absolute measure of free amino acids and proteins. Such constituents as purines and pyrimidines would be included in the trichloroacetic acid-soluble fraction, and the glucosamine content of the cell wall would contribute to the trichloroacetic acid-insoluble component. However, determination of trichloroacetic acid-soluble and -insoluble nitrogen was selected for this study since

[1] The dilute solution was prepared daily by diluting 0.5 ml. of a concentrated stock solution with sufficient 1.0 *N* H_2SO_4 to make 20 ml. Ten grams of $SnCl_2\cdot 2H_2O$ was dissolved in 25 ml. of concentrated HCl to give the stock solution.

it offered a convenient means for obtaining a qualified indication of the amino acid distribution.

Weighed samples (50–100 mg.) of the mycelia were extracted with 5-ml. volumes of 10% trichloroacetic acid in 12-ml. glass-stoppered centrifuge tubes placed horizontally in a rack clamped to the rotary shaker. After shaking for 1 hr., the tubes were centrifuged, and the clear supernatant solutions were decanted into 30-ml. Kjeldahl flasks. The marcs were reextracted with second 5-ml. portions of 10% trichloroacetic acid for 1 hr. These second supernatants were added to the initial extracts, and the volumes of liquid were evaporated to approximately 0.5 ml. each by heating the flasks in a boiling water bath while directing an air stream on the solutions. The concentrated extracts were subjected to standard Kjeldahl digestions, distillations, and titrations for nitrogen determination.

Marcs remaining after removal of the trichloroacetic acid-soluble materials were digested in 30-ml. Kjeldahl flasks and processed to quantitate the trichloroacetic acid-insoluble nitrogen.

Detection of Indole Metabolites—The thin-layer chromatographic system A of Leung *et al.* (12) was suitable for rapid, sensitive evaluation of the hydroxyindole metabolites of *Panaeolus* and *Psilocybe* species. Weighed portions (usually 50 mg.) of the dried, powdered mycelia were extracted by shaking with 5 ml. of methanol in 12-ml. glass-stoppered centrifuge tubes for 1 hr. The mixtures were centrifuged, and the clear supernatant solutions were removed with pipets. The marcs were extracted with second 5-ml. volumes of methanol. Preliminary experiments showed that no detectable quantities of tryptamines were present in the supernatant from a third extraction of mycelium known originally to contain a relatively high level of these indoles. The two extracts were combined in a 50-ml. round-bottom flask and evaporated to dryness under reduced pressure at 40° in a flash evaporator. The soluble metabolites in the residue from each sample were dissolved in 0.5 ml. of methanol, and 10-μl. volumes of the extracts were chromatographed to determine if psilocybin, psilocin, serotonin, or 5-hydroxytryptophan was detectable.

Various volumes (0.1 to 10 μl.) of those extracts containing detectable quantities of one or more of the key metabolites were chromatographed to permit comparison of spot size with that of a known quantity of pure reference material and to permit determination of the volume of extract containing the minimum detectable quantity of the constituent. This information was utilized to estimate the approximate concentration of the metabolite in the mycelium. Table II shows the relative migrations of the four key hydroxyindole derivatives and lists the minimum detectable quantities of the pure compounds under the experimental conditions.

Table II—Thin-Layer Chromatographic Characteristics of Key Hydroxyindole Derivatives[a]

Compound	*Rf*	Minimum Detectable Quantity, mcg.
Psilocybin	0.19	0.05
Psilocin	0.79	0.05
Serotonin	0.63	0.05
5-Hydroxytryptophan	0.57	0.025

[a] The chromatographic adsorbant was a mixture of Silica Gel G:Kieselguhr G (2:1), and the solvent system was *n*-propanol:5% NH_4OH (5:2). The chromatograms were sprayed with 2% *p*-dimethylaminobenzaldehyde in acidic ethanol to produce visible chromophores.

Catalfomo *et al.* (11) reported the absence of hydroxyindole derivatives in the nutrient broth from cultures of *P. cubensis* which had produced such metabolites. Presumably, these constituents are retained endocellularly. However, the broth from one flask in each triplicate set was examined for the possible excretion of this type of metabolite. Two-milliliter samples of the nutrient solutions were evaporated to dryness under reduced pressure, methanolic extracts of the residues were prepared, and the extracts were evaluated chromatographically.

RESULTS AND DISCUSSION

A common inoculum was used for the test flasks of each species in the study to provide maximum uniformity of the fungi prior to their growth in the various media. The test flasks were subjected to constant environmental conditions for the designated

Table III—Average Growth and Distribution of Nitrogen and Phosphate in Cultures of *P. campanulatus*

Culture Series[a]	Mycelial Dry Wt., mg.	Mycelial TCA-sol. N[b] mg./flask	%	Mycelial TCA-insol. N mg./flask	%	Ratio, TCA-sol. N/TCA-insol. N	Added Phosphate,[c] mcg./ml.	Phosphate: Mycelium mcg./mg.	Phosphate: Medium mcg./ml.
5B	538 ± 13	3.03 ± 0.10	0.56	15.2 ± 0.1	2.82	0.20	178	16.0 ± 0.3	4.5 ± 0.7
5C	425 ± 11	2.94 ± 0.16	0.69	15.6 ± 0.1	3.66	0.19	65.0	8.6 ± 0.2	0.60 ± 0.07
5D	175 ± 4	2.80 ± 0.04	1.60	6.2 ± 0.2	3.56	0.45	14.3	6.7 ± 0.2	0.64 ± 0.14
5E	93 ± 3	1.23 ± 0.07	1.31	2.9 ± 0.2	3.14	0.41	3.37	5.4 ± 0.1	0.52 ± 0.11
7B	356 ± 1	3.86 ± 0.13	1.08	10.2 ± 0.3	2.88	0.37	178	23.4 ± 1.0	4.1 ± 0.4
7C	403 ± 5	4.18 ± 0.40	1.04	13.8 ± 0.1	3.44	0.30	65.0	8.2 ± 0.4	0.78 ± 0.05
7D	187 ± 2	2.47 ± 0.05	1.32	6.3 ± 0.1	3.42	0.39	14.3	6.3 ± 0.1	0.38 ± 0.04
7E	98 ± 10	1.09 ± 0.10	1.11	2.6 ± 0.3	2.70	0.41	3.37	5.0 ± 0.2	0.30 ± 0.07
9B	315 ± 5	4.44 ± 0.19	1.41	7.2 ± 0.1	2.28	0.62	178	24.5 ± 0.5	15.2 ± 0.5
9C	330 ± 6	4.14 ± 0.01	1.25	9.2 ± 0.2	2.78	0.45	65.0	10.6 ± 0.4	1.2 ± 0.1
9D	228 ± 9	2.45 ± 0.07	1.07	7.6 ± 0.3	3.31	0.33	14.3	5.3 ± 0.0	0.50 ± 0.04
9E	105 ± 1	1.19 ± 0.04	1.13	2.8 ± 0.1	2.62	0.43	3.37	4.6 ± 0.2	0.35 ± 0.00
11B	313 ± 2	4.17 ± 0.07	1.34	6.9 ± 0.1	2.24	0.59	178	23.5 ± 0.8	21.6 ± 0.4
11C	331 ± 5	3.79 ± 0.07	1.14	8.0 ± 0.3	2.43	0.47	65.0	10.7 ± 0.6	1.3 ± 0.0
11D	261 ± 9	2.62 ± 0.07	1.01	8.3 ± 0.4	3.17	0.32	14.3	4.6 ± 0.1	0.58 ± 0.12
11E	124 ± 10	1.30 ± 0.14	1.00	3.4 ± 0.3	2.72	0.38	3.37	4.2 ± 0.0	0.38 ± 0.04

[a] The code designation of the cultures indicates the incubation period and the composition of the medium. Culture series 5B was incubated for 5 days and utilized Medium B. [b] Trichloroacetic acid. [c] Determined by assay of aliquots of uninoculated media.

TABLE IV—AVERAGE GROWTH AND DISTRIBUTION OF NITROGEN AND PHOSPHATE IN CULTURES OF *P. cubensis*

Culture Series[a]	Mycelial Dry Wt., mg.	Mycelial TCA-sol. N[b] mg./flask	%	Mycelial TCA-insol. N mg./flask	%	Ratio, TCA-sol. N/TCA-insol. N	Added Phosphate,[c] mcg./ml.	Phosphate Mycelium mcg./mg.	Phosphate Medium mcg./ml.
5B	397 ± 8	6.62 ± 1.05	1.68	9.4 ± 0.3	2.37	0.72	190	12.9 ± 0.9	38.0 ± 0.0
5C	432 ± 37	3.21 ± 0.34	0.74	15.3 ± 1.4	3.54	0.21	78.4	9.1 ± 0.4	2.8 ± 0.0
5D	226 ± 2	3.64 ± 0.30	1.61	7.6 ± 1.6	3.37	0.50	16.0	7.3 ± 0.8	1.5 ± 0.3
5E	86 ± 8	0.85 ± 0.05	1.00	3.4 ± 0.4	3.94	0.25	3.50	7.7 ± 0.3	0.48 ± 0.04
7B	313 ± 9	5.38 ± 0.52	1.72	9.6 ± 0.6	3.07	0.57	190	18.0 ± 0.7	51.0 ± 1.3
7C	367 ± 18	4.34 ± 0.19	1.29	15.2 ± 0.7	4.13	0.31	78.4	9.5 ± 0.6	4.9 ± 0.0
7D	270 ± 13	4.06 ± 0.21	1.50	10.8 ± 0.4	3.97	0.38	16.0	6.6 ± 0.1	0.56 ± 0.00
7E	100 ± 14	0.91 ± 0.19	0.90	4.2 ± 0.4	4.24	0.22	3.50	6.9 ± 0.1	0.44 ± 0.01
9B	263 ± 6	3.61 ± 0.74	1.37	9.9 ± 0.5	3.77	0.36	190	18.0 ± 2.0	61.0 ± 1.40
9C	347 ± 5	5.82 ± 0.44	1.68	13.8 ± 0.9	3.97	0.43	78.4	11.3 ± 0.4	1.1 ± 0.1
9D	324 ± 18	4.52 ± 0.17	1.40	11.6 ± 0.8	3.55	0.39	16.0	5.7 ± 0.1	0.63 ± 0.04
9E	98 ± 2	0.77 ± 0.06	0.78	4.3 ± 0.1	4.40	0.18	3.50	7.1 ± 0.2	0.48 ± 0.04
11B	257 ± 4	4.07 ± 0.23	1.58	9.0 ± 0.2	3.52	0.45	190	15.0 ± 2.0	70.0 ± 1.3
11C	324 ± 19	5.69 ± 0.18	1.77	11.8 ± 0.2	3.64	0.49	78.4	14.0 ± 2.0	2.8 ± 0.0
11D	325 ± 7	4.92 ± 0.26	1.51	12.5 ± 0.4	3.85	0.39	16.0	5.5 ± 0.3	0.83 ± 0.04
11E	113 ± 10	0.83 ± 0.09	0.73	4.9 ± 0.5	4.30	0.17	3.50	6.0 ± 0.5	0.35 ± 0.00

[a] The code designation of the cultures indicates the incubation period and the composition of the medium. Culture series 5B was incubated for 5 days and utilized Medium B. [b] Trichloroacetic acid. [c] Determined by assay of aliquots of uninoculated media.

incubation periods, and triplicate sets of flasks were analyzed for each variable to minimize the possibility of using extreme values in the formulation of indicative growth and metabolic patterns.

Analytical data for the distribution of nitrogen and phosphate in the cultures are given in Tables III, IV, and V. Growth of all three fungi was related grossly to the phosphate content of the media. The reduced growth rates in Medium D suggested the possibility that phosphate-deficient metabolic patterns might be detectable in cultures grown in this nutrient solution. The very poor growth in Medium E was probably a combined result of limited availability of phosphate and of unavailability of some factor(s) from the yeast extract which was omitted from this medium.

The data showed that phosphate was readily removed from the media by all three species. *P. campanulatus* was particularly efficient in concentrating phosphate in the mycelium. *P. cyanescens* demonstrated the least tendency to concentrate phosphate in the fungal cells, and when this fungus was grown in Medium B, the residual phosphate in the culture broth clearly indicated that this nutrient was available in excess of the requirements of the fungus and in excess of the strain's capacity to store phosphate. *P. campanulatus* and *P. cubensis* grew equally well in Media B and C, indicating the additional phosphate in Medium B was not contributing to the growth of the fungi.

Distribution of phosphate in the various culture broths confirmed the anticipated exocellular release of phosphate from the older cells. Detection of this phenomenon should be easiest in cultures which develop rapidly in phosphate-rich media and which are harvested after longer incubation periods. *P. cubensis* grown in Medium B provided the most pronounced example observed during this investigation.

Evaluation of data on mycelial phosphate, especially that obtained with mycelia from phosphate-poor cultures, requires special attention. Calculation of the total phosphate per flask will reveal more phosphate than was present in the nutrient medium. This situation reflects the endogenous phosphate which was incorporated into the culture flasks with the inoculum. The quantity of phosphate introduced with the inoculum was insignificant for phosphate-rich cultures, but it represented an increasingly larger proportion of the total in flasks with media containing lesser amounts of KH_2PO_4.

TABLE V—AVERAGE GROWTH AND DISTRIBUTION OF NITROGEN AND PHOSPHATE IN CULTURES OF *P. cyanescens*

Culture Series[a]	Mycelial Dry Wt., mg.	Mycelial TCA-sol. N[b] mg./flask	%	Mycelial TCA-insol. N mg./flask	%	Ratio, TCA-sol. N/TCA-insol. N	Added Phosphate,[c] mcg./ml.	Phosphate Mycelium mcg./mg.	Phosphate Medium mcg./ml.
5B	443 ± 5	3.01 ± 0.09	0.68	11.9 ± 0.2	2.68	0.25	164	8.7 ± 0.2	66.0 ± 3.0
5C	452 ± 16	2.78 ± 0.14	0.62	11.7 ± 0.2	2.59	0.24	65.6	7.1 ± 0.4	3.3 ± 0.0
5D	241 ± 6	1.78 ± 0.09	0.74	7.1 ± 0.0	2.96	0.25	15.2	4.9 ± 0.0	0.74 ± 0.09
5E	100 ± 1	0.52 ± 0.06	0.52	3.0 ± 0.0	3.00	0.17	2.90	4.7 ± 0.1	0.30 ± 0.04
7B	413 ± 5	2.23 ± 0.07	0.54	11.6 ± 0.3	2.82	0.19	164	8.9 ± 0.4	69.0 ± 1.3
7C	426 ± 5	2.18 ± 0.15	0.51	12.5 ± 0.2	2.92	0.18	65.6	7.3 ± 0.2	5.5 ± 0.2
7D	311 ± 7	2.00 ± 0.22	0.64	8.5 ± 0.2	2.74	0.23	15.2	4.0 ± 0.1	0.67 ± 0.09
7E	136 ± 2	0.52 ± 0.15	0.55	3.7 ± 0.1	2.71	0.14	2.90	4.0 ± 0.0	0.49 ± 0.18
9B	365 ± 3	1.84 ± 0.17	0.50	12.8 ± 0.2	3.50	0.14	164	9.3 ± 0.4	68.0 ± 2.3
9C	386 ± 7	1.88 ± 0.25	0.49	12.9 ± 0.9	3.36	0.15	65.6	8.5 ± 0.1	5.3 ± 0.3
9D	300 ± 7	1.74 ± 0.06	0.58	8.3 ± 0.5	2.75	0.21	15.2	4.1 ± 0.1	0.72 ± 0.09
9E	135 ± 8	0.73 ± 0.04	0.55	4.3 ± 0.1	3.19	0.17	2.90	4.0 ± 0.2	0.39 ± 0.04
11B	362 ± 4	2.02 ± 0.10	0.56	11.7 ± 0.2	3.23	0.17	164	9.6 ± 0.2	71.0 ± 1.7
11C	362 ± 14	1.85 ± 0.05	0.51	11.4 ± 0.6	3.14	0.16	65.6	8.8 ± 0.2	5.1 ± 0.0
11D	260 ± 10	1.62 ± 0.15	0.62	7.6 ± 0.1	2.91	0.21	15.2	4.3 ± 0.1	0.81 ± 0.02
11E	148 ± 11	0.73 ± 0.08	0.49	4.2 ± 0.3	2.85	0.17	2.90	3.8 ± 0.1	0.49 ± 0.09

[a] The code designation of the cultures indicates the incubation period and the composition of the medium. Culture series 5B was incubated for 5 days and utilized Medium B. [b] Trichloroacetic acid. [c] Determined by assay of aliquots of uninoculated media.

The quantities of trichloroacetic acid-soluble and -insoluble nitrogen in the mycelia were considered indicative of the free amino acid pool and the protein content, respectively. Various carbohydrate metabolites normally constitute a major portion of the mycelial dry weight, and data on the nitrogenous fractions were evaluated on the basis of nitrogen content per unit weight of mycelium and per flask to reveal changes in the distribution of nitrogenous constituents which were independent of changes in the carbohydrate metabolites. Ratios of the trichloroacetic acid-soluble and -insoluble nitrogen were particularly useful for easy detection of a relative redistribution of nitrogenous metabolites between the two fractions.

Comparison of the maximum mycelial dry weights and the incorporation of nitrogen into trichloroacetic acid-insoluble metabolites suggested that *P. cubensis* reached maximum growth prior to the maximum formation of proteins. A similar, but less pronounced, tendency appeared to exist for *P. cyanescens*. A contrasting pattern in which maximum growth and protein formation were more nearly concurrent was noted for *P. campanulatus*. The majority of the mycelial nitrogen occurred in the bound form in the three species under all of the experimental conditions. The highest concentrations of soluble nitrogen and the highest ratios of soluble to insoluble nitrogen were observed with cultures of *P. cubensis* and *P. campanulatus*. *P. cyanescens* appeared to have a limited tendency for accumulation of soluble nitrogenous metabolites.

The most striking increases in soluble nitrogen during the progressive incubation periods were observed in mycelia of *P. campanulatus* grown in Media B and C and of *P. cubensis* grown in Medium C. The increase in soluble nitrogen in the mycelium of *P. cubensis* was accompanied by a corresponding decrease in the total insoluble nitrogen in the fungal tissue, and it appeared that the net increase in free amino acid nitrogen was probably a result of protein catabolism. However, only a modest net increase in soluble nitrogen was associated with a marked decrease in the total insoluble nitrogen in the mycelia of *P. campanulatus* growth in Media B and C; the decreased total mycelial nitrogen indicated exocellular excretion of nitrogen metabolites by this species during the longer incubation periods.

Chromatographic examination of the mycelial samples for psilocybin, psilocin, 5-hydroxytryptophan, or serotonin revealed detectable quantities of psilocybin and psilocin in *P. cubensis* grown in certain media for appropriate incubation periods (Table VI). None of the key metabolites was detected in mycelia of *P. campanulatus* or *P. cyanescens* or in any of the nutrient broths. When the indoles were not detected following chromatographic evaluation of 10 μl. of an extract prepared from 50 mg. of dried mycelium, the levels of accumulation of psilocybin, psilocin, and serotonin were less than 0.005%, and the concentration of 5-hydroxytryptophan was less than 0.0025%. Other components in the mycelial extracts prevented good resolution when more than 10-μl. quantities were applied to the chromatograms. Minimum concentrations of the compounds which could have been detected in the media were 1.25 mcg./ml. of psilocybin, psilocin, and serotonin and 0.63 mcg./ml. of 5-hydroxytryptophan.

TABLE VI—OCCURRENCE AND ESTIMATED ACCUMULATION OF PSILOCYBIN AND PSILOCIN IN MYCELIUM OF *P. cubensis*

Culture Series[a]	Psilocybin, mcg./mg.	Psilocin, mcg./mg.
5B	—[b]	—
5C	0.1	—
5D	0.1	—
5E	—	—
7B	0.5	—
7C	0.5	0.1
7D	0.5	—
7E	—	—
9B	2.0	0.2
9C	0.5	—
9D	0.05	—
9E	—	—
11B	2.0	—
11C	1.0	—
11D	0.2	—
11E	—	—

[a] The code designation of the cultures indicates the incubation period and the composition of the medium. Culture series 5B was incubated for 5 days and utilized Medium B. [b] None detectable.

Accumulation of psilocybin in the mycelium of *P. cubensis* appeared to have some relationship to phosphate nutrition and general nitrogen metabolism. Formation of this metabolite occurred predominantly after maximum growth, and the results with Medium B suggested that excess phosphate in the mycelium may delay or suppress secondary tryptamine metabolism in this fungus. The formation of secondary nitrogenous constituents after growth-associated somatic protein synthesis has ceased to remove large quantities of amino acids from the soluble nitrogen pool is consistent with theoretical considerations and corresponds to the pattern recently reported with alkaloid formation in *Claviceps* strain SD58 (20). Catalfomo *et al.* (11) utilized a nutrient solution similar to Medium C in studies of *P. cubensis* and reported maximum psilocybin production after a 7-day incubation period and maximum growth after 9 days; the long incubation period required to reach maximum growth suggests that relatively small quantities of inoculum were utilized in the investigation, and a mixture of cells representing widely different stages of metabolic activity may account for the apparent maximum psilocybin content during the growth phase of the organism. The quantity of inoculum employed in this investigation was sufficient to permit rapid growth of the fungus when other conditions were favorable, and the observed tendency for psilocybin formation during the stationary phase of the culture appears valid.

CONCLUSIONS

The studies revealed that saprophytic mycelium of *P. cubensis* was characterized by a relatively high soluble nitrogen component and by the capacity to accumulate secondary tryptamine metabolites under selected conditions. Mycelium of the closely related *P. cyanescens* lacked the capacity to accumulate appreciable quantities of soluble nitrogenous compounds or detectable quantities of key tryptamines. Experimental design precluded interpretation of cause and effect, so there is no direct evidence that a

limited free amino acid pool in *P. cyanescens* is responsible for the failure of the fungus to accumulate psilocybin in saprophytic cultures. However, the results do suggest that the isolate of *P. cyanescens* is less responsive to phosphate nutrients and has narrower metabolic tolerance than *P. cubensis* NRRL A-9109.

P. campanulatus appeared to be the most metabolically responsive of the species tested. It grew well under the saprophytic conditions and appeared to have an adequate soluble nitrogen pool. These observations suggest the improbability of a limited supply of common amino acids as the basic factor behind the lack of serotonin production in saprophytic cultures. Some key factor controlling the formation or function of tryptophan hydroxylase is probably responsible for the different metabolic capabilities of carpophores and vegetative mycelium of this basidiomycete.

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Basidiomycete cultures—phosphate effect
Phosphate effect—trichloroacetic acid-soluble, -insoluble nitrogen metabolites
Fungal free amino acid pool—hydroxytryptamine derivatives accumulation
Colorimetric analysis—spectrophotometer
TLC—separation, identity

Baeocystin and Norbaeocystin: New Analogs of Psilocybin from *Psilocybe baeocystis*

By A. Y. LEUNG* and A. G. PAUL

Two new 4-phosphoryloxytryptamine derivatives have been isolated from *Psilocybe baeocystis* grown in submerged cultures. Using ultraviolet, infrared, and mass spectral analyses the structures have been determined. Both are analogs of the psychotomimetic, psilocybin, and have been named baeocystin (monomethyl analog) and norbaeocystin (demethyl analog).

PSILOCYBIN was first isolated from a Mexican psychotomimetic mushroom, *Psilocybe mexicana* Heim, by Hofmann *et al.* (1). While the occurrence of this compound and its dephosphorylated derivative, psilocin, has been established in several species of mushrooms, analogs have not yet been described.

The formation of psilocybin in submerged cultures of *Psilocybe baeocystis* Singer and Smith has been reported previously (2). In a communication (3) the authors also have reported the isolation of baeocystin, a monomethyl analog of psilocybin, from this same fungus. The present report deals with details of the procedure for the isolation and characterization

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Accepted for publication June 25, 1968.
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PSILOCYBIN was first isolated from a Mexican psychotomimetic mushroom, *Psilocybe mexicana* Heim, by Hofmann *et al.* (1). While the occurrence of this compound and its dephosphorylated derivative, psilocin, has been established in several species of mushrooms, analogs have not yet been described.

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of baeocystin and of a second analog, norbaeocystin.

EXPERIMENTAL

Plant Materials—Fungal tissue was obtained from submerged cultures of the organism grown in the medium previously reported (2). Mycelial pellets were collected on a Büchner funnel, washed with distilled water, and freeze dried.

Isolation Procedure—The ground plant material (23.2 g.) was defatted with petroleum ether (30–60°) in a Soxhlet extractor (Fig. 1). After removal of solvent at room temperature, the tissue was transferred to a conical flask and extracted with successive portions of methanol at room temperature, using a magnetic stirrer, until a concentrated aliquot of an extract gave little or no color reaction with Ehrlich's reagent. The combined extracts (4 l.) were concentrated to dryness under vacuum at 40° using a Rinco rotating evaporator. The residue was mixed with 20 g. of dry powdered cellulose which had been extracted with water-saturated 1-butanol. The mixture was added to the top of a 3.5-cm. o.d. column packed to a height of 94 cm. with powdered cellulose which had been extracted with water-saturated 1-butanol for several days. The column was developed with this same solvent and 20-ml. fractions were collected at a rate of approximately 40 ml./hr. (Eluate I) using an automatic fraction collector.

Thin-layer chromatography (TLC) (2) of Eluate I indicated that Fractions 46–76 contained psilocin, 77–84 contained psilocybin and traces of psilocin, 85–149 contained a mixture of psilocybin, baeocystin, and norbaeocystin, and 150–305 contained these three compounds grossly contaminated with glycine. Fractions 85–149 were combined (Fraction 1) as were Fractions 150–305 (Fraction 2).

The solvent was removed from Fraction 1, the residue was mixed with 20 ml. of warm methanol, and the mixture filtered. The filtrate was concentrated to approximately 5 ml. at room temperature and again filtered. The filtrate was placed in a freezer at −23° for 8 days. The rosette crystals which formed were removed by filtration, washed with cold methanol, and dried in a desiccator. The mother liquid was concentrated, filtered, and returned to the freezer. Additional crystalline material was obtained giving a total yield of 140 mg. (m.p. 199–204° dec.). TLC analysis indicated it to be a mixture of psilocybin, baeocystin, and norbaeocystin.

Solvent was removed from Fraction 2, the residue dissolved in 25 ml. of warm methanol, and the solution concentrated to approximately 6 ml. The concentrate was filtered and the filtrate placed in the freezer for several hours. The heavy precipitate which formed was removed by filtration. TLC analysis indicated that it was glycine. The filtrate was further concentrated to 2 ml. and placed in the freezer for 3 days. No crystalline material was obtained.

The filtrate was combined with the mother liquid from Fraction 1 and this combined solution was mixed with 5 g. of extracted powdered cellulose. The mixture was dried and transferred to a powdered cellulose column (3.5 × 97 cm.) which had previously been extracted with water-saturated 1-butanol. The column was developed with this solvent and fractions richest in psilocybin, baeocystin, and norbaeocystin, but free of glycine, were combined (Fraction 3).

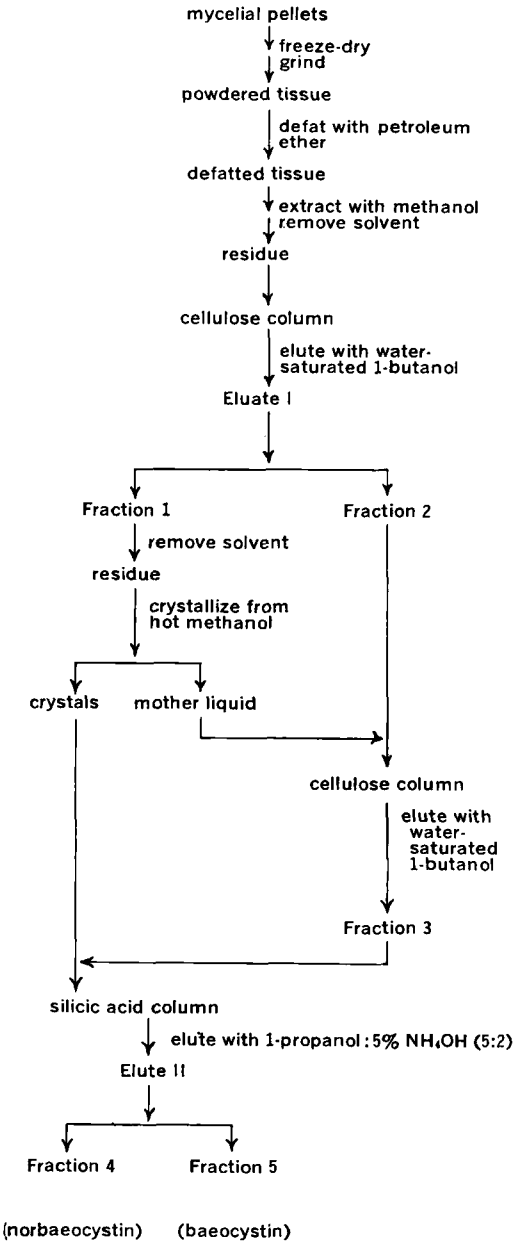


Fig. 1—Isolation procedure.

Solvent was removed from Fraction 3 and the residue was dissolved in a small volume of hot methanol. One hundred milligrams of crystalline material isolated from Fraction 1 was added to the solution and additional hot methanol was added to completely dissolve the material. This solution was transferred to a column of activated silicic acid (3 × 45 cm.). The column was developed with 1-propanol–5% ammonium hydroxide (5:2), using pressure, at a rate of approximately 9 ml./hr.

Eluate II was collected in 3-ml. fractions. TLC analysis of Eluate II indicated that Fractions 25–66 contained norbaeocystin, 70–175 contained baeocystin, and 180–270 contained psilocybin. Fractions 25–66 were combined (Fraction 4), as were Fractions 70–175 (Fraction 5).

The solvent was removed from Fraction 4 and the residue was dissolved in approximately 7 ml. of hot methanol. The solution was concentrated to approximately 1 ml., filtered, further concentrated to approximately 0.5 ml., and placed in the freezer. Crystals which formed at the end of 40 days were removed by filtration, washed with cold methanol–water (1:1), and dried in a desiccator. The yield of norbaeocystin (m.p. 188–192° dec.) was 2 mg.

The solvent was removed from Fraction 5 and the residue was dissolved in approximately 9 ml. of hot methanol. This solution was concentrated to approximately 1.5 ml., filtered, and the filtrate placed in the freezer. After 3 days, crystals which formed were removed by filtration and dried in a desiccator. The yield of baeocystin (m.p. 254–258° dec.) was 6.5 mg.

The extraction was repeated using 58.9 g. of fnngal tissue. The procedures followed were identical with those described except that it was nccessary to chromatograph Fraction 2 on a cellulose column prior to combining this fraction with the mother liquid of Fraction 1, in order to free it of glycine. Fraction 4 yielded 4 mg. of norbaeocystin and Fraction 5 yielded 8 mg. of baeocystin.

Characterization—Both norbaeocystin and baeocystin exhibited identical color reactions on TLC with Ehrlich's reagent and with a modified phosphate reagent (4) as did psilocybin. On two-dimensional TLC, using solvent systems *A* and *B*, these compounds chromatographed close to psilocybin but far from all the other indole compounds tested (2). The R_f values of norbaeocystin were 0.17 ± 0.02 (solvent *A*) and 0.58 ± 0.02 (solvent *B*), while those of baeocystin were 0.16 ± 0.01 (solvent *A*) and 0.46 ± 0.02 (solvent *B*).

The UV spectra[1] of baeocystin and norbaeocystin were superimposable and were identical with that of psilocybin, showing absorption maxima at 221, 268, 280, and 290 mμ (Fig. 2), indicating that they all have the same chromophore, a 4-phosphoryloxytryptamine nucleus. Slightly different chromophores such as those present in psilocin or bufotenine have distinctly different absorption maxima (1, 5).

The IR spectrum[2] of norbaeocystin showed sharp absorption peaks at 3,376 cm.$^{-1}$ and 1,640 cm.$^{-1}$ (Fig. 3) and that of baeocystin showed sharp absorption peaks at 3,275 cm.$^{-1}$ and 1,640 cm.$^{-1}$ (Fig. 4) which are absent in the IR spectrum of psilocybin (1). These absorption bands are indicative of primary or secondary amines.

The low resolution mass spectrum[3] of norbaeocystin showed a weak molecular ion peak at m/e = 256, corresponding to I. Both low- and high-resolution mass spectra[4] showed a strong peak at

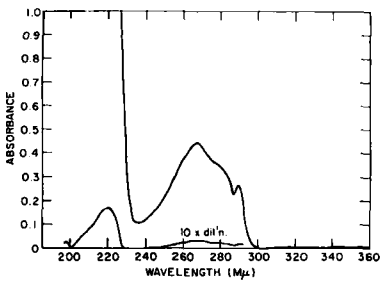
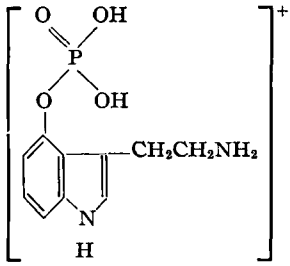


Fig. 2—Ultraviolet absorption spectrum of baeocystin and norbaeocystin (20 mcg./ml. methanol).

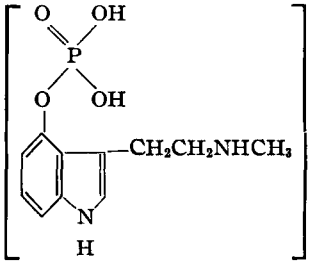
m/e = 176 due to the dephosphorylated species. The empirical formula of this species was shown by high-resolution mass spectral data to be $C_{10}H_{12}N_2O$ with an accurate mass of 176.0957 (calcd., 176.0950).



I

The low-resolution mass spectrum of baeocystin showed a weak molecular ion peak at m/e = 270, corresponding to II. Both low- and high-resolution mass spectra showed a strong peak at m/e = 190 due to the dephosphorylated species. The empirical formula of this species was shown by high-resolution mass spectral data to be $C_{11}H_{14}N_2O$ with an accurate mass of 190.1102 (calcd., 190.1106).

Similarly, psilocybin gave a weak molecular ion peak at m/e = 284 and a strong peak of the dephosphorylated species at m/e = 204.



II

In addition, baeocystin and psilocybin showed strong peaks at m/e = 44 [$CH_2{=}\overset{+}{N}HCH_3$] and m/e = 58 [$CH_2{=}\overset{+}{N}(CH_3)_2$], respectively. However, the corresponding peak of norbaeocystin at m/e = 30 [$CH_2{=}\overset{+}{N}H_2$] lacked prominence. This may be attributed to the lack of resonance of this ion as contrasted with the other two.

Apart from these differences, the mass spectra of all three compounds showed prominent peaks at

[1] Beckman DK-2A ratio recording spectrophotometer.
[2] Perkin-Elmer 337 grating infrared spectrophotometer.
[3] The authors are indebted to Dr. M. Grostic, The Upjohn Co., Kalamazoo, Mich., for the low-resolution mass spectral data.
[4] The authors are indebted to Dr. F. Vane, Hoffmann-LaRoche, Inc., Nutley, N. J., for the high-resolution mass spectral data.

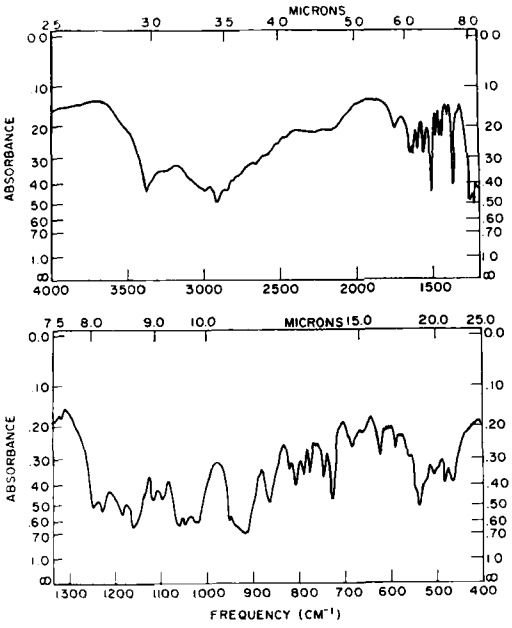


Fig. 3—Infrared spectrum of norbaeocystin (0.9% in KBr).

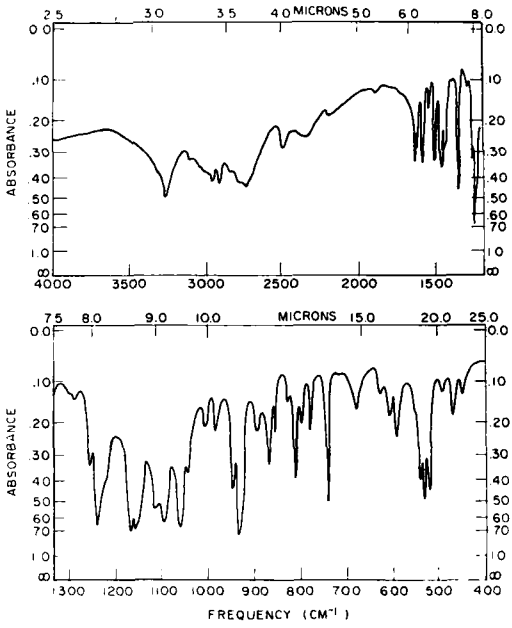


Fig. 4—Infrared spectrum of baeocystin (1.0% in KBr).

m/e = 160, 159, 146, 130, and 117. The proposed fragmentation patterns of these compounds are similar (Fig. 5).

These data indicate that baeocystin is the monomethyl analog (II) and that norbaeocystin is the demethyl analog (I) of psilocybin.

DISCUSSION AND CONCLUSIONS

Despite the simplicity of the structure of psilocybin, there have been no convincing reports on the occurrence of its analogs in nature. The only reports which suggest their presence in fungi have been made by Eugster (6) and by Stein *et al.* (7). From

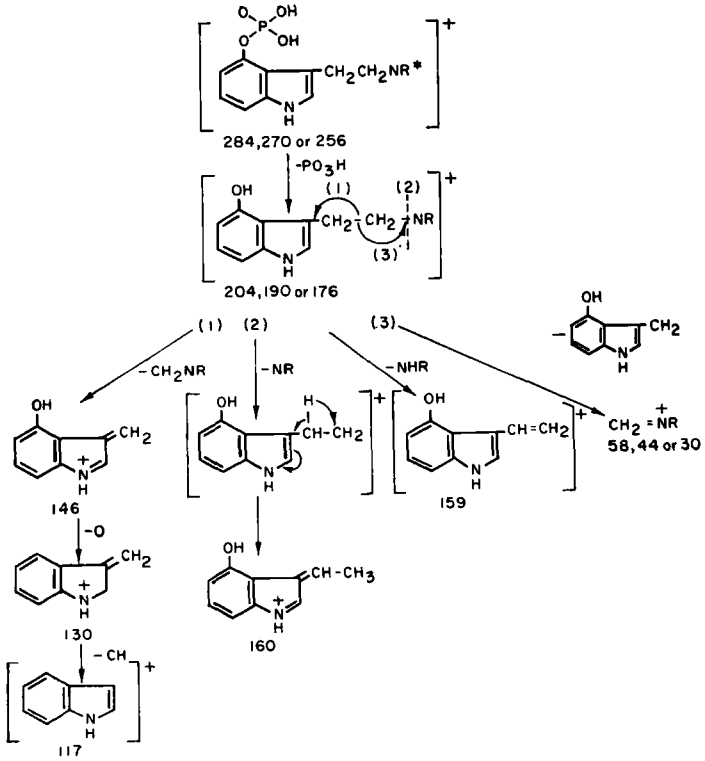


Fig. 5—Proposed fragmentation patterns of psilocybin, baeocystin, and norbaeocystin. R = $(CH_3)_2$, psilocybin; R = HCH_3, baeocystin; R = H_2, norbaeocystin.

Amanita muscaria (Fr.) Hook, Eugster isolated an extremely unstable 4-substituted indole derivative having UV spectrum practically identical with that of psilocybin. Stein *et al.* also isolated an unknown compound from *Panaeolus venenosus* Murr. This compound also had an almost identical UV spectrum as psilocybin, with absorption maxima at 220, 265, and 288 mμ. Its high melting point (>250° dec.) suggests its identity with baeocystin.

The occurrence of baeocystin and norbaeocystin in *P. baeocystis* indicates that this fungus can serve as an appropriate organism for studying the biosynthesis and the metabolism of psilocybin. Earlier studies by Agurell *et al.* (8, 9) indicated that *Psilocybe cubensis* uses the following pathway in the biosynthesis of psilocybin: tryptophan → tryptamine → *N*-methyltryptamine → *N,N*-dimethyltryptamine → psilocin → psilocybin. Recent studies by Agurell and Nilsson (10) suggest that this fungus can also use an alternative route wherein phosphorylation precedes methylation. The high concentration of 4-hydroxytryptamine incorporated into psilocybin and the detection of psilocybin-like compounds were cited as evidence for this alternative pathway. The presence of psilocybin analogs in *P. baeocystis* suggests that the alternative pathway is probably the major route utilized by this fungus. Hence, it should be an ideal organism for further study of the alternative route.

The isolation of baeocystin and norbaeocystin offers a possibility of testing the serotonin hypothesis of mental illness (11). Norbaeocystin and baeocystin are the closest known serotonin analogs which have one of their enzyme-susceptible groups protected. Whether or not this may have any biochemical or pharmacological significance remains to be studied.

SUMMARY

The detection of unknown tryptamine derivatives in *P. baeocystis* has led to the isolation of two new compounds from submerged cultures of this fungus. The isolation procedure involved column chromatography of a methanol extract of the fungal tissue on powdered cellulose and on activated silicic acid. The structures of these compounds have been determined to be the monomethyl and demethyl analogs of psilocybin by TLC characteristics, color reactions, UV, IR, and mass spectral analyses. These compounds have been named baeocystin (monomethyl) and norbaeocystin (demethyl).

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Keyphrases

Psilocybin analogs—*Psilocybe baeocystis*
Baeocystin—isolation, identification
Norbaeocystin—isolation, identification
TLC—separation, identity
UV spectrophotometry—identity
IR spectrophotometry—identity
Mass spectroscopy—structure

N-Acyl Analogs of *N*-(2-Cyanoethyl)cyclohexylamine with CNS Activity

By W. D. ROLL

A series of substituted aryl amides of *N*-(2-cyanoethyl)cyclohexylamine was synthesized and screened for their effect on the central nervous system. Members of this series exhibit a pronounced effect on the spontaneous motor activity of mice, usually accompanied by varying degrees of activity on blood pressure in rats.

SUBSTITUTED AMIDE analogs of *N*-(2-cyanoethyl)cyclohexylamine (Abbott Laboratories) appear to act on the central nervous system either by stimulation or depression in small animals depending on the dosage. The compounds reported herein exert a depressant action on the spontaneous motor activity of mice at a dosage of 4

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Amanita muscaria (Fr.) Hook, Eugster isolated an extremely unstable 4-substituted indole derivative having UV spectrum practically identical with that of psilocybin. Stein *et al.* also isolated an unknown compound from *Panaeolus venenosus* Murr. This compound also had an almost identical UV spectrum as psilocybin, with absorption maxima at 220, 265, and 288 mμ. Its high melting point (>250° dec.) suggests its identity with baeocystin.

The occurrence of baeocystin and norbaeocystin in *P. baeocystis* indicates that this fungus can serve as an appropriate organism for studying the biosynthesis and the metabolism of psilocybin. Earlier studies by Agurell *et al.* (8, 9) indicated that *Psilocybe cubensis* uses the following pathway in the biosynthesis of psilocybin: tryptophan → tryptamine → *N*-methyltryptamine → *N,N*-dimethyltryptamine → psilocin → psilocybin. Recent studies by Agurell and Nilsson (10) suggest that this fungus can also use an alternative route wherein phosphorylation precedes methylation. The high concentration of 4-hydroxytryptamine incorporated into psilocybin and the detection of psilocybin-like compounds were cited as evidence for this alternative pathway. The presence of psilocybin analogs in *P. baeocystis* suggests that the alternative pathway is probably the major route utilized by this fungus. Hence, it should be an ideal organism for further study of the alternative route.

The isolation of baeocystin and norbaeocystin offers a possibility of testing the serotonin hypothesis of mental illness (11). Norbaeocystin and baeocystin are the closest known serotonin analogs which have one of their enzyme-susceptible groups protected. Whether or not this may have any biochemical or pharmacological significance remains to be studied.

SUMMARY

The detection of unknown tryptamine derivatives in *P. baeocystis* has led to the isolation of two new compounds from submerged cultures of this fungus. The isolation procedure involved column chromatography of a methanol extract of the fungal tissue on powdered cellulose and on activated silicic acid. The structures of these compounds have been determined to be the monomethyl and demethyl analogs of psilocybin by TLC characteristics, color reactions, UV, IR, and mass spectral analyses. These compounds have been named baeocystin (monomethyl) and norbaeocystin (demethyl).

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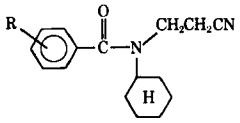
TABLE I—*N*-ACYL ANALOGS OF *N*-(2-CYANOETHYL)CYCLOHEXYLAMINE



Compd. No.	R	M.p., °C.	UV[a] λ, mμ	UV[a] ε	IR C=O	IR CN	Yield, %	Formula	Anal., % Calcd.	Anal., % Found
1	*p*-CH_3O-	77–78	282	1679	1,630	2,265	76.8	$C_{17}H_{22}N_2O_2$	C, 71.30 H, 7.74	C, 71.33 H, 7.78
2	*m*-CH_3O-	Oil[b]	282	2040	1,630	2,265	75.0	$C_{17}H_{22}N_2O_2$	C, 71.30 H, 7.74	C, 71.77 H, 7.83
3	*o*-CH_3O-	43–44	282	2611	1,630	2,265	74.5	$C_{17}H_{22}N_2O_2$	C, 71.30 H, 7.74	C, 71.45 H, 7.80
4	*p*-Cl-	93–94	266	1500	1,630	2,265	79.4	$C_{16}H_{19}ClN_2O$	C, 66.08 H, 6.58	C, 66.20 H, 6.50
5	*m*-Cl-	48–49	266	757	1,630	2,265	75.0	$C_{16}H_{19}ClN_2O$	C, 66.08 H, 6.58	C, 66.12 H, 6.63
6	*o*-Cl-	56–57	266	391	1,630	2,265	78.4	$C_{16}H_{19}ClN_2O$	C, 66.08 H, 6.58	C, 66.14 H, 6.60
7	*p*-CH_3-	74–75	264.5	1220	1,630	2,265	72.5	$C_{17}H_{22}N_2O$	C, 75.52 H, 8.20	C, 75.80 H, 8.25
8	*m*-CH_3-	48–49	264.5	1785	1,630	2,265	71.5	$C_{17}H_{22}N_2O$	C, 75.52 H, 8.20	C, 75.89 H, 8.29
9	*o*-CH_3-	56–57	264.5	451.6	1,630	2,265	72.5	$C_{17}H_{22}N_2O$	C, 75.52 H, 8.20	C, 75.92 H, 8.30

[a] Measured in 95% EtOH. [b] Purified by chromatography (eluted with petroleum ether) on silica gel, n_D^{25} 1.5390. TLC showed single spot.

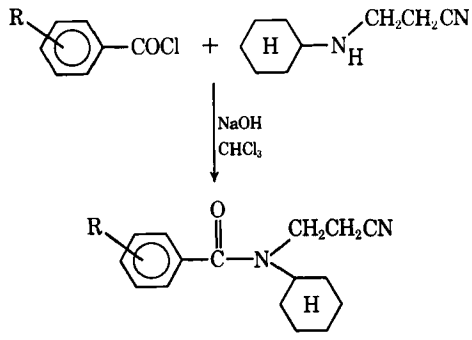
mg./kg. and, in addition, they produce a fall in blood pressure in rats. The methoxybenzyol analogs (Compounds 1, 2, 3) caused the most profound effects on both the central nervous system and the blood pressure; the chlorobenzoyl analogs (Compounds 4, 5, 6) demonstrated similar but intermediate activity; and the toluoyl analogs (Compounds 7, 8, 9) appeared to have the least effect. Within each group the *para*-substituted derivative was the most potent compound.

At a dosage of 2 mg./kg. the *para*- and *meta*-methoxybenzoyl analogs (Compounds 1 and 2) produced an increase in the spontaneous activity of mice, and the former compound antagonized the depressant action of perphenazine. The *para*-chlorobenzoyl analog (Compound 4) also increased the spontaneous motor activity of mice at this dosage.

EXPERIMENTAL

Analyses for carbon and hydrogen were obtained with a Coleman carbon-hydrogen analyzer. Melting points were determined using a Mettler FP-1 melting and boiling point apparatus. IR absorption spectra were obtained with a Perkin-Elmer model 137-B spectrophotometer and UV data were obtained with a Bausch and Lomb Spectronic 600.

Syntheses—The compounds were prepared (Scheme I) by a modification of the Schotten-Baumann (1, 2) reaction. A mixture of 15 ml. of chloroform, 0.01 mole of *N*-(2-cyanoethyl)cyclohexylamine, 60 ml. of 5% sodium hydroxide, and 0.01 mole of acyl halide was shaken in a separator at room temperature until the exothermic reaction was complete. The chloroform layer was washed with water, dried with anhydrous sodium sulfate, and evaporated *in vacuo* to give viscous yellow oils which crystallized on standing for a period of several weeks. The crude products were recrystallized from aqueous ethanol to give the pure products listed in Table I.



where R = *o*, *m*, *p*-OCH_3 (Compounds 3, 2, 1)
o, *m*, *p*-Cl (Compounds 6, 5, 4)
o, *m*, *p*-CH_3 (Compounds 9, 8, 7)

Scheme I

Pharmacology—The depressant/excitatory effects of the compounds were determined in mice with actophotometers (Metro Industries, Inc.). The experimental design and statistical treatment of data were that described by Watzman and co-workers (3). The total body movements of single animals were recorded at 30-min. intervals over a period of 2 hr. and the mean square root of the actual number of counts was plotted against time (Figs. 1–4). Twelve animals were used to study the effect of each compound at each dosage level.

Direct blood pressure measurements were conducted in Wistar rats under urethan anesthesia, 1.2

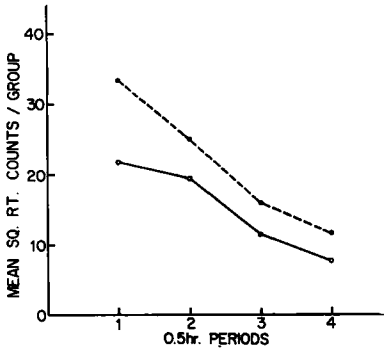


Fig. 1—The effect of N-(p-*methoxybenzoyl*)-N-(2-*cyanoethyl*)*amine at elevated dosage, 4 mg./kg. Key:* ●, *propylene glycol.*

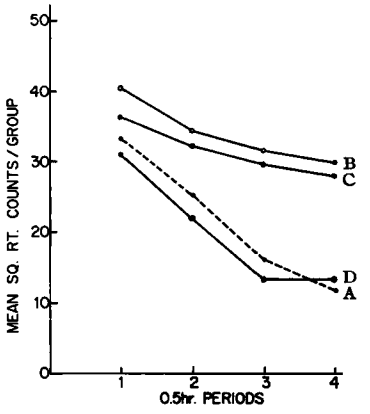


Fig. 2—The effects of N-*methoxybenzoyl analogs on the spontaneous motor activity of mice. Key:* A, *propylene glycol;* B, p-*methoxy;* C, m-*methoxy;* D, o-*methoxy.*

g./kg. i.p., with a mercury manometer connected to the carotid artery. Injections were made *via* the femoral vein. The effects of Compounds 1, 2, and 4 (Figs. 5–7) represent the mean responses of eight test animals.

Indirect blood pressure measurements were carried

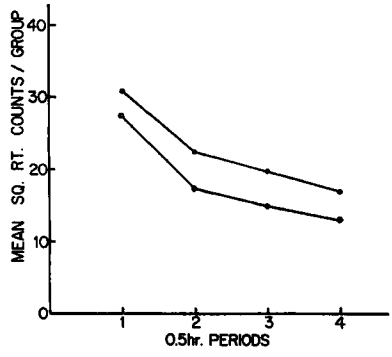


Fig. 3—Spontaneous motor activity of mice: the antagonizing effect of N-(p-*methoxybenzoyl*)-N-(2-*cyanoethyl*)*cyclohexylamine on perphenazine-treated animals. Key:* ◐, *perphenazine;* ●, *perphenazine +* p-*methoxybenzoyl analog.*

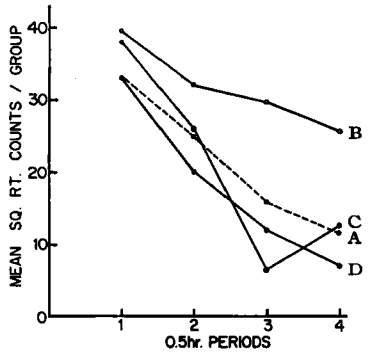


Fig. 4—The effects of N-*chlorobenzoyl analogs on the spontaneous motor activity of mice. Key:* A, *propylene glycol;* B, p-*chloro;* C, m-*chloro;* D, o-*chloro.*

out in normotensive Wistar rats using the photoelectric tensometer (Metro Industries, Inc.). The rats were trained for indirect systolic blood pressure determinations and the methodology was that of Coates *et al.* (4). Each systolic blood pressure value in Fig. 8 represents the mean response of eight animals.

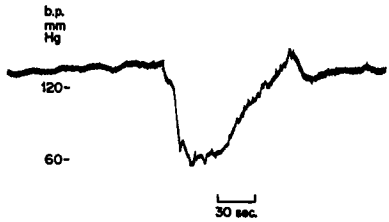


Fig. 5—The effect of Compound 1 on blood pressure in the rat.

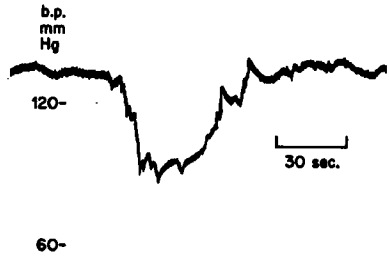


Fig. 6—The effect of Compound 2 on blood pressure in the rat.

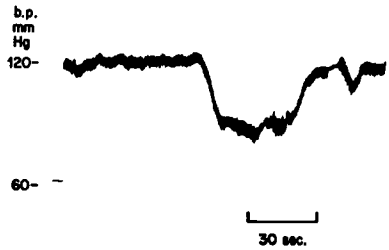


Fig. 7—The effect of Compound 4 on blood pressure in the rat.

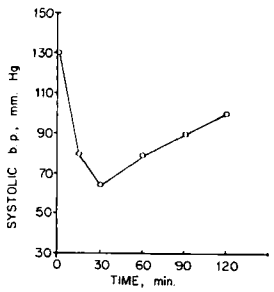


Fig. 8—The effect of Compound 1 on systolic blood pressure, 4 mg./kg. i.p.

RESULTS

The oral administration of 4 mg./kg. of Compound 1, *N*-(*p*-methoxybenzoyl)-*N*-(2-cyanoethyl)cyclohexylamine, in propylene glycol to mice resulted in a significant reduction in the spontaneous motor activity of mice (Fig. 1). A quieting or "taming" effect was seen in the animal at this dosage. Ataxia was clearly discernable in the animal at elevated dosages, *i.e.*, 10 mg./kg. The rat responded even more dramatically to elevated dosages, assuming a cataleptoid posture.

At slightly lower oral dosages, *i.e.*, 2 mg./kg. in propylene glycol, Compounds 1 and 2, *N*-(*m*-methoxybenzyl)-*N*-(2-cyanoethyl)cyclohexylamine, produced excitation in mice, while Compound 3, the *o*-methoxybenzoyl analog caused a slight reduction in total activity (Fig. 2). This dosage level, in the case of Compound 1, rather effectively antagonized the tranquilizing activity of orally, simultaneously administered 0.4 mg./kg. doses of perphenazine (Fig. 3).

Oral doses, 2 mg./kg. in propylene glycol, of Compound 4, *N*-(*p*-chlorobenzoyl)-*N*-(2-cyanoethyl)-cyclohexylamine, produced a high degree of excitation (Fig. 4), but its antagonism to 0.4-mg./kg. doses of perphenazine was insignificant.

Although depression was the predominant symptom of animals receiving 4 mg./kg. of this compound, the degree of reduction in spontaneous motor activity was much less than in the case of Compound 1.

The *m*-chlorobenzoyl analog, Compound 5, caused excitation during the first hour of measurement, but spontaneous activity rapidly decreased during the next 1.5-hr. period. Like Compound 3, the *o*-chlorobenzoyl analog (Compound 6) produced a slight reduction in total activity.

Blood pressure is markedly depressed by Compound 1, and to a somewhat lesser degree by Compounds 2 and 4 by doses of 4 mg./kg. i.v. (Figs. 5–7).

Compound 1 produced a significant hypotensive effect when administered intraperitoneally to unanesthetized normotensive rats (Fig. 8).

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Antitumor Activity of *Juglans nigra* (Black Walnut) Extractives

By UMESH C. BHARGAVA and BERTIS A. WESTFALL

Antitumor activity of compounds present in *Juglans nigra* were studied on spontaneous and/or transplanted tumors in mice. Ellagic acid, juglone, and isolated fractions (strong acids, weak acids, and alkaloids) were injected intraperitoneally for 9–12 days. The results showed that ellagic acid, juglone, and the "strong acids" fraction depressed the tumor growth rate significantly.

CAIN (1) observed that certain water-soluble polyphenolic compounds have the property to retard the growth rate of some experimental rodent tumors; however, no proof was shown experimentally. Furthermore, certain quinone derivatives (2) have been observed to increase the life span and decrease the tumor size in mice. Since some species of walnut (3–5) contain polyphenolic and/or quinone constituents, and also since *Juglans nigra* has been reported to inhibit the growth rate of some other plants (6), it was thought that active principles of walnut

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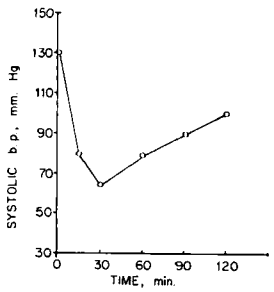

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might inhibit the tumor growth rate in animals (7). This led to the present investigation on the effect of *Juglans nigra* fractions on mammary tumors in mice. Ellagic acid (3) and juglone (8) have been found in *Juglans nigra* and were used in this study. Other pharmacologically active compounds were separated into groups, namely, strong acids, weak acids, and alkaloids, and their pharmacological actions studied. These groups were defined according to their solubility and precipitation tests. Weak acids were soluble in sodium hydroxide solution, but not in sodium bicarbonate solution at pH 7.5. This fraction probably also contained phenolic materials since they exhibit the same properties. Strong acids were soluble in sodium bicarbonate solution at pH 7.5. Other crude extractives which were soluble in acidic solution and gave a precipitation test with reagents such as Wagner's, Meyer's, or Valser's reagents were probably alkaloids. The strong acids and weak acids were extracted from the hulls and the alkaloids from the leaves.

The selection of the type of tumor for the evaluation of antitumor activity is intriguing since many compounds have been reported to inhibit the growth rate of transplanted tumors, but not the spontaneous tumors (9). Therefore, both types of tumors were used.

METHODS

Extraction of Strong Acids and Weak Acids from Hulls—Two kilograms of coarsely ground dried hulls of *Juglans nigra* which were previously macerated with 5% acetic acid was extracted with diethyl ether–butanol mixture (4:1) in a Soxhlet-type apparatus for 48 hr. and the residue obtained after evaporation of the solvent was triturated with 5% acetic acid. The dissolved portion of the residue was filtered and extracted with diethyl ether–butanol mixture (4:1). The aqueous phase was discarded. The above organic solvent layer was washed several times with $NaHCO_3$ solution at pH 7.5 and this was acidified and extracted with diethyl ether–butanol mixture, which on evaporation under vacuum yielded strong acids. The organic layer after extraction with $NaHCO_3$ solution was washed several times with 1 *N* NaOH solution and the alkaline solution was made acidic and extracted like the strong acids. The solvent phase on evaporation under vacuum yielded weak acids. The aqueous phases left after the extraction of strong acids or weak acids were discarded.

Extraction of Alkaloids from Leaves—Two kilograms of dried leaves from *Juglans nigra* was macerated with chloroform–butanol mixture (4:1) containing 5% ammonia solution overnight. Next morning the leaves were extracted for 48 hr. with chloroform–butanol mixture. The organic solvent after extraction was evaporated to semisolid residue under vacuum. The residue was triturated with 5% acetic acid. The acidic solution was rendered alkaline with ammonia solution and extracted with chloroform–butanol mixture. The organic solvent on evaporation under vacuum yielded alkaloids.

Spontaneous Tumor—Mice (40–60 g., Swiss Webster) having mammary adenocarcinoma tumors were purchased from the Taconic Farms and were fed Purina chow and water *ad libitum*. At least a week was allowed after arrival before the animals were used in the experiment.

A daily dose of ellagic acid (50 mg./kg. i.p., solutions pH 11) was injected for 9 days and tumor size (length, width, and height in mm. were multiplied) and mouse weight were measured at 0, 3, 6, and 9 days after the first injection of ellagic acid. Similarly, juglone, strong acids fraction, and weak acids fraction were injected (i.p.) in doses 10 mg./kg., 100 mg./kg., and 100 mg./kg., respectively. The tumor size and mouse weight were measured in the beginning of the experiment and then 3, 6, and 9 days after the first injection of each of the fractions.

Transplantable Tumor—Retired breeder mice (14–16 g., C57BL/6J), purchased from the Jackson Laboratory were used. They were fed Purina chow and water *ad libitum*. Ten days after receiving the animals, each mouse was transplanted subcutaneously with BW-10232 mammary adenocarcinoma in the ventrolateral region.

A daily dose of total alkaloids (50 mg./kg.) was injected (i.p.) and the tumor size and mouse weight were measured at the beginning of the experiment and then 2, 5, 9, and 12 days after the injection of alkaloids.

In another group of mice with transplanted tumors, 10 mg. of ellagic acid/kg. was injected (i.p.) twice a day, the tumor size and mouse weight were measured in the beginning, and then 3, 7, and 10 days after the first injection of ellagic acid.

Control mice were treated in a similar manner as experimental mice except saline was injected intraperitoneally instead of the black walnut fraction. In the case of ellagic acid experiment saline at pH 11 was used. Changes in tumor size and mouse weight were calculated and plotted against time (days). Standard error was calculated using the method of tests of difference or paired data as described by Snedecor (10). Corrected percent mortality of experimental animals was calculated by subtracting percent mortality of controls from percent mortality of experimental animals. Thus the percent of animals which died by chance without the treatment with drugs was eliminated.

RESULTS AND DISCUSSION

Ellagic Acid—The results in Fig. 1 show ellagic acid significantly decreased the rate of tumor growth and increased the weight of spontaneous tumor mice. The changes in tumor size and weight during the 9-day period in ellagic acid-treated mice were 1,457 ± 580 $mm.^3$ and 2.96 ± 1.08 g., respectively, as compared with controls 4,203 ± 1,116 $mm.^3$ and −0.036 ± 0.74 g., respectively, which were statistically significant ($p = 0.03$). There was more growth of tumor in the controls as compared to ellagic acid-treated animals when increase in tumor size per gram of increase in mouse weight was used.

The effect of ellagic acid on transplanted tumor mice was quite different as compared to spon-

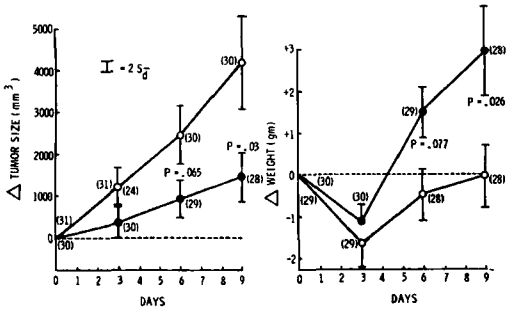

Fig. 1—Effect of ellagic acid on tumor size and body weight in spontaneous tumor mice. Change in tumor size or body weight was plotted against the days after ellagic acid treatment. Each point was obtained from the mean of the animals indicated in the bracket with the standard error. Statistical significance (p *value*) *was calculated by comparing the experimental group with the control. Key:* ○, *control;* ●, *ellagic acid.*

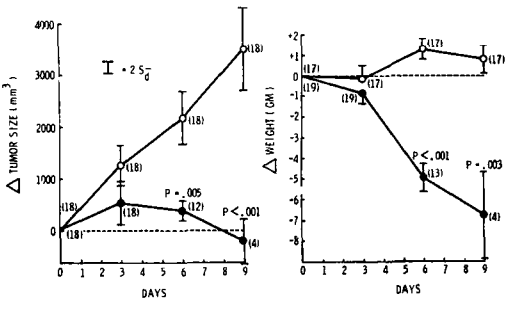

Fig. 3—Effect of juglone on tumor size and body weight in spontaneous tumor mice. Change in tumor size or body weight was plotted against the days after juglone treatment. Each point was obtained from the mean of the animals indicated in the bracket with the standard error. Statistical significance (p *value*) *was calculated by comparing the experimental group with the control. Key:* ○, *control;* ●, *juglone.*

taneous tumor mice. Figure 2 illustrates that ellagic acid had a significant effect ($p = 0.015$) on tumor growth after 6 days of treatment. But this increase in tumor size became insignificant when the mice were treated for 10 days. This might be because the rate of growth of the transplanted tumor was much faster as compared to the spontaneous tumor in respect to tumor volume and size of the animal. Unlike the spontaneous tumor mice, ellagic acid in transplanted tumor mice decreased the body weight ($p < 0.001$).

Percent mortality of mice with transplanted tumor (27.78%) receiving ellagic acid was higher as compared to spontaneous tumor mice (3.44%) receiving that compound.

Juglone—Results obtained from juglone (synthetic) treatment in spontaneous tumor mice are shown in Fig. 3. Juglone decreased significantly the gain in tumor size ($p < 0.001$) and growth in weight ($p = 0.003$) over the period of 9 days of injections. The percent mortality of juglone-treated mice (78.95%) was much higher than any other compounds used. Most of the animals developed acute diarrhea which may have contributed to their death. Therefore, in terms of drug effectiveness, it is questionable that juglone has any specific effect on tumor growth rate since the mouse body weight was also decreased significantly.

Strong Acids, Weak Acids, and Alkaloids—Figure 4 shows the effect of strong acids and weak acids on tumors. Only the strong acids group reduced (slightly) the tumor growth rate and this was statistically significant in the mice receiving this fraction for 3 days. Paper chromatography of the strong acids in isobutyl alcohol–acetic acid–distilled water (40:10:50) solvent system indicates the presence of at least three different compounds (R_f values 0.67, 0.85, and 0.95). The weak acids and alkaloids did not show any significant effect on tumor growth. In the case of alkaloids increase in tumor size and body weight were 7,837 ± 679 mm.^{3} and 3.64 ± 0.45 g., respectively, as compared to controls 7,745 ± 990 mm.^{3} and 3.76 ± 0.52 g., respectively, when the animals were treated with alkaloids for 9 days. The strong acids and weak acids did not show any significantly different effect from controls when weight changes were included in the comparison.

The percent mortality during the period of observation was higher after the animals received the strong acids (25%) than for those receiving weak acids (6.25%) or alkaloids (−5%).

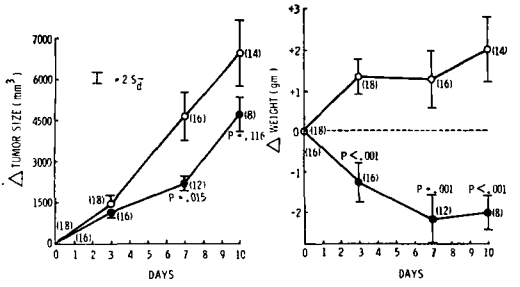

Fig. 2—Effect of ellagic acid on tumor size and body weight in transplanted tumor mice. Change in tumor size or body weight was plotted against the days after ellagic acid treatment. Each point was obtained from the mean of the animals indicated in the bracket with the standard error. Statistical significance (p *value*) *was calculated by comparing the experimental group with the control. Key:* ○, *control;* ●, *ellagic acid.*

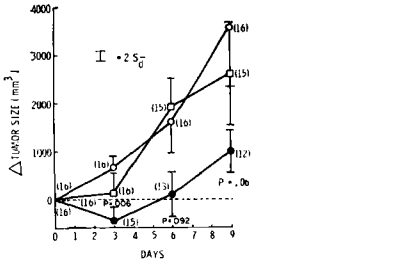

Fig. 4—Effect of strong acids or weak acids on tumor size in mice. Change in tumor size was plotted against days after strong acids or weak acids treatment. Each point was obtained from the mean of the animals indicated in the bracket with the standard error. Statistical significance (p *value*) *was calculated by comparing the experimental group with the control. Key:* ○, *control;* ●, *strong acids;* □, *weak acids.*

It would be worthwhile to isolate the pure compounds from the crude fractions; strong acids, weak acids, and alkaloids and then test them on tumor-bearing mice, because many compounds in the pure state have been reported to be highly active against certain types of leukemias, but were devoid of activity in crude fractions. For example, Svoboda (11) reported the alkaloids, leurocristine and leurosidine, were very active against P-1534 leukemia, but were relatively inactive in crude fractions from which they were isolated. A similar situation was observed by Farnsworth *et al.* (12).

SUMMARY

1. Pharmacologically active compounds present in black walnut were evaluated for antitumor activity on spontaneous and transplanted tumors in mice.

2. Ellagic acid depressed the tumor growth in both spontaneous and transplanted tumor. This effect was more apparent on the spontaneous tumors than on transplanted tumors. Ellagic acid increased the weight of mice with spontaneous tumors but decreased the weight in animals with transplanted tumors.

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4. Strong acids slightly decreased the rate of tumor growth (spontaneous), but weak acids (spontaneous) and alkaloids (transplanted) did not have any significant effect. Furthermore, the weight of the mice was not affected by any of these three groups.

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Keyphrases

Antitumor compounds—*Juglans nigra*
Acidic components, *Juglans nigra*—antitumor activity
Ellagic acid—antitumor activity
Juglone—antitumor activity
Alkaloids, *Juglans nigra*—antitumor activity

Complex Formation Influence on Reaction Rate IV

Studies on the Kinetic Behavior of 3-Carbomethoxy-1-methylpyridinium Cation

By DANA BROOKE* and DAVID E. GUTTMAN†

The kinetics of reaction of the ester, 3-carbomethoxy-1-methylpyridinium cation (NME), were investigated in the pH region 8.0–9.8, in the absence and presence of the electron donor, 8-chlorotheophyllinate anion (CT). Hydrolysis of the ester was shown to be first order with respect to hydroxide ion in this region. The rate of hydrolysis was significantly decreased in the presence of CT. Spectral studies demonstrated the formation of a complex between the ester and CT. The rate of reaction of the complexed ester was about one-fifteenth that of the free ester. Both glycine and TRIS buffers were shown to contribute to the rate of reaction. The contribution due to glycine appeared to be the result of glycinate anion functioning as a general base catalyst. The mechanism of the TRIS reaction appeared to be different from that of glycine. The data suggested that neutral TRIS and the ester reacted by two different pathways to form an unstable ester or a stable amide.

N[1]-ALKYLPYRIDINIUM cations have been extensively used as model compounds in assessing the chemical and physical-chemical properties of pyridine coenzymes. Investigations of the kinetic and complexometric behavior of such cations are, therefore, of general interest. The present investigation was initiated to study the possible effects of complex formation upon the kinetic behavior of the ester, 3-carbomethoxy-1-

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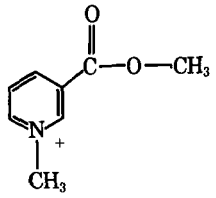
methylpyridinium cation (I). The fact that this ester undergoes hydrolysis in weakly alkaline solutions at conveniently measurable rates made NME an attractive candidate for study from an



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experimental point of view. Moreover, this ester has been shown to form charge transfer complexes with important biological materials such as reduced flavin mononucleotide (1). Others have shown that this ester forms a cyanide-addition compound in weakly alkaline solutions of potassium cyanide (2). However, it was reported that as the ester was hydrolyzed the cyanide-addition compound was destroyed.

As will be seen, NME undergoes hydrolysis in unbuffered solutions or in solutions buffered with glycine. When tris-(hydroxymethyl)aminomethane (TRIS) was employed as a buffer, the ester underwent specific base-catalyzed hydrolysis, aminolysis due to neutral TRIS, and esterolysis due to neutral TRIS and hydroxide ion. In each reaction, the reactivity of the ester was significantly reduced by complex formation with CT. The complexed ester was found to be approximately 5% as reactive as the ester.

EXPERIMENTAL

Materials—The iodide salt of NME, m.p. 124–125° (uncorr.) and the chloride salt of N^1-methylnicotinic acid (NM acid), m.p. 244–245° (uncorr.) were prepared by established methods (3, 4). 8-Chlorotheophylline was obtained from the Aldrich Chemical Co. Solutions of this compound as the anion were prepared by dissolving appropriate amounts in water and using sufficient sodium hydroxide to adjust the pH to values of 8.0 or greater. All other chemicals used were of reagent grade.

Spectral Studies of Complex Formation—Solutions of CT were found to exhibit large increases in absorbance in the visible region of the spectrum on the addition of NME, a species with no visible spectrum. This effect, which is indicative of complex formation, is shown in Fig. 1. Studies of this phenomenon were conducted at 30° using a Beckman, model DB, spectrophotometer equipped with a thermostated cell compartment. Ionic strengths of solutions were brought to desired values with sodium chloride. Absorbance measurements were made as soon as possible after the solutions were prepared to avoid loss of NME due to hydrolysis. In all solutions the total NME concentration $(NME)_t$, was negligibly small compared to the CT concentration. The absorbance value of each solution was determined at a wavelength of 380 mμ and corrected for the contribution of CT to yield the absorbance, A_c, due to complex. Values of $(NME)_t/A_c$ at a particular CT concentration were calculated from the slopes of plots of A_c against $(NME)_t$, all of which were linear and passed through the origin. Plots of $(NME)_t/A_c$ against the reciprocal of CT concentration, the well known Benesi-Hildebrand treatment (5), were used to estimate complex stability constants.

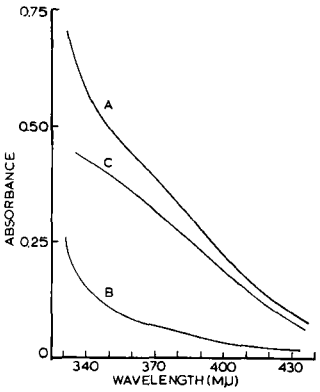

Fig. 1—Spectrum of a solution containing 0.002 M NME and 0.1 M CT (Curve A) compared to that of a solution containing only 0.1 M CT (Curve B). Curve C is the difference spectrum of the two solutions.

Fluorometric Assay for NME—An assay procedure for NME was based on the conversion of ester to N^1-methylnicotinamide (NMN) by means of an ammonolysis reaction. The NMN thus formed was determined by a fluorometric procedure (6, 7). Under the conditions of the assay it was found that about 5% of the ester was converted to NMN and the remaining ester was hydrolyzed. The ammonolysis reaction was found to be complete after 2 hr. and the NMN produced was found to be stable under the reaction conditions. Solutions containing 1×10^{-5} to 7×10^{-5} M NME were assayed by placing 3-ml. samples into 50-ml. volumetric flasks containing 5 ml. of a stock solution of 0.5 M $(NH_4)_2SO_4$ which had been adjusted to a pH of 8.97. The flasks were stoppered and allowed to stand to permit conversion of ester to NMN. After 4 hr., 5 ml. of redistilled acetone containing 2×10^{-4} M manganous chloride was added to each flask, followed by 3 ml. of 3 M sodium hydroxide solution. The flasks were allowed to stand for 30 min. Then 3.5 ml. of 4 N HCl was added to each flask and the flasks were allowed to stand 30 min. Finally, the flasks were made to volume with 2.5% monobasic potassium phosphate. Blanks were prepared in exactly the same manner but contained no NME. The fluorescence of the resulting solutions was measured using a Turner, model 110, fluorometer equipped with a 365-mμ excitation filter and a 436-mμ emission filter. It was found that NM acid, introduced into the samples, did not influence the fluorescence readings of the resulting solutions. Similarly, TRIS and neutral salts used in reaction solutions did not affect the assay. The total fluorescence, F_t, of known solutions was found to be linear with NME concentration and passed through the fluorescence reading of the blank, F_b.

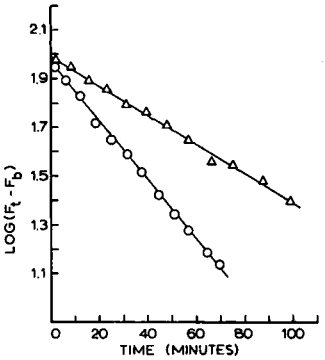

Fig. 2—Example kinetic plots obtained by fluorometrically determining residual NME in solutions buffered with 0.03 M TRIS to pH values of 8.71 (○) or 8.52 (△). Initial concentration of NME was 7.0 × 10^{-5} M and ionic strength was adjusted to 0.13 with addition of sodium perchlorate.

Kinetic Studies—The kinetics of disappearance of NME from solutions maintained at constant pH, constant temperature, and 0.03 *M* TRIS was followed by determining the residual concentration of NME in 3-ml. samples of the reaction solutions at various times. Reaction solutions adjusted to an ionic strength of 0.13 with sodium perchlorate or sodium chloride were prepared in 100-ml. volumetric flasks and kept at 30° in an oil bath. The pH values of reaction solutions were measured during the course of the reaction and were found not to change significantly. A Leeds and Northrup 7401 pH meter was used to make all pH measurements. The residual concentrations of NME in samples of the reaction solutions were determined fluorometrically. Since (F_t − F_b) was proportional to NME concentration, the logarithm of this term was plotted against time to determine apparent first-order rate constants for the disappearance of ester from reaction mixtures. Sample kinetic plots are shown in Fig. 2.

The rates of reaction of ester in the absence or presence of CT in unbuffered solutions at constant pH and 30° were determined by measuring the rate of acid formation on a Radiometer TTT-1 titrator-pH stat equipped with SBR-2, SBU-1 recorder. Ionic strength was brought to desired values with the addition of sodium perchlorate or sodium chloride. The molar amount of sodium hydroxide required to maintain pH of the reaction mixtures during the course of reaction correlated with the molar amount of NME in the original solutions.

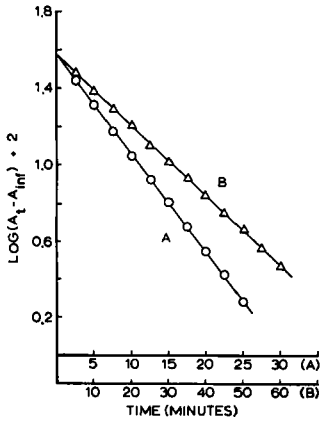

Fig. 3—Kinetic plots prepared by following the absorbance at 380 mμ of reaction solutions prepared to contain 0.005 M NME and 0.1 M CT. Conditions of pH and buffer were: 0.4 M TRIS, pH 9.18 (Curve A), and 0.26 M TRIS, pH 8.63 (Curve B).

The rates of loss of total ester from solutions containing CT, and which were buffered to constant pH with TRIS or glycine buffers of moderate concentration were studied by following the disappearance of absorbance at 380 mμ with time. Solutions at 30° were prepared in 50-ml. volumetric flasks to contain 0.005 *M* NME and appropriate concentrations of buffer and CT. In most cases, ionic strength was controlled by the concentration of CT in the reaction solutions. A portion of the reaction solution was placed in 1-cm. quartz cells and the absorbance at 380 mμ was followed with time using water as a blank. The pH values of reaction mixtures were found not to change significantly during the course of reaction. Representative kinetic plots are shown in Fig. 3.

pKa Values—The values for pKa which were used in calculations were: 8-chlorotheophylline, 5.5 (8); TRIS, 8.10 (9); glycine, 9.78 (10).

RESULTS AND DISCUSSION

Spectral Studies—Spectrophotometric examination of solutions containing both NME and CT suggested that formation of a complex occurred. Figure 1 illustrates the spectrum of a solution of 0.1 *M* CT, the spectrum of a solution containing 0.1 *M* CT and 0.002 *M* NME, and the difference spectrum. Since the ester has no visible spectrum, the large increase in absorbance values of a solution of CT caused by the addition of NME was indicative of complex formation. That a new, broad absorption band appeared at longer wavelengths than the spectrum of either interactant suggests that a charge-transfer complex was formed. This is not surprising since pyridinium cations are well known for their ability to act as acceptors in charge-transfer interactions. Spectral data were analyzed by the method of Benesi and Hildebrand (5) to yield complex stability constants at several ionic strengths. The stability constant at an ionic strength of 0.1 was estimated to be 4.7 M^{-1}. The stability constant at an ionic strength of 0.2 was 4.3 M^{-1}, and at 0.3 was 3.9 M^{-1}.

Complex Formation Influence on Kinetic Behavior—In order to survey the possible effects of complex formation upon the kinetics of hydrolysis of NME, it was first necessary to study these kinetics in the absence of CT. This was done by utilizing two different experimental approaches. In the pH region from 8.0 to 8.8, loss of ester from solutions buffered to a constant pH with 0.03 *M* TRIS was followed by fluorometrically determining the residual concentration of NME as a function of time. In the pH region from 8.6 to 9.6, the rate of acid production in reaction solutions was determined by means of a pH-stat technique. The rate constants obtained by these two methods are presented in Fig. 4 in the form of a pH-rate constant profile. The good correlation between the apparent rate constants for the disappearance of ester and corresponding constants

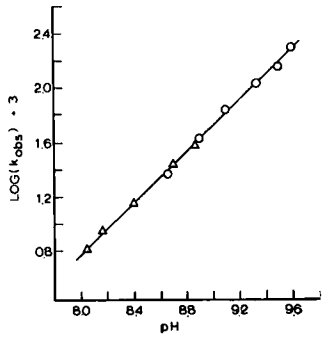

Fig. 4—A plot illustrating the influence of pH on the apparent rate constant for loss of NME from solutions of 0.03 M TRIS (△), and on the apparent rate constant for the production of acid in unbuffered solutions of NME (○).

for the appearance of acid showed that the reaction was solely one of hydrolysis. The slope of the profile in Fig. 4 was 0.97 which indicated that the hydrolysis, under these conditions, was specific base catalyzed. The linearity of the profile suggested that TRIS, in 0.03 *M* concentration, did not contribute significantly to the rates of reaction. The specific base catalytic constant was calculated to be $0.54 \times 10^4\ M^{-1}$ min.$^{-1}$.

The kinetics of ester hydrolysis were also studied in solutions containing 0.1 *M* CT by the pH-stat method. A pH-rate constant profile representing these studies is presented in Fig. 5. That this profile fell below that determined in the absence of CT showed that complex formation did significantly decrease the reactivity of the ester toward hydrolysis. The slope of the profile was found to be 1.05 indicating that the hydrolysis of the ester in solutions containing CT was also specific base catalyzed. That no irreversible reaction occurred between CT and NME was reflected by the fact that the molar quantity of base required to maintain constant pH during the course of reaction corresponded to the initial molar quantity of ester in the reaction solution. The fact that the slope of the profile was not less than 1.0 provided evidence that CT did not act as a general base catalyst.

It was observed, in the course of these studies, that the characteristic yellow color of freshly prepared solutions of CT and NME faded considerably during the course of reaction. Spectral studies showed that the hydrolysis product of NME, N^1-methylnicotinate, also interacted with CT. However, the absorbance at 380 mμ due to this interaction was relatively small compared to that found with a corresponding ester–CT system. The fading, therefore, undoubtedly resulted as a consequence of ester hydrolysis. Kinetic studies were then conducted which took advantage of this effect. Here, the loss of absorbance at 380 mμ of solutions containing NME and CT was used as a reaction parameter to reflect the loss of total NME. The kinetics of loss of NME from solutions containing CT were studied under a variety of conditions by this method. In most cases, TRIS buffers of moderate concentration were used to maintain constant pH. Figure 3 illustrates kinetic plots obtained by this procedure. It can be seen that these plots are linear for at least 3 to 4 half-lives. It was found, however, that under these conditions, where buffer concentrations were much higher than those employed previously, a buffer contribution to rates of reaction was apparent. Figure 6 illustrates the effect on the apparent rate constant of varying the total TRIS concentration under the conditions of constant pH and CT concentration. The value for the apparent rate constant at zero TRIS concentration was interpolated from pH-stat studies on buffer-free solutions (Fig. 5). The apparent rate constants were found to be linear with TRIS concentration indicating that the buffer reaction was first order in TRIS. As will be seen, the TRIS buffer reaction was found to be considerably more complex than one of general base catalysis, and involved a reaction between ester and neutral TRIS, and one between ester, neutral TRIS, and hydroxide ion.

The influence of varying the concentration of CT on the apparent rate constants for disappearance of NME from solutions buffered to a pH of 8.60 with 0.26 *M* TRIS was evaluated. Since CT, in moderate concentrations, was easily salted out of solution, no attempt was made to maintain ionic strength at a constant value in these experiments. The results are summarized in Fig. 7. A modified form of an equation derived by Connors and Mollica (11) was

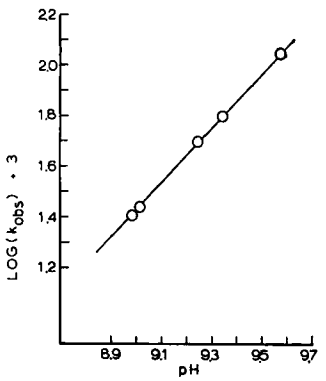

Fig. 5—Illustration of the influence of pH on the apparent rate constant for production of acid in unbuffered solutions of NME containing 0.1 M CT.

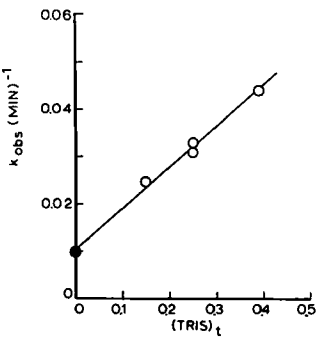

Fig. 6—Plot depicting the influence of total TRIS buffer concentration on the apparent rate constant for loss of NME from solutions of pH 8.60 which contained 0.1 M CT. The open symbols refer to spectral determinations of apparent rate constant. The solid symbol is the apparent hydrolytic rate constant for NME at this pH and CT concentration as interpolated from the data of Fig. 5.

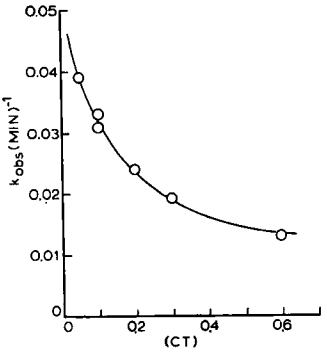

Fig. 7—Illustration of the influence of CT concentration on the apparent rate constant for the loss of NME from solutions buffered to pH 8.60 with 0.26 M TRIS.

used to evaluate these data to determine if complexed ester underwent reaction, and to determine, kinetically, the complex stability constant. It can be shown that

$$\frac{k'(CT') - k^*(CT^*)}{(CT') - (CT^*)} = k_c + \frac{k^* - k'}{K[(CT') - (CT^*)]} \quad \text{(Eq. 1)}$$

where k' = apparent rate constant determined when the CT concentration was (CT'), k^* = apparent rate constant determined when the CT concentration was (CT*), k_c = apparent rate constant for loss of complexed ester, K = complex stability constant.

A plot of the data according to Eq. 1, based on a reference (CT*) of 0.05 M, is given in Fig. 8. The intercept is k_c and the reciprocal of the slope is K. The value for the apparent rate constant for loss of free NME can be estimated by setting (CT') to zero and calculating k'. Here, the value for the apparent rate constant for loss of free ester was calculated to be 0.052 min.$^{-1}$ which does not agree with the apparent hydrolytic rate constant of 0.021 min.$^{-1}$ for free ester as interpolated from the pH-rate constant profile prepared in the absence of CT (Fig. 4). The difference between these two apparent rate constants will be shown to be due to the reaction of TRIS with free NME. The value for k_c was estimated to be 0.0035 min.$^{-1}$. Therefore, in reaction solutions containing CT and 0.26 M TRIS at a pH

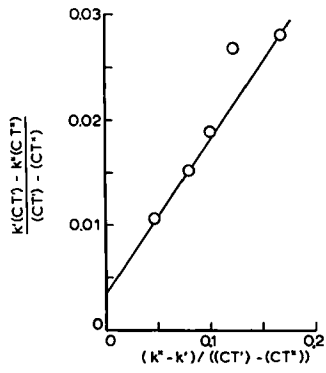

Fig. 8—Data of Fig. 7 plotted according to Eq. 1. See text.

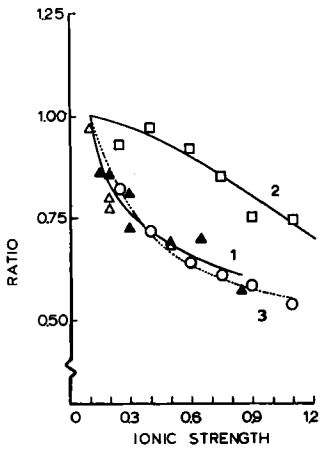

Fig. 9—Plot showing the influence of ionic strength on the apparent rate constant for loss of NME from solutions containing no buffer and no CT (Curve 1, △), on the rate constant for loss of NME from solutions containing 0.1 M CT and 0.26 M TRIS (Curve 2, □), and on the extent of complex formation between NME and CT (Curve 3, ○). The ordinate is the ratio of observed values at a given ionic strength to the observed value at an ionic strength of 0.1. Open symbols refer to the use of sodium perchlorate to adjust ionic strength, closed symbols refer to the use of sodium chloride to adjust ionic strength.

of 8.6, the free ester underwent reaction at about 15 times the rate of the complexed ester.

The value for the complex stability constant, K, was estimated from the kinetic data to be 6.5 M^{-1}. The discrepancy between this value and those estimated from spectral data can be rationalized on the basis that ionic strength effects, resulting from the variation of CT concentration, contributed to the overall effect of CT in the kinetic studies. It is informative, here, to consider the effects of ionic strength variation on the complexometric and kinetic behavior of NME.

The influence of ionic strength variation on the apparent rate constants for loss of NME from unbuffered solutions containing no CT, as determined by pH-stat studies at a pH of 9, and those for the loss of NME from solutions containing 0.1 M CT and 0.26 M TRIS, as determined spectrally, is illustrated in Fig. 9. Curve 1 shows the influence of ionic strength on the apparent rate constants determined in the absence of CT and buffer. Curve 2 shows the influence of ionic strength on the apparent rate constants determined in the presence of CT and buffer. Curve 3 of Fig. 9 reflects the influence of ionic strength on the extent of complex formation between CT and NME. This influence was evaluated by determining the absorbance due to the complex at zero time in those solutions containing CT and buffer. The influence of ionic strength on the apparent rate constants determined in the absence of CT and buffer, and on the extent of complex formation in solutions containing CT and buffer is seen to be qualitatively the same (Curves 1 and 3). That both Curve 1 and Curve 3 decrease and show approximately the same dependence on ionic strength is not unexpected since both curves describe the effect of ionic strength on the reaction of an anion with a cation. However, the dependence

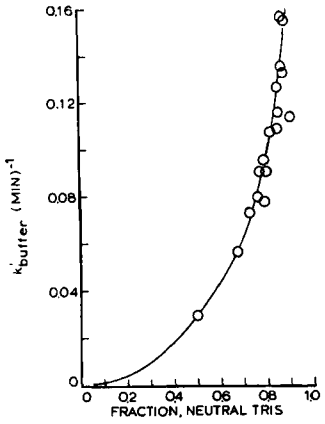

Fig. 10—Plot of the apparent TRIS buffer rate constant observed in solutions containing 0.1 M CT against the fraction of neutral TRIS.

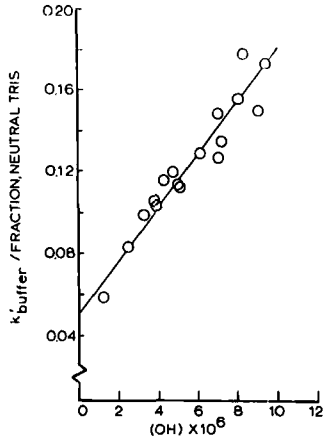

Fig. 11—Illustration of the influence of hydroxide ion concentration on the apparent rate constant for the reaction of neutral TRIS with NME observed in solutions containing 0.1 M CT.

of the apparent rate constants for loss of NME from solutions containing CT and TRIS is seen to be much smaller. This observation can reasonably be explained by considering that although the kinetic parameters for the reaction in these systems might be expected to be decreased by increasing ionic strength, the extent of complex formation would also be expected to be decreased resulting in higher concentrations of free NME. Moreover, the apparent rate constant here reflects, in part, a reaction between neutral TRIS and NME and this reaction would not be expected to be as sensitive to ionic strength variations as would the reaction of the ester with hydroxide ion. The indication from Curve 2 is that, within certain limits, strict control of ionic strength in reaction solutions containing CT and TRIS is not necessary, but that ionic strength variation over wide ranges does result in measurable decreases in apparent rate constants. These considerations also suggest that the observed influence of CT on reaction rate was due to both complex formation and an ionic strength effect and that the latter resulted in the generation of a slightly larger complex stability constant than was expected on the basis of spectral studies.

Buffer Reactions—The reaction of TRIS with NME was examined in solutions containing 0.1 *M* CT at various pH values and at different TRIS concentrations. The ionic strength of each solution was 0.1. The apparent buffer rate constants per mole of TRIS, k'_{buffer}, were calculated by the equation

$$k'_{buffer} = \frac{k_{obs.} - k_{hyd.}}{(buffer)_t} \qquad \text{(Eq. 2)}$$

where $k_{obs.}$ = experimentally determined apparent rate constant, $k_{hyd.}$ = apparent hydrolytic rate constant under the same conditions of pH and CT concentration, but in the absence of TRIS, as interpolated from the data of Fig. 8, $(buffer)_t$ = total buffer concentration.

It was found that a plot of k'_{buffer} against the fraction of neutral TRIS, as shown in Fig. 10, extrapolated to the origin but increased in slope as the fraction increased. Since the buffer reaction was shown to be first order in TRIS (Fig. 6), the data suggested that hydroxide ion was also involved in a buffer reaction. The data were found to give a straight line with positive slope and positive intercept when k'_{buffer} divided by fraction of neutral TRIS was plotted against hydroxide ion concentration. This plot is shown in Fig. 11. These considerations suggested that the TRIS buffer rate equation had terms in neutral TRIS and in netural TRIS and hydroxide ion. The kinetics of TRIS reaction with phenyl ester (9), and with δ-thiolvalerolactone (12) have been observed to give similar rate equations. Bruice and York (9) compared the reaction of TRIS with phenyl esters to that of pentaerythritol (which is essentially TRIS in which the $—NH_2$ group has been replaced by a $—CH_2OH$ group) with phenyl esters and concluded that the reaction of neutral TRIS with esters was one of aminolysis, and that of neutral TRIS and hydroxide ion with esters was one of esterolysis. They found that the rate equation for the reaction of pentaerythritol with esters involved only the term in pentaerythritol and hydroxide ion. This was taken as an indication that the similar term in the rate equation for the reaction of TRIS with esters described esterolysis. They concluded that hydroxide ion reacted with TRIS to form an alkoxide ion which then reacted with the ester.

It would appear from the present investigation that an aminolysis reaction between neutral TRIS and NME also occurred. This is suggested by a comparison of the spectrum of the final (time infinity) reaction mixture which had been prepared with 0.005 *M* NME, 0.1 *M* CT, and 0.4 *M* TRIS to the spectrum of a solution prepared with 0.005 *M* NM acid, 0.1 *M* CT, and 0.4 *M* TRIS. Such a comparison is shown in Fig. 12. That the final reaction solution had absorbance readings which were significantly higher than those of a solution which would be anticipated if all the NME had been converted to acid suggested that a relatively stable product had been formed and that this product interacted to a greater degree with CT than did NM acid. That this reaction product was not an ester such as that which would be expected from an esterolysis reaction was indicated by the stability of the spectrum of the final reaction solution over a period of 4 hr. In contrast to esters, an amide

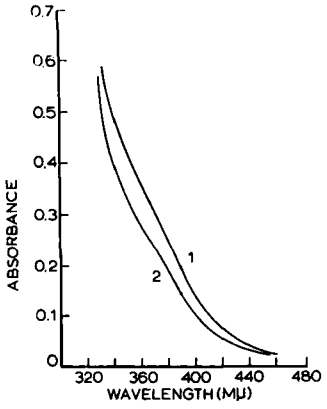

Fig. 12—Spectrum of a time infinity reaction solution which had been prepared to contain 0.005 M NME, 0.1 M CT, and 0.4 M TRIS at pH 9.18 (Curve 1), and that of a solution containing 0.005 M NM acid, 0.1 M CT, and 0.4 M TRIS at pH 9.18 (Curve 2).

formed by the reaction of neutral TRIS with NME would be quite stable under the conditions of the experiment. Such an amide would also be expected to interact with CT to a greater degree than NM acid which would be ionized at the carboxyl group under these conditions. The assumption of stability for the newly formed amide is based on studies of the kinetics of alkaline hydrolysis of NMN (13) in which it was shown that the specific base catalytic constant was 0.23 M^{-1} min.$^{-1}$ (ionic strength 1.0, temperature 30°) which is about 4 orders of magnitude smaller than the specific base catalytic constant for NME. The fact that ammonia reacts with NME to form NMN also supports the conclusion that an aminolytic reaction with TRIS did occur.

The overall reaction mechanism for NME in solutions containing TRIS and CT is quite complex. The fact that NME and its reaction products can possibly interact with CT to form complexes which contribute to the absorbance values of a reaction solution might be expected to make this approach to the determination of apparent rate constants for the loss of NME difficult. However, it can be shown that rate constants determined by following the time course of absorbance of solutions containing NME and CT do accurately reflect the rates of disappearance of NME. A possible overall mechanism describing the reactions of NME is presented by the following equations. Here, the possibility of the various complexes undergoing reaction is neglected since this has been shown to be small.

$$\text{NME} + \text{OH} \xrightarrow{k_1} \text{NM acid} \quad \text{(Eq. 3)}$$

$$\text{NME} + \text{T} \xrightarrow{k_2} \text{NM acid} \quad \text{(Eq. 4)}$$

$$\text{NME} + \text{T} \xrightarrow{k_3} \text{amide}' \quad \text{(Eq. 5)}$$

$$\text{NME} + \text{OH} + \text{T} \xrightarrow{k_4} \text{ester}' \quad \text{(Eq. 6)}$$

$$\text{ester}' + \text{OH} \xrightarrow{k_5} \text{NM acid} \quad \text{(Eq. 7)}$$

$$\text{ester}' + \text{T} \xrightarrow{k_6} \text{NM acid} \quad \text{(Eq. 8)}$$

$$\text{ester}' + \text{T} \xrightarrow{k_7} \text{amide}' \quad \text{(Eq. 9)}$$

where the various k_i's represent specific rate constants for the indicated rate processes, and amide′ = the amide formed by TRIS aminolysis of NME, ester′ = the ester formed by TRIS esterolysis of NME, T = neutral TRIS. Here, processes represented by Eqs. 3 and 7 refer to specific base catalysis, those by Eqs. 4 and 8 refer to possible general base catalysis, those by Eqs. 5 and 9 refer to aminolysis, and that represented by Eq. 6 refers to esterolysis. If it is assumed that all four of the pyridinium cations interact with *CT* to contribute to the absorbance of the reaction solution, A_t, and if it is assumed that only amide′ and NM acid remain in the reaction solution at infinite time, and that only these interact with CT to contribute to the final absorbance, $A_{\text{inf.}}$, then it can be shown that the function $(A_t - A_{\text{inf.}})$ is described by:

$$A_t - A_{\text{inf.}} = B_1 \exp.(-C_1 t) + B_2 \exp.(-C_2 t) \quad \text{(Eq. 10)}$$

where B_1 and B_2 are constants at constant pH, and constant CT and TRIS concentrations, and $C_1 = f_1[k_1(\text{OH}) + k_2(\text{T}) + k_3(\text{T}) + k_4(\text{T})(\text{OH})]$, $C_2 = f_2[k_5(\text{OH}) + k_6(\text{T}) + k_7(\text{T})]$, t = time. Here f_1 refers to the fraction of free NME in the reaction solution, and f_2 refers to the fraction of free ester′ in the reaction solution. From Eq. 10 it would appear that a plot of log $(A_t - A_{\text{inf.}})$ against time would exhibit curvature. However, plots of the experimental data, as shown in Fig. 3, were found to be linear for at least 3–4 half-lives. This indicates that either the C_1 term or the C_2 term of Eq. 10 is effectively zero. Since apparent rate constants determined from plots of log $(A_t - A_{\text{inf.}})$ against time were found to be dependent on (OH), (T), and (T) (OH), it is clear that the C_1 term cannot be zero. Thus, Eq. 10 can be approximated by:

$$A_t - A_{\text{inf.}} = B_1 \exp.(-C_1 t) \quad \text{(Eq. 11)}$$

Apparent rate constants evaluated from semilog plots of $(A_t - A_{\text{inf.}})$ *versus* time reflect, as can be seen from Eq. 11, the rate of loss of NME since C_1 is defined by processes which involve only NME. Therefore, conclusions drawn from experiments of this kind do not appear to be invalidated by the complexity of the reaction mechanism.

Based on the above considerations, and with the assumption that reaction rates of complexes are insignificantly small, the rate equation which describes loss of total NME from buffered solutions containing CT and TRIS can be written:

$$-\frac{d(\text{NME})_t}{dt} = \frac{(\text{NME})_t}{1 + K(\text{CT})} \times [k_1(\text{OH}) + (k_2 + k_3)(\text{T}) + k_4(\text{T})(\text{OH})] \quad \text{(Eq. 12)}$$

Here the term $(\text{NME})_t/[1 + K(\text{CT})]$ is equivalent to $f_1(\text{NME})_t$, and is the concentration of free NME in the reaction solution. The values for the parameters of Eq. 12 are: $K = 4.7\ M^{-1}$, $k_1 = 0.54 \times 10^4\ M^{-1}$ min.$^{-1}$, $(k_2 + k_3) = 0.078\ M^{-1}$ min.$^{-1}$, $k_4 = 1.88 \times 10^4\ M^{-2}$ min.$^{-1}$. The value for the complex stability constant, K, is that obtained from spectral studies at an ionic strength of 0.1. The rate constant k_1 was calculated from the data of Fig. 4. The sum of the rate constants $(k_2 + k_3)$

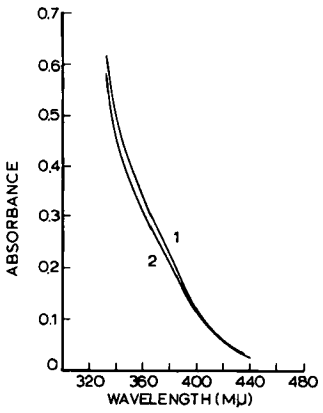

Fig. 13—Spectrum of a time infinity reaction solution which had been prepared to contain 0.005 M NME, 0.1 M CT, and 0.4 M glycine at pH 9.80 (Curve 1), and that of a solution which contained 0.005 M NM acid, 0.1 M CT, and 0.4 M glycine at pH 9.80 (Curve 2).

was calculated from the intercept of Fig. 11. Here, it will be recognized that the intercept was $f_1(k_2 + k_3)$. Similarly, the value for k_4 was calculated from the slope of Fig. 11, the slope being f_1k_4. With these values it is possible to predict the apparent rate constant, at 30°, for the disappearance of total NME at any concentration of hydroxide ion, CT, and neutral TRIS. Thus, in a system which contains no CT and which is maintained at a pH of 8.6 with 0.26 *M* TRIS buffer, the apparent rate constant is calculated to be 0.051 min.$^{-1}$. This is in excellent agreement with the value of 0.052 min.$^{-1}$ which was estimated from the Connors and Mollica treatment of data obtained under the same conditions of pH and TRIS concentration by extrapolating to zero CT concentration. This agreement supports the overall mechanism which has been proposed.

The reaction of NME in the presence of 0.1 *M* CT was also studied in glycine buffers by following the decrease of absorbance with time. Kinetic plots were linear for at least 3–4 half-lives. The results of these studies suggested that glycinate anion catalyzed the hydrolysis of NME. Buffer rate constants for various conditions of pH and glycine concentration were calculated from Eq. 2. A plot of k'_{buffer} against the fraction of glycinate anion was linear and passed through the origin indicating that the buffer reaction was first order with respect to glycinate anion. Figure 13 compares the spectrum of a final reaction solution to that which would be anticipated if all the NME had been hydrolyzed. It is seen that there was little difference. This suggests that glycinate anion did not participate to any significant extent, in an aminolysis reaction, but functioned as a general base that catalyzed the hydrolysis. The general base catalytic constant for glycinate anion was estimated to be 0.39 M^{-1} min.$^{-1}$. This value is 5–6 times larger than the rate constant for the reaction of neutral TRIS with NME ($k_2 + k_3$). Thus, if neutral TRIS functioned as a general base catalyst in the hydrolysis of NME (that is, if k_2 is not zero), its catalytic activity was small compared to that of glycinate anion. Presumably, the greater catalytic efficiency of glycinate anion is due to its greater basicity.

Conclusion—In the present investigation it has unequivocally been shown that complex formation between CT and NME reduced the reactivity of the ester toward specific base-catalyzed hydrolysis. Evidence was obtained to suggest that reactions involving NME and buffer species were similarly affected by complex formation. The reduced reactivity of complexed ester can be rationalized on the basis of at least two effects. Donation of charge by the complexing agent to the pyridinium ring undoubtedly reduces the electron withdrawing ability of the ring resulting in a much lower degree of "acyl activation" of the ester carbonyl. In addition, the attack of nucleophiles on the carbonyl function of complexed NME is almost certainly hindered, on a steric basis, by the presence of complexing agent. It has also been shown that when the manifestations of complex formation between reactant and complexing agent, and between products and complexing agent differ greatly, this difference can be conviently used to follow rates of reaction. Using this approach, the rather involved kinetics of reactions of NME in buffered aqueous media were investigated.

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Keyphrases

Complex formation—reaction rate influence
3-Carbomethoxy-1-methylpyridinium cation—reaction kinetics
8-Chlorotheophylline effect—3-carbomethoxy-1-methylpyridinium hydrolysis
Buffer systems—3-carbomethoxy-1-methylpyridinium hydrolysis
Colorimetric analysis—spectrophotometer
Fluorometry—analysis

Heterogeneous Catalysis of Aspirin Degradation in Chloroformic Solution and Its Relationship to the Determination of Salicylic Acid in Buffered Aspirin Products

By DAVID E. GUTTMAN

Difficulties and spurious results were encountered when buffered aspirin tablets were subjected to the official test for salicylic acid. The present investigation was concerned with this problem and with a characterization of the behaviors of aspirin and salicylic acid in chloroformic solution in contact with chloroform-insoluble agents which are commonly used as buffering agents in buffered aspirin tablets. Two phenomena were studied which can have manifestations of analytical importance. Both aspirin and salicylic acid were found to be adsorbed by solids such as magnesium carbonate, aluminum glycinate, aluminum hydroxide, and magnesium trisilicate. Rate studies showed that chloroformic solutions of aspirin in contact with solid buffers were unstable and generated at a relatively rapid rate a compound with the chromatographic characteristics of salicylic acid. The rate of generation was shown to be due neither to the water or ethanol content of the chloroform nor to the presence of dissolved buffer. Rather, it was demonstrated that catalysis was due to solid surfaces. The addition of citric acid monohydrate to such systems resulted in an almost complete abolition of adsorption and catalysis.

A LIMIT TEST for free salicylic acid in aspirin tablets is described in the USP XVII (1) and is based on the procedure which was developed and described by Weber and Levine (2). A sample preparation is formulated by treating powdered tablets with chloroform and introducing the resulting mixture to a partition chromatographic column. The aqueous stationary phase of the column contains urea and ferric chloride. Salicylic acid is retained on the column in the form of a urea–iron–salicylate complex while other components are eluted with chloroform. Salicylic acid is finally removed by elution with an ethereal solution of acetic acid which decomposes the complex and releases salicylic acid. The absorbance of the salicylic acid fraction is subsequently determined at 306 mμ.

Special problems exist with buffered aspirin tablets, and a modified procedure is recommended by the compendium. During the course of studying this latter procedure, interesting and unusual behaviors were observed which can be of analytical importance. As it will be shown, two effects are operant in systems encountered in this test which, depending on conditions, can result in apparent salicylic acid contents which are significantly lower or higher than the true content.

Received April 10, 1968, from the Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214

Accepted for publication June 21, 1968.

The author wishes to thank Mrs. Gail W. Salomon for her competent technical assistance in this study.

RESULTS

Spurious and nonreproducible results were obtained when a synthetic mixture[1] formulated to simulate an analytical sample prepared from buffered aspirin tablets was subjected to the USP limit test for free salicylic acid. Usually low recoveries were obtained. However, it was found that if the chloroformic sample preparation was allowed to stand prior to the chromatographic procedure, a recovery which was significantly higher than theory was obtained.

Low recoveries can be explained by an adsorption phenomenon. Experiments designed to evaluate this possibility are summarized by Fig. 1. It is apparent that significant adsorption of salicylic acid occurred when chloroformic solutions were contacted with chloroform-insoluble agents which are commonly employed as buffers in aspirin tablets. It is interesting to observe that the affinity of salicylic acid for magnesium carbonate and aluminum glycinate is high but that the capacity of these solids for the acid is rather low. That salicylic acid is tenaciously held by magnesium carbonate and aluminum glycinate is indicated by the fact that it was impossible to completely elute adsorbed material by repeated contacts with fresh solvent.

Further experimentation showed that high recoveries of salicylic acid from samples of the synthetic mixture resulted from a surprisingly rapid transformation of aspirin to a product having the chromatographic characteristics of salicylic acid. The nature of this phenomenon is shown in Fig. 2 which depicts kinetic studies which were conducted on various chloroform–aspirin systems. It is seen that the rate of production of salicylic acid in a one-

[1] Aspirin, 3 g.; magnesium carbonate, 0.9 g.; aluminum glycinate, 0.45 g.; salicylic acid, 0.03 g.

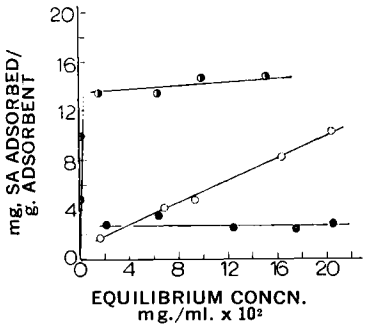


Fig. 1—Adsorption of salicylic acid (SA) from chloroformic solution by various solids. Key: ◐, magnesium carbonate, 10 mg./ml.; ●, aluminum glycinate, 10 mg./ml.; ○, aluminum hydroxide and magnesium trisilicate mixture obtained by powdering antacid tablets, 20 mg./ml. The studies were conducted at room temperature, 25 ± 2°.

phase system of aspirin in chloroform or in chloroform which had been saturated by prolonged contact with magnesium carbonate was slow. In contrast, a rapid rate of production was observed when solid magnesium carbonate was suspended in the aspirin solution. It is apparent that a transformation of aspirin occurred which was surface catalyzed. The curves indicate that, in the solid-containing systems, an equilibration reaction occurred which was first-order with respect to the aspirin concentration. That the plateauing of the curves was due to an approach to equilibrium and not to depletion of catalyst is suggested by the observation that addition of fresh magnesium carbonate to a system which had attained the plateau concentration of salicylic acid did not result in further generation of salicylic acid. Addition of more aspirin to such a system was, however, followed by the appearance of a substantial additional amount of salicylic acid. It was further found that no difference in behavior could be detected between systems prepared with reagent grade chloroform, water-saturated chloroform, or

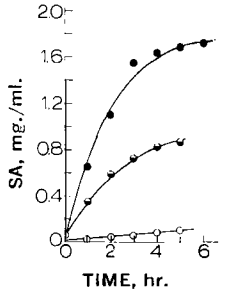


Fig. 2—Generation of salicylic acid (SA) with time in chloroformic solution. Key: ○, a one-phase system containing aspirin at an initial concentration of 20 mg./ml. prepared with reagent grade chloroform or reagent grade chloroform which was saturated by prolonged shaking with magnesium carbonate; ◒, a two-phase system containing aspirin at an initial concentration of 10 mg./ml. and magnesium carbonate at a concentration of 10 mg./ml.; ●, a two-phase system containing aspirin at an initial concentration of 20 mg./ml. and magnesium carbonate at a concentration of 10 mg./ml. The studies were conducted at room temperature, 25 ± 2°.

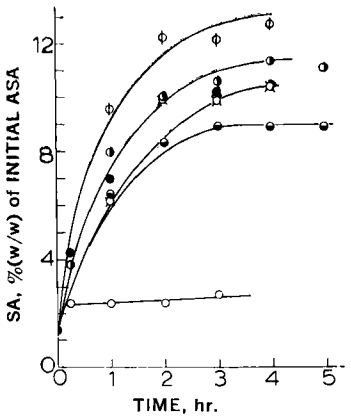


Fig. 3—Generation of salicylic acid (SA) with time in chloroformic solution in contact with suspended solids. The systems were prepared with buffered aspirin tablets to contain an initial concentration of aspirin of 20 mg./ml. Key: ϕ, tablets containing aluminum and magnesium hydroxides; ◑, tablets containing magnesium carbonate and calcium carbonate; ⧖ ●, tablets containing aluminum hydroxide, glycine, and magnesium carbonate; ◒, tablets containing magnesium carbonate and aluminum glycinate; ○, tablets containing calcium phosphate, sodium bicarbonate, and citric acid. The studies were conducted at room temperature, 25 ± 2°.

freshly distilled reagent grade chloroform. Similarly no significant difference in behavior was found between such systems and one prepared with a 5% (v/v) solution of ethanol in chloroform.

Figure 3 illustrates similar rate studies conducted with aspirin–buffer–chloroform systems prepared from commercially available buffered aspirin products. A number of different buffering components were encountered in this study. With one exception, rapid generation of salicylic acid with time was observed in these systems. The exception was a system prepared from tablets which contained, in addition to alkalinizing agents, citric acid. This observation motivated further studies on the influence of citric acid on the kinetics of salicylic acid

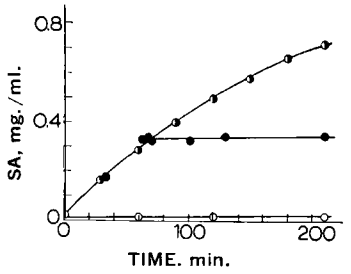


Fig. 4—Influence of citric acid on the rate of generation of salicylic acid (SA) in chloroformic solution in contact with magnesium carbonate. The magnesium carbonate was at a concentration of 5 mg./ml. and aspirin at an initial concentration of 10 mg./ml. Key: ◑, no citric acid added; ○, citric acid added at 0 time to yield a concentration of 5 mg./ml.; ●, citric acid added at 60 min. to yield a concentration of 5 mg./ml. The studies were conducted at room temperature, 25 ± 2°.

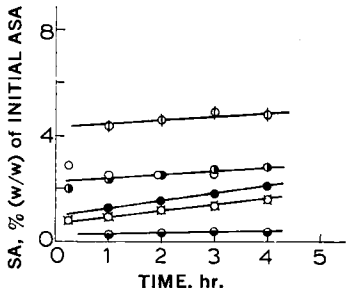


Fig. 5—Influence of citric acid on the rate of production of salicylic acid (SA) in chloroformic solution prepared from buffered aspirin tablets. Each system was prepared to contain aspirin at an initial concentration of 20 mg./ml. A weight of citric acid equal to the weight of sample was added to the powdered sample prior to the addition of chloroform. Key: same as Fig. 3. The studies were conducted at room temperature, 25 ± 2°.

formation. That the influence is dramatic is shown by Fig. 4. In contrast to the relatively rapid increase in salicylic acid content with time which was observed with magnesium carbonate-containing systems, a system formulated to contain equal concentrations of citric acid and magnesium carbonate was found to be essentially unreactive. Similarly, the addition of citric acid to a magnesium carbonate system after 1 hr. of reaction resulted in an almost immediate cessation of salicylic acid production. It was visually observed that the addition of citric acid had a pronounced effect on the nature of the suspension. Marked flocculation of particles was apparent immediately after the addition of citric acid. Citric acid which was oven-dried immediately before addition to a reaction medium had a much less marked effect in inhibiting salicylic acid production.

Citric acid treatment was established to be equally effective in inhibiting salicylic acid production in systems prepared from buffered aspirin tablets as shown by studies which are depicted in Fig. 5. Here, an aliquot of powdered tablet was triturated with an equal weight of citric acid and then treated with sufficient chloroform to obtain the desired aspirin concentration. A comparison of this figure with Fig. 3 shows that in all systems, citric acid was effective in markedly reducing the catalytic ability of suspended solids.

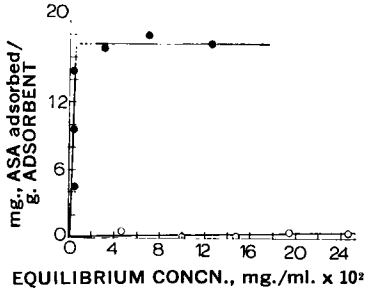


Fig. 6—Adsorption of aspirin (ASA) from chloroformic solution by magnesium carbonate and by a mixture of magnesium carbonate and citric acid. Key: ●, magnesium carbonate 10 mg./ml.; ○, magnesium carbonate 10 mg./ml. and citric acid 10 mg./ml. The studies were conducted at room temperature, 25 ± 2°.

An insight into the mechanism by which citric acid functioned to inhibit the surface-catalyzed transformation of aspirin is provided by adsorption studies which are summarized in Fig. 6. Aspirin, it is seen, was strongly adsorbed to magnesium carbonate. However, a mixture of citric acid and magnesium carbonate possessed little adsorptive capacity for aspirin. It is also of significance that treatment of solids with citric acid also significantly reduced their capacities for adsorbing salicylic acid. Experiments which illustrate this effect are summarized in Table I.

DISCUSSION

Instability of aspirin in chloroformic solution was noted by Levine (3) who reported that aspirin anhydride and salicylic acid were formed, and that anhydride formation was accelerated in the presence of weakly alkaline reagents. He did not, however, comment on the phenomenon of surface catalysis which was observed in this study. Davidson and Auerbach (4) investigated the behavior of aspirin in nonaqueous media and found that in the presence of dissolved base, the compound functioned as an effective acetylating agent. They postulated the existence of a cyclic intermediate which results from the intramolecular nucleophilic attack by the ionized carboxyl group on the ester carbonyl. The resulting intermediate decomposed to yield either aspirin or salicyloylacetic anhydride. Considerable discussion has appeared in the literature relative to the hydrolytic behavior of aspirin in aqueous solution and particularly concerned with the mechanism of intramolecular catalysis which is apparent in the pH region of approximately 4 to 8 (5–13). Recently, Fersht and Kirby (14, 15) have presented convincing evidence to suggest that intramolecular nucleophilic attack to form a cyclic intermediate and subsequent formation of the anhydride is not the most probable mechanism. Rather they concluded that the mechanism of hydrolysis is general base catalysis of attack by water by the carboxylate anion. They did state, however, that a few percent of hydrolysis could occur by the nucleophilic mechanism and their studies do not rule out the possibility that such a mechanism is operant in nonhydroxylic solvents.

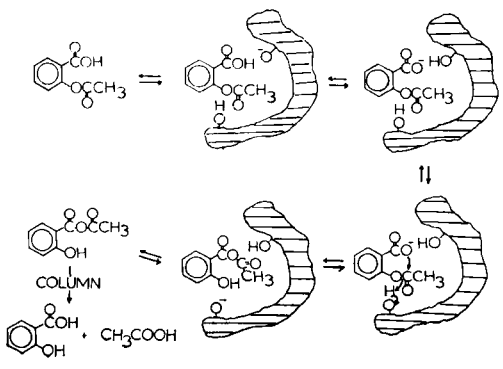
The present investigation has demonstrated that finely divided solids do catalyze a conversion of aspirin to a product which, by the analytical method employed, is determined as salicylic acid. The reaction occurred under essentially anhydrous conditions and adsorption of the aspirin is apparently a prerequisite for the transformation. Although insufficient data is available to definitively characterize the mechanism of reaction, the speculative scheme presented as Scheme I does appear to be consistent with the data. Aspirin is portrayed as being adsorbed to a negatively charged adsorption site where a protolytic reaction occurs to yield aspirin anion. Nucleophilic attack by carboxyl group on the ester carbonyl then occurs to form the cyclic intermediate. The intermediate decomposes

TABLE I—INFLUENCE OF CITRIC ACID ON THE ADSORPTION OF SALICYLIC ACID FROM CHLOROFORMIC SOLUTION BY VARIOUS SOLIDS

Concentration of Citric Acid, mg./ml.	Initial Concentration of Salicylic Acid, mg./ml. × 10^2	Equilibrium Concentration of Salicylic Acid, mg./ml. × 10^2
Magnesium Carbonate, 10 mg./ml.		
0	5	0.0282
0	10	0.018
0	15	1.65
0	20	6.56
0	25	10.2
0	30	15.3
10	5	5.2
10	10	9.7
10	15	15.3
10	20	19.8
10	25	24.5
10	30	30
Aluminum Glycinate, 10 mg./ml.		
0	5	2.25
0	10	6.54
0	15	12.5
0	20	17.6
0	25	22.6
0	30	27.2
10	5	4.18
10	10	8.43
10	15	14.4
10	20	19
10	25	23.7
10	30	29.4
Aluminum Hydroxide Magnesium Trisilicate,[a] 20 mg./ml.		
0	5	1.69
0	10	6.9
0	15	6.9
0	20	10.6
0	25	8.58
0	30	9.49
20	5	3.27
20	10	8.06
20	15	13
20	20	17
20	25	22
20	30	29

[a] Powdered antacid tablets.

to aspirin or to the anhydride with the involvement, in the latter case, of a grouping on the adsorption side functioning as a general acid. The salicyloylacetic anhydride so formed is retained on the chromatographic column by virtue of its phenolic hydroxyl group, and it is rapidly hydrolyzed on the column to acetic and salicylic acid.



Scheme I

It is possible that citric acid monohydrate, which is essentially chloroform insoluble, inhibits the reaction by releasing water of hydration to the surface of the adsorbent and undergoing a neutralization reaction in the hydrate layer which modifies the surface characteristics of the adsorbent so as to destroy adsorption sites.

Since citric acid monohydrate was shown to be effective in inhibiting the formation and adsorption of salicylic acid in the systems of interest, a logical extension of this study was to evaluate the possibility of incorporating a citric acid treatment in the analytical determination of salicylic acid in systems containing aspirin and chloroform-insoluble buffers. The results obtained by such a treatment, using a synthetic mixture, are shown in Table II. The citric acid treatment involved triturating an aliquot of the sample mixture with an equal weight of citric acid monohydrate prior to the addition of chloroform and subsequent chromatography. The results show that, in contrast to the official procedure, the citric acid procedure resulted in almost quantitative recovery of salicylic acid. It should be noted that additional experimentation demonstrated that citric acid treatment was not effective in displacing salicylic acid from metallic salts which are known to form in buffered aspirin products. A modification of this procedure is, however, quite effective in estimating the total nonaspirin salicylate content of buffered products and will be described in a subsequent communication.

EXPERIMENTAL

Materials—Aspirin, salicylic acid, magnesium carbonate (basic), citric acid monohydrate, reagent grade solvents were all obtained from the Fisher Scientific Co. The magnesium carbonate was dried at 120° for 72 hr. prior to use. All other chemicals were used as received. Aluminum glycinate was kindly provided by Dr. John H. Wood, Bristol-Myers Products. Magnesium trisilicate–aluminum hydroxide mixtures were obtained by powdering commercially available antacid tablets. All buffered aspirin tablets which were studied were obtained from local pharmacies.

Procedures—*Adsorption Studies*—Stock solutions of salicylic acid or aspirin in chloroform were prepared to obtain a range of concentrations. Ten-milliliter aliquots of such solutions were placed in 3-

TABLE II—DETERMINATION OF THE SALICYLIC ACID CONTENT OF A SYNTHETIC MIXTURE CONTAINING ASPIRIN, SALICYLIC ACID, MAGNESIUM CARBONATE, AND ALUMINUM GLYCINATE (ASPIRIN 3 g.; SALICYLIC ACID 0.03 g.; MAGNESIUM CARBONATE 0.9 g.; ALUMINUM GLYCINATE 0.45 g.)

Salicylic Acid Theory, %	Salicylic Acid Found, %	
	USP Procedure	Citric Acid Procedure
1.0	0.215	0.975
	0.365	0.975
		1.09
		0.984

dr. glass prescription vials. A weight of adsorbent, usually 100 mg., was added to each vial which was then stoppered. The stoppers were firmly taped and the vials were agitated on a rotating device for 1 hr. Solids were allowed to sediment and an aliquot from each vial was removed and filtered using a Swinney adaptor to a hypodermic syringe. An accurately measured volume of the clear filtrate was appropriately diluted and assayed spectrophotometrically. A Beckman DU spectrophotometer was used throughout. A wavelength of 306 mμ was used for salicylic acid studies while 278 mμ was employed in the case of aspirin solutions. In the citric acid experiments, the citric acid and solid buffer were added as a powdered mixture. These studies were conducted at room temperature, 25 ± 2°.

Rate Studies with Magnesium Carbonate—A solution of aspirin in chloroform was prepared in a 100-ml. volumetric flask by dissolving the required amount of aspirin in sufficient chloroform to make 100 ml. The required weight of magnesium carbonate was then added. A magnetic stirring bar was introduced to the flask, and the mixture was stirred continually by use of a magnetic stirrer. At various time intervals aliquots were removed and filtered. Preliminary experiments showed that filtration resulted in an immediate cessation of salicylic acid production. An accurately measured volume of the filtrate was assayed for salicylic acid by the chromatographic method described under free salicylic acid in the monograph for aspirin tablets in the 17th revision of the USP (1) beginning with the phrase, under *Procedure*, "Pass 50 ml. of chloroform in several portions...." In the citric acid experiments, a weight of citric acid equivalent to the weight of magnesium carbonate was added when the magnesium carbonate was added to the aspirin solution. These and all other rate studies were conducted at room temperature, 25 ± 2°.

Rate Studies with Systems Formulated from Buffered Aspirin Tablets—Buffered aspirin tablets were powdered and a weight equivalent to 2 g. of aspirin was placed in a 100-ml. volumetric flask. Chloroform was added to volume and a stirring bar was placed in the flask. Sampling and assay were conducted as described above. In the citric acid experiments, a weight of citric acid equivalent to the weight of powdered tablets was triturated with the powdered tablets and the mixture was placed in the volumetric flask.

Salicylic Acid Content of the Synthetic Mixture—A synthetic mixture was prepared by intimately mixing 3 g. of aspirin, 0.03 g. of salicylic acid, 0.9 g. of magnesium carbonate, and 0.450 g. of aluminum glycinate. An aliquot of the mixture was assayed for salicylic acid content by the procedure specified for free salicylic acid, for tablets which are coated or contain buffers, in the 17th revision of the USP (1). The citric acid method consisted of accurately weighing a portion of the mixture which was equivalent to 400 mg. of aspirin. The portion was placed in a glass mortar and triturated with 200 mg. of citric acid monohydrate using a glass pestle. Twenty milliliters of chloroform was added to the mixture and it was stirred by means of a stirring bar and magnetic stirrer for 15 min. The mixture was filtered and the filtrate was collected in a 50-ml. volumetric flask. The mortar and pestle were washed with two 10-ml. portions of chloroform which were passed through the filter and collected in the volumetric flask. Chloroform was added to volume. Exactly 10 ml. of the resulting solution was used as a sample and assayed by the free salicylic acid test procedure described in the aspirin tablet monograph in the 17th revision of the USP (1) beginning with the phrase, under *Procedure*, "Pass 50 ml. of chloroform in several portions...."

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Keyphrases

Aspirin, buffered—heterogeneous catalysis of degradation
Chloroform—aspirin-catalyzed degradation
Salicylic acid, free—analysis
Citric acid effect—aspirin stability, buffered
Adsorbents, effect—free salicylic acid analysis

Chlorpromazine and Epinephrine Hyperglycemic Mechanisms

By TAGHI GHAFGHAZI*, TOM S. MIYA, J. H. MENNEAR, and R. K. CHALMERS

Chlorpromazine administration resulted in hyperglycemia in the intact, but not in the adrenalectomized, rat. However, chlorpromazine potentiated epinephrine-induced hyperglycemia in both intact and adrenalectomized animals. β-Adrenergic blockade prevented chlorpromazine-induced as well as epinephrine- and isoproterenol-induced hyperglycemia. α-Adrenergic blockade potentiated epinephrine-induced hyperglycemia. A significant reduction of tolerance to glucose loading was exhibited in both intact and adrenalectomized rats after treatment with chlorpromazine. The results indicate that the adrenals are necessary for chlorpromazine-induced hyperglycemia and that chlorpromazine impairs peripheral utilization of glucose.

PREVIOUS REPORTS in the literature have indicated the ability of chlorpromazine to produce hyperglycemia in several animal species including man. Courvoisier *et al.* (1) were the first to observe the hyperglycemic effect of chlorpromazine. After administration of chlorpromazine an increase in blood glucose levels has been observed in mice and hamsters (2), in dogs (3), and in rabbits (4, 5). Although Norman and Hiestand (2) were not able to find a significant effect of chlorpromazine on blood glucose in rats, other workers (6–8) have demonstrated this phenomenon. Hyperglycemia has also been reported during chlorpromazine therapy in humans (9, 10).

Several mechanisms are probably involved in chlorpromazine-induced hyperglycemia. It is partly the result of an indirect effect due to the release of catecholamines following the fall of body temperature produced by this drug (7). Chlorpromazine in low doses, which have no effect on body temperature, has been shown to decrease the tolerance to glucose load (11).

Chlorpromazine-induced hyperglycemia in the rat has been shown to be blocked by phentolamine, an α-adrenergic blocking agent (7). In the same species, it has been reported that the glycemic effect of chlorpromazine as well as of epinephrine could also be blocked by a β-adrenergic blocking agent such as pronethalol (8). Antonis *et al.* (12) have shown that the hyperglycemic responses to epinephrine in man can be blocked by simultaneously employing both α- and β-adrenergic blocking agents. The rise of blood glucose was not prevented by either blocking agent alone.

In this study, the effect of different adrenergic blocking agents on chlorpromazine-induced hyperglycemia was investigated. Experiments were also conducted to study the effect of concomitant administration of epinephrine and chlorpromazine in intact and adrenalectomized rats. The general objective of the study was to elucidate the mechanism of action of chlorpromazine-induced hyperglycemia.

EXPERIMENTAL

Material and Methods—Male albino Holtzman rats weighing approximately 250 g. and fasted 16–20 hr. with water *ad libitum* were employed. Blood glucose determinations were carried out by the glucose oxidase method[1] on blood taken from the orbital sinus. After bilateral adrenalectomy the animals were maintained on commercial food pellets and 0.9% saline *ad libitum* for 7 days prior to drug treatment.

RESULTS

Chlorpromazine Potentiation of Epinephrine Hyperglycemia—Graded hyperglycemic responses were obtained with 5, 10, 15, and 20 mg./kg. of chlorpromazine (Fig. 1).

The potentiating effect of chlorpromazine pretreatment on epinephrine-induced hyperglycemia is illustrated in Fig. 2. Chlorpromazine at 5 mg./kg. produced only slight hyperglycemia but significantly increased epinephrine-induced hyperglycemia.

Effect of MJ-1999[2] and Phenoxybenzamine[3] Pretreatment on Drug-Induced Hyperglycemia—MJ-

Received May 22, 1968, from the Department of Pharmacology-Toxicology, School of Pharmacy & Pharmacal Sciences, Purdue University, Lafayette, IN 47907

Accepted for publication on July 2, 1968.

Presented to the Pharmacology and Biochemistry section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

Supported in part by grants MH8803 and GM15005.

* Iranian Government Fellow.

[1] Glucose oxidase enzyme (Glucostat), Worthington Biochemical Corp., Freehold, N. J.

[2] MJ-1999 [4'-(2-isopropylamino-1-hydroxyethyl) methane sulfonanilide HCl] was kindly supplied by Mead Johnson Laboratories, Evansville, Ind.

[3] Phenoxybenzamine (Dibenzyline) was kindly supplied by Smith, Kline & French Laboratories, Philadelphia, Pa.

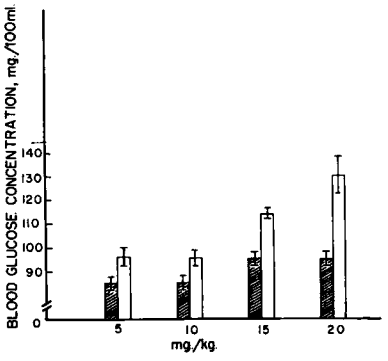

Fig. 1—Effect of intraperitoneal doses of chlorpromazine on fasting blood glucose 1 hr. after treatment. Each bar represents the average and standard errors derived from 6 animals. Shaded bars indicate control determinations.

1999 produced a significant hypoglycemia ($p < 0.005$) and blocked chlorpromazine-induced hyperglycemia (Fig. 3). Phenoxybenzamine raised blood glucose from 75 to 84 mg.% ($p < 0.1$—not significant) and did not block the chlorpromazine-induced hyperglycemia (Fig. 4). The hyperglycemia induced by isoproterenol and epinephrine was blocked by MJ-1999 pretreatment (Fig. 5).

Phenoxybenzamine alone at 10 mg./kg. produced an insignificant hyperglycemia and when administered with epinephrine, potentiated the epinephrine-induced hyperglycemia (epinephrine HCl, 30 mcg./kg., 150.0 ± 2.4 mg.% *versus* phenoxybenzamine plus epinephrine, 203.6 ± 3.6 mg.%; $p < 0.001$).

Figure 6 illustrates the lack of hyperglycemic response from chlorpromazine administration to adrenalectomized rats whereas epinephrine produced a significant hyperglycemia ($p < 0.005$). When chlorpromazine was administered prior to epinephrine, it markedly potentiated ($p < 0.001$) the epinephrine hyperglycemia. All hyperglycemic re-

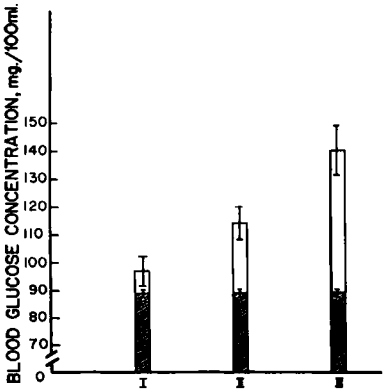

Fig. 2—Effect of chlorpromazine (CPZ) pretreatment on epinephrine HCl-induced hyperglycemia. Blood glucose determined 30 min. after epinephrine (30 mcg./kg.) and 90 min. post-CPZ (5 mg./kg.) administration. Shaded area indicates saline control blood glucose levels. Key: I, CPZ; II, Epi.; III, Epi. + CPZ. Means from 4 rats.

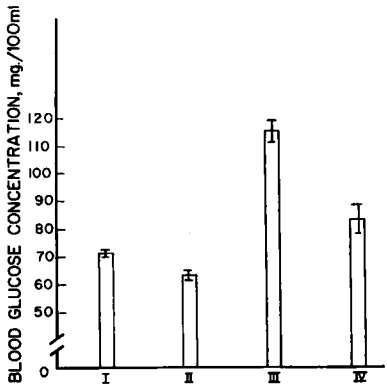

Fig. 3—Effect of MJ-1999 pretreatment on CPZ-induced hyperglycemia. Blood glucose determined 90 min. after CPZ (15 mg./kg.) and 120 min. post-MJ-1999 (10 mg./kg.) pretreatment. Key: I, saline control; II, MJ-1999; III, CPZ; IV, CPZ + MJ-1999. Means from 5 rats.

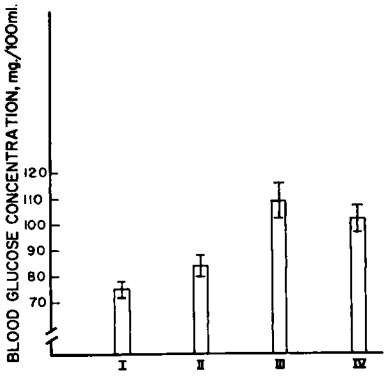

Fig. 4—Effect of phenoxybenzamine (DZ) pretreatment on CPZ-induced hyperglycemia. Blood glucose determined 90 min. after CPZ (15 mg./kg.) and 150 min. post-phenoxybenzamine (10 mg./kg.) injection. Key: I, saline control; II, DZ; III, CPZ; IV, CPZ + DZ. Means from 4 animals.

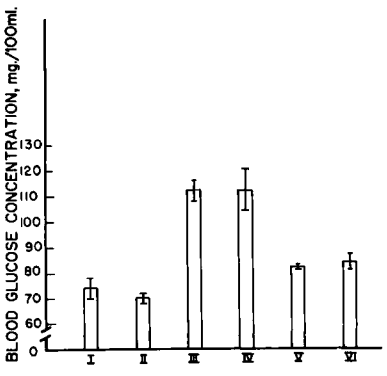

Fig. 5—Inhibition of isoproterenol- and epinephrine-induced hyperglycemia by MJ-1999. MJ-1999 (10 mg./kg., i.p.) administered 30 min. prior to isoproterenol (1 mg./kg.) or epinephrine 30 mcg./kg.). Blood glucose determined 60 min. after MJ-1999. Key: I, saline control; II, MJ-1999; III, Epi.; IV, Isoproterenol; V, Epi. + MJ-1999; VI, Isoproterenol + MJ-1999. Means from 4 animals.

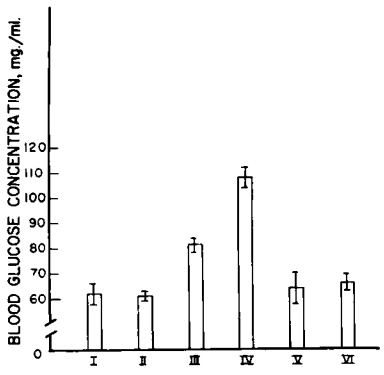

Fig. 6—Effect of MJ-1999 on CPZ potentiation of epinephrine-induced hyperglycemia in adrenalectomized rats. Epinephrine HCl (30 mcg./kg.) injected 60 min. after CPZ (15 mg./kg.) and MJ-1999 injected 30 min. prior to CPZ. Blood glucose determined 30 min. after epinephrine. Key: I, saline control; II, CPZ; III, Epi.; IV, Epi. + CPZ; V, Epi. + MJ-1999; VI, Epi. + CPZ + MJ-1999. Means from 5 animals.

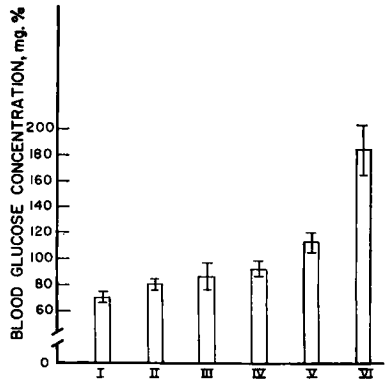

Fig. 8—Effect of phenoxybenzamine (DZ) and chlorpromazine (CPZ) on glucose load in intact rats. CPZ (15 mg./kg.) and DZ (10 mg./kg.) administered 60 min. prior to glucose loading and blood glucose determined 2 hr. after loading. Key: I, saline control; II, DZ; III, CPZ; IV, glucose; V, Glucose + DZ; VI, Glucose + CPZ. Means from 4 animals. Glucose load 2 g./kg., i.p.

sponses were blocked by MJ-1999 in both intact and adrenalectomized rats.

Effect of Chlorpromazine and Phenoxybenzamine on Glucose Tolerance—The hyperglycemia following glucose loading was significantly increased ($p < 0.001$ at 2-hr. interval) in chlorpromazine-treated adrenalectomized (Fig. 7) and intact (Fig. 8) rats ($p < 0.001$). This decreased tolerance to glucose load may indicate impairment of peripheral glucose utilization by chlorpromazine. Since phenoxybenzamine had also potentiated epinephrine-induced hyperglycemia it was of interest to observe its influence on glucose tolerance. As shown in Fig. 8, tolerance to glucose loading was not altered by phenoxybenzamine.

DISCUSSION

Although the blood pressure rise induced by epinephrine can be blocked by chlorpromazine (1, 5), contradictory observations have been made with respect to the glycemic action of epinephrine and chlorpromazine. Gupta *et al.* (5) found that chlorpromazine-induced hyperglycemia was enhanced by epinephrine in rabbits, but Courvoisier *et al.* (1) found no effect of chlorpromazine on epinephrine-induced hyperglycemia. However, this study in rats indicates a definite potentiation of epinephrine hyperglycemia by chlorpromazine pretreatment. From these studies it may be concluded that in the rat the responsible receptors for blood pressure and hyperglycemia affected by epinephrine are not identical. It appears that in the rat the β-adrenergic receptors are involved in the hyperglycemic action since it can be blocked by a β-adrenergic-blocking agent such as MJ-1999 and not by phenoxybenzamine (α-adrenergic-blocking agent). Epinephrine-induced hyperglycemia was markedly potentiated by phenoxybenzamine pretreatment. This potentiation probably resulted from the blockade of α-adrenergic receptors by phenoxybenzamine which increases the effect of epinephrine on the β-adrenergic receptors. This is analogous to the well known "epinephrine reversal" phenomenon in regard to blood pressure responses after α-receptor blockade. Like phenoxybenzamine, the chlorpromazine potentiation of epinephrine-induced hyperglycemia may also be explained, in part, on the basis of the "epinephrine reversal" phenomenon. It has been shown in this study and by others (11) that chlorpromazine pretreatment reduces the tolerance to glucose loading in rats. It would appear that chlorpromazine potentiation of epinephrine-induced hyperglycemia is probably the result of a twofold action, (*a*) the impairment of peripheral utilization of glucose, and (*b*) the production of α-adrenergic blockade.

Although it appears that several mechanisms are involved in the glycemic action of chlorpromazine, the present data indicate that the primary mechanism by which chlorpromazine induces hyperglycemia is through the release of epinephrine. The chlorpromazine potentiation of epinephrine-induced hyperglycemia is independent of the adrenal glands and may be considered a secondary mechanism.

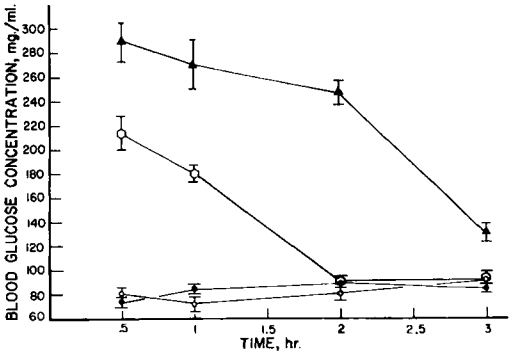

Fig. 7—Effect of chlorpromazine on glucose load in fasted adrenalectomized rats. CPZ (15 mg./kg., i.p.) administered 60 min. before glucose loading (2 g./kg., i.p.). Key: ○, CPZ alone; ○, glucose alone; ▲, CPZ + glucose; ●, saline control. Means from 4 rats.

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Keyphrases

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Chlorpromazine potentiation—epinephrine hyperglycemia
β-Adrenergic receptors—relation to hyperglycemia

Potential Antineoplastic Agents: *N*-(2-Benzoxazolyl)amino Acid Esters

By SHYAM B. ADVANI* and JOSEPH SAM

The investigation of the preparation of *N*-(2-benzoxazolyl)aminoacids and esters from the reaction of aminoacids and esters with either 2-chlorobenzoxazole or 2-benzoxazolinone is described.

TUMORS, because of their parasitic nature, depend upon the host for nutrition and growth. Moreover, because of a limited blood supply, tumor cells depend solely on highly effective transport mechanisms for nutrients. Transport mechanisms involving the accumulation of aminoacids in tumor cells have been studied extensively (1). Tumor cells, being more dependent than the normal tissues on the surrounding fluids for an adequate supply of amino acids, are susceptible to attack by substances which interfere with amino acid transport. Ethionine not only was found to concentrate to a greater extent in tumor cells than methionine, but also was found to possess antineoplastic activity (2). Cycloleucine (1-aminocyclopentanecarboxylic acid) was reported to inhibit growth of tumors by inhibiting transport of other amino acids (3–5). Nitrogen mustards of phenylalanine were found to possess high antitumor activity, probably because of increased transport to the tumor cells (6).

The marked antineoplastic activity of some amino acids prompted the preparation of *N*-(2-benzoxazolyl)amino acids and esters (III, Table I) from 2-chlorobenzoxazole (I) and aminoacids or ethyl esters of aminoacids (II). No reaction was observed when 2-chlorobenzoxazole (I) was treated with either tyrosine or leucine in dry ethanol or benzene according to the method of Montgomery (7). Glycine, on the other hand, gave the desired product (III*c*). The esterification of III*c* in ethanol gave ethyl *N*-(2-benzoxazolyl)glycinate (III*b*). When phenylalanine in ethanol was treated with I in the presence of triethylamine, the ester (III*d*) rather than the acid was isolated. Whereas most amino acids did not react with I, the esters of the amino acids gave satisfactory results. When dimethylformamide (DMF) was used as a solvent in the reactions above, 2-dimethylaminobenzoxazole was isolated as a product (8).

The condensation of I with ethyl *p*-aminobenzoate gave ethyl *N*-(2-benzoxazolyl)-*p*-aminobenzoate (III*h*). The corresponding acid (III*i*) was obtained either from the sodium hydroxide hydrolysis of III*h* or from the reaction of I with

Received May 22, 1968, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Mississippi, University, MS 38677
Accepted for publication June 27, 1968.
Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
Abstracted in part from a thesis submitted by S. B. Advani to the Graduate School, University of Mississippi, in partial fulfillment of Doctor of Philosophy degree requirements.
This investigation was supported by grant CA 07857 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
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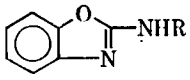
TABLE I—*N*-(2-BENZOXAZOLYL)AMINO ACIDS AND ESTERS



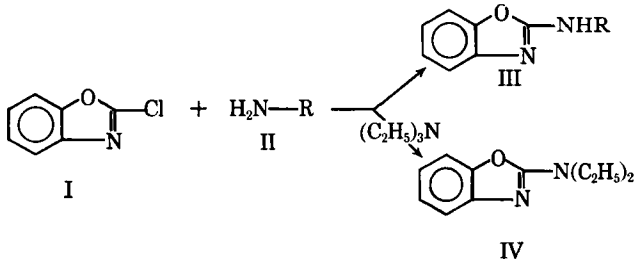
No.	R	Method	Yield, %	M.p., °C.	Molecular Formula	Anal., % Calcd.	Found
III*a*	—CH($CO_2C_2H_5$)CH_2-*p*-C_6H_4OH	A, B[a]	52, 71	161–162[b]	$C_{18}H_{18}N_2O_4$	C, 66.25 H, 5.56 N, 8.58	C, 66.38 H, 5.68 N, 8.69
III*b*	$CH_2CO_2C_2H_5$	D, E	38	104–104.5[c]	$C_{11}H_{12}N_2O_3$	C, 59.99 H, 5.49 N, 12.72	C, 60.20 H, 5.61 N, 12.56
III*c*	CH_2CO_2H	F[d]	31	196–197.5[e]	$C_9H_8N_2O_3$[f]	C, 56.25 H, 4.20 N, 14.58	
III*d*	—CH($CO_2C_2H_5$)$CH_2C_6H_5$	A, F[g]	45, 51	128–129[h]	$C_{18}H_{18}N_2O_3$	C, 69.66 H, 5.85 N, 9.03	C, 69.96 H, 6.15 N, 9.25
III*e*	—CH($CO_2C_2H_5$)$CH_2C_3H_3N_2$[i]	C	70	207–208.5[b]	$C_{15}H_{17}ClN_4O_3$[j]	C, 53.57 H, 5.06 N, 16.66	C, 53.40 H, 5.23 N, 16.07
III*f*	—CH($CO_2C_2H_5$)$CH_2CH_2SCH_3$	B[k]	41	198–199[h] 139–139.5[h,l]	$C_{20}H_{21}N_5O_{10}S$[l]	C, 45.88 H, 4.01 N, 13.38	C, 45.43 H, 3.99 N, 13.11
III*g*	$CH_2CH_2CO_2H$	G	49	175.5–177[m]	$C_{10}H_{10}N_2O_3$	C, 58.25 H, 4.89 N, 13.59	C, 58.20 H, 5.34 N, 13.55
III*h*	—*p*-$C_6H_4CO_2C_2H_5$	B[a]	73	202[b]	$C_{16}H_{14}N_2O_3$	C, 68.08 H, 5.00 N, 9.92	C, 68.45 H, 5.18 N, 9.76
III*i*	—*p*-$C_6H_4CO_2H$	F,[n] H, I	36, 71, 88	328–329[b]	$C_{14}H_{10}N_2O_3$[o]	C, 66.14 H, 3.96 N, 11.02	
III*j*	—*o*-OH-*p*-$C_6H_3CO_2H$	F[n]	26	251.5–252[b]	$C_{14}H_{10}N_2O_4$	C, 62.22 H, 3.73 N, 10.37	C, 62.02 H, 3.91 N, 10.29
III*k*	—*p*-$C_6H_4CH{=}CHCO_2H$	H	70[c]	269.5–270.5[h]	$C_{16}H_{12}N_2O_3$	C, 68.57 H, 4.32 N, 9.99	C, 68.76 H, 4.36 N, 9.84

[a] Benzene used as the solvent. [b] Recryst. from ethanol. [c] Recryst. from ethanol–water. [d] Refluxed 72 hr. [e] Recryst. from benzene–ethanol. [f] Identified through III*b*. [g] Refluxed 92 hr. [h] Recryst. from benzene. [i] $C_3H_3N_2$ = 4-imidazolyl. [j] Hydrochloride. [k] Toluene used as the solvent. [l] Picrate. [m] Recryst. from chloroform. [n] Refluxed 4 hr. [o] Identified through III*h*.

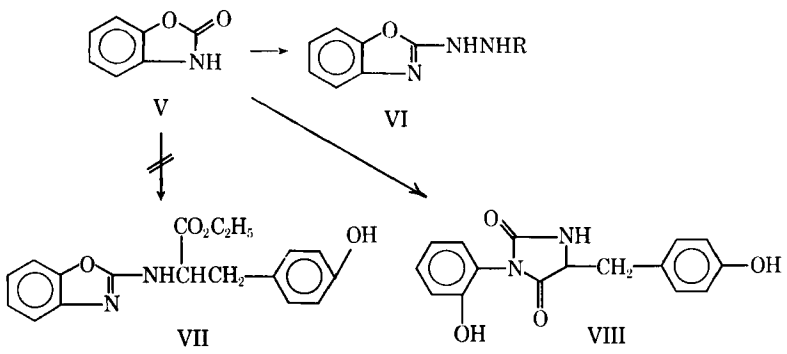
p-aminobenzoic acid. The treatment of I with histidine ethyl ester gave III*e*.

The reaction of 2-chlorobenzoxazole (I) with ethyl tyrosinate in the presence of triethylamine in benzene solution gave approximately a 50% yield of product (III*a*). When the same reaction was conducted in the absence of triethylamine the yield was increased to over 70%. Although no exhaustive study was made, it is speculated that the low yield of III (Table I) obtained with triethylamine in the reaction of I with II (Methods A, D, F, and G) was due to the competitive reaction of triethylamine with 2-chlorobenzoxazole to give 2-diethylaminobenzoxazole (IV). Some of the latter was isolated when conditions described under Method G were utilized. Also, 2-diethylaminobenzoxazole (IV) was the only product obtained when triethylamine was used to remove hydrogen chloride evolved in the reaction (Method J) between I and diethyl glutamate, ethyl valinate, ethyl leucinate, and ethyl alaninate, respectively. The results were in agreement with the observation noted earlier (9) with 2,5-dichlorobenzoxazole and trimethylamine.

An alternate method attempted for the preparation of III (VII) involved the reaction of 2-benzoxazolinone with ethyl tyrosinate. Bower and associates (10) reported favorable results in the preparation of 2-hydrazinobenzoxazoles (VI) by fusion of a hydrazine with V. The fusion of ethyl tyrosinate with 2-benzoxazolinone, however, gave 1-(2-hydroxyphenyl)-4-(4-hydroxybenzyl)imidazolidine-2,5-dione (VIII), which was identified by elemental analysis, infrared, and NMR data. Similar rearrangements have been



I IV



observed (11) in the reaction of ethyl anthranilate with 2-benzoxazolinone.

Infrared data of the compounds are reported in Table II.

Screening of the compounds described in this study currently is being coordinated by the Cancer Chemotherapy National Service Center.

EXPERIMENTAL[1]

Commercially available *dl*-aminoacids (except L-(+)-glutamic acid and *l*-leucine) were utilized in this study; the esters were prepared according to the Fisher esterification method (12).

N-(2-Benzoxazolyl)aminoacid Esters—*Method A*—A solution of 0.025 mole of 2-chlorobenzoxazole, 0.025 mole of aminoacid ester, 6.5 g. (0.065 mole) of triethylamine, and 100 ml. of dry benzene was refluxed for 24 hr. The solution was cooled and the triethylamine hydrochloride removed by filtration. The filtrate was evaporated to dryness under reduced pressure. The residual material either was crystallized and/or recrystallized from a suitable solvent.

Method B—A solution of 0.02 mole of 2-chlorobenzoxazole and 0.02 mole of aminoacid ester in 300 ml. of benzene or toluene was refluxed for 72 hr. The solution was concentrated under reduced pressure on a water bath. The residual material was recrystallized from a suitable solvent.

Method C—A solution of 0.02 mole of aminoacid ester in 200 ml. of benzene or toluene was treated dropwise, while stirring, with a solution of 0.02 mole of 2-chlorobenzoxazole in 10 ml. of ethanol and then refluxed for 48 hr. The solution was allowed to stay at room temperature for 12 hr. and then filtered. The filtrate was concentrated under reduced pressure. The residual material was triturated with acetone, removed by filtration, and then recrystallized.

Ethyl N-(2-Benzoxazolyl)glycinate (IIIb)—*Method D*—The procedure described by Sheehan *et al.* (13) was followed. To a solution of 2.5 g. (0.02 mole) of ethyl glycinate hydrochloride, 3.8 g. (0.025 mole) of 2-chlorobenzoxazole, and 60 ml. of anhydrous chloroform, maintained at −15° (ice and sodium nitrite), was added over a period of 40 min. with vigorous stirring a solution of 4.0 g. (0.04 mole) of triethylamine in 40 ml. of chloroform. Thereafter, while stirring, the temperature was maintained at −15° for 10 min. and then slowly raised to 0°, held at this temperature for another 10 min., and then maintained at room temperature for 1 hr. The solvent was distilled under reduced pressure; the residue was washed with cold water to remove triethylamine hydrochloride and any unreacted ethyl glycinate hydrochloride and then recrystallized.

The nuclear magnetic resonance spectrum showed a four-proton aromatic multiplet (7.1–7.15), a four-proton methylene multiplet (4.1–4.5), and a three-proton methyl triplet (1.2–1.5).

Method E—A solution of 100 mg. of III*c* in 10 ml. of ethanol was treated with an excess of hydrogen chloride and then refluxed for 2 hr. Distillation of the solvent under reduced pressure left an oil which was dissolved in tetrahydrofuran and treated with an excess of triethylamine. The mixture was filtered to remove triethylamine hydrochloride and then again concentrated under reduced pressure. A solution of the residual oil in 5 ml. of ether was allowed to evaporate slowly at room temperature; 25 mg. of solid was obtained, m.p. 104–106°. A mixture of the products obtained by Methods D and E showed no depression of the melting point.

N-(2-Benzoxazolyl)aminoacids—*Method F*—A mixture of 0.05 mole of aminoacid, 10.0 g. (0.1 mole) of triethylamine, and 0.05 mole of 2-chlorobenzoxazole in 200 ml. of ethanol was refluxed for 4–96 hr. The unreacted aminoacid was removed by filtration and the filtrate concentrated under reduced pressure on a water bath. The residual material was treated with

TABLE II—INFRARED SPECTRAL DATA (cm.$^{-1}$)

No.	N—H Str.	C=N C=O Str.	C=C Str.	1,2-Disubst. C=C Str.
III*a*	3,250	1,730	1,650	
			1,585	
III*b*	3,300	1,730	1,640	740
			1,570	
III*d*	3,330	1,725	1,575	745
III*e*	3,330	1,740	1,575	745
III*f*	3,350	1,740	1,675	760
			1,630	715
III*g*	3,330	1,700	1,650	740
			1,575	
III*i*	3,350	1,670	1,600	740
			1,550	
III*j*	3,450	1,650	1,575	
IV	3,350	1,700	1,640	740
			1,575	

[1] All melting points were taken on a Fisher-Johns melting-point apparatus and are uncorrected. Infrared spectra were determined on a Perkin-Elmer model 137 Infracord spectrophotometer using KBr pellets. Nuclear magnetic resonance spectra were determined on a Varian A-60A spectrometer using tetramethylsilane as the internal standard. Chemical shifts are recorded as δ values.

acetone and filtered. Concentration of the filtrate under reduced pressure on a water bath and crystallization and/or recrystallization of the residual material gave product.

Method G—The above procedure was utilized with the exception that benzene was used as the solvent in place of ethanol. A 20% yield of 2-diethylaminobenzoxazole also was obtained.

Method H—A mixture of 0.1 mole of 2-chlorobenzoxazole, 0.1 mole of aminoacid, 200 ml. of benzene, and 20 ml. of ethanol was refluxed on a steam bath for 24 hr. The solid was removed by filtration and washed with warm ethanol to remove any unreacted aminoacid.

N-(2-Benzoxazolyl)-p-aminobenzoic Acid (IIIi)—*Method I*—A mixture of 1.0 g. (0.004 mole) of ethyl *N*-(2-benzoxazolyl)-*p*-aminobenzoate and 50 ml. of 10% sodium hydroxide solution was refluxed on a steam bath for 1 hr. The clear solution was cooled and treated with 10% hydrochloric acid. A white microcrystalline precipitate was obtained.

2-Diethylaminobenzoxazole (IV)—*Method J*—A solution of 7.7 g. (0.05 mole) of 2-chlorobenzoxazole, 10.0 g. (0.05 mole) of diethyl glutamate, and 10.0 g. (0.1 mole) of triethylamine in 200 ml. of dry benzene was refluxed for 24 hr. and then allowed to stay at room temperature for 2 hr. The solution was concentrated under reduced pressure on a water bath. The residue was treated with 50 ml. of acetone and filtered to remove triethylamine hydrochloride. The filtrate was distilled at 140°/1.5 mm. to give 14.1 g. (88%) of oil, $n_D^{25} = 1.5527$. The hydrochloride was prepared in the usual manner and recrystallized from toluene, m.p. 136–137°. The NMR spectrum showed a four-proton aromatic multiplet (6.7–7.4), a four-proton methylene quartet (3.1–3.6), and a six-proton methyl triplet (1.0–1.4).

Anal.—Calcd. for $C_{11}H_{15}ClN_2O$: C, 58.31; H, 6.63; N, 12.39. Found: C, 58.18; H, 6.69; N, 12.40.

Similar results were obtained with ethyl valinate, ethyl leucinate, and ethyl alaninate.

Method K—A solution of 7.7 g. (0.05 mole) of 2-chlorobenzoxazole and 7.3 g. (0.1 mole) of diethylamine in 100 ml. of benzene was refluxed for 24 hr. The diethylamine hydrochloride was removed by filtration; the filtrate was treated as in Method A to yield 9.4 g. (83%) of product, m.p. 135–137°. A mixture of the products obtained by Methods J and K showed no depression of the melting point.

Method L—A solution of 7.7 g. (0.05 mole) of 2-chlorobenzoxazole and 10.0 g. (0.1 mole) of triethylamine in 100 ml. of benzene was refluxed for 2 hr. The triethylamine hydrochloride was removed by filtration; the filtrate was treated as in Method A to give 9.1 g. (82%) of product, m.p. 136–137°. A mixture of the products obtained by Methods J, K, and L showed no depression of the melting point.

1 - (2 - Hydroxyphenyl) - 4 - (4 - hydroxybenzyl)-imidazolidine-2,5-dione (VIII)—A mixture of 3.5 g. (0.017 mole) of ethyl tyrosinate and 2.0 g. (0.015 mole) of 2-benzoxazolinone was fused in an oil bath, preheated to 180° for 15 min. The fused material was allowed to cool to room temperature and then recrystallized from a benzene–alcohol mixture to give 2.3 g. (52%) of product, m.p. 201–201.5°.

The infrared absorption spectrum showed carbonyl absorption at 1775 $cm.^{-1}$ and 1680 $cm.^{-1}$.

Anal.—Calcd. for $C_{16}H_{14}N_2O_4$: C, 64.42; H, 4.73; N, 9.39. Found: C, 64.26; H, 4.98; N, 9.27.

The NMR spectrum showed a one-proton imino singlet (8.3), an eight-proton aromatic multiplet (6.3–7.5), a one-proton methine triplet (4.4–4.6), a two-proton methylene doublet (3.1–3.2), and a broad hydroxyl peak (2.5–3.5).

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Keyphrases

N-(2-Benzoxazolyl)amino acids, esters—synthesis
IR spectrophotometry—structure
NMR spectroscopy—identity

Synthesis of 3′-Deoxynucleosides III

Synthesis of 9-(3-Deoxyaldofuranosyl) Adenines Derived from 3-Deoxy-D-mannose and 3-Deoxy-D-galactose

By J. PROKOP and DANIEL H. MURRAY

The diisopropylidene derivative of 3-deoxy-D-mannose was selectively hydrolyzed to 1,2-*O*-isopropylidene-3-deoxy-D-mannofuranose. In one series of reactions, this compound was converted *via* benzoylation and acetolysis to a suitably blocked sugar, which on condensation with chloromercuri-6-benzamidopurine (using titanium tetrachloride), followed by deacylation, yielded 9-(3-deoxy-α-D-mannofuranosyl) adenine. In a second series of reactions, 1,2-*O*-isopropylidene-3-deoxy-D-mannofuranose was converted through periodate oxidation and borohydride reduction to the corresponding derivatives of 3-deoxy-D-arabinose, convertible by the above standard reactions to 9-(3-deoxy-α-D-arabinofuranosyl)adenine. Inversion of 1,2-*O*-isopropylidene-5,6-di-*O*-methanesulfonyl-3-deoxy-D-galactofuranose with sodium benzoate led to 5,6-di-*O*-benzoyl-1,2-*O*-isopropylidene-3-deoxy-L-mannofuranose from which 9-(3-deoxy-α-L-mannofuranosyl) adenine was similarly prepared.

IN RECENT YEARS, there has been interest in the structure and stereochemistry of the sugar moiety of adenine nucleosides required for inhibitory activity. Among these are adenine compounds bearing various fraudulent sugars (1–3), including 3-deoxyaldofuranoses. In the present report are described the syntheses of 9-(3-deoxy-α-D-mannofuranosyl)adenine, 9-(3-deoxy-α-D-arabinofuranosyl) adenine, and 9-(3-deoxy-α-L-mannofuranosyl) adenine.

Acetonation of 3-deoxy-D-mannose (I, Scheme I) (4) gave the corresponding 1,2:5,6-di-*O*-isopropylidene derivative (II) as a distillable liquid in a yield of 79%. Selective hydrolysis using 0.01 *N* HCl converted II to the syrupy 1,2-*O*-isopropylidene compound (III) in a yield of 67% (based on unrecovered II). The unhydrolyzed diacetone derivative, as well as some deoxymannose produced by complete hydrolysis, could be recovered and reused. Benzoylation of the crude monoacetone compound gave the 5,6-dibenzoate (IV) as a noncrystallizing syrup, whose elemental analysis suggested it to be pure within acceptable analytical limits, although the infrared spectrum indicated that a small amount of benzoic anhydride was present. Acetolysis of the dibenzoate to the anomeric syrupy 1,2-diacetate (V) was followed by coupling with chloromercuri-6-benzamidopurine (VI) (1) in the presence of titanium tetrachloride to give the crude, blocked nucleoside (VII) as a glass. Deacylation was achieved with methanolic sodium methoxide and the crude crystalline nucleoside (VIII) rapidly separated from the cold (0°) solution in a yield of 53%. The anomeric carbon of this nucleoside was assigned the α configuration by application of the *trans* rule[1] (5). In addition, the positive rotation (+52°) is consistent with a number of furanosyl adenines of the α-D-series and is substantially more positive than would be expected for the β-D-anomer.[2]

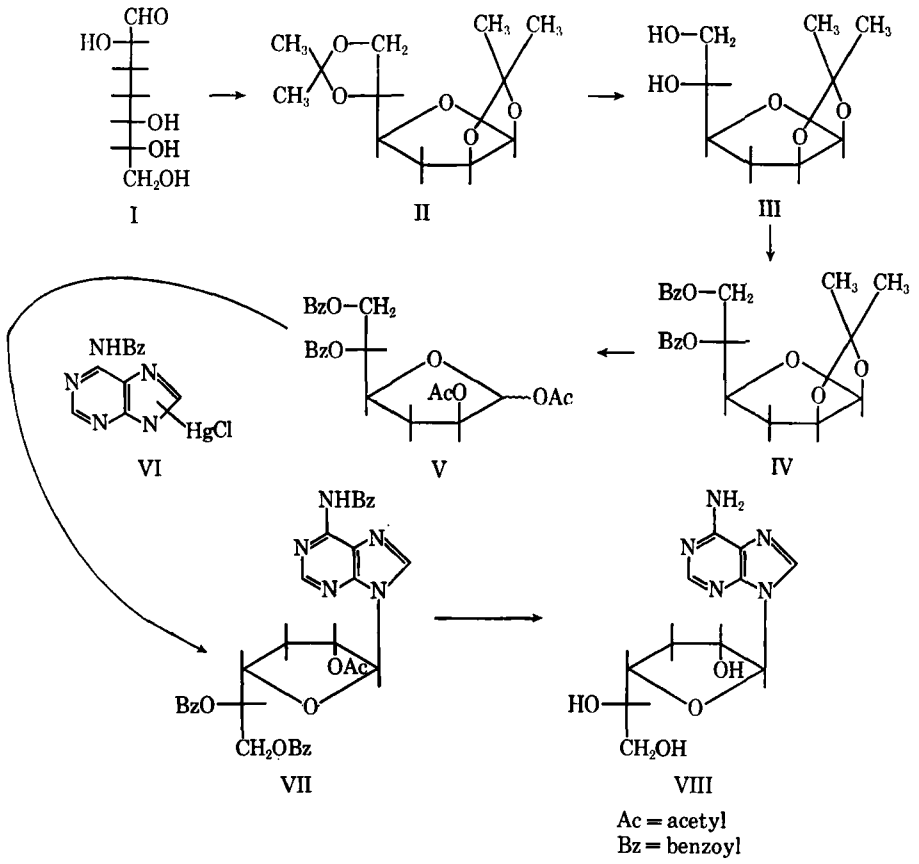
The starting material for the related pentose nucleoside (XIV, Scheme II) was 1,2-*O*-isopropylidene-3-deoxy-D-mannofuranose (III). Oxidation of III with sodium periodate gave a colorless, mobile liquid which reduced Benedict's solution and showed infrared bands at 1,735 cm.$^{-1}$ (C=O, strong) and 2,750 cm.$^{-1}$ (C—H, weak), characteristic of an aldehyde group. Although a low carbon analysis was obtained,[3] the *aldehydo*-pentose structure (IX) is suggested for this compound. Reduction of this aldehyde with sodium borohydride gave 1,2-*O*-isopropylidene-3-deoxy-D-arabinofuranose (X) as a colorless liquid in an overall yield of 60% from the crude monoacetone hexose (III). Benzoylation of the monoacetone pentose gave an 83% yield of the 5-*O*-benzoyl derivative (XI) which was obtained crystalline. This compound

Received January 31, 1968, from the School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication June 28, 1968.
This investigation was supported by Public Health Service grant CY-4198 from the National Cancer Institute.

[1] The terms *cis* and *trans* as used in this paper refer to the configuration of substituents on the C1 and C2 positions of the sugar moiety. Similar condensations in this laboratory, with a variety of sugars, have shown thus far that when the *cis* nucleoside is produced, its yield is, at best, relatively small.

[2] The reader is referred to the extensive tables included in *Reference 6*.

[3] This appears to be related to the presence of impurities resulting from the oxidation of a crude sample of III (a weak hydroxyl bond was also observed in the infrared). Further purification of this compound, beyond simple distillation, was not attempted.

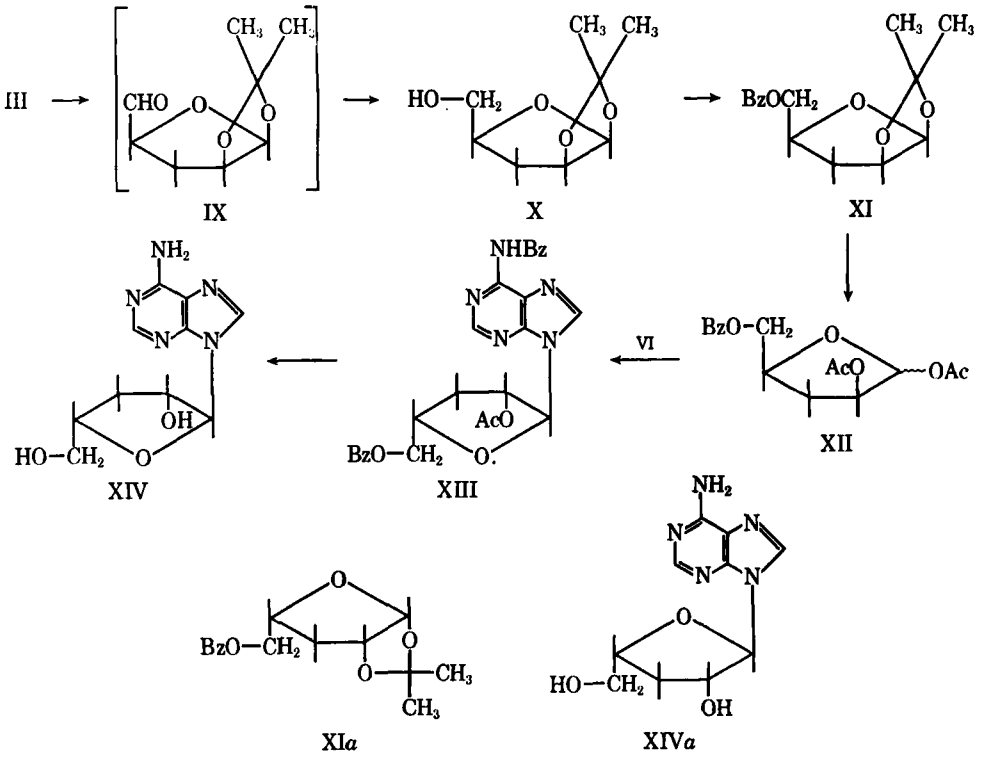


Scheme I

showed an infrared spectrum which was identical in all respects to that of 5-*O*-benzoyl-1,2-*O*-isopropylidene-3-deoxy-L-arabinofuranose (XI*a*) originally prepared from 1,2-*O*-isopropylidene-3-deoxy-D-galactofuranose (1). The constants shown in Table I substantiate the enantiomorphic relationship of these two pentose derivatives. Acetolysis of XI gave the syrupy 1,2-diacetate (XII) in 60% yield. This was converted to the blocked nucleoside (XIII) *via* the titanium tetrachloride procedure and removal of the blocking groups with methanolic sodium methoxide resulted in the separation of the crystalline nucleoside (XIV) in a yield of 31% (from crude XII). This nucleoside was found to be, as expected, the enantiomorph of 9-(3-deoxy-α-L-arabinofuranosyl) adenine (XIV*a*) (1). The infrared spectra of these compounds were superimposable and their constants are as shown in Table I.

In a recent publication, Rembarz (7) has formulated diisopropylidene-3-deoxy-D-mannose as a 1,2:4,6-substituted derivative, *i.e.*, as a pyranose. The optical rotation which is reported for this compound (7), as well as that of the 1,2-monoacetone derivative, closely approaches the determined values of compounds II and III, respectively. The syntheses reported in this paper are consistent throughout with the assignment of a 1,2:5,6-substituted furanose structure for compound II. There is no current explanation for this apparent discrepancy.

The route to the third nucleoside (XX) began with 1,2-*O*-isopropylidene-3-deoxy-D-galactofuranose (XV, Scheme III) (1) which, after conversion to the 5,6-di-*O*-methanesulfonyl derivative (XVI) was inverted using sodium benzoate in dimethylformamide to give syrupy 5,6-di-*O*-benzoyl-1,2-*O*-isopropylidene-3-deoxy-L-mannofuranose (XVII) in a combined yield of 13%. Acetolysis gave XVIII as a syrup which was coupled with chloromercuri-6-benzamidopurine. The acyl groups were removed from the crude blocked nucleoside (XIX) with sodium methoxide in methanol, and 9-(3-deoxy-α-L-mannofuranosyl) adenine (XX) readily crystallized from the reaction in an overall yield of 39% from XVII. The enantiomorphic retionship of this nucleoside with VIII was readily established by consideration of their constants (Table I), and their superimposable infrared spectra.



Scheme II

EXPERIMENTAL[4]

1,2:5,6-Di-O-isopropylidene-3-deoxy-D-mannofuranose (II)—A mixture of 4.00 g. (24.4 mmoles) of finely powdered 3-deoxy-D-mannose (I) (4), 10 g. of anhydrous copper sulfate, and 100 ml. of reagent acetone containing 80 mg. of concentrated sulfuric acid was stirred continuously in a stoppered flask at room temperature for 24 hr. The mixture was filtered, the solid washed with 50 ml. of acetone, and the combined filtrate and washing was stirred for 1 hr. with 4 g. of powdered barium carbonate then filtered through diatomaceous earth.[5] After washing the cake with acetone (3 × 25 ml.), the combined filtrate and washings were evaporated to dryness *in vacuo* at 50° to give 5.69 g. (95.7%) of crude product as a pale yellow liquid of moderate viscosity. Distillation at 0.05 mm. pressure gave a clear colorless liquid with b.p. 60–61°; yield, 4.67 g. (78.5%). For analysis a sample was redistilled at 54°/0.04 mm.; $[\alpha]_D^{20}$ + 24° [c 1.46 $(CH_2Cl)_2$]; $\bar{\nu}_{max.(cm.^{-1})}^{film}$ 1,380 (*gem*-dimethyl), and no absorption at 3,400 (OH).

Anal.—Calcd. for $C_{12}H_{20}O_5$: C, 59.00; H, 8.25. Found: C, 59.06; H, 8.27.

1,2-O-Isopropylidene-3-deoxy-D-mannofuranose (III)—To 445 ml. of 50% aqueous methanol at 40°, 0.01 *N* with respect to hydrochloric acid, was added 8.87 g. (36.3 mmoles) of distilled 1,2:5,6-di-*O*-isopropylidene-3-deoxy-D-mannofuranose (II) and the solution stirred continuously at this temperature for 90 min. The reaction was neutralized with 1 *N* aqueous sodium hydroxide to the phenolphthalein end point, and evaporated *in vacuo* at 40° to a syrup, which was partitioned between 200 ml. of water and 50 ml. of chloroform. The organic phase was separated, dried over magnesium sulfate, filtered, and evaporated to dryness *in vacuo* (50°) to give 0.80 g. (8.8%) of a clear colorless liquid whose infrared spectrum was identical to that of the starting material (II).

The aqueous phase was evaporated to dryness *in vacuo* at 40°, and the syrupy residue was further dried by the addition of absolute ethanol (100 ml.) followed by its removal *in vacuo* (40°). The syrupy residue was extracted with hot chloroform (3 × 30 ml.). The combined extracts were stored overnight at 5° during which time a turbidity developed. Filtration through diatomaceous earth and evaporation to dryness *in vacuo* at 50° gave the crude product as a clear colorless syrup which was sufficiently pure for the next step; yield, 4.55 g. (67.4%

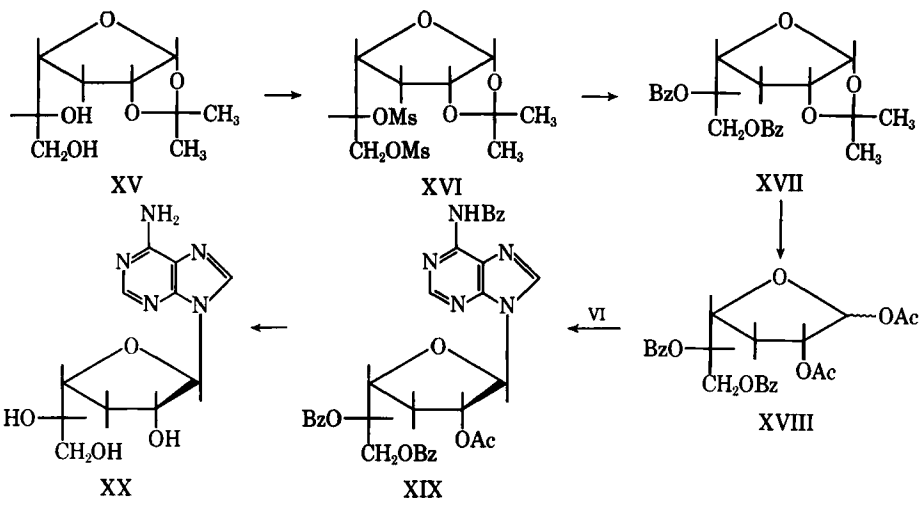
TABLE I—MELTING POINTS AND SPECIFIC ROTATIONS

Compound	M.p., °C.	$[\alpha]_D^{20}$ °
XI	87	+42
XIa	86.5–87[a]	−44[a]
XIV	242.5–243	+54
XIVa	242.5–243[a]	−51[a]
VIII	211–212	+52
XX	212–213	−53

[a] *Reference 1.*

Melting points were determined in an oil bath and are uncorrected. Optical rotations and infrared spectra were determined using a Rudolph model 80 polarimeter and Perkin-Elmer infrared, respectively.

[5] Celite, Johns-Manville, New York, N. Y.



Scheme III

based on unrecovered starting material). For analysis, a sample was sublimed at 85°/0.05 mm. to give a clear, colorless syrup; $[\alpha]_D^{20}$ +24° [c 1.02 $(CH_2Cl)_2$]; $\tilde{\nu}_{max.(cm.^{-1})}^{film}$ 3,500 (OH), 1,380 (*gem*-dimethyl).

Anal.—Calcd. for $C_9H_{16}O_5$: C, 52.93; H, 7.90. Found: C, 52.63; H, 7.83.

The chloroform-insoluble syrupy residue (above) readily crystallized from ethanol on seeding with 3-deoxy-D-mannose.

5,6-Di-O-benzoyl-1,2-O-isopropylidene-3-deoxy-D-mannofuranose (IV)—To a well-stirred solution of 1.51 g. (7.39 mmoles) of crude 1,2-*O*-isopropylidene-3-deoxy-D-mannofuranose (III) in 15 ml. of reagent pyridine at 0° was added 2.22 ml. (19.2 mmoles) of benzoyl chloride dropwise, over a period of 5 min. After being stirred for an additional hour at 0°, the mixture was stored at room temperature for 24 hr. The reaction was quenched by the addition of 4 drops of water and poured slowly into 200 ml. of rapidly stirred ice water. After 45 min., the mixture was extracted with chloroform (3 × 40 ml.). The combined extracts were washed with aqueous saturated sodium bicarbonate (40 ml.) and water (40 ml.), dried over magnesium sulfate, filtered, and evaporated to dryness *in vacuo* at 50°. The last traces of pyridine were removed by the addition of toluene (2 × 15 ml.), followed by its subsequent removal *in vacuo* (50°) to give a practically clear pale yellow syrup. The turbidity was removed by solution of the syrup in 40 ml. of 95% ethanol, filtration through a layer of diatomaceous earth and activated carbon,[6] and evaporation of the filtrate to dryness *in vacuo* (50°), finally at 0.05 mm. pressure gave the product as a clear practically colorless syrup; yield, 2.97 g. (97.4%); $[\alpha]_D^{20}$ −28° [c 2.0 $(CH_2Cl)_2$]; $\tilde{\nu}_{max.(cm.^{-1})}^{film}$ 1,725 (benzoate C=O), 1,600 (phenyl), 1,380 (*gem*-dimethyl), 1,275 (benzoate C—O—C), 710 (phenyl).

Anal.—Calcd. for $C_{23}H_{24}O_7$: C, 66.98; H, 5.87. Found: C, 66.82; H, 5.76.

1,2-Di-O-acetyl-5,6-di-O-benzoyl-3-deoxy-D-mannofuranose (V)—To a well-stirred solution of 2.80 g. (6.68 mmoles) of 5,6-di-*O*-benzoyl-1,2-*O*-isopropylidene-3-deoxy-D-mannofuranose (IV) in 27 ml. of glacial acetic acid and 2.7 ml. of acetic anhydride was added 1.50 ml. of concentrated sulfuric acid dropwise, while maintaining the temperature at 15–20°. After stirring for 1 hr., the reaction was stored at room temperature for 24 hr. The solution was poured into 150 ml. of rapidly stirred ice water and, after 75 min., the mixture was extracted with chloroform (3 × 40 ml.). The combined extracts were washed with aqueous saturated sodium bicarbonate (80 ml.) and water (80 ml.), dried over magnesium sulfate, filtered and evaporated to dryness *in vacuo* at 50°, finally at 0.05 mm. pressure, to give a clear practically colorless syrup which was sufficiently pure for the next step; yield, 2.11 g. (68.1%); $\tilde{\nu}_{max.(cm.^{-1})}^{film}$ 1,750 (acetate C=O), 1,725 (benzoate C=O), 1,600 (phenyl), 1,280 (benzoate C—O—C), 1,220 (acetate C—O—C), 710 (phenyl).

Anal.—Calcd. for $C_{24}H_{24}O_9$: C, 63.15; H, 5.30. Found: C, 62.02; H, 5.67.

9-(3-Deoxy-α-D-mannofuranosyl)adenine (VIII)—A mixture of 1.79 g. (3.93 mmoles) of crude 1,2-di-*O*-acetyl-5,6-di-*O*-benzoyl-3-deoxy-D-mannofuranose (V), 2.33 g. (4.91 mmoles) of chloromercuri-6-benzamidopurine (VI), 2.4 g. of diatomaceous earth, and 170 ml. of ethylene dichloride was distilled until 25 ml. of distillate had been collected. To the partially cooled mixture was added a solution of 0.54 ml. (4.9 mmoles) of titanium tetrachloride in 7 ml. of ethylene dichloride, and the reaction was heated under reflux for 24 hr. After cooling, 65 ml. of aqueous saturated sodium bicarbonate was added; the mixture was stirred vigorously for 4 hr. and filtered through diatomaceous earth. After washing the cake with chloroform (3 × 20 ml.), the organic phase was separated from the filtrate and evaporated to near dryness *in vacuo* at 40°. A solution of the residue in 40 ml. of chloroform was washed with 40 ml. of 30% aqueous potassium iodide and 40 ml. of water, dried over magnesium sulfate, filtered, and evaporated to dryness *in vacuo* at 50° to give the

[6] Nuchar, West Virginia Pulp and Paper Co., New York, N. Y.

crude blocked nucleoside (VII) as a yellow foam which hardened to a glass; yield, 2.10 g. (84.0%); $\bar{\nu}^{KBr}_{max.\ (cm.^{-1})}$ 3,300 (NH), 1,750 shoulder (acetate C=O), 1,725 (benzoate C=O), 1,690 shoulder (amide C=O), 1,600, 1,575 (C=N and C=C), 1,280 (benzoate C—O—C), 1,225 shoulder (acetate C—O—C), 1,090, 1,070, 1,025 (sugar C—O—C), 710 (phenyl).

A solution of 1.99 g. of crude blocked nucleoside (VII) in 50 ml. of 0.1 *N* methanolic sodium methoxide was heated under reflux for 2.5 hr. The cooled solution was neutralized with glacial acetic acid and stirred at 0° for 2 hr. which caused the separation of the crude nucleoside. The product was collected on a filter, washed with 2 ml. of cold methanol and dried, to give a light brown, crystalline solid; yield, 0.557 g. (53.3% from the 1,2-diacetate, V); m.p. 208–211°. For analysis, a sample was recrystallized from water with light charcoaling and again from 80% aqueous methanol to give small, white needles, m.p. 211–212°; $\lambda^{pH\ 1}_{max.\ (m\mu)}$ 257 (ϵ 14,600), $\lambda^{pH\ 13}_{max.\ (m\mu)}$ 260 (ϵ 15,000), $\lambda^{H_2O}_{max.\ (m\mu)}$ 259 (ϵ 14,700); $\bar{\nu}^{KBr}_{max.\ (cm.^{-1})}$ 3400–3100 (broad OH and NH), 1,605, 1,565 (C=C and C=N); $[\alpha]^{20}_D$ +52° (c 1.15 H_2O).

Anal.—Calcd. for $C_{11}H_{15}N_5O_4$: C, 46.97; H, 5.38; N, 24.90. Found: C, 46.86; H, 5.40; N, 24.72.

1,2-O-Isopropylidene-3-deoxy-D-threo-pentodialdofuranose (IX)—To a well-stirred solution of 2.03 g. (9.95 mmoles) of crude 1,2-*O*-isopropylidene-3-deoxy-D-mannofuranose (III) in 60 ml. of water was added 2.13 g. (9.95 mmoles) of sodium metaperiodate and the pH adjusted to between 6.0 and 6.5 (pH paper) with 0.1 *N* sodium hydroxide solution. After 1 hr., during which the pH was adjusted when necessary with sodium hydroxide solution, the solution was extracted with chloroform (5 × 60 ml.). The combined extracts were washed with 60 ml. of water, dried over magnesium sulfate, filtered, and evaporated to dryness *in vacuo* at 40° giving the crude product as a clear, colorless, mobile liquid which was sufficiently pure for the next step; yield, 1.49 g. (87.2%). For analysis, a sample was sublimed at 60°/0.8 mm. to give a clear, colorless liquid; $\bar{\nu}^{film}_{max.\ (cm.^{-1})}$ 3,550 (OH), 2,750 (aldehyde C—H), 1,735 (aldehyde C=O), 1,380 (*gem*-dimethyl).

Anal.—Calcd. for $C_8H_{12}O_4$: C, 55.80; H, 7.03. Found: C, 51.63; H, 6.99.

1,2-O-Isopropylidene-3-deoxy-D-arabinofuranose (X)—To a stirred solution of 0.57 g. (15 mmoles) of sodium borohydride in 20 ml. of water was added a solution of 1.27 g. (7.38 mmoles) of crude 1,2-*O*-isopropylidene-3-deoxy-D-*threo*-pentodialdofuranose (IX) in 20 ml. of methanol. After 1 hr., the solution was neutralized with dilute aqueous acetic acid, then evaporated to near dryness *in vacuo* at 40°. The residue was dissolved in 25 ml. of water and extracted with chloroform (5 × 25 ml.). The combined extracts were washed with 25 ml. of water, dried over magnesium sulfate, filtered, and evaporated to dryness *in vacuo* at 50° to give the crude product as a clear, colorless liquid; yield, 0.996 g. (77.5%). An analytical sample was prepared by sublimation at 80°/0.7 mm. to give a clear, colorless liquid; $\bar{\nu}^{film}_{max.\ (cm.^{-1})}$ 3,500 (OH), 1,380 (*gem*-dimethyl).

Anal.—Calcd. for $C_8H_{14}O_4$: C, 55.16; H, 8.10. Found: C, 54.98; H, 7.73.

5-O-Benzoyl-1,2-O-isopropylidene-3-deoxy-D-arabinofuranose (XI)—To a well-stirred solution of 0.844 g. (4.85 mmoles) of crude 1,2-*O*-isopropylidene-3-deoxy-D-arabinofuranose (X) in 12 ml. of reagent pyridine at 0° was added 0.73 ml. (6.3 mmoles) of benzoyl chloride dropwise, over a period of 4 min. After standing at room temperature for 22 hr., the mixture was poured into 130 ml. of vigorously stirred ice water. The crude product separated as a white solid which was collected on a filter, washed with cold water (5 × 10 ml.), and dried *in vacuo* over phosphorus pentoxide; yield, 1.12 g. (82.9%); m.p. 76–78°. An analytical sample was prepared by recrystallization twice from methanol–water (1:3) to give small needles; m.p. 87°; $\bar{\nu}^{KBr}_{max.\ (cm.^{-1})}$ 1,720 (benzoate C=O), 1,600 (phenyl), 1,385 (*gem*-dimethyl), 1,265 (benzoate C—O—C), 707 (phenyl); $[\alpha]^{20}_D$ + 42° (c 1.18 $CHCl_3$).

Anal.—Calcd. for $C_{15}H_{18}O_5$: C, 64.73; H, 6.52. Found: C, 64.62; H, 6.57.

1,2-Di-O-acetyl-5-O-benzoyl-3-deoxy-D-arabinofuranose (XII)—To a well-stirred solution of 0.973 g. (3.50 mmoles) of crude 5-*O*-benzoyl-1,2-*O*-isopropylidene-3-deoxy-D-arabinofuranose (XI) in 17 ml. of glacial acetic acid and 1.92 ml. of acetic anhydride was added dropwise 1.06 ml. of concentrated sulfuric acid, while maintaining the temperature between 10 and 20°. After standing at room temperature overnight, the solution was poured into 150 ml. of ice water with vigorous stirring for 40 min., then extracted with chloroform (5 × 20 ml.). The extracts were combined, washed with 100 ml. of aqueous saturated sodium bicarbonate and 50 ml. of water, dried over magnesium sulfate, filtered, and evaporated to dryness *in vacuo* at 50° to give the crude product as a clear, colorless syrup; yield, 0.681 g. (60.2%); $\bar{\nu}^{film}_{max.\ (cm.^{-1})}$ 1,750 (acetate C=O), 1,720 (benzoate C=O), 1,600 (phenyl), 1,270 (benzoate C—O—C), 1,220 (acetate C—O—C), 710 (phenyl).

Anal.—Calcd. for $C_{16}H_{18}O_7$: C, 59.62; H, 5.63. Found: C, 59.12; H, 6.08.

9-(3-Deoxy-α-D-arabinofuranosyl) adenine (XIV)—A mixture of 0.473 g. (1.47 mmoles) of crude 1,2-*O*-acetyl-5-*O*-benzoyl-3-deoxy-D-arabinofuranose (XII), 0.872 g. (1.84 mmoles) of chloromercuri-6-benzamidopurine (VI), 1.0 g. of diatomaceous earth, and 80 ml. of ethylene dichloride was distilled until 25 ml. of distillate had been collected. To the partially cooled mixture was added a solution of 0.20 ml. (1.8 mmoles) of titanium tetrachloride in 3 ml. of ethylene dichloride, and the mixture heated under reflux for 24 hr. While the mixture was still warm, 30 ml. of aqueous saturated sodium bicarbonate was added with vigorous stirring. After 2 hr., the mixture was filtered through diatomaceous earth and the cake washed with chloroform (3 × 10 ml.). The organic phase was separated from the filtrate and evaporated to near dryness *in vacuo* at 40°. A solution of the residue in 15 ml. of chloroform was washed with 15 ml. of 30% aqueous potassium iodide and 15 ml. of water, dried over magnesium sulfate, filtered, and evaporated to dryness *in vacuo* at 50° to give the crude, blocked nucleoside, (XIII), as a yellow syrup; yield, 0.697 g. (94.8%); $\bar{\nu}^{film}_{max.\ (cm.^{-1})}$ 1,745 (acetate C=O), 1,720 (benzoate C=O), 1,700 (amide C=O), 1,605, 1,580 (purine ring and phenyl), 1,270 (benzo-

ate C—O—C), 1,220 (acetate C—O—C), 1,020, 1,070, 1,085 (sugar C—O—C), 708 (phenyl).

A solution of 0.675 g. of crude, blocked nucleoside (XIII) in 15 ml. of 0.1 *N* methanolic sodium methoxide was heated under reflux for 2.5 hr. The cooled solution was neutralized with glacial acetic acid, then stirred at 0° for 2 hr. which caused the product to separate as a pale brown crystalline solid. This was collected on a filter, washed with 5 ml. of methanol and dried; yield, 0.111 g. (31%) from the diacetate (XII); m.p. 240°. For analysis, a sample was recrystallized from 90% aqueous ethanol with light charcoaling, and again from 95% ethanol to give a white crystalline solid; m.p. 242.5–243°; $\lambda_{max.\ (m\mu)}^{pH\ 1}$ 257 (ϵ 15,000), $\lambda_{max.\ (m\mu)}^{pH\ 13}$ 260 (ϵ 15,200), $\lambda_{max.\ (m\mu)}^{H_2O}$ 259 (ϵ 15,000), $\tilde{\nu}_{max.\ (cm.^{-1})}^{KBr}$ 3,500–3,150 (broad OH, NH), 1,610, 1,570 (C=C and C=N); $[\alpha]_D^{20}$ + 54° (c 0.70 *N* HCl).

Anal.—Calcd. for $C_{10}H_{13}N_5O_3$: C, 47.80; H, 5.22; N, 27.88. Found: C, 47.56; H, 5.35; N, 27.78.

1,2-O-Isopropylidene-5,6-di-O-methanesulfonyl-3-deoxy-D-galactofuranose (XVI)—To a well-stirred solution of 2.76 g. (13.5 mmoles) of crude 1,2-*O*-isopropylidene-3-deoxy-D-galactofuranose (XV) (1) in 25 ml. of reagent pyridine at 0° was added, dropwise over a period of 10 min., 2.66 ml. (35.1 mmoles) of methanesulfonyl chloride. After being stirred for an additional hour at 0°, the mixture was stored at 5° for 24 hr. and poured into 650 ml. of vigorously stirred cold aqueous sodium bicarbonate. The crude product which separated was collected on a filter, washed with cold water (3 × 50 ml.), and dried; yield, 3.88 g. (79.8%); m.p. 115–117°. Recrystallization from 70% aqueous ethanol gave 3.20 g. of thin wiry needles, m.p. 116–117°. For analysis, a sample was recrystallized from 80% aqueous ethanol; m.p. 116–117°; $\tilde{\nu}_{max.\ (cm.^{-1})}^{KBr.}$ 1,380 (*gem*-dimethyl), 1,180 (sulfonate); $[\alpha]_D^{20}$ −62° [c 1.05 $(CH_2Cl)_2$].

Anal.—Calcd. for $C_{11}H_{20}O_9S_2$: C, 36.66; H, 5.59; S, 17.79. Found: C, 36.68; H, 5.53; S, 17.68.

5,6-Di-O-benzoyl-1,2-O-isopropylidene-3-deoxy-L-mannofuranose (XVII)—A well-stirred mixture of 3.00 g. (8.33 mmoles) of 1,2-*O*-isopropylidene-5,6-di-*O*-methanesulfonyl-3-deoxy-D-galactofuranose (XVI), m.p. 116–117°, 6.00 g. (41.7 mmoles) of sodium benzoate and 80 ml. of dimethylformamide was heated under reflux for 6 hr. The cooled dark mixture was diluted with 160 ml. of water and shaken with 120 ml. of ether to give a turbid mixture from which the two layers could not be separated. Filtration through diatomaceous earth removed the suspended material and, after washing the cake with additional ether (3 × 25 ml.), the ether phase was readily separated from the filtrate. The aqueous phase was further washed with ether (2 × 80 ml.) which was then combined with the original ether phase, washed with aqueous saturated sodium bicarbonate (100 ml.) and water (100 ml.), dried over magnesium sulfate, filtered, and evaporated to dryness *in vacuo* at 50° to give the crude product as a brown syrup; yield, 1.30 g. (37.9%). A solution of this syrup in 10 ml. of benzene was passed through 50 g. of alumina F-20 (grade 2)[7] using benzene as the eluant. The first 200 ml. of eluate was evaporated to dryness *in vacuo* at 50° to give a light amber syrup whose infrared spectrum indicated the desired product but with some contamination. The next 600 ml. of eluate, processed in the same manner, gave a pale yellow syrup with the desired spectrum; yield, 0.556 g. (16.2%); $[\alpha]_D^{20}$ +29° [c 1.27 $(CH_2Cl)_2$]; $\tilde{\nu}_{max.\ (cm.^{-1})}^{film}$ 1,725 (benzoate C=O), 1,600 (phenyl), 1,380 (*gem*-dimethyl), 1,280 (benzoate C—O—C), 710 (phenyl). This material was not purified further but used directly in the next step.

1,2-Di-O-acetyl-5,6-di-O-benzoyl-3-deoxy-L-mannofuranose (XVIII)—To a well-stirred solution of 0.515 g. (1.25 mmoles) of 5,6-di-*O*-benzoyl-1,2-*O*-isopropylidene-3-deoxy-L-mannofuranose (XVII) in 5 ml. of glacial acetic acid and 0.94 ml. (10 mmoles) of acetic anhydride was added 0.28 ml. of concentrated sulfuric acid dropwise, while maintaining the temperature between 15 and 20°. After being stored overnight at room temperature, the solution was poured into 50 ml. of vigorously stirred cold 10% aqueous sodium acetate. After a further 30 min. of stirring, the mixture was extracted with chloroform (3 × 15 ml.). The combined extracts were washed with aqueous saturated sodium bicarbonate (2 × 30 ml.) and water (30 ml.), dried over magnesium sulfate, filtered, and evaporated to dryness *in vacuo* at 50° to give a slightly turbid practically colorless syrup which was sufficiently pure for the following step; yield, 0.454 g. (79.7%); $\tilde{\nu}_{max.\ (cm.^{-1})}^{film}$ 1,750 (acetate C=O), 1,725 (benzoate C=O), 1,600 (phenyl), 1,280 (benzoate C—O—C), 1,220 (acetate C—O—C), 710 (phenyl).

9-(3-Deoxy-α-L-mannofuranosyl)adenine (XX)—A mixture of 0.378 g. (0.829 mmole) of crude 1,2-di-*O*-acetyl-5,6-di-*O*-benzoyl-3-deoxy-L-mannofuranose (XVIII), 0.493 g. (1.04 mmoles) of chloromercuri-6-benzamido-purine (VI), 0.5 g. of diatomaceous earth, and 55 ml. of ethylene dichloride was distilled until 25 ml. of distillate had been collected. To the partially cooled mixture was added a solution of 0.11 ml. (1.0 mmole) of titanium tetrachloride in 5 ml. of ethylene dichloride, and the mixture heated under reflux for 29 hr. While the mixture was still warm, 20 ml. of aqueous saturated sodium bicarbonate was added, the mixture stirred vigorously for 2 hr., and then filtered through diatomaceous earth. After washing the cake with chloroform (3 × 10 ml.), the organic phase was separated from the filtrate and evaporated to near dryness *in vacuo* at 40°. A solution of the residue in 15 ml. of chloroform was washed with 15 ml. of 30% aqueous potassium iodide and 15 ml. of water, dried over magnesium sulfate, filtered, and evaporated to dryness *in vacuo* at 50° to give the crude blocked nucleoside (XIX) as a yellow glass; yield, 0.396 g. (77.0%); $\tilde{\nu}_{max.\ (cm.^{-1})}^{KBr}$ 3,300 (NH), 1,750 shoulder (acetate C=O), 1,725 (benzoate C=O), 1,605, 1,580 (C=N and C=C), 1,280 (benzoate C—O—C), 1,090, 1,070, 1,025 (sugar C—O—C), 710 (phenyl).

A solution of 0.394 g. of the crude blocked nucleoside (XIX) in 10 ml. of 0.1 *N* methanolic sodium methoxide was heated under reflux for 2.5 hr. The cooled solution was neutralized with glacial acetic acid and stored at 5° overnight. The crude crystalline nucleoside, which had separated from solution, was collected on a filter, washed with cold methanol (2 × 5 ml.), and dried; yield, 0.111

[7] Alcoa Activated Alumina, Aluminum Company of America, East St. Louis, Ill.

g. (63.8%); m.p. 210–212° (dec.). For analysis, the product was recrystallized from 90% aqueous methanol with light charcoaling, and again from 80% aqueous methanol; m.p. 212–213°; $\lambda_{max.\ (m\mu)}^{pH\ 1}$ 257 (ε 15,200), $\lambda_{max.\ (m\mu)}^{pH\ 13}$ 260 (ε 15,200), $\lambda_{max.\ (m\mu)}^{H_2O}$ 259 (ε 15,000); $\bar{\nu}_{max.(cm.^{-1})}^{KBr}$ 3,400–3,100 (broad OH, NH), 1,610, 1,575 (C=C and C=N); $[\alpha]_D^{20}$ −53° (c 1.01 H_2O).

Anal.—Calcd. for $C_{11}H_{15}N_5O_4$: C, 46.97; H, 5.38; N, 24.90. Found: C, 46.84; H, 5.36; N, 25.50.

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Keyphrases

9-(3-Deoxyaldofuranosyl) adenines—synthesis
IR spectrophotometry—structure
Optical rotation—identity

Determination of Caffeine in Plasma by Gas Chromatography

By F. L. GRAB* and J. A. REINSTEIN†

A gas chromatographic method has been developed for the rapid, precise, and specific determination of caffeine in plasma. The method overcomes the major drawbacks of previous methods for the determination of caffeine in body fluids, which were: difficult isolation from interfering materials, a substantial blank error and low sensitivity. A standard response curve relating the signal-height ratio of caffeine to that of an internal standard, hexobarbital, permits quantitation of the amount of caffeine present. The method involves extraction of caffeine from plasma with chloroform, after the aqueous phase was adjusted to pH 11.5–12.0. The chloroform extract was evaporated to dryness and the sample was redissolved in carbon disulfide. Two milliliters of plasma was used and caffeine was determined at a concentration of 0.25 mcg./ml.

CAFFEINE, due to its widespread occurrence in beverages, is probably the drug consumed most today. There is, however, no rapid and sensitive method available for measuring this drug in biological fluids. Connors (1) reviewed many methods for the detection of xanthines, but these were generally not specific for caffeine. The older methods involved extraction of caffeine followed by a gravimetric or volumetric assay (4). These were succeeded by the colorimetric murexide reaction of Tanaka and Ohkubo (5). In addition a number of reagents that form colors have been employed by the authors and by others in an attempt to develop more specific tests for the xanthines. These were used in conjunction with paper (1–3, 13) and thin-layer chromatography (17). The reagents include mercuric chloride, potassium ferrocyanide, mercuric acetate, Dragendorff's reagent, and an alkaline phosphotungstate reagent. Visualization of the spots has also been achieved under UV light at 254 mμ using fluorescent thin-layer plates (10, 12, 17). Other methods include argentimetric (2), iodometric (1), solvent extraction (2, 8), ion exchange (2, 12), nonaqueous titrations (1), Kjeldahl (1), and spectrophotometric (2, 6–9, 11, 14, 15) methods. These have proved successful in measuring qualitatively, and in some cases

Received May 22, 1968, from the School of Pharmacy, University of California, San Francisco Medical Center, San Francisco, CA 94122
Accepted for publication June 18, 1968.
Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
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quantitatively, pure xanthines. They were used in detecting mixtures of some xanthines and xanthines mixed with a number of other drugs.

Previously, the analytical method of choice in determining caffeine in biological systems has been spectrophotometry. The original development was made by Ishler *et al.* (6) and since then most of the determinations of caffeine in blood, tissue, and urine were based on this approach (2, 7–9, 21–23). However, these methods do not possess the sensitivity or specificity required for estimating concentrations of this drug produced by the ingestion of normal amounts of caffeine-containing beverages (2, 8, 21, 23). The authors have attempted to use a spectrophotometric assay but meaningful results could not be obtained at the concentration range of interest. The blank values were too high (relative to caffeine blood levels) and variable.

Gas chromatographic assays have been reported for many drugs in biological fluids (16), and xanthines have been examined in a number of GC systems (18, 19, 24–29). Such systems combine separation and quantitation of components and appear to offer distinct advantages over other methods of analysis.

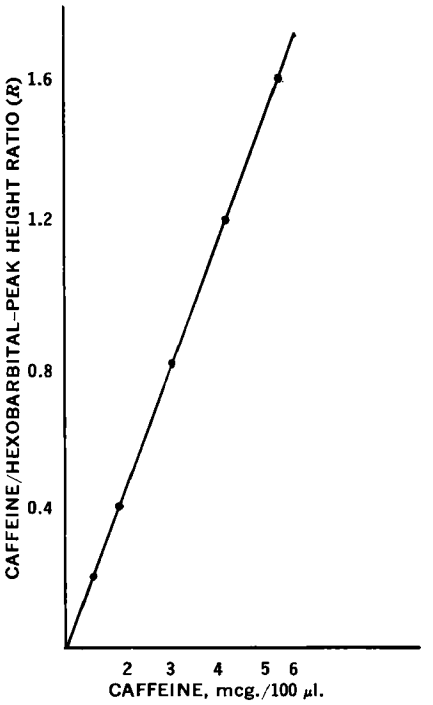

Fig. 2—Standard gas chromatogram response curve caffeine; internal marker, hexobarbital.

EXPERIMENTAL

Equipment—A Varian Aerograph series 1200 gas chromatograph with a flame-ionization detector and a Varian model 20 recorder set at 20 in. $hr.^{-1}$ was used. The column employed was a 1.9 m. (6 ft.) stainless steel helix, 0.32 cm. ($^1/_8$ in.) o.d. packed with 3% OV 17 on Chromosorb W 100–120 mesh AW/-DMCS H.P. (Applied Science lot 4223). The N_2 gas flow rate was 30 ml./min. Operating temperatures were: oven, 200°; injection port, 260°; and detector, 260°. Attenuations from 0.1 × 8 to 0.1 × 64 were used.

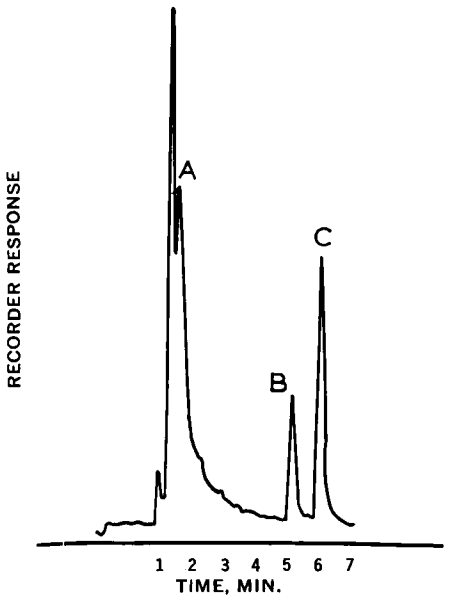

Fig. 1—Typical chromatogram. Key: A, carbon disulfide; B, hexobarbital (R_t = *4.3); C, caffeine* (R_t = *5.2). Column 3% OV 17.*

Reagents—(*a*) Caffeine (Eastman Organic) purified by vacuum sublimation; (*b*) hexobarbital (Winthrop), the internal standard; (*c*) reagent grade carbon disulfide (MCB) further purified by distillation; (*d*) heparin solution, 1,000 units/ml. (free of preservatives); (*e*) 2.5 *N* sodium hydroxide (Fisher Certified Reagent); (*f*) technical grade chloroform (Baker) freshly distilled.

Standard Curve—Standard curves were obtained by preparing solutions of caffeine 0.75–6.0 mcg./100 µl. and hexobarbital 2.0 mcg./100 µl. in carbon disulfide (20). Both caffeine and hexobarbital give sharp peaks with some slight tailing (Fig. 1). The standard curve given by the peak-height ratio of caffeine/hexobarbital *versus* caffeine concentration was linear and reproducible (Fig. 2).

Preparation of Plasma Samples—A 5-ml. sample of blood was taken, added to 2 drops of heparin, and spun down in a centrifuge (IEC International Clinical Centrifuge) for 10 min. at 2500–3000 r.p.m. Two milliliters of plasma was pipeted into a 25-ml. round-bottom screw-cap Pyrex tube with a Teflon-lined cap. The pH was adjusted to 11.5–12.0 with 2.5 *N* sodium hydroxide. To this 10 ml. of chloroform was added. The tubes were then agitated for 15 min. and then centrifuged (IEC International Centrifuge series 2) for 10 min. at 1,000 r.p.m. Using a 10-ml. hypodermic syringe with a 10-cm. (4 in.) cannula, 75% of the chloroform layer was withdrawn and placed into a conical ground-glass

stoppered 15-ml. centrifuge tube. The chloroform extract was then evaporated to dryness in a 60–80° water bath, mounted on a steam cone. Bumping was prevented by placing an antibumping chip, previously boiled in carbon disulfide, into the tube, just prior to driving off the chloroform. One milliliter of hexobarbital solution (2 mcg./ml.) in carbon disulfide was pipeted into the tube. This solution was then concentrated to approximately 100 µl. by heating in the water bath at 40–60°. A 2-µl. sample of this concentrate was then injected onto the column.

RESULTS AND DISCUSSION

Prior to using the present assay to determine plasma levels of caffeine after the ingestion of a cup of coffee, attempts were made to measure the concentration of a known and unknown amount of caffeine in plasma. An accurately measured amount of caffeine was added to two plasma samples, one containing an unknown amount of caffeine and the other containing no caffeine. The plasma samples were treated as described under *Experimental*. A linear plot was obtained for both plasma samples when the caffeine/hexobarbital-peak height ratio was plotted against caffeine concentration (Fig. 3). Theoretically both graphs should be parallel, differing only in the intercept with the y axis. This was the case. The intercept was equal to the concentration of caffeine present in the plasma samples before the additions were made. The sample which contained no caffeine originally passes through the origin, while the other intercepts the y axis at a peak-height ratio of 0.2. This corresponds to a caffeine concentration of 0.5 mcg./ml.

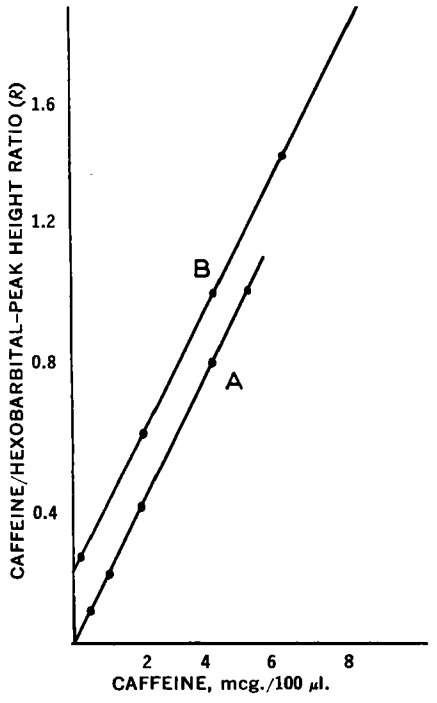

Fig. 3—Calibration curve of caffeine extracted from 2 ml. of plasma. Key: A, no caffeine present in original plasma sample; B, unknown amount of caffeine present in original plasma sample.

TABLE I—RECOVERY OF CAFFEINE ADDED TO PLASMA

Caffeine Added, mcg./ml.	Caffeine Found, mcg./ml.	Recovery, %
0.0	0.00	—
0.5	0.49	98
1.0	1.01	101
2.0	1.95	98
2.5	2.56	102

The standard curve (Fig. 2) can be compared to the calibration curve (Fig. 3A), since the two graphs share a common abscissa. The slope of the latter is 75% of the former. Taking into account that a 75% aliquot of the plasma extract was withdrawn, the values at each level of caffeine are identical for both curves. This indicates that the caffeine was quantitatively extracted (Table I).

The above procedure was repeated in five other subjects who had abstained from caffeine-containing beverages for 36 hr. Plasma samples were obtained and a calibration curve was determined for each. The curves passed through the origin and possessed the same slope. This indicates that the method is reliable and may be used for estimating caffeine plasma levels.

Absorption of Caffeine—Using the assay procedure, the absorption of caffeine, following the ingestion by three subjects of coffee containing 100 mg. of the drug, was studied. Caffeine was detected within 5 min. after ingestion. Peak plasma levels of 1.5–1.8 mcg./ml. were seen 50–75 min. after the dose. It was found that absorption could be described by a pseudo first-order process. The intersubject variation in the half-life of absorption was 8–17 min., while the intrasubject variation was much smaller. Apparent metabolic half-lives varied from 3.5–6 hr. in two subjects and from 6–10.5 in a third (*e.g.*, Fig. 4). Detailed data on the absorption and metabolism of caffeine in the body will be reported in a subsequent paper.

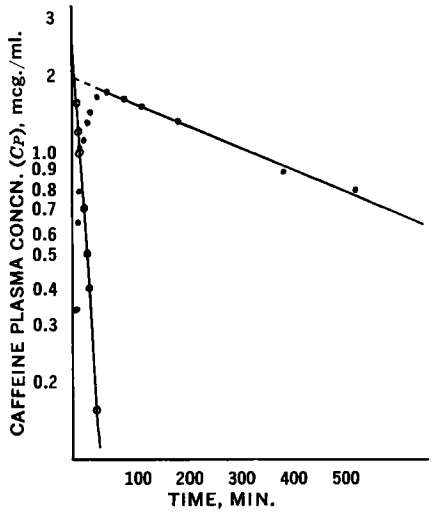

Fig. 4—Caffeine concentration after the ingestion of coffee containing 100 mg. of the drug. A graphical estimation of the metabolic and absorption (from the residual plot) half-lives are shown. Key: ●, observed data, $t_{1/2}^{m}$ = 6.0 hr.; ○, residual data, $t_{1/2}^{a}$ = 10.4 min.

SUMMARY

A gas chromatographic method has been developed for the determination of caffeine in plasma. A standard response curve relating signal-height ratios of caffeine to hexobarbital permits a quantitative estimation of the amount of caffeine present. The method is more rapid and sensitive than any previously reported. Plasma levels of caffeine after the ingestion of one cup of coffee have been studied and concentrations down to 0.25 mcg./ml. have been accurately determined.

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Keyphrases

Caffeine in plasma–analysis
Plasma analysis–caffeine
GLC–analysis
Hexobarbital–internal standard

Oxidation of Organic Compounds with Xenon Trioxide I

Stoichiometric and Kinetic Studies Involving Cinnamyl Alcohol, Cinnamaldehyde, and Cinnamic Acid

By HAROLD J. RHODES, REGINA P. SHIAU, and MARTIN I. BLAKE

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STUDIES INVOLVING the preparation and oxidizing properties of xenon trioxide in aqueous solution have been cited in an earlier paper (1), which described the determination and stoichiometry involved in the oxidation of certain primary, secondary, and tertiary aliphatic and aromatic alcohols.

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Accepted for publication July 23, 1968.

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SUMMARY

A gas chromatographic method has been developed for the determination of caffeine in plasma. A standard response curve relating signal-height ratios of caffeine to hexobarbital permits a quantitative estimation of the amount of caffeine present. The method is more rapid and sensitive than any previously reported. Plasma levels of caffeine after the ingestion of one cup of coffee have been studied and concentrations down to 0.25 mcg./ml. have been accurately determined.

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trioxide oxidation of *cis*- and *trans*-cinnamyl alcohol, *trans*-cinnamaldehyde, and *trans*-cinnamic acid. The stoichiometry of the oxidation of these compounds to carbon dioxide and water by xenon trioxide was determined. In addition, for *trans*-cinnamaldehyde, the following were determined: the reaction order with respect to xenon trioxide and aldehyde, the pH profile for the reaction, and the effect of increasing ionic strength of phosphate buffer on the reaction rate.

EXPERIMENTAL

Apparatus—The pH of reaction mixtures and buffers was determined with a Beckman pH meter, model 76A.

Solutions and Reagents—Highly purified water, which was used for preparing all solutions and dilutions, was obtained by passing the vapors through a column of cupric oxide heated to 800°.

Xenon trioxide solution (0.02 *M* and 0.09 *M*) was prepared by diluting a concentrate (0.2 *M*) with highly purified water. The preparation of the concentrated xenon trioxide solution by hydrolysis of xenon hexafluoride has been described by Appelman and Malm (2).

The following stock solutions of the compounds studied were prepared: 0.0020 *M cis*- and *trans*-cinnamyl alcohol, 0.0020 *M*, 0.0060 *M*, and 0.0080 *M trans*-cinnamaldehyde, and 0.0020 *M trans*-cinnamic acid.

Phosphate buffer solutions used in the kinetic studies were prepared in concentrations of 0.20 *M*, 0.50 *M*, and 0.75 *M* and at pH values ranging from 2 to 6.8 from standard buffer solutions (Fisher Scientific Co., Fair Lawn, N. J.), monobasic potassium phosphate, dibasic potassium phosphate, phosphoric acid, and potassium hydroxide.

Sodium thiosulfate solution (0.0050 *N*) was prepared by diluting a standard 0.020 *N* solution (Fisher Scientific Co., Fair Lawn, N. J.).

Sulfuric acid solution, 10% w/v, was prepared by diluting the concentrated form with highly purified water.

Potassium iodide and starch test solution were obtained from Fisher Scientific Co., Fair Lawn, N. J.

All chemicals and solvents were reagent grade.

Procedure—The stoichiometry involved in the xenon trioxide oxidation of *cis*- and *trans*-cinnamyl alcohol, *trans*-cinnamaldehyde, *trans*-cinnamic acid to carbon dioxide and water was determined by the following procedure, which is a modified version of the one described in a previous paper (1). In each case, exactly 1.0 ml. of 0.02 *M* xenon trioxide, a known excess, was added by micropipet to a series of 50-ml. glass-stoppered conical flasks. Aliquots of the 2.0×10^{-3} *M* substrate stock solution, ranging in volume from 0 to 1,000 μl., followed by a sufficient quantity of highly purified water to bring the total volume to 10 ml., were then added to the flasks. The flasks were stoppered and allowed to stand at room temperature. Since the time required for complete oxidation depends on the nature of the substrate and its concentration, preliminary studies were run to determine these values. Complete oxidation was noted in 9 days for the alcohol, 12 for the aldehyde, and 13 for the acid. After the reaction had gone to completion, the excess xenon trioxide was determined by the method of Appelman and Malm (2), which involves acidifying the reaction solution with 4 ml. of diluted sulfuric acid (10% w/v), adding potassium iodide (1 g.), and titrating the triiodide formed with standard sodium thiosulfate (0.020 *N* or 0.005 *N*) from a 10-ml. buret graduated to 0.01 ml. The end point sharpness was increased by addition, as it was approached, of three drops of starch T.S. to serve as an indicator. The amount of xenon trioxide consumed in the oxidation was determined by subtracting the amount found in excess from that found in a flask to which no substrate was added initially (blank). Thus, the millimoles of xenon trioxide reacting were determined from the normality of the thiosulfate solution and the difference in the volumes consumed by the blank and the sample. The millimoles of xenon trioxide consumed in the oxidation was then plotted against the millimoles of substrate taken. The slope and intercept of the plot were determined by regression analysis (1).

The reaction between *trans*-cinnamaldehyde and xenon trioxide was used for the kinetic studies, rather than the alcohol, because of its more favorable rate of oxidation. All experiments were carried out at 30 ± 0.1° employing a large excess of the aldehyde relative to xenon trioxide. The reaction rate was followed by noting the change in xenon trioxide concentration. Xenon trioxide was determined as described previously. The reaction was carried to at least 50% completion. The procedure is described below.

Aliquots of a 6.0×10^{-3} *M trans*-cinnamaldehyde stock solution containing 0.2 *M* phosphate buffer (pH 5.35), ranging in volume from 60–180 ml., were pipeted into a series of 500-ml. glass-stoppered conical flasks. With the exception of the last stock solution, a sufficient quantity of 0.2 *M* phosphate buffer (pH 5.35) was subsequently added to each to bring the volume to 180 ml. The flasks were then covered with glass stoppers and placed in a thermostatically controlled heating bath previously adjusted to 30 ± 0.1°. Xenon trioxide (200 μl., 0.09 *M*) was added to each at zero time. Aliquots (15 or 20 ml.) of the reaction mixture were removed at six time intervals ranging from 0.5 to 9 hr. The reaction was quenched by adding 1 g. of potassium iodide. The quantity of xenon trioxide was determined as previously described.

To determine the reaction order with respect to xenon trioxide the concentration of xenon trioxide remaining in the reaction mixture was plotted against reaction time on semilogarithmic graph paper. The slope, determined by calculating the least-square regression line, was used to obtain the rate constant. The half-life of the reaction was calculated from the rate constant by the expression: $t_{0.5} = 0.693/k$.

To determine the reaction order with respect to *trans*-cinnamaldehyde, the rate constants were determined at various aldehyde concentrations.

The pH profile was determined at 30 ± 0.1° in 0.2 *M* phosphate buffer at six pH intervals (1.4–6.8). Xenon trioxide solution (200 μl., 0.09 *M*) was added at zero time to each of six buffered solutions of *trans*-cinnamaldehyde (100 ml., 0.0060 *M*), previously adjusted to the desired pH and temperature. The pH of the reaction mixture was then measured with a pH meter. The reaction was followed to at least 50%

completion. The rate constants of the reactions, determined as described previously, were then plotted against pH.

The following procedure was used to determine the effect of the ionic strength of the phosphate buffer on the reaction rate of xenon trioxide with *trans*-cinnamaldehyde. Aliquots (200 ml.) of the stock solution (8.0×10^{-3} *M*) were pipeted into three volumetric flasks (250 ml.). Monobasic potassium phosphate was added to each in sufficient quantity to make the final phosphate buffer concentration 0.75 *M*, 0.50 *M*, and 0.20 *M*, respectively. The solutions were then adjusted to pH 5.0 with potassium hydroxide and diluted with water to bring the volume to 250 ml. An aliquot (100 ml.) of each solution was then reacted with xenon trioxide solution (200 µl., 0.09 *M*) at zero time at a temperature of 30 ± 0.1°. The reaction rates were determined as described previously. The reaction was followed to at least 50% completion. The rate constants and half-lives were calculated. The rate constants were then plotted against the ionic strength of the solution on semilogarithmic graph paper.

RESULTS AND DISCUSSION

The results of the oxidation of *cis*- and *trans*-cinnamyl alcohol, *trans*-cinnamaldehyde, and *trans*-cinnamic acid by xenon trioxide are shown graphically in Fig. 1, where the number of millimoles of xenon trioxide consumed is plotted against the number of millimoles of the alcohol, aldehyde, and acid taken. In each case the relationship is linear. Upon extrapolation, the straight line plots intercept the ordinate above the origin. The slope and *y*-intercept for each plot were calculated by least-square regression analysis. The analytical data are recorded in Table I. In the stoichiometric studies the substrates were oxidized completely to carbon dioxide and water. Therefore, where the *x*- and *y*-axis values represent the millimoles of substrate and xenon trioxide consumed, respectively, the slope for each plot corresponds to the stoichiometric ratio of the reactants involved in the oxidation. The ratio is characteristic of the compound oxidized. The theoretical values for the stoichiometric ratios (Table I) were derived on the basis of the chemical equation representing the complete oxidation of the particular compound. This is illustrated with the following example where 7 moles of xenon trioxide are required to oxidize 1 mole of cinnamaldehyde:

$$C_6H_5CH{=}CHCHO + 7XeO_3 \rightarrow 9CO_2 + 4H_2O + 7Xe$$

The experimental results show less than 4% deviation from the theoretical values.

Intercepts with the ordinate greater than zero were noted in all cases. This deviation may be attributed to secondary processes resulting from the formation of lower oxidation state xenon species such as xenon monoxide, XeO, which can react with water to form peroxide, and this in turn is capable of reacting with xenon trioxide to form xenon dioxide, XeO_2. Xenon dioxide is highly unstable, decomposing to its component elements spontaneously. Appelman (3) has reported on the reactive properties of XeO and XeO_2 in connection with their interaction with XeF_2. It is not likely that upon binary collision the substrate will give up six electrons in one step to a single xenon trioxide molecule. A stepwise transfer of electrons probably occurs which will result in lower oxidation states of xenon capable of reacting with water as indicated above and thus producing intercepts greater than zero.

Table I—Summary of the Stoichiometric Data

Substrate	*y*-Intercept, moles × 10^3	Slope, *m*	Theoretical Slope,[a] *m'*	Deviation, %
Cinnamyl alcohol	0.9169	7.423	7.333	1.23
trans-Cinnamaldehyde	0.1971	7.204	7.000	2.91
trans-Cinnamic acid	0.2314	6.415	6.667	3.77

[a] Based on compound being completely oxidized to carbon dioxide and water.

The kinetic study of the oxidation of *trans*-cinnamaldehyde with xenon trioxide was carried out under such conditions that the quantity of aldehyde taken was in large excess relative to xenon trioxide. Therefore it can be assumed that the concentration of aldehyde remained essentially constant throughout the course of the reaction. The reaction rate may be expressed by the equation:

$$-\frac{d(XeO_3)}{dt} = k(XeO_3)$$

A plot of the xenon trioxide concentration remaining in the reaction mixture *versus* time on semilogarithmic graph paper should be a straight line. This is shown in Fig. 2. Thus, the reaction is first order with respect to xenon trioxide. In view of the fact that the determinations were conducted under conditions where the concentrations of the species other than xenon trioxide were kept essentially constant, the reaction is actually pseudo-first order.

In order to establish the reaction order with respect to *trans*-cinnamaldehyde, a series of experiments was conducted in which the concentration of the aldehyde was varied while the initial concentration of xenon trioxide was kept constant. The data, as shown in Table II, indicate no significant change in the reaction rate as the concentration of the aldehyde was increased three fold. This indicates that

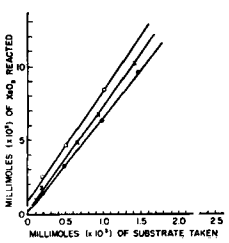

Fig. 1—The stoichiometry of cinnamyl alcohol, trans-*cinnamaldehyde, and* trans-*cinnamic acid with xenon trioxide. Key:* O, *cinnamyl alcohol;* ×, *trans-cinnamaldehyde;* ●, trans-*cinnamic acid.*

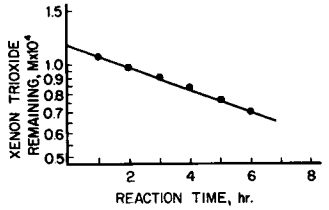

Fig. 2—Rate plot for the oxidation of trans-*cinnamaldehyde with xenon trioxide at pH 5.35.*

TABLE II—THE FIRST-ORDER REACTION RATES FOR THE OXIDATION OF *trans*-CINNAMALDEHYDE WITH XENON TRIOXIDE ($1.0802 \times 10^{-4} M$) AT $30 \pm 0.1°$ AND pH 5.35

Initial Concentration of *trans*-Cinnamaldehyde, moles $\times 10^4$	Rate Constant, $\times 10^2$ hr.$^{-1}$	Half-life, hr.
21.4495	8.74	7.92
32.1743	8.22	8.42
42.8990	8.14	8.51
53.6238	8.53	8.12
64.3485	8.20	8.45

the reaction is independent of the aldehyde concentration within the concentration range studied. The reaction is, therefore, zero order with respect to the aldehyde. A possible explanation may be that the concentration of the aldehyde already present in the reaction mixture was so great that any further increase in concentration did not elicit a discernible effect on the reaction rate. Another possibility may be the heterogeneity of the mixture. For instance, the solubility of *trans*-cinnamaldehyde is about 1.08×10^{-2} *M*; the concentration used in this study ranged from 2 to 6×10^{-3} *M*. However, because of the monopotassium phosphate, some of the aldehyde may be "salted out" of solution. Although this would result in a lower concentration of aldehyde in solution, the "salted out" aldehyde would serve as a source of replenishment for that consumed by the oxidant and thus maintain its concentration in solution nearly constant.

The reaction rates for the oxidation of *trans*-cinnamaldehyde with xenon trioxide were determined in 0.2 *M* phosphate buffer at various pH values ranging from 1 to 7. The study was restricted to acid conditions since xenon trioxide tends to disproportionate in alkaline media to form perxenate and xenon gas. A plot of the rate constant variation with pH is shown in Fig. 3. It was noted that the reaction rate increased with pH to a maximum at pH 4.3, then decreased to a minimum at pH 5.5, and increased sharply above pH 5.5. The pH profile is in general agreement with the observations of Jaselskis and Vas (4) who studied the oxidation of certain diols with xenon trioxide. They noted poorer oxidation at lower pH values. The maximum at pH 4.3 as shown in Fig. 3 cannot be fully explained at this time. The reaction at this pH may be catalyzed by the $H_2PO_4^-$ species of the phophate buffer system. Calculations of the ionic species present as a function of pH indicate that the concentration of this ion is maximal at pH 4.6. The species of the xenon trioxide in aqueous solutions may also vary with pH and may represent an important factor in the oxidation reactions studied here. Further studies are required to clarify more fully the pH effects noted here.

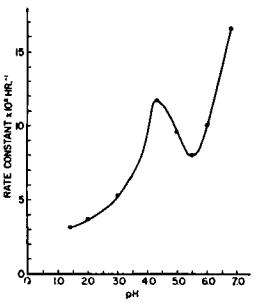

Fig. 3—Effect of pH on rate of oxidation of trans-*cinnamaldehyde with xenon trioxide.*

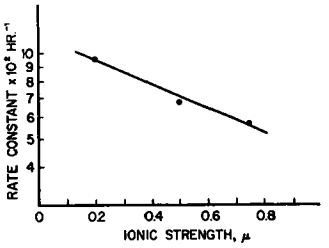

Fig. 4—Effect of buffer concentration on the rate of reaction of trans-*cinnamaldehyde with xenon trioxide at pH 5.35.*

The effect of buffer concentration on the rate of oxidation of *trans*-cinnamaldehyde is shown graphically in Fig. 4. The reaction rate was found to decrease with increase in buffer concentration. In calculating the ionic strength for the reaction mixtures, the contribution of species other than the buffer was negligible. The effect of buffer concentration on the reaction rate may be attributed to a primary salt effect. Since the activity coefficients of the ions present may be affected by increased ionic strength, this may be reflected in a change in rate constant.

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Keyphrases

Xenon trioxide oxidation—organic compounds
Cinnamic compounds—xenon trioxide oxidation
Stoichiometry—cinnamic compounds, oxidation
Kinetics—cinnamic compounds, oxidation
pH dependency—oxidation rate
Ionic strength, phosphate buffer—oxidation rate

X-Ray Contrast Media

Glutaric and Iminodiacetic Acids as Carriers of Iodinated Phenyl Ring

By GAD SHTACHER*

Iodinated derivatives of 3-phenyl-glutaric acid and *N*-benzyl-iminodiacetic acid were prepared. The free acids and their dimethyl esters were screened as potential intravenous and oral cholangiographic agents in cats and compared with presently available agents. The oral agents were present mostly in the intestines showing good X-ray intensity, with little or no visualization of the gall bladder and bile ducts. The X-ray intensity of the intravenous agents was not comparable to that obtained with sodium iodipamide. Attempted triiodination of 2-(3′-aminophenyl)-trimethylenedinitrilo-tetraacetic acid failed, probably due to steric factors.

IN SEARCH FOR improved X-ray contrast agents in intravascular radiography, attention was drawn to the biological properties of polyaminopolycarboxylic acids (1). Ethylenediaminetetraacetic acid (EDTA), the prototype of the synthetic polyaminopolycarboxylic acid group, is not metabolized in mammals, and following its parenteral administration, is very rapidly and almost completely eliminated unaltered through the kidneys. In considering the physicochemical properties of EDTA and structurally related compounds, their chemical stability as well as their high water solubility at physiologic pH are additional essential properties required from intravascular radiopaque media. The chelating property of EDTA has in fact been utilized a few years ago, to study the usefulness of lead-EDTA chelate as an X-ray contrast medium in animals and in man (2). This complex, however, proved to be quite unsafe due to release of free lead ions *in vivo*, thus resulting in heavy metal poisoning. Since the pharmacological as well as toxic effects of EDTA, and in particular its marked nephrotoxicity, have been attributed to its powerful chelating ability, which leads to the removal of essential metal ions, it seemed worthwhile to prepare lower and higher homologs of it, namely, iminodiacetic acid and trimethylenedinitrilo tetraacetic acid, respectively, linked to iodinated phenyl rings. The chelate stability constants of these complexing agents with various metal ions are a thousand to a million times lower than those of EDTA (3), thus practically eliminating the danger of essential metals depletion. On the other hand, however, these compounds retain the characteristic biological and physicochemical properties of polyaminopolycarboxylic acids mentioned above. It was also hoped that a combination of an iodinated nucleus with the amino acid side chains would favor the hepato-biliary elimination of the iodinated compounds, following their intravascular administration. It has been repeatedly shown that presence of iodine atoms in organic compounds markedly enhance their biliary excretion, with a concomitant fall in their renal excretion (4). As part of the reaction sequence leading to the preparation of 2-substituted phenyl-trimethylenedinitrilo-tetraacetic acid, iodinated derivatives of 3-phenyl-glutaric acid were also prepared, since their structure resembles to some extent that of 3-(3′-amino-2′,4′,6′-triiodophenyl)-2-ethylpropionic acid (iopanoic acid), currently used for oral cholecystography. Glutaric acid substituted in the α-position with a 3-amino-2,4,6-triiodobenzyl group was recently synthesized as a potential cholecystographic agent, but was found to be quite toxic (5). It was hoped that symmetrically substituted glutaric acid will show lower toxicity combined with enhanced choleretic property.

Received May 1, 1968, from the Research Laboratories, The Hospital for Special Surgery, New York, NY 10021

Accepted for publication July 5, 1968.

The generous support of this work by Mallinckrodt Chemical Works, St. Louis, Mo., is gratefully acknowledged.

The author expresses his sincere appreciation to Doctors George Brooke Hoey and Philip E. Wiegert, Pharmaceutical Division, Mallinckrodt Chemical Works, for their interest and encouragement.

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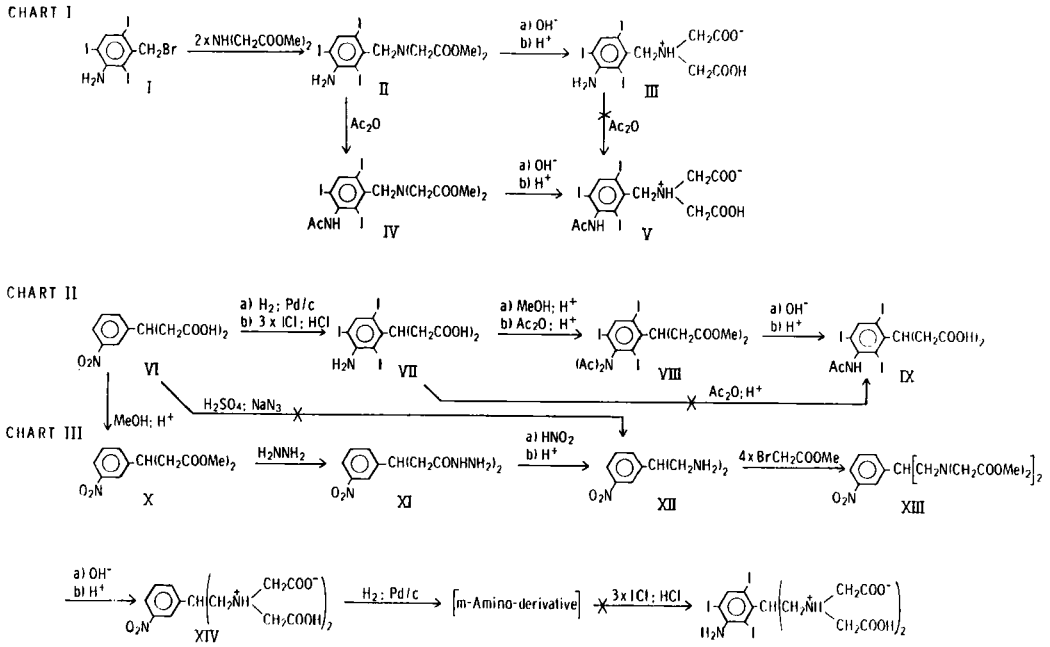
CHEMISTRY

Charts I, II, and III (Scheme I) represent the synthetic approaches to *N*-(3-acetamido-2,4,6-triiodobenzyl)-iminodiacetic acid (V), 3-(3′-acetamido-2′,4′,6′-triiodophenyl)-glutaric acid (IX), and 2-(3′-nitrophenyl)-trimethylenedinitrilo tetraacetic acid (XIV), respectively. 3-Amino-2,4,6-triiodobenzyl bromide (I) was prepared from 3-nitrobenzaldehyde in three steps consisting of catalytic reduction using



Scheme I

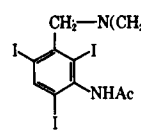
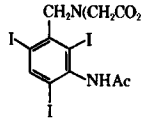
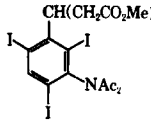
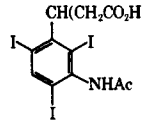
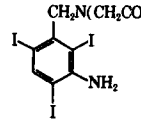
Adams' platinum catalyst (6), iodination with iodine monochloride, followed by treatment with phosphorus tribromide (7). 3-(3′-Nitrophenyl)-glutaric acid (VI), the starting material in Charts II and III (Scheme I) was readily obtained from 3-nitrobenzaldehyde by piperidine-catalyzed condensation of the aldehyde with 2 moles of ethyl acetoacetate, followed by alkaline hydrolysis of the resulting ethyl-3-nitrobenzylidenebis-acetoacetate (8). The most practical approach to the synthesis of 2-(3′-nitrophenyl)-trimethylenedinitrilo tetraacetic acid (XIV), appeared in the alkylation of 2-(3′-nitrophenyl)-trimethylene-diamine (XII) either with chloroacetic acid, or with methyl bromoacetate, followed by saponification of the resulting tetramethyl ester. The nitroaryl diamine (XII) was successfully prepared by the Curtius degradation (9) starting with the corresponding dimethylester (X), and proceeding through the dihydrazide intermediate (XI). Attempts to synthesize the diamine (XII) directly from 3-(3′-nitrophenyl)-glutaric acid by the Schmidt reaction (10) failed. The final step in the reaction sequence of Chart III, namely, triiodination of 2-(3′-aminophenyl)-trimethylenedinitrilo-tetraacetic acid, using either iodine monochloride or potassium iododichloride as iodinating agents, was unsuccessful. Titration of an aliquot sample of the iodination reaction mixture, indicated that only one-third of the theoretical quantity of iodine monochloride had been consumed. It thus appears that steric hindrance exerted by the trimethylenedinitrilo-tetraacetic acid side chain, probably blocked positions 2 and 6 on the benzene ring which were otherwise available for iodine substitution. Direct acetylation of either *N*-(3-amino-2,4,6-triiodobenzyl)-iminodiacetic acid (III), or 3-(3′-amino-2′,4′,6-triiodophenyl)-glutaric acid (VII) to give the corresponding ar-acetamido derivatives, always led to a tarry product, presumably due to the formation of cyclic carboxylic anhydrides which later polymerized. Blocking of the carboxyl groups by methyl ester formation, enabled the acetylation reaction to proceed smoothly in good yield, after which saponification of the acetylated products yielded the desired acetamido-triiodophenyl-carboxylic acids.

PHARMACOLOGY[1]

The acute toxicites of the test compounds are listed in Table I. The test compounds were screened as cholangiographic agents; three of them (IV, VIII, and IX) as oral agents, and three (III, V, and IX) as intravenous agents. Four mongrel cats were used for each compound tested. For intravenous cholangiographic (i.v.c.) screening the cats were fasted 24 hr. prior to dosing. Sixteen hours before the test, 75 ml. of evaporated milk mixed with 25 ml. of tap water was fed to the animals. All animals were dosed with 66 mg. iodine/kg. body weight. For oral cholangiographic screening the same procedure as for the i.v.c. screening was followed. The only variation was the fasting time which was extended to 32 hr., with the evaporated milk fed 6 hr. prior to dosing. Compounds IV and VIII were administered orally at a dose of 60 mg. iodine/kg. body weight. The X-ray intensity of the intravenous agents III, V, and IX was not comparable to that obtained with sodium iodipamide, the reference compound for intravenous cholangiography. The oral agents were present mostly in the intestines of the cats, showing good X-ray intensity. Only one cat treated with Compound IV showed good absorption with excretion of the test compound by way of the biliary system. However, visualization of the bile duct was vague and incomplete.

[1] Private communication. Van Derike, D. R., Valenti, A., and Hoey, G. B., Mallinckrodt Chemical Works, Pharmaceutical Division, St. Louis, Mo.

TABLE I—ACUTE TOXICITY AND DEGREE OF RADIOGRAPHIC VISUALIZATION OF THE GALL BLADDER AND BILE DUCT OF THE VARIOUS CONTRAST MEDIA

Compd. No.	Structure	LD_{50} (g./kg.)[a]	Cholecystography Screening[b]
IV		>10.0[c]	Not absorbed[c]
V[d]		≈6.0[e]	Poorly visualized[e]
VIII		—	No visualization[c]
IX		≈3.0[e]	Poorly visualized[e] No visualization[c]
III[d]		2.4[e]	Poorly visualized[e]

[a] Mice. [b] Cat. [c] Oral administration. [d] Exist in zwitterionic form. [e] Intravenous administration.

EXPERIMENTAL[2]

2,4,6-Triiodo-3-aminobenzyl Bromide (I)—This was prepared in 72% yield by treating the corresponding alcohol with phosphorus tribromide, according to Hebký and Karásek (7). Dioxane was found to be a better solvent for crystallization of (I) than chlorobenzene or tetrahydrofuran, m.p. 164–165° [lit. (7) m.p. 165°].

N-(2,4,6-Triiodo-3-aminobenzyl)-iminodiacetic acid Dimethyl Ester (II)—A mixture of 56.4 g. (0.1 mole) of 2,4,6-triiodo-3-aminobenzyl bromide (I), and 32.2 g. (0.2 mole) of iminodiacetic acid dimethyl ester (11) dissolved in 350 ml. of dry dioxane was stirred and refluxed for 3 hr. The reaction mixture was protected from moisture and CO_2 by soda-lime tube. The hydrobromide salt of iminodiacetic acid dimethyl ester began to separate within a few minutes following refluxing. At the end of the reaction, the reaction mixture was cooled, the hydrobromide salt collected by filtration (22.7 g., 94% yield), and the filtrate was concentrated *in vacuo*. The residue (the crude product) was obtained as a dark brown viscous oil which gradually solidified upon cooling. Two crystallizations of the crude product from methanol, furnished 43.2 g. (67% yield) of an analytically and chromatographically pure substance, as long colorless plates, m.p. 89.5–90.5°.

Anal.—Calcd. for $C_{13}H_{15}I_3N_2O_4$: C, 24.25; H, 2.35; I, 59.10; N, 4.35; mol. wt. 644.0. Found: C, 24.40; H, 2.39; I, 59.35; N, 4.43; mol. wt. 647.5. Infrared absorption spectra (CCl_4) $\lambda_{max.}$: 2.89 μ and 2.97 μ (medium, N—H stretching vibrations), 3.40 μ (medium, aliphatic C—H stretching vibration), 5.70 μ (very strong, C=O stretching vibration of ester), 6.27 μ (strong, probably overlapping of N—H deformation absorption and aromatic ring vibrations), 6.92 μ (strong, aromatic C=C stretching vibration), 8.40 μ (very strong, C—O—C stretching vibration of ester).

N-(2,4,6-Triiodo-3-aminobenzyl)-iminodiacetic Acid (III)—Saponification of the *tert*-imino dimethyl-ester (II) was performed in aqueous methanol, using 2 *N* NaOH in 10% excess. The saponification was completed after 10–15 min. of reflux. The alkaline reaction mixture was cooled, extracted with ether, and acidified carefully with concentrated HCl to pH ~ 1. The precipitated imino acid was filtered, washed with water, and crystallized from a mixture of acetic acid–water. The pure product was obtained as colorless fine needles, m.p. 178–179° dec.

Anal.—Calcd. for $C_{11}H_{11}I_3N_2O_4$: C, 21.45; H, 1.80; I, 61.81; N, 4.55; mol. wt. 615.97. Found, C, 21.21; H, 2.01; I, 62.10; N, 4.48; mol wt.: 618.0.

N-(2,4,6-Triiodo-3-acetamidobenzyl)-iminodiacetic Acid Dimethyl Ester (IV)—*N*-(2,4,6-Triiodo-3-aminobenzyl)-iminodiacetic acid dimethyl ester, 32.2 g. (0.05 mole), was acetylated with a mixture of 70 ml. of acetic anhydride, 70 ml. of glacial acetic acid, and 1 ml. of concentrated H_2SO_4. The reaction mixture was heated in an oil bath at 70° for 3 hr., cooled, and poured into a mixture of ice water. The heavy brown oil which separated was extracted with chloroform. The chloroform after drying over anhydrous $MgSO_4$ was evaporated *in vacuo*, and the residue (the crude product) was obtained as a viscous yellow oil. On TLC (solvent, ethyl acetate), the crude product contained approximately 10–20% of the starting material. The crude product gradually crystallized upon cooling, and after two crystallizations from benzene, 20.5 g. (60% yield) of an analytically and chromatographically pure substance were obtained as colorless tiny plates, m.p. 108–109°.

Anal.—Calcd. for $C_{15}H_{17}I_3N_2O_5$: C, 26.26; H, 2.50; I, 55.50; N, 4.08; mol. wt. 685.06. Found: C, 26.31; H, 2.37; I, 55.80; N, 4.19; mol. wt. 689.5. Infrared absorption spectra $\lambda(CHCl_3)_{max.}$: 2.93 μ (medium, N—H stretching absorption of secondary amide), 3.33 μ (strong, aliphatic, C—H stretching vibrations), 5.73 μ (very strong, C=O stretching vibrations of ester), 5.88 μ (strong, C=O absorption of anilide), 6.94 μ (strong, aromatic C=C stretching vibration), 8.37 μ (very strong C—O—C stretching vibration of ester).

N-(2,4,6-Triiodo-3-acetamidobenzyl)-iminodiacetic Acid (V)—The free amino acid was obtained by saponification of the corresponding dimethyl ester (IV) in aqueous methanol (2 *N* NaOH, 10% excess), at 70° for 5 hr. The alkaline reaction mixture was cooled, extracted with ether, and carefully acidified to pH ~ 1 with concentrated HCl. The brown

[2] Melting points were determined on a Fisher-Johns melting point apparatus. All melting and boiling points are reported as uncorrected values. Infrared spectra (Figs. 1 & 2) were recorded on a Perkin-Elmer Infrared spectrophotometer model 137, calibrated by polystyrene film. Chromatography was carried out on layers of Silica Gel H(E. Merck AG., Germany) at a thickness of 0.25 mm. The compounds were detected by iodine vapor.

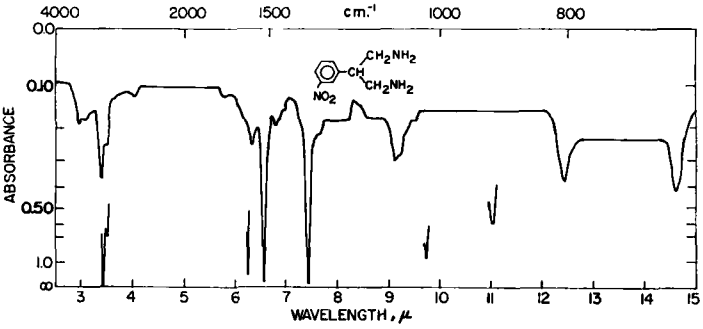


Fig. 1—Infrared absorption spectrum of 2-(3′-nitrophenyl)-trimethylenediamine (XII), 10 mg./ml. in chloroform.

heavy oil which separated was washed several times with water and dried over NaOH pellets *in vacuo*. The resulting solid product was then crystallized from a mixture of acetone–ether to give colorless fine needles, m.p. 128–130° dec. Upon storing, the product slightly decolorized.

Anal.—Calcd. for $C_{13}H_{13}I_3N_2O_5 \cdot 0.5\,H_2O$: C, 23.41; H, 2.11; I, 57.09; N, 4.20; mol. wt. 667.1. Found: C, 23.42; H, 2.16; I, 56.65; N, 4.08; mol. wt. 670.5.

Thin-layer chromatography (MeOH–AcOEt, 9:1), indicated the presence of a trace impurity with a higher R_f value. Attempts to obtain V by direct acetylation of III yielded only tarry material.

3-(3′-Nitrophenyl)-glutaric acid (VI)—This was prepared in 85% yield by a previously reported two-step procedure (8), m.p. 205–207° [lit. (8) m.p. 204–205°].

3-(3′-Amino-2′,4′,6′-triiodophenyl)-glutaric acid (VII)—A suspension of 10 g. of VI (0.04 mole) in 100 ml. of 2 *N* HCl was shaken with hydrogen and 1 g. of 5% Pd/C in a Parr low-pressure hydrogenator. After the theoretical quantity of hydrogen for reduction of the nitro group had been absorbed (*ca.* 2 hr.), the solution was filtered to remove the catalyst and the filtrate was diluted to 1 l. with 0.5 *N* HCl. Iodine monochloride, 23 g. (0.141 mole) in 30 ml. of concentrated HCl was added in one portion to the stirred solution at 55°. Appearance of the product as a heavy yellow to light brown granular precipitate began after about 4 hr. To complete the reaction, heating and stirring were continued for 6 days, a second batch of 23 g. of ICl in 30 ml. of concentrated HCl being added on the third day. After cooling the reaction mixture, the precipitated product was filtered, washed with water, and crystallized from a mixture of methanol–water. Recrystallization from glacial acetic acid yielded 14.2 g. (59% yield) of light cream-colored crystalline product, analytically and chromatographically pure, m.p. 224–226° dec.

Anal.—Calcd. for $C_{11}H_{10}I_3NO_4$: C, 21.98; H, 1.68; I, 63.36; N, 2.33; mol. wt. 600.96. Found: C, 22.05; H, 1.70; I, 63.50; N, 2.25; mol. wt. 597.7.

The product is soluble in methanol, ethanol, and *n*-propanol; it is only slightly soluble in isopropanol and acetone, and insoluble in chloroform, ethyl acetate, benzene, and toluene. Since direct acetylation of VII to yield 3-(3′-acetamido-2′,4′,6′-triiodophenyl)-glutaric acid (IX) failed to give the desired product, IX was obtained by acetylation of the dimethyl ester of 3-(3′-amino-2′,4′,6′-triiodophenyl)-glutaric acid, and subsequent saponification.

3-(3′-Diacetamido-2′,4′,6′,-triiodophenyl)-glutaric Acid Dimethyl Ester (VIII)—A mixture of 12 g. (0.02 mole) of the glutaric acid derivative (VII) in 100 ml. of absolute methanol containing 1 ml. of concentrated H_2SO_4 was gently refluxed for *ca.* 18 hr. The resulting red clear solution was cooled and then poured into 100 ml. of ice water. The dimethyl ester which separated as a heavy red-brown oil was extracted with chloroform. The chloroform extract was consecutively washed with sodium bicarbonate 10% solution and water, and then dried over anhydrous $MgSO_4$. After evaporation of the chloroform under reduced pressure, 10.1 g. (80% yield) of the product was obtained as a very viscous, clear, red-colored oil, which was chromatographically pure (solvent: methanol). The oily product did not crystallize during a long storage period in the cold and was used as such for the acetylation reaction. The dimethyl ester 12.6 g. (0.02 mole) was acetylated with 60 ml. of acetic anhydride containing 0.5 ml. of concentrated H_2SO_4. The reaction mixture was heated in an oil bath at 70° for 18 hr., cooled, and poured into 200 ml. of ice water. The heavy red-brown oil which separated was extracted with chloroform. The chloroform, after being washed with water and dried over anhydrous $MgSO_4$, was evaporated under reduced pressure, and the residue

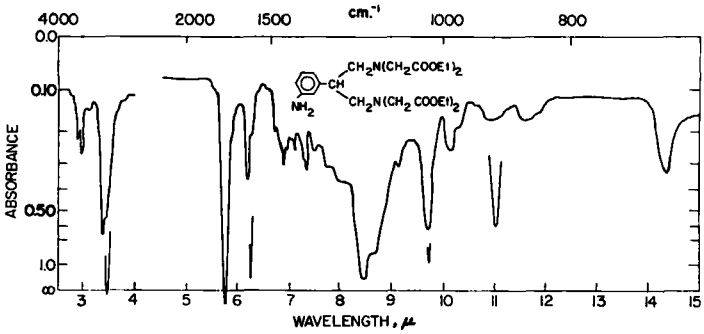


Fig. 2—Infrared absorption spectrum of 2-(3′-aminophenyl)-trimethylenedinitrilo-tetraacetic acid, tetraethyl ester, 10 mg./ml. in carbon tetrachloride.

(the crude product) was obtained as a viscous, clear, red-colored oil which gradually crystallized on storing in an ice box. Two crystallizations of the crude product, one from methanol and one from ether gave 12.1 g. (85% yield) of an analytically and chromatographically pure substance, in the form of colorless long plates, m.p. 102–103°.

Anal.—Calcd. for $C_{17}H_{18}I_3NO_6$: C, 28.63, H, 2.54; I, 53.40; N, 1.96. Found: C, 28.54; H, 2.49; I, 53.25; N, 1.92.

The product is soluble to a varying extent in most organic solvents.

3-(3′-Acetamido-2′,4′,6′-triiodophenyl)-glutaric Acid (IX)—A suspension of 21.4 g. of VIII (0.03 mole) in 60 ml. of 2 *N* NaOH, was stirred and heated to 80° for 24 hr. Complete solution occurred after 2 hr. The alkaline reaction mixture was then cooled, extracted with ether, and acidified with concentrated HCl. A heavy, cream-colored semisolid separated. The aqueous solution was decanted and the viscous precipitate washed twice with distilled water and dried over KOH pellets *in vacuo* at 50° overnight. The resulting cream-colored crude product weighed 18.3 g. and melted in the range of 140–160°. Two crystallizations from glacial acetic acid furnished 13 g. (67% yield) of analytically and chromatographically pure substance, in the form of colorless fine needles, m.p. 233–235° dec. Mixed m.p. with VII (the nonacetylated derivative), 195–205° dec.

Anal.—Calcd. for $C_{13}H_{12}I_3NO_5$: C, 24.28; H, 1.88; I, 59.22; N, 2.18; mol. wt., 643.0. Found: C, 24.28; H, 1.88; I, 59.35; N, 2.16; mol. wt., 645.5.

The product is freely soluble in DMF, only moderately soluble in hot methanol, ethanol, and acetic acid, and insoluble in water and most other organic solvents

3-(3′-Nitrophenyl)-glutaric Acid Dimethylester (X)—A mixture of 25.3 g. (0.1 mole) of VI in 150 ml. of absolute methanol containing 4.5 ml. of concentrated H_2SO_4 was gently refluxed for *ca.* 20 hr. Fifty milliliters of methanol was then distilled off, and the resulting clear dark red solution was cooled in ice water. Within minutes, the entire solution became a thick crystalline paste of the dimethyl ester. The brown-colored crystalline product, after filtration and thorough wash with water, was taken in chloroform. The aqueous filtrate was extracted with chloroform and the combined extracts were consecutively washed with sodium bicarbonate 10% solution and water and then dried over anhydrous calcium chloride. After evaporation of the chloroform under reduced pressure, the residue was distilled in high vacuum. The pure dimethyl ester was obtained as a yellowish clear distillate which crystallized on standing. Yield: 22.8 g. (81%), b.p. 163° (0.07 mm.), m.p. 65–66°. Crystallization from isopropyl ether or methyl-cyclohexane gave colorless needles of the product, without affecting the melting point. The dimethyl ester is soluble in most organic solvents except for ether, isopropyl ether, and methyl-cyclohexane from which it crystallizes on cooling. The NMR spectrum of (X) in $CDCl_3$ (60 Mc., TMS reference standard), showed a doublet centered at δ 2.75 of spacing 7 c.p.s. due to the four protons of the two methylene groups, a singlet at 3.59 due to the overlapping of six protons of the two OCH_3 groups, triplet of triplets centered at 3.80 of spacing 8 c.p.s due to the benzyl proton, and two multiplets each accounting for two aromatic protons centered at 7.38 and 7.96, respectively.

Anal.—Calcd. for $C_{13}H_{15}NO_6$: C, 55.51; H, 5.38; N, 4.98. Found: C, 55.62; H, 5.28; N, 5.03.

3-(3′-Nitrophenyl)-glutaryl Hydrazide (XI)—A solution of 28.1 g. (0.1 mole) of the dimethyl ester (X) in 150 ml. of methanol was added dropwise over a period of 0.5 hr. to a boiling stirred solution of 8 g. (0.25 mole) of hydrazine in 30 ml. of methanol. After completion of the addition, boiling and stirring were continued for another 0.5 hr. "Seeding," or little scratching of the hot solution caused immediate crystallization of the product. After cooling in ice water, the white crystalline paste of the hydrazide was filtered and washed with methanol and ether. After drying in air and at 80°, the hydrazide weighed 26.7 g. (95% yield), and was analytically pure, m.p. 184–186°. Crystallization from water or ethanol did not change the melting point. Since the preparation of hydrazides in the nitroaromatic series sometimes involves complications due to the reducing action of hydrazine, the presence of nitro group in the product (XI) was confirmed by infrared spectroscopy. The infrared spectra of 3-(3′-nitrophenyl)-glutaryl hydrazide contained the expected characteristic group bands (two very strong absorption bands at 6.57 μ and 7.42 μ characteristic of the valence vibrations of —C—NO_2). The hydrazide is soluble in hot water and hot dioxane, and only slightly soluble in methanol, ethanol, or propanol. It is insoluble in chloroform, benzene, and ethyl acetate.

Anal.—Calcd. for $C_{11}H_{15}N_5O_4$: C, 46.97; H, 5.38; N, 24.90. Found: C, 46.91, H, 5.52; N, 25.01.

2-(3′-Nitrophenyl)-trimethylenediamine (XII)—A mixture of 28.1 g. (0.1 mole) of the hydrazide (XI) in 44 ml. of concentrated HCl, 87 g. cracked ice, and 100 ml. of chloroform was diazotized at −5° with 18 g. of sodium nitrite dissolved in 33 ml. of water. After completion of the reaction (*ca.* 30 min.) the chloroform layer was separated and the aqueous layer was extracted twice with 30-ml. portions of chilled chloroform. The combined chloroform extracts were dried over anhydrous calcium chloride. The dried solution of 2-(3′-nitrophenyl)-glutaryl azide was added to 100 ml. of dry toluene and warmed on a steam bath. As soon as the chloroform began to distil out, evolution of nitrogen commenced and continued at a vigorous rate. After all the chloroform had been distilled out, the solution was refluxed strongly for about 1 hr. to complete the decomposition of the azide. To the stirred, hot, dark brown solution of the isocyanate, 60 ml. of concentrated HCl was added cautiously and immediately carbon dioxide began to evolve copiously. After the carbon dioxide evolution had ceased, the reaction mixture was refluxed for 24 hr. and then was concentrated to dryness *in vacuo* on a water bath. The residue of brown crystalline 2-(3′-nitrophenyl)-trimethylenediamine dihydrochloride was suspended in 100 ml. of absolute ethanol, and the suspension was gently refluxed for a few minutes. After cooling to room temperature the salt was filtered and washed with absolute ethanol and absolute ether. After air drying and drying at 80° *in vacuo* for a few hours the crude product weighed 17.5 g. (65% yield) and melted above 275°. A small portion of the dihydrochloride salt was crystallized from methanol–ethyl acetate mixture. The colorless crystalline

product after drying at 80° *in vacuo* melted above 275°.

Anal.—Calcd. for $C_9H_{15}Cl_2N_3O_2$: C, 40.31; H, 5.64; N, 15.67; Cl, 26.45. Found: C, 40.25; H, 5.58; N, 15.85; Cl, 26.15.

The dihydrochloride salt (17.5 g.) was dissolved in 20 ml. of water and the solution was made strongly alkaline with 30% NaOH. The free diamine was extracted with six 15-ml. portions of chloroform, dried, filtered, and distilled *in vacuo* under N_2. The diamine (XII) was thus obtained as a clear yellow oil which on exposure to light turned dark red, b.p. 135–137° (0.05 mm.). Yield: 9.76 g. (77% based on the dihydrochloride salt). Total yield: 50%.

On standing overnight in the refrigerator, the diamine solidified. 2-(3′-Nitrophenyl)-trimethylenediamine (XII) is soluble in warm water and in most organic solvents except for ether, methylcylohexane, and carbon tetrachloride. Because of the pronounced tendency of this diamine to form a carbonate on exposure to the atmosphere its analysis is rather poor but still indicative.

Anal.—Calcd. for $C_9H_{13}N_3O_2$: C, 55.37; H, 6.71; N, 21.53; equiv. wt. 97.61. Found: C, 54.81; H, 6.99; N, 20.83; equiv. wt. 99.25.

The infrared spectra of the freshly prepared diamine possessed characteristic bands in common for the functional groups. $\lambda_{max.}$ ($CHCl_3$): 3.05 μ (medium, N—H stretching vibrations), 3.40 μ (strong, C—H stretching vibrations), 6.29 μ (medium, probably overlapping of N—H deformation absorption and aromatic ring vibrations), 6.56 μ and 7.40 μ (very strong, valence vibrations of —C—NO_2), 12.41 μ and 14.60 μ (medium, out-of-plane deformation vibrations of three adjacent aromatic hydrogens).

2-(3′-Nitrophenyl)-trimethylenedinitrilo Tetraacetic Acid Tetramethyl Ester (XIII)—To a well-stirred suspension of 64.3 g. (0.42 mole) of methyl bromoacetate and 44.5 g. (0.42 mole) of anhydrous Na_2CO_3 in 100 ml. of acetonitrile, a solution of 19.5 g. (0.1 mole) of 2-(3′-nitrophenyl)-trimethylenediamine (XII) in 25 ml. of acetonitrile was added over about 0.5 hr. at 20–25°, while passing nitrogen through the reaction mixture. The reaction mixture was then stirred overnight and later refluxed for another hour. The inorganic salt was filtered and the solvent and excess of methyl bromoacetate were removed under reduced pressure on water bath. The crude product, obtained as a clear brown viscous oil, weighed 46 g. (95% yield) and on TLC (solvent: ethyl acetate) gave one principal spot indicating at least 90% purity. A small sample of the crude amino ester upon distillation in high vacuum, b.p. 210–215° (0.05 mm.), yielded analytically pure substance as a yellow viscous oil. Infrared absorption spectra (CCl_4) $\lambda_{max.}$: 3.32 μ (strong, aliphatic C—H stretching vibrations), 5.74 μ (very strong, C=O stretching vibrations of ester), 6.54 μ and 7.43 μ (strong, —C—NO_2 valence vibrations), 8.38 μ and 9.71 μ (very strong and strong, C—O—C stretching vibrations of acetoxyl group), 14.61 μ (medium, aromatic C—H deformation vibration).

The NMR spectrum of XIII in $CDCl_3$ (60 Mc. TMS reference standard) showed one rather broad peak at δ 3.10 with a shoulder at a higher field, due to the four protons of the two methylene groups, a singlet at 3.48 due to the overlapping of eight protons of four methylene groups, a singlet at 3.69 due to the overlapping of 12 protons of the four OCH_3 groups, and two multiplets each accounting for two aromatic protons centered at 7.53 and 7.95, respectively.

Anal.—Calcd. for $C_{21}H_{29}N_3O_{10}$: C, 52.17; H, 6.05; N, 8.69. Found: C, 52.59; H, 6.24; N, 8.37.

The tetraethyl ester, b.p. 230–234° (0.04 mm.), yellow viscous oil.

Anal.—Calcd. for $C_{25}H_{37}N_3O_{10}$: C, 55.65; H, 6.91; N, 7.79. Found: C, 55.25; H, 6.85; N, 8.09.

2-(3′-Nitrophenyl)-trimethylenedinitrilo Tetraacetic Acid (XIV)—Crude 2-(3′-nitrophenyl)-trimethylenedinitrilo tetraacetic acid tetramethyl ester (XIII) (48.4 g., 0.1 mole) dissolved in 100 ml. of methanol, was directly saponified with a NaOH solution (20 g., 0.5 mole, dissolved in 125 ml. of water). The hydrolysis of the four ester functions was completed within a few minutes, on boiling the reaction mixture. The clear dark red alkaline solution, was treated with active charcoal and refluxed for another 15 min., then cooled, filtered, extracted with ether, and carefully acidified with 42.7 ml. of concentrated HCl (11.7 *M*). The free amino acid (XIV) precipitated from the acidic (pH $\sim$ 1) aqueous solution as tiny particles. The amino acid was filtered, thoroughly washed with water and acetone, and then dried at 80° for a few hours. The amino acid (XIV) obtained in this way as a white, finely divided crystalline powder is already analytically pure and crystallization from a mixture of DMF–water does not change its decomposition point. Yield: 36 g. (84%), m.p. 224–225° dec.

Anal.—Calcd. for $C_{17}H_{21}N_3O_{10}$: C, 47.77; H, 4.95; N, 9.83; equiv. wt. 213.68. Found: C, 47.61; H, 4.85; N, 9.76; equiv. wt. 212.50.

The amino acid is only slightly soluble in hot water and insoluble in most organic solvents except for DMF. 2-(3′-Nitrophenyl)-trimethylenedinitrilo tetraacetic acid was also prepared by a direct adaptation of Dwyer and Garvan's method (12): alkylation of 2-(3′-nitrophenyl)-trimethylenediamine (XII) (0.1 mole), with an excess of sodium chloroacetate (0.6 *M*) in strongly alkaline solution at room temperature. However, longer reaction time (*ca.* 4 days) was necessary and lower yields (*ca.* 40%) were obtained compared with the previous method.

Attempted Preparation of 2-(3′-Amino-2′,4′,6′-triiodophenyl)-trimethylenedinitrilo Tetraacetic Acid—A solution of 4.27 g. (0.01 mole) of the nitrophenyl derivative (XIV) in 35 ml. of 1 *N* HCl, was hydrogenated at room temperature in a Parr low-pressure hydrogenator, using 0.5 g. of 5% Pd/C. After the theoretical quantity of hydrogen for reduction of the nitro group had been absorbed, the solution was filtered to remove the catalyst. The filtrate was used for the iodination step without isolating the intermediate aminophenyl derivative. Employing either iodine monochloride or potassium iododichloride as iodinating agents did not yield the desired aminotriiodophenyl derivative. Titration of an aliquot sample of the iodination reaction mixture, indicated that only one-third of the theoretical quantity of iodine monochloride had been consumed, even after prolonged reaction time (1 week).

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Keyphrases

X-Ray contrast agents—synthesis, X-ray intensity
3-Phenyl-glutaric acid, iodinated derivatives—synthesis
N-Benzyl-iminodiacetic acid, iodinated derivatives—synthesis
NMR spectroscopy—structure
IR spectrophotometry—structure
Toxicity testing—X-ray contrast agents

Assay of Vitamin E in Multivitamin Products Using Thin-Layer Chromatography

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Early work in this field was directed toward the assay of vitamin E in the presence of only vitamin A, culminating in the hydrogenation procedure reported by Fisher *et al.* (2), and included in the United States Pharmacopeia XVII (3). Paper chromatography has also been applied to vitamin A and E mixtures (4). Recent gas-liquid techniques for identification and estimation of tocopherols have been described (5–7) and applied by Pillsbury *et al.* (8) and by Bowman and West (9) to pharmaceuticals. Several recent references are found pertaining to thin-layer chromatography of tocopherols *per se* (10), in biologic materials (11), in oils and plant tissues (12, 13), and in serum (14). Castrén has reported the determination of vitamin E in pharmaceuticals using TLC separations (silica gel) followed by quantitative estimation of the developed spots by planimetry and visual observation (15, 16).[1]

METHOD

The method developed for multivitamin formulations containing vitamin E in potencies of from 0.15

Received May 22, 1968, from Distillation Products Industries, Division of Eastman Kodak Company, Rochester, NY 14603
Accepted for publication July 18, 1968.
Presented to the Drug Standards, Analysis and Control Section, APhA, Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
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to 15 I.U. or more per dosage unit consists of three major steps:

(a) Saponification, extraction, and concentration of the alpha tocopherol into a relatively small volume of ether.

(b) Separation of the alpha tocopherol from other ingredients reactive to ferric chloride by TLC.

(c) Determination of the alpha tocopherol by ferric chloride–α,α'-dipyridyl colorimetry.

Reagents

Ethanol, absolute; potassium hydroxide, pellets, 85%; hydrochloric acid, 37%; ethyl ether, anhydrous, ACS reagent grade; sodium sulfate, anhydrous granular; toluene, Eastman White Label 325; α,α'-dipyridyl, 0.25% in ethanol; ferric chloride, 0.1% in ethanol; 2′,7′-dichlorofluorescein, 0.004% in ethanol.

Materials

Thin-layer[2] plates, 20 × 20 cm., silica gel with fluorescent indicator, 250 μ; thin-layer developing apparatus, sandwich type; colorimeter, Evelyn, or other similar instrument having a constant, low-intensity light source and a direct reading, instantaneously responding photocell and galvanometer, equipped with a 515 mμ filter; UV light source, 2,540 Å.

Procedure

(a) Saponification—In a 125-ml. saponification flask obtain a sufficient quantity of the dosage form to result in a total of preferably 8 to 16 I.U.[3] of vitamin E as follows:

Capsules—Place the required number into the flask. Add 10 ml. water, warm, and swirl until digested.

Tablets—Precrush the required number in a mortar and transfer to the flask using 10 ml. water.

Liquids—Using a suitable pipet, transfer the required volume into the flask, and add 5 ml. water.

Add 30 ml. of ethanol, attach an air condenser, and heat with steam to vigorous reflux. Slowly drop 5 g. KOH pellets through the condenser and reflux for 20 min. Remove from heat and immediately add 10 ml. HCl dropwise through the condenser. Cool and transfer to a 500-ml. separator using 50 ml. distilled water followed by three 50-ml. portions of ether. Shake well for 2 min. Separate the ether layer and wash with four 100-ml. portions of distilled water. Dry the extract by passing it through a funnel containing sodium sulfate on filter paper, washing the cake well after passage with additional ether. Reduce the ether volume sufficiently to allow transfer to a 10-ml. or 25-ml. volumetric flask. Make to a final concentration of alpha tocopherol of about 0.5 mg./ml.

(b) Chromatography—Using a suitable pipet, stripe a sufficient quantity of the ether solution (usually 100 to 300 μl.) containing 80 to 120 mcg. alpha tocopherol on a silica gel plate previously activated for 20 min. at 100°. The stripe should be placed parallel to and approximately 2 cm. from one edge of the plate and should extend for a distance of approximately 14 cm. Place a pilot spot of alpha tocopherol next to the unknown. Develop the chromatogram with a migrating solvent of toluene to a distance of about 15 cm. Remove the plate, dry thoroughly with nitrogen, and spray with 2′,7′-dichlorofluorescein solution. Find the alpha tocopherol zone corresponding to the standard at R_f of about 0.5 by observing under UV light (2,540 Å.). Outline the correct band with pencil and scrape the adsorbent into a 125-ml. flask. Add 10 ml. ethanol, swirl for 3 min., and pass through filter paper (Eaton-Dikeman 617 is satisfactory) into a 25-ml. volumetric flask (low actinic) calibrated at 23 ml. or into a 25-ml. graduated cylinder (low actinic, ground glass stopper). Repeat with three 4-ml. portions of alcohol and make to a final volume of 23 ml.

(c) Colorimetry—Add successively to the flask or cylinder 1 ml. dipyridyl solution and 1 ml. ferric chloride solution. Stopper the container and mix well. After 2.5 min., read the percent transmittance of the solution using a 515-mμ filter in the colorimeter. From a calibration chart, determine the corresponding amount of alpha tocopherol, in mcg.[4] Calculate the potency as follows:

a = mcg. alpha tocopherol, from calibration chart
b = final volume of ether extract, ml.
c = volume of ether extract striped, μl.

$$\frac{a \times b}{c} = d = \text{mg. alpha tocopherol in final ether volume}$$

$$\frac{d \times f}{n} = \text{potency, I.U./dosage unit}$$

where f is the weight-unit relationship (17) for *d*-alpha tocopherol (f = 1.49) and *dl*-alpha tocopherol (f = 1.1) and n is the number of dosage units assayed.

DISCUSSION

Analyses in these laboratories of vitamin E in commercial multivitamin capsules and tablets had shown that the USP XVII assay procedure written for decavitamins was in some instances unsatisfactory. The USP procedure removes the interference due to vitamin A but other reducing substances lead to assay results for vitamin E that are sometimes several times too high. Clearly, some means of separation or a more specific assay method was needed. It seemed that TLC would have the capability of separating alpha tocopherol from the other reactants and still allow common methods of determination to be used.

Investigations were done to determine an adsorbent-solvent system to separate alpha tocopherol from interfering substances in capsules and tablets. Information in the literature suggested that an optimum system would be found among two adsorbents (silica gel and alumina) and six solvents (hexane–ethanol 9:1, ether, toluene, toluene–ether 1:1, cyclohexane–ether 4:1, cyclohexane–ethanol 9:1). Test strips of Eastman Chromagram sheets

[2] Suitable are E. Merck plates, Silica Gel F-254.

[3] The following example for tablets labeled to contain 0.15 I.U./tablet illustrates deviations from the procedure when this very low potency product was assayed.
Saponification: 10 tablets crushed and transferred to flask with 30 to 40 ml. water, 110 ml. ethanol, and 7 g. KOH added for reflux. 14 ml. HCl used for acidification. Ether extract transferred to a 10-ml. volumetric flask (concentration of alpha tocopherol of 0.1 mg./ml.).
Chromatography: 1 ml. of ether solution (containing 100 mcg. alpha tocopherol) striped.

[4] For procedures regarding colorimetry, see Lehman, R. W., *J. Pharm. Sci.*, **53**, 201(1964).

(6060 silica gel, 6063 alumina) (Eastman Kodak Co., Rochester, N. Y.) were developed with these solvents using the unsaponifiable fractions from multivitamin-mineral formulations. Reducing substances were visualized by ferric chloride-dipyridyl spray. Table I shows typical R_f values, these obtained after chromatographing the unsaponifiable fraction of a multivitamin-mineral tablet labeled to contain 0.15 I.U. vitamin E/tablet. The best separations were obtained with alumina/toluene, with silica gel/toluene, and with silica gel/cyclohexane–ether. The authors chose silica gel/toluene for further investigation.

Silica gel was quite effective for separating alpha tocopherol from other tocopherols. The unsaponifiable fraction of a mixed tocopheryl acetate concentrate was chromatographed. Of the total tocopherols in the concentrate, 87% was alpha and 13% was a mixture of nonalpha tocopherols. Gas-liquid chromatography (GLC) applied to the eluted alpha tocopherol band showed that only 0.1% nonalpha tocopherols remained.

Some loss of alpha tocopherol, presumably through oxidation, during the separation procedure can be expected. To determine the loss, replicate quantitative chromatograms of *d*-alpha tocopherol were run on 250-μ silica gel plates and the recoveries compared with the unchromatographed input. The results from 10 determinations (Table II) indicated that a 94.7% apparent recovery ±1.6% *SD* of alpha tocopherol could be expected.

The presence of reducing materials in the thin-layer adsorbent that co-elute with alpha tocopherol would result in an inflated recovery value. Several experiments were done in a manner similar to that noted above but in which ether only was striped. Assay of the eluted adsorbent band corresponding in size and position to an alpha tocopherol band from 250-μ silica gel gave values corresponding to about 3 mcg. alpha tocopherol. Since approximately 100 mcg. amounts of alpha tocopherol are striped in the recommended procedure, this value amounts to some 3% of the total and is included in the 94.7% apparent recovery. The corresponding true recovery value was about 92%.

In order to determine whether alpha tocopherol could in fact be recovered from multivitamin preparations, a formulation was obtained typical of one designed for capsules but without any added vitamin E.[5] TLC on Eastman Chromagram sheets (silica gel) of the unsaponifiable fraction indicated that no vitamin E could be detected by visualization with ferric chloride-dipyridyl spray solution. Without addition of alpha tocopherol, USP assay (without hydrogenation) indicated that 6 I.U. of apparent vitamin E/g. was present in the material. Vitamin E, as *d*-alpha tocopheryl acetate concentrate, was added at 2, 3, and 15 I.U./g. before saponification. Recoveries as obtained by the recommended procedure, shown in Table III, were from 98 to 100% of input after correcting for the apparent losses of alpha tocopherol during separation.

The results of the assay procedure applied to multivitamin capsules, tablets, and liquids are given in Table IV. It can be seen that the total apparent amounts of vitamin E in the unsaponifiable fractions (unhydrogenated) ranged from about 1.5 to some 20 times the labeled amount. After chromatography, the assay results (corrected) were found to be generally near label claim: in 14 of the 18 products, they lay between 94 and 110% of this figure.

[5] Kindly supplied by R. P. Scherer Corp., Detroit, Mich.

Table I—R_f Values for Alpha Tocopherol on Test Strips, 100 μ

Test Strip	R_f
Alumina	
Hexane–ethanol 9:1	0.79
Ether	0.82
Toluene	0.37
Toluene–ether 1:1	0.77
Cyclohexane–ether 4:1	0.34
Cyclohexane–ethanol 9:1	0.76
Silica Gel	
Hexane–ethanol 9:1	0.76
Ether	0.95
Toluene	0.61
Toluene–ether 1:1	0.85
Cyclohexane–ether 4:1	0.63
Cyclohexane–ethanol 9:1	0.80

It was of interest to attempt to determine the nature of the interfering substances in these formulas and some work was done in this area. The authors' knowledge of vitamin A would lead them to infer that, since they did not completely remove it by hydrogenation, higher ratios of vitamin A:alpha tocopherol should result in higher apparent amounts of alpha tocopherol in the unsaponifiable fractions. Table V shows that this is generally true. It should be remembered that not only vitamin A but also butylated hydroxyanisole (BHA) and, to some extent, butylated hydroxytoluene (BHT) blended with the vitamin A as antioxidants would be expected to interfere. Figure 1 illustrates a silica gel chromatogram used to aid in identifying these substances. The spot from the unsaponifiable fraction corresponding closely in R_f to that of BHA was confirmed as such by GLC and IR spectral data. The spot closest to the solvent front contained BHT and anhydrovitamin A as shown by GLC and UV ab-

Table II—Replicate Recoveries of *d*-Alpha Tocopherol by Thin-Layer Chromatography

Silica Gel, 250 μ, %
93.8
94.6
96.1
97.0
95.4
93.0
92.6
95.7
96.5
92.7
94.7% mean ± 1.6% *SD*

Table III—Recoveries of Vitamin E Added to Multivitamin Formulation

Input I.U./g.[a]	Recovery, %	
	Found	Corrected[b]
15	95	100
3	94	99
2	93	98

[a] *d*-Alpha tocopheryl acetate concentrate. [b] Correction factor 0.95.

TABLE IV—VITAMIN E CONTENT OF MULTIVITAMIN FORMULAS POTENCY, I.U. PER DOSAGE UNIT

Sample Number	Label Potency	Total Emmerie-Engel	Chromatographed Emmerie-Engel		
			Uncorrected	Corrected[a]	% Label
Capsules					
1	15	25.2	14.8	15.6	104
2	15	26.2	14.6	15.4	103
3	5	10.5	4.1	4.3	86
4	5	12.4	4.8	5.1	102
5	5	7.3	4.5	4.7	94
6	3.7	6.8	3.6	3.8	103
7	3	11.7	2.9	3.1	103
8	2	4.0	2.1	2.2	110
9	2	7.4	2.1	2.2	110
10	1	1.3	0.97	1.0	100
11	0.5	8.1	0.45	0.47	94
Tablets					
12	15	22.7	15.0	15.8	105
13	10	13.7	11.9	12.5	125
14	7.5	11.2	7.3	7.7	103
15	3	4.6	3.0	3.2	107
16	0.15	2.2	0.16	0.17	113
Liquids					
17	50	172.8	44.7	47.1	94
18	5	103.5	6.0	6.3	126

[a] Correction factor 0.95.

sorption, respectively (the anhydrovitamin A is formed upon acidification of the mixture with hydrochloric acid). A summary of the R_f values of known interfering substances is found in Table VI.

The authors have observed the presence of other reducing agents that have not been identified. For example, a thin-layer chromatogram of a multivitamin unsaponifiable fraction showed the presence of at least four reactive ingredients (R_f of 0, 0.25, 0.58, 0.62) in addition to alpha tocopherol and antioxidants. In another example, a thin-layer chromatogram of a hydrogenated multivitamin unsaponifiable fraction revealed four strongly interfering materials (R_f of 0–0.1). This latter example illustrates one instance where grossly high results were obtained with the USP XVII procedure.

TABLE V—COMPARISON OF VITAMIN A: ALPHA TOCOPHEROL RATIO WITH TOTAL APPARENT VITAMIN E

Units Vitamin A/Unit Vitamin E (Label)	Total Emmerie-Engel % of Label
Capsules	
1,000	145
1,350	184
1,670	166
1,670	175
2,500	200
3,000	133
5,000	210
5,000	248
6,250	370
8,300	390
50,000	1,620
Tablets	
400	137
1,330	149
1,670	151
1,670	154
33,400	1,470

Thus, it is possible that some seven or more reducing materials, some of which are not removed by hydrogenation, can be present in a multivitamin formulation.

The use of 250-μ thin-layer plates was necessary in some cases since overloading of 100 μ thicknesses was encountered upon chromatographing sufficient amounts of certain capsule unsaponifiable fractions to provide 80 mcg. or more alpha tocopherol. Overloading appeared to be common with those formulations having large amounts of diluent oils. Three alternate methods that allow smaller amounts to be chromatographed appear as possibilities to eliminate overloading if 100-μ thicknesses are to be employed: (*a*) more sensitive reagents, such as bathophenanthroline (12, 18) or 2,4,6-tri(2′-pyridyl)-*S*-triazine (18, 19), replacing α,α'-dipyridyl; (*b*) alkaline extraction after saponification thereby eliminating most of the fatty acids; or (*c*) reducing the volume of the colorimetric reaction.

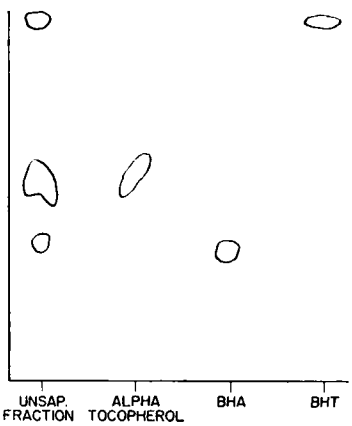

Fig. 1—Identification of some interfering materials

TABLE VI—R_f VALUES OF INTERFERING SUBSTANCES 250 μ SILICA GEL–TOLUENE

Substance	R_f
BHT	0.97
Anhydrovitamin A	0.97
Alpha tocopherol	0.49
BHA	0.38
Beta tocopherol	0.32
Gamma tocopherol	0.30
Delta tocopherol	0.20

Assays Other Than Multivitamins—Thin-layer separation should be applicable to most types of formulations in addition to multivitamins. One such application was to a liquid tonic formula labeled to contain 20 I.U. vitamin E, as *d*-alpha tocopheryl acetate/oz. Assay of the unsaponifiable fraction prior to chromatography indicated 440 I.U. apparent vitamin E/oz. TLC revealed the large amount of reducing substances. Assay of the eluted alpha tocopherol band gave a reasonable result of 20.6 I.U./oz.

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Keyphrases

Vitamin E analysis—multivitamin products
TLC—separation, analysis
UV light—α-tocopherol zone visualization
Colorimetric analysis—spectrophotometer

Alkaloids of *Ochrosia maculata* Jacq. (*Ochrosia borbonica* Gmel.)

Isolation of the Alkaloids and Study of the Antitumor Properties of 9-Methoxyellipticine

By GORDON H. SVOBODA, GERALD A. POORE, and MARILYN L. MONTFORT

Inclusion in the authors' phytochemical screening program of plants of the family *Apocynaceae* related to *Catharanthus roseus* G. Don seemed logical in view of the success realized with the Madagascan periwinkle. Screening of the appropriate extracts of *Ochrosia maculata* elicited both oncolytic and neurosedative activities. The former was found to be associated with 9-methoxyellipticine, the latter with reserpine. While 9-methoxyellipticine possesses experimental antitumor activity of a somewhat lesser order than some of the available clinically active agents, it does exhibit a broader spectrum than most of these compounds. Its moderate degree of potency as an antitumor agent is expressed by its activity against several of the solid mouse neoplasms maintained in these laboratories.

THE GENUS *Ochrosia*, family *Apocynaceae*, consists of approximately 36 species of trees or woody shrubs native to tropical Asia, Oceania, and the Mascarene and Seychelle Islands. *Ochrosia maculata* Jacq., a local name for which is "bois jaune" because of the bark and wood being yellow and bitter (1), is a tree 6–12 m. (20–40 ft.) in height, bearing 3 leaves (rarely 4) in a whorl, being oblong to oblong-lanceolate, 13.5–15.2 cm. (3–6 in.) long, obtuse or somewhat acute, glossy, and often spotted. The flowers are white, being

Received April 4, 1968, from the Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN 46206
Accepted for publication July 11, 1968.
The authors thank the following individuals for their aid in the course of this investigation: D. R. Bedwell, R. E. Martello, and A. T. Oliver for laboratory assistance; Dr. H. A. Rose, R. E. Staten, and D. O. Woolf, Jr., for physical data; Miss E. D. Liljeblad for aid in preparing the manuscript. Special thanks are extended to R. W. Kattau for mouse behavior studies, and to Mrs. V. P. Bothwell, J. W. Mattingly, P. E. Craig, and E. F. Prijller for antitumor testing.

TABLE VI—R_f VALUES OF INTERFERING SUBSTANCES 250 μ SILICA GEL–TOLUENE

Substance	R_f
BHT	0.97
Anhydrovitamin A	0.97
Alpha tocopherol	0.49
BHA	0.38
Beta tocopherol	0.32
Gamma tocopherol	0.30
Delta tocopherol	0.20

Assays Other Than Multivitamins—Thin-layer separation should be applicable to most types of formulations in addition to multivitamins. One such application was to a liquid tonic formula labeled to contain 20 I.U. vitamin E, as *d*-alpha tocopheryl acetate/oz. Assay of the unsaponifiable fraction prior to chromatography indicated 440 I.U. apparent vitamin E/oz. TLC revealed the large amount of reducing substances. Assay of the eluted alpha tocopherol band gave a reasonable result of 20.6 I.U./oz.

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about 0.8 cm. (1/3 in.) across, with calyx lobes ovate. The fruit is bright red and reputedly poisonous.

The various species of this genus have been rather severely handled by taxonomists, producing a confusing state of botanical nomenclature and classification. The following classification of Pichon (2, 3) is the one usually accepted: family, *Apocynaceae*, subfamily, *Plumerioideae;* tribe, *Rauvolfieae;* subtribe, *Ochrosiinae.*

The botanical name now usually considered as being correct for this plant is *Ochrosia maculata* Jacq., having a distribution restricted to the Mascarene and Seychelle Islands.[1] *O. borbonica* Gmel. is considered to be a direct synonym, as is *Cerbera undulata* (4). However, *O. borbonica* has in the past been considered as being a synonym for the Pacific *O. oppositifolia* (Lamk.) K. Schum., a distinct species which is stated to occur from the Mascarene Islands to Polynesia. Consequently, the early chemical investigations recorded as being performed on *O. borbonica* should probably be referred to as *O. maculata* rather than to *O. oppositifolia*, inasmuch as the early French workers were interested in the use of the Mascarene group species as a tonic and febrifuge.

Planchon (5) has referred to the bark and leaves of *O. maculata* (described as *O. borbonica*) as having been used in Réunion, Mauritius, and the Mascarene Islands as a bitter tonic, stomachic, and febrifuge. Ochrosine, a substance isolated by Boissard, has been found by Vinson (5) to be tonic and analeptic. Carrieu [quoted by Barquissau (5)] found that when the bark was used medicinally, it was not toxic even in high doses and that it was no more effective in the treatment of intermittent fevers than were other bitter tonics. It appeared to be cardiotonic, acting as a diuretic and augmenting the impulsive force of the heart. Vinson considered it to be a good remedy for anemia.

A defatted ethanolic extract of the bark[2] showed moderate experimental antitumor activity against adenocarcinoma 755, as well as a CNS depressant and vasodilator activity in the mouse behavior test (6). Subsequent application of a sequential extraction technique using *n*-hexane, benzene, ether, and ethanol provided extracts which elicited other biological activities, *e.g.*, weak serotonin-like effects, anti-inflammatory, diuretic, and nonreserpine-like CNS depressant responses. It was, however, the antitumor activity and the CNS depressant effects which prompted the major phytochemical effort which ensued.

9-Methoxyellipticine has been isolated from this species by two other groups of investigators (7, 8) and its effect against the L-1210 leukemia has been described (7). Other species of this genus have also yielded this alkaloid as indicated by the following documentation: Section 1, *Lactaria: O. elliptica* Labill. (9, 10); *O. coccinea* (Teijsm. and Binnend.) Miq. [*Excavatia coccinea* (Tejsmann and Binnendijk) Mgf.] (10); *O. moorei* F. Muell. (11); Section 2, *Echinocaryon: O. glomerata* Valeton (11); *O. oppositifolia* (Lamk.) K. Schum. (12); Section 3, *Phragmochrosia:* contains only one species, *O. apoensis* Elmer, which has not yet been examined.

Reserpine has been obtained from the leaves of *O. poweri* Bailey (11) and from the bark of *O. coccinea* (13). Its isolation from *O. maculata* is herein reported for the first time.

A number of different extraction and purification techniques were investigated, including the selective extraction and gradient pH techniques which proved to be so useful in the investigation of the alkaloids of *Catharanthus roseus* G. Don (14, 15). However, neither of these proved to be of any special value in the present investigation. While a number of probe runs utilizing a variety of techniques were used, the following procedure was the one eventually considered to be the best for the authors' purposes.

Extraction of the bark with ammoniacal benzene yielded an extract from which relatively pure 9-methoxyellipticine deposited during concentration. Eventual extraction of the bark with ethanol yielded an extract which showed both antitumor and CNS depressant activities. Additional quantities of 9-methoxyellipticine were isolated therefrom using classical techniques. It was, however, necessary to utilize column chromatography to isolate reserpine. All fractions were monitored with TLC and biological activities were checked primarily with the X-5563 plasma cell myeloma and with mouse behavior patterns.

EXPERIMENTAL

Coarsely ground bark (100 kg.) was extracted by stirring with one 400-l. portion of benzene and 4 l. of concentrated ammonium hydroxide. Two subsequent benzene extractions of 400 l. each were run and combined with the first. Concentration *in vacuo* yielded two crops of yellow-gold needles, 7.612 g. and 3.150 g., at 4 l. and 800-ml. volumes, respectively. Interpretation of the physical data (X-ray, UV, and IR spectra) indicated that these two crops of crystals were mainly 9-methoxyellipticine. TLC using the system of ethyl acetate–ethanol (9:1) on Silica Gel G indicated 9-methoxy-

[1] Personal communication from Dr. Julian A. Steyermark, Botanical Institute, Caracas, Venezuela, based on information provided by the Royal Botanic Gardens at Kew.

[2] The drug used in this investigation was obtained from the Meer Corporation, New York, N. Y.

ellipticine to be the major component in both cases.

Concentration of the benzene crude mother liquor (CML) *in vacuo* yielded 279.3 g. of a dark brown syrup which was found to possess moderate anti-inflammatory activity (carageenin-induced edema), a weak reserpine-like CNS depression in mice, and antitumor activity against the adenocarcinoma 755.

A 10-g. aliquot of this crude mother liquor was dissolved in 500 ml. of $EtCl_2$, an equal volume of 1% H_2SO_4 was added, and the $EtCl_2$ was removed *in vacuo*. The acid H_2O-insoluble material was filtered off and held for further examination. The aqueous acid phase was extracted with 500-ml. portions of $EtCl_2$ at pH levels of 1.0, 3.5, and 8.2. Only the lower two showed weak reserpine-like depression. While TLC indicated the possible presence of reserpine, none could be isolated in crystalline form. The insoluble material cited above showed activity against the AC755 and TLC indicated that it was probably caused by the presence of 9-methoxyellipticine or a close relative. None was isolated in crystalline form.

Extraction of the bark with three 400-l. portions of ether gave 75.71 g. of a dark brown syrup which showed weak reserpine-like CNS depression and oncolytic activity against the X-5563 myeloma. A work-up of a 10-g. aliquot in a manner analogous to that just described did not yield any crystalline isolates.

Final extraction of the drug involved the use of three 400-l. portions of 95% ethanol. The combined extracts were concentrated *in vacuo* to approximately 11 l. A 50-ml. aliquot of this concentrate yielded 17.36 g. of dried residue which elicited a weak CNS depression in mice and a serotonin-like bluing of the skin. Some activity was also shown against the P-1534 leukemia.

A 2.2-l. aliquot of the ethanol extract concentrate, containing approximately 763.8 g. of extractive, was extracted with one 8.8-l. and two 4.4-l. portions of 1% H_2SO_4. The combined aqueous acid extracts were extracted at the existing pH of 0.7 with one 16-l. and two 8-l. portions of $EtCl_2$, yielding 15.0 g. of a dark brown gum. Although this extract elicited a weak reserpine-like depression in mice, the alkaloid could not be isolated therefrom directly. A 10-g. aliquot, when chromatographed on 300 g. of partly deactivated Alcoa F-20 alumina (Table I), yielded 0.099 g. of off-white, rod-like crystals from methanol which proved to be reserpine.

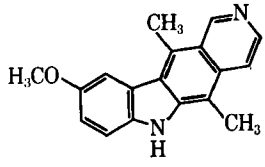
Two subsequent $EtCl_2$ extractions were performed at pH levels of 3.2 and 8.6 as previously described. The residue from the pH 3.2 extract (13.78 g.) proved to be of no interest and was eventually discarded. Concentration *in vacuo* of the $EtCl_2$ at pH 8.6 to an approximate volume of 1.2 l. yielded 16.30 g. of insoluble material which was shown to be mainly 9-methoxyellipticine. Concentration of the crude mother liquor yielded 24.9 g. of dried material which was eventually discarded.

A flow diagram for the extraction scheme is presented in Scheme I.

Both the roots and leaves (1.5 kg. each) were extracted in an analogous manner. The yields of ammoniacal benzene, ether, and ethanol extracts from the roots were 1.33, 1.0, and 13.36 g., respectively. A weak diuretic activity was observed for the ethanol extract, the other two extracts being relatively inactive in all tests. The yields of the corresponding extracts from the leaves were 47.67, 26.99, and 47.95 g., respectively. Both the ammoniacal benzene and ether extracts showed slight anti-inflammatory activity and the latter showed slight antibacterial activity as well. The ethanol extract displayed slight antibacterial activity. No other biological responses were noted. The benzene extract was subjected to column chromatography on Al_2O_3. No crystalline entities were obtained. Details of the above work are not warranted.

9-Methoxyellipticine—Recrystallization of 26.94 g. of crude alkaloid from hot methanol (600 ml./g.) yielded 13.36 g. of first crop material as fine blades showing both parallel extinction and oblique extinction with negative elongation. A second crop of 2.36 g. was obtained from the mother liquor. The physical data for this recrystallized material agreed in all respects with those for the authentic alkaloid.[3] Such data have been sufficiently well documented so as to preclude repetitive presentation here.

The structure for 9-methoxyellipticine (I) has been reported as 9-methoxy-5,11-dimethyl-6*H*-pyrido-[4,3-*b*]-carbazole (10).



I

Reserpine—Methanol recrystallization of the reserpine obtained from the column previously described yielded 0.051 g. of colorless rods. Physical measurements, as well as biological effects and potency, were identical in all respects to those for authentic reserpine previously isolated in these laboratories.

BIOLOGICAL PROPERTIES

The experimental antitumor properties exhibited by 9-methoxyellipticine are of a somewhat lesser order than some of the presently available clinically active compounds and are certainly less than those of acronycine (17). It does, however, exhibit a broader spectrum than most clinically active agents in use today. Its moderate degree of potency is expressed in its activity against a number of solid mouse neoplasms maintained in these laboratories.

Methodology—Procedures for animal tumor testing in these laboratories have been previously described (18) and consist essentially of subcutaneous trocar implantation of solid tumors in the axillary region and intraperitoneal inoculation of ascitic and leukemic cells with standard cell inocula. Treatment is normally initiated 24 hr. later, exceptions being made for the X-5563 myeloma, S-91 melanoma, Ridgeway osteogenic sarcoma, and the Shionogi carcinoma 115. In the case of the X-5563 myeloma treatment was initiated 3 days postimplantation, whereas with the other three systems it was initiated

[3] Kindly supplied by Dr. C. C. G. Culvenor, C.S.I.R.O., Melbourne, Australia. Also see *Reference 16*.

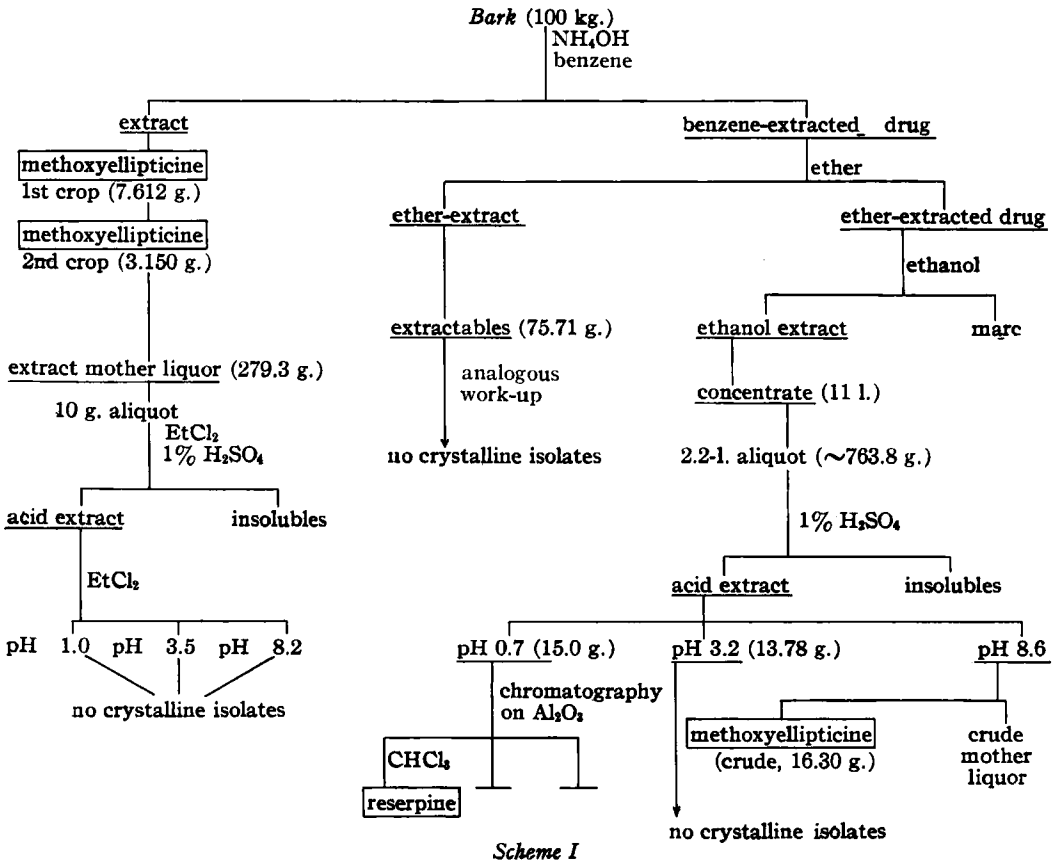
TABLE I—CHROMATOGRAPHY OF WEAK BASE FRACTION FROM EtOH EXTRACT

Fraction	Eluting Solvent	Volume, ml.	Wt., g.	Compound	Wt., g.	Crystallizing Solvent
1	Chloroform	205	0.25	Oil	—	—
2	Chloroform	90	1.17	Reserpine	0.051	Methanol
3	Chloroform	90	1.27	Reserpine	0.048	Methanol
4–19	Chloroform	6,645	2.29	Amorphous residues	—	—
20–30	Chloroform–methanol (99:1)	2,685	0.29	Amorphous residues	—	—
31–38	Chloroform–methanol (19:1)	3,480	0.31	Amorphous residues	—	—
39	Methanol	1,050	0.91	Amorphous residues	—	—

5 days postimplantation, this delay being occasioned because of the very slow rate of initial growth.

Activity of the alkaloid is expressed in terms of tumor inhibition of the solid tumor treated *versus* the control (T/C). When evaluating results for the leukemias and ascites forms, activity is expressed in terms of percent prolongation of life of the treated animals *versus* the controls (T/C).

Preparation of 9-Methoxyellipticine for Testing—This alkaloid was relatively insoluble in almost all of the standard testing vehicles, but this circumstance did not prevent its being tested. A suitable suspension was prepared by grinding the compound with small volumes of a nonionic dispersant[4] to obtain a suitable uniform suspension and diluting with additional diluent to the desired dosage volume. (The dispersant is a polyoxyethylated fatty acid that is H_2O-miscible and nontoxic when diluted to the proper concentration of 1:10 with either sterile distilled H_2O or sterile physiological saline solution.) 9-Methoxyellipticine was insoluble in saline, sesame oil, olive oil, gum acacia, and carboxymethylcellulose and no satisfactory suspensions therein could be obtained.



Scheme I

ANTITUMOR ACTIVITY OF 9-METHOXYELLIPTICINE

The authors' experience with this alkaloid has shown it to possess activity against a relatively broad spectrum of mouse neoplasms, 10 of 17 systems having responded to a somewhat narrow range of dose levels. Furthermore, it was demonstrated to be active in certain cases by both the

[4] Marketed as Emulphor by General Aniline and Film Corp., Melrose Park, Ill.

TABLE II—EXPERIMENTAL TUMOR SPECTRUM OF 9-METHOXYELLIPTICINE

Tumor	Host, 10 Animals	I.p. Dosage, mg./kg./day	Av. Wt. Change, g., T/C	Av. Tumor Size, mm., T/C	Av. Life, T/C	Activity,[a] %
L-1210 leukemia	DBA/2	12 × 1 × 10	−0.6/−0.2	—	16.7/15.5	0
L-1210 leukemia III	DBA/2	12 × 1 × 10	−0.5/+2.8	—	13.9/11.0	26
C-1498 leukemia	C57B1/6	18 × 1 × 10	+0.1/+0.3	—	18.8/14.0	34
P-1534 leukemia	DBA/2	24 × 1 × 10	−1.7/+1.0	—	20.0/15.8	26
AKR leukemia	AKR	18 × 1 × 10	−3.6/+1.4	—	11.2/13.2	0
B-82 leukemia, solid	C58B1/6	18 × 1 × 10	−0.3/+1.6	20.8/14.9	—	0
Freund ascites	CAF[1]	18 × 1 × 10	+3.5/+8.8	—	17.6/17.1	0
S-180 ascites	CAF[1]	24 × 1 × 10	−3.1/−0.3	—	10.0/11.3	0
Taper hepatoma, ascites	C3H	18 × 1 × 10	+3.0/+3.6	—	13.7/10.6	29
Adenocarcinoma 755	C57B1/6	60 × 1 × 10	−4.4/+2.8	5.6/18.0	—	69 (7)
Mecca lymphosarcoma	AKR	12 × 1 × 10	+1.0/+2.6	14.9/22.0	—	32 (10)
X-5563 myeloma	C3H	12 × 1 × 10	−3.8/−2.2	4.2/9.4	—	55 (7)
Gardner lymphosarcoma	C3H	18 × 1 × 10	−1.5/+6.0	15.3/31.8	—	52 (4)
C3H mammary	C3H	24 × 1 × 10	−2.8/+3.6	18.8/30.7	—	39 (7)
High malignancy clone	C3H	18 × 1 × 10	−4.6/−0.1	3.9/9.2	—	58 (9)
S-91 melanoma	DBA/1	24 × 1 × 10	−5.2/+0.6	0/5.5	—	100 (7)
Ridgeway osteogenic sarcoma	AKR	24 × 1 × 14	−2.5/+1.3	8.9/7.9	—	0
Walker 256, carcinosarcoma	Rat	30 × 1 × 10	−2.0/+4.1	8.9/14.3	—	38 (5)[b]
Walker 256, ascites	Rat	30 × 1 × 10	+11.6/51.4	—	21.3/11.6	84 (2)[b,c]

[a] The number in parentheses indicates survivors on solid tumors, indefinite survivors at 45 days on leukemia or ascitic tests. [b] Five rats used. [c] Two indefinite survivors at 29 days postinoculation. Inoculated with ascites form.

intraperitoneal and oral routes. The corresponding dose ranges were from 12–24 mg./kg. and 24–36 mg./kg., respectively.

This alkaloid is active against a lymphocytic leukemia and several solid tumors which are not responsive to the oncolytic alkaloids from *Catharanthus roseus* G. Don. Its inability to show significant activity against most of the ascites systems lends credence to the assumption that its antitumor activity may not be due to direct cytotoxic action.

Activity Against the L-1210 III Leukemia—Unlike many other antitumor agents there does not appear to be a system of choice in determining its efficacy. It may be noteworthy, however, to cite its activity against the L-1210 III leukemia. This particular tumor was made resistant to 6-mercaptopurine by workers at the Sloan-Kettering Institute of Cancer Research. 9-Methoxyellipticine is one of the few compounds tested in these laboratories which has shown any activity against this neoplasm, both intraperitoneally and orally.

Activity Against the Walker Rat Tumor 256—This test was conducted in young Sprague-Dawley rats, the tumor being maintained in both the solid and ascites form. Significant activity was demonstrated against both forms, indefinite survivors being obtained in the case of the ascites form, 4 of 10 rats having survived for 29 days since the time of implantation. At the time of sacrifice no ascites fluid could be detected in any of the animals.

Lack of Activity Against 7,12-Dimethylbenz(a)-anthracene-induced Tumors—Huggins *et al.* (19) have shown that a single oral dose of this carcinogen, dissolved in sesame oil, administered to virgin

TABLE III—DOSE RESPONSE, ORAL THERAPY OF 9-METHOXYELLIPTICINE

Tumor	Dosage mg./kg./day	Av. Weight Change, g., T/C	Av. Tumor Size, mm., T/C	Av. Life, T/C	Activity,[a] %
L-1210 leukemia III	18 × 1 × 10	+0.7/+2.0	—	14.1/10.4	36
	24 × 1 × 10	−0.5/+2.0	—	13.9/10.4	34
	36 × 1 × 10	−2.4/+2.0	—	13.1/10.4	26
P-1534 leukemia	18 × 1 × 10	−0.5/+1.1	—	17.2/15.0	0
	24 × 1 × 10	−0.8/+1.1	—	15.9/15.0	0
	36 × 1 × 10	+0.8/+1.1	—	19.8/15.0	32
X-5563 myeloma	6 × 1 × 10	−1.6/−2.0	5.9/10.7	—	45 (8)
	12 × 1 × 10	−5.0/−2.0	6.8/10.7	—	37 (10)
	18 × 1 × 10	−5.2/−2.0	6.9/10.7	—	36 (10)
Gardner lymphosarcoma	18 × 1 × 10	+1.3/+6.3	25.6/31.8	—	0
	24 × 1 × 10	−3.7/+6.3	18.0/31.8	—	43 (7)
	36 × 1 × 10	−2.9/+6.3	15.7/31.8	—	51 (6)
C3H mammary	18 × 1 × 10	−1.8/+6.3	20.1/29.9	—	33 (6)
	24 × 1 × 10	−6.4/+6.3	6.5/29.9	—	78 (1)
	36 × 1 × 10	−/+6.3	−/29.9	—	N.S.[b]
High malignancy clone	18 × 1 × 10	−3.5/−3.6	2.6/7.2	—	64 (9)
	24 × 1 × 10	−4.8/−3.6	4.2/7.2		42 (8)
	36 × 1 × 10	−5.8/−3.6	0.9/7.2	—	88 (10)

[a] The number in parentheses indicates survivors on solid tumors, indefinite survivors at 45 days on leukemia or ascitic tests. [b] No survivors at end of treatment period.

Sprague-Dawley rats 50 days old induced breast tumors within 21–120 days of feeding. When testing against these tumors, 9-methoxyellipticine was found to be inactive at 30 mg./kg. i.p. and 60 mg./kg. *per os*. Furthermore, some weight loss and toxicity were evidenced in both regimens.

The biological data are summarized in Tables II and III.

SUMMARY

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Keyphrases

Alkaloids—*Ochrosia maculata*
9-Methoxyellipticine—isolation, identity
Antitumor activity—9-methoxyellipticine
TLC—separation
X-ray crystallography—identity
UV spectrophotometry—identity
IR spectrophotometry—identity

Synthesis and Preliminary Evaluation of a New Quinuclidine Derivative as a Radioprotective Agent

By WARREN SHAPIRO, MARTIN F. TANSY, and SAMUEL ELKIN

7,10-Ethano-1-thia-4,7-diazaspiro [4.5] decane dihydrochloride was prepared by the condensation of 3-quinuclidone hydrochloride with 2-aminoethanethiol hydrochloride. The compound was shown to have potential radioprotective properties against a lethal dose of X-radiation in mice. Optimum protection was obtained in a group of mice pretreated intraperitoneally with 0.20 mg./g. of 7,10-ethano-1-thia-4,7-diazaspiro [4.5] decane dihydrochloride 15 min. prior to exposure, where 58.3 percent survived. A toxicity study showed the i.p. LD_{50} of the compound to be 295 mg./kg. with a dose range of 257–339 mg./kg. ($p < 0.05$).

INTEREST IN quinuclidine, 1-azabicyclo[2.2.2]-octane, and its derivatives has been fairly recent. Although this molecule was first synthesized in 1909 (1), it received little attention until World War II. At that time, because of the shortage of quinine and the need for substitutes, quinuclidine became an important tool in the chemistry of synthetic antimalarials.

The greatest amount of synthetic work has been done on 3-substituted quinuclidines. Pharmacologic activity reported to date has included spasmolytic (2, 3), central nervous system stimulation (4), and ganglionic blockade (5).

Received May 24, 1968, from School of Pharmacy, Temple University, Philadelphia, PA 19140
Accepted for publication July 19, 1968.
Abstracted in part from a thesis presented by Warren B. Shapiro to the Graduate School, Temple University School of Pharmacy, in partial fulfillment of Master of Science degree requirements.
This investigation was partially supported by grant MH-07702 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
The authors acknowledge the assistance of Dr. David L. Mann, Temple University School of Pharmacy, in determining the LD_{50} of the described compound and to Dr. Alan D. Conger, Temple University School of Medicine, for his guidance in the radiation studies.

Sprague-Dawley rats 50 days old induced breast tumors within 21–120 days of feeding. When testing against these tumors, 9-methoxyellipticine was found to be inactive at 30 mg./kg. i.p. and 60 mg./kg. *per os*. Furthermore, some weight loss and toxicity were evidenced in both regimens.

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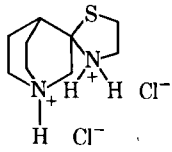
In 1951, Bacq and his co-workers, in Belgium, reported on the efficacy of a decarboxylation product of cysteine as a protective substance against X-radiation (6). The compound variously known as β-mercaptoethylamine, 2-aminoethanethiol, and 2-aminoethylmercaptan, is now commonly referred to as cysteamine. It is considerably more effective than cysteine on a weight basis, but it is also more toxic.

It appears there is a high degree of structural specificity with regard to the radioprotective activity of the compounds of the cysteine-cysteamine group. The sulfur atoms must be present as free sulfhydryl groups, disulfide groups, or derivatives which can undergo rapid intramolecular rearrangement under physiological conditions to give cysteamine derivatives possessing a free SH group (7).

Kaluszyner *et al.* (8) suggest that thiazolidines decompose *in vivo* to mercaptoamines and exert protection in this manner. It was therefore decided to synthesize a spiro thiazolidine derivative of quinuclidine as a potential precursor of free sulfhydryl groups. The following compound was prepared:



Such a compound could be an active radioprotectant without the toxicity of some of the aforementioned agents.

EXPERIMENTAL

All melting points are uncorrected. Analysis was performed by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

3-Quinuclidone Hydrochloride—Prepared by the method of Grob and Zergenyi (10).

Preparation of 7, 10-Ethano-1-thia-4,7-diazaspiro [4.5]decane Dihydrochloride–Into a 300-ml. round-bottom flask equipped with a stirrer, condenser, drying tube, and heating mantle was added 1.36 g. (0.02 mole) of 2-aminoethanethiol hydrochloride and 150 ml. of dry benzene. This mixture was heated until solution was complete and 1.88 g. (0.02 mole) of 3-quinuclidone hydrochloride was added. The mixture was refluxed with stirring for 20 hr. during which time a white precipitate formed. The mixture was filtered and filtrate discarded. The residue was recrystallized three times from dry methanol and anhydrous ether to yield a white crystalline product. Yield, 4.0 g. (77.8%); m.p. 250–255°.

Anal.—Calcd. for $C_9H_{18}Cl_2N_2S$: C, 42.02; H, 7.05; Cl, 27.57; N, 10.89; S, 12.47. Found: C, 42.00; H, 7.23; Cl, 27.51; N, 10.61; S, 12.42.

PHARMACOLOGY

Toxicity—The i.p. LD_{50} of 7,10-ethano-1-thia-4,7-diazaspiro [4.5] decane dihydrochloride was determined by the method of Litchfield and Wilcoxon (11). Six groups of 10 mice were used. The i.p. LD_{50} and dosage range in mg./kg. were determined by a rapid graphic method (see Table I and Fig. 1).

Radiation and Protection—Approximately 400 CBF male mice, 8–10 weeks of age at radiation time, arranged into groups of 12, weighing 17–27 g., were used for these experiments. The hybrid strain was obtained from the Cumberland View Farms Colony, Clinton, Tenn. They were kept in the laboratory for an acclimatization period of 7 days before use. The estimated X-ray LD_{100} for these animals is 825 r. For X-ray exposure, 12 mice were placed in perforated 50-ml. plastic tubes, radially positioned around a circular leucite turntable and rotated at 24 r.p.m. directly under the X-ray tube. Whole-body X-radiation was administered with a General Electric Maxitron 300 X-ray unit at 300 kVp and 20 ma. with 0.625 mm. Cu and 3.30 mm. Al added filtration and at a rate of 353 r/min. as measured in air with a Victoreen dosimeter. The target-to-animal distance was 50 cm. and the dose rate given is the value near the center of the mouse. The room temperature was maintained close to 25° throughout these experiments. After a single exposure to X-radiation, the mice were housed in suspended galvanized metal cages, two/cage, and they were observed over a

TABLE I—SOLUTION OF THE DOSE-EFFECT CURVE OF 7,10-ETHANO-1-THIA-4,7-DIAZASPIRO-[4.5]DECANE DIHYDROCHLORIDE

Dose, mg./kg.	Dead/Tested[a]	Observed % Dead[b]	Expected % Dead	Observed Minus Expected	Contribution to χ^2[c]
450	8/10	80	84	4	0.012
400	4/10	40	77	37	0.75
350	6/10	60	67	7	0.023
300	5/10	50	52	2	0.0016
250	4/10	40	31	9	0.035
200	0/10	0 (7.2%)	7.5	0.3	0.000

[a] Total animals = 60; No. of doses, $K = 6$; animals/dose = 60/6 = 10. [b] LD_{50} from graph (Fig. 1): LD_{84} = 450 mg./kg., LD_{50} = 295 mg./kg., LD_{16} = 220 mg./kg. Slope = $S = [(LD_{84}/LD_{50}) + (LD_{50}/LD_{16})]/2 = [(450/295) + (295/220)]/2 = 1.43$. $f LD_{50} = S^{2.77}/\sqrt{N'}$, $= S^{2.77}/\sqrt{50}$, $= 1.15$. N' = number of animals tested whose expected effects are between 16 and 84. $N' = 50$. LD_{50} range: $LD_{50} \times f LD_{50} = (295) \times (1.15) = 339$ mg./kg.; $LD_{50} \div f LD_{50} = (295)/(1.15) = 257$ mg./kg.; LD_{50} and 19/20 confidence limits: 295(257–339) mg./kg. [c] Total: 0.822. $\chi^2 = 0.822 \times 10 = 8.22$. df, $n = K - 2 = 4$. $(\chi)^2$ from Table II (12) for n of 4 = 9.49; 8.22 is less than 9.49, therefore the data are not significantly heterogeneous.

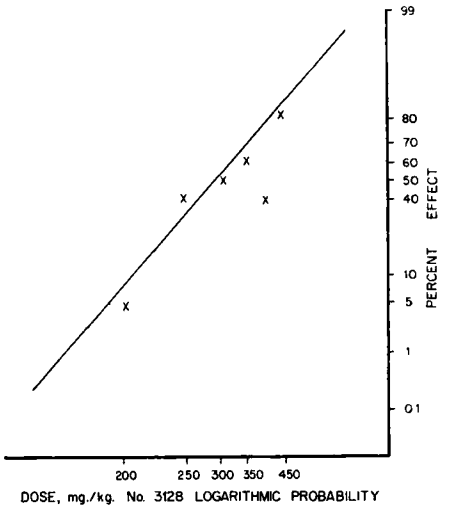


Fig. 1—Dose effect line of 7,10-ethano-1-thia-4,7-diazaspiro[4.5]decane dihydrochloride.

period of 1 month. The pellet form of Purina laboratory chow and tap water was available *ad libitum*.

In the first series of experiments each of a group of 12 mice was injected i.p. with 0.02 mg./g. of 7,10-ethano-1-thia-4,7-diazaspiro [4.5]decane dihydrochloride (dissolved in isotonic saline; final at pH 3.5) while a second group received 0.2 mg./g. of the test compound.

As already indicated these doses of the test compound were below the threshold of toxic, *i.e.*, lethal, dose for mice, administered by this injection route. A third group was injected intraperitoneally with an equivalent volume (0.2 ml.) of saline only. A fourth group was injected *via* the same route with cysteamine hydrochloride (0.24 mg./g.). The latter served as reference. A fifth group of control mice was kept at all times to determine what percentage, if any, of the radiation mortality could be attributed to the well-known "cage effect." Twenty-four hours later, the first four groups were exposed together to an X-ray dose of 825 r. This experimental series was then repeated with four additional groups of mice of comparable number injected intraperitoneally with the same test compound in identical concentrations, the same reference, and physiological saline just 15 min. before they were exposed to the 825 r of X-rays. In the third series of experiments the protective action of a single postradiation intraperitoneal injection of the test compound against radiation lethality was determined. The next three series of experiments were patterned after the first, except that these mice received the test substance, reference, and vehicle by oral intubation.

RESULTS AND DISCUSSION

7,10-Ethano-1-thia-4,7-diazaspiro[4.5]decane dihydrochloride was prepared by the condensation of 3-quinuclidone hydrochloride with 2-aminoethanethiol[1] in dry benzene.

The IR spectra of carbonyl groups of saturated aliphatic six-membered ring ketones usually show an absorption band between 1725 cm.$^{-1}$ and 1705 cm.$^{-1}$ (9). The spectrum of 3-quinuclidone hydrochloride shows this characteristic carbonyl absorbance at 1725 cm.$^{-1}$. The absence of this band in the spectrum of 7,10-ethano-1-thia-4,7-diazaspiro[4.5]decane dihydrochloride is indicative of structural change. The structure was confirmed by elemental analysis.

The toxicity study showed the i.p. LD_{50} of 7,10-ethano-1-thia-4,7-diazaspiro[4.5]decane dihydrochloride to be 295 mg./kg. with a dose range of 257–339 mg./kg. ($p < 0.05$) in mice.

In the first series of radiation experiments optimum protection was obtained in the group of mice pretreated with 0.20 mg./g. of 7,10-ethano-1-thia-4,7-diazaspiro[4.5]decane dihydrochloride administered intraperitoneally 15 min. before the beginning of exposure to 825 r where 58.3% survived (see Table II and Fig. 2). However, in the group of mice pretreated with 0.02 mg./g. of the test compound 15 min. prior to exposure, there was no significant increase in survival. At the doses of 0.20 mg./g. and 0.02 mg./g., 7,10-ethano-1-thia-4,7-diazaspiro-[4.5]decane dihydrochloride was not effective in increasing survival when it was administered intraperitoneally 24 hr. before or immediately after exposure of mice to 825 r. All of the saline-treated control mice died within 18 days of exposure to 825 r. The entire group of untreated control mice survived 30 days indicating that none of the deaths in the exposed groups of mice were due to the "cage effect."

At doses of 0.20 mg./g. and 0.02/mg./g., 7,10-ethano-1-thia-4,7-diazaspiro[4.5]decane dihydrochloride was not effective in increasing survival when it was administered by oral intubation 15 min. before, 24 hr. before, or immediately following expo-

TABLE II—COMPARATIVE EFFECTIVENESS OF ORAL AND PARENTERAL DOSES OF 7,10-ETHANO-1-THIA-4,7-DIAZASPIRO[4.5]DECANE DIHYDROCHLORIDE WITH MEA AGAINST A LETHAL DOSE OF X-RADIATION (12 MICE/GROUP)

	i.p.		Intubation	
	Days Surviving	No. Mice	Days Surviving	No. Mice
Saline, radiated	15	1	17	0
	18	0		
Saline, not radiated	30	12	30	12
MEA	30	11	30	6
0.2 mg./g., postradiation	30	1	22	0
0.02 mg./g., postradiation	15	0	19	0
0.2 mg./g., 15 min. before radiation	30	7	21	0
0.02 mg./g., 15 min. before radiation	13	0	20	0
0.2 mg./g., 24 hr. before radiation	15	0	17	0
0.02 mg./g., 24 hr. before radiation	15	0	21	0

[1] Obtained commercially from the Aldrich Chemical Co. Inc., Milwaukee, Wisc.

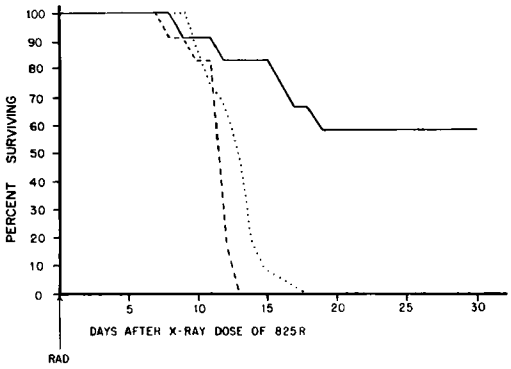


Fig. 2—Radioprotection by 7,10-ethano-1-thia-4,7-diazaspiro [4.5] decane dihydrochloride injected i.p. in mice 15 min. before X-radiation. Key: —, 0.2 mg./g. drug; · · ·, control; - - -, 0.02 mg./g. drug.

sure of mice to 825 r. Since the absorption and fate of the test compound has not yet been established, it is not known whether the failure to protect was due to the route of administration or the lack of absorption of the compound in the form of the dihydrochloride salt.

The mechanism(s) by which 7,10-ethano-1-thia-4,7-diazaspiro [4.5] decane dihydrochloride enhances the radiation resistance of rodents is not clear. However, the fact that the agent provides protection when it is administered intraperitoneally 15 min. before radiation exposure, places it in the same category as the classical chemical radioprotectors such as cysteine, cysteamine, or AET which are only effective when administered immediately before radiation.

The synthesis and radioprotective action of several analogs of 7,10-ethano-1-thia-4,7-diazaspiro[4.5]-decane dihydrochloride will be reported in a later publication.

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7,10-Ethano-1-thia-4,7-diazaspiro[4.5]decane dihydrochloride—synthesis
IR spectrophotometry—structure
Antiradiation activity—quinuclidine derivative

Preliminary Pharmacology of Ellagic Acid from *Juglans nigra* (Black Walnut)

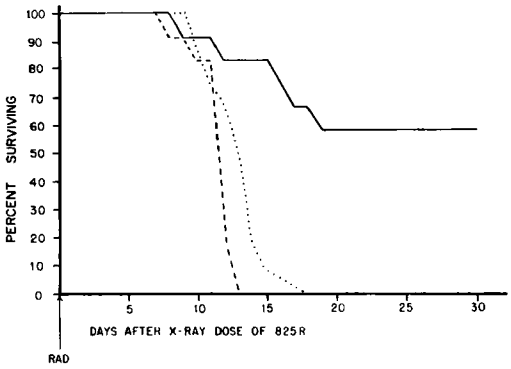
By U. C. BHARGAVA, B. A. WESTFALL, and D. J. SIEHR*

A crystalline compound was isolated from *Juglans nigra* which by comparison with the known compound was found to be ellagic acid. The ellagic acid injected (i.p.) produced significant sedation, ataxia, potentiated sodium pentobarbital sleeping time, and protected mice from death after electroconvulsive shock. Intravenous injection of ellagic acid caused a fall in blood pressure and an elevation of the T wave, whereas the heart and respiration rate initially increased followed by a decrease. The ellagic acid produced no significant effect on isolated duodenal and uterus segments of the rat.

DURING THE isolation of sedative principles from *Juglans nigra*, a crystalline compound was obtained and characterized as ellagic acid by comparing it with the known compound. Ellagic acid is a polyphenolic constituent of many plants such as the pericarp tissue of some castor varieties, cashew nut shell, and some walnut species. *Juglans nigra* is a naturally grown plant in eastern and central North America.

Westfall *et al.* (1) observed that the ether ex-

Received February 26, 1968, from the Department of Pharmacology, School of Medicine, University of Missouri, Columbia, MO 65201, and the *Department of Chemistry, School of Science, University of Missouri at Rolla, Rolla, MO 65401
Accepted for publication July 12, 1968.
This work was supported by grant NIH NY-4295 from the National Institutes of Health, Bethesda, Md.
The authors thank Mr. Paul Langley for spectrophotometric assistance.



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Keyphrases

Radioprotective agent—quinuclidine derivative
7,10-Ethano-1-thia-4,7-diazaspiro[4.5]decane dihydrochloride—synthesis
IR spectrophotometry—structure
Antiradiation activity—quinuclidine derivative

Preliminary Pharmacology of Ellagic Acid from *Juglans nigra* (Black Walnut)

By U. C. BHARGAVA, B. A. WESTFALL, and D. J. SIEHR*

A crystalline compound was isolated from *Juglans nigra* which by comparison with the known compound was found to be ellagic acid. The ellagic acid injected (i.p.) produced significant sedation, ataxia, potentiated sodium pentobarbital sleeping time, and protected mice from death after electroconvulsive shock. Intravenous injection of ellagic acid caused a fall in blood pressure and an elevation of the T wave, whereas the heart and respiration rate initially increased followed by a decrease. The ellagic acid produced no significant effect on isolated duodenal and uterus segments of the rat.

DURING THE isolation of sedative principles from *Juglans nigra*, a crystalline compound was obtained and characterized as ellagic acid by comparing it with the known compound. Ellagic acid is a polyphenolic constituent of many plants such as the pericarp tissue of some castor varieties, cashew nut shell, and some walnut species. *Juglans nigra* is a naturally grown plant in eastern and central North America.

Westfall *et al.* (1) observed that the ether ex-

Received February 26, 1968, from the Department of Pharmacology, School of Medicine, University of Missouri, Columbia, MO 65201, and the *Department of Chemistry, School of Science, University of Missouri at Rolla, Rolla, MO 65401
Accepted for publication July 12, 1968.
This work was supported by grant NIH NY-4295 from the National Institutes of Health, Bethesda, Md.
The authors thank Mr. Paul Langley for spectrophotometric assistance.

tract from hulls of *Juglans nigra* has a depressant property in different species of animals. Perhaps the use of walnuts as a vermifuge in the past also might be related to the immobilizing effect of the extracts on worms (2). Fiedler and Hildebrand (3) studied the pharmacology of ellagic acid to some extent and showed that infusion of ellagic acid caused a fall in blood pressure and an increase in respiration in the guinea pig. Similar results were found in the dog by Botti and Ratnoff (4). Blumenberg *et al.* (5) fed rats with ellagic acid (50 mg./kg., orally) up to a maximum of 45 days. Histological examination of liver and other tests showed no evidence of toxicity in animals.

In this present study, some additional pharmacological properties of ellagic acid were established.

EXPERIMENTAL

Isolation and Properties of Ellagic Acid—Two kilograms of hulls of *Juglans nigra* were extracted (6) with diethyl ether–butanol mixture (4:1). The extract was evaporated under vacuum and triturated with 5% acetic acid. The acetic acid solution was extracted several times with diethyl ether–butanol mixture. The organic solvent phase was collected and washed several times with $NaHCO_3$ solution at pH 7.5 and the alkaline solution was acidified and extracted with diethyl ether–butanol mixture. During the evaporation of the organic solvent phase under vacuum, a small quantity of dark brown powder was obtained. It was washed with cold ethanol, dried, and was crystallized from a large volume of acetone as pale yellow crystals (10 mg. crystals per kg. of hulls). In succeeding experiments, it was found that the yield of the yellow crystalline material could be increased (35 mg. crystals per kg. of hulls) with reproducible results, if the residue was triturated with 40% glacial acetic acid and the solvent, after filtration, evaporated in a flash evaporator. Most of the crude crystals separated during the evaporation. Additional crystalline material was recovered on cooling. The crude material was recrystallized from pyridine.

The high melting point[1] (dec. >360°), the blue coloration with ferric chloride, a positive Greissmeyer reaction (7), its behavior on a paper chromatogram (8), and the IR and UV absorption spectra of the crystalline compound isolated from black walnut hulls were properties identical to those of an authentic sample[2] of ellagic acid. IR and UV absorption spectra were compared using a Beckman IR 7, and a Cary model 14 spectrophotometer, respectively.

Anal.[3]—Calcd. for $C_{14}H_6O_8 \cdot 2H_2O$: C, 49.7; H, 2.96. Found: C, 48.61; H, 3.21.

This material was almost insoluble in the usual organic solvents and water, but dissolved in hot pyridine and produced a yellow solution in aqueous NaOH. For injection walnut ellagic acid was dissolved in 0.1*N* NaOH solution and the pH adjusted to 10 for most of the preparations. A high pH was essential to keep ellagic acid in solution.

Animals—Swiss Webster male mice (30–40 g.) and Wistar male (220–280 g.) and nonpregnant female rats (170–220 g.) were used. Mice were housed in groups of five and rats in four. On their arrival from the farm, they were fed Purina chow and water *ad libitum*. At least a week was allowed after the shipment before the animals were used. The rats were anesthetized with sodium pentobarbital (50 mg./kg. i.p.) for blood pressure measurement, and were killed by concussion for isolated tissue preparations.

Spontaneous Activity—A "jiggle" type activity cage was used for measuring activity. The vibrations originated by the movements of the mouse in the cage were picked up by a phonograph-type crystal pick-up cartridge and converted into electrical impulses which were recorded automatically by a counter. Ninety minutes after ellagic acid administration (1–20 mg./kg. i.p.) a mouse was placed in each activity cage and after 30 sec. activity counts were measured for 5 min.

Ataxia—For measuring muscular incoordination, a rotating rod of diameter 3.5 cm., with a speed of 5 r.p.m. and located 10 in. above the table was used (9). Immediately after measuring the spontaneous activity, the mouse was transferred to the rotating rod and the time required for falling off (before 5 min.) for each mouse was recorded. Two opportunities were given to each mouse on the rotating rod, and if the given mouse remained for more than 5 min. in either of the two attempts, "yes" was recorded, otherwise "no." From these observations the proportion of mice remaining on the rotating rod was calculated.

Sodium Pentobarbital Sleeping Time—Ninety milligrams of ellagic acid/kg. of body weight was injected (i.p.) 90 min. prior to sodium pentobarbital administration. Then a dose of 50 mg./kg. of sodium pentobarbital was injected (i.p.) for narcosis. After the mice lost the righting reflex, they were left on their backs and their tails were pinched 1 cm. distal to the anus every 5 min. Pressure of pinching the tails was kept approximately the same every time. The sodium pentobarbital sleeping time was the time from loss of the righting reflex to return of the righting reflex.

Anticonvulsive Activity—At the peak response period which was 90 min. after the ellagic acid administration (180 mg./kg. i.p.), electroconvulsive shock of 24 ma. for 0.2 sec. (a.c.) was applied through ear clips (with electrode paste) to each mouse (10). The abolition of seizure (tonic extensor) was indicative of anticonvulsant effect of drug. Usually after such electroshock, the untreated mouse dies. Therefore, the number of animals protected by ellagic acid from this type of death was calculated.

Blood Pressure—Blood pressure in rats was measured through the femoral artery using a Statham pressure transducer. Respiration was recorded by inserting one end of tubing into the trachea and connecting the other end to a volumetric pressure transducer (PT-5-A). Lead II was used for recording the ECG. All responses were recorded

[1] Melting point was determined by the Galbraith Laboratories, Inc., Knoxville, Tenn., using Arthur Thomas apparatus.

[2] An authentic specimen of ellagic acid was purchased from Aldrich Chemical Co., Milwaukee, Wisc.

[3] Microanalysis of ellagic acid was also conducted by the Galbraith Laboratories.

by polygraph (Grass). Heart rate was calculated from the ECG. Five milligrams of ellagic acid/kg. was injected into the femoral vein and not more than 0.5 ml. solution was injected at one time.

Rat Duodenum—Two duodenal segments, approximately 3 cm. long, were taken about 1 cm. distal from the pyloric region of the stomach. These were suspended in Tyrode's solution and maintained at a temperature of 37°. The solution was aerated by means of a steady stream of O_2. One preparation was used as control and another as experimental. Recordings of duodenal activity of five animals were obtained by means of a force displacement transducer attached to polygraph (Grass). After obtaining normal responses, ellagic acid (0.01 mg./ml.) was added to one of the preparations and the other was considered as a control.

Rat Uterus—A uterine segment 3 cm. in length was removed from each uterine horn of 5 animals and they were treated in similar manner as the duodenal segments except 0.05 mg./ml. ellagic solution was added instead of 0.01 mg./ml.

RESULTS AND DISCUSSION

A crystalline product was isolated from the hulls of *Juglans nigra* whose elemental analysis, melting point, UV, and IR spectra were identical to those of synthetic ellagic acid as shown in Figs. 1 and 2. The crystalline material could not be separated from synthetic ellagic acid when the substances were cochromatographed on paper.

Injection of ellagic acid in amounts from 1 mg./kg. to 20 mg./kg. caused a progressive increase in sedation as shown in Fig. 3. When the animals were placed on the rotating rod, there was a significant decrease in the proportion of mice (which received ellagic acid) remaining on the rotating rod as illustrated in Fig. 4. Ellagic acid injected (90 mg./kg. i.p.) 90 min. prior to sodium pentobarbital

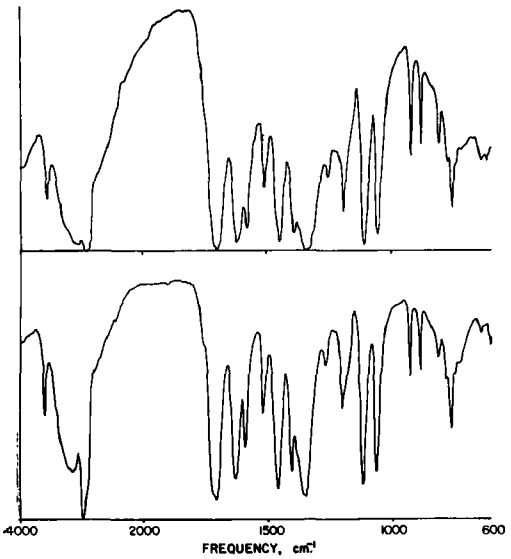

Fig. 1—IR spectra of natural and synthetic ellagic acid in mineral oil. Key: top, synthetic ellagic acid; bottom, ellagic acid from black walnut.

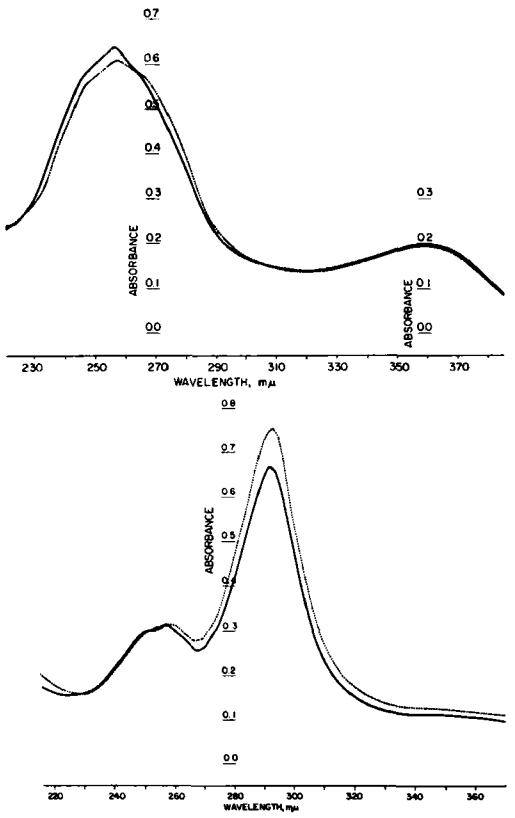

Fig. 2—UV absorption spectra of natural and synthetic ellagic acid in absolute alcohol and NaOH solution. Key: Top (absolute alcohol); bottom (in NaOH solution); . . ., synthetic ellagic acid; —, ellagic acid.

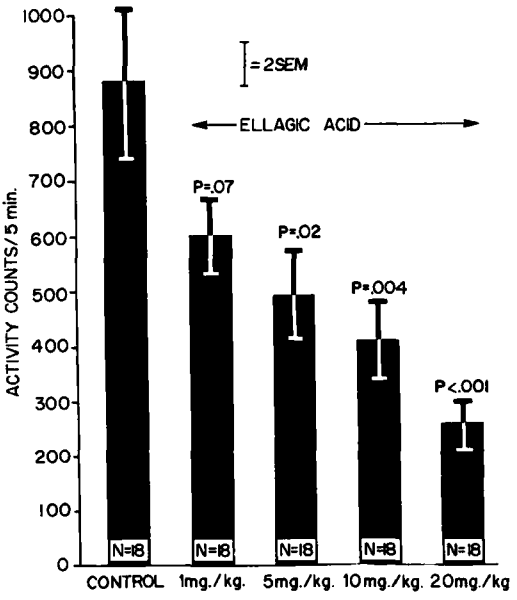

Fig. 3—Effect of ellagic acid on spontaneous activity in mice. Each bar was obtained from the mean ± S.E.M. of the 18 animals. Statistical significance (p value) was calculated by comparing the experimental group with the control.

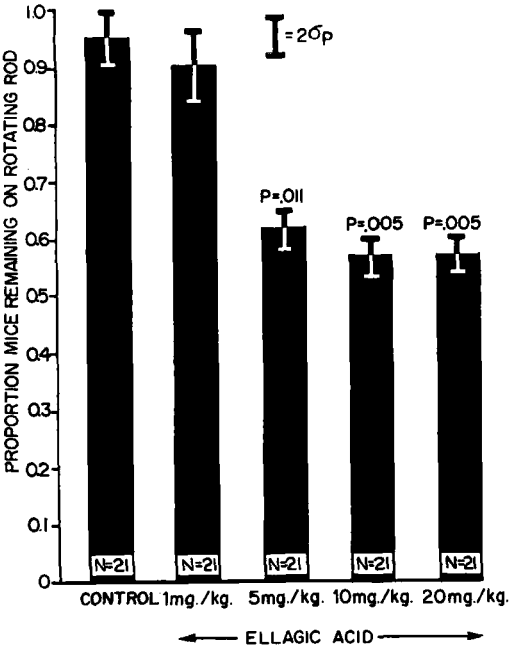

Fig. 4—Effect of ellagic acid on muscular incoordination in mice. Each bar was obtained from the proportion ± S.E.P. of the 21 animals which received "yes." Statistical significance (p *value) was calculated by comparing the experimental group with the control.*

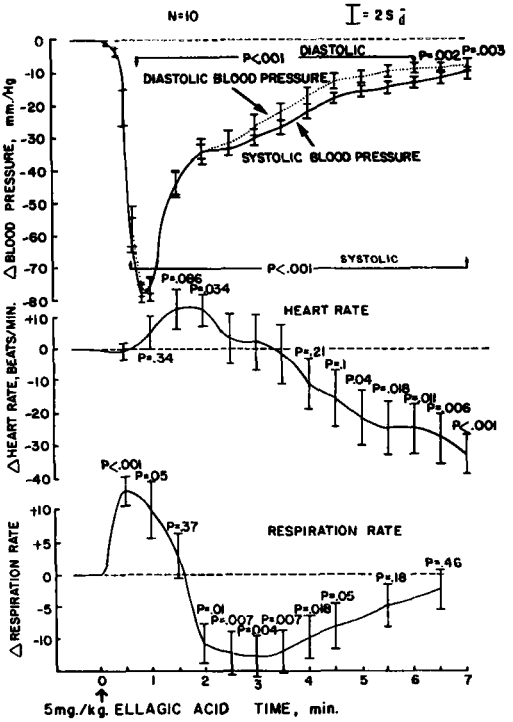

Fig. 5—Effect of ellagic acid on blood pressure, heart rate, and respiration rate in rats. Each point was obtained from the mean ± SE of the 10 animals. Statistical significance (p *value) was calculated by testing the differences.*

injection appeared to potentiate significantly ($p < 0.001$) the sleeping time (control: 34.5 ± 3.6 min. S.E.M.; experimental: 64.5 ± 4.2 min. S.E.M.), but without sodium pentobarbital, the mice receiving ellagic acid did not sleep at all. Ellagic acid (180 mg./kg. i.p.) did not show any anticonvulsive activity (χ^2 test). However, the number of mice remaining alive after electroconvulsive shock was increased. Seven out of 17 animals remained alive in the controls as compared to 14 out of 17 animals in the experimental group. These types of action seem to be more like tranquilizers than sedatives. Furthermore, as there are very few drugs which protect from electroshock, ellagic acid might be potentially useful in this area.

As illustrated in Fig. 5, intravenous injection of ellagic acid (5 mg./kg.) in rats caused a fall in blood pressure, whereas an equal volume of saline at pH 10 produced no change in pressure. In most of the animals there was a lag period of approximately 20 sec. before blood pressure began to fall and the maximum drop in blood pressure was approximately 1 min. after drug administration. Five minutes later blood pressure approached a steady level which was somewhat less than a control level. Heart rate first increased and then decreased and the decrease was more pronounced. Respiration rate first increased then decreased and after 5 min. no significant difference from the control was observed. During the blood pressure fall, the amplitude of respiration was also increased. In the ECG, the T wave was elevated approximately after 2 min. of ellagic acid administration. Similar results were obtained by other workers using guinea pig and dog (3, 4).

There was no apparent effect observed when ellagic acid in a concentration of 0.01 mg./ml. was added in the tissue bath of duodenal segments. There was a slight increase in amplitude and rate of contractions of rat uterine strips in some of the animal preparations used, but there was inconsistency in these results. Therefore, not much weight could be put on this experiment unless a large number of animals were studied and the animals divided according to estrus and nonestrus cycle, since the sexual cycle can play an important part in response to uterine tone and frequency of contractions (11).

Pharmacological tests of ellagic acid on a broader scale are currently in progress. In order to evaluate other active principles present in *Juglans nigra*, crude fractions (6) such as strong acids and weak acids were also injected into rats (i.v.). Like ellagic acid both strong acids and weak acids (20 mg./kg. i.v.) produced a fall in blood pressure, elevation of the T wave, and respiration rate initially increased, then decreased. However, after injection of strong acids the onset of the blood pressure fall was much slower and the maximum drop observed occurred between 3 and 3.5 min. after administration of strong acids, whereas after injection of weak acids the return of blood pressure toward normal was much slower.

SUMMARY

1. A crystalline compound isolated from black walnut hulls was found to be ellagic acid.

2. Intraperitoneal injection of ellagic acid in mice produced significant sedation, ataxia, potentiation of sodium pentobarbital sleeping time, and protected the animals from death after electroconvulsive shock.

3. Intravenous injection of ellagic acid decreased blood pressure and elevated the T wave. Whereas heart and respiration rate initially increased then decreased.

4. *In vitro* studies indicated that ellagic acid appeared to have no apparent effect on activity of the duodenal segment and uterus of the rat.

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Keyphrases

Ellagic acid—*Juglans nigra*
Pharmacologic screening—ellagic acid
IR spectrophotometry—identity
UV spectrophotometry—identity

Influence of Dimethyl Sulfoxide (DMSO) on the Percutaneous Absorption of Salicylic Acid and Sodium Salicylate from Ointments

By JOSEPH M. STELZER, JR.*, JOHN L. COLAIZZI, and PAUL J. WURDACK

Dimethyl sulfoxide (DMSO), 15 percent by weight, was incorporated into selected ointment bases containing 10 percent salicylic acid or 11.6 percent sodium salicylate. Percutaneous absorption was studied by determining salicylate blood levels in New Zealand white rabbits at regular intervals for 8 hr. following application of the ointment to the shaved intact skin and confinement by a specially designed bandage. DMSO in hydrophilic ointment and hydrophilic petrolatum produced more rapid drug absorption and higher salicylate blood levels than the control systems. Polyethylene glycol ointment and a polyoxyethylene (20) stearyl ether gel with DMSO did not produce any significant change in the absorption pattern. The salicylate blood levels obtained from percutaneous absorption of sodium salicylate in hydrophilic ointment containing DMSO were lower than with control systems. In the case of hydrophilic petrolatum, there were no significant differences in absorption patterns of sodium salicylate with or without DMSO. Sodium salicylate did not appear to be absorbed from polyethylene glycol ointment whether or not DMSO was included.

AMONG FACTORS which influence percutaneous absorption, Barr (1) included such factors as the condition of the skin, the thermodynamic properties of the medicament, the effects of moisture, the effects of surfactants, the effects of pH, and the effects of the vehicle. He indicated that organic solvents such as ether, chloroform, benzene, and acetone penetrate the skin with ease and enhance the percutaneous absorption of a drug.

The object of the following research was to determine whether dimethyl sulfoxide (DMSO), a relatively nontoxic organic solvent, would alter the percutaneous absorption patterns of salicylic acid and sodium salicylate when incorporated

Received April 25, 1968, from the Department of Pharmacy, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15213
Accepted for publication July 17, 1968.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
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into hydrophilic ointment USP, polyethylene glycol (PEG) ointment USP, hydrophilic petrolatum USP, and a polyoxyethylene (20) stearyl ether[1] (PSE) gel system.

Biologically, DMSO has demonstrated a broad spectrum of activities including the enhancement of penetration through plant and animal membranes (2). DMSO has been suggested for possible use as an analgesic agent, anti-inflammatory adjunct, bacteriostatic agent, diuretic, tranquilizer, potentiator of other compounds, and penetrant carrier (3). DMSO was reported to facilitate the transport of sodium salicylate, sodium sulfadiazine, aminophylline, Evans blue dye, and sodium heparin across the mucous membrane of a dog's bladder (4). By recording the presence or absence of a physiologic reaction in adult humans after topical application, Stoughton and Fritsch (5) presented evidence that DMSO caused an increase in cutaneous absorption in the order of 25-fold for hexopyrronium ion in 20% DMSO, 25-fold increase for naphazoline hydrochloride in 50% DMSO, and a fivefold increase for fluocinolone acetonide in 25% DMSO. Kligman (2) concluded from his experimentation that if DMSO is to be clinically useful, quite concentrated solutions, perhaps 80 to 90% would be required to accelerate the rate of topical medicaments such as corticosteroids through the skin.

The mechanism of increased membrane permeability effected by DMSO is essentially unknown. Jacob *et al.* (4) has pointed out that the carrier mechanism brought about by DMSO might be related to the facts that it is a weak base and that it forms complexes with various substances. The attraction of DMSO for water should also be considered as a possible factor involved in altered membrane permeability in the presence of DMSO.

Dimethyl sulfoxide is relatively nontoxic. Block (6) indicates that it is nonirritating and nontoxic to skin of rabbits. Jacob and Rosenbaum (7) have summarized some of the known toxicologic information on DMSO. It has been reported that a topical application of 90 to 100% DMSO with occlusive covering to human skin resulted in a papulovesicular reaction after 1 hr. of occlusion which disappeared in a few hours. The vesiculating ceases abruptly when the concentration is reduced to 80% or lower (2). In later toxicity studies, Rubin and Barnett (8) noted unusual lenticular changes in animals tested. Changes in the lens were characterized by division of the lens into two zones, a central area simulating a lens nucleus and a peripheral area. These changes were noted after 90 days of dermal application of 4 ml. 100% DMSO/kg. of body weight/day in rabbits and swine. No such changes have been noted in humans (9, 10). Kleberger (11) applied DMSO to the skin of five human subjects in daily doses ranging from 30 to 800 ml. No change in lens structure was noted. He suggested that the refractive changes seen in animals might be due to the extremely large doses of DMSO received.

[1] Marketed as Brij 78, Atlas Chemical Industries, Inc., Wilmington, Del.

EXPERIMENTAL

Preparation of Ointments—Salicylic acid[2] and sodium salicylate,[2] previously reduced to fine powders in a ball mill, were each passed through a No. 80 mesh sieve and dried at 50° in a heated vacuum desiccator[3] for at least 48 hr. before use. The ointments prepared contained 10% (w/w) salicylic acid or 11.6%[4] (w/w) sodium salicylate and 15% (w/w) of medicinal grade dimethyl sulfoxide.[5] Each ingredient was accurately weighed and incorporated into the various ointment bases. Bases used were hydrophilic ointment USP XVII, hydrophilic petrolatum USP XVII, polyethylene glycol ointment USP XVII, and a polyoxyethylene (20) stearyl ether gel system.[6]

Test Animals—The test ointment containing DMSO was compared to a control ointment that was similar in all respects to the test ointment except that it did not contain DMSO. Each set of ointments was applied to two pairs of New Zealand white rabbits weighing between 2.8 and 3.6 kg. The rabbits were randomly selected without regard to sex. Each rabbit was used only four times and received the same ointment base in each test. Only one pair of rabbits was utilized during any one experimental day due to space and time limitations. The two ointments compared on that day differed with respect to the presence of DMSO in the one, and its absence in the other. Within each pair, the rabbits were alternated weekly. The rabbit receiving the test ointment for the first test run received the control ointment for the second run and *vice versa*. For two of the four experimental runs each rabbit received the test ointment with DMSO, while for the other two runs, the same rabbit received the control ointment without DMSO. A 7-day rest period ensued before reapplication of ointments. The animals were maintained on Purina rabbit chow and water *ad libitum* and housed individually in an animal room maintained at a temperature of approximately 20° and at a relative humidity of approximately 50%. Twenty-four hours prior to the application of the ointment to the

[2] Analytical reagent grade, Mallinckrodt Chemical Works, St. Louis, Mo.

[3] Model No. 68351, Precision Scientific Co., Chicago, Ill.

[4] 11.6% sodium salicylate is equivalent to 10% salicylic acid.

[5] Generously supplied by Crown Zellerbach Corp., Camas, Wash.

[6] The gel was prepared by adding 1 part water to 3 parts PSE. The water and PSE were separately heated to 70° on a steam bath. The two were then removed from the heat and added together with stirring until gel was formed.

TABLE I—BLOOD SALICYLATE CONCENTRATIONS OBTAINED FROM OINTMENTS CONTAINING SALICYLIC ACID[a]

Time, hr.	Hydrophilic Ointment, mg. % Test[b]	Hydrophilic Ointment, mg. % Control[c]	Hydrophilic Petrolatum, mg. % Test[b]	Hydrophilic Petrolatum, mg. % Control[c]
0.5	2.68 ± 0.83	0.75 ± 0.22	3.53 ± 0.51	0.55 ± 0.34
1	6.33 ± 1.89	2.38 ± 0.83	6.80 ± 0.53	1.01 ± 0.39
2	10.44 ± 2.42	3.91 ± 0.65	8.71 ± 0.49	1.63 ± 0.32
3	10.41 ± 1.61	5.49 ± 0.86	9.68 ± 0.80	2.50 ± 0.29
4	9.21 ± 1.54	5.45 ± 1.09	8.72 ± 0.31	3.32 ± 0.64
5	7.53 ± 2.27	5.81 ± 1.39	7.52 ± 0.96	3.38 ± 0.39
6	6.24 ± 2.21	4.23 ± 1.03	6.20 ± 0.97	3.56 ± 0.66
7	5.31 ± 2.07	4.06 ± 1.07	5.01 ± 0.84	3.73 ± 0.76
8	3.80 ± 1.46	2.80 ± 0.88	5.00 ± 0.74	3.82 ± 1.03
	PEG Ointment, mg. % Test[b]	PEG Ointment, mg. % Control[c]	PSE Gel, mg. % Test[b]	PSE Gel, mg. % Control[c]
0.5	0.34 ± 0.17	0.03 ± 0.03	1.18 ± 0.73	0.76 ± 0.36
1	0.09 ± 0.03	0.13 ± 0.05	1.89 ± 1.35	0.66 ± 0.29
2	0.31 ± 0.03	0.12 ± 0.05	2.54 ± 1.00	1.27 ± 0.57
3	0.47 ± 0.16	0.50 ± 0.14	2.51 ± 0.87	2.50 ± 1.19
4	0.83 ± 0.54	0.30 ± 0.07	2.66 ± 1.10	1.84 ± 0.87
5	0.69 ± 0.30	0.38 ± 0.22	2.11 ± 0.78	1.45 ± 0.69
6	1.14 ± 0.58	0.52 ± 0.10	2.38 ± 0.55	1.91 ± 0.69
7	1.08 ± 0.49	0.19 ± 0.07	1.36 ± 0.30	1.41 ± 0.43
8	0.92 ± 0.49	0.94 ± 0.36	1.50 ± 0.49	2.01 ± 0.92

[a] Average of eight determinations with standard error of the mean. [b] Contains 10% salicylic acid and 15% DMSO in ointment base. [c] Contains 10% salicylic acid in ointment base.

rabbit, hair was removed with an animal clipper[7] from the skin of the ears and the dorsal area between the forelegs and hind legs on both sides of the spine.

Application of Ointment—A specially prepared bandage restricted and controlled the area of contact between the ointment and the skin of the rabbit. The edges of an 8.35 × 14.7 cm. piece of aluminum foil were doubled over and flattened 1 cm. on each side to produce a rectangular plate measuring 6.35 × 12.7 cm. with a 1-cm. reinforced margin. An accurately weighed 5.00-g. sample of the selected ointment was uniformly spread over one surface of the plate whose opposite side was centered on an 8.9 × 20 cm. strip of adhesive tape. The entire assembly was then applied to the shaved dorsal skin of the rabbit and adjusted to conform to the contours of the area. To insure adequate contact between the ointment and the skin and to minimize contamination, the assembly was covered with two elastic bandages.[8] The bandage arrangement employed was adapted from Stolar *et al.* (12). The ointment remained in contact with the skin for the 8-hr. experimental period, during which time the rabbit did not receive food or water.

Procedure—Blood samples were withdrawn and tested for blood salicylate concentration. One-half milliliter of blood was withdrawn from the marginal ear vein of the rabbit at the following time periods: prior to application of ointment, 0.5 hr. after ointment application, and at hourly intervals for 8 hr. after application of the ointment. The 0.5 ml. of blood was collected in a syringe[9] containing 0.1 ml. of heparin USP,[10] 5,000 units/ml. This blood and heparin mixture was added to 5.0 ml. of the combined protein precipitant-color reagent in a centrifuge tube and analyzed for salicylate content according to the method described by Trinder (13). After centrifugation and filtration the colorimetric analysis for salicylate was performed at a wavelength setting of 540 mμ using a Spectronic "20"[11] spectrophotometer. The absorbance reading obtained from the blood sample withdrawn prior to application was considered the zero reading and was subtracted from the other readings to account for any absorbance contributed by any constituents in the blood other than the salicylic acid. The salicylate content of the sample was obtained from a standard curve previously prepared by treating 0.1 ml. of heparin and 0.5-ml. quantities of sodium salicylate solutions containing the equivalent of 10, 20, 30, 40, and 50 mg. % of salicylic acid, with 5.0 ml. of color reagent.

Statistical inferences drawn concerning the results obtained in this study were based upon a randomized blocks design analysis of variance at a 95% level of significance, using two treatments and four blocks (14).

RESULTS

DMSO, 15% by weight, was incorporated into the four selected ointment bases (Table I) containing 10% (w/w) salicylic acid.

A stiff, white ointment resulted when hydrophilic ointment was combined with 10% salicylic acid. The addition of 15% DMSO altered the consistency, producing a slightly softer ointment. Figure 1 illustrates the altered percutaneous absorption pattern of salicylic acid obtained upon the addition of DMSO to hydrophilic ointment. The statistical analysis of the results illustrated in Fig. 1 showed that the salicylate blood levels obtained with the ointments containing DMSO were significantly higher than with the controls for the determinations made 1, 2, 3, and 4 hr. following application of ointment. Statistical analysis of the data illustrated in Figs. 2 and 3 indicates that the addition of DMSO in a 15% (w/w) concentration produced no significant change in the absorption pattern of salicylic

[7] Model No. A-2 with size 40 head, John Oster Manufacturing Co., Milwaukee, Wisc.
[8] Readi-flex bandages generously supplied by Parke, Davis & Co., Detroit, Mich.
[9] Glaspak disposable tuberculin syringe, Becton, Dickinson, and Co., Rutherford, N. Y.
[10] Generously supplied by the Upjohn Co., Kalamazoo, Mich.
[11] Bausch and Lomb, Rochester, N. Y.

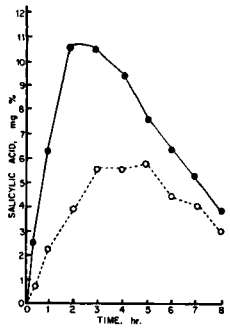

Fig. 1—Effect of DMSO on percutaneous absorption of 10% salicylic acid from hydrophilic ointment. Key: ●—, 15% DMSO added; ○- - -, control.

acid when added to PEG ointment or to PSE gel, both containing 10% salicylic acid. PEG ointment with 10% salicylic acid was a stiff, glossy white ointment that softened slightly when 15% DMSO was added. When 10% salicylic acid was combined with the PSE gel system, the gel liquified. After standing undisturbed for 15 min. the system again congealed. The addition of 15% DMSO caused the gel to liquify again and at room temperature it remained in this condition. This resultant liquid system, when refrigerated at 10–15°, congealed.

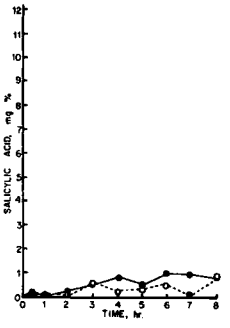

Fig. 2—Effect of DMSO on percutaneous absorption of 10% salicylic acid from polyethylene glycol ointment. Key: ●—, 15% DMSO added; ○- - -, control.

Figure 4 indicates that a more rapid drug absorption and higher salicylate blood levels were achieved when 15% DMSO was added to hydrophilic petrolatum containing 10% salicylic acid. The statistical analysis of the study involving the percutaneous absorption of salicylic acid from hydrophilic petrolatum showed that the blood levels obtained with the ointments containing DMSO were significantly higher than with the controls up to and including the determinations performed 5 hr. after initial ointment application. The absorption was approximately four times greater than that obtained when no DMSO was present. Hydrophilic petrolatum

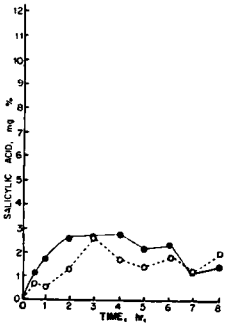

Fig. 3—Effect of DMSO on percutaneous absorption of 10% salicylic acid from a polyoxyethylene (20) stearyl ether gel. Key: ●—, 15% DMSO added; ○- - -, control.

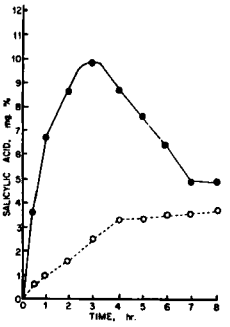

Fig. 4—Effect of DMSO on percutaneous absorption of 10% salicylic acid from hydrophilic petrolatum. Key: ●—, 15% DMSO added; ○- - -, control.

containing 10% salicylic acid formed a smooth, translucent ointment. Upon addition of 15% DMSO no change in the ointment consistency was noticed. After standing for 24 hr., droplets of DMSO appeared on the surface of the ointment. The droplets of liquid could be reincorporated into the ointment by additional mixing.

Hydrophilic ointment and hydrophilic petrolatum both containing 11.6% sodium salicylate and 15% DMSO, were compared to control ointments without DMSO, as indicated in Table II. The blood level patterns obtained in these studies with sodium salicylate are shown in Fig. 5 (hydrophilic ointment) and Fig. 6 (hydrophilic petrolatum). Statistical analysis showed that DMSO appeared to significantly decrease percutaneous absorption of sodium salicylate from hydrophilic ointment after about 5 hr. from the time of ointment application. The results of the study with hydrophilic petrolatum showed no statistically significant differences between the ointments containing DMSO and the controls. The preparations containing the hydrophilic ointment as the base were opaque white and had the same smooth consistency. The two sodium salicylate ointments prepared with hydrophilic petrolatum were smooth and white with no noticeable change in consistency resulting from the addition of 15% DMSO.

A stiff, glossy white ointment resulted when sodium salicylate in an 11.6% (w/w) concentration was added to polyethylene glycol ointment. The addition of 15% DMSO caused a slight softening of the ointment. Sodium salicylate did not appear to be significantly absorbed from polyethylene glycol ointment whether or not DMSO was present.

DISCUSSION

A statistical analysis of the results of this study indicated that DMSO functioned as a penetrant carrier, enhancing the percutaneous absorption of salicylic acid from both hydrophilic ointment and hydrophilic petrolatum. DMSO appeared, however, to have a reverse effect on the percutaneous absorption of sodium salicylate from hydrophilic ointment. In the case of sodium salicylate, the salicylate blood levels observed when DMSO was incorporated into the hydrophilic systems were lower than with the control ointment. Figures 2 and 3 indicate that DMSO appeared to produce only a slight increase in the percutaneous absorption pattern of salicylic acid from polyethylene glycol ointment and the polyoxyethylene (20) stearyl ether gel, but this increase was not found to be statistically significant. In addition, the slight

TABLE II—BLOOD SALICYLATE CONCENTRATIONS OBTAINED FROM OINTMENTS CONTAINING SODIUM SALICYLATE[a]

Time, hr.	Hydrophilic Ointment, mg. %—Test[b]	Hydrophilic Ointment, mg. %—Control[c]	Hydrophilic Petrolatum, mg. %—Test[b]	Hydrophilic Petrolatum, mg. %—Control[c]
0.5	0.20 ± 0.20	0.19 ± 0.12	0.13 ± 0.13	0 ± 0
1	0.40 ± 0.40	0.44 ± 0.16	0 ± 0	0 ± 0
2	0.19 ± 0.12	0.56 ± 0.21	0.13 ± 0.07	0.33 ± 0.2
3	0.44 ± 0.16	1.20 ± 0.48	0.13 ± 0.13	0 ± 0
4	0.51 ± 0.28	2.23 ± 0.34	0.29 ± 0.21	0.61 ± 0.15
5	0.66 ± 0.27	2.95 ± 0.63	0.41 ± 0.19	1.40 ± 0.39
6	0.91 ± 0.19	3.19 ± 0.25	0.19 ± 0.12	1.46 ± 0.33
7	0.88 ± 0.05	3.39 ± 0.23	0.74 ± 0.22	2.09 ± 0.48
8	1.38 ± 0.33	4.03 ± 0.47	1.11 ± 0.43	2.83 ± 0.56

[a] Average of eight determinations with standard error of the mean. [b] Contains 11.6% sodium salicylate and 15% DMSO in ointment base. [c] Contains 11.6% sodium salicylate in ointment base.

decrease that resulted in the percutaneous absorption pattern when sodium salicylate was incorporated with DMSO and hydrophilic petrolatum was found not to be statistically significant. DMSO appeared to have no effect on sodium salicylate absorption from polyethylene glycol ointment.

The 10% salicylic acid in the ointment systems was completely solubilized by the quantity of DMSO added to the ointment, but only about one-third of the quantity of 11.6% sodium salicylate was solubilized in the same volume of DMSO. The addition of DMSO resulted in significantly increased absorption for both hydrophilic ointment and hydrophilic petrolatum which might be attributed to the ability of DMSO to solubilize salicylic acid and then serve as a penetrant carrier for it.

Salicylic acid, which is lipid-soluble, penetrates more readily through the skin than the nonlipid-soluble sodium salicylate (15). Skin membrane permeability is thought to be determined largely by the lipid-protein structure of the cellular membrane. It is known that various organic solvents penetrate the skin with ease and enhance percutaneous absorption of lipid-soluble drugs (16). Solvents can increase permeability by solubilizing lipoidal materials of the cell wall (15). This solubilization may be induced by DMSO, thereby facilitating the passage of both the DMSO and dissolved drugs. Substances that are lipid-soluble penetrate the cell wall because of its lipoidal nature, on the other hand the uptake of water by cell membrane protein provides entry for water-soluble substances (17). Dimethyl sulfoxide is extremely hygroscopic and causes dehydration of the skin. This dehydration may free the skin membrane of water, permitting a more rapid absorption of the lipid-soluble salicylic acid through the membrane. However, dehydration would produce a reverse effect on water-soluble sodium salicylate since the sodium salicylate would have a tendency to remain in the water which was extracted from the skin into the ointment base, and thus not pass through the membrane. Kligman (2) indicated that DMSO will cross the dermal barrier rapidly and in high concentrations. Substitution of DMSO for bound water in the protein barrier, thereby causing reversible configuration changes of these proteins, was suggested by Rammler and Zaffaroni (18) as a possible mode of action. This displacement of protein-bound water could possibly further account for the fact that DMSO caused a reduction, rather than an increase, in the percutaneous absorption of sodium salicylate from hydrophilic ointment as observed in this study.

As discussed by Stolar *et al.* (12), the type of ointment base does exert a very significant influence on the extent of percutaneous absorption of both salicylic acid and sodium salicylate. Higuchi (19) suggested that activity coefficient plays a major role in absorption. Drugs held firmly by the vehicle, such as when the drug forms a complex with the vehicle, exhibit a low activity coefficient. Therefore the rate of release from such a drug-vehicle combination is slow. An example of this phenomenon is provided by the complexation of salicylic acid with PEG ointment thus providing a reservoir which releases the drug very slowly (20). The data of this present study indicated that DMSO apparently has little effect upon the release of salicylic acid from this complex with PEG polymers. The negligible results obtained with sodium salicylate and PEG ointment might also be due to similar type complexation. The PSE gel systems

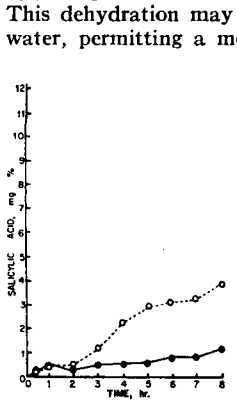

Fig. 5—Effect of DMSO on percutaneous absorption of 11.6% sodium salicylate from hydrophilic ointment. Key: ●—, 15% DMSO added; ○- - -, control.

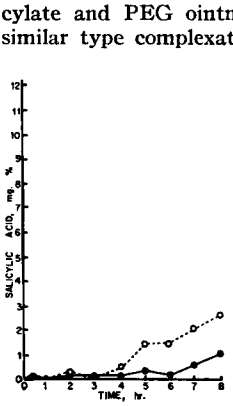

Fig. 6—Effect of DMSO on percutaneous absorption of 11.6% sodium salicylate from hydrophilic petrolatum. Key: ●—, 15% DMSO added; ○- - -, control.

responded in a somewhat similar manner as the PEG ointments. Relatively low salicylate blood levels were obtained with this gel, but it appeared that the blood levels obtained were somewhat higher than with PEG ointment. This may be due to a less intense complexation occurring in the case of the surfactant polymer than in the case of PEG.

SUMMARY

1. Dimethyl sulfoxide, in a 15% concentration, enhanced the percutaneous absorption of salicylic acid from hydrophilic ointment USP XVII and hydrophilic petrolatum USP XVII.

2. Dimethyl sulfoxide, in a 15% concentration, hindered the percutaneous absorption of sodium salicylate from hydrophilic ointment USP XVII.

3. Salicylic acid is slowly released from polyethylene glycol ointment USP XVII and a polyoxyethylene (20) stearyl ether gel system.

4. Dimethyl sulfoxide, in a 15% concentration, had little effect upon the release of salicylic acid or sodium salicylate from polyethylene glycol ointment USP XVII.

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Keyphrases

Salicylic acid, sodium salicylate absorption—ointments
Dimethyl sulfoxide effect—salicylic acid, sodium salicylate absorption
Ointment preparation—salicylic acid, sodium salicylate
Colorimetric analysis—spectrophotometer

Analysis of Steroids VIII

Determination of Conjugated Ketosteroids in Pharmaceutical Preparations Using the Sodium Borohydride Method

By SÁNDOR GÖRÖG

A simple and rapid ultraviolet spectrophotometric method has been developed for the determination of Δ^4-3-keto- and $\Delta^{1,4}$-3-ketosteroids in pharmaceutical preparations. The method consists of the reduction of the C-3 carbonyl group with sodium borohydride, followed by the determination of the decrease of absorbance due to the reduction measured by differential spectrophotometry. Other active components or excipients of the preparation whose absorbances are not changed by sodium borohydride do not interfere with the determination.

Δ^4-3-KETO AND $\Delta^{1,4}$-3-KETO bonding systems very often occur in various kinds of steroid drugs. Their simplest determination is carried out spectrophotometrically. In some cases the direct spectrophotometric measurement can be done without any difficulties with the aid of the intensive absorption band at about 241 mμ, log $\epsilon = 4.2$ (1).

The spectrophotometric determination is disturbed either by the spectra of other components present in the pharmaceutical preparation or by the excipients if they have light absorption. In

Received December 15, 1967, from the Laboratory for Physical Chemistry, Scientific Department, Chemical Works of G. Richter, Budapest X, Hungary.
Accepted for publication May 27, 1968.
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such cases the spectrophotometric measurements are generally combined with chromatographic separations (2), or the spectrum is shifted toward the longer wavelengths by means of a reaction by one of the condensation types, where selective measurements can be made (3, 4).

In this paper a new method is presented which permits the quantitation of conjugated ketosteroids in the presence of accompanying substances with absorbing properties. A method for the specific spectrophotometric determination of conjugated ketosteroids (5) has been recently published. The method essentially is the reduction of the C-3 carbonyl group of the Δ^4-3-ketosteroids with sodium borohydride. This results in the disappearance of the conjugation band in the ultraviolet spectra of the compound. Thus, if the spectrum of the test substance is taken and a solution of the same concentration as the sample is used as a blank, but treated with sodium borohydride, the absorbance measured at about 241 mμ gives the contents of conjugated ketosteroids, even in the presence of impurities showing absorption characteristics, provided that the latter do not change their spectra on treatment with sodium borohydride. In this case the light absorption due to the impurities is cancelled out, and the measured absorbance is characteristic of the contents of conjugated ketosteroids.

This principle has been successfully applied to check the contents of active ingredients in a number of pharmaceutical preparations.

EXPERIMENTAL

The assay of some characteristic examples from among the preparations of Chemical Works of G. Richter Ltd., Budapest, will be described.

Reagents and Apparatus—The sodium borohydride and solvents, methanol and ethanol, used were of analytical grade. Sodium borohydride was weighed into the reaction flask as a solid; in each instance 0.1 g. ± 5% was used. Hydrochloric acid, 1 *N*, 0.01 *N*, aqueous solution. Sodium hydroxide, 1 *N*, aqueous solution. Spectromom 202 spectrophotometer was used in this study.

Determination of 19-Nortestosterone Phenylpropionate in Oil-Injectable Formulations—Thoroughly shake 1.00 ml. of the injection solution, containing 25 mg. of the active ingredient,[1] for 5 min. with 100 ml. of methanol and allow to stand until the oil has settled. Transfer 5 ml. of the stock solution to a 100-ml. volumetric flask, add 5 ml. of methanol followed by 0.1 g. of sodium borohydride. After the sodium borohydride has been dissolved allow the mixture to stand for 15 min. and dilute to volume with methanol.

Weigh 0.1 g. of sodium borohydride into another 100-ml. volumetric flask. Allow to stand with 10 ml. of methanol for 15 min. and then boil for 2 min. After cooling add 5 ml. of the stock solution and dilute to volume with methanol. Determine the absorbance of this solution at 240 mμ using the former solution as a blank.

The concentration of the active ingredient in the sample is calculated from the following equation:

$$\text{mg. of 19-nortestosterone phenylpropionate per ml. formulation} = 2000A/a$$

where a = 42.1, the absorptivity of the active ingredient at 240 mμ.

Determination of Progesterone in Injectable Formulations[2]—*Composition A*—An oil solution containing 12.5 mg. of progesterone and 2.5 mg. of estradiol monobenzoate per ml. with benzyl alcohol as the preservative.

Treat 2 ml. of the injection solution exactly as described above for 19-nortestosterone phenylpropionate.

The concentration of progesterone is calculated as follows:

$$\text{mg. of progesterone per ml. formulation} = 1000A/a$$

where a = 54.1, the absorptivity of progesterone at 240 mμ.

Composition B—An aqueous solution containing 12.5 mg. of progesterone and 2.5 mg. of estradiol monobenzoate per ml. and containing emulsifiers.

Dilute 2 ml. of the injection with sufficient methanol to make 100 ml. Treat this stock solution exactly as described above for 19-nortestosterone phenylpropionate. Determine the absorbance at 250 mμ.

The concentration of progesterone is calculated from the equation for Composition A. In this case a = 42.7, the absorptivity of progesterone at 250 mμ.

Determination of Prednisolone in Ointments—The contents are 0.5% prednisolone and 0.2% methyl *p*-hydroxybenzoate in a hydrophilic ointment.

Dissolve 2.5 g. of the formulation by heating with 50 ml. of ethanol. Dilute the solution with ethanol to make 100.0 ml. To a 10-ml. aliquot of this stock solution add 1 ml. of 1 *N* sodium hydroxide and 0.1 g. of sodium borohydride and reflux for 1 hr. After cooling add 5 ml. of 1 *N* hydrochloric acid and dilute the solution with ethanol to make 100 ml.

Carefully add 5 ml. of 1 *N* hydrochloric acid to a mixture of 0.1 g. sodium borohydride and 1 ml. of 1 *N* sodium hydroxide. When the evolution of hydrogen has ceased, add 10 ml. of the stock solution and dilute with ethanol to 100 ml. Determine the absorbance of the solutions at 243 mμ, using the solution boiled with sodium borohydride as the reference.

The prednisolone content is calculated as follows:

$$\% \text{ prednisolone} = 100A/a$$

where a = 41.0, the absorptivity of prednisolone at 243 mμ in ethanol solution and W is the weight in g. of the sample.

[1] Nerobolil, Richter tradename.

[2] Limovan, Richter tradename.

TABLE I—SPECTROPHOTOMETRIC ANALYSIS OF STEROIDS IN DIFFERENT FORMULATIONS

Formulation Investigated	No. of Tests	Contents of Active Ingredient Found[a]	Declared
19-Nortestosterone phenylpropionate in oil injection	6	24.62 ± 0.17 mg./ml.	25 mg./ml.
Progesterone in oil injection	4	12.42 ± 0.10 mg./ml.	12.5 mg./ml.
Progesterone in aqueous emulsion injection	5	12.66 ± 0.24 mg./ml.	12.5 mg./ml.
Prednisolone in ointment	7	0.491 ± 0.0059%	0.5%
Prednisolone in tablets	5	1.975 ± 0.014 mg./tab.	2 mg./tab.

[a] Average ± standard deviation.

Determination of Prednisolone in Tablets—The active ingredients are 100 mg. of phenylbutazone and 2 mg. of prednisolone per 0.3-g. tablet.[3]

Extract 1 g. of the finely pulverized tablet by shaking for 2 hr. with 60 ml. of 0.01 *N* hydrochloric acid. Filter the extract into a 100-ml. volumetric flask, wash with 3 × 10 ml. of 0.01 *N* hydrochloric acid, and dilute to volume with 0.01 *N* hydrochloric acid. To a 10-ml. aliquot of the stock solution add 2 ml. of 1 *N* sodium hydroxide followed by 0.1 g. of sodium borohydride. Heat the solution for 1 hr. on a steam bath. After cooling add 4 ml. of 1 *N* hydrochloric acid and dilute the solution with water to make 50 ml.

Dissolve 0.1 g. of sodium borohydride in 10 ml. of water in another 50-ml. volumetric flask. Carefully add 4 ml. of 1 *N* hydrochloric acid followed by 2 ml. of 1 *N* sodium hydroxide and 10 ml. of the stock solution. Dilute to volume with water. The absorbance of the solution is determined at 248 mμ against the solution heated with sodium borohydride.

The following equation is used for the calculation:

$$\text{mg. of prednisolone per tablet} = \frac{500A \times \text{av. wt./tablet}}{a \times \text{wt. of the sample}}$$

where a = 39.7, the absorptivity of prednisolone at 248 mμ in aqueous solution.

RESULTS

Table I summarizes typical analyses of five formulations.

From the data of Table I it is seen that the standard deviation does not usually exceed ±1% with the exception of the aqueous emulsion containing progesterone, where the standard deviation has increased 1.9% owing to the difficulties described in the *Discussion*, and the difference from the declared contents of active ingredients is less than ±2%.

DISCUSSION

In every case stock solutions were prepared by adequate extraction of the formulations. An aliquot was taken from the stock solution and submitted to reduction by sodium borohydride. This solution was used as a blank. The test solution was prepared by adding an aliquot of the same volume of the stock solution to sodium borohydride previously decomposed either with hydrochloric acid or by boiling with methanol, and the solution obtained was diluted in the same way as the blank.

The reduction of Δ^4-3-ketosteroids was accomplished according to the method described previously (5): in methanol solution, with a large excess of sodium borohydride, at room temperature for 15 min. With $\Delta^{1,4}$-3-ketosteroids only a partial reduction takes place under such conditions. For this reason, in the case of formulations containing prednisolone, the reduction was carried out at the boiling point. Since sodium borohydride decomposes very quickly in methanol under these conditions, ethanol or water was used instead of methanol as a solvent and sodium hydroxide was employed as a stabilizer. In this manner a quantitative reaction can also be achieved in the above-mentioned cases.

The pH value of the test solution does not affect the results. Therefore, in the case of Δ^4-3-ketosteroids the measurements were made in mild alkaline solution produced by the decomposition products of sodium borohydride. In the case of prednisolone in aqueous medium, the measurements were made in the solution which had been made alkaline to stabilize the sodium borohydride; in ethanolic medium, owing to the poor solubility of the decomposition products of sodium borohydride in alkaline ethanol, the measurements were made after acidifying with hydrochloric acid.

The only prerequisite to obtaining correct results in this manner is that the other active ingredients and excipients used should not change their absorbance at about 241 mμ on treatment with sodium borohydride. This condition was fulfilled with the preparations studied.

Special mention must be made of the oil, *Oleum helianthi*, very often used as a solvent for steroid hormones. The active ingredient of the oil injection was extracted simply by shaking the injection with a 50–100-fold quantity of methanol. After the separation of the oil, the methanol solution was tested. It was found that the active ingredient can be completely extracted by a single extraction.

However, other substances showing absorption are also extracted from the oil by methanol. Therefore direct measurement is impossible even in the absence of other absorbing components (19-nortestosterone phenylpropionate injection). Curve a, Fig. 1, indicates the spectrum of this formulation; it was taken after extraction and dilution (as described in *Experimental*) but without treatment with sodium borohydride. It can be seen that the spectrum shows large distortion in comparison with the spectra of Δ^4-3-ketosteroids. Curve b is the spectrum of the solution reduced with sodium borohydride, showing the spectrum of the substances extracted from the oil (background), while the differential spectrum (Curve c) which was taken as described in *Experimental*, is in good agreement with the spectrum of 19-nortestosterone phenylpropionate (6).

There is only a small irregularity in the differential

[3] Rheosolone, Richter tradename.

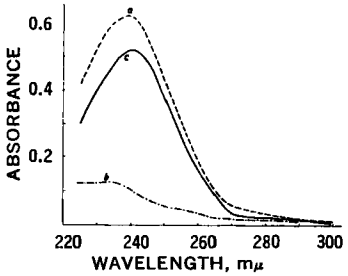

Fig. 1—Determination of 19-nortestosterone phenylpropionate in oily injection. Key: a, spectrum of diluted stock solution without treatment with sodium borohydride; b, spectrum after treatment with sodium borohydride; c, differential spectrum of the solutions a and b.

curve, namely, the shoulder of small intensity that occurs at about 280 mμ, where 19-nortestosterone phenylpropionate has no measurable absorption at this concentration.

The differential spectra of the methanolic extracts of various oil samples treated with sodium borohydride were recorded to clarify this question. It was found that a differential spectrum appears with a small but reproducible intensity. The maximum of this spectrum is at about 280 mμ, and the differential absorbance measured at 240 mμ can be neglected under the given conditions.

This effect can be more distinctly seen on Curve c, Fig. 2, where the differential spectrum of the oil-injectable formulation of progesterone is presented. In this case the concentration of the active ingredient is half that in the case of 19-nortestosterone phenylpropionate injection, and thus the relative concentration of impurities is doubled. In this case the error was not yet significant, but oily injections containing smaller quantities of active ingredient cannot be tested with the desirable precision. The absorption band, $\lambda_{max.}$ 230 mμ (7), of estradiol monobenzoate and the aromatic fine structure of benzyl alcohol $\lambda_{max.}$ 252, 258, 264, 268 mμ, can readily be recognized on Curve b, Fig. 2. The latter also can be seen on Curve a.

In the cases described above the background eliminated by differential spectrophotometry at 240 mμ did not amount to 50% of the differential

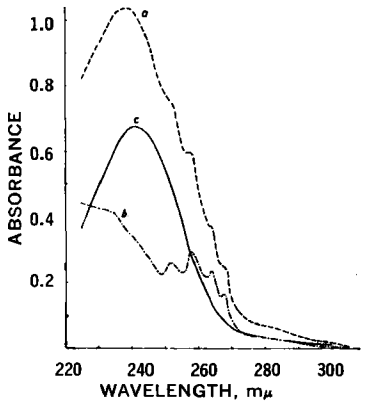

Fig. 2—Determination of progesterone in oil injection. See Fig. 1 for key.

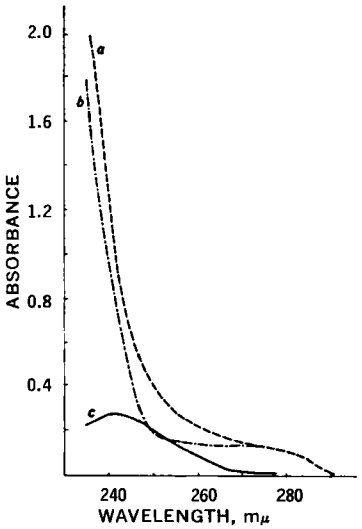

Fig. 3—Determination of progesterone in aqueous emulsion injection. See Fig. 1 for key.

absorbance. However, the measurement could also be accomplished with the aqueous emulsion injection containing progesterone, where the value of the background (Curve b, Fig. 3), owing to the auxiliaries used in the preparation of the emulsions, is a multiple of the value of the differential spectrum (Curve c). In this case the measurement, because of the high absorbance of the blank, was not made at the peak but at the slope of the spectrum of progesterone, namely at 250 mμ. The error in this case slightly exceeded that observed with the other preparations tested.

In the case of the prednisolone-containing ointment, the interfering effect of methyl *p*-hydroxybenzoate had to be prevented. The spectrum of this compound [$\lambda_{max.}$ 258 mμ, a = 109.2 (8)] can be identified in Curve b, Fig. 4. The differential spectrum (Curve c) shows appropriate agreement with the spectrum of prednisolone.

In the test with tablets containing prednisolone and phenylbutazone, the problem was due to the

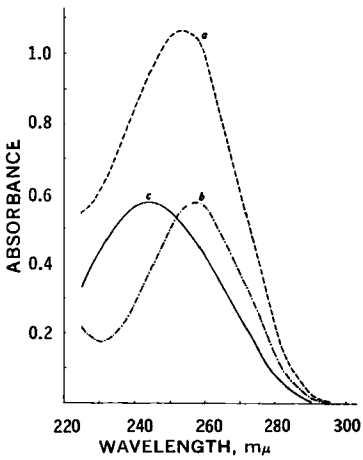

Fig. 4—Determination of prednisolone in ointment. See Fig. 1 for key.

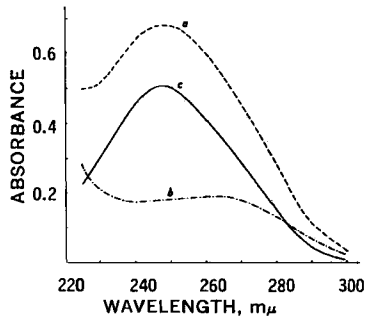

Fig. 5—Determination of prednisolone in tablets. See Fig. 1 for key.

presence of a 50-fold quantity of phenylbutazone having nearly the same absorbance at about 240 mμ as the prednisolone. In the presence of both active ingredients (alcohol extraction), the measurements, owing to the high background absorbances, could only be made in dilute solutions. The sensitivity and precision of the method was extremely decreased by this circumstance. To avoid this, an attempt was made to select the extraction. This could be achieved by the use of a 0.01 *N* solution of hydrochloric acid. In this solution prednisolone dissolves satisfactorily for the purpose given, while phenylbutazone being present at this acidity quantitatively in the keto form is dissolved only slightly. Thus the extraction of prednisolone becomes nearly selective. The small amount of phenylbutazone extracted has carbonyl groups of acid hydrazide type, which do not react with sodium borohydride, therefore the differential curve (Curve c, Fig. 5) is suitable for the correct estimation of prednisolone. The background (Curve b) is characteristic of the spectrum of phenylbutazone measured in alkaline solution [$\lambda_{max.}$ 264 mμ (9)].

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Keyphrases

Steroids—analysis
Ketosteroids, conjugated—determination in pharamaceuticals
Sodium borohydride reduction—steroid analysis
UV spectrophotometry—analysis

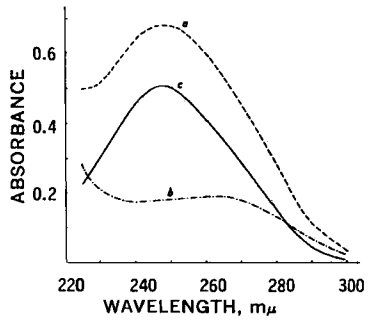
Effect of Physostigmine upon the Output of Catecholamines from the Adrenal Gland of the Rat

By C. L. KAUL and R. S. GREWAL

An investigation has been made on the effect of physostigmine on the output of catecholamines from the adrenal gland of the rat. Physostigmine (20 mcg. i.v.) causes three- to fourfold increase in the catecholamines output from the adrenal gland of the rat. This effect is mediated centrally as no increase was seen in the pithed animals. It is concluded that this peripheral release of catecholamines does not play any significant role in the hypertensive response of physostigmine in the rat.

INTRAVENOUS administration of physostigmine causes an appreciable rise in blood pressure in an urethan-anesthetized rat (1, 2). This pressor response of physostigmine has been mainly attributed to central adrenergic stimulation and is absent or much less in pithed animals (2). Although the hypertensive response of physostigmine is mainly central, there is some evidence to suggest that some peripheral action may also be involved (2–4). Medaković and Varagić (5) have also postulated that liberation of epinephrine and norepinephrine from the adrenals does not seem to play a significant role in the hypertensive response to physostigmine, as the response to physostigmine was the same in normal or adrenalectomized animals.

Received May 22, 1968, from the CIBA Research Centre, Goregaon, Bombay 63, India.
Accepted for publication July 19, 1968.



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INTRAVENOUS administration of physostigmine causes an appreciable rise in blood pressure in an urethan-anesthetized rat (1, 2). This pressor response of physostigmine has been mainly attributed to central adrenergic stimulation and is absent or much less in pithed animals (2). Although the hypertensive response of physostigmine is mainly central, there is some evidence to suggest that some peripheral action may also be involved (2–4). Medaković and Varagić (5) have also postulated that liberation of epinephrine and norepinephrine from the adrenals does not seem to play a significant role in the hypertensive response to physostigmine, as the response to physostigmine was the same in normal or adrenalectomized animals.

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Physostigmine is known to increase the output of epinephrine (10–15-fold) from the cat adrenals (6). According to these authors, this effect is mediated centrally, as no increase in the output of epinephrine was demonstrated when the splanchnic and other nerve supply to the adrenal glands was cut. Since physostigmine is known to cause central adrenergic stimulation in the rat, it is possible that this effect is conveyed to the periphery through established sympathetic pathways and one would expect it to cause a release of catecholamines from the rat adrenals (as has been shown in the case of cats). This release of catecholamines could possibly contribute to the hypertensive effect of physostigmine.

The purpose of the present work was to see if physostigmine increased the output of catecholamine from the adrenal gland in the rat and if this output contributes to its hypertensive response. Therefore it was also thought desirable to compare the pressor response of epinephrine, norepinephrine, and physostigmine in normal and adrenalectomized animals to see if there are any differences in the response of the cardiovascular system under these conditions. Finally the effect of β-blocker on the hypertensive response of physostigmine in normal and adrenalectomized rats was also investigated.

METHODS

Adult male rats (C.F. strain) weighing from 300–340 g. (Series 1–3) and 250 g. (Series 4) were used. They were anesthetized with urethan (1.5 g./kg.). Four series of experiments were carried out in these animals. In the first series the pressor response to 20 mcg. of physostigmine was measured in normal and adrenalectomized animals. In the second series pressor response to intravenous injection of epinephrine and norepinephrine was measured in normal and adrenalectomized rats. In both these sets of experiments the blood pressure was recorded from a common carotid artery by a mercury manometer writing on a smoked kymograph. In the third series of experiments the total catecholamines were measured as norepinephrine from the venous effluent of the main adrenal vein (side branch tied) during control period and after intravenous injection of 20 mcg. of physostigmine. In these experiments both the jugular veins were cannulated, one for injection of the drugs and the other for giving continuous infusion of heparinized saline (0.1 ml./min.) till the end of the experiment. Blood 0.5–0.7 ml., was collected in about 30 min. from a cannulated adrenal vein in a cold centrifuge tube, containing heparin. The plasma was separated by centrifugation (about 2,000 r.p.m.) and it was assayed against norepinephrine on the blood pressure of a pithed rat. As the volume of the plasma was very small, the unknown plasma sample was bracketed between two or three doses of standard norepinephrine. In some experiments where the volume of plasma was higher, two lines of a Latin square were carried out for assay. Three samples were usually collected from each rat, one at 0 hr. before giving physostigmine and the other two at 30 and 60 min. after giving physostigmine. Control experiments were carried out without physostigmine and the samples were collected at 0, 30, and 60 min. and were assayed against norepinephrine to see if there were any increase in the amount of catecholamines released during this time.

To confirm whether the effect of physostigmine was central or peripheral, a set of experiments (Series 4) was done using pithed animals and in this set also three samples of blood were collected and assayed as described above. In these experiments the blood flow was very low and a longer period of collection (40 min.) was necessary to collect approximately the same volume of blood so that an assay could be carried out and the rate of heparinized saline infusion had also to be increased (0.2–0.3 ml./min.).

For the estimation of total catecholamines from the adrenal gland, extraction of the tissues was carried out by perchloric acid and the extract after centrifugation was assayed on the spinal cat (blood pressure) using epinephrine as a standard.

Drugs used—Epinephrine acid tartrate; *l*-norepinephrine-*d*-hydrogen tartrate; physostigmine salicylate; and propranolol.

All concentration and doses of these drugs refer to their salts except for epinephrine and norepinephrine where it is expressed as a free base. Doses of physostigmine are expressed per rat.

RESULTS

Results of the first group of experiments show that pressor responses to 20 mcg. of physostigmine in adrenalectomized animals is slightly but significantly higher than the controls (Table I). There was however no significant difference in the pressor responses of epinephrine or norepinephrine in normal and adrenalectomized animals at different time intervals (Table II).

The effect of physostigmine on the catecholamine output is shown in Table III. From this table it can be seen that there is a 3–4-fold increase in the amount of catecholamine output measured as norepinephrine. This increase in the catecholamine output was seen 0.5 hr. after intravenous injection of physostigmine and persisted up to 1 hr. when the last sample of blood was collected. In a set of control experiments where no physostigmine was given, there was some increase in the amount of catecholamine output in the blood collected at 1 hr. However, the difference between the two means was not statistically significant. In pithed animals, the control levels of catecholamines were lower than the normal animals and physostigmine produced no significant increase in the output of

TABLE I—BLOOD PRESSURE RESPONSE (mm. Hg) TO PHYSOSTIGMINE (20 mcg.) IN NORMAL AND ADRENALECTOMIZED RATS[a]

Treatment	B.P. Rise, mm. Hg
Normal	54.20 ± 3.19 (14)[b]
Adrenalectomized	66.3 ± 2.73 (10)[c]

[a] All values are the means ± SEM. [b] Figures in the brackets indicate the number of observations. [c] $p < 0.01$.

TABLE II—BLOOD PRESSURE RESPONSES (mm. Hg) TO EPINEPHRINE (0.5 mcg.) AND NOREPINEPHRINE (0.5 mcg.) IN NORMAL AND ADRENALECTOMIZED RATS[a]

Normal					
0 hr.		0.5 hr.		1 hr.	
Epinephrine	Norepinephrine	Epinephrine	Norepinephrine	Epinephrine	Norepinephrine
Normal					
39.30 ± 4.47	42.60 ± 5.77	39.10 ± 4.99	34.00 ± 4.49	40.10 ± 6.76	31.60 ± 4.54
Adrenalectomized					
52.60 ± 4.22	43.30 ± 8.92	54.00 ± 7.44	35.30 ± 8.08	40.30 ± 5.09	31.30 ± 5.54

[a] All values are the means ± SEM of determinations on 6 rats.

TABLE III—CATECHOLAMINE OUTPUT FROM THE ADRENAL VEIN IN CONTROL RATS AND RATS GIVEN PHYSOSTIGMINE[a]

Treatment	Drug	Dose, mcg.	Concn., mcg./ml., Calcd. as Norepinephrine		
			0 hr.	30 min.	60 min.
Control	—	—	0.16 ± 0.03 (8)[b]	0.22 ± 0.04 (9)	0.26 ± 0.05 (9)
Normal rats	Physostigmine	20	0.20 ± 0.02 (15)	0.65 ± 0.11 (12)[c]	0.86 ± 0.18 (11)[c]
Pithed rats	Physostigmine	20	0.023 ± 0.002 (6)	0.027 ± 0.006 (6)	0.021 ± 0.006 (6)

[a] All values are the means ± SEM. [b] Figures in the brackets indicate the number of observations. [c] $p < 0.001$.

catecholamines (Table III). Since the catecholamine concentration was lower in the plasma of the pithed animals, it was also of interest to see if pithing itself produced any significant effect on the catecholamine content of the rat adrenals. The results of these experiments are shown in Table IV. From this table it can be seen that there is a significant reduction in the catecholamine content of adrenal gland after pithing.

The experiments reported with propranolol (Table V) show that in both normal and adrenalectomized animals, propranolol potentiated the hypertensive response of physostigmine slightly. In adrenalectomized animals, with the higher dose of propranolol the increase in the pressor response was significantly higher than the normal rats (65%).

TABLE IV—THE EFFECT OF PITHING[a] ON THE CATECHOLAMINE CONTENT OF RAT ADRENALS[b]

Treatment	Concn., mcg./g.
Control	931.0 ± 40.4 (21)[c]
Pithed	676.0 ± 30.0 (11)[d]

[a] The animals were pithed under light ether anesthesia and were kept on artificial respiration for 0.5 hr. before the adrenals were taken out. [b] All values are the means ± SEM. [c] Figures in the brackets indicate the number of observations. Six adrenals from 3 rats were used for each observation. [d] $p < 0.001$.

Varagić *et al.* (7) have also reported slight increase in the pressor response of physostigmine in rats given propranolol (21.7 mg./kg.).

DISCUSSION

The results presented in this paper show that intravenous administration of physostigmine in normal rats increases the output of catecholamines from the adrenals. This increase in the output of catecholamines was absent in pithed animals, indicating thereby that physostigmine produces the effect centrally and no peripheral action is involved. Similar results have been reported by Stewart and Rogoff (6) in cats.

The lower concentration of catecholamines found in the plasma of pithed animals is probably related to the fact that a significant reduction in the total catecholamine content of the adrenal gland occurs after pithing. It perhaps also indicates that central adrenergic mechanisms have a certain tonic influence on the release of adrenal medullary hormones and in the absence of this central influence, as happens in pithing, the amount of hormone release is less. Another contributory factor would be reduction in the blood supply to the adrenals consequent to the lowering of blood pressure after pithing. Harvey *et al.* (8) have also reported low values of plasma catecholamines in pithed dog

TABLE V—EFFECT OF PROPRANOLOL (100 AND 200 mcg./kg.) ON THE HYPERTENSIVE RESPONSE OF PHYSOSTIGMINE (20 mcg.) IN NORMAL AND ADRENALECTOMIZED RATS[a]

Treatment	Dose, mcg./kg.	B.P. Response, mm. Hg	
		Before Propranolol	After Propranolol
Normal	100	55.61 ± 5.09 (6)[b]	71.10 ± 10.98 (6)
Normal	200	54.30 ± 6.10 (6)	65.20 ± 3.70 (5)
Adrenalectomized	100	59.10 ± 5.60 (6)	76.30 ± 5.96 (6)
Adrenalectomized	200	52.60 ± 6.90 (6)	87.30 ± 9.70 (6)[c]

[a] All values are the mean ± SEM. [b] Figures in the brackets indicate the number of observations. [c] $p < 0.05$.

and these low values have been partly attributed by these authors to the pithing of the animals.

The peripheral release of catecholamines (epinephrine and norepinephrine) does not seem to play any significant role in the hypertensive response of physostigmine (5, 9). This observation is based on the fact that these authors did not find any difference in the pressor response of physostigmine in normal as well as in the adrenalectomized animals. They further suggested (9) that although physostigmine is known to increase the output of epinephrine from the adrenals of the cat (6) it is unlikely that this effect occurs in the rat. These results contradict their statement that physostigmine will not increase the peripheral release of catecholamines from the adrenals of the rat. The results presented in this paper clearly demonstrate such an effect in the rat.

The authors have shown that catecholamines are being released following injection of physostigmine and if these were contributing to the hypertensive response then one would have seen a bigger rise of blood pressure following physostigmine injection in normal animal with intact adrenals than in adrenalectomized animals. In fact the experiments showed an increase in the pressor response of physostigmine in adrenalectomized animals. Therefore these results show that release of catecholamines from the adrenal glands does not contribute to the hypertensive response of physostigmine.

Some doubt also exists in the literature as to whether the adrenal medullary secretion plays any significant role in the cardiovascular regulations (10). Since most of the catecholamine present in the adrenal medulla is adrenaline (85%) (11), it is possible that its effect on β-adrenergic receptors in certain vascular beds leads to vasodilation and masks the vasoconstriction effect. This could probably explain why a significant increase in the pressor response of physostigmine in the adrenalectomized animals as compared to controls is seen. This could not be attributed to the change of sensitivity of the cardiovascular system, after adrenalectomy, since pressor responses of epinephrine and norepinephrine were the same in both normal and adrenalectomized animals. This is further supported by experiment with propranolol where an increase in the pressor response of physostigmine occurred. The slight but significant ($p < 0.05$) increase in the pressor response in the adrenalectomized animals is difficult to explain with the data available.

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Keyphrases

Physostigmine—catecholamine output
Adrenal gland catecholamine output—physostigmine effect
Central mediation—physostigmine activity

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By MARÍA GABALDÓN, JUAN SÁNCHEZ, and ANTONIO LLOMBART, JR.

A technique is described for the study of the utilization of diethylstilbestrol (DES) by rat liver *in vitro*. The disappearance of DES and the seasonal variations were determined on liver slices and homogenates. A chromatographic identification of diethylstilbestrol monoglucuronide (DESGA) as a reaction product in the assays of slices was carried out. The effect of liver preincubation with saline phosphate buffer, of phenylmercuric acetate, and of uridine diphosphate glucuronic acid on DES utilization by liver was studied. The possibility of a DES utilization pathway other than monoglucuronide formation is discussed. A thin-layer chromatographic study was carried out with DES and DESGA.

DIETHYLSTILBESTROL (α,α' - diethylstilbenediol) is an orally active synthetic estrogen that has been utilized in therapeutics to produce prostatic tumor regression; it inhibits glutamate dehydrogenase by breaking the enzyme into inactive subunits (1), uncouples oxidative phosphorylation with mitochondrial volume expansion (2), and inhibits electron transport between cytochromes *b* and *c* (3).

Received January 31, 1968, from the Servicio de Cancerología Experimental, Facultad de Medicina, Paseo de Valencia al Mar, Valencia, Spain.
Accepted for publication July 17, 1968.
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In spite of its important activity at physiological and enzymatic levels, and of its use in medicine and veterinarian practice, very little is known about its metabolism and mechanism of action. Reports by Zondek (4), Engel (5), Riegel (6), and Hopwood (7) showed that liver *in vitro* is capable of utilizing DES; however, in no case is the nature of the reaction involved nor the type of product formed mentioned, only the loss of estrogenic activity of DES after the incubation period with liver being ascertained by means of biological assay.

Other studies point to the role of DES as a tyrosinase (8) and horseradish peroxidase (9) substrate but no mention is made of the reaction products, except to the appearance of water soluble metabolites.

Experiments with ^{14}C-labeled DES have demonstrated that this compound, like the natural estrogens, enters the enterohepatic circulation and is eliminated to a high degree through feces (10). Knoche (11) using ^{14}C- and ^{3}H-labeled DES in rats studied the *in vivo* oxidation with production of labeled H_2O and CO_2 and four radioactive metabolites.

The excretion of DES in the urine as a monoglucuronide (DESGA) is well known (12), but the enzymatic mechanism of glucuronide formation has not been subjected to detailed investigation. Zimmerberg (13) studied the quantitative DES utilization by rat liver slices and attributed substrate disappearance to conjugation processes with glucuronic acid and the sulfate but the reaction products were not identified. Hartiala (14) employing chromatography, indirectly pointed out the formation of DESGA in rat duodenal mucosa slices. Dutton and Storey (15) showed the formation of a small amount of glucuronide in rat liver homogenates.

The present report presents a systematic study of DES disappearance following incubation period with rat liver slices and homogenates. Starting from the incubation mixture, the technique employed allows for the determination of DES without DESGA interference or any other phenolic compounds. The amount of DES consumed was calculated by difference with a blank incubated without substrate and added after protein precipitation.

A thin-layer chromatographic study of DES and DESGA was performed for qualitative as well as semiquantitative identification of reaction products.

EXPERIMENTAL

Chemicals—DES and uridine diphosphate glucuronic acid (UDPGA) were provided by Sigma Chemical Co. and tris(hydroxymethyl)aminomethane (tris) and phenylmercuric acetate, by Schuchardt. All solvents were redistilled and diethyl ether was maintained free from peroxides by storage over ferrous sulfate and was freshly distilled before use.

Animals—Wistar male rats of 240–280 g. were sacrificed by cervical dislocation and the livers immediately removed and washed with 0.15 *M* KCl–3.2×10^{-4} *M* $KHCO_3$ previously cooled to 0°. Slices of 250 mg. each were obtained with a Stadie-Riggs hand microtome and 15% homogenates were prepared with 0.15 *M* KCl–3.2×10^{-4} *M* $KHCO_3$ in a Potter-Elvhjem homogenizer cooled to 0°.

Preparation of DESGA—DESGA was obtained from the urine of rabbits fed DES at a daily rate of 500 mg. for 5 days. Following the procedures given by Dodgson *et al.* (12), a chromatographically pure product of m.p. 176° was obtained after three crystallizations from benzene–acetone. The presence of glucuronic acid in the product was demonstrated by hydrolysis with concentrated HCl vapors and elution of the hydrolyzed product on Silica Gel FH_{254} plate with ethyl acetate–2-propanol–water (6:2.5:1.5). Two spots were found: a red spot on the site of application similar to the one yielded by DES when given the same treatment, and another spot with the same R_f as glucuronic acid.

Thin-layer Chromatography—The plates used in the thin-layer chromatography were Silica Gel G and Merck precoated Silica Gel FH_{254}. They were heated for 30 min. at 110° before use. The Merck precoated plates were observed at 254 mμ.

For the detection of glucuronic acid, 2% aniline phthalate in water-saturated *n*-butanol was used; for the detection of DES the following methods were applied: (*a*) direct observation after 2 days of exposure to daylight; (*b*) 1% I_2 in methanol; (*c*) 50% H_2SO_4 v/v followed by heating for 3 min. at 100°; (*d*) equal volumes of 1% $FeCl_3$ and 1% potassium ferricyanide mixed before use followed by application of 10% HCl, and (*e*) 0.5 *N* Folin-Ciocalteu's reagent and exposure to ammonia vapor. The last four detecting agents can be also used for the detection of DESGA.

Solubility Determination—Aliquots of a DES solution containing 2 mg./ml. in acetone were placed in tubes and evaporated to dryness. Five-milliliter portions of the solutions in which DES solubility was to be determined were added to the residue. The tubes were hermetically sealed and shaken at 25° for 24 hr. The contents were filtered only where an excess of DES occurred and the latter ascertained in triplicate by the method outlined below.

Calibration Curve for DES—A modified Tubis technique (16) has been employed for the determination of DES. A standard curve was obtained by treatment of 1 ml. DES solution containing from 0 to 65 mcg./ml. in 30% ethanol with 4 ml. of 4% $Na_2CO_3 \cdot 10H_2O$ in 0.1 *N* NaOH and 0.25 ml. of 1 *N* Folin-Ciocalteu's reagent. The contents were shaken and readings taken at 540 mμ after 45 min. at 20°. A linear function was obtained over the range studied.

DES Determination in the Incubation Mixture—One milliliter of 20% trichloroacetic acid and 4 ml. of ethanol were added to 5 ml. of the incubation

mixture, over a 10-min. period and the contents filtered. Five milliliters of a freshly prepared 2% $NaHCO_3$ was added to an aliquot of 5 ml. from the filtrate, toward formation of the sodium salts of DESGA and of the other acids so as to prevent their removal by diethyl ether. The mixture was extracted twice with 10-ml. portions of diethyl ether. The ether was removed on the water bath and the aqueous alcoholic residue evaporated under vacuum at a temperature below 45°. The solid residue was lacking in any substance which could interfere with subsequent DES determination.

Two milliliters of 30% ethanol, 8 ml. of 4% $Na_2CO_3 \cdot 10H_2O$ in *N* NaOH, and 0.50 ml. of *N* Folin-Ciocalteu's reagent were added to the solid residue. The contents were shaken and readings taken at 540 mμ after 45 min. at 20°. Reagent volumes double those described for the calibration curve were used so that readings should be within the limits of the standard curve. In order that the amount of DES determined could be related to total DES the former was multiplied by 2 (correction factor for using an aliquot of 5 ml. from the filtrate) and by 2 again (correction factor for using double the amount of reagents as in the calibration curve). The amount of DES utilized by the liver was calculated as the difference between the blanks incubated without DES and with DES after precipitation with trichloroacetic acid and the samples incubated with DES for 90 min. at 37°. Assays were done in triplicate.

Isolation of DESGA from the Incubation Mixture—An incubation volume five times greater than in the assays of slices was used in conjunction with the DESGA analysis. The reaction was stopped with 5 ml. of 12 *N* H_3PO_4 and 20 min. later, 20 ml. of ethanol was added and following a period of 20 min. the mixture was centrifugated and concentrated to a volume of 10 ml. Four extractions were carried out with 10-ml. portions of diethyl ether and the pooled ethereal layers were washed with 2 ml. of 0.01 *N* H_3PO_4 and then concentrated to 5 ml. They were extracted with three 5-ml. portions of a saturated $NaHCO_3$ solution. The aqueous portion was washed with 3 ml. of ether, acidified with 2.7 ml. of 12 *N* H_3PO_4, and then extracted with four 20-ml. portions of ether. The pooled etheral layers were washed with 4 ml. of 0.01 *N* H_3PO_4 and evaporated to dryness. The residue was dissolved in 80% ethanol and an aliquot applied to the Silica Gel FH_{254} plate. The plate was completely developed with benzene–ethanol (6:4) in order to separate contaminating substances with less polarity; once dry it was developed with ethyl acetate–2-propanol–water (6:2.5:1.5) or *n*-butanol–acetic acid–water (6:2:2). A spot of the same R_f as the standard DESGA, which is absent in the blank, was detected.

TABLE I—R_f VALUES OF DES AND DESGA

	R_f in Solvent Systems[a]					
	1	*2*	*3*	*4*	*5*	*6*
DES	0.39	1.00	1.00	0.72	0.90	0.95
DESGA	0.00	0.82	0.30	0.00	0.00	0.90

[a] Solvent systems employed were: *1*, benzene–ethanol (9:1); *2*, 1-butanol–acetic acid–water (6:2:2); *3*, ethyl acetate–2-propanol–water (6:2.5:1.5); *4*, chloroform–ethanol (8:2); *5*, benzene–ethanol (6:4); *6*, methanol–acetic acid–water (8:1:1).

TABLE II—DES SOLUBILITY IN AQUEOUS ALCOHOLIC SOLUTIONS[a]

Solvent	Solubility, mg./ml.
Water	0.012
10% Ethanol	0.030
20% Ethanol	0.083
30% Ethanol	0.496
30% Propylene glycol	0.036
40% Propylene glycol	0.135
50% Propylene glycol	0.420
60% Propylene glycol	1.212
70% Propylene glycol	3.572

[a] Percentages refer to v/v.

RESULTS

Thin-layer Chromatography—Table I presents R_f values for DES and DESGA with the different solvents used. The detection limits for DES with the methods applied were: for *1*, *4*, and *5*, 0.60–0.90 mcg.; for *2*, 0.20 mcg., and for *3*, 1.00–1.30 mcg. (see *Experimental*).

Solubility Determinations—Table II presents DES solubilities in water solutions of ethanol and propylene glycol.

Utilization by Liver—Table III shows DES utilization by rat liver slices in the presence and absence of phenylmercuric acetate, and inhibitor of UDP-glucuronate glucuronyltransferase (acceptor-unspecific), EC 2.4.1.17 (17) and the seasonal variations observed. Table IV depicts DES utilization by rat liver homogenates in the presence and absence of UDPGA, and the seasonal variations observed.

DISCUSSION

The amount of DES consumed by rat liver slices cannot be exclusively attributed to monoglucuronide formation since the actual amount of the latter detected was far lower than the theoretically expected one, should all DES have been transformed into DESGA. The utilization of DES in the presence of inhibitor and by liver previously inactivated by preincubation further supports the same assumption. The difference between the amount of DES utilized in the absence and presence

TABLE III—DES UTILIZATION BY RAT LIVER SLICES[a]

Conditions	DES Consumed, mcg. Mean ± *SD*	p[d]
Spring		
Without inhibitor (10)[b]	44.40 ± 7.85	
With inhibitor (10)	35.12 ± 7.30	<0.025
Without inhibitor (8)[c]	17.32 ± 10.10	<0.01
Autumn		
Without inhibitor (7)	44.24 ± 8.50	
With inhibitor (7)	40.64 ± 7.96	>0.2

[a] The composition of the incubation mixture was as follows: NaCl, 96.3 m*M*; KCl, 4 m*M*; $NaHCO_3$, 6.3 m*M*; $MgCl_2 \cdot 6H_2O$, 9.9 m*M*; $Na_2HPO_4 \cdot 2H_2O$, 19.8 m*M*; HCl, 4 m*M*; DES, 0.149 m*M*; phenylmercuric acetate, 0.5 m*M*; 3% v/v propylene glycol; liver slice, 250 mg.; final volume, 5 ml.; pH, 7.4; incubation with shaking at 37° for 90 min. [b] Numbers in parentheses indicate number of animals used. [c] Assay done after slice preincubation with saline phosphate buffer for 24 hr. at 37°. [d] Level of significance, Student *t* test; *p* value compared with the assay performed in the absence of inhibitor.

TABLE IV—DES UTILIZATION BY RAT LIVER HOMOGENATES[a]

Conditions	DES Consumed, mcg. Mean ± SD	p[c]
Spring		
With UDPGA (7)[b]	21.40 ± 8.17	
Without UDPGA (7)	7.36 ± 5.56	<0.01
Autumn		
With UDPGA (7)	0.00	
Without UDPGA (7)	0.00	

[a] The composition of the incubation mixture was as follows: Tris, 35 m*M*; $MgCl_2 \cdot 6H_2O$, 10.5 m*M*; UDPGA, 0.67 m*M*: DES, 0.149 m*M*: 3% v/v propylene glycol; 15% homogenate, 1 ml.; final volume, 5 ml.; pH, 7.4; incubation with shaking at 37° for 90 min. [b] Numbers in parentheses indicate number of animals used. [c] Level of significance, Student *t* test; *p* value compared with the assay performed in the presence of UDPGA.

of inhibitor is statistically significant ($p < 0.025$) in the experiments carried out in spring and relates to the amount of DESGA detected. The same experiments carried out in autumn did not yield statistically significant differences ($p > 0.2$).

Jellinck (8) using ^{14}C-labeled DES and tyrosinase pointed out that after the incubation of the mixture production of water-soluble metabolites ensued, a phenomenon that was accentuated by the presence of proteins, not affected by thiol reagents and totally inhibited by potassium cyanide. The latter finding is of interest considering that Zimmerberg (13) obtained complete DES recoveries from incubation mixtures of rat liver slices and cyanide. It is possible that in the present report the DES disappearance in the presence of phenylmercuric acetate could be due to an oxidative process that would transform DES into a water-soluble metabolite and which might explain the incomplete recovery of DES by ether extraction.

The DES disappearance in the liver system inactivated by preincubation at 37° for 24 hr. can be interpreted as an adsorption phenonenon on proteins. Similar retention phenomena upon inactive proteins have been described for DES by Riegel *et al.* (6) with boiled liver and in the case of estrone by Jellinck (18, 19) with boiled liver, albumin, and hemin.

As far as the rat liver homogenates is concerned, the difference between the amount of DES consumed in the presence and absence of UDPGA is statistically significant ($p < 0.01$) and it seems logical to attribute this difference exclusively to the formation of glucuronide. The disappearance of DES in the absence of UDPGA, which is very variable, can be due to residual levels of UDPGA or other unknown pathways of utilization. Non-enzymatic phenomena do not seem to play any role at all, as there has not been any case of DES disappearance in experiments undertaken in autumn.

Seasonal variations, although previously described, are not too well known. Dutton (20) and Hartiala (21) have pointed out greater glucuronyltransferase activity in winter than in spring with *o*-aminophenol as substrate. Periodic variations have been observed in relation to synthetic hypoglycemic drugs (22) the toxicity being greater from December to March. No explanation for the phenomenon observed in this study can be advanced at the present time.

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Keyphrases

Diethylstilbestrol—rat liver utilization *in vitro*
Metabolite—diethylstilbestrol monoglucuronide
Solubility—diethylstilbestrol
TLC—analysis
Colorimetric analysis—spectrophotometer

Spray-Congealed Formulations of Sulfaethidole (SETD) and Waxes for Prolonged-Release Medication

Effect of Modifiers

By Y. RAGHUNATHAN and CHARLES H. BECKER

An investigation was undertaken to examine the effects of the addition of certain substances—*viz.*, low molecular weight polyethylene, ethylcellulose 20 cps., and glyceryl ester of hydrogenated rosin, to carnauba wax, hydrogenated castor oil, and synthetic wax-like ester, on the *in vitro* dissolution patterns of spray-congealed particles of sulfaethidole (SETD) and wax-modifier combinations. The pneumatically atomized and congealed particles of the drug-wax-modifier suspensions were observed to possess log-normal particle-size distributions. These were characteristic for a particular wax. The addition of low molecular weight polyethylene was observed to increase the particle size significantly for all three wax formulations. The *in vitro* dissolution patterns appear to be second order, being functions of the effective surface area and the weight of drug available for dissolution. The dissolution patterns of SETD from the three wax formulations were observed to be characteristic for each wax and dissolution medium. The addition of low molecular weight polyethylene was observed to retard the dissolution rates of SETD from hydrogenated castor oil and synthetic wax-like ester formulations. The addition of ethylcellulose tended to increase the dissolution rate from carnauba wax and synthetic wax-like ester formulations. The addition of glyceryl ester of hydrogenated rosin tended to decrease the dissolution rate of the drug from carnauba wax and hydrogenated castor oil formulations.

ALTHOUGH THE USE of spray-congealing techniques for modifying the physical, chemical, or physiological behavior of drugs has been in use for more than 10 years, information of direct value in the spray congealing of pharmaceutical systems has not appeared in the literature until very recently. Scott *et al.* (1) were probably the first pharmaceutical investigators to conduct an extensive study on the production factors influencing the size and distribution of spray-congealed particles. In their work, stearic acid-ethylcellulose mixtures were spray congealed using a specially instrumented spray dryer and a centrifugal wheel atomizer. The variables studied included atomizer wheel, feed rate, and feed viscosity.

A series of studies has been undertaken in these laboratories on spray-congealed particles prepared from a suspension of sulfaethidole (SETD) in melted waxes by atomizing through a compressed air-nozzle assembly. Cox (2) studied the effect of various production variables such as siphon height and atomizing pressure on the dissolution behavior and size of spray-congealed particles of SETD-white wax and SETD-synthetic wax-like ester. John and Becker (3) studied the effect of the surfactant sorbitan monooleate on the particle size and dissolution behavior of similar spray-congealed particles. Cusimano and Becker (4) prepared spray-congealed particles of SETD and different waxes and observed that their dissolution behavior depended on the chemical composition of the wax and the composition of the dissolution medium. The *in vitro* dissolution data obtained by using the rotating-bottle method (5) in these investigations could not be fitted to existing theoretical models for dissolution (6, 7) because these are based on the assumption that the total surface area of the dissolving solid does not change significantly during the period of observation. The dissolution model utilized was first proposed by Cox (2).

$$C = C_e[1 - (1 + t/d)^{-c}] \qquad \text{(Eq. 1)}$$

where C is the weight of drug in solution at a particular time t, C_e is the weight of drug in solution at equilibrium, and c and d are constants for a particular formulation.

The purpose of the present investigation was to

Received May 22, 1968, from the College of Pharmacy, University of Florida, Gainesville, FL 32601
Accepted for publication July 12, 1968.
Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
Abstracted in part from a dissertation presented by Y. Raghunathan to the Graduate School of the University of Florida, Gainesville, in partial fulfillment of Doctor of Philosophy degree requirements.
The authors express their appreciation to Dr. John I. Thornby, Statistical Consultant at the J. Hillis Miller Health Center, University of Florida, Gainesville, for his assistance in programming the dissolution data. The Assistance of the University of Florida Computing Center is also gratefully acknowledged.

determine the effect of the presence of certain modifiers in the spray-congealed particles of SETD and waxes on the *in vitro* dissolution behavior of the drug in acid pepsin medium pH 1.1 (simulated gastric fluid USP) and alkaline pancreatin medium pH 8.3 [test fluid B of Robinson and Swintosky (8)]. Additional experiments were also conducted to study the *in vitro* dissolution pattern of SETD from tablets made with these spray-congealed powders with and without disintegrants and from control tablets made with a mixture of SETD and spray-congealed powders of waxes with modifiers. The results of these will be reported separately.

EXPERIMENTAL

Experimental Design and Choice of Materials—The main consideration given in the selection of the waxes for this investigation was that the resulting congealed particles should be free flowing, so that they could be added to tablet granulations, if needed. The candidate modifiers should dissolve completely in these waxes when melted. Accordingly, carnauba wax, hydrogenated castor oil,[1] and a synthetic wax-like ester[2] were selected as the waxes. They are designated W_1, W_2, and W_3, respectively. The three modifiers used were: a low molecular weight polyethylene,[3] ethylcellulose 20 cps.,[4] and glyceryl ester of hydrogenated rosin.[5] These are designated M_1, M_2, and M_3, respectively, and were used in three levels of concentration. The content of SETD was kept constant in all the formulations at 25% w/w. The exact drug content of each product was determined by subsequent assay. The content of wax in the different formulations varied depending upon the concentration of the modifier added. The concentrations of the modifiers expressed as % w/w were: 0, 5, and 10 of low molecular weight polyethylene (M_1); 0, 1, and 2.5 of ethylcellulose (M_2); and 0, 5, and 10 of glyceryl ester of hydrogenated rosin (M_3). The concentration levels are designated as C_5, C_{10}, C_1, $C_{2.5}$ *etc.*, where the subscript indicates the % w/w of the modifier.

Preparation of Spray-Congealed SETD and Wax Particles—The appropriate quantity of wax was first melted in a stainless steel beaker. The modifier was then added and dissolved in the wax by stirring over very low heat. The stainless steel beaker was then transferred to a constant-temperature bath and maintained at the desired temperature for spray congealing (100 ± 2° for carnauba wax and castor wax formulations and 80 ± 2° for synthetic wax-like ester formulations). A sufficient quantity of SETD was next added and mixed with a variable speed mixer until a homogeneous dispersion was achieved. The mixer ran constantly during the spray congealing operation so that any settling of the drug would be avoided. The batch size was maintained constant at 2,000 g. The melted mix was atomized through an external-mixing pneumatic nozzle having an orifice diameter of 0.25 cm. (0.1 in.). The apparatus has been described previously (3). A Teflon siphon tube, wrapped with a heating tape, maintained the melted wax-drug mixture at the desired temperature while it passed up to the pneumatic nozzle. Dry air was passed through the nozzle at a pressure of 40 p.s.i. This was sufficient to deliver the melted wax-drug mixture to the nozzle and then bring about atomization. Since the air was not heated prior to entering the nozzle, the latter was equipped with a heater and a thermocouple monitoring system so that it could be maintained at the desired temperature during the spraying operation. This prevented interruption due to clogging of the nozzle by the congealing of melted wax drug. The nozzle orifice was directed into the collection chamber such that the particles of melted wax-drug congealed in the air. This resulted in a very fine powder which was collected and then passed through a No. 30 U.S. Standard sieve before conducting any of the subsequent experiments.

Particle-Size Measurements—A microscope fitted with an ocular micrometer was used to determine the particle-size distribution of each sample. The ocular micrometer was calibrated using the 45 × objective and a stage micrometer, the scale of which was 2 mm. in length. The calibration was such that one division of the micrometer scale of the eyepiece was equal to 0.267 μ.

A small sample of the material was withdrawn from the powder jar containing the product, after the latter had been thoroughly mixed by shaking for about 3 min. The sample was placed on a clean slide and a cover slip placed over it. The slide was mounted on the microscope and the 45 × objective brought into focus. In order to obtain a representative distribution, all particles within a microscopic field were measured and at least five different fields, each a representative of a particular section of the slide, were examined.

Assay of Spray-Congealed SETD-Wax Particles—Each product was formulated to contain about 25% SETD by weight. Chemical analyses were then performed on each product to determine the exact SETD content.

About 0.5 g. of the spray-congealed product, accurately weighed, was dissolved in 50 ml. of hot chloroform and the SETD was extracted with 5 portions of 0.10 *N* HCl. The acid extract was then assayed for SETD content by the Bratton-Marshall (9) procedure using a Klett-Summerson photoelectric colorimeter with a No. 54 filter.

A standard curve for SETD was also prepared and was found to be valid to a concentration of 2 mg. % SETD. Appropriate blank and standard solutions were run with all samples assayed.

In Vitro Dissolution Studies—The dissolution behavior of all spray-congealed products was determined using a rotating-bottle apparatus (5), and a procedure similar to that of Robinson and Swintosky (8). Rosen (10) has shown a direct rank relationship between *in vitro* dissolution data using the rotating-bottle method and the respective *in vitro* data in his studies on sustained-release preparations of prochlorperazine-S^{35}.

[1] Marketed as Castorwax M.P. 87 by Baker Castor Oil Co., Bayonne, N. J.
[2] Marketed as Glycowax S-932 by Glyco Chemicals, Inc., New York, N. Y.
[3] Marketed as Epolene E/10 by Eastman Chemical Products, Inc., Kingsport, Tenn.
[4] Marketed as Ethocel 20 CPS by Dow Chemical Co., Midland, Mich.
[5] Marketed as Stabelite Ester 5 by Hercules Powder Co., Wilmington, Del.

About 0.5 g. of the product, equivalent to 0.1200 g. of SETD as determined by prior assay, was accurately weighed into 90-ml. screw-capped bottles. Sixty milliliters of acid pepsin medium pH 1.1 (simulated gastric fluid USP), previously warmed to 37 ± 1°, was added to each bottle. Duplicate samples were allowed to rotate end over end at a speed of 40 to 45 r.p.m. in a water bath maintained at 37 ± 1°. The samples were removed at 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 2 hr. The samples were filtered immediately and an appropriate aliquot of the filtrate was then assayed for SETD content. Samples were also run for extended periods of time, 24 and 48 hr. However, equilibrium concentration was not attained in acid pepsin medium during this period.

Dissolution studies were also conducted in an alkaline environment in a similar manner. Again, the equivalent of 0.1200 g. of SETD was weighed into each bottle, and 60 ml. of alkaline pancreatin medium (pH 8.3) was added. Samples were removed for assay at 0.25, 0.5, 0.75, 1, 2, 3, 4, and 6 hr. Studies were also conducted for a period of 24 hr. at which time equilibrium was attained in most of the samples.

RESULTS AND DISCUSSION

Spray-Congealed SETD-Wax Products—All the spray-congealed SETD-wax products were fine powders. Carnauba wax and hydrogenated castor oil products were more free-flowing than the synthetic wax-like ester products. The latter showed a tendency to become lumpy on storage. However, the lumps could be broken up easily by shaking the container for a few minutes.

An examination of the production rates of the various formulations showed that the synthetic wax-like ester products, in general, could be sprayed at a faster rate than hydrogenated castor oil and carnauba wax formulations, in that order. This correlates with the relative hardness of the waxes. The synthetic wax-like ester was found to be the least hard of the three waxes and carnauba wax, the hardest, as determined by penetrometer[6] studies. All three modifiers increased the hardness when added to the synthetic wax-like ester formulation. The additives also slowed the rate of production. The same type of behavior was also noticeable in hydrogenated castor oil formulations. However, the difference in hardness among the carnauba wax formulations with the addition of modifiers was not so clearly detectable by the penetrometer.

Particle-Size Analysis—The size frequency data of the spray-congealed formulations were converted to a weight basis by assuming the shape and density factors to be constant throughout the size range according to the procedure described by Edmundson (11). The relative weight in each size interval was obtained by cubing the midpoint value of the interval and multiplying by the frequency. The cumulative frequency distributions by weight were found to be log-normally distributed. Figure 1 is a plot of the particle sizes of SETD-carnauba wax and SETD-carnauba wax with 10% low molecular weight polyethylene. The effect of the addition of this modifier to the formulation on the particle size is evident.

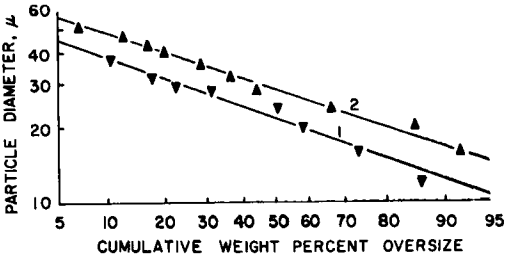

Fig. 1—Particle-size distribution (by weight) of spray-congealed particles of SETD-carnauba wax (1) and SETD-carnauba wax with 10% low molecular weight polyethylene (2).

A statistical analysis of the particle-size data of all the spray-congealed formulations showed that SETD-carnauba wax formulations were generally smaller particles with an average geometric weight mean diameter (dg_w) of 24.92 μ and an average geometric standard deviation (σ) of 1.52. SETD-hydrogenated castor oil formulations had an average dg_w of 29.14 μ and an average σ of 1.53, while SETD-synthetic wax-like ester formulations had an average dg_w of 27.53 μ and σ of 1.57. The addition of low molecular weight polyethylene to the waxes resulted in larger spray-congealed particles. As the modifier concentration was increased, the particle-size increase was observed to be linear and statistically significant. The average increase in dg_w when 10% of low molecular weight polyethylene was added to the waxes was 6.75 μ with no significant change in the standard deviation. The increase in dg_w with the addition of ethylcellulose or glyceryl ester of hydrogenated rosin was not very significant in the concentrations considered in the present study. There appeared, however, to be a decrease in the standard deviation in the formulations containing ethylcellulose, indicating the formation of uniform particles.

Microscopic examination of representative samples from the spray-congealed products showed that all the formulations yielded particles that appeared to be spherical in shape with smooth nonporous surface. Existence of some clusters of the particles was also noted in all the formulations.

Analysis of Dissolution Data—An inspection of the values of constant c in Eq. 1 obtained in the previous studies from this laboratory showed that the values were close to unity. The substitution of the constant c by 1 in Eq. 1 can simplify the equation as follows:

$$C = C_e[1 - (1 + t/d)^{-1}] \qquad \text{(Eq. 2)}$$

Equation 2 may be rearranged as follows:

$$\frac{C}{(C_e - C)} = \frac{t}{d} \qquad \text{(Eq. 3)}$$

Dividing Eq. 3 by C_e gives the integrated form of a second-order equation

$$\frac{C}{(C_e - C)C_e} = \frac{t}{d \cdot C_e} = k_2 t \qquad \text{(Eq. 4)}$$

[6] Precision Universal Penetrometer, Precision Scientific Company, Chicago, Ill.

The first derivative of Eq. 4 being

$$\frac{dC}{dt} = k_2(C_e - C)^2 \qquad \text{(Eq. 5)}$$

The dissolution rate from a solid by the Noyes-Whitney equation (6) is given as follows:

$$\frac{dC}{dt} = k \cdot S[(X - C) - (X - C_e)] \qquad \text{(Eq. 6)}$$

where S is the surface area of the dissolving solid, assumed constant, and X is the initial weight of the solid present. It is reasonable to assume that the effective surface area available at any time is approximately proportional to the amount of solid drug remaining to be dissolved. Thus, effective surface area may be written in terms of C as follows:

$$S = k_S[(X - C) - (X - C_e)] \qquad \text{(Eq. 7)}$$

where k_s is the proportionality constant.

When $C = C_e$, then $S = 0$. Thus effective surface should be nil at equilibrium concentration and no more of the solid dissolves. Substituting the right-hand side expression of Eq. 7 into Eq. 6,

$$\frac{dC}{dt} = k \cdot k_S(C_e - C)^2$$

or,

$$\frac{dC}{dt} = k_2(C_e - C)^2 \qquad \text{(Eq. 8)}$$

Thus an apparent second-order rate of dissolution should be observed with changing surface area. The initial dissolution rate, $k \cdot C_e^2$, may be obtained from Eq. 8 by substituting the initial conditions: when $t = 0$, then $C = 0$.

Figure 2 shows the plot of $C/[(C_e - C)C_e]$ *versus* t for the dissolution of plain SETD powder in acid pepsin medium. Figures 3, 4, and 5 show similar second-order plots of the dissolution data

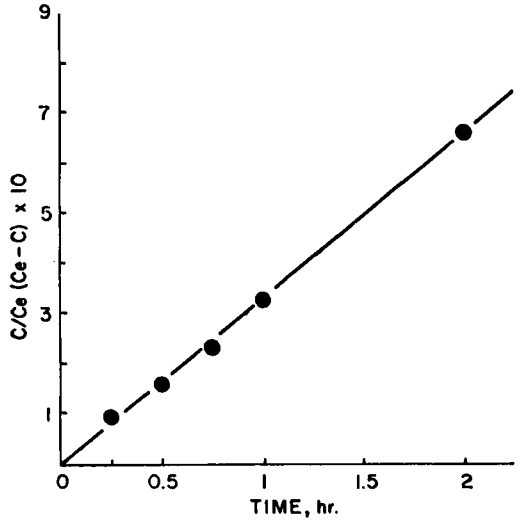

Fig. 2—Plot of $C/[(C_e - C)C_e]$ *as a function of time for the dissolution of plain SETD powder in acid pepsin medium.*

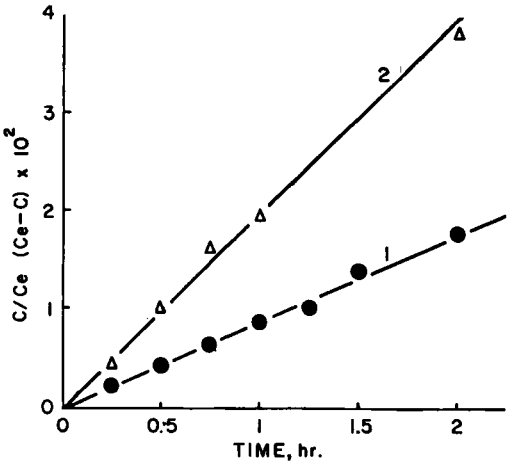

Fig. 3—Plot of $C/[(C_e - C)C_e]$ *as a function of time for the dissolution of SETD from spray-congealed particles of SETD-hydrogenated castor oil in acid pepsin medium (1) and alkaline pancreatin medium (2).*

of some of the spray-congealed formulations, indicating that the drug release pattern remains essentially the same as in the powder with only the rates being modified.

Figures represent cumulative percent of SETD released with time, of some of the spray-congealed formulations of hydrogenated castor oil showing the effect of the addition of low molecular weight polyethylene, ethylcellulose, and glyceryl ester of hydrogenated rosin, respectively. The points are experimental, whereas the lines are drawn from the predicted value obtained by fitting Eq. 2 by a modified Gauss-Newton nonlinear least-

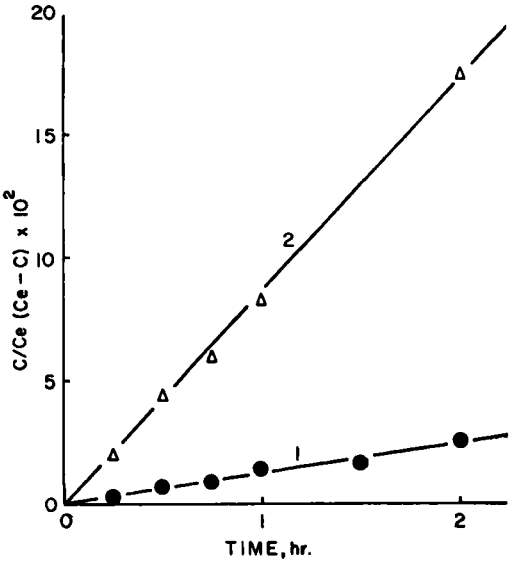

Fig. 4—Plot of $C/[(C_e - C)C_e]$ *as a function of time for the dissolution of SETD from spray-congealed particles of SETD-carnauba wax with 2.5% ethylcellulose in acid pepsin medium (1) and alkaline pancreatin medium (2).*

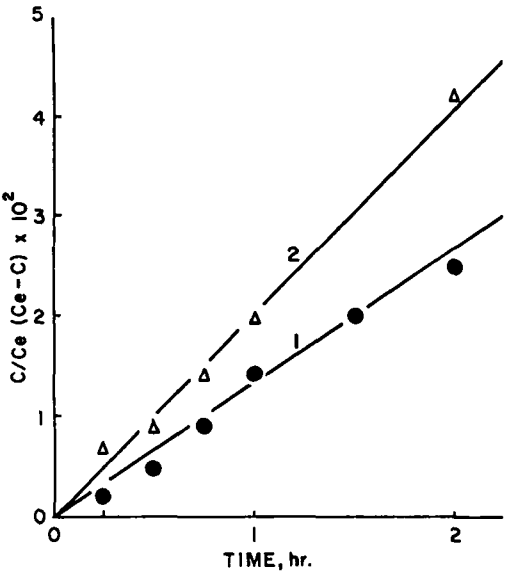

Fig. 5—Plot of $C/[(C_e - C)C_e]$ *as a function of time for the dissolution of SETD from spray-congealed particles of SETD-carnauba wax with 10% glyceryl ester of hydrogenated rosin in acid pepsin medium (1) and alkaline pancreatin medium (2).*

squares procedure (12). The mean standard deviation for the fit was 1.43% for the acid pepsin dissolution data and 1.90% for the alkaline pancreatin dissolution data.

Effect of Modifiers on the Dissolution Pattern of SETD-Carnauba Wax Formulations—Table I gives dissolution data of SETD-carnauba wax formulations with different modifiers. In general, it can be seen that the percentage of SETD released at equilibrium in alkaline pancreatin medium was higher than in acid pepsin medium. These observations are also reflected in higher second-order rate constants and higher initial dissolution rates. This may be mainly due to solubilizing or disintegrating effect of the alkaline pancreatin medium on carnauba wax, resulting in exposure of more effective surfaces of drug for dissolution. The effect of the addition of low molecular weight polyethylene in 10% concentration to the wax decreased the percentage of SETD released by about 5% in acid pepsin medium at the end of 2 hr. This is also reflected in the lower values observed for the second-order rate constant and the initial dissolution rate.

In alkaline pancreatin medium the percentage released at the end of 1 hr. was about 6% less, while at the end of 6 hr. the difference was more

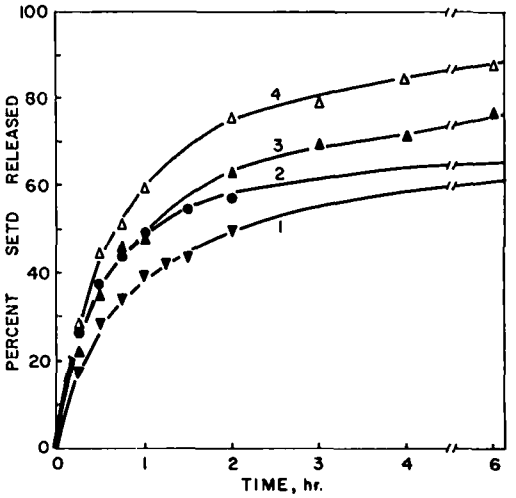

Fig. 7—Plot of cumulative percent of SETD released versus time in acid pepsin medium (1, 2) and alkaline pancreatin medium (3, 4) from SETD-hydrogenated castor oil (2, 4) and SETD-hydrogenated castor oil with 2.5% ethyl cellulose (1, 3).

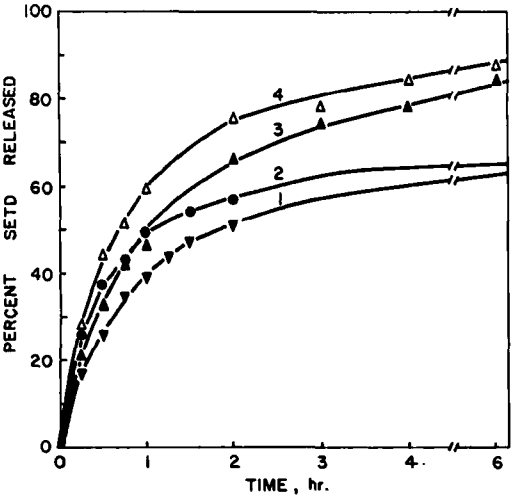

Fig. 6—Plot of cumulative percent of SETD released versus time in acid pepsin medium (1, 2) and alkaline pancreatin medium (3, 4) from SETD-hydrogenated castor oil (2, 4) and SETD-hydrogenated castor oil with 10% low molecular weight polyethylene (1, 3).

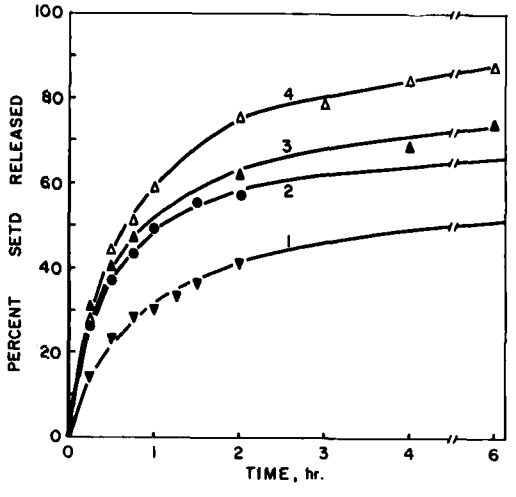

Fig. 8—Plot of cumulative percent SETD released versus time in acid pepsin medium (1, 2) and alkaline pancreatin medium (3, 4) from SETD-hydrogenated castor oil (2, 4) and SETD-hydrogenated castor oil with 10% glyceryl ester of hydrogenated rosin (1, 3).

TABLE I—DISSOLUTION DATA[a] EXPRESSED AS PERCENT SETD RELEASED FROM SETD-CARNAUBA WAX FORMULATIONS

Time, hr.	W_1		$W_1M_1C_{10}$		$W_1M_2C_{2.5}$		$W_1M_3C_{10}$	
	A[b]	B[c]	A	B	A	B	A	B
0.25	11.9	45.31	9.89	47.30	14.01	58.22	7.88	40.98
0.50	20.48	61.06	17.34	59.29	23.42	69.22	14.15	54.40
1	32.05	73.91	27.80	67.89	35.25	76.44	23.47	65.06
2	44.66	82.59	39.83	73.19	47.16	80.65	35.03	72.12
4	—	87.75	—	76.16		82.93	—	74.97
6	—	89.61	—	77.21		83.71	—	78.51
At equilibrium	73.33	93.59	70.20	79.39	85.48	85.33	68.83	80.90
k_2[d]	0.872	3.343	0.778	6.196	1.146	8.388	0.626	4.230
ID[e]	68.13	421.69	55.25	562.36	83.75	879.27	42.74	398.58

[a] Predicted values from least-square line. [b] A = acid pepsin medium. [c] B = alkaline pancreatin medium. [d] Apparent second-order rate constant $\times 10^2$ mg.$^{-1}$hr.$^{-1}$. [e] Initial dissolution rate mg. hr.$^{-1}$.

than 12%. The addition of low molecular weight polyethylene appeared to decrease the solubility of the wax matrix by the alkaline pancreatin medium resulting in only 79.39% release at equilibrium. This results in a higher rate constant and higher initial dissolution rate. The addition of ethylcellulose in 2.5% concentration to the wax increased the release of SETD in acid pepsin medium slightly, as can be seen from the slightly higher values for the rate constant and the initial dissolution rate. The dissolution was not very significantly affected, as noted by the percent of SETD release at the end of 2 hr. The amount of SETD released at equilibrium in the alkaline pancreatin medium was lower than the value observed with no modifier. This is reflected by the higher rate constant and higher initial dissolution rate. Under the microscope the spray-congealed particles containing this modifier were observed to contain short fibers. The presence of these fibers appeared to have a wicking effect resulting in increased initial release.

The addition of 10% glyceryl esters of hydrogenated rosin decreased the release of drug in acid pepsin medium by about 9% at the end of 2 hr. Again, the rate constant and the initial dissolution rate reflect this decrease. The release was also observed to be more gradual in the alkaline pancreatin medium with lower equilibrium value resulting in a higher rate constant and lower initial dissolution rate.

Effect of Modifiers on the Dissolution Pattern of SETD in SETD-Hydrogenated Castor Oil Formulations—Table II gives the dissolution data of SETD-hydrogenated castor oil formulations with different modifiers. In general, it can be seen as in the previous table, the amount of SETD released at equilibrium in alkaline pancreatin medium was higher than in acid pepsin medium. However, the initial dissolution rates are generally about the same, so that these result in lower rate constants. This is a very desirable feature from the standpoint of sustained-release formulations. The presence of low molecular weight polyethylene in 10% concentration appeared to retard the release of SETD by about 10% in acid pepsin medium and alkaline pancreatin medium as seen from the 1-hr. data and with almost no retardation in equilibrium values. This is well brought out in the lower rate constants and initial dissolution rates. It is conceivable that a higher concentration of low molecular weight polyethylene may further retard the initial dissolution rate, yet giving a complete release at the end of 6 hr. and hence warrants further study.

The addition of 2.5% ethylcellulose also retarded the dissolution of drug in both acid pepsin medium and alkaline pancreatin medium so that a more uniform release resulted. This can be seen from the closer values of the rate constants in the two media.

The addition of 10% glyceryl ester of hydrogenated rosin resulted in a release pattern similar to that observed in the case of product containing 2.5% ethylcellulose. The initial dissolution rate and the equilibrium values in acid pepsin medium were lower than with ethylcellulose resulting in a rate constant that was slightly higher than with ethylcellulose products. The initial dissolution rate and the rate constant in alkaline pancreatin were also higher.

TABLE II—DISSOLUTION DATA[a] EXPRESSED AS PERCENT SETD RELEASED FROM SETD-HYDROGENATED CASTOR OIL FORMULATIONS

Time, hr.	W_2		$W_2M_1C_{10}$		$W_2M_2C_{2.5}$		$W_2M_3O_{10}$	
	A[b]	B[c]	A	B	A	B	A	B
0.25	25.34	27.29	16.3	20.10	16.56	22.85	13.09	25.62
0.50	37.38	42.75	26.76	33.27	26.84	35.93	21.53	38.88
1	49.04	59.65	39.29	49.48	38.93	50.36	31.75	52.47
2	58.10	74.34	51.28	65.42	42.78	63.01	41.63	63.57
4	—	80.99	—	77.98	—	72.06	—	71.10
6	—	84.78	—	83.31	—	75.68	—	74.02
At equilibrium	71.29	98.63	73.83	96.50	70.80	84.14	60.45	80.64
k_2[d]	2.580	1.293	1.283	0.909	1.221	1.476	1.525	1.924
ID[e]	188.82	181.11	100.76	121.88	103.77	150.54	80.24	180.22

[a] Predicted values from least-square line. [b] A = acid pepsin medium. [c] B = alkaline pancreatin medium. [d] Apparent second-order rate constant $\times 10^2$ mg.$^{-1}$ hr.$^{-1}$. [e] Initial dissolution rate mg. hr.$^{-1}$.

TABLE III—DISSOLUTION DATA[a] EXPRESSED AS PERCENT SETD FROM SETD-SYNTHETIC WAX-LIKE ESTER FORMULATIONS

Time, hr.	W_3 A[b]	W_3 B[c]	$W_3M_1C_{10}$ A	$W_3M_1C_{10}$ B	$W_3M_2C_{2.5}$ A	$W_3M_2C_{2.5}$ B	$W_3M_2C_{10}$ A	$W_3M_2C_{10}$ B
0.25	10.83	23.5	2.49	5.24	14.59	29.90	16.07	37.21
0.50	18.74	36.08	4.62	9.90	24.03	44.19	26.38	53.99
1	29.53	49.26	8.09	17.85	35.52	58.06	38.86	69.71
2	41.46	60.27	12.95	29.80	46.67	68.87	50.99	81.59
4	—	67.85	—	44.78	—	75.94	—	89.19
6	—	70.82	—	53.80	—	78.62	—	92.04
At equilibrium	69.60	77.61	32.42	90.10	68.05	83.04	88.49	98.35
k_2[d]	0.883	1.865	0.854	0.228	1.337	1.463	1.260	2.063
ID[e]	61.58	161.81	12.93	26.71	89.17	222.02	98.61	287.36

[a] Predicted values from least-square line. [b] A = acid pepsin medium. [c] B = alkaline pancreatin medium. [d] Apparent second-order rate constant $\times 10^2$ mg.$^{-1}$hr.$^{-1}$. [e] Initial dissolution rate mg.hr.$^{-1}$.

Effect of Modifiers on the Dissolution Pattern of SETD in SETD-Synthetic Wax-like Ester Formulations—Table III gives the dissolution data of SETD-synthetic wax-like ester formulations with different modifiers. Here again, in general, the release of SETD was observed to be higher in the alkaline pancreatin medium than in the acid pepsin medium. The release in acid pepsin medium without modifier was about 41% in 2 hr. This was observed to be similar to the release pattern of carnauba wax. The rate constants and initial dissolution rates were also close. The release in alkaline pancreatin medium was, compared to the other two waxes, less complete being only about 70% at the end of 6 hr. This is reflected in a comparatively higher rate constant even though the initial dissolution rate is fairly low.

The addition of 10% low molecular weight polyethylene retarded the release of SETD in acid pepsin medium to about 13% in 2 hr. and about 54% in alkaline pancreatin medium in 6 hr. The retardation can also be seen in the lower values for the rate constant and initial dissolution rate.

The addition of 2.5% ethylcellulose to the synthetic wax-like ester increased the amount of SETD released by about 5% in 2 hr. in acid pepsin medium. The increase in release in alkaline pancreatin medium was also similar. These are also noticed by the higher rate constant in acid pepsin medium and higher initial dissolution rate in alkaline pancreatin medium.

The presence of 10% glyceryl ester of hydrogenated rosin in synthetic wax-like ester formulation resulted in more complete release in acid pepsin and alkaline pancreatin media as noticed by slightly higher rate constants and higher initial dissolution rates.

The analysis of the dissolution data reveals that for a good sustained-release effect from formulations such as spray-congealed particles for dissolution, the rate constant should be small and fairly unaffected by the pH or composition of the medium. They should also possess small initial dissolution rates and high equilibrium values. The purpose of this investiation has been, in addition to studying the effect of modifiers on SETD-wax formulations, to gain some insight into some of the factors controlling the dissolution process.

SUMMARY AND CONCLUSIONS

1. Atomization through the use of pneumatic nozzle and siphon tube was found to provide discretely formed, apparently nonporous spheres which possessed a log-normal particle-size distribution. These were observed to be characteristic for a particular wax formulation. The addition of low molecular weight polyethylene was observed to increase the geometric weight mean diameter of the particles significantly for all the wax formulations. The geometric weight mean diameter was unaffected by the addition of ethylcellulose and glyceryl ester of hydrogenated rosin in the concentration used in this investigation.

2. The release pattern from drug-wax particles appears to be second order, being a function of the amount of drug remaining undissolved and the effective surface area of the drug particles, thus agreeing basically with the Noyes-Whitney equation for dissolution.

3. The release rates from SETD-carnauba wax formulations were generally observed to be higher in the alkaline pancreatin than in acid pepsin medium. The addition of 10% low molecular weight polyethylene and 10% glyceryl ester of hydrogenated rosin reduced the initial dissolution rates and the value of the second-order rate constant in acid pepsin medium. The rate constant and the initial dissolution rates were higher in the alkaline pancreatin medium. The addition of 2.5% ethyl cellulose increased the initial dissolution rate and the rate constants in acid pepsin medium and alkaline pancreatin medium.

4. The second-order rate constant for SETD-hydrogenated castor oil formulation was lower in alkaline pancreatin medium than in acid pepsin medium as a result of more complete release at equilibrium in the former medium. However, the initial dissolution rates were almost the same in both media. The addition of 10% low molecular weight polyethylene tended to bring the values for the rate constants in both the media closer to each other. The initial dissolution rates were also much reduced as compared to the product containing no modifier. The release value at equilibrium in alkaline pancreatin medium was also noted to be more than 96%. The addition of 2.5% ethylcellulose and 10% glyceryl ester of hydrogenated rosin also decreased the value of the rate constants and the initial dissolution rate in acid pepsin medium. The rate constants were higher in alkaline pancreatin medium. The initial dissolution rate for the product with ethylcellulose was slightly reduced while it was essentially the same for the product with glyceryl esters of hydrogenated rosin.

5. The release rates of SETD from SETD-synthetic wax-like ester formulations were higher in the alkaline pancreatin medium than in the acid pepsin medium. The initial dissolution rates and the rate constants were also similarly affected. The addition of 10% low molecular weight polyethylene decreased the dissolution rate in alkaline pancreatin medium considerably. The initial dissolution rates in both the acid and alkaline medium were also considerably reduced. The addition of 2.5% ethylcellulose resulted in higher initial dissolution rates with the values of the rate constants also higher and closer to each other. The addition of 10% glyceryl ester of hydrogenated rosin increased the initial dissolution rates and the values of the rate constants.

6. The analysis of the data suggests that for a good sustained-release effect from formulations such as spray-congealed particles, the rate constants should be small and independent of pH and composition of the medium with low initial dissolution rates and high equilibrium values in alkaline pancreatin medium. This investigation attempted to gain some insight in that direction.

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Keyphrases

Spray-congealed formulations

Sulfaethidole–wax—prolonged release formulations

Modifiers, sulfaethidole-wax formulation—*in vitro* dissolution

Particle size—modifiers effect

Colorimetric analysis—spectrophotometer

Preparation and Properties of Some Relatives of Noscapine

By J. SAM, A. V. LOPEZ*, and R. M. SHAFIK

Fusion of phenylacetic acids with phthalic anhydrides resulted in the formation of benzylidenephthalides. Ammonolysis of the phthalides yielded 3-hydroxy-3-benzylphthalimidines which were easily dehydrated to 3-benzylidenephthalimidines. Bromination of 3-hydroxy-3-benzylphthalimidine with *N*-bromosuccinimide yielded 3-(α-bromobenzylidene)phthalimidine. Reaction of the latter with pyrrolidine gave the α-pyrrolidino derivative which on treatment with dilute hydrochloric acid resulted in 3-(α-hydroxybenzylidene)phthalimidine. Treatment of tetrahalobenzylidenephthalides with sodium methoxide produced 1,3-indandione derivatives. Mild sedative properties were noticed in compounds tested.

NOSCAPINE (I), formerly known as narcotine (1), is the most abundant of the opium alkaloids after morphine. Despite the fact that it comes from a plant rich in narcotic alkaloids it possesses none of the undesirable effects of narcotics; however, it possesses mild central nervous system activity similar to that of papaverine (2). Winter and Flataker (3) during their search for antitussive agents, discovered that noscapine was very effective in this respect. Further studies (4, 5) showed that in addition to its central effect noscapine also has bronchodilation activity. These factors prompted the in-

Received July 24, 1967, from the Department of Pharmaceutical Chemistry, The University of Mississippi, University, MS 38677

Accepted for publication July 17, 1968.

This investigation was supported in part by grant MH 05293 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and in part during a tenure of a fellowship (R.M.S.) of the Mississippi Heart Association.

Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.

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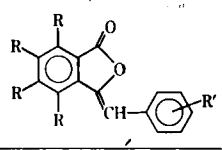
TABLE I—3-BENZYLIDENEPHTHALIDES (II)

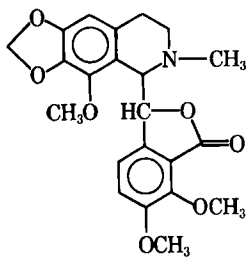


No.	R	R′	M.p., °C.	Yield, %	Molecular Formula	Anal., % Calcd.	Found
II*a*	H	H	98–100[a]	79	$C_{15}H_{10}O_2$[b]		
II*b*	Cl	H	290–291[c]	63	$C_{15}H_6Cl_4O_2$[d]	C, 50.0 H, 1.68 Cl, 39.4	C, 50.2 H, 1.77 Cl, 39.3
II*c*	Br	H	288–289[c]	70	$C_{15}H_6Br_4O_2$	C, 33.5 H, 1.14 Br, 59.4	C, 33.6 H, 1.26 Br, 59.3
II*d*	Cl	3,4-diCH_3O	221–223[c]	75	$C_{17}H_{10}Cl_4O_4$	C, 48.6 H, 2.39 Cl, 33.8	C, 49.0 H, 2.60 Cl, 33.5
II*e*	Br	3,4-diCH_3O	238–240[c]	65	$C_{17}H_{10}Br_4O_4$	C, 34.1 H, 1.68 Br, 53.5	C, 33.9 H, 1.91 Br, 52.5
II*f*	Cl	2-CH_3O	269–270[c]	60	$C_{16}H_8Cl_4O_3$	C, 49.3 H, 2.04 Cl, 36.4	C, 49.2 H, 2.29 Cl, 36.3
II*g*	Br	2-CH_3O	301–302[c]	60	$C_{16}H_8Br_4O_3$	C, 33.8 H, 1.42	C, 33.7 H, 1.70
II*h*	Cl	4-CH_3O	270–271[c]	75	$C_{16}H_8Cl_4O_3$	C, 49.3 H, 2.04 Cl, 36.4	C, 49.8 H, 2.26 Cl, 36.9
II*i*	Br	4-CH_3O	284–286[c]	75	$C_{16}H_8Br_4O_3$	C, 33.8 H, 1.42 Br, 56.3	C, 33.8 H, 1.59 Br, 56.3

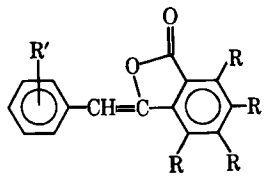
[a] Recryst. from ethanol. [b] *References 15, 16.* [c] Recrysd. from 2-ethoxyethanol (Cellosolve). [d] *Reference 17.*

vestigation of a series of substituted 3-benzylidenephthalides (II, Table I) and some of their nitrogen isosters (3-benzylidenephthalimidines). The 3-hydroxy-3-benzylphthalimidines (III) also possess a structural resemblance to chlorthalidone (IV) (6) which is a potent diuretic agent of long duration (7) and effective in the treatment of patients with cardiac edema (8). The latter, however, contains the sulfonamide group characteristic of diuretics. The 1,3-indandiones (X) were prepared for possible anticoagulant and anti-inflammatory activity (9–15).

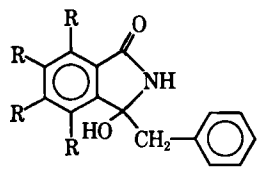
3-Benzylidenephthalides (II, Table I) were prepared using a slight modification of the method described by Gabriel (16) by fusing at high temperature a mixture of the appropriate phenylacetic acid and phthalic anhydride with a catalytic amount of anhydrous sodium acetate. The ammonolysis of 3-benzylidenephthalides with ammonium hydroxide solution resulted in the formation of 3-hydroxy-3-benzylphthalimidines (III) and not 2-phenylacetylbenzamide (V) as was reported by Gabriel (16). The structures (III) were substantiated by nuclear magnetic resonance spectra (NMR) and by the ease with which dehydration occurred on heating at 250–300° to give 3-benzylidenephthalimidines



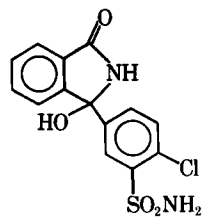
I



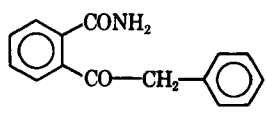
II



III

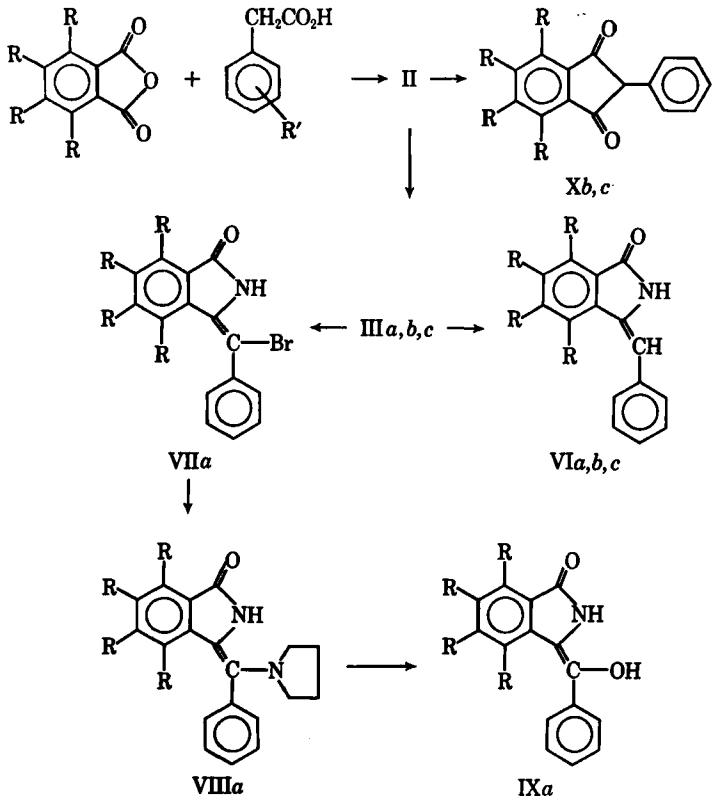


IV



V

(VI). Treatment of III*a* with *N*-bromosuccinimide gave 3-(α-bromobenzylidene)phthalimidine (VII*a*). Refluxing VII*a* with excess pyrrolidine



Scheme I

resulted in the formation of 3-(α-pyrrolidinobenzylidene)phthalimidine (VIII*a*) which upon treatment with dilute hydrochloric acid provided 3-(α-hydroxybenzylidene) phthalimidine (IX*a*). 3 - Hydroxy - 3 - benzyl - 4,5,6,7 - tetrahalophthalimidines could not be brominated in the same way using *N*-bromosuccinimide; dehydration to the corresponding benzylidenephthalimidines (VI*b*,*c*) occurred. Reaction of 3-benzylidene-4,5,6,7-tetrahalophthalides (II*b* and II*c*) with sodium methoxide yielded the tetrahaloindandiones (X*b* and X*c*). The IR spectral data of the compounds reported in this study are summarized in Table II.

PHARMACOLOGICAL RESULTS[1]

Compounds II*b*, II*c*, VI*b*, and VII*a* were administered by intraperitoneal injection to 25–30-g. albino mice as suspensions in 10% acacia solution. The mice were observed during the 3 hr. following injection and rechecked at 12 and 24 hr. No deaths were observed with any of the compounds among groups of 3–5 mice at maximum dosages of 1.5–2.0

TABLE II—INFRARED SPECTRAL DATA (cm.$^{-1}$)[a]

No.	OH	NH	C=O	C=C
II*a*			1,780	1,660
II*b*			1,800	1,645
II*c*			1,790	1,640
II*d*			1,770	1,640
II*e*			1,765	1,640
II*f*			1,790	1,640
II*g*			1,790	1,645
II*h*			1,785	1,640
II*i*			1,780	1,640
III*a*	3,350	3,200	1,690	
III*b*	3,550	3,300	1,700	
III*c*	3,450	3,320	1,700	
VI*a*		3,200	1,700	1,650
VI*b*		3,200	1,710	1,640
VI*c*		3,350	1,710	1,640
VII*a*		3,200	1,710	1,655
VIII*a*[b]		3,150	1,670	1,610
IX*a*	3,070	3,180	1,700	1,605
X*b*			1,740, 1,700	
X*c*			1,730, 1,705	

[a] The compounds in this table all exhibited characteristic aromatic absorptions, single band from 1,625–1,560 and multiple bands in region of 900–680. [b] Pyrrolidine methylene groups exhibit a doublet at 2,950–2,850.

[1] The authors are grateful to Dr. Marvin Davis, Department of Pharmacology, School of Pharmacy, The University of Mississippi, for the pharmacological data.

g./kg. With each of the compounds the mice showed inactivity, apparently being sedated slightly for more than 12 hr. None showed loss of righting, grasping, or pain reflexes and they remained reactive to loud sound stimuli. Ptosis was evident at 1.5 g./kg. with either IIc or VIIa.

Compounds IIa, IId, and IXa were administered by intramuscular injection to male albino mice, weighing from 25–50 g. with an average of 35 g., as a 10% suspension in 5% acacia soluion at maximum dosages of 1 g./kg. During the observation period of 24 hr. the animals were housed in small single-animal isolation cages. No food or water was available. The animals were observed for 2 hr. after injection and rechecked at 4, 6, 18, and 24 hr. Only slight sedation was observed with IIa and IId; no noticeable effects were seen with IXa and no deaths were observed with either of the three compounds.

EXPERIMENTAL[2]

3-Benzylidenephthalides (II, Table I)—Utilizing a method similar to that described by Gabriel (16), an intimate mixture of 0.67 mole of the proper phthalic anhydride, 0.80 mole of the desired phenylacetic acid, and 2.3 g. of anhydrous sodium acetate was heated at a temperature of 250–300° for 2–4 hr. The crude materials were purified by recrystallization from appropriate solvents to yield 60–79% of products.

The NMR spectrum of IIa showed a one-proton methine singlet (6.4) and a nine-proton aromatic multiplet (7.2–8.0).

3-Hydroxy-3-benzylphthalimidine (IIIa)—A solution of 75 g. (0.337 mole) of 3-benzylidinephthalide (IIa) in 100 ml. of ethanol and 100 ml. of concentrated ammonium hydroxide solution was refluxed for 4 hr. The resulting solution was poured over 200 g. of crushed ice and the solid was collected and recrystallized from water to yield 70 g. (87%) of product, m.p. 165–166°.

Anal.—Calcd. for $C_{15}H_{13}NO_2$: C, 75.3; H, 5.48; N, 5.86. Found: C, 75.4; H, 5.40; N, 6.00.

The NMR spectrum showed a one-proton hydroxyl singlet (2.9), a two-proton methylene singlet (3.4), a one-proton imino singlet (5.5), a five-proton aromatic singlet (7.2), and a four-proton aromatic multiplet (7.4–7.7).

3-Hydroxy-3-benzyl-4,5,6,7-tetrachlorophthalimidine (IIIb)—A mixture of 66 g. (0.183 mole) of 3-benzylidene-4,5,6,7-tetrachlorophthalide (IIb), 300 ml. of 2-ethoxyethanol, and 300 ml. of concentrated ammonium hydroxide solution was heated at 60–70° for 4 hr. The resulting pinkish reaction mixture was filtered; the filtrate was poured onto 200 g. of crushed ice and the solid was collected and recrystallized from ethanol to yield 20 g. (30%) of product, m.p. 215–217°.

Anal.—Calcd. for $C_{15}H_9Cl_4NO_2$: C, 47.8; H, 2.41; Cl, 37.6; N, 3.72. Found: C, 48.2; H, 3.00; Cl, 38.0; N, 3.61.

The NMR spectrum showed a one-proton hydroxyl singlet (2.9), a two-proton methylene multiplet (3.3–4.0), a five-proton aromatic singlet (7.2), and a one-proton imino singlet (8.6).

3-Hydroxy-3-benzyl-4,5,6,7-tetrabromophthalimidine (IIIc)—The above procedure was followed using 10 g. (0.018 mole) of 3-benzylidene-4,5,6,7-tetrabromophthalide (IIc), 300 ml. of 2-ethoxyethanol, and 300 ml of concentrated ammonium hydroxide solution. The yield of product was 7 g. (70%), m.p. 243–244°.

Anal.—Calcd. for $C_{15}H_9Br_4NO_2$: C, 32.5; H, 1.64; Br, 57.6. Found: C, 32.2; H, 1.79; Br, 57.3.

3-Benzylidenephthalimidine (VIa)—One gram (0.0042 mole) of 3-hydroxy-3-benzylphthalimidine (IIIa) was heated in an open vessel at 200–230° for 1 hr. The crude material which solidified on cooling was recrystallized from ethanol to yield 0.83 g. (91%) of product, m.p. 182–184°.

Anal.—Calcd. for $C_{15}H_{11}NO$: C, 81.4; H, 5.00; N, 6.33. Found: C, 80.9; H, 4.96; N, 6.85.

The NMR spectrum showed a one-proton methine singlet (6.5), a nine-proton aromatic multiplet (7.2–8.0), and a one-proton imino singlet (8.6).

3-Benzylidene-4,5,6,7-tetrachlorophthalimidine (VIb)—Two grams (0.0053 mole) of 3-hydroxy-3-benzyl-4,5,6,7-tetrachlorophthalimidine (IIIb) was heated in an open vessel at 300° for 30 min. The crude material which solidified on cooling was recrystallized from 2-ethoxyethanol to yield 1.8 g. (95%) of product, m.p. 331–332°.

Anal.—Calcd. for $C_{15}H_7Cl_4NO$: C, 50.2; H, 1.97; Cl, 39.5; N, 3.90. Found: C, 50.4; H, 1.88; Cl, 39.5; N, 4.06.

3-Benzylidene-4,5,6,7-tetrabromophthalimidine (VIc)—The above procedure was followed using 2 g. (0.0036 mole) of 3-hydroxy-3-benzyl-4,5,6,7-tetrabromophthalimidine (IIIc). The yield of product was 1.5 g. (70%), m.p. 304–305°.

Anal.—Calcd. for $C_{15}H_7Br_4NO$: C, 33.6; H, 1.31; Br, 59.5; N, 2.60. Found: C, 33.4; H, 1.32; Br, 59.3; N, 2.66.

3-(α-Bromobenzylidene)phthalimidine (VIIa)—The procedure described by Ziegler (18) for allylic brominations was followed using 40 g. (0.168 mole) of 3-hydroxy-3-benzylphthalimidine (IIIa), 28 g. (0.17 mole) of *N*-bromosuccinimide, and 1 l. of anhydrous chloroform. The mixture was refluxed for 12 hr., concentrated to 200 ml., and then allowed to cool to room temperature. Both succinimide and the product precipitated from solution. The succinimide was removed by washing the mixture with three successive portions of 100 ml. of boiling water. The residual material was recrystallized from ethanol to yield 29 g. (60%) of product, m.p. 211–213°.

Anal.—Calcd. for $C_{15}H_{10}BrNO$: C, 60.0; H, 3.36; Br, 26.6; N, 4.67. Found: C, 60.2; H, 3.13; Br, 26.9; N, 4.78.

The NMR spectrum showed a nine-proton aromatic multiplet (7.2–7.9) and a one-proton imino singlet (8.6).

3-(α-Pyrrolidinobenzylidene)phthalimidine (VIIIa)—A mixture of 15 g. (0.05 mole) of 3-(α-bromobenzylidene)phthalimidine (VIIa) and 125 ml. of pyrrolidine was refluxed for 6 hr. after which time water was added to the solution until precipitation occurred. The crude yellow product was collected and recrystallized from ethanol to yield 7 g. (50%) of product, m.p. 176.5–178°.

[2] Melting points were taken in open glass capillaries using a Thomas-Hoover melting point apparatus and are uncorrected. IR spectra were determined on a Perkin-Elmer Model 137 Infracord spectrophotometer using potassium bromide pellets. NMR spectra were determined on a Varian A-60A spectrometer, using tetramethylsilane as an internal standard; chemical shifts are recorded as δ values.

Anal.—Calcd. for $C_{19}H_{18}N_2O$: C, 78.6; H, 6.25; N, 9.65. Found: C, 79.1; H, 6.21; N, 9.39.

The NMR spectrum showed a four-proton methylene multiplet (1.7–2.1), a two-proton methylene triplet (2.9–3.2), a two-proton methylene triplet (3.3–3.6), a nine-proton aromatic multiplet (6.8–7.6), and a one-proton imino singlet (11.0).

A picrate was prepared in the usual manner and recrystallized from ethanol; m.p. 198–200°.

Anal.—Calcd. for $C_{25}H_{21}N_5O_8$: C, 57.8; H, 4.07; N, 13.50. Found: C, 57.8; H, 4.12; N, 13.30.

3-(α-Hydroxybenzylidene)phthalimidine (IXa)—A mixture of 4 g. (0.0137 mole) of 3-(α-pyrrolidinobenzylidene)phthalimidine (VIII*a*) and 400 ml. of a 10% hydrochloric acid solution was heated on a steam bath for 2 hr. The white solid was collected and recrystallized from ethanol to yield 2 g. (61%) of product, m.p. 185–187°.

Anal.—Calcd. for $C_{15}H_{11}NO_2$: C, 75.9; H, 4.67; N, 5.90. Found: C, 75.9; H, 4.60; N, 5.74.

The NMR spectrum showed a broad hydroxyl peak (3.5–4.0), a nine-proton aromatic multiplet (7.2–7.9), and a one-proton imino singlet (8.9).

2-Phenyl-4,5,6,7-tetrachloro-1,3-indandione (Xb)—The procedure described by Zalukajevs (15) for the preparation of 2-phenyl-1,3-indandione was followed using 3.6 g. (0.01 mole) of 3-benzylidene-4,5,6,7-tetrachlorophthalide (II*b*) and 50 ml. of 2-ethoxyethanol. The mixture was heated until solution occurred and while still hot was added to 30 ml. of methanol in which 0.5 g. of sodium had been dissolved. The solution was allowed to cool to room temperature and then diluted with 50 ml. of methanol and 150 ml. of water, respectively. The solution was then acidified with concentrated hydrochloric acid whereupon precipitation of a crude yellow material occurred. The material was collected by filtration and recrystallized from glacial acetic acid to yield 3 g. (83%) of product, m.p. 222–223°.

Anal.—Calcd. for $C_{15}H_6Cl_4O_2$: C, 50.0; H, 1.68; Cl, 39.4. Found: C, 50.4; H, 1.19; Cl, 39.6.

2-Phenyl-4,5,6,7-tetrabromo-1,3-indandione (Xc)—The above procedure was followed using 5.37 g. (0.01 mole) of 3-benzylidene-4,5,6,7-tetrabromophthalide (II*c*). The crude material was recrystallized from glacial acetic acid to yield 3.75 g. (70%) of product, m.p. 257–258°.

Anal.—Calcd. for $C_{15}H_6Br_4O_2$: C, 33.5; H, 1.14; Br, 59.4. Found: C, 33.7; H, 1.22; Br, 59.5.

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Keyphrases

Noscapine-related compounds—synthesis
Sedative activity—noscapine-related compounds
NMR spectroscopy—structure
IR spectrophotometry—structure

Analysis of Pharmaceuticals by Ultraviolet Densitometry on Thin-Layer Chromatograms I

Parabens in Gels and Creams

By HERBERT SCHRIFTMAN

Precision of about 1 percent and excellent recovery are attainable by quantitative densitometry of ultraviolet chromophores separated by thin-layer chromatography on plates containing a phosphor. Quenching of the fluorescent background affords a sensitive measure of concentration. Substrates with native or derived fluorescence can be determined on plates by fluorometry using either a nonfluorescent background or a phosphor that fluoresces at different wavelengths. This report describes application of these and similar techniques to the determination of parabens in pharmaceutical and cosmetic products. The densitometry technique requires simple solvent extraction of the paraben-preserved product and chromatography on silica gel thin layers containing phosphor. Use of a 4:1 pentane–acetic acid system provided resolution of the parabens from each other, and no interference was encountered with hydrocortisone acetate, partial sodium salt of *n*-lauryl β-imino diproprionic acid, EDTA, polyethylene glycol, hydroxyethyl cellulose, hydroxypropyl methylcellulose, D & C Green No. 5 dye, and various waxes and fats. Preliminary investigations for applying direct ultraviolet densitometry to other types of thin layers are mentioned.

IN A RECENT comprehensive review (1) on the use of TLC in pharmaceutical analysis, Comer and Comer attributed the major disadvantages of quantitative TLC to the "lack of precision and accuracy of direct measurement instrumentation or the tedious nature of removal of absorbent containing the desired compounds." This report describes direct, accurate, and precise determination of paraben mixtures on TLC plates by UV densitometry, which eliminates the aforementioned difficulties. The procedures for the parabens are exemplary, for the general technique may be extended to many other mixtures containing UV chromophores.

Mixtures of methyl and propyl parabens are widely used as preservatives in foods, drugs, and cosmetics, and several procedures have been reported for the separation and determination of these esters. Paper (2), column (3), and thin-layer (4) chromatographic methods have been reported in which the separated parabens are quantitated by spectrometry after elution. Donato (5) described a method combining UV spectrometry for determining total parabens with gas chromatography for determining their ratio. A subcommittee of the PMA Quality Control Section has recently evaluated a gas chromatographic assay for parabens which makes use of the internal standard technique (6).

In the method described here, the parabens are spotted and separated by ascending TLC using Brinkmann silica gel plates with phosphor and a pentane–acetic acid (4:1) solvent system, after a relatively simple solvent extraction from the product. The separated parabens are analyzed *in situ* by photoelectric densitometry on a Photovolt recording TLC densitometer. The total absorbance of each spot expressed as the total area under the density curve is measured by K & E planimeter or alternately by a Photovolt recording integrator, and compared by direct proportion to a reference spot concomitantly analyzed.

Application of this procedure to a pharmaceutical gel is evaluated and found to be comparable in precision, speed, and accuracy to Donato's (5) method.

Besides having applicability to other products containing parabens (cosmetic creams, pharmaceutical ointments, and antacid suspensions), the technology described here has been extended to other mixtures containing UV chromophores. Investigations for applying direct UV densitometry to the assay of other drug products as well as using other types of TLC plates and sheets (*i.e.*, cellulose) and other media possessing UV transparency (*i.e.*, agarose—a new agar gel used for thin-layer electrophoresis) are in progress.

Received May 22, 1968 from the Pharmaceutical Research and Development Laboratories, Warner-Lambert Research Institute, Morris Plains, NJ 07950

Accepted for publication July 17, 1968.

Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

The author wishes to express his gratitude to P. R. Amin for some of the technical assistance, and to Dr. L. Chafetz for his encouragement and advice.

EXPERIMENTAL

Reagents—ACS grades of absolute methanol, diethyl ether, anhydrous sodium sulfate, and glacial acetic acid and chromatoquality grade of pentane (Matheson) were employed.

Equipment—Brinkmann Silica Gel GF TLC plates (20 × 20 cm.) with fluorescent indicator, micropipet[1] (0.001 and 0.002 ml.), Photovolt densitometer model 530 with recording and integrator attachments, 15-ml. screw-cap vials, and appropriate chromatographic containers were used.

General Procedure—Aliquots of the sample containing the equivalents of 2.5 to 15 mg. of esters are transferred to a separator adapted for centrifugation where the esters are extracted with several portions of 5-ml. volumes of ether. The ether phases are collected, washed with distilled water, and filtered through sodium sulfate and Whatman No. 1 paper into a 50-ml. beaker. Another 5-ml. ether wash of the filter is collected and the combined ether solution is evaporated gently on a steam bath with a stream of air. The residue is dissolved in exactly 1.0 ml. of methanol and transferred into a 15-ml. screw-cap vial. The appropriate quantity of the solution is spotted on an activated and dried Brinkmann plate. The quantity spotted ranged from three times 0.001 to five times 0.002 ml. Concomitantly, a standard containing equivalent quantities is spotted. Due to uncontrollable high humidity conditions in this laboratory, it was found necessary to redry the activated plates in a vacuum desiccator after the spotting operation. Special care was also required for maintaining a low water content in the solvent systems, *i.e.*, keeping a beaker of silica gel (indicating type) in the chromatography tank. As a matter of course, all ensuing experiments were conducted in this manner. The dried plate is placed in the tank containing a mixture of 160 ml. of pentane and 40 ml. of acetic acid; and the solvent is permitted to ascend the plate about 15 cm. (in about 30–45 min.). The plate is air-dried and scanned face down with the recording TLC densitometer using a short-wave UV light (254 mμ) and a phototube detector fitted with a 485-mμ color filter. The total absorbance of each spot expressed as the total area under the density curve is measured and compared by a K & E planimeter or alternately by an integrator. Propylparaben migrates faster than methylparaben, and their relative migrations in comparison with other parabens are illustrated in Fig. 1. Figure 2 shows a typical separation of the methylparaben and propylparaben spots with their corresponding recorded and integrated density curves.

Specific Assays—*Pharmaceutical Gels*—About 5 g. of gel accurately weighed (equivalent to about 7.5 and 2.5 mg. of methyl and propylparaben, respectively) is dissolved in a beaker with 5 ml. of a methanol–water mixture (1:1). The solution is quantitatively transferred with two additional 5-ml. portions of solvent into a separator adapted for centrifugation. Four 5-ml. volumes of ether are employed for the subsequent extraction and centrifugation steps, discarding the lower aqueous phases and collecting the upper ether layers into a regular separator. The collected extracts are washed twice with water and filtered through sodium sulfate and Whatman No. 1 paper into a 50-ml. beaker. Another 5-ml. ether wash of the filter is collected and the combined ether solution is evaporated gently on a steam bath with a stream of air. The residue is dissolved in exactly 1.0 ml. of methanol and transferred into a 15-ml. screw-cap vial. Three times 0.001 ml. of this solution is spotted on a Brinkmann TLC plate and analyzed as described above in the *General Procedure*. Concomitantly, a standard containing 75 and 25 mg. of methyl and propylparaben, respectively, per 10 ml. of methanol is spotted and compared.

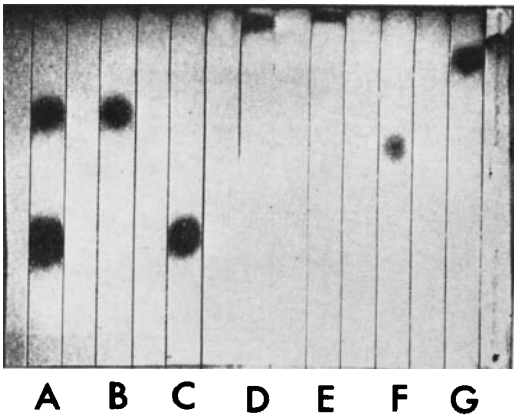

Fig. 1—Relative migration of parabens. Key: A, *methyl and propylparaben;* B, *propyl;* C, *methyl;* D, *hexyl;* E, *heptyl;* F, *ethyl;* G, *butyl paraben.*

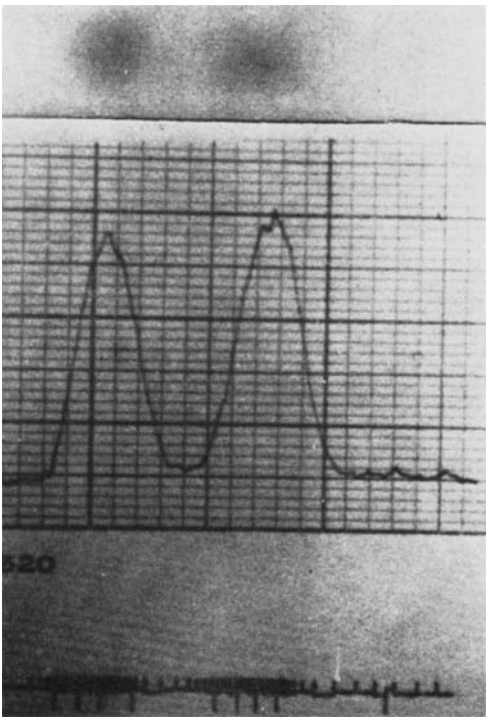

Fig. 2—Typical separation of the methylparaben and propylparaben spots with their corresponding recorded and integrated density curves.

[1] Microcaps, Drummond Scientific Co.

TABLE I—ANALYSIS OF GEL

Sample	Wt., g.	% of Declared Value—Methyl Paraben	% of Declared Value—Propyl Paraben
1	5.3405	98.8 (98.3, 99.2)	101.9 (102.6, 101.1)
2	6.1025	99.2 (98.8, 99.6)	99.5 (98.1, 100.8)
3	5.0380	102.2	100.0
4	5.2115	102.6	100.7
5	5.1450	99.7	99.9
6	5.4061	101.2	100.6

Statistical Evaluation

Methyl Paraben		Propyl Paraben	
average	= 100.5	average	= 100.3
σ	= 1.6	σ	= 0.8
3σ	= 4.8	3σ	= 2.4

Cosmetic Cream—Approximately 10 g. of sample accurately weighed into a beaker is well mixed and stirred with 25 ml. of hot methanol on a steam bath. The hot mixture is filtered through glass wool into a 50-ml. conical flask. Additional 5- to 10-ml. volumes of hot methanol are used for transferring the contents of the beaker and washing the filter. The solution is chilled in refrigerator or ice bath and filtered cold through Whatman No. 1 paper. Flask and filter paper are washed with additional quantities (2 × 5 ml.) of chilled methanol. The combined filtrate is concentrated to one-half its volume on a Rinco rotary vacuum evaporator. The cooling, filtering, and evaporation steps are repeated and the final residue is dissolved in methanol and transferred quantitatively into a 10-ml. volumetric flask. Five times 0.002 ml. is applied to Brinkmann plates and analyzed as described above. The standard solution in this example contains 15 and 10 mg. of methylparaben and propylparaben, respectively, per 10 ml. of methanol.

RESULTS AND DISCUSSION

The analytical data for the gel was comparable in precision and accuracy with the values previously reported in other chromatographic methods (2–5). For example, our σ values of 1.6 and 0.8 (see Table I) are comparable to Donato's (5) results of 1.2 and 3.1 (calculated from his data) for methylparaben and propylparaben. In addition, Donato's GLC method requires the formation of the trimethylsilyl ether derivatives, determination of their ratio, and finally UV spectrophotometric measurement of the total parabens. In the method presented here, the separated parabens are still intact and available for further study or identification. In addition, the recovery data for a gel placebo formulation, to which known quantities are added, is in the range of 97.2–101.6% of theory (see Table II). Similar precision and accuracy were obtained with the cosmetic creams.

TABLE II—RECOVERY DATA[a]

Sample	Recovery, % Methyl Paraben	Recovery, % Propyl Paraben
1	97.2	100.3
2	99.1	101.6

[a] 7.5 mg. and 2.5 mg. of methyl and propyl paraben, respectively, were added for each 5 g. of gel placebo.

No interferences or difficulties were encountered from the excipients in the above formulations which included partial sodium salt of *n*-lauryl β-imino dipropionic acid (Deriphat 160), EDTA, polyethylene glycol, hydroxyethyl cellulose (Natrosol, Hercules), hydroxypropyl methylcellulose NF (Methocel), D & C Green No. 5 dye, and various waxes and fats.

OTHER APPLICATIONS

Preliminary investigations for applying direct UV densitometry to other multicomponent drug products and other types of layers are listed below. Detailed and comprehensive reports of these applications are in preparation and will be available at a later date.

1. Antacid suspensions containing parabens were assayed using the method outlined above except that cellulose thin layers were employed and in most instances the extraction technique was further simplified.

2. Gel chromatography and electrophoresis techniques were developed in which multicomponent products were separated and analyzed *in situ* in a specially designed gel tray. Using these trays the analyst could also observe periodically or continuously the progress of the migration and separation of the compounds.

3. Cellulose thin layers were substituted for paper in the various chromatographic and electrophoretic analyses of pharmaceutical products reported by this author (7–10). In those instances, the papers were developed by various chromogenic reagents, and then analyzed by color densitometry. In the current study, the thin-layer chromatograms and electrophoretograms are evaluated by UV densitometry without any prior treatment.

4. Oxolinic acid (a new synthetic antimicrobial agent specially effective against *Proteus* in urinary tract infections) and its various analogs were analyzed by the technology described here, based on their fluorescent properties.

SUMMARY

TLC and UV densitometry have been successfully applied for analyzing the paraben contents of pharmaceutical gels and cosmetic creams. The procedure compares favorably in precision, accuracy, and speed with present acceptable technology, *i.e.*, GLC. Application of UV densitometry to the

assay of other products and drugs as well as the use of other thin-layer media is indicated and will be reported at a later date.

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Keyphrases

Parabens in gels, creams—analysis
UV densitometry on TLC—analysis
UV light, phototube detector—scanning

3-Substituted-2-benzoxazolinones

By JOSEPH SAM, J. L. VALENTINE, and C. W. RICHMOND

The preparation of 2-amino-5-trifluoromethylbenzoxazole, 5-trifluoromethyl-2-benzoxazolinone and their chemical precursors are described. The reaction of appropriately substituted 2-benzoxazolinones with substituted alkyl halides provided the 3-substituted 2-benzoxazolinones.

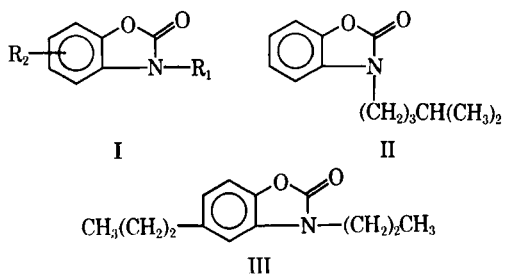
PREVIOUS INVESTIGATIONS (1–4) on the medicinal value of 3-substituted-2-benzoxazolinones (I, Table I) prompted the present work on other 3-substituted derivatives. Lespagnol and co-workers (5, 6) studied the time required for 2-benzoxazolinone and some 3-substituted derivatives to produce immobilization in fish. Swinyard and associates (7) demonstrated that the 3-substituted derivatives prepared by Lespagnol were active in antagonizing maximal electroshock states. Subsequently, Close and co-workers (2) studied a number of 3-substituted derivatives for their analgesic activity. Two of these derivatives (II and III) were tested clinically and proved to be less active than indicated by the animal studies

The 2-benzoxazolinones (XI) utilized in this study were prepared according to procedures described in earlier reports; however, additional studies were conducted on the preparation of 5-trifluoromethyl-2-benzoxazolinone (VI).

Previously, the preparation of VI *via* two routes was reported (1). One of these routes involved the condensation of phosgene with 2-amino-4-trifluoromethylphenol (V), whereas the alternate route involved the use of the intermediates 2-nitro-4-trifluoromethylphenyl ethyl carbonate (VII) and the corresponding amino derivative (VIII) *in situ*. Compounds VII and VIII now have been isolated and the structures elucidated. (Scheme I.)

The preparation of VII was accomplished by condensing IV with ethyl chloroformate. Catalytic reduction of VII gave VIII which upon treatment with aqueous hydrochloric acid yielded VI.

The pronounced biological properties of 5-chloro-2-aminobenzoxazole (8) prompted the authors to investigate the preparation of 5-trifluoromethyl-2-aminobenzoxazole (X). The con-



I II III

Received April 29, 1968, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Mississippi, University, MS 38677
Accepted for publication July 18, 1968.
Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
Abstracted in part from theses submitted by J. L. Valentine and C. W. Richmond to the Graduate School, The University of Mississippi, in partial fulfillment of Master of Science and Doctor of Philosophy degree requirements, respectively.
The authors are grateful to the A. H. Robins Company for financial support of the project.

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3-Substituted-2-benzoxazolinones

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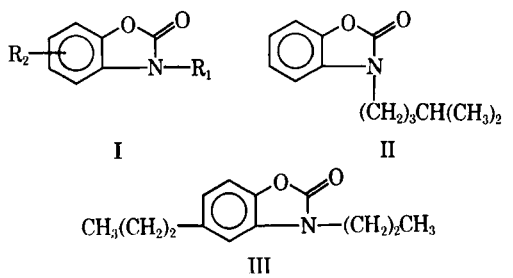

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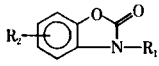
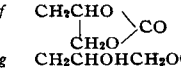
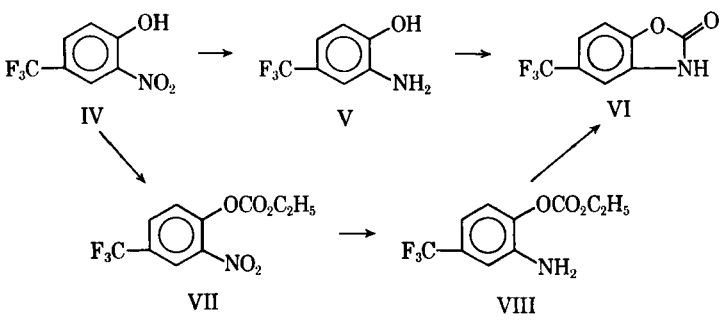
The authors are grateful to the A. H. Robins Company for financial support of the project.

TABLE I—3-SUBSTITUTED-2-BENZOXAZOLINONES



No.	R_1	R_2	Recryst. From[a]	Method	Yield, %	M.p., °C.	Formula	Anal. Calcd.	Anal. Found
I*a*	$CH_2CH_2SCH_3$	5-F	W	A	56	71–72	$C_{10}H_{10}FNO_2S$	C, 52.9 H, 4.4 N, 6.2	C, 53.2 H, 4.6 N, 6.5
I*b*	$CONH_2$	H	E–W	D	84	144–146	$C_8H_6N_2O_3$	C, 53.9 H, 3.4 N, 15.7	C, 54.2 H, 3.6 N, 15.0
I*c*	$CON(CH_3)_2$	H	W	E	53	68–69	$C_{10}H_{10}N_2O_3$	C, 58.2 H, 4.9 N, 13.6	C, 58.3 H, 5.2 N, 13.6
I*d*	$(CH_2)_3OH$	H	—	A	81	—	$C_{10}H_{11}NO_3$[b]		
I*e*	$CH_2CHOHCH_2OH$	H	B	A	70	73–75	$C_{10}H_{11}NO_4$	C, 57.4 H, 5.3 N, 6.7	C, 57.5 H, 5.5 N, 6.7
I*f*		H	E	F	68	184–185	$C_{11}H_9NO_5$	C, 56.2 H, 3.7 N, 6.0	C, 56.4 H, 3.7 N, 5.9
I*g*	$CH_2CHOHCH_2OCONH_2$	H	W	G	50	136–137	$C_{11}H_{12}N_2O_5$	C, 52.4 H, 4.8 N, 11.1	C, 52.1 H, 4.7 N, 10.7
I*h*	$(CH_2)_3OCONH_2$	H	E–W	H	50	142–143	$C_{11}H_{12}N_2O_4$	C, 55.9 H, 5.1 N, 11.9	C, 56.2 H, 5.2 N, 11.8
I*i*	$CH_2CH(CH_3)_2$	H	E–W	A	60	63–64	$C_{11}H_{13}NO_2$	C, 69.1 H, 6.9 N, 7.3	C, 69.2 H, 6.9 N, 7.5
I*j*	CH_2CCH	H	E–W	A	70	97–98	$C_{10}H_7NO_2$	C, 69.4 H, 4.1 N, 8.1	C, 69.2 H, 4.0 N, 8.2
I*k*	$(CH_2)_2CH_3$	H	—	A	72	—	$C_{10}H_{11}NO_2$[c]	C, 67.8 H, 6.3 N, 7.9	C, 67.8 H, 6.2 N, 8.0
I*l*	CH_2CHCH_2	5-Cl-6-Br	M	A	72	70–71	$C_{10}H_7BrClNO_2$	C, 41.6 H, 2.4 N, 4.9	C, 41.6 H, 2.4 N, 4.7
I*m*	$(CH_2)_3OCH_3$	5,6-diCl	E	I	82	90–91	$C_{11}H_{11}Cl_2NO_3$	C, 47.8 H, 4.0 N, 5.1	C, 47.7 H, 4.0 N, 4.9
I*n*	$(CH_2)_3N(CH_3)_2$	5-CF_3	E	A	68	180–181	$C_{19}H_{18}F_3N_5O_9$[d]	C, 44.8 H, 3.5 N, 13.5	C, 44.2 H, 3.3 N, 13.6
I*o*	$(CH_2)_3Cl$	H	M	B	89	64–65	$C_{10}H_{10}ClNO_2$	C, 56.7 H, 4.8 N, 6.7	C, 56.6 H, 4.7 N, 6.4
I*p*	$(CH_2)_3Cl$	5,6-diCl	E	B	82	135–136	$C_{10}H_8Cl_2NO_2$	C, 42.7 H, 2.9 N, 5.0	C, 42.5 H, 3.0 N, 5.1
I*q*	$(CH_2)_3Cl$	5-Cl-6-Br	B	B	91	130–131	$C_{10}H_8BrCl_2NO_2$	C, 37.0 H, 2.5 N, 4.3	C, 37.2 H, 2.6 N, 4.1
I*r*	$(CH_2)_3Cl$	5-CF_3	M–W	B	90	47–48	$C_{11}H_8F_3ClNO_2$	C, 47.3 H, 3.2 N, 5.0	C, 47.2 H, 3.1 N, 5.2
I*s*	$(CH_2)_3NC_8H_4O_2$	H	D–W	C	81	179–181	$C_{18}H_{14}N_2O_4$[e]	C, 67.1 H, 4.4 N, 8.7	C, 67.4 H, 4.5 N, 8.7
I*t*	$(CH_2)_3NC_8H_4O_2$	5-Cl-6-Br	C	C	81	214–215	$C_{18}H_{12}BrClN_2O_4$[e]	C, 49.6 H, 2.8 N, 6.4	C, 49.4 H, 2.9 N, 6.2
I*u*	$(CH_2)_3NC_8H_4O_2$	5,6-diCl	C	C	81	219–220	$C_{18}H_{12}Cl_2N_2O_4$[e]	C, 55.3 H, 3.1 N, 7.2	C, 55.1 H, 3.2 N, 7.1

[a] W = water, E–W = ethanol–water, E = ethanol, B = benzene, M = methanol, D–W = dioxane–water, C = ethyl cellosolve, M–W = methanol–water. [b] B.p. 135–140°/0.05 mm., analysis as I*h*. [c] B.p. 122–124°/0.05 mm., n_D^{25} 1.5365. [d] Picrate, free base, b.p. 138–142°/2.5 mm., n_D^{26} 1.4892. [e] Phthalimido.



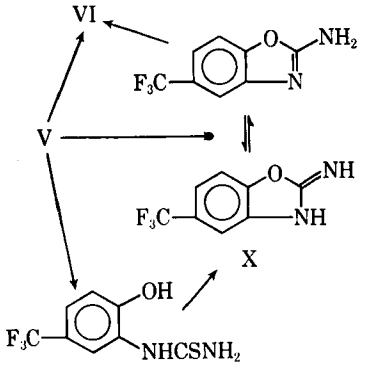
Scheme I

TABLE II—ACUTE INTRAPERITONEAL TOXICITY

No.[a]	LD_{50}, mg./kg.
I*a*	>1,000
I*c*	>1,000
I*e*	>1,000
I*h*	>1,000
I*j*	400–800
I*l*	400–800
I*m*	>1,000
I*n*	100–300
I*p*	>1,600
I*q*	>1,600
I*s*	>1,000
I*t*	>1,000
IV	800–1,600
V	>1,000
VI	200–400
VII	800–1,600
VIII	>1,000
IX	>1,000

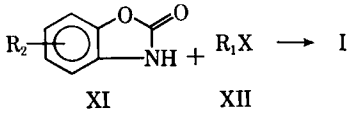
[a] Refers to compound numbers found in Table I and in the text.

densation of V with ammonium thiocyanate yielded 2-hydroxy-5-trifluoromethylphenyl thiourea (IX) which upon treatment with yellow lead oxide gave X. The preparation of X also was accomplished *via* condensation of V with cyanogen bromide. Refluxing X in dilute acid provided an alternate route to VI. (Scheme II.)



Scheme II

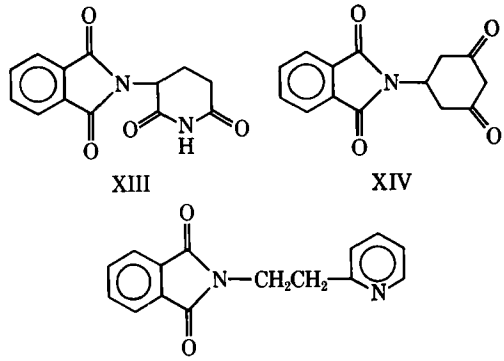
The 3-substituted-2-benzoxazolinones (I, Table I) were obtained from the condensation of the potassium salt of the requisite 2-benzoxazolinone (XI) with an appropriately substituted alkyl halide (XII). (Scheme III.)



Scheme III

The treatment of I*e* with phosgene gave I*f* which upon subsequent reaction with ammonia yielded I*g*. The opening of carbonates such as I*f* with ammonia to give the primary carbamate (*e.g.*, I*g*) has been rigorously demonstrated (9). The reaction of I*p* with sodium methoxide and I*o*, I*p*, and I*q* with potassium phthalimide provided the corresponding methoxy (I*m*) and phthalimido (I*s*, I*t*, and I*u*) derivatives, respectively, whereas the reaction of I*d* with phosgene, followed by ammonia gave the corresponding carbamate (I*h*).

The phthalimide derivatives are structurally related to the medicinally active thalidomide (XIII) (10), *d,l*-3-phthalimidoglutarimide (XIV) (11), and *N*-(2-pyridyl)ethylphthalimide (XV) (12).



PHARMACOLOGICAL RESULTS[1]

Fasted female Pharmakon mice (19–28 g.) were used for this study. The test animals were observed for 4 hr. posttreatment for signs of toxicity and for observable pharmacological effects. Thereafter, the animals were observed daily for 3 days. All compounds were administered intraperitoneally. Table II summarizes the acute intraperitoneal toxicity.

Compounds I*a*, I*c*, I*e*, I*h*, I*j*, I*n*, and I*t* showed good muscle relaxation at 1,000 mg./kg. accompanied by less motor activity, slow respiratory rate, and depression. The degree of muscle relaxation was less at 300 and 100 mg./kg. with all the above compounds exclusive of I*e* which exhibited these properties only at 1,000 mg./kg. Compounds V, VIII, and IX showed good depression at 100 mg./kg. with less muscle tone and motor activity; however these activities reached a minimum at 30 mg./kg. Compound I*m* had weak depressant properties at 100 mg./kg. but depressant activity was abolished at 30 mg./kg. Compound I*s* exhibited depression, catatonia, and reduced motor activity at 1,000, 300, 100, and 30 mg./kg.

[1] The authors are grateful to Dr. John Ward, A. H. Robins Co., Richmond, Va., for the pharmacological data.

TABLE III—INFRARED SPECTRAL DATA OF 3-SUBSTITUTED-2-BENZOXAZOLINONES[a]

No.[b]	Methylene[c]	Carbonyl	Other Groups
Ia	2,925, 1,470	1,775	—CH_3; 1,460, 1,380; —SCH_3; 1,325
Ib	—	1,800, 1,750	—$CONH_2$; 3,500, 3,300
Ic	—	1,775, 1,700	—$CON(CH_3)_2$; 1,145
Id	2,925, 1,480	1,750	—OH; 1,175, 1,095
Ie	2,925, 1,480	1,750	di-OH; 1,100, 1,085
If	2,900, 1,480	1,775	—OCOO—; 1,180, 1,090
Ig	2,950, 1,480	1,775, 1,680	—$OCONH_2$; 1,125; —OH; 1,100
Ih	1,490	1,775, 1,725	—$OCONH_2$; 1,110
Ii	2,975, 1,480	1,775	—CH_3; 1,380, 1,360, 1,145
Ij	2,900, 1,480	1,760	—$CH_2C{\equiv}CH$; 1,430
Ik[d]	2,900, 1,470	1,750	—CH_3; 3,000, 1,460, 1,350
Il	1,470	1,775	—$CH_2CH{=}CH_2$; 1,430
Im	2,925, 1,470	1,775	—CH_3; 1,450, 1,380; —OCH_3; 1,350
In[d]	2,925, 1,470	1,775	—CH_3; 2,750, 1,460;, —$N(CH_3)_2$; 1,160, 1,120
Io	2,850, 1,480, 725	1,775	—CH_2Cl; 680
Ip	2,900, 1,470, 725	1,775	—CH_2Cl; 675
Iq	2,900, 1,480, 720	1,775	—CH_2Cl; 675
Ir	2,925, 1,480, 725	1,775	—CH_2Cl; 680
Is	2,925, 1,480, 725	1,775, 1,710	—
It	2,925, 1,480, 710	1,775, 1,700	—
Iu	2,950, 1,480, 725	1,775, 1,710	—

[a] The compounds in this table all exhibit characteristic aromatic absorptions, single peak from 1,630–1,600, multiplet peaks in regions of 1,225–950 and 900–700. [b] Refers to compound numbers found in Table I. [c] Absorption at 2,975–2,900 of weak intensity. [d] Liquid film.

EXPERIMENTAL[2]

Syntheses—5-Fluoro-2-benzoxazolinone (1), 2-benzoxazolinone (13), 5-chloro-6-bromo-2-benzoxazolinone (14), 5,6-dichloro-2-benzoxazolinone (14), and 2-nitro-4-trifluoromethylphenol (15) were prepared according to reported procedures.

3-Substituted-2-benzoxazolinones—*Method A*—A modification of the procedure described by Close *et al.* (2) was followed. A solution of 0.05 mole of KOH in 200 ml. of ethyl cellosolve was treated with 0.05 mole of an appropriate 2-benzoxazolinone (Table I) and thereafter stirred at room temperature for 2 hr. In one portion was added 0.05 mole of an appropriate alkyl halide. The entire mixture was stirred and refluxed for 4 hr. The solid was removed by filtration and the filtrate concentrated under reduced pressure on a steam bath. The residue was distilled and the resultant distillate was recrystallized from an appropriate solvent (Table I).

3-(3-Chloropropyl)-2-benzoxazolinones—*Method B*—The procedure used in Method A was followed using 0.05 mole of an appropriate 2-benzoxazolinone, 0.05 mole of KOH, and 0.4 mole of 1-bromo-3-chloropropane.

3-(3-Phthalimidopropyl)-2-benzoxazolinones—*Method C*—A warm solution of 0.05 mole of a requisite 3-(3-chloropropyl)-2-benzoxazolinone in 200 ml. of ethyl cellosolve was stirred and treated, in one portion, with 9.26 g. (0.05 mole) of potassium phthalimide and thereafter refluxed for 4 hr. The resulting precipitate (KCl) was removed by filtration and the filtrate concentrated under reduced pressure on a steam bath. The residual solid was then recrystallized from a suitable solvent (Table I).

3-Carbamyl-2-benzoxazolinone — *Method D*—A modification of the procedure described by Mooradian (16) was followed. A solution of 5.4 g. (0.04 mole) of 2-benzoxazolinone and 7.5 g. (0.05 mole) of *N,N*-diethylaniline in 40 ml. of chloroform was added dropwise at room temperature to a solution of 5.0 g. (0.05 mole) of phosgene in 75 ml. of toluene. The reaction mixture was stirred for 24 hr., saturated with ammonia gas, and filtered. The filtrate was washed with 5% HCl and water, respectively, and then concentrated under reduced pressure. The residual solid was recrystallized.

3-(N,N-Dimethylcarbamoyl)-2-benzoxazolinone—*Method E*—To a stirred solution of 6.75 g. (0.05 mole) of 2-benzoxazolinone in 50 ml. of pyridine at 0–5° was added dropwise 5.35 g. (0.05 mole) of *N,N*-dimethylcarbamoyl chloride. The mixture was stirred for 1 hr. at 0–5° and then refluxed for 1 hr. The solution was cooled to room temperature and thereafter treated with 50 ml. of anhydrous ether. The solid which separated was removed by filtration; the filtrate was concentrated under reduced pressure and the residual solid recrystallized.

4-(2-Benzoxazolinon-3-yl)methyl-1,3-dioxolan-2-one—*Method F*—A modification of the procedure described by Lunsford *et al.* (17) was followed. To a cooled solution (5–10°) of 10.45 g. (0.05 mole) of *Ie* in 300 ml. of anhydrous benzene and 200 ml. of diethylene glycol was added dropwise a solution of 5.0 g. (0.05 mole) of phosgene in 100 ml. of anhydrous benzene. The resulting solution was stirred and maintained at 5–10° for 1 hr. The solution, after being allowed to attain room temperature, was treated with 7.95 g. (0.1 mole) of pyridine and stirred for an additional 10 hr. The reaction mixture was washed three

[2] All melting points were taken on a Fisher-Johns melting point apparatus and are corrected. IR spectra (Table III) were obtained with a Perkin-Elmer model 137B Infracord spectrophotometer using KBr pellets; values are recorded as cm.$^{-1}$. The NMR spectra were determined on a Varian A-60A spectrometer using Me_4Si as an internal standard; chemical shifts are recorded as δ values.

times with water, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residual solid was recrystallized.

3 - (2 - Benzoxazolinon - 3 - yl) - 2 - hydroxypropyl Carbamate—*Method G*—To 100 ml. of concentrated ammonium hydroxide solution at 0° was added 8 g. (0.02 mole) of *If*. The solution was stirred at 0° for 1 hr. and then concentrated under reduced pressure. The resultant solid was recrystallized.

3 - (2 - Benzoxazolinon - 3 - yl)propyl Carbamate—*Method H*—A solution of 3.5 g. (0.035 mole) of phosgene and 5.5 g. of *N,N*-diethylaniline in 100 ml. of benzene was treated dropwise with a solution of 6.0 g. (0.03 mole) of *Id* in 100 ml. of anhydrous benzene. The solution was maintained at 5–10° for 4 hr., then allowed to warm to room temperature and thereafter saturated with ammonia. The benzene was distilled under reduced pressure; the resultant solid was washed with water and then recrystallized.

3 - (3 - Methoxypropyl) - 5,6 - dichloro - 2 - benzoxazolinone—*Method I*—A solution of 1.2 g. (0.05 mole) of sodium ribbon and 14.0 g. (0.05 mole) of *Ip* in 200 ml. of methanol was refluxed for 30 min. The solution was concentrated to one-fourth the original volume and treated with 250 ml. of water. The precipitate was filtered and recrystallized.

2-Amino-4-trifluoromethylphenol (V)—The procedure described by Pettit and Tatlow (15) was modified by using 0.1 g. of platinum oxide. From 20.7 g. (0.1 mole) of 2-nitro-4-trifluoromethylphenol there was obtained 17.3 g. (98%) of product, m.p. 126.5–127° after recrystallization from water [lit. (15) m.p. 121–122°]; $\nu_{max.}$ 3,000 (NH_2), 1,150, 1,200 (phenolic OH); NMR (in d_2-DMSO), 3-proton singlet at 6.8 (aromatic CH), 2-proton singlet at 7.0 (NH_2), 1-proton singlet at 3.2–3.4 (phenolic OH).

2-Nitro-4-trifluoromethylphenyl Ethyl Carbonate (VII)—A modification of the procedure described by Sam *et al.* (1) was followed. A cooled solution (5–10°) of 2.94 g. (0.062 mole) of NaOH and 17.5 g. (0.089 mole) of IV in 40 ml. of water was treated with 7.96 g. (0.074 mole) of ethyl chloroformate and thereafter maintained at 5–10° with stirring for 1 hr. The solid was removed by filtration and recrystallized from ethanol–water to give 16.6 g. (95%) of product, m.p. 87.5–88°; $\nu_{max.}$ 1,750 (C=O); NMR (in d_5-pyridine), 3-proton multiplet at 7.75–8.0 (aromatic CH), 2-proton multiplet at 4.15–4.4 (CH_2), 3-proton multiplet at 1.2–1.4 (CH_3).

Anal.—Calcd. for $C_{10}H_8F_3NO_5$: C, 43.02; H, 2.88; N, 5.01. Found: C, 43.15; H, 2.72; N, 5.24.

2-Amino-4-trifluoromethylphenyl Ethyl Carbonate (VIII)—A solution of 13.9 g. (0.05 mole) of VII in 100 ml. of ethanol was hydrogenated at 40 p.s.i. using 0.1 g. of platinum oxide catalyst until the theoretical amount of hydrogen had been absorbed (*ca.* 2 hr.). The catalyst was removed by filtration and the ethanol distilled under reduced pressure. The residual dark yellow solid was recrystallized from benzene to give 13.0 g. (93%) of product, m.p. 194–195°; $\nu_{max.}$ 3,200 (NH_2), 1,675 (C=O); NMR (in d_5-pyridine), 3-proton triplet at 7.1–7.3 (aromatic CH), 2-proton multiplet at 4.1–4.5 (CH_2), 3-proton multiplet at 1.05–1.35 (CH_3), 2-proton singlet at 8.83 (NH_2).

Anal.—Calcd. for $C_{10}H_{10}F_3NO_3$: C, 48.20; H, 4.05; N, 5.62. Found: C, 48.44; H, 4.24; N, 5.83.

2-Hydroxy-5-trifluoromethylphenyl Thiourea (IX)—The procedure described by Sam and Plampin (14) was followed. From 17.8 g. (0.1 mole) of V and 9.2 g. (0.12 mole) of ammonium thiocyanate there was obtained 12.2 g. (68%) of product, m.p. 151–152°, after recrystallization from water; $\nu_{max.}$ 3,200 (NH, NH_2), 1,625 (C=O), 1,175, 1,125 (phenolic OH); NMR (in d_2-DMSO), 3-proton multiplet at 7.16–7.73 (aromatic CH), 2-proton singlet at 8.66 (NH_2), 1-proton singlet at 9.25 (NH), 1-proton broad singlet at 10.73–10.96 (phenolic OH); upon D_2O exchange the absorptions at 8.66, 9.25, and 10.73–10.96 were absent.

Anal.—Calcd. for $C_8H_7F_3N_2OS$: C, 40.67; H, 2.99; N, 11.89. Found: C, 40.78; H, 3.17; N, 11.73.

2-Amino-5-trifluoromethylbenzoxazole (X)—*Method J*—The method described by Sam and Plampin (14) for the preparation of 2-aminobenzoxazoles was followed using 11.8 g. (0.05 mole) of IX and 22.2 g. (0.1 mole) of yellow lead oxide. The product (9.3 g., 67%) was recrystallized from water, m.p. 146–147°; $\nu_{max.}$ 3,200 (NH_2 or 2NH); NMR (in d_2-DMSO), 5-proton multiplet at 7.33–7.83 (aromatic CH, NH_2, or 2NH); D_2O exchange showed that two protons were exchanged.

Anal.—Calcd. for $C_8H_5F_3N_2O$: C, 47.53; H, 2.49; N, 13.86. Found: C, 47.59; H, 2.23; N, 13.74.

Method K—The method described by Sam and Plampin (14) for the preparation of 2-aminobenzoxazoles was followed using 16.9 g. (0.15 mole) of cyanogen bromide and 17.7 g. (0.1 mole) of V. The product (12.5 g., 71%) was recrystallized from water, m.p. 146–147°. A mixture of the products obtained from Methods *J* and *K* showed no depression of the melting point. The IR and NMR spectra were identical.

5-Trifluoromethyl-2-benzoxazolinone (VI)—*Method L*—The method described by Sam and Plampin (14) was followed using 15.0 g. (0.07 mole) of X. The product (13.5 g., 82%) was recrystallized from water, m.p. 169–170° [lit. (1) 169–170°]; $\nu_{max.}$ 1,750 (C=O); NMR (in *d*-$CHCl_3$), 3-proton multiplet at 7.2–7.5 (aromatic CH), 1-proton singlet at 10.29 (NH).

Method M—A solution of 5 g. (0.018 mole) of VIII in 250 ml. of 20% aqueous HCl was refluxed for 18 hr. and then cooled to room temperature. The precipitate was filtered and recrystallized from water to give 2.0 g. (45%) of product, m.p. 169–170°. A mixture with the product obtained in Method *L* showed no depression of the melting point. The IR and NMR spectra were identical.

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Keyphrases

3-Substituted-2-benzoxazolinones—synthesis
2-Amino-5-trifluoromethylbenzoxazole—synthesis
5-Trifluoromethyl-2-benzoxazolinone—synthesis
Pharmacological screening—3-substituted-2-benzoxazoles
IR spectrophotometry—structure
NMR spectroscopy—structure

Drug Standards

Quantitative Determination of Gallamine Triethiodide

By PETER P. ASCIONE, JOHN B. ZAGAR, and GEORGE P. CHREKIAN

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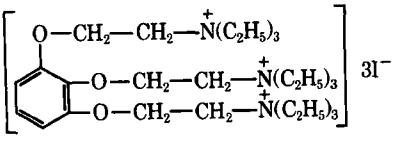

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Pharmaceutical dosage form and the crystalline powder are currently being assayed by the procedure outlined in the NF XII (3). This procedure is based on the Volhard volumetric titration of the iodide present in the molecule. However this procedure is capable of measuring halogens other than the iodide portion of the molecule, thereby lacking specificity for gallamine. A spectrophotometric determination was investigated but it was found to be a function of the iodide portion of the molecule rather than the active triethylammonium groups.

The purpose of this investigation was to develop a rapid and more specific procedure which could be employed in quality control laboratories for analyzing gallamine triethiodide both in

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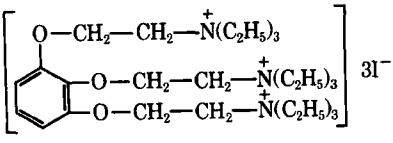

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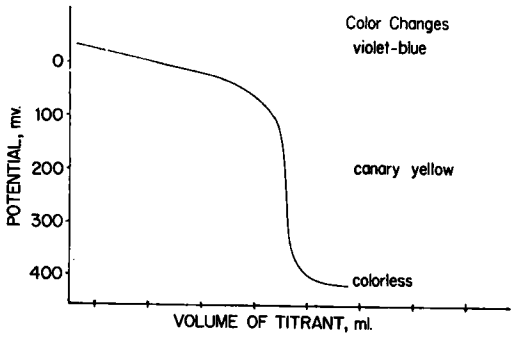

Fig. 1—Titration of gallamine triethiodide showing potentiometric inflection and color change of bromophenol blue indicator.

pharmaceutical dosage form and as a crystalline powder.

EXPERIMENTAL

Apparatus—Precision-Dow Recordomatic titrimeter equipped with a glass electrode and a sleeve-type calomel electrode. The calomel electrode was modified by replacing the aqueous saturated solution of potassium chloride in the bridge with saturated solution of potassium chloride in methanol.

Reagents and Solutions—Chloroform; *p*-dioxane, *N,N*-dimethylformamide (DMF); glacial acetic acid; 1% bromophenol blue in DMF; 6% mercuric acetate in glacial acetic acid; 0.1 *N* perchloric acid in glacial acetic acid [standardized against primary standard potassium acid phthalate by the procedure of the USP XVII (4)]; phosphate buffer: dissolve 38 g. of monobasic sodium phosphate and 2 g. of anhydrous dibasic sodium phosphate in water to make 1,000 ml.; bromocresol green dye solution: dissolve 200 mg. of bromocresol green in 100 ml. of water by adding 6.4 ml. of 0.1 *N* sodium hydroxide and dilute to 250 ml. with water, wash the solution with five 10-ml. portions of chloroform, and filter; standard preparation: dissolve in water a suitable quantity of gallamine triethiodide reference standard and dilute quantitatively and stepwise to obtain a solution having a concentration of 100 mcg. per ml.

Nonaqueous Method—*Assay Procedure*—Weigh accurately 0.2 g. of gallamine triethiodide into a 250-ml. beaker and dissolve in 10 ml. of DMF. Add 5 ml. of 6% mercuric acetate; then add 150 ml. of *p*-dioxane and mix the solution with the aid of magnetic stirring. The titration is followed potentiometrically using 0.1 *N* perchloric acid in glacial acetic as the titrant. In addition to the potentiometric determination, visual titrations can also be carried out using 6 drops of 1% bromophenol blue in DMF as the indicator, which changes from purple to canary yellow at the stoichiometric end point. Each milliliter of 0.1 *N* perchloric acid is equivalent to 29.72 mg. of gallamine triethiodide.

Colorimetric Acid-Dye Extraction Method—*Assay Preparation*—Pipet an accurately measured volume of gallamine triethiodide injection and dilute quantitatively and stepwise to obtain a solution having a concentration of 100 mcg./ml. in water.

Procedure—Pipet 5 ml. of assay preparation into a 125-ml. separator. To a second 125-ml. separator pipet 5 ml. of standard preparation. To each separator add 10 ml. of phosphate buffer solution and 5 ml. bromocresol green solution. Extract each solution with four 20-ml. portions of chloroform, collecting the chloroform extracts in 100-ml. volumetric flasks. Add chloroform to volume in each flask and mix. Concomitantly determine the absorbances of both solutions in 1-cm. cells at 416 mμ on a suitable spectrophotometer, using chloroform as the blank.

RESULTS AND DISCUSSION

Gallamine triethiodide possesses three quaternary nitrogen groups, each exhibiting basic characteristics in nonaqueous media. These three basic groups are susceptible to titration by perchloric acid. The nonaqueous method specifically and distinctively measures the quaternary nature of this compound.

The solvent of choice for determining the purity of crystalline gallamine triethiodide was *p*-dioxane. However due to the limited solubility of this compound, it was first necessary to effect solution using DMF. Using the ratio of DMF to *p*-dioxane as outlined under *Experimental*, a well-defined potentiometric end point shown in Fig. 1 was obtained when titrated with 0.1 *N* perchloric acid in acetic acid.

The bromophenol blue end point of the titration was found to appear sharply and occurred at the stoichiometric end point. This was determined potentiometrically with the indicator in the system as shown in Fig. 1. To obtain a stoichiometric end point it was necessary to add mercuric acetate to the solution of gallamine triethiodide prior to titration (5).

Noted in Table I are the results obtained by the nonaqueous method and acid-dye extraction procedure on various lots of crystalline powder and batches of dosage form. The methods are rapid, simple, more specific, and provide quantitative results.

The colorimetric acid-dye method is based on the quaternary nitrogen groups each having the basic characteristics in a dye extraction (6). These three basic groups react with bromocresol green at pH 5.3 and are distinguished because of their solubility in chloroform.

TABLE I—SUMMARY OF ANALYTICAL DATA FOR ANALYSIS OF CRYSTALLINE POWDER AND INJECTABLE DOSAGE FORM OF GALLAMINE TRIETHIODIDE

	Method		
	Potentiometric Titration, %	Visual Titration, %	Colorimetric Acid-Dye Extraction, mg./ml.
Crystalline Powder			
Lot 1	100.3	99.7	
Lot 2	99.5	99.6	
Lot 3	99.0	98.7	
Lot 4	98.6	99.3	
Injectable Form 20.6 mg./ml.			
Batch 1			21.1
Batch 2			21.3
Batch 3			20.5
Batch 4			20.1

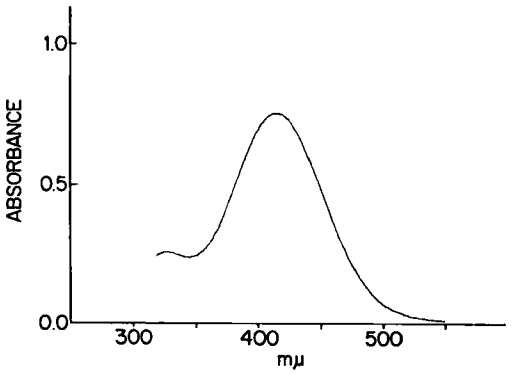

Fig. 2—Spectrum of acid-dye complex of gallamine triethiodide.

The absorption spectrum of the acid-dye chloroform extract was determined on a Cary model II recording spectrophotometer. Figure 2 shows the absorption spectra in the region 550–350 for the gallamine acid-dye extract. The wavelength where maximum absorption occurred was 416 mμ.

Various concentrations of gallamine triethiodide were evaluated by the acid-dye extraction procedure for the purpose of preparing a standard calibration curve. A typical linear Beer's law plot was obtained when the absorbance of the acid-dye extract *versus* concentration of gallamine triethiodide was plotted in the range of 2 to 10 mcg./ml.

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Titration, nonaqueous—analysis
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Perchloric acid—titrant
Colorimetric analysis—spectrophotometer
Bromocresol green-gallamine triethiodide—complex

Technical Articles

Instrumentation of a Rotary Tablet Machine

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The Manesty 45 station Rotapress was instrumented to measure compressional force, ejection force, and lower punch pulldown force. Various positions in the pressure linkage were monitored to determine the optimum location for compressional force measurement. The upper part of the compression column just below the link to the upper roller carriage was chosen for installation of strain gauges for compressional force measurement. Ejection force was measured by installing a force washer beneath the head of the bolt which holds the ejection cam in place. The ejection cam was not modified in any way. Lower punch pulldown force was measured by using a bolt containing internally mounted strain gauges to replace one of the three bolts normally holding the pulldown cam in position. Examples of the results obtained by compressing antacid tablets at various speeds and forces are given. Data collected over a 24-day period on the compression of these tablets under regular production conditions are given.

ROTARY TABLET machine instrumentation has been described by Knoechel (1–3) and Wray (4). Sites for gauging and methodology have been developed for certain types of American-made machines such as the Stokes BB-2, Stokes 541, and other similar presses. It has become apparent that measurement of tableting forces is a helpful tool in the development of tablet formulations and trouble shooting. Furthermore, the use of tablet machine instrumentation makes for better production control thus giving

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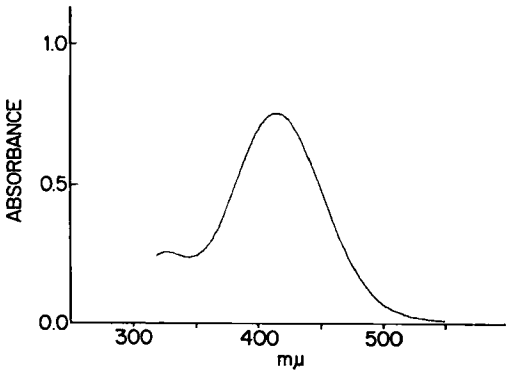

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more uniform, better quality products. Causes for variabilities in weights, hardness, and gauge can be found and minimized. Instrumentation is the first step in the development of a fully automated tablet press.

The Manesty 45 Rotapress has been used for several years by this production department on high volume items. Normally the press is run at 3,000–4,000 tablets per minute (TPM). Since this press was achieving more importance in the daily operation, it was appropriate to choose it for instrumentation studies. The mechanism of tablet compression of the Rotapress is somewhat different from other machines, thus experimentation on gauging sites for compressive force measurement on this machine was necessary. In addition, methods for measuring strain on cams were developed.

INSTRUMENTATION OF THE MACHINE

Compressional Force—A schematic diagram of the compression mechanism is shown in Fig. 1. The large metal column shown on the right-hand side of Fig. 1 supports one end of both the top and bottom roll carriages. The column in turn is bolted at two places to the corner post. It is not attached to the horizontal part of the machine which supports the turret. The left-hand side of Fig. 1 shows the upper roll carriage is attached to the top pressure link and the bottom roll carriage to a different support, the bottom pressure link. Thus one side of the pressure mechanism is fixed (the right side in Fig. 1), and the other side is variable. Connection of the top and bottom linkages on the left by various parts allows pressure adjustment (tablet thickness) and variable penetration of the upper punch. When a tablet is produced the force is exerted through the column and through the pressure links. Therefore, strain gauges were attached to various positions on these parts as illustrated in Fig. 1.

Location A, top pressure link above eye. Location B, bottom pressure link at the narrowest point. Location C, upper section of compression column. Location D, lower section of column just above the table. Location E, upper section of column just below the pin supporting the upper roll carriage. Location F, at the bottom of the compression column just above pin that supports lower roll carriage.

These positions are shown in Fig. 1. Half bridge configurations were used for all screening work. Actually at some positions on the column it is impossible to place gauges on both sides because of the abutment with the corner post. It is best to split the placement of the full Wheatstone bridge circuit between two sides of a column in order to eliminate measurement of column bending.

Of the six positions monitored, Location E in Fig. 1 was the most promising. Judgment was made on the uniformity of the signal, the accessibility for installation of a full bridge on both sides of the member

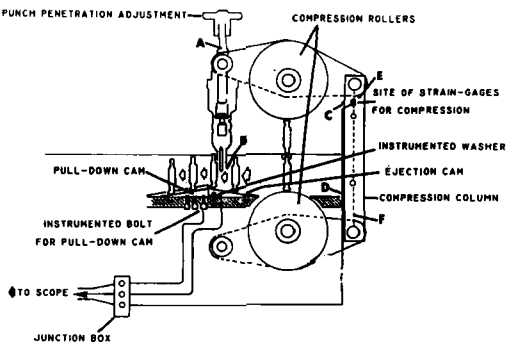

Fig. 1—Schematic diagram of the compression mechanism of the Manesty 45 Rotapress. Note various sites for placement of strain gauges for compressional force measurement. Sites for placement of force washer and instrumented bolt are indicated for measurement of ejection force and lower punch pulldown force, respectively.

being monitored, and the signal strength. All positions except E and C showed varying degrees of harmonies or extraneous signals aside from that resulting from the compression stroke. These signals from the upper position of the compression column, Locations E and C, give readings of about one-tenth the force reading on a punch. The sensitivity was thought to be satisfactory since readings of 20 to 40 microstrain (μst.) were obtained during ordinary tableting operations. Opposite Position C, the pressure bar is butted against the corner post of the machine and is thus inaccessible. For this reason, Location E, the position just beneath the upper roll carriage pin, was chosen. The column at this position is accessible from both sides and allows installation of a half bridge on both sides of the column. During the experimentation on finding the best location, it was thought that the deep milling marks on the pressure bar might be the cause of poor signal. Because of this, Location E was smoothed down to a fine mirror finish before the final gauge installation was made. This resulted in the achievement of excellent signals from this position.

It was necessary to calibrate the compressional response from the column to actual pounds of force. To do this an upper and lower 1.59 cm. ($^5/_8$ in.) FFBE punch was instrumented with strain gauges in a full Wheatstone bridge configuration. The lower shank portion of the punches was machined away to give four flat surfaces for strain gauge installation. These punches were statically compressed on a Carver press and a linear relation was obtained by plotting punch response in microstrain to applied pressure in pounds. The Carver press was fitted with Bourdon type gauges of various ranges which had been checked for calibration by a dead weight tester. The punches were then fitted into the tablet machine and a simultaneous reading from the punches and compression column was recorded in microstrain. This was also a linear relationship. It was then possible to relate the column microstrain reading to pounds using the gauged punches as a secondary standard.

Calibration Results—Graphs of compressional force *versus* the microstrain reading obtained from the compression column were made and are shown in Fig. 2. The results of the co-variance analysis are

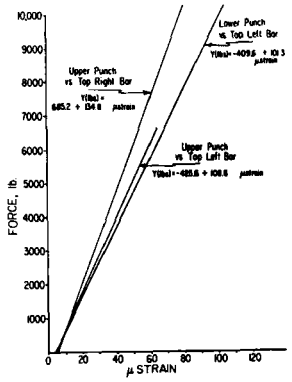

Fig. 2—Calibration of compressional force.

given below and shown in Fig. 2 also.

Upper punch *versus* top left bar
Y (lb.) $= -425.6 + 108.8\ X$ (μst.) (Eq. 1)

Lower punch *versus* top left bar
Y (lb.) $= -409.6 + 101.3\ X$ (μst.) (Eq. 2)

Upper punch *versus* top right bar
Y (lb.) $= -685.2 + 134.9\ X$ (μst.) (Eq. 3)

Close agreement in response of the left bar to equal pressures on upper and lower punch was achieved. At 4,000 lb., for instance, there is a 250-lb. deviation of the upper punch from the lower punch. The response of the right bar was calibrated only against the upper punch. The right bar is less sensitive than the left bar, thus giving a substantially less μstrain reading. The reason for this at present is unaccountable.

The response from the right bar was monitored for antacid tablets. Thus Eq. 3 was used for calculation of force. For example, if an average reading of 40 μ-strain is obtained on compression of these tablets, then the force is calculated as follows:

Force in lb. $= -685.2 + 134.9$ lb./μst. (40 μst.)
Force $= 4711$ lb.

The negative intercept obtained in the above equation is not physically meaningful. Lower pressure readings would indicate the lines in Fig. 2 would actually go through the origin. Therefore Eqs. 1, 2, and 3 may be used for calculation of true force, but are not truly definitive because of the negative intercept.

Ejection Force—Because of the high operating speeds of the Manesty press and for the sake of maintaining a conventional machine, an attempt was made to measure ejection forces without fabrication of a special ejection track. It was theorized that the use of a force washer under the bolt holding the ejection track in place might be a satisfactory method. A photograph of the ejection track and force washer is illustrated in Fig. 3. When tested this method was found to work satisfactorily.

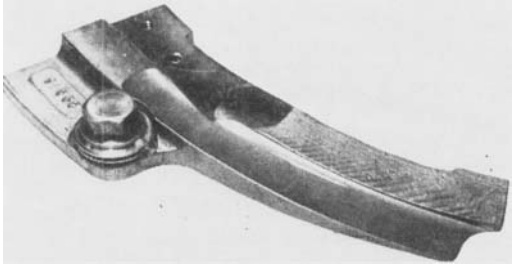

Fig. 3—Photograph of ejection track and force washer.

The force washer is a product of Lockheed Electronics (Houston, Tex.). It measures 2.16 cm. (0.85 in.) in diameter and is 0.81 cm. (0.32 in.) thick. Thus it is small enough to insert under the bolt and not interfere with machine performance to the slightest degree. One drawback of the washer is that it employs only one active strain gauge in the circuit. Additional active gauges would provide better electrical balance and temperature compensation. Similar force washers using a full Wheatstone bridge circuit have been obtained from Lockheed and have operated satisfactorily.

Ejection Force Measurement—The force washer was installed beneath the bolt that holds the ejection cam in place. Two plastic shims were used beneath the cam to obtain proper ejection height. The cam was shimmed uniformly throughout its length.

Three experiments were used to test the usefulness of this method. First, the ejection response was monitored during the run-in period. At this time no tablets were being produced. The plungers holding the lower punches were tightened to normal. Under these conditions, no ejection response was noted. This meant that the ordinary travel of the punches over the ejection cam gave no strain measurement when tablets were not being made. Next, tablets were made by hand turning the machine. The force washer was monitored for response at the time of compression. This tested whether or not compressional force was transmitted through the machine to the force washer. No response was recorded at the time of compression, thus verifying the feasibility of the force washer.

An experiment was run on an antacid tablet in which the upper punch penetration was deep, medium, and shallow. In order to compensate for the various penetrations, the lower roller was moved up or down by use of tablet thickness adjustment. Tablets of about the same hardness and thickness were produced under all three conditions and ejection forces were monitored. It was found that at deep penetration the lower punches were striking the ejection cam at a very early point and no ejection response was apparent. However, at medium and shallow penetration, good ejection responses were achieved.

Lower Punch Pulldown Force (LPPF) Measurement—The utility of this force measurement had been demonstrated for the Stokes BB-2-27 and will be described in a separate paper (5). Frequently, while making prolonged runs, lower punches may begin to bind and stick either on the punch shank or at the punch tip and die wall interface. Among conditions causing sticking are inadequate removal of overflowing granulation, excessive binding properties of the material being tableted, and poor or faulty lubrication of the formulation or the machine. Strains resulting from the above conditions are often the cause of excessive punch and cam wear or tooling and machine failure.

An instrumented bolt was made for the Manesty 45 Rotapress and is the same design as reported in

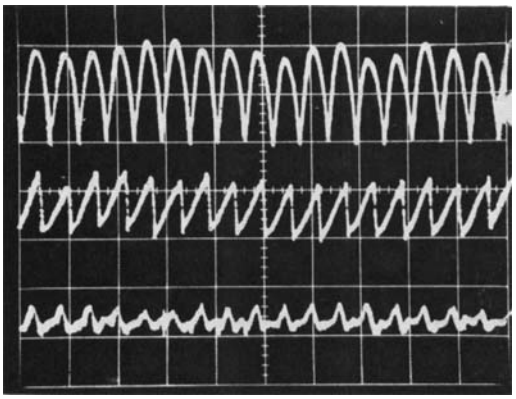

Fig. 4—Oscilloscopic tracing of various force measurements obtained on Manesty 45 Rotapress. Uppermost tracing: compressional force measurement; each large division is about 2,000 lb. of force. Middle tracing: ejection force; each large division is 10 μstrain. Lower tracing: lower punch pulldown force; each large division is 10 μstrain.

Reference 5. The bolts used for holding all cam tracks in the Manesty 45 press are 1.27 cm. (1/2 in.) in diameter and have 12 threads to the inch (Whitworth thread). Bolts having this thread system are difficult to obtain in this country. The bolts which were instrumented were obtained from Manesty. Later, when it was decided to obtain bolts having internally mounted gauges, it was necessary to buy bolts of slightly longer lengths in order for the bolts to be calibrated. These bolts were sent to Strainsert, Bryn Mawr, Pennsylvania, for installation of strain gauges.

The instrumented bolt was placed in the position nearest the ejection cam. This position gave the most sensitive and reproducible result (5). Therefore, this position was adopted for permanent measurement.

TESTING OF THE INSTRUMENTATION AND THE NATURE OF THE DATA OBTAINED

Compression, Ejection, and Cam Track Force Measurement—All data reported pertain to antacid tablets weighing about 1.33 g. produced from 1.59 cm. (5/8 in.) flat-faced bevel-edged punches and dies.

Typical data resulting from compressional force and various cam strains are shown in Fig. 4. The uppermost tracing in Fig. 4 is the typical compression response. A reading of about 40 μstrain was obtained in this instance; this is equivalent to about 4,700 lb. of force. The middle tracing represents the response obtained from ejection force measurement using the Lockheed force washer. The ejection force was not transposed to pounds of force, but was normally read and recorded as μstrain. The particular washer used in this work has a rating of 0–250 lb. The lowermost tracing in Fig. 4 is normal LPPF as seen at the beginning of a run or during good running conditions. The average response in this particular tracing is about 6 μstrain. Force rating of the Strainsert bolts which were eventually used was 0–100 lb.

Monitoring Antacid Tablets at Various Machine Settings—In order to determine the extent of several machine factors on the compression of antacid tablets, a number of short runs were made at different machine settings. The factors studied were speed, pressure setting, and depth of upper punch penetration. The summary of the results is shown in Table I.

It was found that the depth of upper punch penetration has a major effect on magnitude of ejection force. During deep penetration the lower punch strikes the ejection cam at its foremost position. This occurs because the lower roller carriage is moved downward to compensate for the deeper penetration of the upper punch. At medium or shallow penetration of the upper punch into the die, the lower punch will strike the ejection cam at a higher position. It was found that at shallow penetration an ejection response of about 30 μstrain was obtained while at deep penetration a response of about 8 μstrain was obtained. If the ejection cam had been shimmed at the furthermost point, an increased response may have been obtained. Normally tablets are produced using only minimal penetration of upper punch, so a satisfactory response was obtained under conditions of machine usage.

The data indicate that both higher ejection force and higher LPPF are obtained under higher compressional forces. This concurs with results of other studies (3, 5). Faster speeds generally show increased ejection force and LPPF as compared with slower speeds.

Tablet hardness (average of four determinations) was plotted against compressional force (Fig. 5) for this single batch of granulation and a generally linear relationship was found. Without mathematical treatment of the data it can be seen that a

TABLE I—SUMMARY OF ANTACID TABLET COMPRESSION DATA

Condition	Compression Force, lb.[a]	Ejection Force, μst.[a]	Lower Punch Pull-down Force, μst.[a]	Weight, g.[a]	Hardness, kg.[b]
Shallow penetration, high pressure, fast speed	4,653	36.1	6.5	1.326	13.8
Shallow penetration, high pressure, slow speed	4,572	32.9	6.1	1.323	13.9
Shallow penetration, low pressure, fast speed	3,844	29.8	5.5	1.335	8.9
Shallow penetration, low pressure, slow speed	3,709	26.4	4.7	1.336	9.4
Deep penetration, high pressure, fast speed	4,302	8.0	6.5	1.335	11.4
Deep penetration, high pressure, slow speed	5,057	9.6	5.4	1.334	14.8
Deep penetration, low pressure, fast speed	4,006	8.4	5.4	1.331	10.9
Deep penetration, low pressure, slow speed	3,574	8.0	3.2	1.323	7.1

[a] Average of 10 readings. [b] Average of four readings.

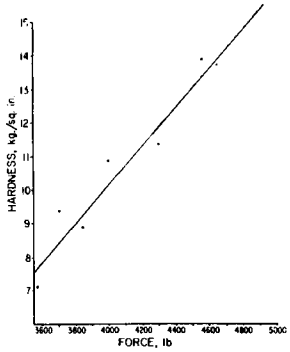

Fig. 5—Tablet hardness as a function of compressional force for a single lot of granulation.

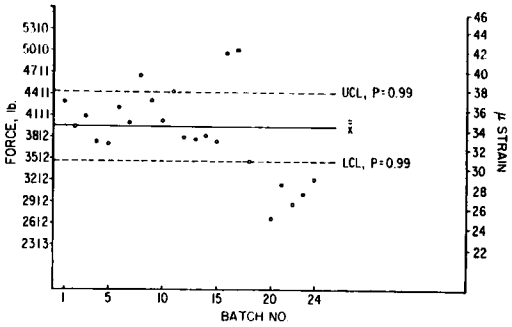

Fig. 6—Average compressionl force for various batches of antacid tablets. Eight of the 24 consecutive batches fall outside the p = *0.99 control limits.*

compressional force range of 3,550–4,650 lb. produces tablets in the 7.5–14 kg. hardness range. Compressional force of 3,800–4,150 lb. produced tablets in the 9–11 kg. hardness range.

ROUTINE MONITORING OF TABLETS IN PRODUCTION

Experimental Design—The normal production of antacid tablets in the plant was monitored by means of the instrumented Rotapress. The study was carried out for 24 working days which provided data for 24 separate batches of granulation produced from a continuous process. Usually photographs of the oscilloscopic tracings were taken at four equally spaced intervals throughout the day. Five peaks were read for compressional force analysis. At the same time tablets were collected. The weight and hardness of four tablets at each time interval were measured. The weights were determined analytically, and hardnesses were measured using an air actuated Strong-Cobb hardness tester.

Results of Daily Monitoring the Production of Antacid Tablets—The means and variabilities of the compressional force, weight, and hardness were calculated. Three separate variabilities were considered: (*a*) variability of single observations; (*b*) variability of time period averages; and (*c*) variability among batches.

Coefficients of variation of compressional force have been reported to range between ±5% and ± 40% (2). It has been shown that small deviations in tablet weight will account for similar small deviations in compressional force (6). Additional factors such as overload setting, machine speed, force flow feed *versus* standard feed, and a multiplicity of granulation factors greatly add to the variability of compressional force. Usually weight is controlled very precisely, therefore contributing little to overall compressional force variability. Of more significance may be the nature of the granulation itself and its level of loading into the machine.

A summary of compressional force means and variabilities is given in Table II. A relatively low coefficient of variation, 5.2%, is obtained for individual compressional force readings. However, the coefficient of variation of time period averages is 9.0% and for batches is 27%. An *F* ratio test ($p = 0.01$) shows significant differences for any two variabilities tested in Table II. In Fig. 6, the average compressional force per batch is plotted using control limits of $p = 0.99$. The limits were calculated from the standard deviation of time period averages. It can be seen that eight of twenty-four batches or 33% fall outside the limits. Five of the batches that are outside the limits were also the last five batches studied. The variability of compressional force (2,350–4,450 lb.) during these runs seems unusually high. The tablets prepared were satisfactory with respect to weight and hardness specifications. It is apparent that the granulation process being continuous adds the majority of the variability to compressive force. The precise nature of this variability has not been determined. Granulation properties such as mesh size, bulk volume, and flowability may be varying enough during normal processing to affect compressional force. Other tablet properties which might be of importance, *i.e.*, density and porosity, were not measured. However measurement of these factors may elucidate to some degree other basic correlations which are not reported in this study.

In Fig. 7, the average tablet weight is seen to be within the control limits ($p = 0.99$ using the time period standard deviation). The coefficient of variation for tablet weights is about 1% thus indicating that weight has a relatively small contribution to compressive force variability. Tablet hardness is shown in Fig. 8. It shows a long downward trend and subsequent fall (Batch 16) below the control limits ($p = 0.99$). It also shows some cyclical variation which occurs every 4 or 5 days. The lack of more detailed processing data precludes an accurate

TABLE II—SUMMARY OF COMPRESSION FORCE DATA OF TWENTY-FOUR BATCHES OF ANTACID TABLETS

Type of Observation	No. of Observations	Compression Force Average, lb.	*SD*, lb.	Coefficient of Variation, %
Individual	565	3,497	216	5.2
Time period	113	3,497	378	9.0
Batches	24	3,497	1150	27

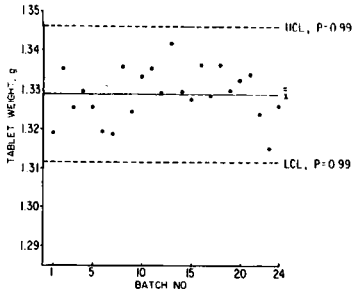

Fig. 7—Average tablet weight for various batches of antacid tablets. None of the batches fall outside the p = 0.99 control limits.

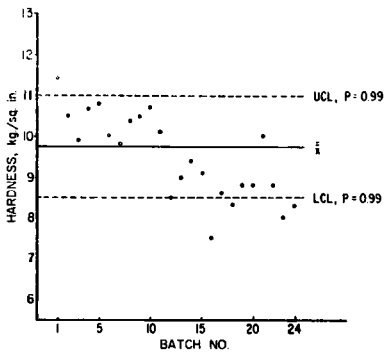

Fig. 8—Average tablet hardness for various batches of antacid tablets. Note downward trend and cyclical variation which occurs every 4 or 5 days.

explanation for this cyclical performance. Tests for correlation between pairs of responses, compressive force, weight, and hardness were made. The only significant correlation was between hardness and weight.

CONCLUSION

The instrumentation of the Manesty 45 Rotapress was described. Locations for the measurement of compressional force were investigated, and the top of the compression column was found to be a satisfactory location. A method for the measurement of ejection force and lower punch pulldown force was developed. Compression of antacid tablets at various machine conditions was described, and it was found that compressional force (CF) has a measurable effect on tablet properties. The results of monitoring CF, weight, and hardness during routine production were reported. A wide variability in CF was found but tablet weight and hardness were nevertheless positively correlated.

The instrumentation of this machine has provided a means of better understanding the tableting process as well as the drawing of correlations between machine conditions and tablet characteristics. It is thought that overall product quality might be improved by employing an instrumented tablet machine in regular production runs. The instrumented machine will help bridge the gap between pilot scale-ups and actual production runs. The combined use of smaller instrumented rotary tablet machines for pilot sized lots together with the use of the instrumented Rotapress in actual production provides a means of formulation scale-up not previously available. Formulation characteristics and defects are better understood with the use of tablet machine instrumentation.

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Keyphrases

Rotary tablet machine
Instrumentation—Manesty 45 Rotapress
Compressional force—determination
Ejection force—determination
Punch, lower—pulldown force
Diagram—tablet machine instrumentation

New Approach to the Fusion Method for Preparing Granular Effervescent Products

By RICHARD B. MURRAY

General methods for the preparation of effervescent granules are discussed briefly and a new adaptation of an old procedure is presented. The use of a special mixer in simplifying the initial blending, heating, and screening steps of granulations made by the fusion method is described. No external heat source is required, nor are liquids in any form used to accomplish the granulating. The instrumentation, basic formulation data, the method for monitoring the fusion process in the mixer, and the advantages of the new procedure are reported. Process data for granulation batch sizes of 60 and 300 kg. are given.

EFFERVESCENT SALTS have been known and used for about 130 years. Their popularity with the medical profession and the public has varied widely during this period. The preference for this dosage form declined markedly after reaching a peak during the early 1900's, but a significant number of ethical and proprietary products have since created or maintained considerable popularity in the market place.

Effervescent combinations consisting of a medicinal agent, a dry, nontoxic organic acid such as citric or tartaric acid, and an alkali metal carbonate or bicarbonate obviously cannot be wet granulated with aqueous solvent systems in the usual manner. With the exception of an experimental technique described by Coletta and Kennon (1) in 1964 which utilized the Wurster air-suspension apparatus, methods for preparing effervescent granules generally follow one of three approaches. The common goal of all three approaches is the introduction of just enough water to initiate a reaction between the acidic and basic components of a formulation, thereby massing it. The "wet method" utilizes a nonsolvent liquid such as alcohol, containing a small percentage of water. The "steam method" makes use of steam to add controlled amounts of water to the mixing material. The "fusion method" employs heat to liberate water of crystallization from components such as hydrous citric acid to effect moistening.

The fusion method normally presents two major production problems. The first is the need for specialized equipment such as steam-heated hot plates, ovens, or externally heated coating pans which are required to trigger the fusion process. The second problem involves the means of monitoring the progress of the fusion reaction and determining the point at which it is to be terminated.

This paper describes the use of a standard production mixer with no external heat supply for the preparation of granular effervescent salts by the fusion method. The required heat was generated solely by the resistance of the mixer contents to turbulent, high-speed mixing.

EXPERIMENTAL

Equipment—The mixer used for development studies was a Littleford-Lodige,[1] model FM100D, Fig. 1. This particular unit has a total capacity of 3.3 cu. ft. and a working capacity of 2.1 cu. ft. It has stainless steel contact parts and is equipped with a 5-hp. drive motor coupled to the plow shaft, with a variable speed drive which allows plow speed to be varied from 90 to 225 r.p.m. The mixer also has one high-speed chopper assembly powered by another 5-hp. motor. Rubbing plates were not utilized.

Carbon dioxide formed during the fusion reaction was vented by way of the liquid input tube. The optional vent pipe available for Lodige mixers, fitted with a filter boot, will also effectively relieve pressure and prevent material loss.

Instrumentation—The mixer was instrumented to measure three variables: the rotation speed of the plow shaft, the current demand of the plow drive motor, and the temperature of the mixing chamber. The factory-installed tachometer and ammeter were sufficiently accurate to measure the plow speed and electrical current demand. The mixing chamber temperature was monitored using a Simpson model 389-3L temperature tester equipped with a No. 0010 thermistor probe fixed in place at the nozzle end of the liquid input tube. Positioning the probe in this highly turbulent area of the chamber assured constant contact with the mixing materials.

Preliminary Tests—The suitability of various effervescent formulations for granulation by the fusion process was tested on a small scale using covered

Received May 22, 1968, from the Pharmaceutical Product Development Department, Mead Johnson Research Center, Evansville, IN 47721

Accepted for publication July 11, 1968.

Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

The author expresses his appreciation to Mr. Gary E. Moore for his technical assistance and to Dr. Eugene E. Hamlow for his constructive review of the manuscript.

[1] Marketed by Littleford Bros., Inc., Cincinnati, Ohio.

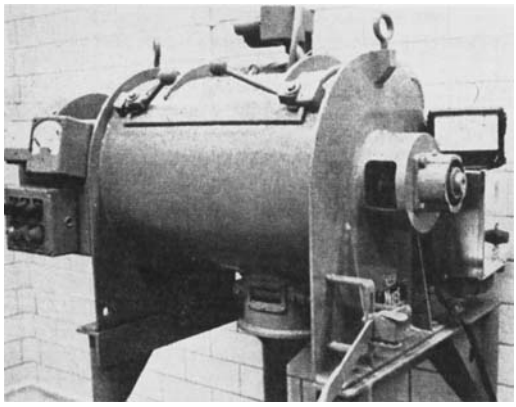

Fig. 1—Littleford-Lodige mixer, model FM100D, equipped with tachometer, ammeter, and temperature tester.

trays in a standard drying oven at 49° (120°F.). Blended ingredients were spread to a uniform depth of about 2 cm. Test blends which reached the desired degree of wetness within 1 hr. in the oven were generally suitable for scale-up and evaluation in the FM100D Lodige mixer. If a blend was slow to react, the proportion of hydrous citric acid was increased and the mixture was rechecked. Attempts to use a laboratory size M20E Lodige mixer for granulating were unsuccessful. Temperature rise sufficient to start the fusion reaction could not be attained due to the absence of a high-speed chopper and to the relatively small capacity of the mixing chamber.

Formulation and Process Data—The same basic effervescent granulation was prepared in each of the following examples to better illustrate the effects of mixer size and speed. The formulation which was granulated consisted of 50% potassium bicarbonate, 30% anhydrous citric acid, and 15% hydrous citric acid, plus small amounts of sweeteners, flavors, and water-soluble dyes.

Trial A—The model FM100D Lodige mixer was charged with 60 kg. of material. Mixing was commenced using a plow speed of 220 r.p.m. with the chopper operating. Current demand and temperature were recorded at 30-sec. intervals (see Figs. 2 and 3). As anticipated, the temperature and time relationship proved to be nearly linear throughout the process. The initiation and the progress of the fusion process were monitored by observing the drive-motor current demand. The current remained constant at 12.5 amp. for the first 4 min., then increased as the fusion reaction progressed and resistance to mixing increased. The fusion reaction apparently commenced when the temperature of the mixing material was just over 32° (90°F.). A suitable degree of wetness was reached in 8 min., at which time the current demand was 20 amp., an increase of 7.5 amp. over the initial power requirement. The temperature of the mixer contents reached 38° (101°F.).

The granulated material was immediately discharged and spread on trays for drying in forced air ovens at 49° (120°F.). The wet mass produced in the Lodige did not require wet screening. The dried granulation was passed through a No. 12 mesh stainless steel screen and, when intended for tableting, blended with a suitable lubricant.

Trial B—Sixty kilograms of the basic effervescent formulation was granulated in the FM100D Lodige mixer with the plow speed reduced to 110 r.p.m. and the chopper operating. The current demand remained constant at 8 amp. and the temperature increased to about 32° (90°F.) during the first 7 min. of mixing (Figs. 2 and 3). The fusion reaction began at this point and was terminated after 14 min. total mixing time. The final temperature was 41° (106°F) and the final current demand was 15.5 amp. The increase from initial to terminal power requirements again amounted to 7.5 amp.

Trial C—Scale-up to a production batch size was done in a model FKM600D Lodige mixer (Fig. 4). This unit has a working capacity of about 13 cu. ft. The plows are driven by a 15-hp. motor through a variable speed drive and the mixer is equipped with two high-speed chopper units individually powered with 5-hp. motors. A thermistor probe was in-

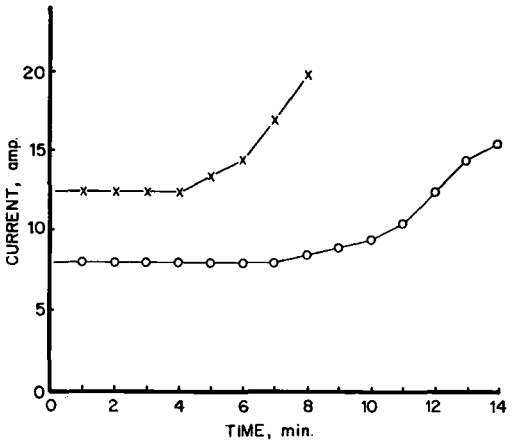

Fig. 2—Drive motor current demand during processing; FM100D Littleford-Lodige mixer. Key: plow speed X—X, 220 r.p.m.; O—O, 110 r.p.m. (separate trials).

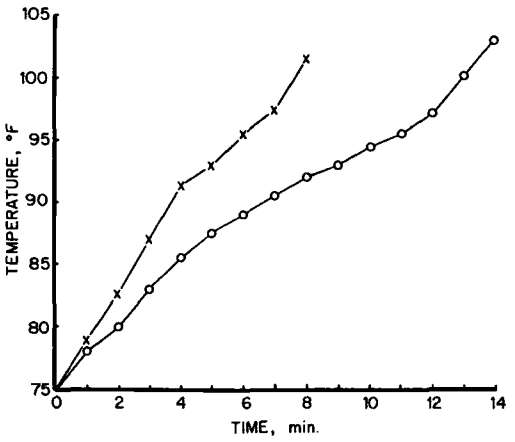

Fig. 3—Temperature of the mixer contents during processing; FM100D Littleford-Lodige mixer. Key: plow speed X—X, 220 r.p.m.; O—O, 110 r.p.m. (separate trials).

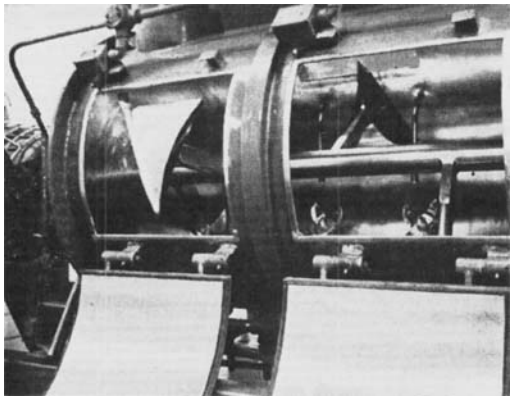

Fig. 4—Littleford-Lodige mixer, model FKM600D, showing plows, chopper heads, and liquid input tubes.

stalled at the end of one liquid input tube extending into the mixing chamber.

The mixer was charged with 300 kg. of the same effervescent formulation which was used in the preceding trials. Mixing was initiated with both chopper units operating. Plow speed was adjusted to 110 r.p.m. and the drive-motor current demand was recorded at regular intervals. The temperature in the mixer was also recorded throughout the run (Figs. 5 and 6). As noted in experiments using the FM100D Lodige mixer, the temperature and time relationship was relatively linear. The fusion reaction began after the material had mixed for 10 min. as indicated by the increase in the current required.

The granulation developed the desired consistency after 13 min., at which time the temperature was 42° (109°F.) and the drive-motor current demand had reached 32 amp. The granulation was then discharged from the mixer, spread on trays, allowed to cool to room temperature to slow the fusion reaction, and dried.

DISCUSSION

These data illustrate an interesting difference in the power requirement between the FM100D and FKM600D Lodige mixers during the granulating cycle. The current demand of the smaller unit was constant until the fusion reaction began to take place. With the large mixer, however, current demand at the beginning of the the cycle was high, then dropped gradually until the fusion reaction caused a return to the initial level. No attempt has been made to investigate this observation, but it is surmised that changes in interparticular friction due to attrition, increasing mix homogeneity, and free moisture level may be involved.

The most critical decision to be made during the preparation of effervescent granules by the fusion method, regardless of the system being utilized, is the determination of the point at which to terminate the massing reaction. Human judgment must still be relied upon, but operator experience is less important when the Lodige mixer is used for the processing. The onset and extent of the reaction can be monitored by observation of the variation in current required by the mixer. A terminal amperage which yields granulations having the desired characteristics can be established using information from a number of trial batches. This value, however, will be a valid indication of the reaction state of the granulation only if other factors which affect drive-motor current demand are held constant. These factors include plow speed, batch size, and product composition.

The terminal temperature and the total mix time have been observed to vary significantly from batch to batch. Therefore, these were not considered to be dependable indicators of the extent of the fusion reaction.

Empirical decisions can still be made by the operator if he questions the condition of a particular batch. He may discharge and examine small quantities of material from the mixer while it continues in operation.

The Lodige mixer combines into one operation the blending of raw materials and the heating to initiate the fusion reaction. Wet screening of the mass is often eliminated. Since the mixer is equally suited to the preparation of standard wet granulations and dry blends, it need not remain idle when production of effervescent products is not underway. Although the method described in this paper is strictly a batch operation, effervescent formulations have been

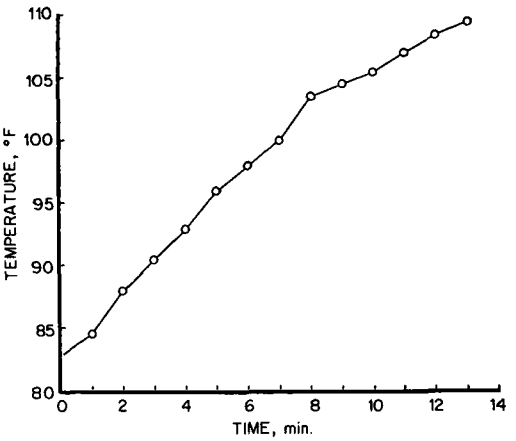

Fig. 5—Temperature of the mixer contents during processing; FKM600D Littleford-Lodige mixer.

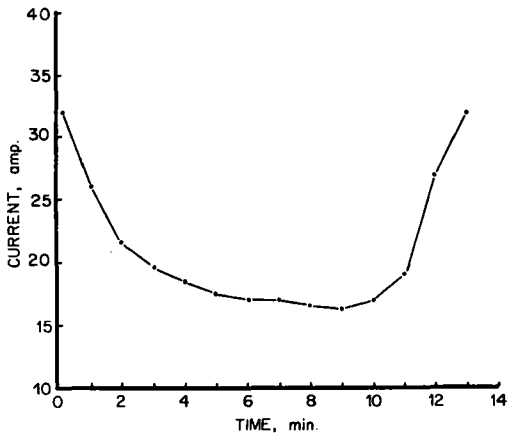

Fig. 6—Drive motor current demand during processing; FKM600D Littleford-Lodige mixer.

routinely blended and granulated at the rate of 700 kg./hr. using a single model FKM600D Lodige mixer.

SUMMARY

A new approach to the preparation of effervescent granules by the fusion method has been described. The use of Littleford-Lodige mixers, both pilot plant and production models, was discussed with illustrated examples. Advantages of the procedure, which requires neither an external heat source nor a granulating solution, were presented on the basis of both development and production experience.

REFERENCE

(1) Coletta, V., and Kennon, L., *J. Pharm. Sci.*, 53, 1524(1964).

Keyphrases

Effervescent products, granular—preparation
Fusion method—effervescent products
Water of crystallization—moisture source

Evaluation of Tableting Tool Life Records

By CHARLES J. SWARTZ and JOACHIM ANSCHEL

A study of tableting tool life records, accumulated over a period of 8 years, has served to develop and define relationships between tool performance and the many operating variables that exist in routine tablet production. The effects of these numerous factors on punch and die life have led to conclusions involving techniques for extending tool usability. Outstanding among these are narrowing of working tolerances for tools and extension of product runs on individual machines rather than frequent interchange of machine and tools.

BEGINNING IN 1959, performance records were kept at Ciba on punches and dies in use with standard tableting machines. A previous publication (1) describing all facets of a punch and die control program indicated the need for correlating these records of tool wear with many variables affecting compression. The tool life record keeping was briefly described in the same paper. These performance records have now been collected for 8 years, and the results obtained represent several billion compressions. This paper will review and evaluate the information derived from these records and attempt to define relationships which may be of practical value to research and production technologists.

Specially designed record cards for sets of punches and dies (Fig. 1) were maintained in the Pharmaceutical Manufacturing Division and reviewed jointly by Pharmacy Research and Development and Production personnel. The cards contain information on (*a*) the total number of tablets compressed with any given set of tools, (*b*) the product prepared, (*c*) the machines the tools were used with, (*d*) the type of steel, (*e*) the dies employed, and (*f*) the reasons for which the tools were eventually discarded. Cards were also kept for the tablet compressing machines with which these tools were used. The record cards further proved helpful for in-use inventory control of tools since they serve as a concise summary of available stock.

Received May 22, 1968, from the Development and Control Department, Ciba Pharmaceutical Company, Summit, NJ 07901

Accepted for publication July 25, 1968.

Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

EXAMINATION OF RECORDS

The record cards disclosed the principal reasons for termination of punch use which included damages to the tips and heads, rolled-in or burred edges, pitted faces, worn or distorted monograms, distorted or flattened bisections, undersized tip lands, and scored, pitted heads. Less frequent reasons for termination of use were distortions and eccentricities of the barrel (shank). Examination of die records showed that badly worn and scored die bores were the main reasons for rejection after use. On the other hand, carbide-lined dies outlived their usefulness principally as a result of distortion and burring of the die screw groove.

EFFECTS OF VARIABLES ON LIFE OF PUNCHES

The records show that some of the damage to tools can be attributed to normal wear, while other contributory variables are not related to regular operation. The effects of these variables will be discussed in the following section.

routinely blended and granulated at the rate of 700 kg./hr. using a single model FKM600D Lodige mixer.

SUMMARY

A new approach to the preparation of effervescent granules by the fusion method has been described. The use of Littleford-Lodige mixers, both pilot plant and production models, was discussed with illustrated examples. Advantages of the procedure, which requires neither an external heat source nor a granulating solution, were presented on the basis of both development and production experience.

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Keyphrases

Effervescent products, granular—preparation
Fusion method—effervescent products
Water of crystallization—moisture source

Evaluation of Tableting Tool Life Records

By CHARLES J. SWARTZ and JOACHIM ANSCHEL

A study of tableting tool life records, accumulated over a period of 8 years, has served to develop and define relationships between tool performance and the many operating variables that exist in routine tablet production. The effects of these numerous factors on punch and die life have led to conclusions involving techniques for extending tool usability. Outstanding among these are narrowing of working tolerances for tools and extension of product runs on individual machines rather than frequent interchange of machine and tools.

BEGINNING IN 1959, performance records were kept at Ciba on punches and dies in use with standard tableting machines. A previous publication (1) describing all facets of a punch and die control program indicated the need for correlating these records of tool wear with many variables affecting compression. The tool life record keeping was briefly described in the same paper. These performance records have now been collected for 8 years, and the results obtained represent several billion compressions. This paper will review and evaluate the information derived from these records and attempt to define relationships which may be of practical value to research and production technologists.

Specially designed record cards for sets of punches and dies (Fig. 1) were maintained in the Pharmaceutical Manufacturing Division and reviewed jointly by Pharmacy Research and Development and Production personnel. The cards contain information on (*a*) the total number of tablets compressed with any given set of tools, (*b*) the product prepared, (*c*) the machines the tools were used with, (*d*) the type of steel, (*e*) the dies employed, and (*f*) the reasons for which the tools were eventually discarded. Cards were also kept for the tablet compressing machines with which these tools were used. The record cards further proved helpful for in-use inventory control of tools since they serve as a concise summary of available stock.

Received May 22, 1968, from the Development and Control Department, Ciba Pharmaceutical Company, Summit, NJ 07901

Accepted for publication July 25, 1968.

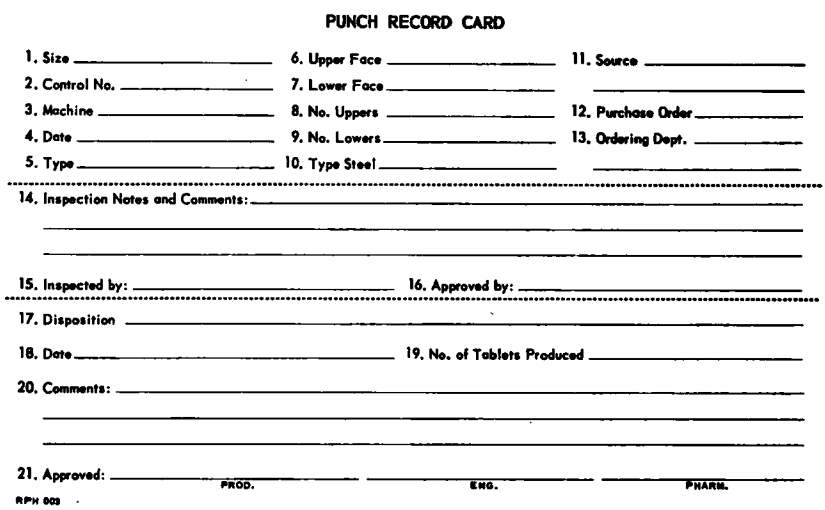
Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

EXAMINATION OF RECORDS

The record cards disclosed the principal reasons for termination of punch use which included damages to the tips and heads, rolled-in or burred edges, pitted faces, worn or distorted monograms, distorted or flattened bisections, undersized tip lands, and scored, pitted heads. Less frequent reasons for termination of use were distortions and eccentricities of the barrel (shank). Examination of die records showed that badly worn and scored die bores were the main reasons for rejection after use. On the other hand, carbide-lined dies outlived their usefulness principally as a result of distortion and burring of the die screw groove.

EFFECTS OF VARIABLES ON LIFE OF PUNCHES

The records show that some of the damage to tools can be attributed to normal wear, while other contributory variables are not related to regular operation. The effects of these variables will be discussed in the following section.



PUNCH RECORD CARD

1. Size _____ 6. Upper Face _____ 11. Source _____
2. Control No. _____ 7. Lower Face _____ _____
3. Machine _____ 8. No. Uppers _____ 12. Purchase Order _____
4. Date _____ 9. No. Lowers _____ 13. Ordering Dept. _____
5. Type _____ 10. Type Steel _____ _____

14. Inspection Notes and Comments: _____

15. Inspected by: _____ 16. Approved by: _____

17. Disposition _____

18. Date _____ 19. No. of Tablets Produced _____

20. Comments: _____

21. Approved: _____ PROD. _____ ENG. _____ PHARM.

RPH 003

Fig. 1—Punch record card.

Operating Variables—As machines become older, the clearances between moving parts become enlarged. These negative effects are tied to tool life performance and are reflected in the data on machine life. The primary reason for this reduction in tool life is probably centered around excessive clearances developed over years of usage. Enlarged dimensions show up most prominently in worn punch sockets, roughened cams which damage punch heads, or worn die pockets. After years of wear, the deflection of an upper punch at the die table can increase to more than double the value initially obtained in a new machine.

Other detrimental effects may result from faulty lubrication of machines as well as neglect of other machine maintenance features. Cam design as well as cam track tolerances have a direct effect on wear but these factors are more difficult to document. It should be pointed out that the cams themselves can also fail because of unusual wear but these effects will not be documented here.

Damage to punch heads is usually due to machine failures for one or more of the following reasons: (*a*) defective weight adjustment cam, (*b*) excessive ejection force, (*c*) tearing and friction resulting from lack of lubrication, (*d*) tight pressure rolls.

Shape of Punch Tips—In evaluating the reasons for failure of the numerous punches involved in the study, the most common one was "rolled-in" punch tips. This was most evident in the relatively short life of flat-beveled edge, deep concave, and modified ball-shaped tools. To overcome part of the problem with flat-beveled edge punches, the addition of a 0.005-cm. (0.002 in.) blended-in flat on the punch tip edges has been found to increase the life of the tools.

The condition of the dies employed in any compressing operation also has an important effect on punch tip life. In instances where punches are employed with worn dies, granulation is very often extruded around the edges of the punches. The constant abrasion due to powder extrusion will considerably accelerate tip wear. An insufficient or roughened die bore chamber will obviously play a large role in feathering the edges of the tips. Worn punch sockets in the upper punch guide bores also contribute to setting up the "rolled-in" problem.

Type of Steel Used—Although no definitive proof arose from an examination of the records, it can be assumed that the type of steel used and its treatment during processing can play an important role in extending tool life. The hardness, carbon content, nature of heat treatment, and inherent structural features of the steel all have a role in determining the suitability of a particular steel for certain production needs.

With one high volume product run on a 35-station machine, four groups of punches manufactured from different types of steel were evaluated. At the end of the test period, which encompassed approximately 1,900,000 compressions per punch, there was no discernible difference in the wear noted on any of the tools. All four groups displayed tip damage to about the same extent. A larger sample would have to be studied in order to obtain any meaningful results.

Storage and Care of Tooling—Rusting and pitting of tool steel is a relatively frequent occurrence if proper preventive precautions are not taken. Careful maintenance of humidity control in the tool storage area is an excellent step in this direction. Following routine use and prior to storage, the tools are cleaned ultrasonically, inspected visually, oiled down, and placed into specially constructed wooden tool boxes preventing direct contact between punches. Tools are reinspected at regular intervals by specially trained mechanics. The inspection includes selected dimensional checks in addition to hardness and visual observations for gross imperfections. If required, recommendations for rework or destruction are made at these intervals.

Effect of Granulation Characteristics—The nature of the granulation to be compressed is obviously a key variable in any evaluation of tool life. The records disclosed many examples of these effects. A tableting operation can be considered a three-component system consisting of machine, tools, and the granulation. Abrasion or corrosion by many of the chemicals involved were well established, and a few specific instances might serve to demonstrate this. In one clear illustration, it is possible to show the effects on tool life if only the conditions of manufacture of the granulation were changed. Figure 2

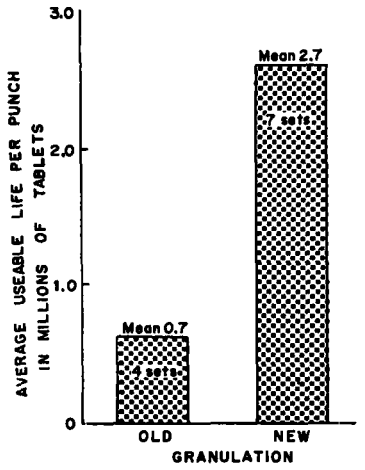


Fig. 2—Tool life associated with change in granulating procedure.

shows the average tool life before and after the modification of the granulation procedure. An almost fourfold increase in tool life resulted on the same machine under similar pressure conditions. The significant change can, therefore, be wholly attributed to modification of the granulation technique.

The variation in tool life from product to product can be noted in Fig. 3. With Product A, the punch life was consistently low, ranging between 0.3 and 0.7 million compressions per tool. In this instance, the short life of the tools was found to be related to a combination of the properties of the product compressed and the deep concave shape of the punches used with carbide insert dies. The hygroscopic and acidic nature of the granulation further contributed to shortening the tool life.

Machine Variables—Punches employed for the other products showed a large variation in tool life which is not as readily explained as for Product A. Products B and E in Fig. 3 provide an interesting comparison since the nature of the granules and excipients is somewhat similar in both. One product (Product E) was compressed on the same tableting machine on a long-run basis with stops only for cleaning and maintenance. It has been the authors' experience that this practice tends to increase punch life. Frequent removal of the punches from the machine and interchange of punches with different dies will increase the wear on the tools involved (Product B).

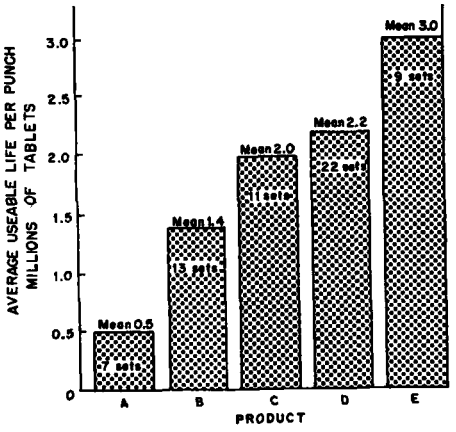


Fig. 3—Variation in average tool life for different products.

Tool life in excess of 4 million tablets per punch (Product C with a mean of 2 million) was achieved by employing the punches with a newer tableting machine of improved design (Manesty Rotapress 55). This machine has closer tolerances on the upper punch guides. As observed by stroboscopic light, punches on this higher speed machine seem to flow more smoothly through the cam tracks. Reduced vertical travel accommodates the rotational speeds required to produce up to 5,000 tablets/min.

With an average tool life of 2.2 million compressions for Product D the extension from 1–2 million to 2–3 million in some of the later sets of tools can be attributed to the replacement of the machine heads by specially designed ones with tighter punch socket clearances. This correlates well with the performance noted above with tooling for Product C. Details of this particular modification were reported in an earlier communication (1).

EFFECTS OF VARIABLES ON DIE LIFE

Although useful working life of dies varied considerably from product to product, it was much more consistent than that of punches within each product class. The records were not extensive enough for graphical representation, but several points of interest were noted. Die life ranged from 200 thousand to 12 million compressions per die and in all these instances dies were no longer usable when the wear rings (grooves) in the die bore became excessive. Carbide-lined dies, as expected, extended tool life by threefold or more but the effect of carbide-lined dies on punches must be weighed. As noted earlier, "rolled-in" tips were the main reason for punch failure and constant honing of the punch tips on carbide insert dies accelerates this process. The weakest portion of the carbide insert die is its supporting steel casing. The act of repeated tightening of these dies into die tables causes burring of the die screw groove as well as eventual deformation in this area leading to "popping" of the dies during running of the presses.

DISCUSSION

With many of the products studied, improvements in tool life were noted for more recent sets. There are many factors explaining these changes which have been described earlier. But as a result of the control program for incoming tools, it has been possible to consider the dimensional specifications of the punches and dies to be constants. Changes to tighter tolerances on new machines seem to be the greatest contributing factor. The smoother flow pattern of upper punch movement in newer machines must certainly aid in the extension of punch life.

Tableting tools are manufactured in accordance with precise specifications and are readily damaged by improper handling. Machine operator carelessness or lack of experience can contribute to a considerable reduction in tool life. On the other hand, better lubricants and improvements in tablet formula-

tion technology have increased the life span of tableting tools.

Keeping and reviewing of tool life records can lead to considerable savings in time and money in tablet manufacture. Under optimum conditions, life records of controlled tableting tools can show the maximum life expectancy of the equipment. When tool life is below these expected standards, reasons can usually be determined from the knowledge gained rather than just intuition. Benefits derived from the program have been substantial, and as a consequence, it will be continued as a valuable control procedure.

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Keyphrases

Tableting tool life records—evaluation
Punches—variables affecting wear
Dies—variables affecting wear
Record card—punches

Notes

Effect of Paralyzation with Dimethyltubocurarine on Cortical After-Discharge Duration

By ROBERT N. STRAW

Paralyzation with dimethyltubocurarine iodide increased the duration of cortical after-discharge in a manner similar to that previously reported for gallamine triethiodide. The after-discharge duration was determined from EEG records obtained from cats fitted with electrodes.

PARALYZATION WITH gallamine triethiodide, but not with decamethionium bromide or succinylcholine chloride, increases the duration of cortical after-discharge (1–3). The purpose of this investigation was to examine the effects of paralyzation with dimethyltubocurarine iodide on after-discharge duration.

METHODS

Five adult cats of both sexes were used in these experiments. Stainless steel screw electrodes were threaded into the skull under sodium pentobarbital anesthesia as previously described (1, 3). Preliminary trials to determine the threshold for a bilateral cortical after-discharge began after a 2-week recovery period. Each cat was stimulated with a 5-sec. train of 1-msec., monophasic square wave pulses at 50 p.p.s. (delivered from a Grass S-4 stimulator *via* a Grass SIU-4B stimulus isolation unit and Grass CCU-1A constant current unit) starting from 2.5 ma. and with increments of 2.5 ma. At least 5 min. elapsed between stimuli. The threshold was redetermined 2 days later starting with a current 5 ma. below the previously determined threshold. The threshold value obtained at this second trial was then used in each experimental trial. Throughout all experimental trials the cats, restrained only by the confines of their cage, were stimulated once on every other day.

The medial ectosylvian gyrus was chosen as the cortical area to be stimulated since, according to Garner and French (4), it has a relatively low seizure threshold. In addition, a generalized clonic overt seizure always accompanies the bilateral after-discharge elicited by stimulation of this area (5).

The stereotaxic coordinates (6) of the bipolar stimulating electrodes were $A = 10; L = 16; A = 2; L = 14$. Bipolar EEG recordings were made from both the stimulated site and the contralateral homologous area. In addition, EEG recordings were made from the posterior lateral and posterior sigmoidal gyri. All EEG recordings were made on a Grass model III electroencephalograph.

In the paralyzation experiments, the animals were given dimethyltubocurarine iodide (1 mg./kg.) intravenously. This dose was selected because it produces apparent total neuromuscular blockade of sufficient duration to conduct a trial under complete paralysis. Artificial respiration (Harvard pump set at 16 strokes/min.) was immediately started, the tidal volume being based on the animals' weights.

The after-discharge duration was determined from the EEG record. Analyses were performed on the data using a randomized complete block analysis of variance or by use of a Student's t comparison (7). For all tests, $p < 0.05$ was selected as the significant probability.

Received April 24, 1968, from the Department of Pharmacology, The Upjohn Company, Kalamazoo, MI 49001
Accepted for publication June 25, 1968.
The author wishes to acknowledge the technical assistance of Mr R. E. Williams.

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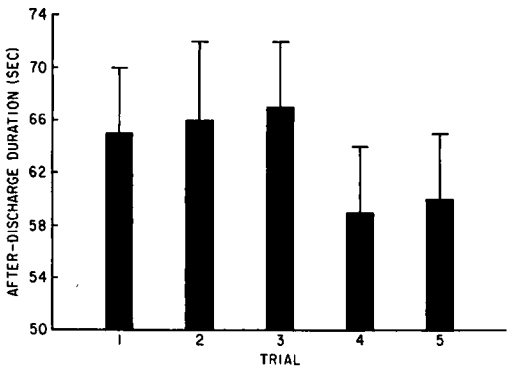

Fig. 1—Plot of mean duration versus *trial. Each value is the mean of 5 cats used throughout these experiments. The vertical line is the limit of the standard error.*

RESULTS

Figure 1 illustrates the trial-to-trial variability in the mean duration during the preparalyzation trials. An analysis of variance on these data showed no evidence for a difference among trials. The F ratio for trials was 2.41. There was, however, a significant difference among cats. The coefficient of variability for this analysis was 7.8%.

Table I documents the effect of paralyzation with dimethyltubocurarine on the duration of cortical after-discharge. As can be seen, the duration was significantly longer in the paralyzed state than in the nonparalyzed control trial.

In all trials (both control and paralyzed), all animals were in the alert state prior to stimulation. In the control (nonparalyzed) trials this was judged on the basis of overt behavior and the EEG record. During the trial under dimethyltubocurarine paralysis the state of arousal was determined from the EEG record.

DISCUSSION

The data from this investigation demonstrate that dimethyltubocurarine increases the duration of cortical after-discharge in a manner similar to that previously reported (1–3) for gallamine.

It has been previously speculated (3) that the mechanism whereby gallamine increases the duration of cortical after-discharge is *via* blockade of phasic inhibitory bursts arising in the muscle spindles during a motor seizure. As decamethonium and succinylcholine stimulate, rather than block, afferents arising in the muscle spindles (8), no increase would be expected following paralysis with these agents.

In contrast, Halpern and Black (2) have attributed the increase in cortical after-discharge duration following gallamine to a central action of this agent. However, as no increase in duration occurs following gallamine in an *encéphalé isolé* preparation (3) the effect of this agent would appear to be mediated from a more peripheral site(s).

As dimethyltubocurarine also blocks muscle spindles (8) one would expect an increase in duration following paralysis with this agent if the above "peripheral" hypothesis is correct. Such an increase was demonstrated in this report.

However, central effects of tubocurarine have been reported by several investigators (*cf.* 9–15). While there seems to be little doubt that tubocurarine exerts a central effect when applied directly to the brain (*cf.* 9, 10), conflicting reports as to central effects following intravenous injection have been presented. Some investigators (*cf.* 11–13) have found no changes in the CNS following administration of the drug while others (*cf.* 9, 14, 15) have reported various effects, primarily of a stimulant nature.

Thus, the effect of dimethyltubocurarine on cortical after-discharge duration cannot, at this time, be stated with certainty to be of either peripheral or central origin. It is obvious that further definitive work is necessary to fully describe the mechanisms whereby the neuromuscular blocking agents affect cortical after-discharge duration.

TABLE I—EFFECT OF PARALYZATION WITH DIMETHYLTUBOCURARINE IODIDE ON CORTICAL AFTER-DISCHARGE DURATION[a]

	Control[b]	Paralyzed
Mean	60	72[c]
SE	4.5	8.0

[a] Values are duration, in sec. [b] Trial immediately preceding that in which animal was paralyzed. [c] Significantly different from control; Student's t, paired comparison, $p < 0.05$.

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Keyphrases

Dimethyltubocurarine iodide
Paralyzation—dimethyltubocurarine
Cortical after-discharge duration—induced paralysis
EEG monitoring—after-discharge duration

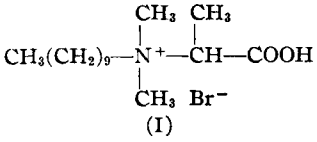
Measurement of the Apparent Dissociation Constants of Some Betaine Salts by Spectropolarimetry

By SHARON BONKOSKI and J. H. PERRIN

It is shown that spectropolarimetry can be used to measure dissociation constants in the same manner as spectrophotometry when the rotations of the species involved in the equilibrium differ sufficiently. The salts of *N*-decyl-*N*, *N*-dimethyl alanine (betaines) have a pKa′ of approximately 1.75 at 25° in an ionic strength of 0.10.

IT HAS LONG been realized that the optical rotation of acids depends upon their degree of ionization; for example, Britton and Moss (1) found *l*-malic acid to have a molecular rotation of −0.325 while its di-anion has a value of −1.60 at the Na_D line. They found the rotations to be additive for various mixtures of the undissociated and dissociated acid species, but the small differences in rotation make dissociation constant estimation difficult by this method. However, rotations of optically active acids are greatly enhanced at lower wavelengths, and in the last few years commercial instrumentation has become available, permitting reproducible measurements of optical rotatory dispersion (ORD) down to 200 mμ. The type of ORD curve obtained for an optically active acid depends upon the absorbing groups present and their position relative to the optically active centers, as well as the number and positions of the optically active centers; however, one expects some anomalous behavior due to the absorption of the carboxylic acid group in the region of 210–215 mμ (2).

Hydrobromide and hydrochloride salts of the ampholytic surfactant *N*-decyl-*N*, *N*-dimethyl alanine (I) behave as monobasic acids, the nitrogen



(I)

being quaternary. The salts exhibit anomalous ORD curves as expected for amino acid derivatives, and have been shown to have the first peak of a probable Cotton effect with zero rotation just below 220 mμ, the region of absorption due to carboxyl groups (3). Both the acids and their anions have peaks in the region of 230 mμ, and there are sufficient differences in the molecular rotations of the two species to permit pKa determinations at wavelengths below 235 mμ.

EXPERIMENTAL

Preparation of Betaines—The salts of the betaines were prepared as previously described, from optically active dimethyl alanines and decyl bromide (3, 4), and the purities checked by NMR, ORD, and melting points (3).

ORD Measurements—All measurements were made at 25 ± 0.2° in a Cary model 60 spectropolarimeter (Applied Physics Corp., Monrovia, Calif.). The pH measurements were made using a Beckman research pH meter (Beckman Instruments, Fullerton, Calif.). Preliminary pH measurements of the betaine solutions suggested that the apparent dissociation constants (pKa′) were below 2.0, and so the rotation of the undissociated acid was obtained in 1.2 *M* hydrochloric acid and the rotation of the anion in 0.1 *M* sodium hydroxide. Sodium chloride–hydrochloric acid buffers of ionic strength (μ) 0.10 were used for the measurements between pH 1.2 and 2.1. All measurements were made in a 5-cm. cell and the solution scanned from 250 to 220 mμ. In any pKa′ determination the concentration of the betaine was kept constant.

RESULTS AND DISCUSSION

These betaine salts form micelles (the critical micelle concentration is estimated to be 1×10^{-2} moles/l. from *Reference 3*), and it has been found (5) that the degree of ionization changes abruptly on micelle formation, and in the investigations reported here betaine concentrations of approximately 2×10^{-3} moles/l. were used to avoid this complication. A typical set of ORD curves is shown in Fig. 1 using L-decyl-*N*-*N*-dimethyl alanine hydrobromide. The molecular rotation of the undissociated acid at 225.5 mμ is seen to be approximately 1,000 while its anion has a value of approximately 90. The pKa′ can be calculated when the rotations of the two species as well as the rotations in several buffers are known at any wavelength, in the same manner as is used with spectrophotometric data (6). Let α_{HA} be the observed rotation of the unionized moiety, $[\alpha]_{HA}$ its specific rotation and C_{HA} its concentration. Then $\alpha_{HA} = KC_{HA}[\alpha]_{HA}$ where $K = 0.01$ (pathlength in decimeters). Similarly for the anion (A) $\alpha_A = KC_A[\alpha]_A$ for a mixture of the two species the

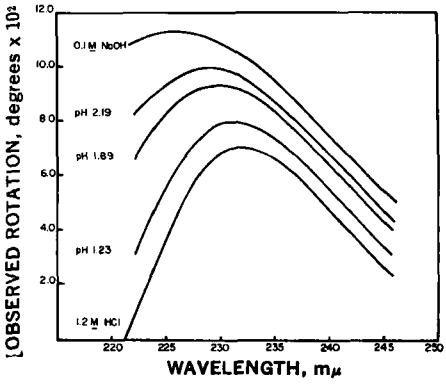


Fig. 1—Typical set of ORD curves.

Received April 4, 1968, from the School of Pharmacy, University of Wisconsin, Madison, WI 53706
Accepted for publication June 11, 1968.

observed rotation is the sum of the above at the same wavelength

$$\alpha = \alpha_{HA} + \alpha_A = KC_{HA}[\alpha]_{HA} + KC_A[\alpha]_A \quad \text{(Eq. 1)}$$

$$\alpha = KC[\alpha] = K(C_{HA} + C_A)[\alpha] \quad \text{(Eq. 2)}$$

where C is the total concentration of the compound and $[\alpha]$ its specific rotation. Combining Eqs. 1 and 2 gives

$$\frac{C_A}{C_{HA}} = \frac{[\alpha] - [\alpha]_{HA}}{[\alpha]_A - [\alpha]}$$

In any pKa′ determination the concentration of the compound is kept constant and observed rotations can be substituted for specific rotations and the pKa″s are given by (6).

$$\text{pKa}' = \text{pH} - \log\left(\frac{[\alpha] - [\alpha]_{HA}}{[\alpha]_A - [\alpha]}\right)$$

Measurements were made in at least eight buffers and the pKa″s were calculated from the rotations at several wavelengths. The results are summarized below.

Betaine	pKa′ (μ = 0.10)
L chloride	1.75 ± 0.02
L bromide	1.75 ± 0.02
D chloride	1.76 ± 0.03
D bromide	1.73 ± 0.02

The results show the pKa′ to be independent of the configuration of the betaine and the nature of the anions investigated.

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Keyphrases

Betaine salts
Dissociation constants—betaine salts
Spectropolarimetry—dissociation constants

Improved Method for Preparing 3-Azabicyclo[3.2.1]octane Hydrochloride and the Synthesis of its Phenothiazine Derivatives

By N. D. POTTI and W. LEWIS NOBLES

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SEVERAL COMPOUNDS derived from azabicyclic amines were reported to possess useful and interesting pharmacodynamic and chemotherapeutic properties (1–3). The primary purpose of this study was to elaborate an easier method for the preparation of 3-azabicyclo[3.2.1]octane (III). A few methods have been reported in the literature (2, 4–7) for the synthesis of this compound or its derivatives; however, these methods either are time-consuming and laborious or they make use of special equipment. The synthesis of two new phenothiazine derivatives of this complex amine is also reported.

The Schmidt reaction of norcamphor (I) was reported (7) to yield 10–28% of the lactam (II), which on reduction gave the desired amine (III). (Scheme I.)

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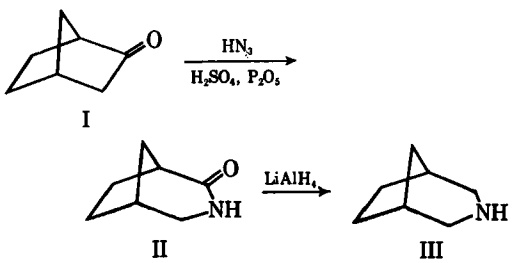

Scheme I

Received March 4, 1968, from the University of Mississippi, University, MS 38677
Accepted for publication June 11, 1968.

observed rotation is the sum of the above at the same wavelength

$$\alpha = \alpha_{HA} + \alpha_A = KC_{HA}[\alpha]_{HA} + KC_A[\alpha]_A \quad \text{(Eq. 1)}$$

$$\alpha = KC[\alpha] = K(C_{HA} + C_A)[\alpha] \quad \text{(Eq. 2)}$$

where C is the total concentration of the compound and $[\alpha]$ its specific rotation. Combining Eqs. 1 and 2 gives

$$\frac{C_A}{C_{HA}} = \frac{[\alpha] - [\alpha]_{HA}}{[\alpha]_A - [\alpha]}$$

In any pKa′ determination the concentration of the compound is kept constant and observed rotations can be substituted for specific rotations and the pKa″s are given by (6).

$$\text{pKa}' = \text{pH} - \log\left(\frac{[\alpha] - [\alpha]_{HA}}{[\alpha]_A - [\alpha]}\right)$$

Measurements were made in at least eight buffers and the pKa″s were calculated from the rotations at several wavelengths. The results are summarized below.

Betaine	pKa′ (μ = 0.10)
L chloride	1.75 ± 0.02
L bromide	1.75 ± 0.02
D chloride	1.76 ± 0.03
D bromide	1.73 ± 0.02

The results show the pKa′ to be independent of the configuration of the betaine and the nature of the anions investigated.

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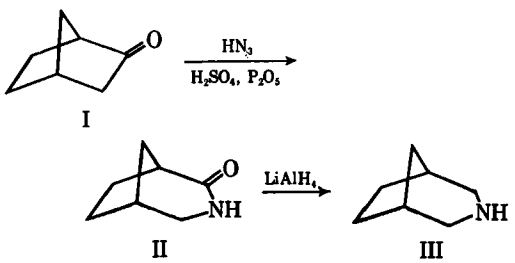

Scheme I

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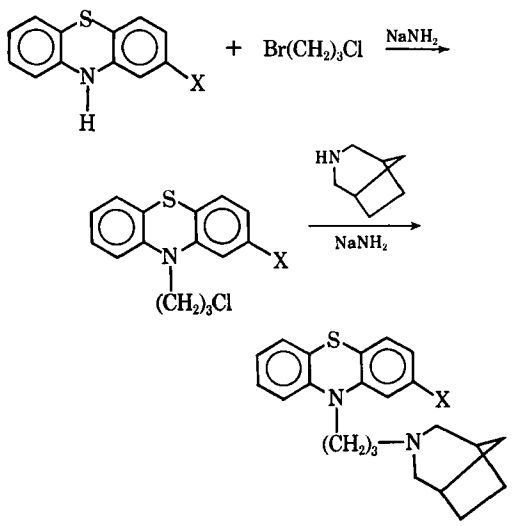
(8) gave about 16% of the lactam. The reduction of the lactam with lithium aluminum hydride proceeded smoothly to yield the amine (III) in almost quantitative yields.

Cyclic ketones, in general, undergo the Schmidt reaction to give the respective cyclic lactam in very good yields (8–10). The bicyclic ketone norcamphor differs considerably in this respect. It may be noted that a good percentage of cyclopentene-3-acetonitrile has been formed as a minor product (7). This is to be expected when one considers norcamphor as a branched aliphatic ketone, since such ketones are reported (11) to give nitriles as by-products in the Schmidt reaction.

Several aliphatic and alicyclic amine derivatives of phenothiazine were reported (12), but only a few compounds derived from azabicyclic amines were reported (13,14). These compounds were found to possess chlorpromazine-like activity. The compounds described herein were synthesized by Scheme II and were isolated as the oxalate, since other salts offered difficulty in crystallization.



X = H, OCH_3

Scheme II

EXPERIMENTAL[1]

3-Azabicyclo[3.2.1]octane-2-one (II)—*Method A*—In a 2-l., three-neck flask equipped with a stirrer, a thermometer, and a dropping funnel, a paste of 45 g. (0.7 mole) of sodium azide in 45 ml. of warm water was made. Five-hundred milliliters of chloroform was added and stirred while the temperature was maintained between 0–5°, with the help of an ice-salt bath. Forty-five milliliters of concentrated sulfuric acid was added dropwise, while the temperature was kept in the temperature range mentioned above. Stirring was continued for another 0.5 hr. after the completion of the addition of the acid. The temperature was not allowed to rise above 5° throughout this addition. The chloroform solution of hydrazoic acid was decanted into a 1-l. conical flask. The residue was washed with 100 ml. of chloroform, and the washings were added to the main bulk of the solution. The solution was kept cooled in an ice bath and dried with anhydrous sodium sulfate.

In a 2-l., three-neck flask equipped with a stirrer, a thermometer, and a dropping funnel, 90 ml. of concentrated sulfuric acid was placed. To this, 15 g. of phosphorus pentoxide was added and stirred while 55 g. (0.5 mole) of norcamphor[2] was added slowly, keeping the temperature of the mixture below 30° by occasional cooling with an ice-salt bath. Stirring was continued for another 15–30 min., and 250 ml. of dry chloroform was added. The temperature was maintained between 0–8°, using an ice-salt bath, while the hydrazoic acid solution was added dropwise with vigorous stirring. Stirring was continued for another 4 hr. after the addition, during which time the temperature of the reaction mixture was allowed to come to room temperature. It was made alkaline with 20% sodium hydroxide solution (about 750 ml.) with cooling, so that the temperature was kept below 30°. The chloroform layer was separated; the aqueous layer was extracted with two 250-ml. portions of chloroform and added to the main bulk of the chloroform solution. This chloroform solution was washed with 50 ml. of distilled water and dried over anhydrous sodium sulfate; it was then filtered into a 2-l. distilling flask. The chloroform was removed using a water aspirator. The residue (47 g.) was distilled under vacuum, using an oil bath whose temperature was kept between 130–150°. Twenty-seven grams (43.2%) of the lactam (II) boiling between 110–125° (5 mm.) was collected; this soon solidified into a white mass, which was recrystallized from petroleum ether (b.p. 60–90°); m.p. 93–94°.

Method B—Following the method of Blicke and Doorenbos (8), the Schmidt reaction with norcamphor was performed. In a three-neck, round-bottom flask, fitted with a stirrer and thermometer, 22 g. (0.2 mole) of norcamphor was dissolved in 200 ml. of concentrated hydrochloric acid. The reaction mixture was kept at about 5° and 21 g. (0.32 mole) of sodium azide was added with stirring, which was continued for 5 hr. Solid sodium carbonate was added gradually with stirring until the reaction mixture was alkaline. The organic material was extracted with chloroform, and the extract was dried over anhydrous sodium sulfate. Removal of the solvent left 16.5 g. of a yellow oil, which was distilled under vacuum to yield 4 g. (16%) of a lactam boiling between 110–125° (5 mm.). About 6 g. of a low boiling fraction containing mostly cyclopentene-3-aceto nitrile was also collected.

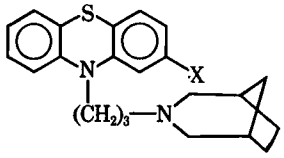
3-Azabicyclo[3.2.1]octane Hydrochloride (III)—The reduction of the lactam was effected essentially by following the method of Elderfield and Losin (7).

In a 3-l., three-neck flask fitted with an efficient condenser, stirrer, and dropping funnel was placed 1.5 l. of absolute ether. With stirring, 16 g. (0.16 mole) of lithium aluminum hydride was added

[1] All melting points are uncorrected. Melting points were determined on a Thomas-Hoover capillary melting point apparatus. Infrared spectra were determined on a Perkin-Elmer model 137 G Infracord spectrophotometer. As sodium azide and hydrazoic acid are highly toxic, the reaction must be carried out in a well-ventilated hood.

[2] Norcamphor was purchased from Aldrich Chemical Company, Inc., Milwaukee, Wisc.

TABLE I—PHENOTHIAZINE DERIVATIVES OF 3-AZABICYCLO[3.2.1]OCTANE



No.[a]	X	Formula	M.p., °C.[b]	Anal., %[c]	Calcd.	Found
1	H	$C_{22}H_{26}N_2S \cdot C_2H_2O_4$	204–205	C	65.48	65.32
				H	6.37	6.76
				N	6.37	6.26
2	OCH_3	$C_{23}H_{28}N_2OS \cdot C_2H_2O_4$	155–156	C	63.91	63.75
				H	6.39	6.44
				N	5.97	5.86

[a] Recrystallized from ethanol. [b] Melting points are uncorrected. [c] Microanalysis through Dr. Alfred Bernhardt, West Germany.

slowly. A solution of 25 g. (0.2 mole) of 3-azabicyclo[3.2.1]octane-2-one (II) in 750 ml. of absolute ether was added to the above, the addition being made in such a way that the reaction mixture refluxed gently. Stirring was continued for another 4–6 hr., and the reaction mixture stood overnight. A minimum quantity of ice was carefully added in small portions to break the complex (the color changed from gray to milky) and allowed to stand for 2 hr. The ether layer was separated from the inorganic material by filtration, and the residue was washed with two 200-ml. portions of ether. The ether solution was dried with anhydrous sodium sulfate. Twenty-two milliliters of concentrated hydrochloric acid (36%) in 100 ml. of ethanol was added with stirring to the above. The ether was removed by evaporation. The yellow viscous liquid was transferred to a 250-ml. round-bottom flask and evaporated to dryness under water vacuum. The crude hydrochloride was recrystallized from an ethanol–acetone mixture. The yield of the pure product was 21.8 g. (70%). The pure amine hydrochloride melted with decomposition above 298°. [Lit. (7) darkened at 240° and decomposed above 300°.] By further treatment of the mother liquor one may isolate 10–15% more of the compound.

The phenothiazine derivatives were prepared by following the method of Yale and Sowinski (15) with some modifications.

3-(10-Phenothiazinyl)-1-{N-(3-azabicyclo-[3.2.1]octyl)}propane Oxalate (Compound 1, Table I)—A mixture of 5.97 g. (0.03 mole) of phenothiazine and 1.4 g. (0.035 mole) of sodamide was refluxed in 100 ml. of dry toluene for 2 hr. To this 16.0 g. (0.12 mole) of 1-bromo-3-chloropropane was added. The refluxing was continued for 18 hr. The solvent and the excess 1-bromo-3-chloropropane were removed by distillation under water vacuum, and the residue was treated with 10 ml. of water and was extracted with 75 ml. of ether. The ether solution was dried with anhydrous sodium sulfate. The ether was removed under vacuum. The residue was dissolved in 100 ml. of dry toluene and dried with anhydrous sodium sulfate. The solution was decanted into a 200-ml. round-bottom flask and refluxed with 1.4 g. (0.035 mole) of sodamide and 3.3 g. (0.03 mole) of 3-azabicyclo[3.2.1]octane for 20 hr. The toluene solution was decanted into a separator and was extracted with dilute hydrochloric acid. The free base was precipitated from the acid solution by sodium carbonate and was extracted with 100 ml. ether. After drying, the ether was removed by evaporation. The residue was dissolved in 20 ml. of ethanol and 2 g. of oxalic acid in 20 ml. of ethanol was added. On cooling, the oxalate separated; on recrystallization from ethanol, this gave 3.60 g. (28%) of product, m.p. 204–205°.

3-[10-(2-Methoxy) Phenothiazinyl]-1-{N-(3-azabicyclo [3.2.1]octyl)}propane Oxalate (Compound 2, Table I)—This compound was prepared by the same procedure as described above, using 6.9 g. (0.03 mole) of 2-methoxy phenothiazine, which yielded 2.8 g. (25%) of the pure oxalate, m.p. 155–156°.

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Keyphrases

3-Azabicyclo[3.2.1]octane HCl—improved synthesis
Phenothiazine derivatives—3-azabicyclo-[3.2.1]octane HCl
Ir spectrophotometry—structure

Neutron Activation Analysis of *l*-Ephedrine and Norephedrine Silver Complexes

By MITCHELL L. BORKE*, PRADEEP B. MADAN†, and BRUCE D. MARTIN

Silver complex pyridinates of *l*-ephedrine and norephedrine were prepared and analyzed by neutron activation. The analytical results have established satisfactory correlations between theoretical and experimental values for percent silver in the two compounds.

DURING THE PAST several years neutron activation analysis (NAA) has become a sensitive, powerful analytical tool effective for approximately two-thirds of the elements. NAA provides an analytical method without destruction of the sample. Activation is carried out by placing the sample in a neutron field; *i.e.*, in a nuclear reactor or a neutron howitzer, where thermal neutrons present create radioactive nuclides through an (n,γ) nuclear reaction. Analysis is usually achieved by measuring the radioactivity of the product nuclides by direct γ-ray spectrometry with a scintillation detector and a γ-ray spectrometer or a multichannel analyzer.

Quantitative measurements are made either by comparison of the sample with a standard or by mathematical calculations based on the knowledge of the neutron flux (1) as well as the efficiency and geometry of the γ-ray detector (2). Sometimes the actual measurement must be preceded by a radiochemical separation procedure. Basic principles and application of NAA are described in the literature as well as in the excellent reference books (3–6).

This paper presents an analytical procedure for the determination of silver in *l*-ephedrine and norephedrine silver complex pyridinates by neutron activation utilizing the $^{109}Ag(n,\gamma)^{110}Ag$ nuclear reaction and equipment available to educational and manufacturing facilities, and requiring a minimum of shielding.

EXPERIMENTAL

Both *l*-ephedrine and norephedrine silver complex pyridinates were prepared according to the method reported by Nakatsuka in 1936 (7) for the synthesis of argentic 8-hydroxyquinolinate.

Neutron Activation Analysis—Solid samples of the silver complex and a standard sample ($AgNO_3$) of approximately the same, but accurately known, weight were placed in separate 2-ml. volumetric flasks, and introduced into the irradiation ports of a neutron howitzer[1] containing 3 Ci of plutonium-beryllium. After 20 min. both the sample and the standard were removed and counted for 10 sec. The counting system consisted of a commercial shielded well scintillation detector with 4.4 × 5 cm. ($1^3/_4 \times 2$ in.) NaI(Tl) crystal.[2] The detector output was fed into a γ-ray spectrometer[3] set on the ^{110}Ag photopeak (0.663 Mev.) and using a 20-Kev. window. Since the irradiation time was sufficiently long to achieve saturation, and the time between the removal of the material from the howitzer and the beginning of counting was maintained constant for both the sample and the standard, no decay correction was necessary.

The silver content was calculated as follows:

$$\% \text{ silver in sample} = \frac{\text{g. of standard} \times \% \text{ silver in standard}}{\text{g. of sample}} \times \frac{\text{c.p.m. (sample)}}{\text{c.p.m. (standard)}}$$

RESULTS AND DISCUSSION

Table I summarizes the results of NAA *l*-ephedrine and norephedrine silver complex pyridinates. The data for the samples and the standards are the averages of 15 determinations.

The activity of the samples (A) was calculated from the relationship

$$A = \bar{n} \pm S_m$$

where $\bar{n}$ is the average count rate and S_m is the standard deviation of the mean (8). Chauvenet's criterion was applied to each set of data in order to determine whether or not any values should be rejected as not being representative of the Poisson distribution. The chi-square test (9) indicated that the counting equipment was operating properly. Because of the short half-life of the radionuclide produced (^{110}Ag), no time was required for any products to decay before counting as noted previously by some authors (10).

The results of this study have established satisfactory correlation between theoretical and experimental values for percent silver in the two compounds as indicated by 1.90 and 1.35% error for *l*-ephedrine and norephedrine silver complex pyridinates, respectively. This investigation primarily demonstrated the usefulness of NAA as a method for the determination of silver content of compounds having pharmaceutical interest. It also showed that by selecting suitable metals for complexation with some organic compounds, NAA can be carried out using isotopic neutron sources. The radioactive product is not excessively active and may be easily handled by laboratory technicians trained in the handling of radioactive isotopes.

Received March 20, 1968, from the Department of Pharmaceutical Chemistry, School of Pharmacy, Duquesne University, Pittsburgh, PA 15219

Accepted for publication June 6, 1968.

Abstracted in part from a thesis submitted by Pradeep B. Madan to the Graduate School, Duquesne University, Pittsburgh, Pa., in partial fulfillment of Master of Science degree requirements, 1968.

* To whom communications should be directed.

† Present address: Microbiological Associates, Inc., P.O. Box 5970, Washington, D. C. 20014.

[1] Neutron-Pac, Irradiator-Howitzer, NAC-64, Nuclear Materials and Equipment Corp., Apollo, Pa.

[2] Model 810B, Baird-Atomic, Inc., Cambridge, Mass.

[3] Model 530, Baird-Atomic, Inc., Cambridge, Mass.

TABLE I—RESULTS OF NEUTRON ACTIVATION ANALYSIS OF *l*-EPHEDRINE AND NOREPHEDRINE SILVER COMPLEX PYRIDINATES

Compound	Sample Wt., g.	Amount Ag, g.	Activity, C.p.m.	% Ag (Exptl.)	% Ag (Theor.)	%[a] Error
l-Ephedrine-Ag pyridinate $(C_{20}H_{27}N_2O_2)Ag(C_5H_5N)$	0.5393	0.0541	38 ± 2	20.6	21.0	1.90
Standard $AgNO_3$	0.5393	0.1665	114 ± 3	—	63.5	—
Norephedrine-Ag-pyridinate $(C_{18}H_{23}N_2O_2)Ag(C_5H_5N)$	0.3918	0.0491	39 ± 2	22.0	22.3	1.35
Standard $AgNO_3$	0.3929	0.1215	113 ± 3	—	63.5	—

[a] $\frac{[\text{Percent Ag (Exptl.)} - \text{Percent Ag (Theor.)}]}{\text{Percent Ag (Theor.)}} \times 100$.

The use of this procedure in a manufacturing facility would provide a convenient analytical method for compounds of this nature.

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Keyphrases

Phenethylamine analogs—analysis
Silver complexes—phenethylamine analogs
Neutron activation—analysis

Identification of Aliphatic Amines from Rates of Cinnamoylation

By WEN-HAI HONG and KENNETH A. CONNORS

The second-order rate constants for reaction between *trans*-cinnamic anhydride and 26 aliphatic amines in acetonitrile solution are reported. The rate constant for this reaction is a discriminating criterion of identity.

EARLIER PAPERS in this series have described the identification of alcohols from rates of alkaline hydrolysis of their 3,5-dinitrobenzoate esters (1) and of sugars from their rates of oxime formation (2). Kinetic studies provide a powerful approach to the characterization of organic compounds because of the marked sensitivity of reaction rate to structure of the reactants. Because of its potential for distinguishing between closely related compounds, and its advantages of sensitivity, simplicity, and speed, rate measurement (actually rate constant measurement) should become a useful adjunct to spectroscopy and to conventional chemical methods of qualitative analysis.[1]

A procedure for the determination of amines has recently been described in which the amine is quantitatively converted to the corresponding cinnamide by acylation with *trans*-cinnamic anhydride (3). During the development of the method some cinnamoylation rates were measured, and it was noted that the acylation rate constant is dependent upon the amine structure. In the present paper this dependence is exploited to provide a new method for the characterization of primary and secondary aliphatic amines.

Received May 6, 1968, from the School of Pharmacy, University of Wisconsin, Madison, WI 53706
Accepted for publication June 25, 1968.
Supported in part by a grant from the Research Committee of the University of Wisconsin and by general research grant FR 05456 from the National Institutes of Health.

[1] An incidental benefit of this approach is the accumulation of extensive collections of rate data for related compounds under common conditions; such data may be put to valuable mechanistic use, and may also provide guidance in the development of quantitative analyses.

EXPERIMENTAL

Materials—*Trans*-cinnamic anhydride (J. T. Baker Chemical Co.) was recrystallized three times from benzene; m.p. 134–136°. Acetonitrile (Fisher Scientific Co., catalog No. A-21) was refluxed over phosphorus pentoxide and then distilled from phosphorus pentoxide through a packed column (4); b.p. 81–81.5°. Amines were purified by distillation.

Apparatus—Spectral measurements were made with a Cary model 14 recording spectrophotometer fitted with a thermostated cell compartment.

Procedure—Acetonitrile solutions of cinnamic anhydride (approximately 4×10^{-6} *M*) and of an amine (5.0×10^{-6} to 2.5×10^{-2} *M*, depending upon

TABLE I—RESULTS OF NEUTRON ACTIVATION ANALYSIS OF *l*-EPHEDRINE AND NOREPHEDRINE SILVER COMPLEX PYRIDINATES

Compound	Sample Wt., g.	Amount Ag, g.	Activity, C.p.m.	% Ag (Exptl.)	% Ag (Theor.)	%[a] Error
l-Ephedrine-Ag pyridinate $(C_{20}H_{27}N_2O_2)Ag(C_5H_5N)$	0.5393	0.0541	38 ± 2	20.6	21.0	1.90
Standard $AgNO_3$	0.5393	0.1665	114 ± 3	—	63.5	—
Norephedrine-Ag-pyridinate $(C_{18}H_{23}N_2O_2)Ag(C_5H_5N)$	0.3918	0.0491	39 ± 2	22.0	22.3	1.35
Standard $AgNO_3$	0.3929	0.1215	113 ± 3	—	63.5	—

[a] $\frac{[\text{Percent Ag (Exptl.)} - \text{Percent Ag (Theor.)}]}{\text{Percent Ag (Theor.)}} \times 100$.

The use of this procedure in a manufacturing facility would provide a convenient analytical method for compounds of this nature.

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Keyphrases

Phenethylamine analogs—analysis
Silver complexes—phenethylamine analogs
Neutron activation—analysis

Identification of Aliphatic Amines from Rates of Cinnamoylation

By WEN-HAI HONG and KENNETH A. CONNORS

The second-order rate constants for reaction between *trans*-cinnamic anhydride and 26 aliphatic amines in acetonitrile solution are reported. The rate constant for this reaction is a discriminating criterion of identity.

EARLIER PAPERS in this series have described the identification of alcohols from rates of alkaline hydrolysis of their 3,5-dinitrobenzoate esters (1) and of sugars from their rates of oxime formation (2). Kinetic studies provide a powerful approach to the characterization of organic compounds because of the marked sensitivity of reaction rate to structure of the reactants. Because of its potential for distinguishing between closely related compounds, and its advantages of sensitivity, simplicity, and speed, rate measurement (actually rate constant measurement) should become a useful adjunct to spectroscopy and to conventional chemical methods of qualitative analysis.[1]

A procedure for the determination of amines has recently been described in which the amine is quantitatively converted to the corresponding cinnamide by acylation with *trans*-cinnamic anhydride (3). During the development of the method some cinnamoylation rates were measured, and it was noted that the acylation rate constant is dependent upon the amine structure. In the present paper this dependence is exploited to provide a new method for the characterization of primary and secondary aliphatic amines.

Received May 6, 1968, from the School of Pharmacy, University of Wisconsin, Madison, WI 53706
Accepted for publication June 25, 1968.
Supported in part by a grant from the Research Committee of the University of Wisconsin and by general research grant FR 05456 from the National Institutes of Health.

[1] An incidental benefit of this approach is the accumulation of extensive collections of rate data for related compounds under common conditions; such data may be put to valuable mechanistic use, and may also provide guidance in the development of quantitative analyses.

EXPERIMENTAL

Materials—*Trans*-cinnamic anhydride (J. T. Baker Chemical Co.) was recrystallized three times from benzene; m.p. 134–136°. Acetonitrile (Fisher Scientific Co., catalog No. A-21) was refluxed over phosphorus pentoxide and then distilled from phosphorus pentoxide through a packed column (4); b.p. 81–81.5°. Amines were purified by distillation.

Apparatus—Spectral measurements were made with a Cary model 14 recording spectrophotometer fitted with a thermostated cell compartment.

Procedure—Acetonitrile solutions of cinnamic anhydride (approximately 4×10^{-6} *M*) and of an amine (5.0×10^{-6} to 2.5×10^{-2} *M*, depending upon

TABLE I—CINNAMOYLATION RATE CONSTANTS FOR ALIPHATIC AMINES

Amine	B.p., °C.[a]	k (M^{-1} sec.$^{-1}$)[b] Mean[c]	Av. dev[d]	pKa
Isopropyl-	33 (32–33)	21.42	0.09	10.63
tert-Butyl-	46 (44–45)	0.436	0.001	10.82
n-Propyl-	49 (47)	134.5	3.6	10.53
Diethyl-	55 (54)	30.3	0.6	10.98
Allyl-	56 (53)	33.8	0.5	9.69
sec-Butyl	63 (62)	16.95	0.07	10.56
Isobutyl-	69 (66–66.3)	115.3	0.0	10.72
n-Butyl-	77 (76)	158.3	0.4	10.60
Isoamyl-	95 (94–95)	156.8	5.9	10.6
n-Amyl-	104 (103–105)	160.8	4.5	10.64
Cyclopentyl-	105 (104–106)	46.0	0.8	9.95
Piperidine	109 (104–105)	1139	17	11.22
Di-*n*-propyl-	110 (109)	23.8	0.1	11.00
n-Hexyl-	128 (129–130)	168.8	5.7	10.64
Morpholine	130 (130–131)	151.6	4.1	8.70
Cyclohexyl-	134 (131–131.2)	25.9	0.3	10.64
Diisobutyl-	139 (137–139)	6.76	0.27	10.68
N-Methyl-cyclohexyl-	146 (146.5–147.5)	17.37	0.01	10.9
N-Ethyl-cyclohexyl-	164 (163–164)	0.629	0.006	11.3
N-Isopropyl-cyclohexyl-	174 (171–172)	0.107	0.004	11.3
n-Octyl-	180 (178–180)	164.4	3.7	10.65
Benzyl-	184 (181–183)	25.97	0.02	9.34
Diisoamyl-	187 (186–189)	3.69	0.05	10.94
β-Phenethyl-	198 (197–198)	58.3	0.5	9.83
Diisooctyl-	— (276)	8.34	0.03	—
Dibenzyl-	300 (285)	1.08	0.02	—

[a] Values in parentheses are the author's values. [b] At 25° in acetonitrile solution. [c] Mean of 2 to 5 determinations. [d] Average deviation = $\Sigma|x_i - \bar{x}|/(n-1)$.

reactivity) were equilibrated at 25.0 ± 0.1°. Fifteen milliliters of each solution was mixed in a 50-ml. beaker and rapidly transferred to a 10-cm. cylindrical cell. The absorbance of the reaction mixture at 305 mμ was followed with time as a continuous trace on the recorder chart.

RESULTS AND DISCUSSION

The reaction studied is

$$(C_6H_5CH{=}CHCO)_2O + RR'NH \xrightarrow{k} C_6H_5CH{=}CHCONRR' + C_6H_5CH{=}CHCOOH$$

For many amines this reaction is very rapid in acetonitrile solution, but the rate can be made experimentally accessible and convenient by employing low concentrations of the reactants. The amine concentration was always in excess, and first-order plots of $\log(A_t - A_\infty)$ against time were obtained.[2] From the slope the apparent first-order rate constant $k_{obs.}$ was calculated. This is related to the second-order rate constant k by $k_{obs.} = k$ [amine], thus permitting evaluation of k. Variation in amine concentration produced a directly proportional change in $k_{obs.}$, showing that the reaction is first order with respect to the amine.

Table I lists 26 amines, in order of increasing boiling point, with the second-order rate constants for their cinnamoylation.[3] The results in Table I show that the cinnamoylation rate constant is a very discriminating criterion of identity, the maximum range in relative rate being about 10^4. (Aromatic amines are much less reactive than aliphatic amines.) In fact, this rate constant alone would permit one to identify most of the amines listed. By taking both boiling point and rate constant, positive identification could be made of every amine studied, except perhaps diethylamine and allylamine (and these can be distinguished by their pKa values).

A Bronsted-type plot of log k against pKa shows considerable scatter. Primary amines with no substituents on the α or β carbon atoms appear to behave well, giving a line with slope 0.78. Most of the deviations from this line can be accounted for in a qualitative way in terms of steric effects, which usually lower k, so that the reactivity (nucleophilicity) of the amine toward the anhydride is less than would be predicted from its basicity. The alicyclic secondary amines react faster than anticipated on the basis of their pKa values. Attempts to correlate the data with the use of Taft polar and steric substituent constants did not give useful results.

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[2] Note that for use as an identification tool, reaction kinetic studies must be designed to give first-order kinetics, because only a first-order rate constant can be evaluated without knowledge of reactant concentration. Since (presumably) the reactant identity will be unknown, its molecular weight and hence its concentration will be unknown.

[3] Pyrrolidine was too reactive to be studied under first-order conditions.

Keyphrases

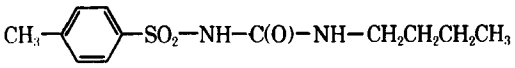
Amines, aliphatic—identification
Cinnamoylation rates—amine identification
UV spectrophotometry—reaction monitoring

Synthesis and Pharmacological Evaluation of Some Tosylbiurets and Tosylthiocarbamates as Potential Hypoglycemic Agents

By DOUGLAS C. KRIESEL and JAMES MENZIE*

The synthesis and preliminary pharmacological screening of some *p*-toluenesulfonylbiurets and alkyl *p*-toluenesulfonylthiocarbamates are described. The biurets were prepared by refluxing tosyl isocyanate with the appropriate urea, and the thiocarbamates were formed by the addition of selected mercaptans to the same isocyanate. None of the tosylbiurets possessed hypoglycemic activity; however, propyl tosylthiocarbamate lowered serum glucose levels in mice to approximately the same extent as tolbutamide.

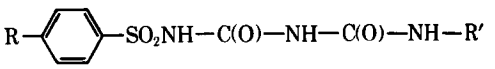
SIGNIFICANT STRIDES in the treatment of diabetes mellitus have been made since the introduction of oral hypoglycemic agents such as the sulfonylureas and the biguanides. In particular, the drug tolbutamide (I) has been successful in the treatment of the maturity-onset form of this disease.



(I)

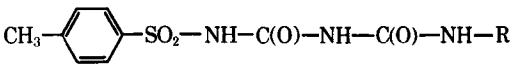
However, there are certain situations where tolbutamide therapy is inadequate; these cases have responded in some instances to other oral hypoglycemic agents. The purpose of this research was to attempt to prepare some hypoglycemic drugs that would be clinically effective in the treatment of diabetes mellitus.

Two types of derivatives of *p*-toluenesulfonyl isocyanate have been made and screened as hypoglycemic agents. Some *p*-toluenesulfonylbiurets were prepared to add to a series of molecules of this type that were previously recorded in the literature (1). These previously synthesized biurets did possess hypoglycemic activity although of a lower order than tolbutamide.



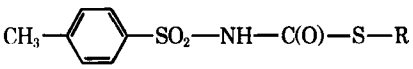
(II) R = NH_2, R′ = *n*-butyl
(III) R = CH_3, R′ = *n*-butyl
(IV) R = CH_3, R′ = 3-methoxy-*n*-propyl

Molecular models have demonstrated that these compounds (II, III, IV) possess a total length of about 2.5 Å. more than the known hypoglycemic agent, tolbutamide. The decrease in hypoglycemic activity might have resulted from the fact that the receptor protein was unable to accommodate for the increase in molecular size offered by the additional two terminal carbon atoms. The synthesis of some closely related tosylbiurets (V, VI, VII, VIII) was carried out to determine whether a steric relationship existed in this series.



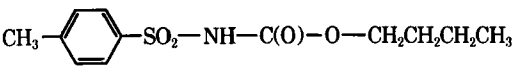
(V) R = H
(VI) R = CH_3
(VII) R = CH_2CH_3
(VIII) R = $CH_2CH_2CH_3$

A series of tosylthiocarbamates, (IX, X, XI, XII), also prepared from *p*-toluenesulfonyl isocyanate, was synthesized for biological testing.



(IX) R = CH_3
(X) R = CH_2CH_3
(XI) R = $CH_2CH_2CH_3$
(XII) R = $CH_2CH_2CH_2CH_3$

It was hoped that through substitution of a divalent sulfur atom for its bioisoster, the NH, in the tosylureas, a favorable change could be made which would lead to more useful molecules. A previously reported analog of the above, *n*-butyl *N*-tosylcarbamate (XIII), has been shown to be slightly active in reducing serum glucose levels in fasted rats (2). In this case, substitution of an



(XIII)

ether oxygen, which is also a bioisoster of the NH group, lowered the hypoglycemic activity of the resultant molecule when compared to tolbutamide.

DISCUSSION

The preparation of the biurets was accomplished by condensing the appropriate urea with tosyl isocyanate. The urea was dissolved in DMSO and added to the isocyanate in benzene to facilitate a homogeneous mixture. Nucleophilic attack by the unsubstituted nitrogen of the urea on the carbonyl of the isocyanate occurred yielding the corresponding tosylbiuret. The question of whether the disubstituted biurets were of the 1,3- or the 1,5- type was answered by obtaining an NMR spectrum for 5-methyl-1-tosylbiuret. A doublet at 2.70 p.p.m. (CH_3) and a quadruplet at 7.10 p.p.m. (NH) showed that coupling was taking place between the methyl group and an adjacent proton.

Received May 22, 1968, from the School of Pharmacy, Southwestern State College, Weatherford, OK 73096
Accepted for publication July 10, 1968.
* Recipient of the Mead Johnson Award for Undergraduate Research in Pharmacy, 1966–1967.

TABLE I—CHARACTERIZATION OF THE DERIVATIVES OF *p*-TOLUENESULFONYL ISOCYANATE

Compound	M.p., °C.	Yield, %	Anal.,% Calcd.	Found
		A. Tosylbiurets		
V	183–185	45.9	C, 42.07	42.24
			H, 4.31	4.52
VI	174–176	68.7	C, 44.28	44.27
			H, 4.83	4.90
			N, 15.49	15.20
VII	165–167	59.9	C, 46.32	46.57
			H, 5.30	5.42
			N, 14.74	14.73
VIII	112–114	37.8	C, 48.14	48.43
			H, 5.73	5.92
		B. Tosylthiocarbamates		
IX	132–134	57.1	C, 44.05	44.27
			H, 4.53	4.67
X	78–80	76.9	C, 46.30	46.32
			H, 5.06	5.19
XI	80–82	40.4	C, 48.32	48.56
			H, 5.54	5.60
XII	60–62	44.4	C, 50.14	50.38
			H, 5.97	5.98

TABLE II—SERUM GLUCOSE LEVELS OF SOME DERIVATIVES OF *p*-TOLUENESULFONYL ISOCYANATE

	Mean Serum Glucose Level, mg.% ± *SD*
Tosylbiurets	
Control (0.1 ml. propylene glycol)	130 ± 32
Tolbutamide, 25 mg./kg.	70 ± 12
V, 25 mg./kg.	160 ± 13
VI, 25 mg./kg.	182 ± 11
VII, 25 mg./kg.	132 ± 16
VIII, 25 mg./kg.	118 ± 15
Tosylthiocarbamates	
Control (0.1 ml. of 5% acacia)	115 ± 22
Tolbutamide, 25 mg./kg.	80 ± 19
IX, 25 mg./kg.	109 ± 23
X, 25 mg./kg.	114 ± 22
XI, 25 mg./kg.	83 ± 22
XII, 25 mg./kg.	110 ± 34

When D_2O was added, the methyl peak appeared as a singlet and the peak at 7.10 p.p.m. disappeared. This could only occur with the 1,5-disubstituted analogs.

Formation of the tosylthiocarbamates readily took place at room temperature when the thiol was added to tosyl isocyanate in the presence of a few drops of the catalyst, pyridine. Crystallization of the appropriate product was accomplished when the mixture in benzene was cooled to 10° and previously cooled cyclohexane was slowly added to reach the cloud point.

EXPERIMENTAL

All melting points were determined with a Thomas-Hoover Unimelt apparatus and are uncorrected. All of the elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

Tosylbiurets—*p*-Toluenesulfonyl isocyanate[1] (3.0 g., 0.015 mole) was dissolved in 30 ml. of dry benzene and added to a solution of the appropriate urea (0.015 mole) previously dissolved in 10 ml. of dimethyl sulfoxide. The mixture was refluxed for 48 hr. and the solution was then concentrated *in vacuo* to a viscous oil. The oil was dissolved in 15 ml. of ethanol and water was added to the cloud point. Cooling in ice water and scratching the sides of the vessel yielded a white crystalline product that was recrystallized from aqueous ethanol (Table I).

Tosylthiocarbamates—*p*-Toluenesulfonyl isocyanate (5.0 g., 0.025 mole) was quickly dissolved in 20 ml. of dry benzene and the appropriate mercaptan (0.025 mole) was added along with two drops of pyridine. The mixture was let stand at room temperature for 24 hr. and then cooled to 10°. Cyclohexane previously cooled to the same temperature was added slowly to reach the cloud point and after briefly scratching the sides of the vessel, the mixture was kept at 10° until crystallization ensued. Recrystallization of the white crystalline solid took place from a mixture of benzene–cyclohexane (Table I).

PHARMACOLOGICAL DATA

Screening for reduction of serum glucose levels was carried out using the Beckman Ultramicro adaptation of the method of Keston and Teller (3). This method involves a coupled enzyme system and utilizes glucose-oxidase-peroxidase and the chromogen, *o*-dianisidine. Initially, the oxidase converts glucose to gluconic acid and hydrogen peroxide; then the H_2O_2 formed is transformed to oxygen by the peroxidase. Finally the oxygen reacts with the chromogen to form a stable color complex which is read spectrophotometrically at 410 mμ. The intensity of the color formed is proportional to the glucose concentration.

The tosylbiurets were screened using mixed white rats that had been fasted for 24 hr. The compound was dissolved in propylene glycol and administered intraperitoneally (approximately 0.1 ml. was given). After 1 hr. a sample of blood was obtained by excising a portion of the tail, after which the sample was quickly centrifuged to obtain the necessary amount of serum.

[1] Purchased from Aldrich Chemial Co. Milwaukee, Wisc.

Fasted mixed white mice were used to screen the tosylthiocarbamates for hypoglycemic activity. The compounds were suspended in 5% acacia and administered intraperitoneally in a dose of 0.1 ml. After 1 hr., a sample of blood was obtained by decapitation and after immediate centrifuging, a serum sample was collected.

No hypoglycemic activity was obtained in the tosylbiuret series signifying that these molecules do not accommodate well with the receptor sites necessary to initiate the release of endogenous insulin.

n-Propyl *N*-tosylthiocarbamate was shown to be approximately as active as tolbutamide when tested in mice according to the enzymatic method described in this communication.

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(3) Beckman Ultramicro Analytical System Instruction Manual UM-IM-2, Technical Bulletin No. 6073D

Keyphrases

Tosylbiurets, thiocarbamates—synthesis

Propyl tosylthiocarbamate—hypoglycemic activity

Colorimetric analysis—spectrophotometer

Antiradiation Compounds XII. Dithiocarbamates of Strongly Basic Acridines and Quinaldines

By WILLIAM O. FOYE, DOUGLAS H. KAY, and PRAMOD R. AMIN*

Dithiocarbamates of strong heterocyclic bases (pKa 8–12) in the acridine and quinaldine series have been obtained, with further examples of the formation of imino-*N*-carbodithioates. Significant radiation protection of mice was found for the dithiocarbamate ester of 9-aminoacridine, whereas dithiocarbamates of both stronger and weaker bases were nonprotective.

Significant radiation protection in mice irradiated with 1000 r (γ-rays) has been found for the dithiocarbamate of a strongly basic heterocyclic imine (1). The base was 1,2-dihydro-1-methyl-2-iminopyridine, obtained by ring-*N*-alkylation of 2-aminopyridine, and has a pKa of 12.2. Dithiocarbamates of the corresponding 4-iminopyridine and 2-iminopyrimidine gave no radiation protection at this radiation level, however. In order to determine structural requirements for radiation protection by dithiocarbamates of strongly basic heterocycles, preparation of dithiocarbamates of this type of compound has been extended to include acridines and quinaldines.

Dithiocarbamates were generally prepared from these bases, for which pKa values ranged from 8–12 (2) in alcohol with or without the presence of additional base such as pyridine or triethylamine. The dithiocarbamates were obtained as esters, salts of the heterocyclic base, or salts of the added base; the nature of the product was unpredictable. Characteristic absorption for dithiocarbamates was observed in the UV at 220, 240–250, 260–275, and 290–310 mμ (3) and in the IR near 1,000 cm.$^{-1}$ (1) for these products, providing further evidence for dithiocarbamate formation of imines. In contrast to dithiocarbamate formation of aliphatic amines which generally takes place quite rapidly (4), some of the products reported here required a number of days to form.

Radiation protection in mice was found for the dithiocarbamate of 9-aminoacridine (pKa = 10.0) *versus* 825 r (X-rays). No protection was reported for several other members of these series made from heterocycles having either greater or lesser basicities.

DISCUSSION

The method of Albert (5) was used to prepare 3-aminoacridine (pKa = 8.0). Treatment of this compound with carbon disulfide in ethanol gave an undefinable product, but in the presence of a large excess of triethylamine, the triethylammonium salt of 3-acridyldithiocarbamate was obtained. In contrast to this behavior, 9-aminoacridine (pKa = 10.0) was converted to ethyl 9-acridyldithiocarbamate when treated with carbon disulfide in ethanol in either the presence or absence of triethylamine. The yield was larger in the former case, however. The UV absorption spectrum in ethanol showed peaks at 243 and 270 mμ for the 3-isomer and 261 and 304 mμ for the 9-isomer.

The procedure of Albert (6) was used to prepare 9-imino-10-methylacridinium iodide (pKa = 11.1). This compound was treated with sodium hydroxide to yield 9-amino-9-hydroxy-10-methylacridine which was dehydrated to yield 9,10-dihydro-9-imino-10-methylacridine. Both the hydroxy and the de-

Received May 22, 1968, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston, MA 02115

Accepted for publication July 9, 1968.

Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

This investigation was supported by research grant RH00297 from the National Center for Radiological Health, U. S. Public Health Service, Bethesda, Md.

* In part.

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Keyphrases

Tosylbiurets, thiocarbamates—synthesis

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Colorimetric analysis—spectrophotometer

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By WILLIAM O. FOYE, DOUGLAS H. KAY, and PRAMOD R. AMIN*

Dithiocarbamates of strong heterocyclic bases (pKa 8–12) in the acridine and quinaldine series have been obtained, with further examples of the formation of imino-*N*-carbodithioates. Significant radiation protection of mice was found for the dithiocarbamate ester of 9-aminoacridine, whereas dithiocarbamates of both stronger and weaker bases were nonprotective.

Significant radiation protection in mice irradiated with 1000 r (γ-rays) has been found for the dithiocarbamate of a strongly basic heterocyclic imine (1). The base was 1,2-dihydro-1-methyl-2-iminopyridine, obtained by ring-*N*-alkylation of 2-aminopyridine, and has a pKa of 12.2. Dithiocarbamates of the corresponding 4-iminopyridine and 2-iminopyrimidine gave no radiation protection at this radiation level, however. In order to determine structural requirements for radiation protection by dithiocarbamates of strongly basic heterocycles, preparation of dithiocarbamates of this type of compound has been extended to include acridines and quinaldines.

Dithiocarbamates were generally prepared from these bases, for which pKa values ranged from 8–12 (2) in alcohol with or without the presence of additional base such as pyridine or triethylamine. The dithiocarbamates were obtained as esters, salts of the heterocyclic base, or salts of the added base; the nature of the product was unpredictable. Characteristic absorption for dithiocarbamates was observed in the UV at 220, 240–250, 260–275, and 290–310 mμ (3) and in the IR near 1,000 cm.$^{-1}$ (1) for these products, providing further evidence for dithiocarbamate formation of imines. In contrast to dithiocarbamate formation of aliphatic amines which generally takes place quite rapidly (4), some of the products reported here required a number of days to form.

Radiation protection in mice was found for the dithiocarbamate of 9-aminoacridine (pKa = 10.0) *versus* 825 r (X-rays). No protection was reported for several other members of these series made from heterocycles having either greater or lesser basicities.

DISCUSSION

The method of Albert (5) was used to prepare 3-aminoacridine (pKa = 8.0). Treatment of this compound with carbon disulfide in ethanol gave an undefinable product, but in the presence of a large excess of triethylamine, the triethylammonium salt of 3-acridyldithiocarbamate was obtained. In contrast to this behavior, 9-aminoacridine (pKa = 10.0) was converted to ethyl 9-acridyldithiocarbamate when treated with carbon disulfide in ethanol in either the presence or absence of triethylamine. The yield was larger in the former case, however. The UV absorption spectrum in ethanol showed peaks at 243 and 270 mμ for the 3-isomer and 261 and 304 mμ for the 9-isomer.

The procedure of Albert (6) was used to prepare 9-imino-10-methylacridinium iodide (pKa = 11.1). This compound was treated with sodium hydroxide to yield 9-amino-9-hydroxy-10-methylacridine which was dehydrated to yield 9,10-dihydro-9-imino-10-methylacridine. Both the hydroxy and the de-

Received May 22, 1968, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston, MA 02115

Accepted for publication July 9, 1968.

Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

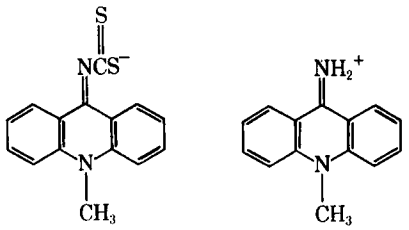
This investigation was supported by research grant RH00297 from the National Center for Radiological Health, U. S. Public Health Service, Bethesda, Md.

* In part.

hydrated compounds were found to have identical IR and UV absorption spectra in ethanol (peaks at 220 and 265 mμ).

Treatment of either of these compounds with carbon disulfide in absolute ethanol yielded 9,10-dihydro - 10 - methylacridyl - 9 - immonium 9,10-dihydro - 10 - methylacridyl - 9 - imino - *N* - carbodithioate (I), which showed UV absorption at 221, 243, 265, and 288 (shoulder) mμ.

6,9-Diamino-2-ethoxyacridine monohydrate (pKa 11.6) was prepared from the lactate salt by treatment with aqueous sodium hydroxide followed by oven drying until the correct weight was lost to give the monohydrate. Treatment of this compound with carbon disulfide in acetone gave bis-(2-ethoxy-6-aminoacridyl-9-ammonium) 2-ethoxyacridyl-6,9-bis(dithiocarbamate)(II).



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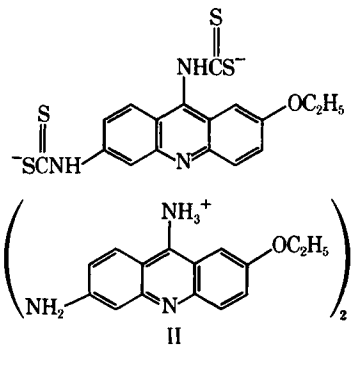
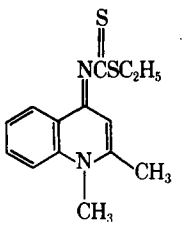


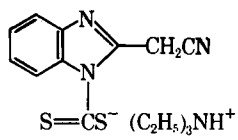
II

Pyridinyl 4-quinaldinyldithiocarbamate was obtained from the reaction at 25° of 4-aminoquinaldine (pKa = 9.4), pyridine, carbon disulfide, and triethylamine, and required 16 days. By analogy to the aminoacridine reactions with carbon disulfide and triethylamine in ethanol, similar treatment of 4-aminoquinaldine gave a product for which the analytical data was reasonable for the ethyl ester of the dithiocarbamate. However, an analytically pure sample was not obtainable.

The preparation of 1,4-dihydro-4-imino-1-methylquinaldine hydriodide (m.p. 280–283°) has not previously been described. However, Ochiai (7) has described an isomer, 4-methylaminoquinaldine (m.p. 239°), its hydriodide (m.p. 285° dec.), and the picrate (m.p. 264° dec.). The free base, 1,4-dihydro-4-imino-1-methylquinaldine (pKa ~ 12.0) was obtained by treating the hydriodide with potassium hydroxide followed by a toluene extraction. Proof that these conditions had not caused rearrangement to 4-methylaminoquinaldine (which was found to occur with certain pyrimidines and pyridines) (1) was shown by the formation of the picrate (m.p. 243–245°) of the free base in toluene. The toluene was removed, and the free base in absolute ethanol was treated with carbon disulfide to yield ethyl 1,4 - dihydro - 1 - methylquinaldine - 4 - imino - *N*-carbodithioate (III).



III



IV

Treatment of 2-benzimidazolylacetonitrile (pKa = 10.7) in absolute ethanol with carbon disulfide and triethylamine readily yielded the triethylammonium salt of the dithiocarbamate (IV).

Antiradiation Properties—Anitradiation screening results of several of the compounds at the Walter Reed Army Institute of Research have been reported through the courtesy of Dr. D. P. Jacobus. Tests were carried out in mice *versus* 825 r (X-rays) with an observation period of 30 days. The dithiocarbamates of 3-aminoacridine, 4-imino-1-methylquinaldine and 2-benzimidazolylacetonitrile provided no protection at this level of radiation dosage. The dithiocarbamate ester of 9-aminoacridine, however, showed fair protection (25–44% survival) at this radiation dosage but no protection at 1000 r (γ-rays) at compound dosage levels of less than 50 mg./kg.

EXPERIMENTAL

Analyses for carbon, hydrogen, and nitrogen were done by Weiler and Strauss, Oxford, England. Sulfur analyses were done by Parr bomb peroxide fusion. Melting points were taken on a Mel-Temp apparatus and are corrected. IR absorption spectra were obtained with a Perkin-Elmer model 137B spectrometer. The authors are indebted to Mr. J. R. Lo for determination of pKa values, obtained with a Beckman research pH meter with glass and calomel electrodes.

Triethylammonium 3-Acridyldithiocarbamate—Six grams (0.031 mole) of 3-aminoacridine (5) was dissolved in 36 ml. of absolute ethanol containing 4.3 ml. (0.061 mole) of carbon disulfide and 50 g. (0.5 mole) of triethylamine. The reaction solution was stirred for 30 min. and then kept at 25° for 3 days. Precipitation occurred during this time. The solid was collected and washed with 10-ml. portions of absolute ethanol, acetone, and anhydrous ether. A yield of 3.2 g. (28%) was obtained, m.p. 123–125°, IR (KBr) 2,650(NH^+), 1,000(C=S) $cm.^{-1}$, UV max. (95% EtOH) 243 (ε84,610), 270 (ε121,540) mμ.

Anal.—Calcd. for $C_{20}H_{25}N_3S_2$: C, 64.96; H, 6.74; N, 11.31; S, 17.35. Found: C, 64.68; H, 6.61; N, 10.81; S, 17.53.

Ethyl 9-Acridyldithiocarbamate Monohydrate—9-Aminoacridine (10.0 g., 0.052 mole) was refluxed with 115 ml. of 95% ethanol and 25 ml. of triethylamine on a water bath. Then 14 ml. of carbon disulfide was added slowly during 20 min., and refluxing was continued for 1 hr., during which

time hydrogen sulfide was evolved and the reaction solution became orange in color.

The reaction mixture was stirred occasionally at room temperature for 2 days. During this time precipitation occurred and hydrogen sulfide was evolved. About 10 g. of a yellow solid was obtained which was recrystallized from 350 ml. of 95% ethanol. About 2.5 g. of solid was insoluble in the ethanol. On cooling and concentration of the filtrate, 3.5 g. (21%) of product was obtained, m.p. 188–190°, IR (mineral oil) 1,000 (C=S) cm.$^{-1}$, UV max. (95% EtOH) 261 (ϵ77,210), 304 (ϵ11,780) mμ.

Anal.—Calcd. for $C_{16}H_{14}N_2S_2 \cdot H_2O$: C, 60.73; H, 5.09; N, 8.85; S, 20.26. Found: C, 60.6; H, 5.3; N, 8.6; S, 20.1.

9,10-Dihydro-10-methylacridyl-9-immonium 9,10-Dihydro-10-methylacridyl-9-imino-N-carbodithioate—Three grams (0.014 mole) of 9,10-dihydro-9-imino-10-methylacridine (6) was dissolved in 12 ml. of absolute ethanol in a flask protected from moisture. Then 3.5 ml. (0.05 mole) of carbon disulfide was added and the reaction solution was gently refluxed above a water bath for 20 min. An orange solid was precipitated during this time.

The mixture was ice cooled and the orange solid was collected and washed with 15 ml. of absolute ethanol; 2.1 g. (63%) of product was obtained, m.p. 241–243°, IR (KBr) 2,050 (NH_2^+), 1,675 (C=N), 995 (C=S) cm.$^{-1}$, UV max. (95% EtOH) 221 (ϵ40,000), 243 (ϵ71,000), 265(ϵ86,270), 288 (shoulder)(ϵ24,000) mμ.

Anal.—Calcd. for $C_{29}H_{24}N_4S_2$: C, 70.70; H, 4.91; N, 11.37. Found: C, 70.93; H, 4.99; N, 11.19.

6,9-Diamino-2-ethoxyacridine Monohydrate—A mixture of 4 g. of sodium hydroxide dissolved in 100 ml. of distilled water, and 15 g. of 6,9-diamino-2-ethoxyacridine lactate monohydrate (0.042 mole) (Aldrich Chemical Co.) was stirred for 2 hr. The yellow solid was collected, washed with three 40-ml. portions of distilled water, and dried at 105–110° until it weighed 11.2 g., m.p. 116–118°, pKa(H_2O) 11.6.

Anal.—Calcd. for $C_{15}H_{16}N_3O \cdot H_2O$: C, 66.15; H, 6.62; N, 15.43. Found: C, 66,39; H, 6.64; N, 15.35.

Bis(2-ethoxy-6-aminoacridyl-9-ammonium) 2-Ethoxyacridyl-6,9-bis(dithiocarbamate)Trihydrate—To 6.0 g. (0.022 mole) of 6,9-diamino-2-ethoxyacridine monohydrate in 200 ml. of acetone, 23 ml. (0.33 mole) of carbon disulfide was added with constant stirring. An orange solid precipitated immediately. The reaction mixture was stirred for 3 hr. and the solid was collected, washed with two 40-ml. portions of acetone, and air-dried, giving 5.0 g. (75%), m.p. 174–175°; IR(KBr) 3,000 (NH_3^+), 1,000 (C=S) cm.$^{-1}$.

Anal.—Calcd. for $C_{47}H_{45}N_9O_3S_4 \cdot 3H_2O$: C, 58.42; H, 5.32; N, 13.04. Found: C, 58.96; H, 4.95; N, 13.00.

Pyridinyl 4-Quinaldinyldithiocarbamate Monohydrate—Five grams (0.032 mole) of 4-aminoquinaldine (Aldrich Chemical Co.) was dissolved in 25 ml. of pyridine, 15 ml. of triethylamine, and 12 ml. (0.17 mole) of carbon disulfide in a stoppered flask and stirred occasionally during 16 days, the last 3 days with the stopper ajar. A yellow solid was slowly deposited and the reaction solution became dark red in color. The solid was collected, washed with three 20-ml. portions of acetone and one 20-ml. portion of anhydrous ether, yielding 3.0 g. (28%), m.p. 290–291° with decomposition beginning about 150°, IR (KBr) 2,750 (NH^+), 993 (C=S) cm.$^{-1}$.

Anal.—Calcd. for $C_{16}H_{15}N_3S_2 \cdot H_2O$: C, 57.97, H, 5.17; N, 12.67. Found: C, 57.78; H, 5.08; N, 13.42.

1,4-Dihydro-4-imino-1-methylquinaldine Hydriodide—Ten grams (0.063 mole) of 4-aminoquinaldine in 60 ml. of methanol was refluxed on a water bath, and 16.0 ml. (0.25 mole) of methyl iodide was added dropwise during 10 min. A white solid precipitated after about 20 min. The mixture was refluxed for 60 min. and then ice cooled. The solid was collected, washed with two 20-ml. portions of hot acetone, and recrystallized from methanol to yield 9.8 g. (49%), m.p. 280–283°.

Anal.—Calcd. for $C_{11}H_{13}IN_2 \cdot H_2O$: C, 41.81; H, 4.72; N, 8.81. Found: C, 42.11; H, 4.75; N, 8.92.

The picrate was crystallized from 95% ethanol, m.p. 243–245°.

Anal.—Calcd. for $C_{17}H_{15}N_5O_7$: C, 50.73; H, 3.76; N, 17.45. Found: C, 51.22, H, 4.03; N, 17.55.

Ethyl 1,4-Dihydro-1-methylquinaldine-4-imino-N-carbodithioate Monohydrate—Four grams (0.013 mole) of 1,4-dihydro-4-imino-1-methylquinaldine hydriodide monohydrate was mixed thoroughly with 3.0 g. of powdered potassium hydroxide in a mortar and extracted in a Soxhlet apparatus with toluene for 2.5 hr. Five milliliters of the toluene extract was treated with 5 ml. of a saturated solution of picric acid in toluene, giving a picrate melting at 243–245°, the value found for the picrate of 1,4-dihydro-4-imino-1-methylquinaldine.

The toluene solution of 1,4-dihydro-4-imino-1-methylquinaldine was evaporated at 25 mm. on a water bath. The tan hygroscopic solid obtained was dissolved in 15 ml. of hot absolute ethanol and the solution was filtered. The warm filtrate was treated with 1 ml. (0.014 mole) of carbon disulfide, and an orange solid slowly precipitated. The mixture was stirred for 1 hr., and the tan solid was collected and washed with 10 ml. of absolute ethanol and two 10-ml. portions of anhydrous ether, giving 1.0 g. (27%), m.p. 134–135°, IR(KBr) 1,650 (C=N), 1,005 (C=S) cm.$^{-1}$.

Anal.—Calcd. for $C_{14}H_{16}N_2S_2 \cdot H_2O$: C, 57.16; H, 6.66; N, 9.51; S, 21.78. Found: C, 56.97; H, 6.12; N, 9.75; S, 21.34.

Triethylammonium 2-Benzimidazolylacetonitrile-1-carbodithioate—2-Benzimidazolylacetonitrile (5.5 g., 0.035 mole)[pKa(H_2O) 10.7] was warmed with 30 ml. of absolute ethanol, 42 ml. (0.6 mole) of carbon disulfide, and 70 ml. (0.56 mole) of triethylamine to effect solution. The solution was allowed to stand at 25° for 2 days with occasional stirring. The orange solid was collected and washed with two 30-ml. portions of anhydrous ether and 25 ml. of absolute ethanol, yielding 9.8 g. (84%), m.p. 132–134°, IR(KBr), 2,650 (NH^+), 2,200 (C≡N), 1,010 (C=S) cm.$^{-1}$.

Anal.—Calcd. for $C_{16}H_{22}N_4S_2$: C, 57.45; H. 6.62; N, 16.76; S, 19.17. Found: C, 57.46; H, 6.71; N, 16.75; S, 19.99.

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Keyphrases

Antiradiation compounds
Acridine dithiocarbamates—synthesis
Quinaldine dithiocarbamates—synthesis
Radioprotective activity—screening
IR spectrophotometry—identity
UV spectrophotometry—identity

Rheological Stability of a Procaine Penicillin G Suspension

By JAMES C. BOYLAN and ROBERT L. ROBISON

The rheology of a 58.6 percent procaine penicillin G suspension was followed for a 24-month period. Samples stored as recommended (5°) were unchanged after 2 years. Samples stored at 26 and 37° increased in viscosity, yield value, and thixotropy with time.

In formulating a product where rheological properties are important, it is not only necessary to ascertain that the freshly prepared product possesses the necessary rheological characteristics, but more importantly whether or not these characteristics change during the recommended shelf life of the product. A specific case where unchanging rheological properties are critical is an injectable suspension of procaine penicillin G (600,000 u./ml.). The rheological parameters of this type of suspension determine (*a*) ease of filling, (*b*) prevention of separation during shipping and storage, and (*c*) injectability. It is difficult to formulate a procaine penicillin G suspension that will be satisfactory under all these varied conditions.

In 1958, Ober and co-workers (1) published an outstanding rheological study of concentrated procaine penicillin G depot preparations. They reported that in aqueous suspensions of 40 to 70% (w/w) procaine penicillin G, the rheological structural breakdown point (the point of maximum torque at very low shearing rate) is the physical parameter that defines the structure of the suspension prior to any applied shearing force. Although the nature of this structure is not known, they reasoned that the existence of this structural breakdown point does allow highly concentrated procaine penicillin G suspensions to fluidize enough to allow passage through a hypodermic needle. Furthermore, it permits good depot formation by virtue of quickly regaining this structure. They also determined what effect particle-size distribution, specific surface of the powder, and percent solids had on these phenomena of structure formation, breakdown, and recovery. By using a variety of techniques, including rheology, a region of satisfactory formulation was ascertained.

Received April 15, 1968, from Eli Lilly and Company, Indianapolis, IN 46206
Accepted for publication June 14, 1968.

It is the purpose here to report the rheological changes obtained with a procaine penicillin G suspension under a variety of storage conditions and to illustrate how critically important storage conditions are to product performance.

EXPERIMENTAL

Materials—The suspension studied[1] had the following formula:

procaine penicillin G (USP)	58.6%
sodium citrate (USP)	4.0%
polysorbate 80 (USP)	0.4%
lecithin	1.5%
butyl parahydroxybenzoate	0.015%
water for injection (USP) *q.s.ad.*	100.0%

All figures are expressed as w/v.

The samples evaluated were disposable syringes taken from a production lot. Storage was under three conditions: 5, 26, and 37°. The marketed product bears a refrigeration storage statement and a 24-month expiration date.

Rheological Evaluation—The viscometer used in this study was a Ferranti-Shirley cone and plate viscometer[2] equipped with a 400 g. cm. spring, an automatic gap-setting device, an X-Y recorder,[3] and a constant-temperature water bath.[4] The use of this viscometer has been described elsewhere (2). Calibration of the instrument was carried out using N.B.S. standard viscosity oils.

Each sample was contained in a 1-ml. disposable syringe. Since the sample size required for this viscometer is approximately 1 ml., the sample was used in its entirety (except for some retention in the syringe). The sample to be evaluated was treated

[1] Duracillin A. S. (Lilly), 600,000 u./ml.
[2] Ferranti Electric Co., Plainview, Long Island, N. Y.
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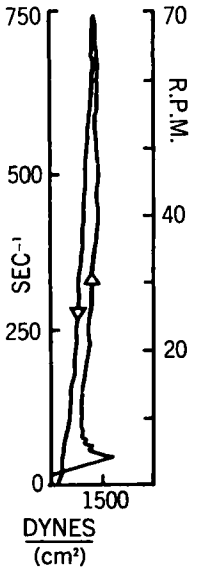

Fig. 1—Rheogram of procaine penicillin G suspension (600, 000 u./ml.) stored 24 months at 5°. Viscosity at 70 r.p.m. is 1.5 poises. Thixotropic area is 1 sq. in. Rheograms obtained initially and after 6, 12, and 18 months are also essentially the same as Fig. 1.

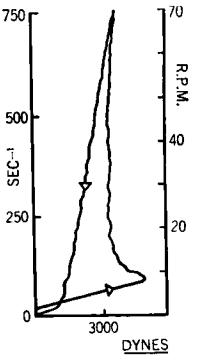

Fig. 2—Rheogram of procaine penicillin G suspension (600, 000 u./ml.) stored 12 months at 26°. Viscosity at 70 r.p.m. is 4.3 poises. Thixotropic area is 3.5 sq. in.

as follows: After the needle guard and syringe plunger were removed, a microspatula was used to help the suspension gently flow out of the plunger end of the syringe. This eliminated the need to expel the sample through the needle and insured minimum preshearing of the sample. As would be expected, if the suspension was expelled through the needle, very little structure remained after expulsion. The plate was immediately raised into position with the cone. Prior to running, the sample was held as close to 25° as practical. The plate was prestabilized at 25 ± 0.1° by circulating water from the water bath. Since, however, the suspension dries rapidly when exposed to air at room temperature, it was necessary to run the sample within 1 min. after raising the plate into position with the cone. The results obtained indicated that temperature control was adequate for reproducible results. All rheograms were obtained using a truncated cone having an angle of 33 min. 26 sec., and a radius of 2 cm. The instrument was set at an upsweep time of 60 sec., a downsweep time of 60 sec., and a maximum r.p.m. of 70.

RESULTS AND DISCUSSION

The freshly prepared suspension filled, shipped, and injected satisfactorily.

The results obtained as the samples aged are definitely related to the storage temperature. Rheograms of suspension stored at 5° (the recommended storage temperature) showed no change after 24 months (the recommended storage time) (see Fig. 1). Suspension stored at either 26 or 37° showed pronounced rheological changes. Figure 2, for example, shows the rheogram of a disposable syringe stored for 12 months at 26°. The yield value, thixotropy, and viscosity have all increased as a result of the higher storage temperature. Material represented by Fig. 2 is still ejectable, but more force and effort are required to do so. At 37° the effect is much more dramatic and occurs much sooner. Within 3 weeks at 37° the yield value of the material is so great that the pen of the X-Y recorder goes off scale. Under this condition the suspension cannot be injected from the syringe into a muscle. Hence, it is mandatory that this product be stored at the recommended temperature.

One additional important fact that this information points out is that elevated temperature conditions cannot be used to predict the rheological properties of this suspension. The only way to determine the rheological characteristics is to store the product at the temperature of interest and wait the necessary time before gathering the data.

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Keyphrases

Procaine penicillin G suspension
Rheology—penicillin G suspension
Aging—rheological properties
Temperature—rheological properties

Preparation of Some 5-Hydroxyimidazolidin-2-ones, 4-Imidazolin-2-ones, and Hydantoins from 3,4-Diphenyl-4-oxazolin-2-one

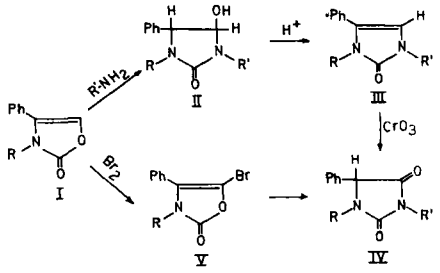
By M. F. SAETTONE and V. NUTI

A series of 1,3,4-trisubstituted 5-hydroxyimidazolidin-2-ones (II) was prepared by reaction of 3,4-diphenyl-4-oxazolin-2-one with primary amines. Compounds II were dehydrated to 4-imidazolin-2-ones (III). The action of bromine on the latter compounds, leading to 1,3,5-trisubstituted hydantoins (IV), was investigated. Compounds IV were also prepared from 5-bromo-3,4-diphenyl-4-oxazolin-2-one and primary amines. The results of some preliminary pharmacological studies are reported.

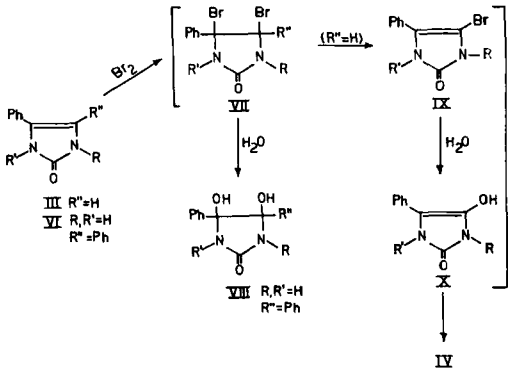
It was reported in a previous paper (1) that 5-unsubstituted 4-oxazolin 2-ones (I, Scheme I) can be converted into 5-hydroxyimidazolidin-2-ones (II) by reaction with primary amines. As a part of the structural proof, Compounds II were dehydrated to the corresponding 4-imidazolin-2-ones (III), and these were in turn oxidized to hydantoins (IV). The preparation of 1,3,5-trisubstituted hydantoins *via* a different route, involving bromination of compounds of Type I at the 5-position and reaction of the bromo derivative (V) with primary amines, was also described.

The recent discovery (2) of the potent central nervous system depressant activity shown by some 1-aminoalkyl-3-aryl derivatives of imidazolidin-2-one, prompted the authors to prepare by their method a number of 5-hydroxyimidazolidin-2-ones (II and Table I) in order to test their biological properties. It also appeared interesting to prepare the Δ^4-unsaturated (III and Table II), and 5-oxo (IV and Table III) analogs of Compounds II, in an endeavor to establish structure-activity relationships between the three related series.

All 1,3,5-trisubstituted 5-hydroxyimidazolidin-2-ones prepared in the present study were obtained by reaction of 3,4-diphenyl-4-oxazolin-2-one (I, R = Ph) with primary amines, in satisfactory yields. The dehydration of the hydroxy derivatives II to 4-imidazolin-2-ones (III), and the conversion of Compounds III into hydantoins (IV), were thoroughly reinvestigated. Best results were obtained (60–95% yields) when the dehydration was carried out with dilute sulfuric acid at room temperature, rather than with boiling acetic acid as described before (1). Chromic acid oxidation proved to be a poor method for conversion of 4-imidazolinones to hydantoins, particularly when applied to compounds bearing a 2-aminoalkyl side chain. However, it was found that bromination of Compounds III followed by crystallization of the resulting unstable bromo-derivatives from aqueous ethanol resulted in smooth conversion into hydantoins. 4-Imidazolinones bearing basic side-chains were transformed by this procedure directly into hydantoin hydrobromides. The reaction (Scheme II) probably involves addition of bromine to the double bond of Compounds III to give the dibromo derivatives VII (R″ = H), elimination of hydrogen bromide from VII to give the unstable 5-bromo derivatives IX, substitution of the bromine atom by an OH group and tautomerization of the resulting 5-hydroxy-4-imidazolinone X to the hydantoin. 4,5-Disubstituted 4-imidazolin-2-ones (such as VI) have been reported (3) to undergo a similar addition of bromine. However, substitution at the 4 and 5 position prevents elimination of hydrogen bromide, and treatment of the unstable adduct VII (R″ = Ph) with water results in formation of a 4,5-dihydroxyimidazolidin-2-one (VIII).



Scheme I

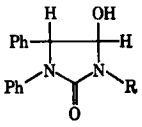


Scheme II

The hydantoins were also prepared by reaction of 5-bromo-4,5-diphenyl-4-imidazolin-2-one (V, R = Ph) with primary amines: this method gave in general higher yields, and in one case (3-allyl-1,5-diphenyl-hydantoin, Compound *25*) was the only possible route for the preparation of the desired compound. Indeed, treatment of the 1-allylimidazolone *14* with bromine gave the dibromohydantoin XI, also prepared by bromination of the hydantoin *25*.

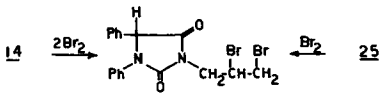
Received May 14, 1968, from the Institute of Pharmaceutical Chemistry, University of Pisa, 56100 Pisa, Italy.
Accepted for publication July 5, 1968.

TABLE I—5-HYDROXYIMIDAZOLIDIN-2-ONES



No.	R	Recrystn. Solvent[a]	Yield, %	M.p., °C.[b]	Molecular Formula	Anal., % Calcd.	Found
1	Ethyl	E	84	163–165	$C_{17}H_{18}N_2O_2$	C, 73.32 H, 6.43 N, 9.92	C, 73.10 H, 6.52 N, 10.00
2	*n*-Propyl	E	94	121–123	$C_{18}H_{20}N_2O_2$	C, 72.92 H, 6.80 N, 9.45	C, 73.20 H, 6.83 N, 9.24
3	Allyl	E	78	160–162	$C_{18}H_{18}N_2O_2$	C, 73.45 H, 6.16 N, 9.52	C, 73.38 H, 6.22 N, 9.43
4	*n*-Butyl	B–PE	64	120–122	$C_{19}H_{22}N_2O_2$	C, 73.52 H, 7.14 N, 9.03	C, 73.72 H, 7.29 N, 9.05
5	Isobutyl	E	95	137–139	$C_{19}H_{22}N_2O_2$	C, 73.52 H, 7.14 N, 9.03	C, 73.31 H, 7.17 N, 9.15
6	Cyclohexyl	E	80	151–153	$C_{21}H_{24}N_2O_2$	C, 74.97 H, 7.19 N, 8.33	C, 75.20 H, 7.20 N, 8.35
7	Cyclopentyl	E	59	145–147	$C_{20}H_{22}N_2O_2$	C, 74.51 H, 6.88 N, 8.69	C, 74.42 H, 6.89 N, 8.64
8	2-Dimethylaminoethyl	E	58	164–166	$C_{19}H_{23}N_3O_2$	C, 70.13 H, 7.12 N, 12.91	C, 69.96 H, 7.21 N, 12.77
9	2-Diethylaminoethyl	E	57	108–110	$C_{21}H_{27}N_3O_2$	C, 71.36 H, 7.70 N, 11.89	C, 71.27 H, 7.72 N, 11.81
10	2-(1-Pyrrolidino)ethyl	E	70	166–168	$C_{21}H_{25}N_3O_2$	C, 71.77 H, 7.17 N, 11.96	C, 71.62 H, 7.40 N, 12.02
11	2-(4-Morpholino)ethyl	E	78	169–171	$C_{21}H_{25}N_3O_3$	C, 68.84 H, 6.86 N, 11.44	C, 68.70 H, 7.10 N, 11.37

[a] E, 95% ethanol; B, benzene; PE, petroleum ether, boiling range 60–80°. [b] Uncorrected.



XI

Pharmacological Results[1]—All compounds were submitted to a primary screening, aimed at detecting CNS and other activities. Compound *11* demonstrated antidepressant activity at oral doses of 300 mg./kg., when tested for the reserpine-induced depression and ptosis in mice (4). However, the degree of reversal was slight. Compounds *19*, *20*, and *21* demonstrated smooth muscle relaxant activity, when tested on the isolated guinea pig ileum (5) at the respective concentrations of 10, 10, and 1 mcg./ml. In addition, Compound *20* exhibited local anesthetic activity at a concentration of 2%, when tested against the abolition of the corneal reflex of the rabbit. All levels of activity were considered not sufficient to warrant further investigation. Interestingly, Compound *22* (Δ^4 unsaturated analog of Compound *11*) and all hydantoins were devoid of any activity.

EXPERIMENTAL[2]

1-Substituted 3,4-Diphenyl-5-hydroxyimidazolidin-2-ones (Table I)—Mixtures containing 2.37 g. (0.01 mole) of 3,4-diphenyl-4-oxazolin-2-one (I, R = Ph) (1) and 0.015 mole of a low-boiling amine (*n*-propyl, allyl, *n*-butyl, isobutyl, or cyclopentylamine) were heated 14 hr. under reflux. Similar mixtures containing higher boiling amines were heated 10–12 hr. at 120°. The mixtures were then poured into water; the product which separated was washed with water, triturated with 50% aqueous ethanol, and collected. A crystallization from 95% ethanol (or from benzene–petroleum ether) gave the pure product.

Compound *1* (R = ethyl) was prepared as follows: a suspension of the finely powdered oxazolone (5 g.) in 33% aqueous ethylamine (50 ml.) was allowed to stand 18 days at room temperature, with occasional swirling. After a few days, a clear solution was obtained from which Compound *1* slowly separated. After addition of water (100 ml.) the product was collected and purified as described before.

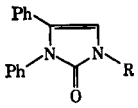
IR spectra: all compounds exhibited a carbonyl absorption band in the 5.98–6.00 μ region.

1-Substituted 3,4-Diphenyl-4-imidazolin-2-ones (Table II)—Solutions of the 5-hydroxyimidazolidinones in 95% ethanol (5 g. in 50 ml.), heated at 50°, were treated with 0.5 ml. of concentrated sulfuric acid, then were allowed to stand 2 hr. at room temperature. The solutions were then concentrated to one-third the original volume (rotary evaporator), and poured into excess water (or excess 10% sodium carbonate in the case of the basic compounds *19*, *20*, *21*, and *22*). The product which separated was collected, washed with water, and crystallized from benzene–petroleum ether.

[1] The authors are grateful to Bristol Laboratories, Syracuse, NY 13201, for the pharmacological data.

[2] All melting points were taken on a Kofler hot stage, and are uncorrected. IR spectra were recorded on Nujol mulls. All microanalyses were performed in the Microanalytical Laboratory of this Institute.

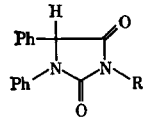
TABLE II—4-IMIDAZOLIN-2-ONES



No.	R	Recrystn. Solvent[a]	Yield, %	M.p., °C.[b]	Molecular Formula	Anal., % Calcd.	Found
12	Ethyl	B-PE	90	115–117	$C_{17}H_{16}N_2O$	C, 77.25 H, 6.10 N, 10.60	C, 77.00 H, 6.33 N, 10.72
13	*n*-Propyl	B-PE	85	121–123	$C_{18}H_{18}N_2O$	C, 77.67 H, 6.52 N, 10.07	C, 77.46 H, 6.79 N, 9.99
14	Allyl	B-PE	85	79–81	$C_{18}H_{16}N_2O$	C, 78.23 H, 5.84 N, 10.14	C, 78.03 H, 6.09 N, 9.92
15	*n*-Butyl	B-PE	95	86–88	$C_{19}H_{20}N_2O$	C, 78.05 H, 6.90 N, 9.58	C, 77.81 H, 7.12 N, 9.72
16	Isobutyl	B-PE	81	106–108	$C_{19}H_{20}N_2O$	C, 78.05 H, 6.90 N, 9.58	C, 77.92 H, 7.16 N, 9.71
17	Cyclohexyl	B-PE	77	125–127	$C_{21}H_{22}N_2O$	C, 79.21 H, 6.96 N, 8.80	C, 79.42 H, 7.20 N, 8.56
18	Cyclopentyl	B-PE	85	129–131	$C_{20}H_{20}N_2O$	C, 78.92 H, 6.62 N, 9.20	C, 78.69 H, 6.83 N, 9.28
19	2-Dimethylaminoethyl	B-PE	60	90–92	$C_{19}H_{21}N_3O$	C, 74.24 H, 6.89 N, 13.67	C, 74.18 H, 7.12 N, 13.71
20	2-Diethylaminoethyl HCl[c]	E-ET	85	218–210	$C_{21}H_{26}ClN_3O$	C, 67.81 H, 7.04 N, 11.32	C, 67.61 H, 6.89 N, 11.32
21	2-(1-Pyrrolidino)ethyl	B-PE	89	110–112	$C_{21}H_{23}N_3O$	C, 75.64 H, 6.95 N, 12.60	C, 75.52 H, 6.97 N, 12.40
22	2-(4-Morpholino)ethyl	B-PE	76	123–125	$C_{21}H_{23}N_3O_2$	C, 72.18 H, 6.63 N, 12.03	C, 72.40 H, 6.88 N, 15.80

[a] B, benzene; PE, petroleum ether, boiling range 60–80°; E, 99.5% ethanol; ET, ethyl ether. [b] Uncorrected. [c] The free base was a viscous oil which could not be induced to crystallize.

TABLE III—HYDANTOINS



No.	R	Recrystn. Solvent[a]	Yield, %[b]	M.p., °C.[c]	Molecular Formula	Anal., % Calcd.	Found
23	Ethyl	E	75 (90)	131–133	$C_{17}H_{16}N_2O_2$	C, 72.84 H, 5.75 N, 9.99	C, 72.84 H, 5.85 N, 9.95
24	*n*-Propyl	E	38 (70)	92–93	$C_{18}H_{18}N_2O_2$	C, 73.45 H, 6.16 N, 9.52	C, 73.24 H, 6.09 N, 9.50
25	Allyl	E	(76)	89–91	$C_{18}H_{16}N_2O_2$	C, 73.95 H, 5.52 N, 9.58	C, 73.80 H, 5.74 N, 9.68
26	*n*-Butyl	E	38 (97)	90–91	$C_{19}H_{20}N_2O_2$	C, 74.00 H, 6.54 N, 9.09	C, 73.84 H, 6.78 N, 9.15
27	Isobutyl	E	47 (89)	119–121	$C_{19}H_{20}N_2O_2$	C, 74.00 H, 6.54 N, 9.09	C, 73.73 H, 6.62 N, 8.91
28	Cyclohexyl	E	55 (45)	130–132	$C_{21}H_{22}N_2O_2$	C, 75.42 H, 6.63 N, 8.38	C, 75.23 H, 6.67 N, 8.46
29	Cyclopentyl	E	24 (69)	113–114	$C_{20}H_{20}N_2O_2$	C, 74.97 H, 6.29 N, 8.74	C, 74.70 H, 6.33 N, 8.87
30	2-Dimethylaminoethyl	E	79 (81)	98–99	$C_{19}H_{21}N_3O_2$	C, 70.56 H, 6.55 N, 13.00	C, 70.38 H, 6.65 N, 12.92
31	2-Diethylaminoethyl	E	65 (90)	107–108	$C_{21}H_{25}N_3O_2$	C, 71.77 H, 7.17 N, 11.96	C, 71.63 H, 7.45 N, 11.99
32	2-(1-Pyrrolidino)ethyl	E	57 (63)	120–122	$C_{21}H_{23}N_3O_2$	C, 72.18 H, 6.63 N, 12.03	C, 71.92 H, 6.81 N, 12.02
33	2-(4-Morpholino)ethyl	E	65 (63)	131–133	$C_{21}H_{23}N_3O_3$	C, 69.02 H, 6.34 N, 11.50	C, 68.64 H, 6.38 N, 11.71

[a] E, 95% ethanol. [b] The yields between parentheses refer to the preparation from 5-bromo-3,4-diphenyl-4-oxazolin-2-one and primary amines. [c] Uncorrected.

Compound *20* separated as an oil, which resisted all attempts at crystallization. It was therefore taken up in ether and characterized as the hydrochloride, m.p. 208–210.°

IR spectra: all compounds exhibited a carbonyl absorption band in the 5.92–5.95 μ region.

3-Substituted 1,5-Diphenylhydantoins (Table III) —*A: Bromination of the 4-Imidazolin-2-ones*—To a solution of the appropriate 4-imidazolinone (0.01 mole) in chloroform (50 ml.) was slowly added, at room temperature, a solution of bromine (1.9 g., 0.012 mole) in chloroform (10 ml.). The solvent was then distilled (evolution of hydrogen bromide) and the oily residue was crystallized from 95% ethanol to afford the hydantoin.

Compound *14* (R = allyl) absorbed two molar equivalents of bromine to afford in 45% yield 3-(2,3-dibromo-1-propyl)1,5-diphenylhydantoin (XI), m.p. 131–133° after crystallization from benzene–petroleum ether. The same compound was obtained on treatment of the allylhydantoin *25* with one molar equivalent of bromine, in 65% yield.

Anal.—Calcd. for $C_{18}H_{16}Br_2N_2O_2$: C, 47.81; H, 3.56; N, 6.19. Found: C, 47.83; H, 3.73; N, 6.00.

Treatment of the 4-imidazolinones *19*, *20* (as the oily base), *21*, and *22* with bromine, followed by crystallization of the oily reaction product from ethanol, afforded the hydrobromides of the hydantoins *30* (m.p. 224–226°; N% Calcd. 10.39. Found: 10.30.); *31* (m.p. 189–191°; N% Calcd. 9.72. Found: 9.56.); *32* (m.p. 258–261° dec.; N% Calcd. 9.72. Found: 9.61)., and *33* (m.p. 287–288° dec.; N% Calcd. 9.41. Found: 9.38.). The salts were converted into the free bases by treatment with 10% sodium carbonate.

B: Treatment of 5-Bromo-3,4-diphenyl-4-oxazolin-2-one (V, R = Ph) with Amines—Mixtures containing 3.16 g. (0.01 mole) of the bromooxazolone (1) and 0.015 mole of the appropriate amine were heated 6 hr. at 100°. Mixtures containing low-boiling amines (*n*-propyl, *n*-butyl, isobutyl, and allylamine) were heated at the reflux temperature of the amine for 8–10 hr. The excess amine was then distilled off at reduced pressure and the crude product was crystallized from 95% ethanol.

IR spectra: all compounds exhibited a typical hydantoin carbonyl absorption (6) (two bands in the regions 5.68–5.69 μ and 5.87–5.89 μ).

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Keyphrases

3, 4 - Diphenyl - 4 - oxazolin - 2 - one derivatives—synthesis
Pharmacological screening—3,4-diphenyl-4-oxazolin-2-one derivatives
IR spectrophotometry—structure

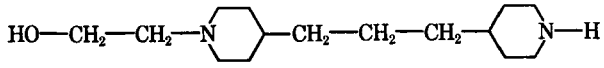
Antibacterial and Antifungal Activity of Certain β-Aminoketones

By RAJENDRA S. VARMA* and W. LEWIS NOBLES

Preliminary biological evaluation for 17 compounds is provided. Eleven compounds in this study exhibited some degree of activity.

UNDER THE CONDITIONS of the Mannich reaction, a series of β-aminoketones dihydrochlorides was synthesized utilizing 1-(*N*-β-hydroxyethyl-4-piperidyl)-3-(4-piperidyl)-propane (I) and several aromatic ketones (1). In this report, preliminary screening results for antibacterial and antifungal activities are described.

Several techniques are available to test for antimicrobial activity. Among the *in vitro* methods are dilution or agar diffusion techniques. The former methods are suitable for assay procedures, but the methods are time consuming for screening of a large number of compounds, and many of them are not satisfactory to determine antifungal activity when filamentous fungi are used as test organisms. This is particularly true if partial inhibition is studied because it is difficult to determine the amount of growth of these fungi (2). Diffusion methods such as those represented by the use of filter paper disks on an agar plate were chosen because of their suitability for water-soluble compounds and their simplicity of operation.



I

Received April 17, 1968, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Mississippi, University, MS 38677

Accepted for publication June 17, 1968.

This investigation was supported in part by grant No. AI-04701 from the National Institutes of Health, Bethesda, Md. The authors express their deep appreciation for the facilities and assistance provided during this work.

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Compound *20* separated as an oil, which resisted all attempts at crystallization. It was therefore taken up in ether and characterized as the hydrochloride, m.p. 208–210.°

IR spectra: all compounds exhibited a carbonyl absorption band in the 5.92–5.95 μ region.

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Compound *14* (R = allyl) absorbed two molar equivalents of bromine to afford in 45% yield 3-(2,3-dibromo-1-propyl)1,5-diphenylhydantoin (XI), m.p. 131–133° after crystallization from benzene–petroleum ether. The same compound was obtained on treatment of the allylhydantoin *25* with one molar equivalent of bromine, in 65% yield.

Anal.—Calcd. for $C_{18}H_{16}Br_2N_2O_2$: C, 47.81; H, 3.56; N, 6.19. Found: C, 47.83; H, 3.73; N, 6.00.

Treatment of the 4-imidazolinones *19*, *20* (as the oily base), *21*, and *22* with bromine, followed by crystallization of the oily reaction product from ethanol, afforded the hydrobromides of the hydantoins *30* (m.p. 224–226°; N% Calcd. 10.39. Found: 10.30.); *31* (m.p. 189–191°; N% Calcd. 9.72. Found: 9.56.); *32* (m.p. 258–261° dec.; N% Calcd. 9.72. Found: 9.61)., and *33* (m.p. 287–288° dec.; N% Calcd. 9.41. Found: 9.38.). The salts were converted into the free bases by treatment with 10% sodium carbonate.

B: Treatment of 5-Bromo-3,4-diphenyl-4-oxazolin-2-one (V, R = Ph) with Amines—Mixtures containing 3.16 g. (0.01 mole) of the bromooxazolone (1) and 0.015 mole of the appropriate amine were heated 6 hr. at 100°. Mixtures containing low-boiling amines (*n*-propyl, *n*-butyl, isobutyl, and allylamine) were heated at the reflux temperature of the amine for 8–10 hr. The excess amine was then distilled off at reduced pressure and the crude product was crystallized from 95% ethanol.

IR spectra: all compounds exhibited a typical hydantoin carbonyl absorption (6) (two bands in the regions 5.68–5.69 μ and 5.87–5.89 μ).

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Keyphrases

3, 4 - Diphenyl - 4 - oxazolin - 2 - one derivatives—synthesis
Pharmacological screening—3,4-diphenyl-4-oxazolin-2-one derivatives
IR spectrophotometry—structure

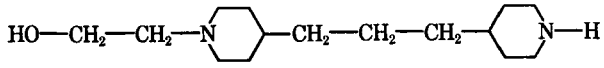
Antibacterial and Antifungal Activity of Certain β-Aminoketones

By RAJENDRA S. VARMA* and W. LEWIS NOBLES

Preliminary biological evaluation for 17 compounds is provided. Eleven compounds in this study exhibited some degree of activity.

UNDER THE CONDITIONS of the Mannich reaction, a series of β-aminoketones dihydrochlorides was synthesized utilizing 1-(*N*-β-hydroxyethyl-4-piperidyl)-3-(4-piperidyl)-propane (I) and several aromatic ketones (1). In this report, preliminary screening results for antibacterial and antifungal activities are described.

Several techniques are available to test for antimicrobial activity. Among the *in vitro* methods are dilution or agar diffusion techniques. The former methods are suitable for assay procedures, but the methods are time consuming for screening of a large number of compounds, and many of them are not satisfactory to determine antifungal activity when filamentous fungi are used as test organisms. This is particularly true if partial inhibition is studied because it is difficult to determine the amount of growth of these fungi (2). Diffusion methods such as those represented by the use of filter paper disks on an agar plate were chosen because of their suitability for water-soluble compounds and their simplicity of operation.



I

Received April 17, 1968, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Mississippi, University, MS 38677

Accepted for publication June 17, 1968.

This investigation was supported in part by grant No. AI-04701 from the National Institutes of Health, Bethesda, Md. The authors express their deep appreciation for the facilities and assistance provided during this work.

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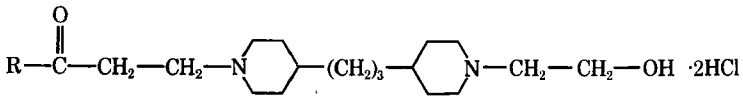
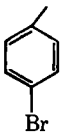
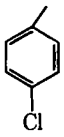
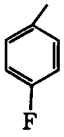
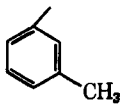
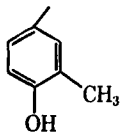
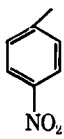
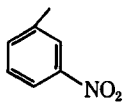
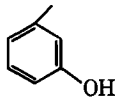
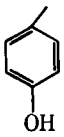
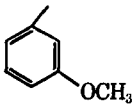
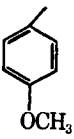
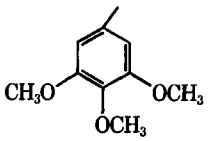
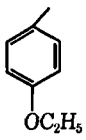
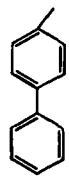
TABLE I—ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF β-AMINOKETONES



R	Microbial Spectrum[a]											
	1	2	3	4	5	6	7	8	9	10	11	12
	−	−	−	−	−	−	−	+	−	−	−	−
	−	−	−	−	−	+	+	−	−	−	−	−
	+	−	+	+	+	−	+	+	+	−	−	−
	−	−	−	−	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	−	−	−	−	−
	−	−	−	+	−	−	+	−	+	−	−	−
	−	−	−	−	−	−	−	−	−	−	−	−
	+	−	+	+	+	−	+	+	+	+	−	+
	−	−	−	−	+	+	−	−	−	−	−	−
	−	−	−	−	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	−	−	−	−	−

(*Continued on next page.*)

TABLE I (*Continued.*)

R	Microbial Spectrum[a] 1	2	3	4	5	6	7	8	9	10	11	12
	−	−	−	−	−	−	+	−	−	−	−	−
	−	−	−	−	+	−	−	+	−	−	−	−
	−	−	−	−	−	−	−	−	−	−	−	−
	−	−	+	+	−	−	+	−	+	+	−	−
	−	−	−	−	−	−	+	+	+	+	−	+
	+	−	+	+	+	+	+	+	−	+	+	−

[a] Microbial spectrum: Gram-positive—1, *Staphylococcus aureus* K257; 2, *Mycobacterium smegmatis*. Gram-negative—3, *E. coli* ATCC 4157; 4, *Pseudomonas aeruginosa*; 5, *Klebsiella pneumoniae* ATCC 8052; 6, *Proteus vulgaris* LBa 155; 7, *Neisseria catarrhalis*; 8, *Saccharomyces sp.*; 9, *Candida albicans* ATCC 10231; 10, *S. epidermidis*; 11, *Aspergillus niger*; 12, *Trichophyton mentagrophytes* ATCC 9129.

EXPERIMENTAL

Materials and Methods—The test organisms included Gram-positive *Staphylococcus aureus* K257, *Mycobacterium smegmatis*; Gram-negative *E. coli* ATCC 4157, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* ATCC 8052, *Proteus vulgaris* Lba 155, *Neisseria catarrhalis*; *Saccharomyces sp.*, *S. epidermidis*, *A. niger*, *Candida albicans* ATCC 10231, and *Trichophyton mentagrophytes* ATCC 9129. The agar medium was inoculated heavily with the test organism and then filter paper disks (6.35 mm.) saturated with two drops of the solution of the test compound (20 mg./ml. in aqueous ethanol or water) were placed on the agar. After 48 hr. of incubation period, the zones of inhibition around the disks were measured. Zone sizes smaller than 6.35 mm. were considered minus activity.

DISCUSSION

Seventeen β-aminoketones substituted at various positions in the aromatic ring were subjected to preliminary antibacterial and antifungal screening procedures. The substituents consisted of 4-fluoro, 4-chloro, 4-bromo, 4-nitro, 4-methyl, 4-phenyl, 4-hydroxy, 4-methoxy, 4-ethoxy, 3-nitro, 3-methoxy, 3-methyl, and 3-hydroxy. The activity against various organisms of these compounds is described in Table I. Hydroxy, fluoro, 4-methyl, 3,4,5-trimethoxy substituted β-amino ketones were completely inactive, whereas compounds with substituents such as chloro, bromo, and 4-nitro demonstrated the highest activity.

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Keyphrases

β-Amino ketones
Antibacterial activity—β-amino ketones
Antifungal activity—β-amino ketones
Paper disk method—analysis

Occurrence of Norcaperatic Acid in *Polyporus fibrillosus*

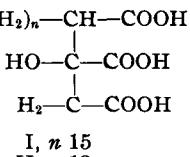
By GERALD SULLIVAN

Norcaperatic acid (α-tetradecylcitric acid) has been isolated from a carpophore of *Polyporus fibrillosus* Karst. in a 22 percent yield and identified by its physical and chemical properties. This is the first reported occurrence of norcaperatic acid in a *Polyporus* species.

THE NATURAL occurrence of aliphatic citric acid derivatives appears to be confined to a limited number of basidiomycetes and a lichen. Agaricic acid (α-hexadecylcitric acid) has been isolated from *Fomes officinales* (Fr.) Faull. [syn.: *Polyporus officinalis* Fr.] (1) and caperatic acid, the mono methyl ester of norcaperatic acid (α-tetradecylcitric acid), from the lichen *Parmelia caperata* L. (2). More recently Miyata *et al.* (3) found the potassium salt of norcaperatic acid to be present in the mushroom *Cantharellus floccosus* Schw. and suggested that this compound may be responsible for the reported toxicity of the mushroom.

Polyporus fibrillosus Karst. [syn.: *Pycnoporellus fibrillosus* (Karst.) Murr. and *Polyporus aurantiacus* Peck] is considered an uncommon mushroom and belongs to the order Polyporales (4). An examination of the literature for substituted citric acid derivatives occurring in the Polyporales revealed that only two such compounds, agaricic (1) and norcaperatic acid (3), had been reported. In addition to these two compounds, Birkinshaw *et al.* (5) reported the isolation of ungulinic acid, which bears a general structural relationship to these substituted citric acid derivatives, from the mycelium of *Polyporus resinosus* Fr. [syn.: *Polyporus benzoinus* (Wahl.) Fr.] which had been grown in saprophytic culture.

The structural formulas of agaricic acid (I) and norcaperatic acid (II) may be represented as:



I, n 15
II, n 13

EXPERIMENTAL[1]

Isolation and Characterization—A carpophore of *Polyporus fibrillosus* Karst. had been collected in the vicinity of Darrington, Wash., in September 1965. The carpophore was orange-red in color and was found attached to a fallen coniferous tree. The carpophore was dried in a forced-air drying oven at 48° for 48 hr., ground to a 60-mesh powder in a Wiley laboratory mill, and subjected to a selective solvent extraction in a Soxhlet apparatus employing a sequence of petroleum ether, ether, chloroform, and 95% ethanol.

A total yield of 31 g. (22%, dry weight basis) of a white microcrystalline material was obtained from the ether extract. The isolated material was found to be insoluble in cold water but soluble in 5% sodium hydroxide, boiling water, acetone, ethanol, and pyridine.

Partial purification of this material was accomplished by repeated recrystallization from boiling water, which resulted in a compound that exhibited a melting range of 132–135°. Final purification was obtained by solubilizing the compound in acetone, mixing with activated carbon, and filtering. The solvent was evaporated under pressure at 50° and the resulting compound recrystallized 3 times from boiling water. The compound was dried at room temperature and a portion of the isolated compound was further dried in a drying pistol for a 24-hr. period at 65°.

Qualitative elemental analysis revealed the absence of nitrogen, sulfur, and halogens and a m.p. of 137–138° was observed. A small amount of the compound was placed on a spatula and ignited in a flame. No residue was observed, indicating that the isolated compound was not a salt. An IR spectrum of the compound in a KBr pellet suggested the presence of (OH), (CH_2), and (C=O) groups: $\nu_{max.}$ 3,500, 2,850, and 1,720 $cm.^{-1}$. The modified Furth-Herrmann color reaction test (3, 6, 7) for aliphatic polycarboxylic acids was applied to the isolated compound and a positive test was obtained when a few milligrams of the compound was dissolved in 3 drops of pyridine and 3 ml. of acetic anhydride added.

The results of the IR spectrum, solubility, ignition, and color reaction tests of the isolated compound indicated that it was probably an aliphatic hydroxy carbonyl compound.

Identification of the Isolated Compound—Authentic samples of both agaricic and norcaperatic acid were obtained and a comparison of the isolated compound with those of the two authentic samples was conducted. The IR absorption spectrum of the isolated compound was found to be identical to that of norcaperatic acid. The observed m.p. (137–138°) of the isolated compound was identical to that of norcaperatic acid and a mixed m.p. with norcaperatic acid resulted with no depression of the original m.p. A parent peak was not obtained from the mass spectra of these compounds but the fragmentation

Received April 25, 1968, from the College of Pharmacy, University of Texas, Austin, TX 78712
Accepted for publication June 28, 1968.
Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
The author thanks Dr. D. E. Stuntz, Department of Botany, University of Washington, for the taxonomic identification of the plant material and Dr. L. R. Brady, College of Pharmacy, University of Washington, for furnishing reference compounds.
This investigation was supported by The University of Texas Research Institute, grant R-822.

[1] The melting points are uncorrected and were determined using a Thomas-Hoover Unit-Melt capillary melting point apparatus. The IR spectra were taken in KBr pellets using a Beckman infrared spectrophotometer, model IR5A. The NMR spectra were determined on a Varian A-60 spectrometer in pyridine with tetramethylsilane as internal standard. The mass spectra were obtained on a C.E.C. No. 21-103, and quantitative elemental analysis was performed by Schwarzkopf Microanalytical Laboratory.

patterns for both norcaperatic acid and the isolated compound were identical. Peaks at m/e = 29, 43, 57, 59, 85, 99, 113, 126, 299, 308, and 326 were consistent with the structure of the molecule. The NMR spectra of both the isolated compound and norcaperatic acid were found to be identical and were in agreement with that reported by Miyata *et al.* (3) for norcaperatic acid. A ratio of 3:29 was obtained by the integration of the combined signals of the protons on the α and γ carbons and the protons on the aliphatic side chain. The absence of a methyl ester function in the infrared, mass, and NMR spectra excluded the possibility of the isolated compound being caperatic acid.

The potassium salt of the isolated compound was prepared as described by Miyata *et al* (3). The observed m.p. (173–174°) of the prepared salt was identical to that reported for the potassium salt of norcaperatic acid. A mixed m.p. with a sample of the reference compound resulted with no depression of the original m.p.

Quantitative elemental analysis of the prepared salt gave the following results.

Anal.—Calcd. for $C_{20}H_{35}KO_7$: C, 56.31; H, 8.27. Found: C, 55.97; H, 8.33.

RESULTS AND DISCUSSION

A 22% yield of norcaperatic acid was obtained from the ether extract of *P. fibrillosus*. The identification of the acid was established by its solubilities, its physical and chemical properties, and by direct comparison with reference compounds. This marks the first reported occurrence of norcaperatic acid in a *Polyporus* species.

The very high concentration of norcaperatic acid in *P. fibrillosus* is reminiscent of the reported concentration of agaricic acid (18%, dry weight basis) in *F. officinalis*. There appears to be no apparent function for these compounds in the respective fungi other than that of waste products. Nord (8) has suggested that many of the metabolites produced in yields exceeding functional requirements, or for which there is no apparent function, accumulate because some of the enzyme systems involved in the oxidative sequence become saturated with respect to their substrates.

The occurrence of norcaperatic acid in the genus *Polyporus* may possibly be restricted to only *P. fibrillosus* since Overholts (4) indicated that this species has no close relatives and numerous other phytochemical investigations (9, 10) have not revealed the presence of this type of acid in other *Polyporus* species.

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Keyphrases

Norcaperatic acid—*Polyporus fibrillosus*
α-Tetradecylcitric acid—isolated, identified
IR spectrophotometry—identity
Mass spectroscopy—identity
NMR spectroscopy—identity

Utilization of the Guggenheim Method in Kinetics

By PAUL J. NIEBERGALL and EDWIN T. SUGITA

The Guggenheim method for the evaluation of rate constants is shown to be applicable to a wide range of problems that are of pharmaceutical interest. These include reaction kinetics in which more than one product is produced from a common reactant, consecutive first-order reactions, dissolution followed by partitioning into a lipid phase, the use of dissolution kinetics to obtain drug solubility, and the analysis of drugs through kinetics.

FIRST-ORDER chemical reactions are frequently followed by directly measuring x, the concentration reacted, and then obtaining the rate constant by plotting the logarithm of $a - x$ *versus* time, where a is equal to the initial concentration of the reactant. Alternately, some physical property, P, which is proportional to concentration may be used, and a plot of log $(P - P_\infty)$ or log $(P_\infty - P)$ *versus* time is used to obtain the rate constant. One objection to this is that overemphasis is placed upon the initial concentration or the time infinity reading of the physical property. A further difficulty arises when either the initial concentration or the final reading of the physical property cannot be obtained. In order

Received May 22, 1968, from the Department of Pharmacy, The Philadelphia College of Pharmacy and Science, Philadelphia, PA 19104
Accepted for publication July 16, 1968.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
This investigation was supported by the United States Public Health Service research grant AI-05321, from the National Institute of Allergy and Infectious Diseases.

patterns for both norcaperatic acid and the isolated compound were identical. Peaks at m/e = 29, 43, 57, 59, 85, 99, 113, 126, 299, 308, and 326 were consistent with the structure of the molecule. The NMR spectra of both the isolated compound and norcaperatic acid were found to be identical and were in agreement with that reported by Miyata *et al.* (3) for norcaperatic acid. A ratio of 3:29 was obtained by the integration of the combined signals of the protons on the α and γ carbons and the protons on the aliphatic side chain. The absence of a methyl ester function in the infrared, mass, and NMR spectra excluded the possibility of the isolated compound being caperatic acid.

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The occurrence of norcaperatic acid in the genus *Polyporus* may possibly be restricted to only *P. fibrillosus* since Overholts (4) indicated that this species has no close relatives and numerous other phytochemical investigations (9, 10) have not revealed the presence of this type of acid in other *Polyporus* species.

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Keyphrases

Norcaperatic acid—*Polyporus fibrillosus*
α-Tetradecylcitric acid—isolated, identified
IR spectrophotometry—identity
Mass spectroscopy—identity
NMR spectroscopy—identity

Utilization of the Guggenheim Method in Kinetics

By PAUL J. NIEBERGALL and EDWIN T. SUGITA

The Guggenheim method for the evaluation of rate constants is shown to be applicable to a wide range of problems that are of pharmaceutical interest. These include reaction kinetics in which more than one product is produced from a common reactant, consecutive first-order reactions, dissolution followed by partitioning into a lipid phase, the use of dissolution kinetics to obtain drug solubility, and the analysis of drugs through kinetics.

FIRST-ORDER chemical reactions are frequently followed by directly measuring x, the concentration reacted, and then obtaining the rate constant by plotting the logarithm of $a - x$ *versus* time, where a is equal to the initial concentration of the reactant. Alternately, some physical property, P, which is proportional to concentration may be used, and a plot of log $(P - P_\infty)$ or log $(P_\infty - P)$ *versus* time is used to obtain the rate constant. One objection to this is that overemphasis is placed upon the initial concentration or the time infinity reading of the physical property. A further difficulty arises when either the initial concentration or the final reading of the physical property cannot be obtained. In order

Received May 22, 1968, from the Department of Pharmacy, The Philadelphia College of Pharmacy and Science, Philadelphia, PA 19104
Accepted for publication July 16, 1968.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
This investigation was supported by the United States Public Health Service research grant AI-05321, from the National Institute of Allergy and Infectious Diseases.

to overcome these difficulties, Guggenheim (1) proposed a method whereby measurements of concentration or of a physical property are made at equal time intervals, eliminating the need for measuring a or P_∞. The method was stated to be useful for "a unimolecular reaction, whether reversible or irreveresible," and the example given was for a simple irreversible unimolecular reaction. Textbooks of chemical kinetics continue to illustrate the use of the Guggenheim method using the same type reaction, and to the best of the authors' knowledge, this is the extent to which the Guggenheim method has been used in research. It has been found however, that the Guggenheim method is applicable to a much larger variety of kinetic systems than is generally realized, and these findings might be of interest to others.

THEORY

In order to extend the usefulness of the Guggenheim approach to first-order kinetics, it is necessary to realize that the type equation used by Guggenheim (1) to illustrate his method is in reality a particular solution to the more general equation type:

$$X = G' - G \exp(-kt) \qquad \text{(Eq. 1)}$$

in which X represents concentration[1] at time t and G', G, and k are constants. Assume that the concentration X is determined at times $t_1, t_2, \ldots t_n$, and that another set of concentration data is obtained at times $t_{1+\tau}, t_{2+\tau}, \ldots t_{n+\tau}$ in which τ is a constant time increment. This results in:

$$X_{t+\tau} = G' - G \exp[-k(t+\tau)] \qquad \text{(Eq. 2)}$$

Subtracting[2] Eq. 1 from Eq. 2 gives:

$$X_{t+\tau} - X = \Delta X = [G - G \exp(-k\tau)] \exp(-kt) \qquad \text{(Eq. 3)}$$

Taking logarithms yields:

$$\log(\Delta X) = \log[G - G \exp(-k\tau)] - kt/2.303 \qquad \text{(Eq. 4)}$$

The slope of the straight line is equal to $-k/2.303$. The intercept of the Guggenheim type plot is generally ignored. However, it can provide some very useful information for a variety of systems. Rearranging the intercept gives:

$$G = \frac{\text{antilog intercept}}{1 - \exp(-k\tau)} \qquad \text{(Eq. 5)}$$

Thus, once k is obtained from the slope of the Guggenheim plot, G can be obtained from the intercept and Eq. 5.

It should be noted that Eq. 1 reduces to the type equation generally used to illustrate the Guggenheim approach, if $G' = 0$. A very common situation of general pharmaceutical interest occurs when $G = G'$ and Eq. 1 becomes:

$$X = G - G \exp(-kt) = G[1 - \exp(-kt)] \qquad \text{(Eq. 6)}$$

Equations 5 and 6 form the basis for the present extension of the Guggenheim approach.

[1] Concentration is used in this paper for the sake of brevity. The equations are equally valid if changes in some physical property are followed in place of concentration.

[2] An interesting alternate solution to Eqs. 1 and 2, which obviates the log transform of Eq. 3 is given by Swinbourne, E. S., *J. Chem. Soc.*, **1960**, 2371.

EXAMPLES

Simultaneous Dissolution and Partitioning—A set of published data which can be used to illustrate the utility of Eqs. 5 and 6 is that of Niebergall *et al.* (2). The physical model involves dissolution into an aqueous phase followed by a first-order transfer into a lipid phase overlayering the aqueous phase. The integrated expression given was:

$$B = \frac{K}{K'}[1 - \exp(-K't)] \qquad \text{(Eq. 7)}$$

in which B represents the weight of drug in the aqueous phase and the constants K and K' are used to evaluate the rate constants k_1 and k_2. This equation is of the same general type as Eq. 6. In this publication, the Guggenheim method was used to obtain K'. However, the potential usefulness of the intercept of the plot was not realized, and a plot of B *versus* $[1 - \exp(-K't)]$ was used to obtain K. Treating the intercept of the data used for the authors' publication according to Eq. 5 yields the following comparison:

	k_1	k_2
Niebergall *et al.* (2)	0.00293	0.0316
Guggenheim approach	0.00292	0.0315

The validity of the Guggenheim plot of Eq. 7 was demonstrated by Figs. 3 and 4 of the authors' previous publication (2).

Two or More Products from a Single Reactant—Guttman and Meister (3) and Galleli and Kostenbauder (4) published studies concerning reactions in which a reactant produced two different products *via* a unimolecular process. The authors have unpublished data from their laboratories for the hydrolysis of penicillin that fits the same reaction pattern. In all instances, the integrated expressions for the appearance of products are of the form:

$$P_1 = \frac{k_1 R_0}{K}[1 - \exp(-Kt)] \qquad \text{(Eq. 8)}$$

$$P_2 = \frac{k_2 R_0}{K}[1 - \exp(-Kt)] \qquad \text{(Eq. 9)}$$

in which R_0 is the initial concentration of reactant, P_1 and P_2 are the concentrations of products, and $k_1 + k_2 = K$. In the literature cited (3, 4) and in the authors' penicillin studies, logarithmic plots of reactant concentration versus time were used to obtain K. Plots of P *versus* $[1 - \exp(-Kt)]$ were then used to evaluate the individual rate constants. Equations 8 and 9 are of the same form as Eq. 6, with the constant G equal to k_1R_0/K or k_2R_0/K, depending upon which product concentration is being followed. The authors therefore reanalyzed their data as a general test of the applicability of the Guggenheim approach to reactions in which more than one product is produced simultaneously from a single reactant *via* a first-order process. A plot of the data is shown in Fig. 1, and a comparison follows:

	k_1	k_2
Previous method	0.00274	0.0108
Guggenheim approach	0.00270	0.0108

In addition to requiring only one plot of the data instead of two, the Guggenheim method has the additional advantage of not requiring the loss of

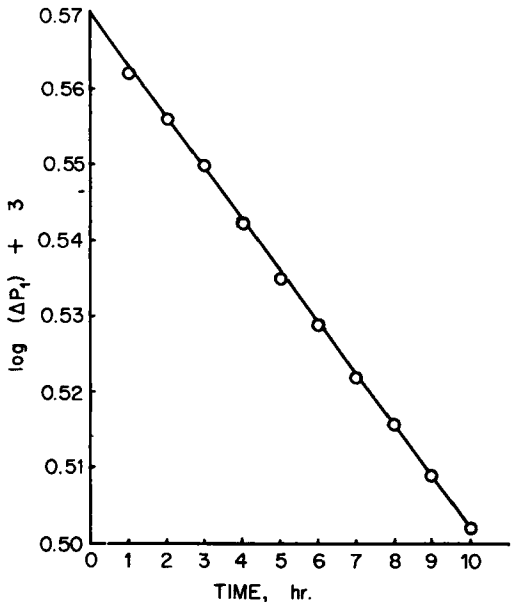

Fig. 1—Guggenheim plot illustrating a reaction in which two products are produced simultaneously via *first-order reactions from a single reactant.*

reactant to be followed. In fact, all of the rate constants can be obtained by following the appearance of only one product. It is preferable from the standpoint of good kinetic procedure to follow the concentration of reactant as well as the concentration of all products as a function of time, in order to insure a valid interpretation of the data. However, from a practical point of view, it is not at all uncommon to find that one or more of the species in question cannot be followed. This was true of the authors' penicillin data in which the penicillin assay was much less reliable than the assay for either of the two products, and many replications were needed to obtain a mean value for K. The authors' therefore calculated k_1 and k_2 using the Guggenheim approach on each of the two products which represented their most reliable data. The two values of k_1 and k_2 thus obtained agreed within 1%, and are being used as their most reliable estimates of the rate constants. The estimate obtained by following the loss of penicillin and the method described in the literature (3, 4) is thus being used only as a check of the validity of their proposed mechanism.

Consecutive First Order-Reactions—A common kinetic pattern which requires rather involved methods of analysis, is that in which a reactant A goes through an intermediate B to final product C with all processes being first order. Integrated expressions for the reactant and intermediate are:

$$A = A_0 \exp(-k_1 t) \quad \text{(Eq. 10)}$$

$$B = \frac{k_1 A_0}{k_2 - k_1} [\exp(-k_1 t) - \exp(-k_2 t)] \quad \text{(Eq. 11)}$$

in which the terms have their usual meanings. Jensen and Lamb (5) treated this type of system by following both A and B as a function of time. The rate constant k_1 was obtained by a logarithmic plot of Eq. 10. The rate constant k_2 was obtained using dimensionless parameters as described by Frost and Pearson (6) in which $\alpha = A/A_0$, $\beta = B/A_0$, $K = k_2/k_1$, and $\beta_{max.} = K^{K/(1-K)}$. A plot of β *versus* $1 - \alpha$ was made, and $\beta_{max.}$ obtained graphically. This enabled K to be obtained and k_2 evaluated by multiplying K by k_1. The rate constants can be obtained much more readily, and without the disadvantage of having to estimate one of the parameters graphically, by use of the Guggenheim approach. Dividing Eq. 11 by Eq. 10 gives:

$$\frac{B}{A} = R = K - K \exp(-K' t) \quad \text{(Eq. 12)}$$

in which $K = k_1/K'$ and $K' = k_2 - k_1$. Equation 12 is seen to be of the same general form as Eq. 6. Thus, if $\log (R_{t+\tau} - R)$ is plotted *versus* time, a straight line should be obtained. The slope of this line yields K' and through use of Eq. 5, K can be obtained from the intercept. Multiplying K by K' gives k_1 which can then be added to K' to give k_2. This reaction scheme was simulated on Pace TR-20 analog computer with the following results:

	k_1		k_2	
k_2/k_1	Computer	Guggenheim	Computer	Guggenheim
0.20	0.050	0.048	0.010	0.011
0.50	0.100	0.100	0.050	0.053
2.00	0.050	0.049	0.100	0.100

Plots of the data for $k_2/k_1 = 0.20$ using different values of τ are shown in Fig. 2. The slopes of the lines are positive, since $k_2 < k_1$. It was found that τ should be taken such that values of R are not used beyond $\beta_{max.}$. The method appears valid beyond this point, but there is a loss in accuracy, yielding constants that differ from the computer constants by 10–15%.

Effect of Varying τ—Values of τ greater than one half-life ($t_{0.5}$) should tend to give more accurate results using the Guggenheim approach, since the greater τ, the greater the value of ΔX at a given time. However, it may not always be practical to take $\tau \gg t_{0.5}$. The authors investigated the effect of varying τ in a number of systems. In general, for all systems studied, it was found that rate constants

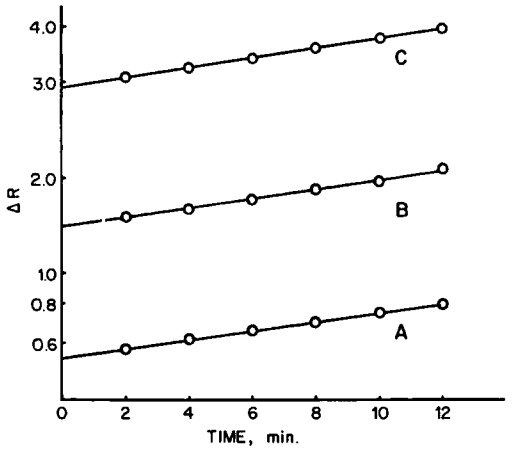

Fig. 2—Guggenheim plot for a series of consecutive first-order reactions in which $k_2/k_1 = 0.20$ *at varying values of* τ. *Key:* A, $\tau = t_{0.25}$; B, $\xi = t_{0.50}$; C, $\tau = t_{0.75}$.

evaluated using $t_{0.25}$ through $t_{0.75}$ differed by less than 5%. This is illustrated in Fig. 2 for the series first-order reaction in which $k_2/k_1 = 0.20$. The choice of τ appears to have no effect upon the slope of the Guggenheim plot. The following estimates of k_1 were obtained:

τ	k_1
$t_{0.25}$	0.047
$t_{0.50}$	0.048
$t_{0.75}$	0.047

Since this is computer simulated data, it is rather idealized. However, it does point out the fact that even if the time interval cannot be equal to one half-life or more, the Guggenheim method should not automatically be discarded as a means of evaluating kinetic data.

Dissolution Rate Estimation of Drug Solubility—Assuming constant surface area, the dissolution rate of a drug may be expressed as:

$$dC/dt = k(C_s - C) = kC_s - kC \quad \text{(Eq. 13)}$$

in which C is the concentration of drug at time t, C_s is taken to be the equilibrium solubility of the drug, and k is the dissolution rate constant. This equation can be integrated through use of an integrating factor and the fact that at time zero, $C = 0$ to give:

$$C = C_s - C_s \exp(-kt) \quad \text{(Eq. 14)}$$

This has the same general form as Eq. 6, and a plot of log (Δ C) *versus* time should yield a straight line with slope equal to $-k/2.30s$. The intercept of the straight line can be used to evaluate C_s through the use of Eq. 5. This was done for the dissolution of salicylic acid pellets of constant surface area in a pH 2.00 buffer at 30°. The value for C_s that was obtained using the Guggenheim approach was 2.25 g./l. as compared to 2.24 g./l. determined by allowing the dissolution to proceed to equilibrium. This information is included only to illustrate a potential application of the Guggenheim approach. Further work must be done to completely evaluate this application.

Kinetic Analysis of Drugs—A second potential application of the Guggenheim method would be the kinetic analysis of drugs (7). For example, if a drug for which no reliable assay exists, degrades *via* a first-order process into a readily assayable product, the integrated expression for the concentration of product as a function of time is:

$$P = A_0[1 - \exp(-kt)] \quad \text{(Eq. 15)}$$

in which P is the concentration of product at time t, A_0 is the initial concentration of the reactant, and k is the observed first-order rate constant. This equation has the same general form as Eq. 6, and the Guggenheim method should be applicable for the estimation of A_0. The authors' tried this for the analysis of penicillin by following the appearance of penicillinic acid at 322 mμ using a Beckman model DU spectrophotometer. The estimated penicillin concentration was 4.06×10^{-4} M as compared to the actual concentration of 4.00×10^{-4} M. Complete details of the procedure utilized are not being given at this time, and the data mentioned are included only to illustrate a potential usefulness for the Guggenheim method. Further studies into the practicality and desirability of using this method for the assay of drugs are currently underway.

These are but a few examples of the variety of kinetic patterns which are capable of being analyzed using the Guggenheim approach. The results obtained thus far suggest that the Guggenheim method should be applicable to any kinetic equation that is of the same form as Eq. 6. Further application for example, might be the analysis of a zero-order process followed consecutively by a first-order process, such as zero-order drug release followed by first-order drug absorption. The fact that the Guggenheim approach is applicable to a given system does not mean that it necessarily is the method of choice. The authors feel however, that in the past the Guggenheim approach has not been utilized to its full capabilities, and have attempted to point out ways in which it might be utilized.

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Keyphrases

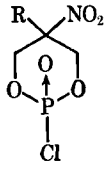
Guggenheim method—kinetic utilization
First-order kinetics—Guggenheim method
Consecutive first-order reaction—Guggenheim method
Drug degradation—Guggenheim method

1,3,2-Dioxaphosphorinane 2-Oxides II. Preparation and Antitumor Activity of Some 2-Alkylamino and 2-Arylamino-5-alkyl-5-nitro-1,3,2-dioxaphosphorinane 2-Oxides

By JOHN H. BILLMAN, RALPH F. MAY, and JANE E. HEARD

A series of 2-substituted-5-alkyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxides consisting of 2-alkylamino and 2-arylamino types have been prepared and submitted for antitumor screening.

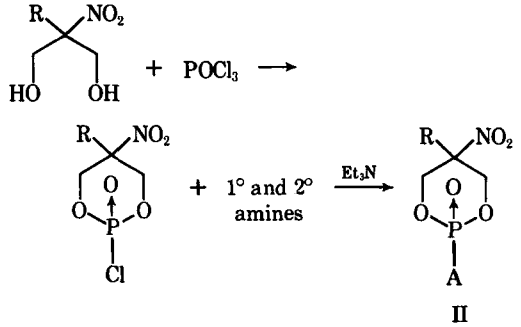
IN THE first publication of this series (1) the authors discussed several miscellaneous 2-substituted derivatives of 2-chloro-5-alkyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxides (I).



I

In view of the fact that different atoms or groups can change the electronic density about the phosphorus atom and by so doing affect the antitumor activity of the 1,3,2-dioxaphosphorinane 2-oxides, it was decided that a desirable grouping to introduce into the 2-position would be substituted amines. Twenty-seven such amides have been prepared and are listed in Tables I and II.

The reaction sequence leading to the preparation of the phosphoramides, II, is shown in Scheme I.



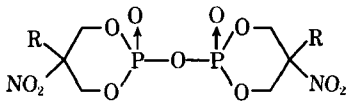
II

R = —CH_3, —CH_2CH_3

A = —NHR, —N⌒$(CH_2)_n$, —NHAr, *etc.*

Scheme I

The aliphatic amines react with the phosphochloridic acids, I, at 0°, whereas the aromatic amines which are less basic require heating. Also, the aliphatic amines form the amides in very good yields while the aromatic ones give poor yields owing to the production of the pyrophosphate, III, the formation of which is described in the first article of this



III

series (1). It appears that whenever the reaction time is greater than 0.5 hr. the pyrophosphate III is formed to a greater extent.

Biological Results—Preliminary antitumor screening data indicated that some of these compounds are exceedingly active towards Walker carcinosarcoma 256 (intramuscular). Upon rescreening one of these compounds, it failed to measure up to the previous high activity. Additional rescreening data are being sought for the other compounds to corroborate the original findings.

EXPERIMENTAL[1]

General Method for the Synthesis of 2-Alkylamino and 2-Arylamino-5-alkyl-5-nitro-1,3,2-dioxaphosphorinane 2-Oxides (Method A)—The preparation of 2-aziridino-5-methyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxide. The 2-chloro-2-methyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxide, 5 g. (0.0232 mole) was dissolved in 125 ml. of acetone and cooled to 0°. To this mixture aziridine, 1.32 g. (0.0232 mole), and triethylamine, 4.68 g. (0.0464 mole), were added dropwise with stirring. The solid hydrochloride formed at once and was removed by filtration. The filtrate was evaporated to dryness *in vacuo* yielding 5.0 g. (97.0% yield) of white solid. Recrystallization from ethanol gave 4.2 g. (82.0% yield) of pure solid product (m.p. 154–156°).

Anal.—Calcd. for $C_6H_{11}N_2O_5P$: N, 12.61; Found: N, 12.86.

2 - (3′ - Chloroanilinium) - 5 - methyl - 5 - nitro-1,3,2-dioxaphosphorinane 2-Oxide (Method B)—Ten grams (0.0464 mole) of 2-chloro-5-methyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxide and 200 ml. of benzene were placed in a 500-ml. three-necked,

Received April 8, 1968, from the Department of Chemistry, Indiana University, Bloomington, IN 47401

Accepted for Publication June 14, 1968.

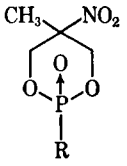
This investigation was supported by a Public Health Service Grant (CA-06448-04) from the U. S. Public Health Service, National Institutes of Health, Bethesda, Md.

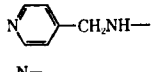
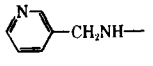
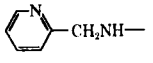
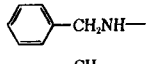
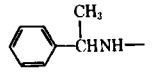
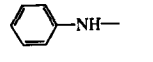
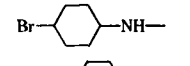
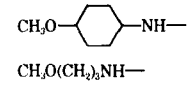
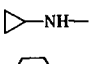
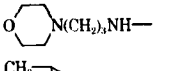
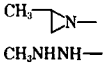
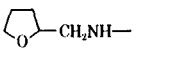
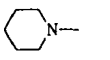
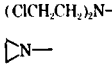
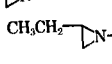
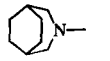
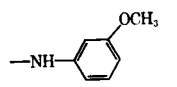
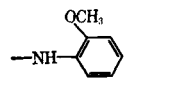
This paper represents part of a Ph.D. thesis submitted to the Graduate School, Indiana University, 1967, and part of a M.A. thesis submitted to the Graduate School, Indiana University, 1968.

The authors wish to thank the Commercial Solvents Corporation for samples of the diols used in this research.

[1] All melting points were taken on a Thomas-Hoover device and are corrected. The infrared data were obtained from spectra prepared on a Perkin-Elmer Infracord. The elemental analyses were performed by Midwest Microlab, Inc., Indianapolis, Ind.

TABLE I—2-SUBSTITUTED AMINO DERIVATIVES OF 2-CHLORO-5-METHYL-5-NITRO-1,3,2-DIOXAPHOSPHORINANE 2-OXIDE



Compd. No.	R	Formula	Yield (pure), %	M.p., °C.	%N Calcd.	%N Found	Infrared Assignment, μ		
1		$C_{10}H_{19}N_2O_5P$	93.0	187–189	10.07	10.03	7.95	9.30	12.25
2		$C_9H_{16}N_2O_5P$	40.6	148–150	10.65	10.32	8.05	9.40	12.25
3		$C_{10}H_{14}N_3O_5P$	45.0	189–191	14.67	14.96	7.95	9.28	12.26
4		$C_{10}H_{14}N_3O_5P$	87.0	175–177	14.67	14.79	7.91	9.34	12.21
5		$C_{10}H_{14}N_3O_5P$	75.5	140–142	14.67	14.48	7.92	9.36	12.34
6		$C_{11}H_{16}N_2O_5P$	75.0	146–148	9.80	9.49	8.10	9.40	12.10
7		$C_{12}H_{17}N_2O_5P$	57.4	169–171	9.35	9.44	8.12	9.40	12.20
8		$C_{10}H_{12}N_2O_5P$	59.4	173–175	10.30	10.15	8.16	9.45	12.00
9		$C_{10}H_{12}BrN_2O_5P$	30.7	208–210	8.00	7.73	7.91	9.43	12.10
10		$C_{11}H_{16}N_2O_6P$	42.6	159–161	9.28	9.19	7.96	9.41	12.05
11	$CH_3O(CH_2)_3NH$—	$C_8H_{17}N_2O_6P$	71.0	105–107	10.45	10.36	8.02	9.40	12.30
12		$C_7H_{13}N_2O_5P$	80.0	147–149	11.89	11.96	8.05	9.30	12.20
13		$C_{11}H_{22}N_3O_6P$	33.3	152–154	13.00	12.99	8.00	9.30	12.45
14		$C_7H_{13}N_2O_6P$	91.2	113–115	11.85	11.88	7.88	9.50	12.50
15	CH_3NHNH—	$C_6H_{13}ClN_3O_5P$	74.2	154–156	16.09	16.19	7.83	9.41	12.25
16		$C_9H_{17}N_2O_6P$	76.0	120–122	10.00	9.76	7.91	9.38	12.31
17		$C_9H_{17}N_2O_5P$	73.5	246–248	10.60	10.58	7.98	9.40	12.30
18	$(ClCH_2CH_2)_2N$—	$C_8H_{15}Cl_2N_2O_5P$	67.0	251–253	8.70	8.43	7.70	9.30	12.63
19		$C_6H_{11}N_2O_5P$	82.0	154–156	12.61	12.86	8.10	9.50	12.30
20		$C_8H_{15}N_2O_5P$	51.5	131–133	11.20	10.94	7.85	9.30	12.20
21		$C_{12}H_{21}N_2O_5P$	85.0	216–218	9.21	9.33	7.95	9.40	12.30
22		$C_{11}H_{16}N_2O_6P$	36.6	181–182.5	9.28	9.36	8.05	9.4, 9.8	12.0
23		$C_{11}H_{16}N_2O_6P$	67.4	141.5–143.5	9.28	9.32	8.1	9.5, 10.0	12.1

(Continued on next page.)

TABLE I (*Continued.*)

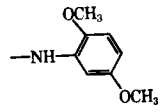
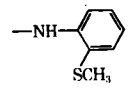
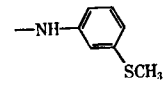
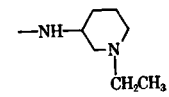
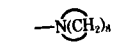
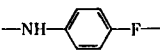
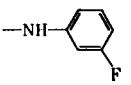
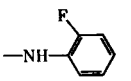
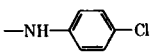
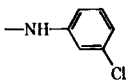
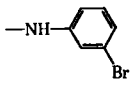
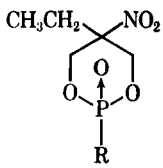
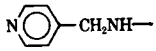
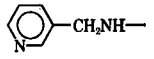
Compd. No.	R	Formula	Yield (pure), %	M.p., °C.	%N Calcd.	%N Found	Infrared Assignment, μ		
24		$C_{12}H_{17}N_2O_7P$	62.5	161–163	8.43	8.54	8.1	9.6, 10.05	
25		$C_{11}H_{15}N_2O_5PS$	49.1	127–128	8.80	8.98	8.0	9.4, 9.95	12.1
26		$C_{11}H_{15}N_2O_5P$	29.9	185–187	8.80	8.61	8.15	9.45, 10.0	12.05
27		$C_{11}H_{21}N_3O_5P$	31.5	110–112	13.69	13.64	8.08	9.60, 10.0	12.42
28		$C_{12}H_{23}N_2O_5P$	62.0	184–186	9.15	9.27	8.3	9.7, 10.2	12.5
29		$C_{10}H_{12}FN_2O_5P$	55.0	164–166	9.65	9.93	8.0	9.5, 10.0	12.25
30		$C_{10}H_{12}FN_2O_5P$	39.6	179–181	9.65	9.90	8.15	9.5	12.13
31		$C_{10}H_{12}FN_2O_5P$	56.5	162–164	9.65	9.56	8.07	9.45, 9.95	12.21
32		$C_{10}H_{12}ClN_2O_5P$	44.7	202–204	9.14	9.38	8.20	9.5, 9.9	12.1
33		$C_{10}H_{12}ClN_2O_5P$	57.5	171.5–173.5	9.14	9.30	8.13	9.50, 9.87	12.10
34		$C_{10}H_{12}BrN_2O_5P$	57.5	185–187	8.00	8.13	8.15	9.5, 10.0	12.1

TABLE II—2-SUBSTITUTED AMINO DERIVATIVES OF 2-CHLORO-5-ETHYL-5-NITRO-1,3,2-DIOXAPHOSPHORINANE 2-OXIDE



Compd. No.	R	Formula	Yield (pure), %	M.p., °C.	%N Calcd.	%N Found	Infrared Assignment, μ		
1		$C_7H_{13}N_2O_5P$	56.8	118–120	11.88	12.20	7.80	9.69	12.45
2		$C_8H_{15}N_2O_5P$	80.0	106–108	11.20	11.42	7.86	9.70	12.50
3		$C_9H_{17}N_2O_5P$	86.5	119–121	10.60	10.74	7.90	9.65	12.40
4		$C_{11}H_{16}N_3O_5P$	47.2	196–198	13.96	13.86	8.00	9.60	12.28
5		$C_{11}H_{16}N_3O_5P$	44.6	173–175	13.96	13.88	8.00	9.61	12.26
6	NH_2—	$C_5H_{11}N_2O_5P$	87.0	204–206	13.31	13.37	7.91	9.25	

round-bottom flask equipped with a Dean-Stark trap, magnetic stirring bar, and heating mantle. After the benzene solution was refluxed for 0.5 hr. to remove all water from the system, 11.8 g. (0.0928 mole) of 3-chloroaniline was added dropwise to the reaction flask. White material precipitated. The reaction mixture was stirred at reflux temperature, overnight, then the reaction was interrupted and the hot solution was filtered immediately to remove the 3-chloroaniline hydrochloride. About 100 ml. of benzene was removed from the filtrate under reduced pressure causing a white precipitate to form. The solution was cooled in a refrigerator and 7.1 g. of crystals was collected by suction filtration. The solvent from the filtrate was evaporated to dryness under reduced pressure leaving a solid which was combined with the crystals previously collected and recrystallized from 2-propanol to give a total of 8 g. (57.5%) of the white crystalline 2-(3'-chloroanilinium)-5-methyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxide, m.p. 171.5–173.5°.

Anal.—Calcd. for $C_{10}H_{12}ClN_2O_5P$: N, 9.14. Found: N, 9.30.

REFERENCES

(1) Billman, J. H., May, R. F., and Heard, J. E., *J. Pharm. Sci.*, 57, 1812 (1968).

Keyphrases

2-Alkylamino-5-alkyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxides—synthesis
2-Arylamino-5-alkyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxides—synthesis
Antitumor activity—1, 3, 2-dioxaphosphorinane 2-oxides
IR spectrophotometry—structure

1,3,2-Dioxaphosphorinane 2-Oxides III. Preparation and Antitumor Evaluation of Some 3,9-Disubstituted-2,4,8,10-tetraoxa-3,9-diphosphaspiro[5.5]-undecane 3,9-Dioxide

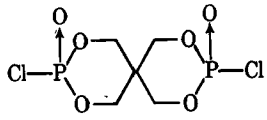
By JOHN H. BILLMAN and RALPH F. MAY

A series of 3,9-aminodisubstituted-2,4,8,10-tetraoxa-3,9-diphosphaspiro [5.5] undecane 3,9-dioxides has been prepared and screened for antitumor activity. Only one of these compounds showed activity toward Walker carcinosarcoma 256.

In view of the fact that some 2-alkylamino and 2-arylamino-5-alkyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxides have been shown to possess some antitumor activity against Walker carcinosarcoma 256 (intramuscular) (1), one might expect that molecules containing two dioxaphosphorinane 2-oxide groupings would possess equal or greater activity than the single ring system.

Since Sweeting (2) had reported the synthesis of 3,9-dichloro-2,4,8,10-tetraoxa-3,9-diphosphaspiro [5.5] undecane 3,9-dioxide, I, it was decided that this dichloride would be an excellent starting material for making compounds that would fulfill the requirement of a double ring system. It was also felt that it would be of interest to see if the spirane moiety would act as a good carrier group and if in some way or other would have an effect on antitumor activities.

The desired amides were prepared by condensing the appropriate amines with the dichloride I in acetonitrile in the presence of triethylamine as outlined in Scheme I and as listed in Table I.



I

Biological Results—Only Compound 6 in Table I showed appreciable activity, and this was toward Walker carcinosarcoma 256. It showed an inhibition of 35% at a dose level of 25 mg./kg., 58% at 100 mg./kg., and 79% at 200 mg./kg. The activity of this compound is probably due to the alkylating groups in the molecule. Most of the other amides had an exceedingly high melting point and were very insoluble in most of the common organic solvents. The lack of antitumor activity for these diamides may be accounted for by their great insolubility.

Received April 8, 1968, from the Department of Chemistry, Indiana University, Bloomington, IN 47401

Accepted for publication June 14, 1968.

This investigation was supported by a Public Health Service grant (CA-06448-04) from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

This paper represents part of a M.A. thesis submitted to the Graduate School, Indiana University, 1966.

The authors wish to thank Dr. O. J. Sweeting for supplying a large sample of 3,9-dichloro-2,4,8,10-tetraoxa-3,9-diphosphaspiro[5.5]undecane 3,9-dioxide (I) which was used in the early part of this work.

round-bottom flask equipped with a Dean-Stark trap, magnetic stirring bar, and heating mantle. After the benzene solution was refluxed for 0.5 hr. to remove all water from the system, 11.8 g. (0.0928 mole) of 3-chloroaniline was added dropwise to the reaction flask. White material precipitated. The reaction mixture was stirred at reflux temperature, overnight, then the reaction was interrupted and the hot solution was filtered immediately to remove the 3-chloroaniline hydrochloride. About 100 ml. of benzene was removed from the filtrate under reduced pressure causing a white precipitate to form. The solution was cooled in a refrigerator and 7.1 g. of crystals was collected by suction filtration. The solvent from the filtrate was evaporated to dryness under reduced pressure leaving a solid which was combined with the crystals previously collected and recrystallized from 2-propanol to give a total of 8 g. (57.5%) of the white crystalline 2-(3'-chloroanilinium)-5-methyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxide, m.p. 171.5–173.5°.

Anal.—Calcd. for $C_{10}H_{12}ClN_2O_5P$: N, 9.14. Found: N, 9.30.

REFERENCES

(1) Billman, J. H., May, R. F., and Heard, J. E., *J. Pharm. Sci.*, 57, 1812 (1968).

Keyphrases

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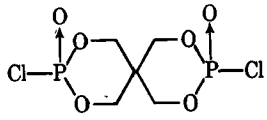
A series of 3,9-aminodisubstituted-2,4,8,10-tetraoxa-3,9-diphosphaspiro [5.5] undecane 3,9-dioxides has been prepared and screened for antitumor activity. Only one of these compounds showed activity toward Walker carcinosarcoma 256.

In view of the fact that some 2-alkylamino and 2-arylamino-5-alkyl-5-nitro-1,3,2-dioxaphosphorinane 2-oxides have been shown to possess some antitumor activity against Walker carcinosarcoma 256 (intramuscular) (1), one might expect that molecules containing two dioxaphosphorinane 2-oxide groupings would possess equal or greater activity than the single ring system.

Since Sweeting (2) had reported the synthesis of 3,9-dichloro-2,4,8,10-tetraoxa-3,9-diphosphaspiro [5.5] undecane 3,9-dioxide, I, it was decided that this dichloride would be an excellent starting material for making compounds that would fulfill the requirement of a double ring system. It was also felt that it would be of interest to see if the spirane moiety would act as a good carrier group and if in some way or other would have an effect on antitumor activities.

The desired amides were prepared by condensing the appropriate amines with the dichloride I in acetonitrile in the presence of triethylamine as outlined in Scheme I and as listed in Table I.



I

Biological Results—Only Compound 6 in Table I showed appreciable activity, and this was toward Walker carcinosarcoma 256. It showed an inhibition of 35% at a dose level of 25 mg./kg., 58% at 100 mg./kg., and 79% at 200 mg./kg. The activity of this compound is probably due to the alkylating groups in the molecule. Most of the other amides had an exceedingly high melting point and were very insoluble in most of the common organic solvents. The lack of antitumor activity for these diamides may be accounted for by their great insolubility.

Received April 8, 1968, from the Department of Chemistry, Indiana University, Bloomington, IN 47401

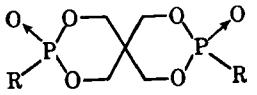
Accepted for publication June 14, 1968.

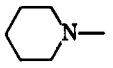
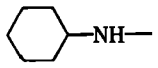
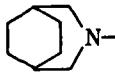
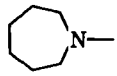
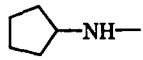
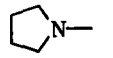
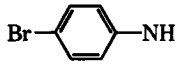
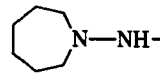
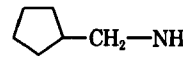
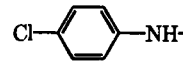
This investigation was supported by a Public Health Service grant (CA-06448-04) from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

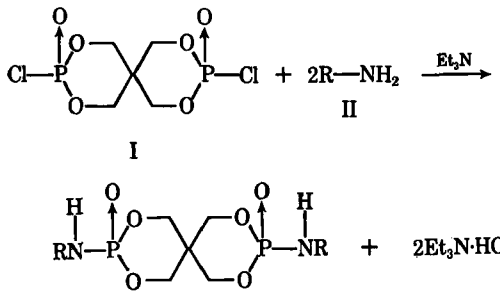
This paper represents part of a M.A. thesis submitted to the Graduate School, Indiana University, 1966.

The authors wish to thank Dr. O. J. Sweeting for supplying a large sample of 3,9-dichloro-2,4,8,10-tetraoxa-3,9-diphosphaspiro[5.5]undecane 3,9-dioxide (I) which was used in the early part of this work.

TABLE I—3,9-DISUBSTITUTED-2,4,8,10-TETRAOXA-3,9-DIPHOSPHASPIRO[5.5]UNDECANE 3,9-DIOXIDES



Compd. No.	R	Formula	M.p., °C.	% N Calcd.	% N Found	Infrared Assignment, μ		
1		$C_{15}H_{28}N_2O_6P_2$	315–315	7.11	7.16	8.05	9.75	
2		$C_{17}H_{30}N_2O_6P_2$	282–284	6.65	6.45	8.15	9.75	3.10
3		$C_{21}H_{36}N_2O_6P_2$	7360	5.91	5.76	8.00	9.75	
4		$C_{17}H_{32}N_2O_6P_2$	313–314	6.64	6.63	8.01	9.78	
5		$C_{15}H_{26}N_2O_6P_2$	272–273	7.15	7.02	8.15	9.80	3.10
6	$ClCH_2CH_2NH$—	$C_9H_{18}Cl_2N_2O_6P_2$	196–197	7.33	7.45	8.15	9.80	3.11
7		$C_{13}H_{24}N_2O_6P_2$	302–304	7.64	7.86	8.00	9.85	
8		$C_{17}H_{18}Br_2N_2O_6P_2$	286–287	4.95	4.89	7.95	9.75	3.11
9		$C_{17}H_{34}N_4O_6P_2$	159–160	12.41	12.06	8.11	9.70	3.11
10		$C_{15}H_{28}N_2O_6P_2$	204–205	6.56	6.34	8.15	9.70	3.08
11		$C_{17}H_{18}Cl_2N_2O_6P_2$	248–250	5.86	5.58	7.95	9.75	3.12



I II III

Scheme I

EXPERIMENTAL[1]

3,9-Disubstituted-2,4,8,10-tetraoxa-3,9-diphosphaspiro[5.5]undecane 3,9-Dioxide—The synthesis of these compounds is illustrated by the procedure for 3,9-dipyrolidino-2,4,8,10-tetraoxa-3,9-diphosphaspiro [5.5] undecane 3,9-dioxide. The spirane I, 5.00 g. (0.0168 mole), was dissolved in 125 ml. of hot acetonitrile, and the temperature was maintained at reflux. Dropwise addition of 4.77 g. (0.0672 mole) of the amine (II) resulted in an immediate precipitate. After 1 hr. of stirring, the solid was removed by filtration and washed with cold acetonitrile to afford 5.5 g. (89.5% yield) of a white solid. The material was recrystallized from a 20:1 mixture of acetonitrile and methanol to give 4.5 g. (73.4% yield) of pure product (m.p. 302–304°).

Anal.—Calcd. for $C_{13}H_{24}N_2O_6P_2$: N, 7.64. Found: N, 7.86.

[1] All melting points below 360° were taken on a Thomas-Hoover device and are corrected. The analyses were performed by Midwest Microlab, Inc., Indianapolis, Ind.

REFERENCES

(1) Billman, J. H., May, R. F., and Heard, J. E., *J. Pharm. Sci.*, **57**, 1809 (1968).

(2) Ratz, R., and Sweeting, O. J., *J. Org. Chem.*, **28**, 1608 (1963).

Keyphrases

3,9 - Disubstituted - 2,4,8,10 - tetraoxa - 3,9-diphosphaspiro[5.5]undecane-3,9-dioxides—synthesis

Antitumor activity—screening

IR spectrophotometry—structure

Centrally Acting Isosteric Mannich Bases

By RONALD E. ORTH*, J. W. BENNETT, ON-HOU MA†, and LARRY YOUNG

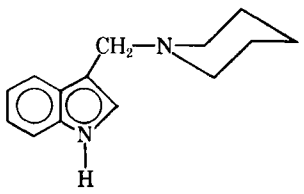
A number of *N*-heteroparaffinomethyl-1*H*-benzazoles is obtained by the Mannich and allied syntheses. Amine salts of all but the indoles are prepared. The degree of motricity and toxic levels are ascertained for the title compounds. Three major molecular modifications investigated cause variations in pharmacodynamic activity: substitution of the isosteric 1*H*-benzimidazole, 1*H*-indazole, and 1*H*-benzotriazole with 1*H*-indole; variation of the *N*-heteroparaffino-side chain moiety; and placement of the *N*-heteroparaffinomethyl substituents in different positions relative to the 1-nitrogen, depending on the particular benzazole. The 3-substituted indoles are most toxic and very active, while the 1-substituted benzimidazoles are best tolerated. Seven benzazoles (at least one from each chemical class) produce unusually high motility. Some general observations are reported. Methylpiperidino derivatives produce the greatest responses studied.

POWELL *et al.* (1) attribute hypertension and *in situ* uterine contraction to 3-(dimethylaminomethyl)indole (gramine). However, Bertaccini and Zamboni (2) report that the indolealkylamines (gramine included) with a single carbon atom lateral side chain have little smooth muscle activity. They find good 5-hydroxytryptamine-like activity exhibited by the indazolealkylamines. Studies by Walshe *et al.* (3) regarding factors influencing cerebral oxidation in relation to hepatic coma, using rat brain cortex slices, indicate gramine and 3-methylindole are active cerebral respiratory inhibitors at concentrations likely to be found in the body. Dubnik *et al.* (4), relating the effect of monoamine oxidase (MAO) inhibitors on brain serotonin of mice in addition to that resulting from inhibition of MAO, find gramine (a MAO-inhibitor) a cause of increased serotonin levels in the mouse brain.

Stimulated by inferences from the literature, this investigation is designed to (*a*) accommodate the synthesis of the title compounds and/or their water soluble salts (Table I) and (*b*) determine the degree of motor involvement and LD_{50}'s they produce in young white mice. Motricity data and subjective observations are noted in Table II.

Three major molecular modifications of gramine are responsible for producing the variations in bioactivity: interchange of the isosteric benzimidazole, 1*H*-indazole, and benzotriazole ring systems for indole; interchange of the *N*-heteroparaffino substituents on the side chain methyleno moiety; and placement of the *N*-heteroparaffinomethyl substituent in different positions relative to the 1-nitrogen, depending on the particular benzazole.

Gramine, synthesized by the Mannich reaction in 1935 (5), is the "open ended" tertiary amino model for the cyclized analogs prepared. Okuda (6), using a Mannich modification, has synthesized 3-(piperidinomethyl)indole (I). The product is found identical to Hellmann's (7) Mannich product, which is formed utilizing the acidic properties of benzenesulfonamide. Henry (8) has prepared the same compound from gramine *N*-oxide.



I

The Mannich reaction in methanol (9) is useful for preparing 1-(*N*-heteroparaffinomethyl)benzimidazole (II). 1-Hydroxymethylbenzimidazole (III) is also formed. The products of the normal Mannich condensation of benzimidazole with HCHO and *N*-heteroparaffins are stable and crystalline. Titration results using $(C_6H_5)_3C^-Na^+$ indicate a side chain attachment to the imidazole nitrogen (Scheme I).

The many attempts by Snyder *et al.* (10) at varying the Mannich reaction conditions to induce

Received April 28, 1967, from the Pharmaceutical Chemistry Department, College of Pharmacy, University of Kentucky, Lexington, KY 40506

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* Present address: College of Pharmacy, University of Houston, Houston, TX 77004. Direct all inquiries.

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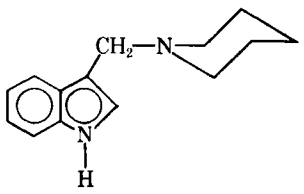

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TABLE I—*N*-HETEROPARAFFINOMETHYLBENZAZOLES

Name	Ref.[a]	M.p. °C.[b]	% N[c] Calcd.	% N[c] Found	%, Yield	Hydrochloride[d] M.p. °C.[b]
3-(Pyrrolidinomethyl)indole	(6)	126–128	14.0	13.8	21	—
3-(3-Methylpiperidinomethyl)indole	(6)	144–146	12.3	11.4	35	—
3-(4-Methylpiperidinomethyl)indole	(6)	107–109	12.3	12.4	30	—
3-(4-Methylpiperazinomethyl)indole	(6)	158–160	18.3	18.1	41	—
1-(Pyrrolidinomethyl)benzimidazole	(9)	—	20.9	20.2	42	187–188
1-(4-Methylpiperazinomethyl)benzimidazole	(9)	116–117	24.3	23.9	87	151–152
1-(2-Methylpiperidinomethyl)benzimidazole	(9)	—	18.3	18.0	92	180
1-(3-Methylpiperidinomethyl)benzimidazole	(9)	64	18.3	18.2	89	d 213, sub. 160
1-(4-Methylpiperidinomethyl)benzimidazole	(9)		18.3	18.3	92	200–220, sub. 188
1-(Pyrrolidinomethyl)benzotriazole	(9)	83	27.7	27.8	80	148–150
1-(3-Methylpiperidinomethyl)benzotriazole	(9)	62–64	24.3	24.3	95	125–130
1-(4-Methylpiperidinomethyl)benzotriazole	(9)	78	24.3	24.5	92	120–122
1-(4-Methylpiperazinomethyl)benzotriazole	(9)	127	30.3	30.1	95	75
1-(2-Methylpiperidinomethyl)1*H* indazole	(12)	...	18.3	18.7	21	MeI 210–212
1-(3-Methylpiperidinomethyl)1*H* indazole	(12)	71–72	18.3	17.9	34	Hygroscopic
1-(Pyrrolidinomethyl) 1*H* indazole	(12)	130–135	20.9	21.2	16	Hygroscopic

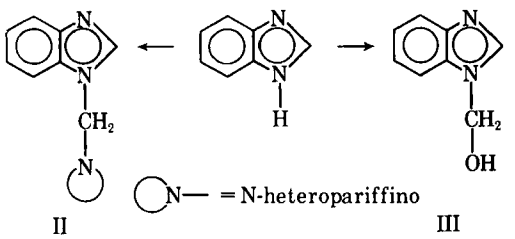
[a] Prepared according to the procedures described in the reference for similar compounds. [b] Uncorrected transition points obtained on Fisher-Johns melting point apparatus. [c] Weiler and Strauss Microanalytical Laboratories, Oxford, England [d] The indoles form red polymers with hydrogen chloride.

indazole to undergo condensation are a classic study of thoroughness. These investigators utilize a circuitous route to prepare 3-piperidinomethyl-1*H*-indazole commencing with methyl-3-indazolecarboxylate. Kochetkov and Dudykina (11) have also prepared this product with as arduous an

TABLE II—PRELIMINARY PHARMACODYNAMICS OF *N*-HETEROPARAFFINOMETHYLBENZAZOLES IN MICE

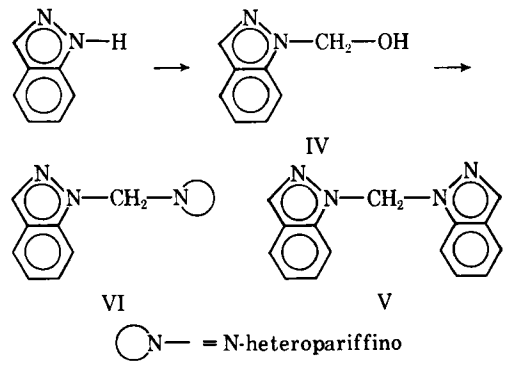
Name[a]	LD_{50}[b]	Dosage and Response[c] Dose	Motricity, 0.5–1 hr.	Dose	Motricity, 0.5–1 hr.	Observations[d]
Placebo	—	0	7–7	0	7–7	Normal behavior
d-Amphetamine sulfate	50/—	4	101–122	—	—	Intense motricity
3-Pyrrolidinomethyl indole	67/35	7	2–4	13	39–1	Cyanosis, convulsions
3-(2-Methylpiperidinomethyl)indole	87/20	8	3–2	17	36–28	Cyanosis, convulsions
3-(3-Methylpiperidinomethyl)indole	—	7	55–8	15	11–3	Normal behavior
1-Pyrrolidinomethyl benzimidazole	870/26	50	18–3	100	11–2	Low response at 250 mg./kg.
1-Piperidinomethyl benzimidazole	818/26	50	13–13	100	17–21	Motricity lost at 250 mg./kg.
1-Morpholinomethyl benzimidazole	960/26	50	11–13	100	4–4	Hypersensitive at 250 mg./kg.
1-(4-Methylpiperazinomethyl)-benzimidazole	368/26	50	6–11	100	5–6	Unsteady at 250 mg./kg.
1-(2-Methylpiperidinomethyl)-benzimidazole	930/26	50	15–3	100	11–3	Low response at 250 mg./kg.
1-(3-Methylpiperidinomethyl)-benzimidazole	806/26	50	9–7	100	6–3	High secretion/all doses
1-(4-Methylpiperidinomethyl)-benzimidazole	1200/26	50	5–53	100	4–4	Hypersensitive/all doses
1-Pyrrolidinomethyl benzotriazole	549–19	50	6–5	100	3–3	Respiratory failure
1-Morpholinomethyl benzotriazole	750/22	50	1–7	100	9–4	Normal at 500 mg.
1-Piperidinomethyl benzotriazole	—	50	3–1	100	2–3	Decreased motricity
1-(3-Methylpiperidinomethyl)-benzotriazole	686/23	50	61–28	100	51–87	Hypersensitive/all doses
1-(4-Methylpiperidinomethyl)-benzotriazole	570/26	50	35–3	100	26–6	Irritable
1-(4-Methylpiperazinomethyl)-benzotriazole	—	50	11–15	100	9–19	Normal
1-(3-Methylpiperidinomethyl)1*H*-indazole	290/20	15	34–14	30	36–112	Hypersensitive at 100 mg.
1-Pyrrolidinomethyl-1*H*-indazole	—	15	21–8	30	2–16	Normal

[a] The indoles are suspended in 0.5% methylcellulose. 1-(4-Methylpiperazinomethyl)benzimidazole is administered as the free base. All of the other benzimidazoles, indazoles, and benzotriazoles are administered as the HCl salt. [b] Administered intraperitoneally. Dose/number of young white mice (20 g.). [c] Three mice are utilized at each dosage level (mg./kg.), requiring an apparatus composed of three squirrel cages mounted on an axis. A spring tension pen, parallel but off-center, projects from the end of the axle to record on a Bird V. H. kymograph (Phipps and Bird, Inc.). The cages are narrow enough to allow unrestricted forward locomotion. Recordings (5 min.) are taken 0.5 hr. and 1 hr. after administration. A Jakar map measurer is used to tabulate the linear, rocking, and rotary tracing distances. [d] The mice are isolated and observed for 1 hr. following LD_{50} measurements. Prodding and tail pinching responses are observed.



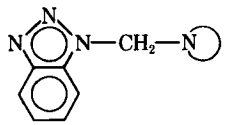
Scheme I—Bachman-Mannich reaction and products.

approach. A facile method of synthesis for 1-(*N*-heteroparaffinomethyl)1*H*-indazole is reported by Pozharskii *et al.* (12). 1-Hydroxymethyl-1*H*-indazole (IV) is first prepared by treating indazole with formalin in HCl. Water-soluble IV and water-insoluble V are obtained and separated. Refluxing IV with the appropriate *N*-heteroparaffin resulted in the formation of the 1-(*N*-heteroparaffinomethyl)-1*H*-indazole (VI) (Scheme II). Methiodide and HCl salts are prepared.



Scheme II—The synthesis of 1-N-heteroparaffinomethyl-1H-indazole and 1,1'-di-indazolylmethane.

The Bachman-Mannich reaction is best used for the synthesis of 1-(*N*-heteroparaffinomethyl)benzotriazoles (VII) (9). Hydrochloride salts are prepared to confer aqueous solubility on these compounds. The benzotriazoles are relatively unstable products and are hydrolyzed in hot water and with $(C_6H_5)_3C^-Na^+$ to give more than 1 mole of active hydrogen/mole of product.



VII

DISCUSSION OF RESULTS

Chemistry—New benzazoles are prepared using adaptations of previously reported procedures. The ease of preparation and percentage yields vary greatly from one class of benzazoles to another. Variation even occurs within a class. For instance, pyrrolidine reacts less favorably with each of the benzazoles than any of the other *N*-heteroparaffins used in this study. The best reactivity and yields, under Mannich conditions, are given in descending order: benzotriazole; benzimidazole; indole. Indazole is not included in this grouping as it (*a*) does not form the 3-substituted Mannich product expected (13), but (*b*) reacts readily with formalin in acid to give two 1-substituted products. Ionization data and electron-density diagrams would lead one to suspect indazole to be more acidic than indole, but less than benzotriazole or benzimidazole (14). Often acylation and alkylation occur at the more electronegative 2-nitrogen, followed by migration of the substituent to the 1-nitrogen position in indazole, to give an isomer which possesses a Kekule-type aromatic ring. Indazole activity may reflect its amphoteric nature, but then benzimidazole is also amphoteric. Electron-density diagrams suggest that the indole nitrogen atom is positive enough that the 1-hydrogen should dissociate to give a magnitude of acidity necessary for a successful Mannich reaction at this position. The 3-position is electronegative and is readily attacked when conditions favor electrophilic substitution. Benzotriazole, with its highly delocalized 1,2,3-triazole electrons, and benzimidazole, with a high degree of delocalization give every indication that their respective acidities are closely related to the excellent yields obtained.

Pharmacodynamics—Placebo i.p. injections of 0.4 ml. of water give the same motricity results as with noninjected animals. A reading of zero indicates no motricity and the extreme value of 122, recorded 1 hr. following the administration of 4 mg./kg. of *d*-amphetamine, create the scale.

The indoles are toxic, and show increased locomotion at 0.2 the LD_{50}. The exceptional indole is the 3-methyl derivative which exhibits questionably high activity at 7 mg./kg. at 0.5 hr. and normal motricity at all other times and doses. The 2-methyl homolog exhibits sustained hypermotricity through both time periods, while the pyrrolidino compound produces hypermotricity at 13 mg./kg. after 0.5 hr. and hypomotricity after 1 hr. The indoles cause death by convulsions and respiratory failure.

The relatively inactive benzimidazoles are the least toxic of the benzazoles studied. The 4-methylpiperidino homolog is the best tolerated of all benzazoles studied, and causes hypermotricity an hour after administration of a 50 mg./kg. dose. One hundred milligrams has no such locomotor stimulant effect. A great variation in class sensitivity is exhibited. Hypersensitivity at all dosage levels is characteristic of this same 4-methylpiperidino product, whereas the 2-methylpiperidino compound increases the pain threshold when 250 mg./kg. is given.

Highest motricity in the well-tolerated benzotriazole series is displayed by the 3-methyl- and 4-methylpiperidino homologs. At low dosages, the 3-methyl derivative exhibits sustained hypermotricity. At 100 mg./kg. the rate of locomotion increases from 51 at 0.5 hr. to 87 after 1 hr. Hypersensitivity is noted at all dosages for this compound. The 4-methyl homolog promotes hypermotricity at 0.5 hr., but not at 1 hr. The mice are irritable at all levels with this product.

1-(3-Methylpiperidinomethyl)-1*H*-indazole is tolerated about four times as well as the indoles and 0.5 as well as the benzotriazoles. It causes the highest motricity of all benzazoles tested. At 0.1 its LD_{50} dose, a response rivaling that of *d*-amphetamine is produced in 1 hr.

The *N*-heteroparaffinomethyl benzazoles of greatest interest are: 3-(pyrrolidinomethyl)indole; 3-(2-

methylpiperidinomethyl)indole; 3-(3-methylpiperidinomethyl)indole; 1-(4-methylpiperidinomethyl)-benzimidazole; 1-(3-methylpiperidinomethyl)benzotriazole; 1-(4-methylpiperidinomethyl)benzotriazole; and 1-(3-methylpiperidinomethyl)-1*H*-indazole. It is interesting to note that in all cases, with one exception, the benzazoles which produce the highest locomotor activity are methylpiperidino derivatives. Death usually occurs coupled with excess secretions, respiratory failure, and cyanosis. Occasionally convulsions are observed.

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Keyphrases

Mannich bases—centrally acting
Benzazole derivatives—Mannich reaction synthesis
Pharmacological screening—benzazoles

Hexahydropyrimidines IX. Synthesis of 2-Substituted-1,3-bis{2-methyl-4-[*N*,*N*-bis(2-chloroethyl)-amino]benzyl}hexahydropyrimidines as Transport Molecules for Tumor Inhibition

By JOHN H. BILLMAN and M. SAMI KHAN

A series of nitrogen mustard hexahydropyrimidines has been prepared by reacting *N*,*N*-bis{2-methyl-4-[*N*,*N*-bis(2-chloroethyl)amino]benzyl}1,3-diaminopropane with various aldehydes and evaluated for antitumor activity against various test systems. Some of the aldehydes used herein for the preparation of hexahydropyrimidines were reported to cause temporary tumor regression in test animals. The majority of compounds were screened against Walker 256 in rats and KB cell culture.

IT IS WELL KNOWN that a majority of tumors contain cells with a lower pH than cells in normal tissues. It has been shown also by Fitch *et al.* (1) that the administration of glucose to tumor-bearing animals can produce an even lower pH value for the tumor cells. Since hexahydropyrimidines are one class of compounds which hydrolyze readily *in vitro* under mild acidic conditions, it is likely that these compounds could be selectively hydrolyzed by the tumor cells to liberate active aldehydes as well as diamines which in themselves might act as antitumor agents. Thus it seems reasonable to expect that properly designed hexahydropyrimidines might act as carrier molecules to direct the nitrogen mustard grouping or other active neoplastic agents into the cellular metabolism.

In an earlier publication (2) the authors have reported the synthesis and the antitumor activity of a number of hexahydropyrimidines containing two aromatic nitrogen mustard groupings in the N-1 and N-3 positions. The primary screening results indicate that all of these compounds were nontoxic at high dose levels and were very active in KB cell culture. (0.1–0.0047 mg./ml.). Against Walker 256 a somewhat moderate activity was observed. In view of these encouraging results, it was of interest to study the effect of the substitution of methyl groups in the 2-positions of the benzene rings containing the nitrogen mustard grouping in order to obtain compounds with better therapeutic indexes and establish more fully their antitumor potentiality. Previous test results have indicated that a methyl group in the 2-position of aldehydes

Received March 15, 1968, from the Department of Chemistry, Indiana University, Bloomington, IN 47401
Accepted for publication July 8, 1968.
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TABLE I—2-SUBSTITUTED-1,3-BIS{2-METHYL-4-[*N,N*-BIS(2-CHLOROETHYL)AMINO]BENZYL}HEXAHYDROPYRIMIDINES

Compd.	R	Reaction Time, hr.	Yield (Pure), %	M.p., °C.	Formula	%Cl Calcd.	%Cl Found	%N Calcd.	%N Found	IR Absorption Bands for Grouping N—C—N, cm.$^{-1}$
1	Phenyl[a]	4	70	128–129	$C_{34}H_{46}Cl_4N_4$	21.79	21.62	8.61	8.88	1080,1160(s),1115,1135(shoulder)
2	2-Hydroxyphenyl[b]	4	60	132–133	$C_{34}H_{46}Cl_4N_4O$	21.27	21.41	8.40	8.64	1070,1140,1160(s),1115(shoulder)
3	2-Hydroxy-5-chlorophenyl[c]	6	71.3	164.5–165.5	$C_{34}H_{44}Cl_6N_4O$	25.29	24.98	7.99	7.92	1070(s),1115,1140(shoulder),1175(w)
4	2-Hydroxy-5-bromophenyl[d]	5	71.6	159–160	$C_{34}H_{44}BrCl_4N_4O$			7.51	7.38	1065(s),1130(shoulder),1170(m)
5	2-Hydroxy-3,5-dichlorophenyl[d]	10	74	150–151	$C_{34}H_{42}Cl_6N_4O$	28.92	28.64	7.61	7.82	1065,1175(s),1120,1135(shoulder)
6	2-Hydroxy-3-allylphenyl[e]	5	64.2	137–138	$C_{37}H_{48}Cl_4N_4O$	20.07	20.20	7.92	7.90	1065(s),1115,1135(shoulder),1155(w)
7	2-Hydroxy-3-methoxyphenyl[f]	12	66.9	87–88	$C_{35}H_{48}Cl_4N_4O_2$	20.37	20.10	8.18	8.02	1065(s),1120,1140(w),1165(m)
8	4-Dimethylaminophenyl[g]	18	79.7	119–120	$C_{36}H_{52}Cl_4N_6$	20.44	20.66	10.09	9.80	1075,1165(s),1120,1125(shoulder)
9	4-Methoxyphenyl[a]	10	85.4	132–133	$C_{35}H_{46}Cl_4N_4O$	20.83	21.26	8.23	8.22	1080,1165(s),1115(shoulder),1135(m)
10	4-Cyanophenyl[c]	18	77.6	154–155	$C_{36}H_{46}Cl_4N_6$	20.99	21.26	10.36	10.56	1080,1160(s),1115,1125(shoulder)
11	4-Fluorophenyl[c]	18	56.7	88–89	$C_{34}H_{44}Cl_4FN_4$			8.37	8.69	1080,1150(s),1120,1135(shoulder)
12	3-Fluorophenyl[b]	18	89.2	125–126	$C_{34}H_{44}Cl_4FN_4$			8.37	8.26	1080(s),1120,1135(shoulder),1175(m)
13	2-Fluorophenyl[c]	10	59.7	119–120	$C_{34}H_{44}Cl_4FN_4$			8.37	8.46	1080(s),1110(shoulder),1130(w),1160(m)

[a] Recrystallized from acetonitrile–methanol (2:1). [b] Recrystallized from acetonitrile. [c] Recrystallized from acetonitrile–methanol (1:1). [d] Recrystallized from acetonitrile–benzene (2:1). [e] Recrystallized from ethanol-benzene (1:1). [f] Recrystallized from acetonitrile. [g] Recrystallized from acetonitrile–ethanol (1:1).

used in the formation of their hexahydropyrimidine rings has enhanced the molecular activity.

The synthesis of the *N,N'*-bis{2-methyl-4-[*N,N*-bis(2-chloroethyl)amino]benzyl}1,3-diaminopropane (IV) reported in Table I was conveniently accomplished according to Scheme I.

The structure of these hexahydropyrimidines was proven by elemental analysis and IR spectra. Bergmann *et al.* (3) state that the IR spectrum of the grouping N—C—N is characterized by three peaks occurring between 1,089 and 1,170 cm.$^{-1}$. The IR spectra of these hexahydropyrimidines VI (Table I) in KBr disks had three similar major peaks between 1,070 and 1,175 cm.$^{-1}$. These maxima presumably correspond to the C—N frequency. The shift in frequency is probably due to the presence of bulky substituents in the 1, 2, and 3 positions and to the strong hydrogen bonding effects. It was noted that when there were electron-donating groups in position 2 of the phenyl ring substituted in position 2 of the pyrimidine, considerable shift to lower frequency was observed. When there were electron-withdrawing groups, on the other hand, absorption always fell within the reported region 1,089–1170 cm.$^{-1}$.

BIOLOGICAL RESULTS

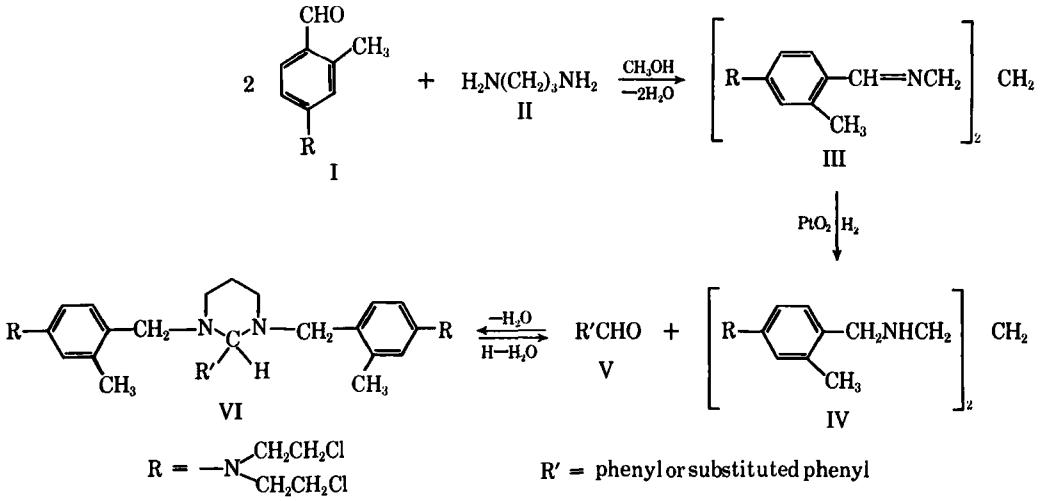
The nitrogen mustard hexahydropyrimidines (Table I) and the diaminedihydrochloride IV, which was reacted with the aldehydes, were submitted to the Cancer Chemotherapy National Service Center (CCNSC) for cytotoxic studies in tissue culture and animal testing for antitumor activity.

Although complete screening results have not been received for all of the compounds, the available figures indicate the hexahydropyrimidines and the diamine have a moderate activity against Walker 256. At a dose level of 50 mg./kg., Compound 1 produced 50% inhibition, Compound 4, 34% inhibition, Compound 10, 79% inhibition, Compound 11, 67% inhibition, Compound 12, 84% inhibition, and Compound 13, 48% inhibition against Walker 256. At a dose level of 15 mg./kg., Compound 8 gave 86% inhibition against Walker 256. Tissue culture activity had been received for only Compounds 1 and 2, which have given reproducible ED_{50} value of 0.19–0.97 mg./ml. in a KB human epidermoid carcinoma according to the criteria established by CCNSC.

EXPERIMENTAL[1]

N,N' - Bis{2 - methyl - 4 - [*N,N* - bis(2 - chloroethyl)amino]benzyl}1,3-diaminopropane (IV)—A solution of 5.2 g. (0.02 mole) of 4-[*N,N*-bis(2-chloroethyl)amino]-*o*-tolualdehyde (I) (4) in 60 ml. absolute methanol was transferred to a 250-ml. hydrogenation bottle and to this solution 0.74 g. (0.01 mole) of 1,3-diaminopropane (II) was added in one batch. The bottle was immediately wel

[1] All melting points were taken on a Fisher-John melting point apparatus and were corrected. The elemental analyses were performed by Midwest Microlaboratories, Inc., Indianapolis, In., and Triangle Chemical Laboratories, Inc., Chapel Hill, N. C. The IR spectra were determined in KBr disks on a Beckman IR-10 spectrophotometer. All evaporations, unless stated otherwise, were conducted in a rotary flask evaporator at water pressure. Aldehydes used were either reagent grade or purified by distillation or recrystallization from appropriate solvents.



Scheme I

stoppered and the mixture was allowed to shake vigorously for 18 hr., at room temperature. At the end of this period, the methanol was removed *in vacuo* to give 5.3 g. (95%) of desired di-Schiff base (III), a yellow oil which could not be induced to crystallize. The crude di-Schiff base, 5.3 g. (0.009 mole) in 200 ml. of absolute ethanol, was then reduced, using 0.1 g. of PtO_2 catalyst, at room temperature in a low-pressure Parr hydrogenator with an initial pressure of 3.15 kg./cm.^{2}. Approximately 40 min. were required to complete the reduction. The catalyst was removed by filtration and the solvent was evaporated *in vacuo*. There was obtained 5.34 g. (100%) of a light yellow oil. A 4.0-g. sample of this oil in 200 ml. of dry ether was converted to 4.4 g. (97.3%) of the dihydrochloride with dry HCl, m.p. 215–218° (dec.). Three crystallizations from hot methanol gave 4.2 g. (92.9%) of an analytical sample, m.p. 219–220° (dec.).

Anal.—Calcd. for $C_{27}H_{40}Cl_4N_4 \cdot 2HCl$: Cl, 33.48; N, 8.81. Found: Cl, 33.26; N, 8.67.

2 - Substituted - 1,3 - bis{2 - methyl - 4 - [N,N-bis(2 - chloroethyl)amino]benzyl} hexahydropyrimidines (Table I)—The 2-substituted derivatives were prepared in the following manner. A 5.62-g. (0.01 mole) sample of *N,N'*-bis{2-methyl-4-[*N,N* - bis(2 - chloroethyl)amino]benzyl}1,3 - diaminopropane (IV) was dissolved in 60 ml. of absolute methanol and transferred to a 250-ml. hydrogenation bottle. To the solution was added 1.06 g. (0.01 mole) of benzaldehyde in 20 ml. of absolute methanol. The bottle was well stoppered and the mixture was shaken vigorously for 4 hr. at room temperature. The crystalline solid which separated from the solution was filtered to give 4.1 g. of desired product. The filtrate obtained from the reaction mixture was reduced to 1/3 of its original volume and allowed to stand overnight in a refrigerator. The crystalline solid was filtered to give an additional 0.7 g. of the product. The overall crude yield was 4.8 g. (73.8%), m.p. 126–129°. Three crystallizations from acetonitrile–methanol (2:1) provided 4.55 g. (70%) of pure sample, m.p. 128–129°.

Anal.—Calcd. for $C_{34}H_{44}Cl_4N_4$: Cl, 21.79; N, 8.61. Found: Cl, 21.62; N, 8.88.

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Keyphrases

Hexahydropyrimidines—synthesis
Antitumor activity—hexahydropyrimidines
IR spectrophotometry—structure

Application of *n*-Butyl Lithium in the Synthesis of Some Methoxy-Substituted Aromatic Carboxylic Acids

By IAN W. MATHISON, RICHARD C. GUELDNER, and DIANNA M. CARROLL

The application of *n*-butyl lithium for the rapid synthesis of methoxy-substituted aromatic carboxylic acids is reported. New compounds associated with this study are described.

SYNTHETIC STUDIES by the authors on isoquinolines as potential cardiovascular agents (1–4) were patterned on the D and E rings of reserpine particularly incorporating the 3,4,5-trimethoxybenzoyl radical. The marked antiarrhythmic activity observed with decahydroisoquinoline compounds possessing this moiety (4), as well as the documented importance of the 3,4,5-trimethoxybenzoyl radical for neurosedative and analgesic activity (5), led the authors to investigate the synthesis of other methoxy-substituted aromatic carboxylic acids in order to ascertain the biological effects induced by various methoxy substitutions in the above series of compounds. The authors report improved, rapid syntheses for some methoxy benzoic acids and a new direct synthesis for tetramethoxyterephthalic acid, using *n*-butyl lithium as the key reactant.

Many studies have been carried out on the applications and anomalies of lithiation of aromatic systems (6). In general, metalation with *n*-butyl lithium occurs *ortho* to ether functions, *i.e.*, —OCH_3. The authors have demonstrated that treatment of 1,2,4,5-tetramethoxybenzene with 2 moles *n*-butyl lithium for 40 min. in tetrahydrofuran, under standard metalation conditions, followed by carbonation of the resulting lithium derivative with solid CO_2, yielded the corresponding tetramethoxyterephthalic acid (I). The reported melting point for a compound presumed to be tetramethoxyterephthalic acid (7), obtained from oxidative degradation of a natural product, halfordin, is considerably higher (20°) than that for the authors' sample. Although analytical data were reported, no spectral data were reported by these workers. The authors' spectral and analytical data were consistent with the proposed structure for their compound. To further substantiate the structure of the above terephthalic acid and thus the entry of two lithium atoms during the above metalation, methylation of the dilithiated product was carried out using dimethyl sulfate. The resulting tetramethoxy-*p*-xylene had the required spectral and analytical data. Oxidation of this *p*-xylene derivative using potassium permanganate yielded the identical tetramethoxyterephthalic acid (I) prepared from the carbonation of the dilithiated 1,2,4,5-tetramethoxybenzene. Additionally the authors examined the product obtained from treatment of 1,2,4,5-tetramethoxybenzene with 1 mole of *n*-butyl lithium. As expected, only the monocarboxylic acid, 2,3,5,6-tetramethoxybenzoic acid, was produced as a result of the introduction of one lithium atom into the aromatic ring followed by carbonation. Purification of the product yielded a compound not previously reported, although Bargellini *et al* (11) reported an unspecified tetramethoxybenzoic acid produced during the action of cyanogen bromide on 1,2,3,5-tetramethoxybenzene. The melting point of their acid compares with the authors' product and its formation from 1,2,3,5-tetramethoxybenzene is explainable by methoxyl migration considering the reaction conditions reported by these investigators. Treatment of 1,2,3,5-tetramethoxybenzene with 1 mole of *n*-butyl lithium yielded the expected 2,3,4,6-tetramethoxybenzoic acid. NMR data verified the structure of this compound although Nierenstein (8) had previously reported a compound of higher melting point. Attempts to introduce two lithium atoms into this molecule to form tetramethoxyisophthalic acid were not successful.

Treatment of 3,5-dimethoxytoluene with 1 mole of *n*-butyl lithium, followed by carbonation of the resulting metalated intermediate, yielded 2,6-dimethoxy-4-methylbenzoic acid; as anticipated the lithium atom was introduced adjacent to the two methoxyl groupings. It was shown that extended reaction times (105 min.) were necessary to obtain good yields in this reaction.

The above studies indicate that metalation of appropriately substituted methoxy benzenes with *n*-butyl lithium, followed by carbonation with solid carbon dioxide is a rapid, direct method for the synthesis of methoxy-substituted aromatic carboxylic acids and is superior to previously reported procedures.

EXPERIMENTAL

Apparatus—All melting points were determined using a Swissco melting point apparatus and are corrected. Elemental analyses were carried out by Drs. G. Weiler and F. B. Strauss, Oxford, England. IR spectra were recorded on a Perkin-Elmer model 137B Infracord spectrophotometer. NMR spectra were recorded on a Varian A60 spectrometer using tetramethylsilane as internal standard.

Tetramethoxyterephthalic Acid (I)—To 1,2,4,5-tetramethoxybenzene (1.1 g.) in tetrahydrofuran (100 ml.) (freshly distilled from lithium aluminum hydride) was added, in small portions, a solution of 15% *n*-butyl lithium in hexane (4.5 ml.) under nitrogen. In approximately 45 sec. a white precipitate formed. The mixture was stirred for an additional 30 min. At this time excess solid carbon dioxide was added and the resulting mixture evaporated on a rotary evaporator. The solid residue was dissolved in distilled water and acidified. Gentle warming of this solution on a steam bath caused the remaining traces of tetrahydrofuran to be removed. The mixture was allowed to cool

Received May 16, 1968, from the Department of Medicinal Chemistry, University of Tennessee College of Pharmacy, Memphis, TN 38103

Accepted for publication July 10, 1968.

The authors would like to acknowledge the generous financial support provided by Marion Laboratories, Inc., Kansas City, Mo., during the course of this continuing research program.

and stand overnight. The precipitated solid was filtered (13 g.) and recrystallized from acetone–ethyl acetate as colorless prisms (11.6 g.), m.p. 249.5–251.5°.

Anal.—Calcd. for $C_{12}H_{14}O_8$: C, 50.35; H, 4.93. Found: C, 50.42; H, 5.04.

The NMR spectrum (deuterated dimethyl sulfoxide) showed two groups of protons in a ratio 1:6, consistent with the —COO*H* and —OC*H*$_3$ protons. The carboxyl protons 6.1–7.5 p.p.m. were considerably upfield from the expected peak position indicating considerable shielding or solvent effects. Addition of D_2O removed this peak position confirming the assignment of a carboxyl proton to this peak. The IR spectrum (KBr) was consistent with the proposed structure (1720 cm.$^{-1}$, —*C*OOH).

Tetramethoxy-p-xylene (II)—To 1,2,4,5-tetramethoxybenzene (2.00 g.) in tetrahydrofuran (100 ml.) was added a solution of 15% *n*-butyl lithium in hexane (8.6 ml.) in a similar manner to that described for Compound I. The reaction was allowed to proceed for 1.5 hr. At this time the mixture was cooled to 0° and dimethyl sulfate (2.7 g.) added to the precipitated mixture. The precipitate immediately dissolved and the resulting solution stirred for an additional 1.5 hr. The solution was then concentrated using a rotary evaporator. Water and 5 *N* sodium hydroxide were added and further concentration was carried out to yield a low melting solid (1.95 g.). This material was crystallized from ethanol–water (10:1) to yield colorless prisms, m.p. 82–83°.

Anal.—Calcd. for $C_{12}H_{18}O_4$: C, 63.70; H, 8.02. Found: C, 63.74; H, 7.93.

The NMR (carbon tetrachloride) showed peaks at 3.7 p.p.m. —OC*H*$_3$ and 2.05 p.p.m. —C*H*$_3$ (2:1) consistent with the proposed structure.

Compound II (0.46 g.), obtained from above, was added to a solution of potassium permanganate (1.4 g.) in water (100 ml.). The mixture was heated to reflux; after 45 min. the solution had decolorized. The solution was cooled and the precipitated manganese dioxide filtered. The filtrate was evaporated to near dryness, made acidic, and the precipitate filtered. The recrystallized precipitate (from water) had a m.p. 251.0–252.0°. Mixture melting point data with Compound I showed no depression of the melting point and thus confirmed this oxidation product to be Compound I.

2,3,5,6-Tetramethoxybenzoic Acid (III)—1,2,4,5-Tetramethoxybenzene (1.1 g.) was treated with 15% *n*-butyl lithium in hexane (2.1 ml.) in tetrahydrofuran (100 ml.) in an identical manner to that previously described for I. Treatment of the lithiated benzene derivative with carbon dioxide followed by an identical workup procedure outlined for I, yielded 1.0 g. solid which was recrystallized from water to yield colorless needles, m.p. 150.5–151.5°.

Anal.—Calcd. for $C_{11}H_{14}O_6$: C, 54.54; H, 5.83. Found: C, 54.39; H, 5.80.

The IR spectrum was consistent with the proposed structure: major peaks (KBr) at 3400 cm.$^{-1}$ (OH), 1750 cm.$^{-1}$ (COOH), and 1060 cm.$^{-1}$ (OCH$_3$).

2,3,4,6-Tetramethoxybenzoic Acid (IV)—1,2,3,5-Tetramethoxybenzene (1.54 g.) was treated with 15% *n*-butyl lithium (3.23 ml.) in tetrahydrofuran in the usual manner. The reaction was allowed to proceed for 20 min. and then carbonated by the addition of solid carbon dioxide. The usual workup yielded a solid (1.14 g.) which was recrystallized from ethanol–benzene as colorless prisms, m.p. 152–152.5° (with decomposition) [lit. value 184–186° (8)].

Anal.—Calcd. for $C_{11}H_{14}O_6$: C, 54.54; H, 5.83. Found: C, 54.79; H, 5.82.

The NMR spectrum (deuterated acetone) showed peaks at 3.8 p.p.m. (—OC*H*$_3$), 5.7 p.p.m. (—COO*H*), and 6.5 p.p.m. (—CH), consistent with the proposed structure.

2,6-Dimethoxy-4-methylbenzoic Acid (V)—3,5-Dimethoxytoluene (3.28 g.) was treated with 15% *n*-butyl lithium (9.0 ml.) under the standard conditions, with cooling, outlined in I. The reaction was allowed to proceed for 105 min. and then terminated by addition of solid carbon dioxide. The tetrahydrofuran was removed *in vacuo* and the resulting solid suspended in water and extracted with chloroform (to remove any unreacted 3,5-dimethoxytoluene). The aqueous layer was acidified and filtered. The filtered solid (2.35 g.) was recrystallized from water as colorless prisms, m.p. 189–190° [lit. value 180–182° (9)].

2,3,4-Trimethoxybenzoic Acid (VI)—1,2,3-Trimethoxybenzene (1.39 g.) was treated in tetrahydrofuran (100 ml.) with 15% *n*-butyl lithium in hexane (3.5 ml.) under the previously described conditions for a period of 45 min. Solid carbon dioxide was added at this time. The tetrahydrofuran was evaporated and the residue suspended in water. The insoluble starting material was filtered and the filtrate acidified. No precipitate appeared. The solution was extracted with chloroform. The chloroform extract was evaporated to yield a viscous mass which was recrystallized from water to yield 0.7 g. colorless prisms, m.p. 98–100° [lit. value 97° (10)]. Further amounts of product were obtained from the aqueous layer by concentration followed by chloroform extraction.

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Keyphrases

Carboxylic acids, methoxy substituted—syntheses
n-Butyl lithium—methoxy-substituted carboxylic acids syntheses
IR spectrophotometry—structure
NMR spectroscopy—structure

Remarks on the Synthesis of Quaternary Cocaine Compounds

Sir:

We recently had the occasion to prepare several quaternary derivatives of cocaine for comparative pharmacological studies (1). Three such derivatives had previously been reported in the literature: methyl iodide (2), allyl bromide (3), and benzyl bromide (4). Of these references, only two describe any physical properties to support the assigned structures (2, 3), and we find a serious discrepancy with one of these (3). The difficulties encountered in the synthesis of these compounds prompted us to report our data concerning synthesis.

The methyl iodide quaternary of cocaine was prepared as described with little problem (2). Comparison of melting points plus supportive IR and NMR spectra and combustion analysis confirmed the structure.

Serious doubt, however, was raised on the reported isolation of the quaternary allyl bromide derivative of cocaine (3). Repetition of the procedure as described yielded a compound in good yield with the identical melting point as that reported, 186–187°. This proved to be the hydrobromide salt of cocaine as confirmed by IR and NMR spectra, combustion analysis, mixture melting point with an authentic sample, and conversion to and isolation of cocaine (free base) by treatment with saturated sodium bicarbonate solution. The latter test for all quaternary derivatives proved most valuable.

We have succeeded in preparing the quaternary allyl cocaine bromide by the reaction of cocaine with excess allyl bromide (1:10) in dimethylformamide in the presence of copper wire. Its melting point is 128–129°, and the structure was confirmed by IR and NMR spectra and combustion analysis. In addition, treatment with saturated sodium bicarbonate yielded no precipitate of cocaine.

However, several attempts to prepare the benzyl quaternary derivative of cocaine resulted only in the isolation of cocaine hydrobromide, m.p. 186–187°. Since the authors reporting this derivative, along with its pharmacology, cited no physical properties (4), the structure should be held in doubt until absolutely confirmed. We have suspected that we have obtained a mixture of the desired compound and the hydrobromide salt in our attempts, but have not as yet succeeded in isolating the quaternary in pure form.

A mixture also was presumably obtained in attempts to synthesize the ethyl quaternary iodide of cocaine. The NMR spectrum and combustion analysis of the reaction product, m.p. 140–144°, suggested that a small fraction of the mixture was the desired compound. The only product ultimately isolated, however, was the hydroiodide salt, m.p. 172–173°.

The other quaternary we prepared was the *n*-propyl iodide of cocaine. Treatment of cocaine with excess *n*-propyl iodide in a closed flask for several weeks gave a small yield of the desired compound, m.p. 155–156°. The structure was confirmed by combustion analysis and by its NMR spectrum.

Thus, caution in the interpretation of results in the preparation of alkyl quaternary halide derivatives of cocaine and of other sterically hindered bases is required. The predominant product using standard procedures is the simple hydrohalide salt of the tertiary base. Supportive evidence other than melting point and water solubility is mandatory.

Methods—All melting points were taken on a Fisher-Johns apparatus and are uncorrected. The IR spectra were recorded on either a Beckman IR-5 or IR-10 spectrophotometer in a KBr pellet. The NMR spectra were recorded on a Varian A-60 model in D_2O. Analyses were performed by Schwartzkopf Analytical Laboratories, Woodside, N. Y.

Methyl Cocaine Iodide—This compound was prepared from cocaine and methyl iodide (1:10), pale yellow platelets, m.p. 165–166°. [Lit. value, 169° (2).] Purification was accomplished from ethanol–ether. The IR spectrum exhibited carbonyl peaks at 1,705 and 1,730 cm.$^{-1}$. The NMR spectrum revealed a multiplet at 7.65 p.p.m. (5 H, aromatic), a quartet at 5.50 p.p.m. (1 H), a broad band at 4.20 p.p.m. (2 H, bridgehead), a singlet at 3.66 p.p.m. (3 H, OCH_3), a multiplet at 3.61 p.p.m. (1 H), 2 singlets at 3.10 and 3.20 p.p.m. [6 H, $N^+(CH_3)_2$], and a broad multiplet at 2.33 p.p.m. (6 H, cycloaliphatic).

Anal.—Calcd. for $C_{18}H_{24}INO_4$: C, 48.54; H, 5.39; N, 3.15. Found: C, 48.23; H, 5.54; N, 2.99.

Allyl Cocaine Bromide—The reaction mixture, containing 3.0 g. (0.01 mole) of cocaine, 24.2 g. (0.20 mole) of allyl bromide, 10 ml. of dimethylformamide, and several pieces of copper wire, was sealed in a 50-ml. flask and stirred by a magnetic bar for a period of 3 days. The product was isolated by filtration and crystallized from ethanol–ether; white monoclinic prisms, m.p. 128–129°, in a yield of 3.2 g. (76%). Its IR spectrum exhibited carbonyl peaks at 1,705 and 1,727 cm.$^{-1}$ and lacked the presence of a strong shoulder from 3,000–2,500 cm.$^{-1}$. The NMR spectrum was identical to that of the methyl quaternary with the exception of new multiplets at 5.74 p.p.m. (3 H, $CH{=}CH_2$) and a singlet at 2.83 p.p.m. (2 H, N^+CH_2—) in place of the singlet at 3.20 p.p.m. (3 H, N^+CH_3).

Anal.—Calcd. for $C_{20}H_{26}BrNO_4$: C, 56.60; H, 6.13; N, 3.30. Found: C, 56.27; H, 6.38; N, 3.40.

***n*-Propyl Cocaine Iodide**—A solution of cocaine and *n*-propyl iodide (1:10) was allowed to stand in a closed flask for 5 weeks. The reaction product was collected on a filter, washed with ether, and crystallized from ethanol–ether yielding fine white crystals, m.p. 155–156°, in a yield of 0.8 g. (17%). The IR spectrum was nearly identical to that of the methyl quaternary except in the fingerprint region. The NMR spectrum was identical to that of the methyl quaternary except for aliphatic absorption at 3.13 p.p.m. (2 H, N^+CH_2—), a multiplet at 1.21 p.p.m. (2 H), and a triplet at 0.59 (3 H, CH_3).

Anal.—Calcd. for $C_{20}H_{28}INO$: C, 50.77; H, 5.92; N, 2.96. Found: C, 50.87; H, 5.76; N, 2.95.

Cocaine Hydrobromide—The reaction as described (3) was conducted. A solution of 3.0 g. (0.01 mole) of cocaine and 6.0 g. (0.05 mole) of allyl bromide in 30 ml. of benzene was heated at reflux with stirring under a nitrogen atmosphere for a period of 5 hr. The resulting precipitate was collected on a filter and crystallized from ethanol–ether. A yield of 3.1 g. (82%) of white crystals was obtained, m.p. 185–186°. (Lit. value, 186–187°). The IR spectrum exhibited a characteristic broad shoulder at 3,000–2,500 cm.$^{-1}$ (N^+H) plus the double carbonyl peaks at 1,732 and 1,705 cm.$^{-1}$. Its NMR spectrum revealed the aromatic protons as a multiplet at 7.65 p.p.m. (5 H), a multiplet of 5.50 p.p.m. (1 H), a singlet at 4.58 p.p.m. (1 H, HOD), a broad band at 4.20 (2 H, bridgehead), a singlet at 3.66 (3 H, OCH_3), a multiplet at 3.56 p.p.m. (1 H), a triplet at 2.93 p.p.m. (3 H, N^+CH_3), and a multiplet at 2.33 p.p.m. (6 H, cycloaliphatic). Treatment of the product with saturated sodium bicarbonate solution led to the isolation of long, white needles of cocaine as the free base, m.p. 96°. A mixture melting point with an authentic sample of cocaine hydrobromide showed no depression, m.p. 185–186°.

Anal.—Calcd. for $C_{17}H_{22}BrNO_4$: C, 53.13; H, 5.73; N, 3.65. Found: C, 53.27; H, 5.86; N, 3.64.

(1) Shriver, D. A., M.S. thesis, University of Iowa, Iowa City, Iowa, August, 1968.
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Received June 17, 1968.
Accepted for publication July 22, 1968.

Keyphrases

Cocaine derivatives, quaternary—synthesis
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Books

REVIEWS

Microbial Transformation of Steroids and Alkaloids. By HIROSHI ILZUKA and ATSHUSHI NAITO. University of Tokyo Press, Tokyo, Japan, 1967. U. S. Agent: University Park Press, State College, Pa. xi + 294 pp. 18.5 × 26 cm. Price $16.50.

One approach to appraise the value of this book is to compare its contents to the two other books covering the same material ("Microbial Transformations of Steroids," by A. Capek *et al.*, Academic Press, New York, 1965, and "Microbial Transformations of Steroids," a handbook, by W. Charney and H. L. Herzog, Academic Press, New York, 1967).

This monograph by Drs. Ilzuka and Naito is useful as a reference source for individuals who would like to find quickly the references to accomplish a certain microbial reaction on steroids and a few alkaloids. It is indexed according to the type of microbial reaction, name of microorganism, type of substances (substrates and products), and authors.

Allyl Cocaine Bromide—The reaction mixture, containing 3.0 g. (0.01 mole) of cocaine, 24.2 g. (0.20 mole) of allyl bromide, 10 ml. of dimethylformamide, and several pieces of copper wire, was sealed in a 50-ml. flask and stirred by a magnetic bar for a period of 3 days. The product was isolated by filtration and crystallized from ethanol–ether; white monoclinic prisms, m.p. 128–129°, in a yield of 3.2 g. (76%). Its IR spectrum exhibited carbonyl peaks at 1,705 and 1,727 cm.$^{-1}$ and lacked the presence of a strong shoulder from 3,000–2,500 cm.$^{-1}$. The NMR spectrum was identical to that of the methyl quaternary with the exception of new multiplets at 5.74 p.p.m. (3 H, $CH{=}CH_2$) and a singlet at 2.83 p.p.m. (2 H, N^+CH_2—) in place of the singlet at 3.20 p.p.m. (3 H, N^+CH_3).

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While being well organized, it has the serious drawback of an incomplete listing of references. Furthermore, there is no information on biochemical mechanisms of microbial reactions.

The book by Capek *et al.* is fairly complete through 1965, relative to references and is similarly indexed. In addition, it covers such useful topics as mechanisms of transformations, practical methods of fermentation, isolation and identification procedures, chromatography, *etc.*, which are especially useful to someone who may have had no personal experience in performing a microbiological transformation. The third book of Charney and Herzog exhaustively examines both the entire scientific and U. S. patent literature through 1967. Furthermore, it also gives an excellent description of the up-to-date mechanisms of the various classes of microbial transformations, while omitting the practical aspects such as care of cultures, fermentation procedures, and separation of products. Without question, this handbook is most useful to those actively engaged in research in this field.

Taking all things into consideration, we rate: 1, Charney and Herzog ($21.00); 2, Capek *et al.* ($6.25); and 3, Ilzuka and Naito ($16.50).

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Principles of Adsorption Chromatography: The Separation of Nonionic Organic Compounds. By LLOYD R. SNYDER. Marcel Dekker, Inc., 95 Madison Avenue, New York, NY 10016, 1968. xvi + 413 pp. 16 × 23 cm. Price $17.50.

This represents Volume 3 in a new series of monographs on Chromatographic Science edited by Giddings and Keller. As the author states, the adsorption process is complex and difficult to understand. The author has attempted a rational organization of the underlying principles, and this volume will enable a serious worker in the field of adsorption chromatography to better understand and predict the separation of complex nonionic organic compounds.

After a brief introduction, the author discussed "The Chromatographic Process and Techniques of Separation," "General Aspects of Adsorption," "Isotherm Linearity," "Bed Efficiency," *etc.* The chapter on "The Role of Sample Structure" is particularly interesting, and one wishes that the author had devoted more effort on this aspect.

Reviewed by William J. Mader
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NOTICES

Proceedings of the 3rd International Pharmacological Meeting, 1966 Sau Paulo, Brazil. Pergamon Press Inc., 44-01 21st St., Long Island City, New York, NY 11101, 1968. 16 × 23.5 cm. The proceedings are presented in 11 volumes with the individual pertinent data as follows. Vol. 1: *Mode of Action of Anti-Parasitic Drugs.* Edited by J. RODRIGUES DA SILVA and M. J. FERREIRA. x + 119 pp. Price $9. Vol. 2: *Pharmacology of Reproduction.* Edited by E. DICZFALUSY. vi + 126 pp. Price $9. Vol. 3: *Clinical Pharmacology.* Edited by R. K. RICHARDS. vi + 113 pp. Price $9. Vol. 4: *Mechanisms of Drug Toxicity.* Edited by H. RASKOVA. vii + 104 pp. Price $9. Vol. 5: *Control of Growth Processes by Chemical Agents.* Edited by A. D. WELCH. vii + 91 pp. Price $8. Vol. 6: *Drugs in Relation to Blood Coagulation. Haemostasis* and *Thrombosis.* Edited by G. V. R. BORN. vii + 126 pp. Price $10. Vol. 7: *Physico-Chemical Aspects of Drug Action.* Edited by E. J. ARIENS. ix + 385 pp. Price $14. Vol. 8: *Salt and Water Balance.* Edited by K. H. BEYER. viii + 109 pp. Price $9. Vol. 9: *Pharmacology of Pain.* Edited by R. K. S. LIM. viii + 250 pp. Price $13. Vol. 11: *Immunopharmacology.* Edited by H. O. SCHILD. ix + 166 pp. Price $13.

III International Pharmacological Congress—International Symposium on Vaso Active Polypeptides: Bradykinin and Related Kinins. Edited by M. ROCHA e SILVA and H. A. ROTHSCHILD. Available from Dept. of Pharmacology, Faculty of Medicine, Ribeirao Preto—S.P., Brazil, 1967. 300 pp. (in English). Price: unbound $10.00; bound $12.50.

Committee on Safety of Drugs. Annual Report for 1966. Her Majesty's Stationery Office, London, England, 1967. Available from Sales Section, British Information Services, 845 Third Ave., New York, NY 10022. 15-page pamphlet. Price 1s. 9d. net. Approx. 40 cents U. S.

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Taking all things into consideration, we rate: 1, Charney and Herzog ($21.00); 2, Capek *et al.* ($6.25); and 3, Ilzuka and Naito ($16.50).

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JOURNAL OF

Pharmaceutical Sciences

A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Council

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JANUARY TO DECEMBER, 1968

JOURNAL OF Pharmaceutical Sciences

November 1968 volume 57, number 11

Review Article

Particle Size of Drugs and Its Relationship to Absorption and Activity

By JULIAN H. FINCHER

THE PARTICLE SIZE of a drug is not a new consideration. Down through the ages the state of subdivision of a drug was and still is a primary factor to be considered in the preparation of esthetical, elegant, and stable dosage forms (1–11). With the evolvement of physical pharmacy, pharmaceutical scientists and medical practitioners have begun to look more critically at this property in an effort to not only learn of its effect in physical systems, but also to gain insight into the influence of particle size in biological systems. This article treats primarily the recent information of the biopharmaceutics (12) of the particle size of drugs.

Several review articles treating particle size in different context have been published since 1963: (*a*) "Effect of Particle Size on Dissolution and Gastrointestinal (GI) Absorption Rates of Pharmaceuticals," in March 1963, by Levy (13); (*b*) "Pharmaceutical Aspects of Fine Particles and Their Evaluation," in September 1963, by Lees (14) (no references were listed); (*c*) "Particle Size in Relation to Formulation," in July 1964, by Dare (15) (partial list of references was presented); (*d*) "Importance of Particle Size in Pharmaceutical Practice," in November 1966, by Lamy (1); (*e*) "Importance of Particle Size in Pharmaceuticals" in February 1967, by Renoz (16). The latter two are not comprehensive reviews but were written for the purpose of calling this property to the attention of the hospital pharmacist and Belgium industrial pharmacist, respectively. Some of the more important principles and considerations discussed in these reviews are worth reiterating here since neither of these articles was published in this journal. From this writer's experience, some are not readily accessible to every library. All are in English, however, except Renoz's (16), which is in French.

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Clinical Aspects—GI—(*a*) Absorption from particulate matter usually results after the drug is in the dissolved state. If the dissolution rate is the rate-limiting step; *i.e.*, the dissolution rate is less than the diffusion rate to the site of absorption and the absorption rate itself, the particle size of the drug is of great importance in the transport from the GI tract to the site of action by way of the blood and lymph (13, 14).

(*b*) When the solubility of a drug is less than 0.1 mg./ml., its physiological availability must be considered and the effect of particle size could be paramount (8). Others claim that particle size is a factor to be considered if the solubility is 1 mg./ml. or less (15).

(*c*) Most drugs are passively absorbed and their rates of absorption are dependent upon the concentration gradients in each case; by increasing the dissolution rate in the GI tract the absorption rate is necessarily increased so long as the dissolution rate is still the limiting step (12–15). It

follows that an increase in the absorption rate results in the entrance of more drug into the blood, and usually more will reach the site of action before metabolism and excretion eliminate the active form of the drug from the body.

(*d*) If a drug is not absorbed enough to be systemically active and if the drug is not soluble enough to be used for its local action in the gut, the reduction of particle size may improve its therapeutic efficacy. A larger particle may be needed to give local action in the lower GI tract (13–15).

(*e*) Reduction of particle size can act as a "double-edged sword." The potential toxic effects due to increased concentration must always be considered in both locally acting and systemically acting drugs (13–15).

(*f*) The stability characteristics of the drug may be altered considerably by reduction of particle size. The resultant increase in surface area places more of the drug molecules in a vulnerable position for rapid degradation by the GI fluids (13–15).

Other general clinical considerations which are directly related to particle-size effects are: (*g*) Localized absorption areas in the gut—if the area of absorption of a given drug is in the stomach or upper region of the gut only, then the reduction of particle size is potentially beneficial, since more of the drug would be in solution in the absorption region. However, if the area of absorption is in the lower portion of the gut, drug absorption may be independent of particle size. After the time required to reach the site of absorption, dissolution would have occurred already when using the drug in a large or a small particle size (13–16). This factor obviously is directly related to the conveyance of the materials through the GI tract—stomach emptying time and peristaltic activity of the gut in general (12).

(*h*) pH of the contents of the GI tract—the average changes of medium pH as a drug is conveyed through the GI tract and has been discussed in a review by Wagner (12). The solubilities of weak acids and weak bases are a function of the pH of the dissolving medium, and in some cases the availability of drug will be independent of particle size due to a dissolution in one compartment and a subsequent precipitation in another. For example, a weak organic base drug would be solubilized rapidly in the form of the amine acid salt in the pH of the stomach, but as the drug passes into the duodenum and small intestines the free base would be precipitated in the contents. Since the large and small particles are each dissolved rapidly and reprecipitated before any appreciable absorption can occur, the absorption rate is independent of the initial particle size (13). A weak acid drug would lend itself to particle-size consideration. In solution, it is largely in the undissociated form in the stomach, and absorption can occur. It would be very insoluble in this pH, however, and a smaller particle could improve the dissolution rate and the availability of the drug for absorption. After the weak acid drug passes into the duodenum and small intestines, it would be much more soluble due to salt formation, but at the same time it would be highly ionic in character. Thus, the availability for absorption is greater, but its ionic character would make the absorption rate less since only a small fraction of the drug molecules are in the undissociated state (12, 13). It is appropriate to mention at this point that the pH at the site of absorption may be different from the pH of the GI lumen contents, and absorption from an ionic solution may not be as limited as the pH-partition theory would indicate.

(*i*) The degree of agitation in the GI contents is related to particle-size effects, and this factor is subject to considerable variability within the individual depending on the physiological state during the time of administration and absorption. No suggestions are offered for the control of this variable, but the agitation intensity would affect the size of the "saturated boundary layer" on each of the drug particles and also the "effective diffusion rate" of the drug to the sites of absorption (13).

(*j*) Measurement of the effects of particle size on drug action—apparently, there is not a good way to measure the dissolution rate of a drug (dosage form) in the stomach and intestinal contents. *In vitro* measurements made in simulated gastric and in simulated intestinal fluids are not necessarily indicative of actual *in vivo* processes. The determination of blood levels is assumed to be a good criterion for drug action in most cases, and since passive excretion by way of the urine is a function of blood level, the excretion rate is usally a good criterion to use (12). In order to measure the amount of drug absorbed, the bound drug and the metabolites, as well as the unchanged drug, must be measured. Thus, with particle size controlled, the absorption, distribution, metabolism, and excretion routes of the drug should be studied first. If particle-size effects need to be determined, the blood levels and excretion rates can be used more effectively as an indirect measure of drug availability, as some function of particle size.

(*k*) Since particle absorption has been reported

(14, 15), one cannot preclude this possibility as an adverse effect in the use of extremely small, insoluble particles. The passage of bacterial cells and oil globules (in emulsified form) through the intestinal wall into the blood has been reported. Particles (globules) up to 0.5 μ are reported to pass through (14, 15). Entrance of very small particles of barium sulfate into the intestinal glands can cause granulomas (14).

Clinical Aspects—Other Than GI—In parenteral therapy, the particle size (surface area) of suspended particles injected i.m. or subcutaneously is an important factor. The absorption of the drug from particles appears to increase with an increase in the specific surface area; however, in certain vehicles where a hydrophobic protective layer is involved the absorption may be more delayed when using very small particles. This may be related to the fact that fine particles tend to cake or form gels in certain vehicles. The viscosity of suspensions increases with a decrease in particle size, and this factor may explain some of the delayed absorption using very small particles (14, 15).

In semisolid systems (ointments and suppositories), the particle size of the insoluble fraction of the drug is an important factor in obtaining the desired results. In these cases an improved dissolution rate leads to greater availability of the drug for absorption in the case of systemic effects, and for higher concentration at the area of application for a better local action (14, 15).

Inhalation therapy has been improved considerably by controlling the particle size of aerosol-generated droplets. The size of the droplet governs the deposition area in the respiratory tract (14, 15). Tables of the droplet size, ranges, and areas of condensation are presented in the aerosol chapters of Husa's and Sprowl's dispensing textbooks (17, 18).

The particle size of practically insoluble drugs in dusting powders, insufflates, and aerosols was predicted to be an important consideration in their dissolution and subsequent local action (14).

Physical and Chemical Aspects of Particle Size—The dissolution rate of drug particles has been discussed thoroughly by Levy (13) and Dare (15) in terms of the modified Noyes-Whitney (19) equation and there is no particular need for a complete discussion here. The modified Noyes-Whitney equation is:

$$\frac{dA}{dt} = KS(C_s - C) \qquad \text{(Eq. 1)}$$

where A = amount of drug in solution, t = time, K = intrinsic dissolution rate constant, S = surface area, C_s = concentration of the drug–solvent boundary on the surface of the particle which is approximately equal to the solubility of the drug in the solvent, and C = concentration of the drug in the dissolution medium at time t.

The equation is useful for the determination of the intrinsic dissolution rate at a constant surface area. It is noted by an examination of the equation that the rate of dissolution dA/dt is directly proportional to the surface area, S, and the concentration differential $(C_s - C)$. The variables which must be controlled in the determination of the intrinsic dissolution rate are surface area, agitation intensity, temperature of the system, and volume of the dissolution medium. This expression is very valuable indeed, but it falls short of expressing the dissolution rate of a multiparticulate system where agitation intensity is more difficult to control. It is even farther from simulating the conditions in the fluids of the GI tract or other tissue fluids at different administration sites.

The closest analogy of the above expression in a living system would be the subcutaneous implantation of pellets made from practically insoluble drugs which exert a therapeutic effect for several months. The change of surface area with time is small, but even in this case the absorption rate is not constant (13).

The Hixson-Crowell (20) cube root law as modified by Parrott (21) is presented:

$$Kt = W_o^{1/3} - W^{1/3} \qquad \text{(Eq. 2)}$$

where W_o = weight of solid particle initially, W = weight of solid particle at time, t, K = the product of intrinsic dissolution constant, solubility, and density.

The weight of a sphere was determined at different times and a linear relationship was obtained. This equation is useful only for the determination of the intrinsic dissolution rate constant and it is limited to those particles whose ratio of dimensions does not change as dissolution proceeds (22). The error would be even more pronounced if different dissolution rates occurred from different faces (22). There is an applicability, however, to those cylindrical particles whose heights are equal to their initial diameter (22). In general, all particles with dimensions which are in the proximity of spheres would first dissolve away those faces and edges, so that by their absence the particles can assume the shape of minimum surface (spherical). In shapes grossly different from spherical, most of the drug material will be dissolved before the spherical shape is approached, if it is possible for the final shape to be nearly spherical. Long needles and irregular

platelets are good examples of grossly different shapes from spherical. The Hixson-Crowell equation has limited applicability to *in vivo* conditions.

Dissolution rates of multiparticulate systems are the best estimate of actual *in vivo* conditions (23). The Hixson-Crowell equation was extended by Niebergall *et al.* (24) to include multiparticulate systems. Their equation is:

$$W_o^{1/3} - W^{1/3} = K''N^{1/3}t \qquad \text{(Eq. 3)}$$

where W_o, W, K'', and t have the same significance as above and where N equals the number of particles.

According to Levy (13) the determination of apparent dissolution rates is necessary since (*a*) highly irregular shaped particles do not maintain constant ratio of dimensions; (*b*) the particle-size distribution is not normal or log-normal and cannot be adequately described mathematically in many cases; (*c*) many particles exhibit different dissolution rates from the different crystal faces (anisotropic in nature); (*d*) agitation intensity is hard to control or define, and it varies with density and particle size—small particles (less than 10 μ) are not subjected to much agitation and the boundary layer is effectively larger for them; (*e*) dissolution rates need to be determined in the presence of the other ingredients of a formulation; (*f*) the functional surface area is different from the specific surface of solid drugs and this difference may be brought about by surface fissures, porosity, surface hydrophobicity, and particle aggregation—surfactants may change the effective surface of a particle.

Agitation intensity and its effect on the dissolution rate, as determined by the method of the rotating disk, has been expressed quantitatively by Levy (13).

$$R = K\ (\text{r.p.m.})^{1/2} = \text{dissolution rate/min.} \qquad \text{(Eq. 4)}$$

where K = a proportionality constant.

In a dissertation by Fincher (25) a review of physical phenomena associated with the reduction of particle size is presented. The reduction of particle size is associated with an enormous increase in specific surface area. The classic example (26) of a cube 1 cm. (10^{-2} m.) on edge being divided into smaller cubes which are 1 mμ (10^{-9} m.) on edge gives an increase in surface area of seven orders of magnitude, *i.e.*, 6×10^{-4} m.^{2}, as compared to 6×10^{3} m.^{2} Properties which play a subordinate role in determining the behavior of large particles will become increasingly important as the particle size is reduced (26).

Drug materials, upon fine subdivision, show an increase in solubility and vapor pressure and a decrease in melting point. Particle size determines color in some cases. For example, antimony changes from red to yellow as the particle size decreases. As particle size is decreased the amount of gas and other types of molecules adsorbed is increased due to the increased surface (26). This phenomenon is utilized in the treatment of flatulence, in toxin absorption in the treatment of poisoning, and in the determination of surface areas (B.E.T. method).

The mechanism of solution involves surface action and by virtue of an increased surface a given solute will dissolve more rapidly (26). Increased solubility is reported to be evident when particle size is in the submicron range. Alexander (27) found a linear relationship between the logarithm of the solubility, S, and the surface area, A, of amorphous silica: $\log S = 0.00480A - 2.043$. As will be noted, the slope is a very small positive number, but as the surface area becomes very large, the increase in solubility is significant. Higuchi (28), in a discussion of the theoretical effects of particle size on solubility noted that the increase in solubility is significant only for very small particles. Even perfect crystals, differing in size, will have different solubilities (28).

The formation of complexes has the effect of increasing molecular dimensions, increasing the charge on the molecule if the complex is soluble, and decreasing the solubility of the drug if the complex is not charged. Complexing is a function of pH, and the pH of GI contents is an important consideration here.

The advantages gained by reducing the particle size below 1 μ are far outweighed by the difficulties encountered in handling and formulating drugs into dosage forms (15). The surface energy of the particles is one property manifesting itself in a very small particle size (below about 5 μ) by their sticking to each other and the sides of the container or by their scattering asunder on contact with an agitator.

Effects of Particle Size on Clinical Response Reported Prior to 1964—Table I is an alphabetical summary of those drugs which have been studied and information published prior to June 1964 (*References 29–57*).

More Recent Reports on Particle Size and Clinical Response—Table II is an alphabetical listing of those drugs which have been studied with respect to particle size and have been reported since 1964 (*References 58–65*).

The effect of the particle size on sulfisoxazole blood levels in dogs was studied by Fincher *et al.* (58), and statistical analyses were performed on the data taken at 0.5, 1, 2, 4, 8, and 12 hr. after

TABLE I—ALPHABETICAL SUMMARY OF DRUGS THAT HAVE BEEN STUDIED IN BIOLOGICAL SYSTEMS WITH RESPECT TO PARTICLE SIZE—STUDIES REPORTED UP TO JULY 1964

Name of Drug	Biological System Used	Particle Size or SSA Range	Method Used to Det. Particle Size of SSA	Criterion Used, Hours of Sampling	Clinical Effect of Reduced Particle Size (Increase SSA)	Dosage Form	Ref.
Aspirin	—	20, 120 mesh, solution	Sieve	Blood level	Increased	Cachet, solution	15
Calomel	Humans	USP (2–50 μ) 0.8 μ, max. (colloidal)	—	Antiseptic action	Increased	Ointment	44, 45
Chloramphenicol	Bacteria	11–60 μ	—	Zones of inhibition	Increased	Ointment	55–57
Chloramphenicol	Rabbits	50–800 μ	Sieve	Blood levels	Increased rates	—	33
Bishydroxycoumarin	Humans	Fine and larger	—	Hemophilia	Increased	Tablets	53
Griseofulvin	Humans	0.4–2.5 m.² per g. (0.8–11.4 μ)	Instrument[b]	Blood levels (4, 8, 12)	Increased absorption	Tablets	29, 30
Griseofulvin	Rats	Common powd. + micronized	—	Serum level (2, 4, 8, 12)	Increased Increased Increased	Suspension Suspension + surfactants Suspension in corn oil	31
Griseofulvin	—	Common powd. + micronized	—	—	—	—	32
p-Hydroxypropiophenone	Humans	Large and small	Microscope	Estrogenic activity	Increased	—	49
Mercury	Rats and rabbits	Smaller particles	—	Wet kidney content	Increased	—	48
Phenolphthalein	Humans	USP and colloidal	—	Purgative	Increased	—	43
Phenothiazine	Lambs	1–10 μ	Instrument[b]	Worm counts in abomasa and intestine (small)	Increased	—	38, 39
Phenothiazine	Moth larvas	4 and 45 μ	—	Toxicity effects	Increased	—	52
Phenothiazine	Sheep	—	—	Nematodes in large intestine	Decreased	—	40, 41
Procaine Penicillin	Humans	5–60 μ	—	Serum levels	Increased Decreased	Suspension in water Suspension in oil	50
Spironolactone	—	Common and micronized	—	Serum levels	Increased	Capsules	51
Sulfadiazine	Humans	Common powd. in tablets, microcrystalline, micronized	—	Plasma levels + urine excretion	Increased	Tablets susp.	35
Sulfadiazine	Humans	Common powd. + micronized	—	Serum levels (1, 2, 4, 6, 24)	Increased	Suspension	34
Sulfaethidole	Rabbits	68–274 μ	Sieve	Blood levels	Increased	—	36
Sulfathiazole	Humans	50–100 mesh and eutectic mixture with urea	—	—	Increased with eutectic	Suspension	37
Sulfur	Humans	Elemental and colloidal	—	Excretion	Increased	—	46
Tetracycline hydrochloride	Humans	1.00 μ–0.95 cm. pellet	Sieve	—	No effect	—	42
Tolbutamide[a]	—	—	—	—	—	—	54
Vitamin A	Humans (infants)	1–2 μ	Microscope	—	Increased	Emulsion	47

[a] See Table II, last item. [b] Fisher subsieve sizer.

oral administration of the drug in capsules. Perhaps, for a better picture of particle-size effect, it would have been better to administer the drug particles in a freshly prepared suspension, since capsules have been known to delay the availability of the drug for absorption up to 30 min., or even more. This fact may explain some of the higher degree of variation at the 0.5-hr. study. The changes of blood level with particle size at each of the times studied can be expressed in semilogarithmic function:

$$\log M = aP + b \qquad \text{(Eq. 5)}$$

where M = blood level in mg. %, P = particle diameter, and a and b are constants representing the slope and intercept, respectively.

The slope of the time constant lines changes from a negative to a positive value between 4 and 8 hr. after administration indicating that blood levels are higher for larger than for smaller particles after about 6 hr. A linear plot of the slope of each time constant line $(d \log M)/dP$, as a function of time, t, in hours produced a *straight* line whose equation is:

$$\frac{d \log M \times 10^3}{dP} = a_1 t + b_1 \qquad \text{(Eq. 6)}$$

where a_1 and b_1 are the slope and intercept equal to 0.781 and −4.32, respectively, for sulfisoxazole. The factor 10^3 was introduced for the convenience of plotting whole numbers. Integration of this equation between limits of yields:

$$10^3 \log \frac{M_2}{M_1} = (a_1 t + b_1) \cdot (P_2 - P_1) \qquad \text{(Eq. 7)}$$

If P_1, T, P_2, and M_1 are known, one can calculate with Eq. 7 the blood level resulting from P_2 at a given time. The proportion of dose absorbed did

TABLE II—ALPHABETICAL SUMMARY OF DRUGS THAT HAVE BEEN STUDIED IN BIOLOGICAL SYSTEMS WITH RESPECT TO PARTICLE SIZE—STUDIES REPORTED SINCE JUNE 1964

Name of Drug	Biological System Used	Particle Size or SSA Range	Method Used to Det. Particle Size of SSA	Criterion Used, Hours of Sampling	Clinical Effect of Reduced Particle Size	Dosage Form	Ref.
Amphotericin	Dog Mice Humans	Not known	—	Toxicity; therapeutic effect	Increased Increased	Parenteral	64
Fluocinolone acetonide	Humans	Coarse and micronized	—	Vasoconstriction	Increased	Ointments	60
Lime	Alfalfa	8–10 mesh 10–20 mesh 20–30 mesh 30–50 mesh 5–100 mesh 100 − + mesh	Sieve	Rate of growth	Increased to 50 mesh	Fertilizer	62
Medroxyprogesterone acetate	Humans	Common (1.2 m.²) and micronized (7.4 m.²) before tableting	—	Urinary excretion (8 hr.)	Increased	Tablets	59
Nitrofurantoin	Dogs	50–60 mesh 200–325 mesh micronized	Sieve	Emesis	Increased	Capsule	63
Nitrofurantoin	Rat	50–80 mesh 80–200 mesh 200–micronized	Sieve	Urinary excretion	Increased	Suspension	63
Nitrofurantoin	Humans	Same as above	Sieve	Urinary excretion	Increased	Capsules	63
Potassium dihydrogen phosphate	Rats	10–50 mesh	Sieve	Cariostatic action	Increased to 20–30 mesh	Diet	61
Sulfisoxazole	Dogs	1.7, 39, and 95 μ	Electronic counter[a]	Blood levels (0.5, 1, 2, 4, 8, 12)	Increased	Capsule	58
Tolbutamide	Humans	6.60, 36.9, 63.1, and 98.6 cm.² per dose of 5 g.	Calculations	Urinary excretion (every 2–12 hr. then every 12 hr.)	Increased	Tablet and capsules	65

[a] Coulter, Coulter Electronics, Franklin Park, Ill.

not change with particle size, but the smaller particles gave quick and higher blood levels which declined rapidly (58). With very insoluble drugs, such as griseofulvin, the absorbed proportion of the dose is increased with a decrease in particle size (29–32).

Smith *et al.* (59) compared the urinary excretion of micronized and nonmicronized medroxyprogesterone acetate after oral administration of tablets, each containing 10 mg. of the drug plus 0.05 mg. of ethinyl estradiol. A crossover testing procedure was used. One tablet of nonmicronized drug was compared with one-half tablet of micronized drug and one tablet of nonmicronized was compared to one tablet of micronized. An average of 2.23 times as much micronized drug was excreted as compared to the nonmicronized in a period of 8 hr. The specific surface area (SSA) of the micronized and nonmicronized was reported to be, before tableting, 7.4 and 1.2 m.²/g., respectively (59), but the method of determination of the SSA was not given. The effect of tableting on the SSA is not known here. Methods for determination of the apparent or effective surface area after incorporation in a tablet or other dosage form are needed in order to obtain meaningful SSA *versus* availability results. One-point determination, evident in the above studies (59), may be justified in some cases, but one still wonders what happened before and after the end point used.

A comparison of the effect of micronized and coarse fluocinolone acetonide particles on the degree of vasoconstriction was done by Barrett *et al.* (60). The coarse particles were partially milled in a triple roller mill during preparation, and the resultant particle size is open to question. White soft paraffin was the base used for the studies and two percentages of the drug were used, *viz.*, 0.025 and 0.01%. In both concentrations, the micronized form produced about two times the effects of the coarse. Ten subjects were used for each test and the ointment was applied to the flexor aspect of the forearm for 16 hr., was washed away, and the degree of vasoconstriction was read 1.5 hr. later.

It is interesting to note that the particle size of potassium dihydrogen phosphate had an effect on the cariostatic activity in rats when fed in their diets (61). The best size for cariostatic action was found to be in the 20–30 mesh range. Since the solubility of KH_2PO_4 is 1 part in about 4.5 parts of water, one wonders how particle size could possibly have any effect on tooth decay except that the larger particles may fracture the teeth more on chewing.

Another interesting study was performed by fertilizing alfalfa with limes of different mesh size (62). In the calcitic form no differences in alfalfa growth were noted with all particle sizes, but with the dolomitic form of particles above 30 mesh did not produce accelerated growth as compared to the control. Below 30 mesh the particles size had no increased effect.

Paul *et al.* (63) studied the relationship of the particle size of nitrofurantoin to emesis in dogs, urinary excretion in rats, dogs, and humans. Twenty dogs were used for each of the emesis studies, four rats and 15 humans were used in the urinary excretion studies for each crystal size. The range of crystal sizes studied was 50–400 mesh. Since the larger crystals caused less emesis in dogs and the smaller crystals gave higher blood levels, an optimum crystal size range was reached, which gave the highest blood levels with minimum nausea. A plot of the percent of dose excreted in the rat as a function of time gave excellent curves, which were very similar to those obtained by Fincher *et al.* (58). The smooth shape of the curves presented is indicative of a well planned and thorough study. No statistical analyses were performed on their (63) data, but in physiological systems 10–15 determinations can give useful data and show the expected results with the degree of variability known.

Bennett *et al.* (64) studied the toxicity and therapeutic effect of the particle size of amphotericin B after injection into the dog, mouse, and man. As the particle size increased, the toxicity level decreased but the therapeutic effect also decreased. The writer did not have access to the original article, and possibly some mistake in transmitting the abstract data has been made, but an i.v. injection was reportedly used which is unusual, if not undesirable in any circumstances.

Nelson *et al.* (65) administered doses of tolbutamide to normal humans in the form of a cylindrical disk (6.60 cm.^{2}) and three granules having initial surface areas of 36.9, 63.1, and 98.6 cm.^{2}/0.5-g. dose. The granules were administered in hard gelatin capsules. Urinary collections were made at intervals of 2 hr. for the first 12 hr. and at 12-hr. intervals up to 60 hr. Each test was performed three times and the average percent of dose excretions for the metabolite gave linear correlation with the surface area of the dose. Their conclusion was that the surface area of a tolbutamide dose can have a significant effect on the extent and the rate of availability.

Recent Studies of Effect of Particle Size on Release of Drugs From Dosage Forms In Vitro—The effect of salicylic acid particles on its diffusion rate from some ointment bases was studied by Kucera and Veber (66). Their exact results are not known to the author since a translation was not available. From the abstract, two grades of salicylic acid were used in the studies—one was the official form and the other was sodium salicylate. The diffusion of both samples was very low from hydrophobic bases, but, from hydrophilic bases the rate of diffusion of the sodium salicylate was very marked (66). This appears to be a study of insoluble drug *versus* a soluble form.

The interdependence of the displacement factor and particle size in suppositories was studied by Adel (67). By reduction of the particle size the value of the displacement factor is decreased. A particle greater than 150 μ should not be used in suppositories based on their conclusion from a study of the displacement factor.

The increase of the dissolution rate of drugs by preparation of solid solutions and/or eutectic mixtures of the drug and urea was attributed to the availability of the drug from such solutions and/or mixtures in a very fine state of subdivision (68–71).

The factors influencing drug release from a prolonged-action matrix were reviewed by Lazarus *et al.*, and the particle size of the drug was one of the primary considerations (72).

The theoretical effect of the distribution of the particle size in a diffusion-controlled process was studied by Higuchi, Rowe, and Hiestand (73). The change of particle-size distribution with time is shown. As dissolution proceeds the smaller particles dissolved very rapidly, leaving only the larger particles in the distribution. The assumptions are (*a*) the dissolution rate is diffusion controlled; (*b*) the diffusion layer thickness is always the same for all particles of the same size and is equal to or greater than the radius; (c) the concentration change in the "sink" is negligible at all times; (*d*) the effective particle shape approximates a sphere.

The dissolution of a single particle is expressed in terms of the particle diameter, a, at any given time, t.

$$a^2 = a_o^2 - \frac{2D\Delta Ct}{\rho} \qquad \text{(Eq. 8)}$$

where a_o = initial diameter, D = Fick's law diffusion rate constant, C = concentration of the dissolved solid, ρ = particle density.

The total dissolution rate of a powder with log-normal distribution is expressed as a function of the number of particles and the mass mean radius of the particles in the distribution. For a detailed discussion and equations see *Reference 73*.

Their theory was tested using micronized methylprednisolone, and a reasonably close agreement was obtained. The differences were believed to be due to the combined effects of agitation, sedimentation, particle shapes, and the variation of solubility with particle size.

Discussion of General Information Relating to Particle-Size Effect on Drug Absorption

and Activity—It has been noted by Dayton *et al.* (74) that with certain drugs their plasma level decline is dependent on the dose; when giving probenecid, diphenylhydantoin, phenylbutazone, and two analogs of phenylbutazone and biscoumacetate to dogs in larger doses, their rate of plasma disappearance was decreased. This phenomenon was not attributed to plasma protein binding, but was found to be caused by a special case of self-inhibition of metabolism. It is pertinent to note in this case that if particle-size reduction leads to a larger percentage of the dose being absorbed, a higher dose is the result, and a longer duration of action would be expected. This discovery also emphasizes the need for knowledge of metabolic processes prior to a study of particle-size effects.

In a review by Levine and Pelikan (75), these writers discussed the mechanisms of drug absorption and excretion. The molecular size, shape, electrical charge, and degree of polarity are properties of the drug molecule that affect its absorption. The properties of the living membrane are not only dependent on the properties of its constituents, but also on their arrangement. A living membrane is in dynamic equilibrium. Changes occur in living membranes when the pH or other environmental factors change. Consequently, when the amount of drug absorbed is directly proportional to the initial concentration, this does not necessarily prove simple diffusion as the total mechanism. Dose effects are indicated in the case of benzomethamine. A plot of the amount of drug absorbed in 3 hr. *versus* the amount of benzomethamine in GI loop gives three straight lines of different slopes. The size of the dose determines which method is used in the absorption process. The middle-sized dose gave the most rapid absorption rate (75). This "dose-effect" is another reason why the absorption rate of the drug must be studied before performing particle-size studies. If particle-size reduction produces a higher dose, which in turn induces another absorption process, the results may be interpreted in the wrong manner.

The effect of molecular size on the diffusion coefficient was considered by Wurster (76). The conclusion was that a large increase in molecular size would be necessary to get an appreciable increase in the diffusion coefficient. The charge on a particle (molecule) can increase the effective molecular size much greater than the physical dimensions *per se*, and can decrease the diffusion process through a biological membrane (77). The diffusion through an aqueous medium would be facilitated, however. Water is, apparently, able to pass through the pores of living membranes easily, but charged ions encounter some difficulty even though some ions may be pulled through by "solvent drag." When a molecule is lipid soluble, this greatly increases the permeability of a substance, *i.e.*, in effect the size of the pores of the membrane increases greatly (75, 77).

Reduction of the particle size of purified cellulose from 74 × 16 μ to 100 mesh had the effect of reducing the "lag-time" of the digestion by rumen cellulolytic bacteria *in vitro*, from 12 to 6 hr. (78).

Time and space will not permit a detailed discussion of other aspects of particle-size studies, but the study of particle size and its effect on drug absorption and action can be divided into several phases each of which is not without its problems. It is assumed that some knowledge of the drug's metabolism and excretion is already accomplished. The phases will be outlined in the order in which they are likely to be encountered, and a few pertinent references given.

Phase I—The aquisition of monosized particles in the range of desired studies. This is usually not feasible nor possible, and a narrow particle-size distribution is the next best possibility (25, 68–71, 79, 80).

Phase II—Characterization of the particles with regard to crystal shape, form, and habit. This is normally done by use of X-ray diffraction, microscopy, and other physical property studies, and it should be checked after formulations are prepared.

Phase III—Selection of the best method for particle-size analysis and determination of the particle-size distribution or specific surface area (2, 25, 81, 82).

Phase IV—Selection of the best dosage form and animal to administer the particles for the purposes of the study.

Phase V—Determination of the effective particle size after incorporation into the dosage form. Phase II may have to be repeated if a character change occurs during the production procedures (13).

Phase VI—Selection of an appropriate criterion to use in the evaluation of the effects of particle size (29–65).

Phase VII—Design of the animal experimentation procedures, including proposed statistical analyses to be performed (29–65).

Phase VIII—The experiment and data analysis.

In vitro dissolution rates may precede animal studies, but unless positive correlation is established with *in vivo* results, they cannot be the sole initial factor for estimation of biological avail-

ability. If a correlation is established, they may be used as the criterion in subsequent batches of the same drug and dosage form. Methods for the determination of dissolution rates *in vitro* have been devised, and in some cases, correlated with *in vivo* studies (13, 68–71, 73, 82–88).

The significance of particle-size effects on the therapeutic response of drugs will be reflected in the new revisions of the official compendia of this country and others (16, 89–91). Effective particle-size limits will probably be an established procedure for those drugs which are practically insoluble. If the particle size of other drugs, which are not so insoluble, can influence the needed therapeutic response, their particle size may also need to be controlled.

The effect of particle size on formulation procedures, as such, are not necessarily a part of this article but a few references are compiled for the benefit of the readers' interest (1–11, 13–16, 66, 67–73, 82–84, 92–111).

A recent discussion of the increase in solubility of small particles (spherical only) has been presented by Smolen and Kildsig (112). The thermodynamic equation—relating vapor pressure to the change of free energy—is the basis for their derivation of another equation which relates solubility to particle size:

$$dG = dNRT \ln \frac{P}{P_o} \qquad \text{(Eq. 9)}$$

where G = surface free energy, P = vapor pressure of a small particle, P_o = vapor pressure of pure drug, N = number of moles of substance in the bulk of drug material.

In Eq. 9, they (112) substituted γdA for dG, $(4\pi r^2)/V$ for dN, and $8\pi r dr$ for dA and obtained

$$RT \ln \frac{P}{P_o} - \frac{2\gamma V}{r} \qquad \text{(Eq. 10)}$$

where V = molar volume, γ = surface energy, r = radius of particle, A = surface area.

Since the same relationship applies using the solubilities, S and S_o, in place of P and P_o, respectively, Eq. 10 can be written

$$\log \frac{S}{S_o} = \frac{2\gamma V}{2.303\, RTr} \qquad \text{(Eq. 11)}$$

The greater solubility of smaller particles is a consequence of a larger molar free energy.

Model of Possible Particle-Size (SSA) Effects on Absorption Rate of a Drug—Consider a system composed of a segment of GI lumen in which a single drug particle is placed. Let

- C_s = concentration of the drug at saturation in the GI fluid (the concentration of the saturated boundary layer)
- C_m = concentration at the membrane site of absorption
- C_b = concentration in the blood (the other side of the membrane site of absorption)
- A_o = initial surface area of the particle at the time of its entrance into the GI segment
- t_o = initial time the particle enters the segment
- A = surface area of the particle at the time it leaves the GI segment
- t = time the particle leaves the GI segment
- a_o = total amount of drug dissolved at the time it enters the GI segment
- a = amount of drug dissolved at the time it leaves the segment
- f_a = agitation intensity factor
- x = mean distance from the center of the lumen to the wall of the lumen—the expected mean location of the particle
- N = total number of particles
- a_T = total amount of drug dissolved in the segment
- $a_{\text{tot.}}$ = total amount of drug dissolved in all segments

The assumptions are (*a*) passive diffusion is the absorption process; (*b*) the rate of absorption is limited by the dissolution rate of the particle.

Under these conditions, the absorption rate is equal to the dissolution rate of the particle, and $C_s >>>> C_m > C_b$. The dissolution rate, da/dt, is a function of the effective surface area of the particle at any time, t, the agitation intensity factor, $f_a(C_s - C_m)$—which includes the solubility effect and the time the particle remains in the segment. It is represented by the equation:

$$\frac{da}{dt} = R_1 (A_o - A)(C_s - C_m) f_a \qquad \text{(Eq. 12)}$$

The mean distance between the center of the lumen need not be considered if the steady-state is reached in a negligible amount of time. R_1 is a proportionality constant. The change in surface area $(A_o - A)$ decreases with time and approaches zero as a limit. The concentration gradient $(C_s - C_m)$ is approximately equal to C_s, a constant, since C_m is reduced to a minimum by the blood sink under the conditions existing. The agitation is assumed to be constant in a given segment of the GI tract. This factor may fluctuate considerably in a biological system in a given segment, but one has no control over this variable

except to assign it an average value which is assumed to be constant.

With the above considerations, the constants, R_1, $(C_s - C_m)$, and f_a, may be combined into one constant, K, the dissolution rate constant in the fluids of the GI segment. Substitution gives[1]

$$\frac{da}{dt} = K(A_o - A) \qquad \text{(Eq. 13)}$$

on rearrangement one obtains

$$da = K(A_o - A)dt \qquad \text{(Eq. 14)}$$

The integral of equation between the limits of t_o and t gives the total amount of drug dissolved in the segment.

$$\int_a^{a_o} da = K \int_t^{t_o} (A_o - A)dt \qquad \text{(Eq. 15)}$$

$$a_o - a = K(A_o - A)(t_o - t) \qquad \text{(Eq. 16)}$$

Since $a_o = 0$ at $t_o = 0$, one can write

$$-a = K(A_o - A)(-t) \qquad \text{(Eq. 17)}$$

Multiplying through by -1, one obtains:

$$a = K(A_o - A)t \qquad \text{(Eq. 18)}$$

With the assumptions made at the onset of this discussion, the amount of drug dissolved from a unit particle during the time it remains in the GI segment in question is a function of the change in surface area. In order to obtain the amount of drug, a_T dissolved from N particles of the same size, the equation would be

$$a_T = NK(A_o - A)t \qquad \text{(Eq. 19)}$$

If the particles are not monosized, the size distribution must be considered. In a log-normal distribution, the initial area of the mass median particle, as suggested by Higuchi *et al.* (73), would be a good estimate of the A_0 value, even though the number of particles would change to some extent with time (73). The more narrow the particle-size distribution, the better the estimate of the mass median particle area.

If a drug is absorbed from more than one segment of the GI tract, each having a different dissolution medium, then each set of constants would have to be determined separately. The total amount of drug dissolved in the GI tract, $a_{\text{tot.}}$, is found by the sum of the a_T values in each GI segment

$$a_{\text{tot.}} = \sum N_i K_i (A_{o_i} - A_i)t_i \qquad \text{(Eq. 20)}$$

where i refers to any GI segment. The stomach, duodenum, and segments of the small intestine, whose medium content is different from the others, could be used as hypothetical segments. It is recognized that, due to duodenal and intestinal secretions, there is a gradual change in any one segment (12), and an average content must be determined. It is also recognized that the emptying time of the stomach and the motility of other segments may be quite variable. The average time the particles remain in a given segment must be determined. Also, the change in the surface area of the particle in any given segment is not an easy determination. X-rays have been used to study movements of particles, but the size or surface-area determination would be a more difficult task. The values of each element of the equation would have to be based on the means of 10 to 15 determinations, especially where physiological variables are involved.

A word about averages. A treatment of a given disease or an abnormal condition is analogous to the make-up of our society. Our society is not made up of averages, but it is made up of individuals who deviate considerably from the mean. Furthermore, the things which are important to our lives at any given time period are not made up of averages, but they are made of those which serve to fulfil the needs of the individual at the time period in question. Thus, average behavior of biological systems under study are good guidelines, but they are not necessarily the all-important answer in an individual system.

Lest anyone get the idea that particle-size control and knowledge of the particle size are the answer to all the ills of dosage form production and drug availability for absorption, the author has this to add: particle size is like a wrench in the tool box. If it is needed to accomplish the goal, then by all means it should be used. If it is not needed, then it should be left in the tool box of knowledge until the occasion arises where it can contribute. In formulation and production one would suspect that particle size is considered regularly. In the case of its effect on drug availability, its use may be less frequent. However, where the consideration of particle size is essential, its effect should be known.

[1] Since the modified Noyes-Whitney equation indicates that the dissolution rate is proportional to the surface area, it is represented in this way in Eq. 13. If the effect of $(A_o - A)$ is some function of $(A_o - A)$ raised to some power e, *i.e.*, $(A_o - A)^e$, the equation would be linear as a log-log function.

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Keyphrases

Particle size, drugs—review
Drug particle-size relationship—absorption, activity
Therapeutic aspects—particle size
Release rates, drug—particle size
Absorption rate model—particle-size effects

Relative Stabilizing Effects of Several High Molecular Weight Polymers on the Stability of a 2 Percent Hexadecane in Water Emulsion

By HELMY A. F. MAHROUS* and A. P. LEMBERGER

The stability characteristics of a 2 percent hexadecane in water emulsion containing 0.09 percent dioctyl sodium sulfosuccinate (AOT) were investigated. Size distribution analyses of the emulsified hexadecane particles within the diameter range of 1–25μ were made over an extended time. The volume concentrations of the emulsified oil in this diameter range were calculated. The mass median, the number median, the volume surface, and the arithmetic mean diameters were also calculated. The data obtained established that the changes in particle-size distribution observed were largely the result of coalescence. This system was then employed as a standard to evaluate the effectiveness of methylcellulose, 25 cps., polyvinylpyrrolidone, polyvinyl alcohol, and polyoxyethylene glycol 6000 in retarding the degree and rate of coalescence of this emulsion. It is suggested that strongly hydrated films at the oil-water interface form effective barriers to coalescence.

THE STABILITY of emulsions is a complex phenomenon in which the electrical forces (1, 2) and the film-film interaction forces play an important role (3). The latter force is important especially in the case of emulsions stabilized with nonionic surfactants.

One important group of nonionic surfactants includes certain high molecular weight polymeric substances. King and Mukherjee (4) reported that these agents produced a coarser but more stable emulsion than those stabilized with soaps. Jellinek (5) found that emulsions stabilized with hydrophilic colloidal substances underwent rapid initial changes. The rate of change observed in these systems decreased after a period of time. In another study methylcellulose was found to stabilize latex particles against coagulation with electrolytes by forming a protective film around the particles. The stabilizing effect of methylcellulose increased as its concentration was raised (6).

One of the most widely used methods for studying the stability of emulsions is size-distribution analysis. Usually size analysis data are presented as number count, number percent, and volume or weight percent distributions. Reliance on one parameter may be misleading, since calculations based on volume or weight percents tend to show the importance of small numbers of large globules, while number counts or distributions will minimize the presence of a few large globules which are important in emulsion stability. Thus, the combination of the number-size distributions and weight-size distributions serves to give a better understanding of the nature of changes which occur in emulsions (7).

Deaggregation in hexadecane in water emulsion systems containing various concentrations of dioctyl sodium sulfosuccinate has been reported by Higuchi *et al.* (8) and by Mourad and Lemberger (3, 9). However, none of these studies involved an investigation of coalescence of larger globules. The purpose of this study was to critically examine the coalescence in a 2% hexadecane in water emulsion containing 0.09% dioctyl sodium sulfosuccinate as a basis for further study of the influence of high molecular weight polymers on the stability of the system. Methylcellulose, polyvinylpyrrolidone, polyvinyl alcohol, and polyoxyethylene glycol 6000 were selected for examination as additives.

EXPERIMENTAL

Materials—The hexadecane used for the preparation of the emulsions was purified by redistillation under reduced pressure. Dioctyl sodium sulfosuccinate[1] (AOT) was further purified by the fractional precipitation method (10) from methanol and a water–methanol solvent mixture. The fraction selected for use was that capable of solubilizing 40–50 molecules of water. Water used was doubly distilled from permanganate in a glass apparatus. The poly-

Received May 22, 1968, from the School of Pharmacy, The University of Wisconsin, Madison, WI 53706
Accepted for publication July 22, 1968.
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Supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

[1] Aerosol OT, 100% pure, American Cyanamid Co., New York, N. Y.

mers used were methylcellulose 25,[2] polyvinylpyrrolidone, polyvinyl alcohol, and polyoxyethylene glycol 6000.[3]

Instruments—The instrument used for the size-distribution analyses was the Coulter counter, model B.[4] The model B has the advantage of counting particles between two different size limits, thus simplifying the reduction of the raw data. The size analyses were conducted by using a 100-μ aperture tube which had been calibrated with a monodisperse latex system at all the instrument settings used in this study.

The samples were tumbled at a constant temperature in a water bath fitted with a rotating mechanism.

Procedure—The emulsions were prepared by the high-voltage electrical dispersion method described by Nawab and Mason (11) and used previously in these laboratories (3, 8, 9) with a slight modification. A second syringe with needle was mounted above the apparatus such that the oil discharged into the water from the lower syringe was continuously replaced with oil from the top syringe thus maintaining a constant pressure head. Six kilovolts were applied to a 25-gauge needle whose tip was evenly cut perpendicular to its axis. The stock emulsion contained 40% v/v of hexadecane and 1.8% AOT. It was placed in a polyethylene bottle and was aged for 1 week in a rotating wheel-type constant-temperature bath at 30° prior to dilution.

Preparation of 2% Methylcellulose Solution—Methylcellulose powder was added to boiling water ($^1/_3$ of the volume of the needed solution) and stirred to wet the powder completely. The remainder of water was added (cooled at 5°) with stirring. The solution was then stored in a refrigerator for 8 days prior to its use.

Preparation of Polyvinyl Alcohol—The PVA in powder form was added with stirring to cold water (20° or less) until a smooth slurry was obtained. This slurry was heated at 75–80° until complete solution was obtained.

Preparation of Polyvinylpyrrolidone and Polyoxyethylene Glycol 6000—Solutions of polyvinylpyrrolidone and polyoxyethylene glycol 6000 were prepared by dissolving the powders in cold water with stirring.

All the above solutions were filtered with suction through a sintered-glass funnel. Microscopic examinations of these solutions indicated that they were free of particulate matter that might be confused with oil globules.

Preparation of the 2% Hexadecane in Water Emulsions—For a given experiment the stock emulsion was diluted 1–20 by volume in order to obtain a 2% hexadecane emulsion containing 0.09% AOT. Sufficient quantities of concentrated aqueous solutions of the polymeric solutions were added to the system during the dilution of the stock emulsion to give the desired final concentrations of these substances in the emulsion. The concentration of the various additives was set at 0.25, 0.5, 1.00, and 2.00% w/v. The samples containing the various concentrations were placed in 15-ml. glass vials and tumbled gently in the constant-temperature bath at 30°. All emulsions studied were prepared by dilution of the same stock emulsion for each experimental run.

For particle-size analysis exactly 0.5 ml. of the emulsion was diluted to 25 ml. with 0.09% sodium chloride injection solution.[5] Four milliliters of this dilution was further diluted to 100 ml. with more of the sodium chloride solution. This suspension of oil globules was then analyzed for its size distribution using the particle counter fitted with a 100-μ aperture tube. The diluted emulsion samples were analyzed for oil globules within the range of 1–25 μ. Each emulsion system studied was run in triplicate determinations.

Size-distribution data were recorded and processed for the calculation of the different diameter values and determination of the volume of oil in each sample. The volume of the oil particles per given sample volume of the emulsion was referred to as V. The raw data obtained from the size-analysis measurements were processed with the aid of an IBM 1604 computer.

RESULTS AND DISCUSSION

The raw counts obtained by the particle counter were processed to obtain the number and the weight percent distributions. A plot of these weight percents *versus* the particle diameters produced weight-size distribution curves. The number percent distribution curves were obtained by plotting the number percents *versus* the particle diameters. The arithmetic mean diameter, $d_{me.}$, and the volume surface diameter, $d_{vs.}$, were calculated from the data. The mass median and the number median diameters were easily obtained from the plots of weight and number percents against the particle diameters, respectively.

Tables I and II show typical weight and number distribution data, respectively, for the 2% hexadecane in water emulsion stabilized with 0.09% dioctyl sodium sulfosuccinate. Table I also includes values for the mass median (d_m), the number median (d_n), the arithmetic mean, and the volume surface diameters. The total volume of the oil counted (V) and the polydispersity ratio d_m/d_n were also recorded in Table I. Size-distribution analyses and the other parameters were recorded as the average of three samples/run. Graphical representations of Tables I and II are shown in Figs. 1 and 2, respectively.

It is apparent from Figs. 1 and 2 that the size distributions within the size range selected for analysis changed with time. Specifically, it is seen that the larger particles disappeared from the system. Since the large particles constitute a larger percentage of the population on a weight basis than on a number basis, one would expect the size distributions of Fig. 1 to be affected to a greater extent over the time of observation.

The comparative change in size distributions based on weight and number percents can be illustrated even more clearly with the differential plots given in Figs. 3 and 4. The larger particles (>6 μ) are disappearing and, as a result, the smaller particles constitute an increasing fraction of the total population of the aged systems.

An approximate index to the behavior of this system upon aging is provided by the mass median

[2] Methocel 25, Dow Chemical Co., Midland, Mich.
[3] Carbowax 6000, Union Carbide and Carbon Corp., New York, N. Y.
[4] Coulter Electronics, Inc., Hialeah, Fla.
[5] Abbott Laboratories, Chicago, Il

TABLE I—TYPICAL DATA OBTAINED FROM WEIGHT PERCENT DISTRIBUTION ANALYSES OF A 2% HEXADECANE IN WATER EMULSION STABILIZED WITH 0.09% AOT

du	1 day	4 days	7 days	11 days	16 days	42 days	94 days	224 days
24.88	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
19.78	4.04	0.30	0.00	0.00	0.00	0.00	0.00	0.00
15.67	12.27	2.30	1.32	0.83	0.69	0.40	0.70	0.64
12.43	24.64	7.20	3.42	1.70	1.21	1.13	1.01	1.03
9.87	38.02	18.78	9.04	4.93	3.22	1.41	1.29	1.25
7.83	52.19	31.66	18.85	11.56	7.50	2.67	1.48	1.43
6.21	64.73	45.81	32.13	22.63	16.69	5.95	2.13	1.64
4.92	75.15	61.30	49.06	38.88	32.06	15.89	6.29	2.06
3.92	84.98	75.35	66.65	59.48	52.98	34.94	20.57	3.26
3.11	91.86	86.69	82.48	78.34	74.43	60.98	47.93	12.15
2.46	95.83	94.20	93.09	91.56	89.66	84.10	77.63	41.88
1.95	98.49	98.24	98.30	97.87	96.96	95.88	94.99	77.16
1.65	99.48	99.50	99.59	99.43	99.21	99.01	98.97	92.54
1.43	99.90	99.95	99.93	99.90	99.85	99.91	99.82	98.66
1.14	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
				Calculated Values				
d_m	8.00	6.70	5.60	4.70	4.10	3.35	3.05	2.30
d_n	2.47	2.62	2.71	2.67	2.58	2.48	2.40	1.95
d_m/d_n	3.23	2.53	2.06	1.80	1.59	1.35	1.26	1.18
$d_{me.}$	2.76	2.72	2.69	2.60	2.49	2.36	2.26	1.83
$d_{vs.}$	5.63	4.53	3.94	3.60	3.33	2.92	1.65	1.05
Volume $\times 10^{-53}$	4.65	4.41	3.75	3.54	2.70	1.86	1.67	1:31

diameter and number median diameter of the particles as read from the appropriate plots. The volume surface diameter calculated from the raw data is also indicative of the behavior of the system. The ratio between the mass median and number median diameters, d_m/d_n, termed the polydispersity by Rowe (12) is also useful as a criterion of emulsion stability. As this ratio approaches unity, the emulsion system is approaching the monodisperse state.

The disappearance of the larger particles could be accounted for either by coalescence or by deaggregation. Significant deaggregation seems the less likely explanation, however, because in this case, one would expect to see a much more pronounced increase in the frequency of the small particles on a number percent basis than actually occurs. This conclusion seems further justified by the marked decrease in cumulative volume of particles counted in the sample taken as the emulsion aged (Table I).

Influence of Additives—The arithmetic mean diameters, volume-surface diameters, the mass median diameters, the number median diameters, and the polydispersity ratios for emulsion systems containing various concentrations of polymeric substances were obtained over a period of aging. These parameters are given in Tables III, IV, V, and VI for methylcellulose, polyvinyl alcohol, polyoxyethylene glycol 6000, and polyvinylpyrrolidone containing systems, respectively. Since each system was prepared from a separately prepared standard emulsion the values for each of these emulsions are also given in the tables. From the data presented it can be seen that all standard systems demonstrated essentially the same behavior.

Examination of Tables III and IV reveals that addition of either methylcellulose or polyvinyl alcohol resulted in a stabilizing effect. Perhaps of most significance is the observation that d_m did not decrease as markedly over the aging period while d_n tended to remain constant or actually to increase slightly in value. Thus, the polydispersity tended to stabilize indicating that the particle-size distribution of the emulsified hexadecane remained essentially the same. This is also confirmed in Figs. 5 and 6 which show the weight percent size distributions of the emulsions containing 2% methylcellulose and

TABLE II—TYPICAL DATA OBTAINED FROM NUMBER PERCENT DISTRIBUTION ANALYSES OF A 2% HEXADECANE IN WATER EMULSION STABILIZED WITH 0.09% AOT

du	1 day	4 days	7 days	11 days	16 days	42 days	94 days	224 days
24.88	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
19.78	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15.67	0.16	0.02	0.01	0.01	0.00	0.00	0.00	0.00
12.43	0.55	0.15	0.05	0.01	0.01	0.01	0.00	0.00
9.86	1.40	0.57	0.25	0.11	0.06	0.01	0.01	0.00
7.83	3.12	1.71	0.94	0.50	0.27	0.05	0.13	0.01
6.21	6.23	4.13	2.80	1.80	1.19	0.30	0.52	0.01
4.92	11.68	9.44	7.36	5.61	4.26	1.48	0.66	0.04
3.92	20.83	19.04	17.46	15.24	12.64	7.57	4.02	0.44
3.12	34.45	34.65	35.27	33.14	29.79	23.16	17.18	2.24
2.47	49.91	55.23	58.96	57.96	54.17	50.78	45.61	15.87
1.95	70.97	77.44	82.43	81.60	77.53	78.41	78.80	48.16
1.65	85.54	90.77	91.04	92.37	92.55	93.09	93.56	74.52
1.44	95.79	98.45	98.26	98.90	98.30	98.54	98.02	91.23
1.14	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

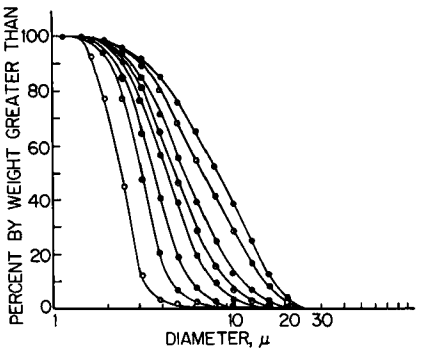

Fig. 1—Typical weight percent–particle-size distribution curves for a 2% hexadecane emulsion containing 0.09% AOT. Key: ◐, 1 day; ◑, 4 days; ●, 7 days; ◓, 11 days; ◒, 16 days; ⊕, 42 days; ⊙, 129 days; ○, 224 days.

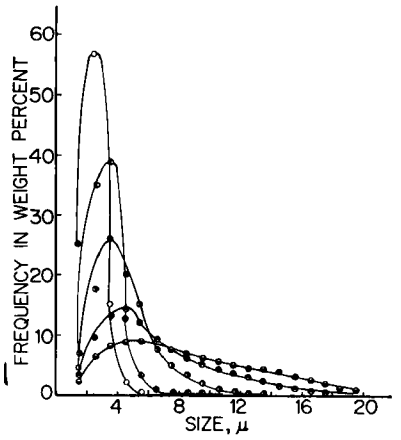

Fig. 3—A plot of frequency in weight percent against size for a 2% N hexadecane emulsion containing 0.09% AOT. Key: ◒, 1 day; ◓, 4 days; ◑, 17 days; ◐, 94 days; ○, 224 days.

2% polyvinyl alcohol over the period of observation. The stabilizing effect of different concentrations of these additives is also indicated by the $d_{vs.}$ values obtained. The volume-surface diameter decreased markedly with time of aging in the standard system, whereas the $d_{vs.}$ tended toward a constant value as the concentration of additive was increased. That these additives not only stabilized the size distribution but also reduced coalescence rates is demonstrated in Figs. 7 and 8. In these figures the reciprocal of the volume of hexadecane in the sample analyzed is plotted as a function of time. The manner of plotting is analogous to that of Smoluchowski (13, 14). It is seen that coalescence occurs rapidly initially in the standard emulsions probably due to the formation of droplets larger than 25 μ in diameter from the larger droplets initially present. Since the sample included particles to a maximum of 25 μ in diameter, the total volume of the emulsified hexadecane particles larger than this would be lost to observation. Increasing concentrations of methylcellulose 25 and polyvinyl alcohol progressively reduced the rate of coalescence and at the maximum concentration used in these studies, the total volume of internal phase remained constant. This observation coupled with the size-distribution analyses data indicate that, principally, the larger globules seem to be stabilized against coalescence to form particles larger than 25 μ.

A similar family of plots for emulsions containing polyvinylpyrrolidone and polyoxyethylene glycol 6000 are given in Figs. 9–12. As one would expect from the data given in the tables of these systems, there is no evidence of stabilization. Neither the polydispersity ratios nor the volume-surface diameters obtained differed significantly from those obtained with the standard emulsion. The weight percent size distributions of the systems containing 2% of these additives are seen to change during the aging period in the same manner as the control emulsions. The plots of $1/V$ against time give families of roughly parallel lines showing that the rate of coalescence in these systems is identical to that of the control emulsions.

The polymeric substances used in this study possess surface-active properties. They reduce surface tension of water and have the ability to be adsorbed at the interfaces (6, 15–17). The mode of the orien-

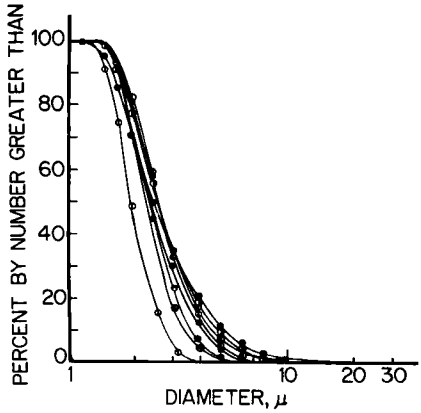

Fig. 2—Typical number percent–particle-size distribution curves for a 2% hexadecane emulsion containing 0.09% AOT. Key: ●, 1 day; ◑, 4 days; ◐, 7 days; ⊙, 11 days; ◒, 16 days; ◓, 42 days; ⊕, 94 days; ⓓ, 224 days.

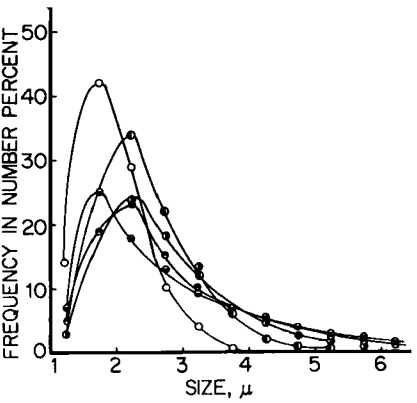

Fig. 4—A plot of frequency in number percent against size for a 2% N hexadecane emulsion containing 0.09% AOT. Key: ◒, 1 day; ◓, 4 days; ◑, 17 days; ◐, 94 days; ○, 224 days.

TABLE III—VARIOUS VALUES CALCULATED FOR THE STANDARD SYSTEM CONTAINING METHYLCELLULOSE

	1 day	4 days	7 days	11 days	16 days	42 days	94 days	224 days
Standard, μ								
$d_{me.}$	2.76	2.72	2.69	2.60	2.49	2.36	2.25	1.83
$d_{vs.}$	5.63	4.53	3.94	3.60	3.33	2.92	2.65	2.05
d_m	8.00	6.70	5.60	4.70	4.10	3.35	3.05	2.30
d_n	2.47	2.62	2.71	2.67	2.58	2.48	2.40	1.94
d_m/d_n	3.23	2.53	2.06	1.80	1.59	1.35	1.26	1.18
Standard + 0.25% methylcellulose, μ								
$d_{me.}$	2.79	2.72	2.62	2.71	2.65	2.65	2.50	2.14
$d_{vs.}$	5.57	4.74	4.24	4.09	3.84	3.48	3.11	2.77
d_m	8.00	6.40	5.45	5.05	4.80	4.00	3.60	2.90
d_n	2.56	2.58	2.60	2.69	2.67	2.74	2.63	2.24
d_m/d_n	3.13	2.51	2.08	1.87	1.79	1.46	1.36	1.29
Standard + 0.50% methylcellulose, μ								
$d_{me.}$	2.69	2.71	2.51	2.58	2.51	2.49	2.43	2.33
$d_{vs.}$	5.57	5.22	4.84	4.69	4.61	4.43	3.99	3.76
d_m	8.15	7.20	6.60	6.05	5.80	5.30	4.70	4.40
d_n	2.42	2.48	2.31	2.44	2.36	2.36	2.36	2.26
d_m/d_n	3.38	2.89	2.86	2.47	2.45	2.24	1.99	1.95
Standard + 1.00% methylcellulose, μ								
$d_{me.}$	2.59	2.59	2.49		2.39	2.48	2.53	2.51
$d_{vs.}$	5.68	5.31	5.04		4.92	4.61	4.23	3.64
d_m	8.60	7.60	7.30	no data	7.00	6.25	5.70	4.70
d_n	2.31	2.34	2.15		2.13	2.27	2.39	2.54
d_m/d_n	3.72	3.24	3.38		3.04	2.74	2.38	1.84
Standard + 2.00% methylcellulose, μ								
$d_{me.}$	2.64	2.65	2.46		2.41	2.54	2.55	2.52
$d_{vs.}$	5.74	5.55	5.28		5.16	4.47	4.76	4.57
d_m	8.10	8.05	7.70	no data	7.40	6.60	6.40	6.00
d_n	2.34	2.36	2.17		2.08	2.27	2.32	2.34
d_m/d_n	3.68	3.40	3.51		3.56	2.90	2.78	2.56

TABLE IV—VARIOUS VALUES CALCULATED FOR THE STANDARD SYSTEM CONTAINING POLYVINYL ALCOHOL (PVA)

	1 day	3 days	6 days	10 days	16 days	42 days	129 days	212 days
Standard, μ								
$d_{me.}$	2.45	2.43	2.42	2.44	2.33	2.25	2.00	1.85
$d_{vs.}$	4.62	4.33	4.08	3.75	3.40	2.89	2.40	2.13
d_m	6.40	5.80	5.30	4.80	4.30	3.40	2.75	2.40
d_n	2.25	2.29	2.31	2.40	2.32	2.33	2.09	1.94
d_m/d_n	2.84	2.53	2.28	2.00	1.85	1.45	1.32	1.23
Standard + 0.25% PVA, μ								
$d_{me.}$	2.33	2.37	2.39	2.42	2.45	2.51	2.42	2.40
$d_{vs.}$	4.51	4.43	4.35	4.13	3.83	3.48	3.01	2.82
d_m	6.40	6.00	5.80	5.42	5.00	4.25	3.55	3.30
d_n	2.10	2.16	2.21	2.28	2.15	2.53	2.57	2.45
d_m/d_n	3.03	2.76	2.62	2.36	2.32	1.67	1.38	1.34
Standard + 0.50% PVA, μ								
$d_{me.}$	2.33	2.35	2.38	2.43	2.43	2.57	2.61	2.48
$d_{vs.}$	4.64	4.55	4.46	4.20	4.03	3.65	3.27	3.06
d_m	6.40	6.25	6.08	5.55	5.30	4.55	3.90	3.60
d_n	2.00	2.24	2.16	2.29	2.31	2.60	2.76	2.64
d_m/d_n	3.19	2.78	2.80	2.42	2.29	1.75	1.41	1.37
Standard + 1.00% PVA, μ								
$d_{me.}$	2.22	2.37	2.34	2.34	2.34	2.49	2.72	2.60
$d_{vs.}$	4.66	4.63	4.68	4.60	4.55	4.27	3.97	4.12
d_m	6.70	6.50	6.40	6.30	6.15	5.60	5.00	4.70
d_n	1.95	2.17	2.09	2.08	2.09	2.32	2.75	2.53
d_m/d_n	3.42	2.98	3.05	3.02	2.94	2.41	1.82	1.85
Standard + 2.00% PVA, μ								
$d_{me.}$	2.28	2.29	2.28	2.36	2.31	2.37	2.66	2.53
$d_{vs.}$	4.81	4.70	4.78	4.75	4.60	4.61	4.42	4.38
d_m	6.90	6.80	6.85	6.80	6.65	6.40	6.15	5.65
d_n	1.99	2.00	1.98	2.10	1.95	2.12	2.53	2.38
d_m/d_n	3.45	3.39	3.45	3.23	3.39	3.01	2.43	2.36

TABLE V—VARIOUS VALUES CALCULATED FOR THE STANDARD SYSTEM CONTAINING POLYOXYETHYLENE GLYCOL 6000

	1 day	3 days	6 days	9 days	15 days	33 days	129 days	212 days
Standard, μ								
$d_{me.}$	2.51	2.39	2.38	2.41	2.43	2.29	2.00	1.76
$d_{vs.}$	4.66	4.38	4.14	3.97	3.65	3.22	2.39	2.00
d_m	6.40	6.00	5.55	5.20	4.70	4.10	2.75	2.25
d_n	2.33	2.20	2.25	2.33	2.42	2.34	2.12	1.87
d_m/d_n	2.74	2.62	2.46	2.22	1.94	1.75	1.30	1.20
Standard + 0.25% polyoxyethylene glycol 6000, μ								
$d_{me.}$	2.48	2.32	2.39	2.34	2.40	2.21	1.78	1.63
$d_{vs.}$	4.70	4.28	4.13	3.93	3.59	3.20	2.13	1.87
d_m	6.20	5.60	5.38	5.00	4.50	3.90	2.35	2.05
d_n	2.31	2.15	2.25	2.27	2.38	2.25	1.86	1.70
d_m/d_n	2.68	2.50	2.38	2.19	1.95	1.73	1.26	1.20
Standard + 0.5% polyoxyethylene glycol 6000, μ								
$d_{me.}$	2.42	2.38	2.42	2.36	2.42	2.23	1.83	1.66
$d_{vs.}$	4.37	4.28	4.00	3.77	3.53	2.99	2.11	1.86
d_m	6.00	5.60	5.20	4.90	4.40	3.70	2.30	2.00
d_n	2.27	2.30	2.31	2.27	2.40	2.19	1.93	1.77
d_m/d_n	2.70	2.68	2.26	2.17	1.83	1.68	1.19	1.13
Standard + 1.00% polyoxyethylene glycol 6000, μ								
$d_{me.}$	2.45	2.36	2.37	2.34	2.38	2.31	1.98	1.86
$d_{vs.}$	4.75	4.29	4.00	3.73	3.54	3.18	2.40	2.19
d_m	6.00	5.65	5.20	4.80	4.50	3.80	2.70	2.36
d_n	2.25	2.19	2.26	2.24	2.37	2.31	2.09	2.00
d_m/d_n	2.68	2.64	2.40	2.14	1.90	1.64	1.28	1.18
Standard + 2.00% polyoxyethylene glycol 6000, μ								
$d_{me.}$	2.34	2.39	2.36	2.36	2.43	2.33	1.90	1.68
$d_{vs.}$	4.39	4.29	3.98	3.84	3.52	3.22	2.24	1.90
d_m	6.00	5.60	5.25	4.90	4.50	3.90	2.45	2.00
d_n	2.18	2.24	2.24	2.28	2.42	2.30	2.02	1.75
d_m/d_n	2.74	2.58	2.34	2.14	1.86	1.69	1.22	1.14

TABLE VI—VARIOUS VALUES CALCULATED FOR THE STANDARD SYSTEM CONTAINING POLYVINYLPYRROLIDONE (PVP)

	1 day	4 days	7 days	12 days	21 days	43 days	80 days	140 days
Standard, μ								
$d_{me.}$	2.52	2.55	2.57	2.59	2.54	2.57	2.53	2.37
$d_{vs.}$	4.69	4.33	4.98	3.92	3.66	3.45	3.26	3.04
d_m	6.20	5.60	5.10	4.80	4.50	4.15	3.90	3.40
d_n	2.43	2.49	2.56	2.61	2.58	2.66	2.65	2.48
d_m/d_n	2.54	2.28	1.99	1.83	1.74	1.56	1.46	1.37
Standard + 0.25% PVP, μ								
$d_{me.}$	2.46	2.34	2.43	2.42	2.35	2.29	2.23	2.11
$d_{vs.}$	4.51	3.96	3.74	3.56	3.42	3.19	3.00	2.88
d_m	6.00	5.00	4.70	4.40	4.20	3.75	3.50	3.20
d_n	2.37	2.39	2.40	2.35	2.33	2.30	2.18	
d_m/d_n	2.52	2.09	1.96	1.82	1.78	1.61	1.52	1.46
Standard + 0.50% PVP, μ								
$d_{me.}$	2.44	2.48	2.50	2.52	2.37	2.40	2.32	2.19
$d_{vs.}$	4.46	4.09	3.89	3.71	3.32	3.27	3.04	2.76
d_m	5.90	5.05	4.75	4.50	4.05	3.80	3.60	3.30
d_n	2.38	2.44	2.47	2.53	2.39	2.46	2.39	2.16
d_m/d_n	2.48	2.06	1.92	1.78	1.68	1.54	1.49	1.50
Standard + 1.00% PVP, μ								
$d_{me.}$	2.38	2.47	2.49	2.51	2.47	2.44	2.38	2.30
$d_{vs.}$	4.42	3.99	3.83	3.68	3.42	3.30	3.04	2.64
d_m	5.80	5.00	4.80	4.50	4.30	3.90	3.60	3.20
d_n	2.41	2.43	2.47	2.52	2.50	2.50	2.42	2.30
d_m/d_n	2.41	2.05	1.94	1.78	1.71	1.56	1.49	1.38
Standard + 2.00% PVP, μ								
$d_{me.}$	2.49	2.50	2.52	2.53	2.50	2.50	2.40	2.27
$d_{vs.}$	4.48	4.06	3.88	3.77	3.59	3.42	3.07	2.79
d_m	5.80	5.00	4.80	4.60	4.40	4.20	3.70	3.20
d_n	2.43	2.47	2.50	2.53	2.53	2.57	2.51	2.39
d_m/d_n	2.38	2.03	1.91	1.81	1.73	1.63	1.47	1.34

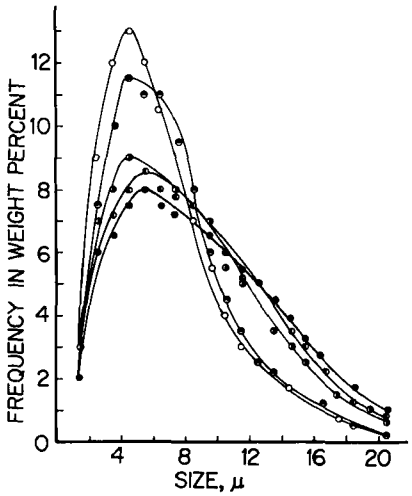

Fig. 5—A plot of frequency in weight percent against size for the system containing 2.0% methylcellulose as an additional stabilizer. Key: ●, 1 day; ◐, 4 days; ◑, 17 days; ◓, 94 days; ○, 227 days.

tation of these substances might be responsible for the differences in their stabilizing effect on the emulsions under study. Since the concentration of AOT in the system (0.09%) is below the critical micelle concentration, it can also be assumed that the monolayer surrounding the oil particles will be of the expanded type. If enough substance is adsorbed at the interface, the expanded monomolecular film may change to a more condensed film which, in turn, may protect the globules against coalescence. It, therefore, could well be that the stabilizing effect of methylcellulose and polyvinyl alcohol was a result of their adsorption and orientation at the interface in such a manner as to ensure the transformation of the expanded AOT film to a more condensed, stronger monomolecular layer. The degree of this transformation appears to be dependent upon the concentration of the additive as had been reported by Saunders (6). He found that the stabilization of latex particles by methylcellulose was dependent upon the concentration of the latter substance. He also reported that latex particles were stable when enough methyl-

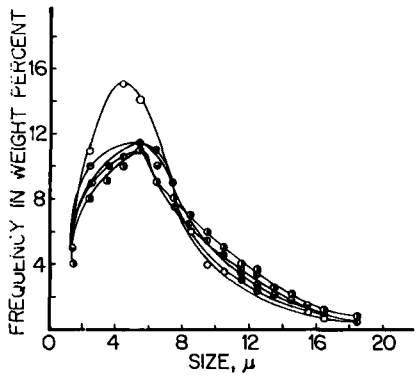

Fig. 6—A plot of frequency in weight percent against size for the system containing 2.00% polyvinyl alcohol as an additional stabilizer. Key: ◑, 1 day; ◐, 3 days; ◓, 16 days; ◒, 129 days; ○, 224 days.

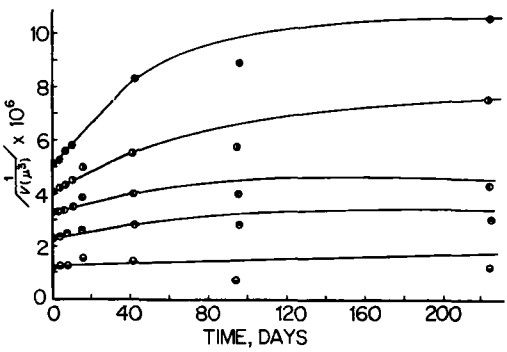

Fig. 7—A plot of the reciprocal of the volume of emulsified particles/50 ml. of emulsion (V) against time for systems containing methylcellulose as an additional stabilizer. Key: ●, standard (+3); ◑, 0.25% (+2); ◐, 0.50% (+1); ◒, 1.00%; ◓, 2.0% (−1). (Values in parentheses indicate a factor added to value of 1/V for plotting purposes only.)

cellulose was added to ensure a condensed monolayer around the particles.

Methylcellulose and polyvinyl alcohol possess hydroxyl groups in their structures. These hydroxyl groups are strongly hydrophilic because of their tendency toward hydrogen bonding with the water molecules in the external phase. Thus, upon adsorption at the interface, the methylcellulose and the polyvinyl alcohol orient themselves in such a way that a hydrated film is formed around the oil globules which tends to protect them against coalescence.

The stabilizing effect of such hydrated films was observed by Van den Temple (18) in his work with paraffin in water emulsions. He described the oil particles as being separated by a water film which might be the result of hydration or solvation of the original film.

On the other hand, polyoxyethylene glycol 6000 and polyvinylpyrrolidone showed no stabilizing effect on the emulsion under study, although one can as-

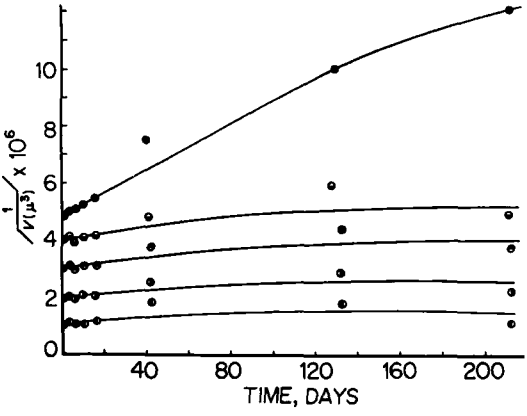

Fig. 8—A plot of the reciprocal of the volume of emulsified particles/50 ml. of emulsion (V) against time for systems containing polyvinyl alcohol as additional stabilizer. Key: ●, standard (+3); ◓, 0.25% (+2); ◒, 0.50% (+1); ◑, 1.00%; ◐, 2.00% (−1). (Values in parentheses indicate a factor added to value of 1/V for plotting purposes only.)

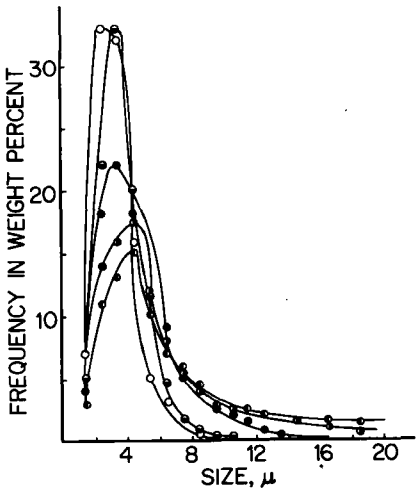

Fig. 9—A plot of frequency in weight percent against size for the system containing 2.00% polyvinylpyrrolidone as an additional stabilizer. Key: ◐, 1 day; ◑, 4 days; ●, 21 days; ◒, 80 days; ○, 140 days.

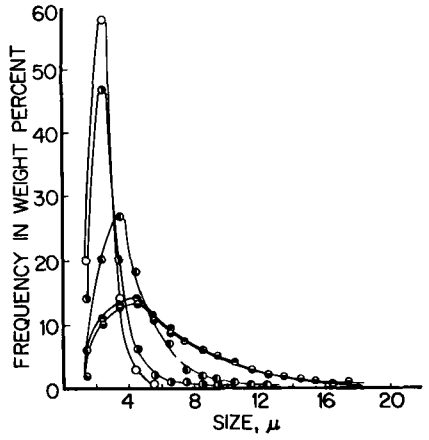

Fig. 10—A plot of frequency in weight percent against size for the system containing 2.00% polyoxyethylene glycol 6000 as an additional stabilizer. Key: ◒, 1 day; ◓, 3 days; ◐, 33 days; ◑, 129 days; ○, 212 days.

sume that water molecules could be attracted to at least some of the ether oxygen atoms by hydrogen bonds. The hydrophilic nature of the polyoxyethylene glycol or polyvinylpyrrolidone may not be sufficient to induce orientation of the molecu'e at the interface in such a definitive manner as with methylcellulose or polyvinyl alcohol.

Eley (17) described the molecule of polyoxyethylene glycol adsorbed at the interface as lying approximately flat in the water surface. He also added that molecules will not be expected to pack well when lying flat in a surface. Thus, it seems reasonable to assume that the polyoxyethylene glycol films formed around the oil globules were not oriented in such a manner as to produce strongly hydrated films. The same may be true for polyvinylpyrrolidone, but further studies are needed to investigate the mode of adsorption of these polymeric substances at the interface before more definitive statements can be made.

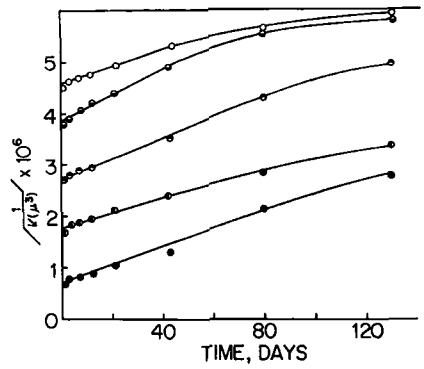

Fig. 11—A plot of the reciprocal of the volume of emulsified particles/50 ml. of emulsion (V) against time for systems containing polyvinylpyrrolidone as an additional stabilizer. ○, standard (+3); ◒, 0.25% (+2); ◓, 0.50% (+1); ◐, 1.00%; ●, 2.00% (−1). (Values in parentheses indicate a factor added to value of 1/V for plotting purposes only.)

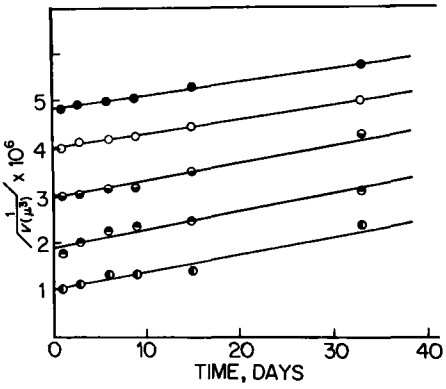

Fig. 12—A plot of the reciprocal of the volume of emulsified particles/50 ml. of emulsion (V) against time for systems containing polyoxyethylene glycol 6000 as an additional stabilizer. Key: ●, standard (+3); ○, 0.25% (+2); ◒, 0.50% (+1); ◓, 1.00%; ◐, 2.00% (−1). (Values in parentheses indicate a factor added to value of 1/V for plotting purposes only.)

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Keyphrases

Emulsion stabilization—polymers
Hexadecane-water emulsion—high mol. wt. polymers, stabilization
Particle-size distribution—emulsions
Polymers, effect—emulsion particle size
Stability, emulsions—aging, polymer effect

Evaluation of Physical and Pharmaceutical Factors Involved in Drug Release and Availability from Chloramphenicol Capsules

By A. J. AGUIAR, L. M. WHEELER, S. FUSARI, and J. E. ZELMER

The importance of physical and pharmaceutical factors involved in chloramphenicol release and availability from four commercial lots of chloramphenicol was evaluated. The study was carried out in three parts: (*a*) deaggregation or dispersion determinations, (*b*) dissolution studies, and (*c*) *in vitro* gut permeation using an everted sac technique. A correlation between drug release as reflected by the deaggregation and dissolution rates and chloramphenicol plasma levels in humans is demonstrated. In this specific instance the results of the study demonstrate that, although the products tested contain analytically the same quantity of chloramphenicol, the rate of release of the antibiotic, and hence its availability from the capsules differs significantly. The commonly accepted scheme which defines drug absorption from dosage forms is modified to include the deaggregation rate.

THE IMPORTANCE of physical properties of drugs in relation to their pharmacological availability and activity has been demonstrated and emphasized repeatedly in the past. Until recently, proportionally little attention was paid to studies related to drug release from pharmaceutical dosage forms. For example, there has been a vast amount of research done to evaluate dissolution rates of drugs from tablets where the geometry of the dissolving surface is kept constant during the dissolution process. However, most tablets and capsules of drugs used in clinical therapy are intended to disintegrate in the gastrointestinal environment after oral administration.

If the tablets or capsules disintegrate, the surface area and the geometry of the dissolving surface will change continuously with time. The dissolution rate which is dependent on the surface area will also change. Therefore, studies in which the dispersion (or deaggregation) as well as the dissolution rates are measured are of greater value in assessing the optimum formula for a capsule or tablet of a drug than simple dissolution measurements. The studies would also allow a closer correlation of drug availability from the dosage forms with plasma levels or other pharmacological criteria of drug efficacy.

The present work is an evaluation of the availability of chloramphenicol from four commercial lots of chloramphenicol capsules. Specifically the study is an attempt to relate pharmaceutical and physical factors with the differences in plasma levels observed when the four capsules were tested in adult human volunteers. The human absorption studies are reported elsewhere (1, 2).

Received April 22, 1968, from the Research and Quality Control Divisions, Parke, Davis & Co., Detroit, MI 48232
Accepted for publication June 11, 1968.
This paper is the second of a series on Clinical Equivalency. See *References 1* and *2* for the first and third papers.
The authors thank Dr. A. W. Kinkel and Dr. E. Holmes for the human absorption data shown in Fig. 5; Miss B. Shawler for assistance in the dissolution measurements; and Dr. J. F. Sadusk and Dr. L. M. Lueck for their constructive suggestions during the course of this work.

EXPERIMENTAL

The capsules tested were four commercial lots of chloramphenicol capsules produced by different manufacturers and were purchased from a local

Keyphrases

Emulsion stabilization—polymers
Hexadecane-water emulsion—high mol. wt. polymers, stabilization
Particle-size distribution—emulsions
Polymers, effect—emulsion particle size
Stability, emulsions—aging, polymer effect

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The importance of physical and pharmaceutical factors involved in chloramphenicol release and availability from four commercial lots of chloramphenicol was evaluated. The study was carried out in three parts: (*a*) deaggregation or dispersion determinations, (*b*) dissolution studies, and (*c*) *in vitro* gut permeation using an everted sac technique. A correlation between drug release as reflected by the deaggregation and dissolution rates and chloramphenicol plasma levels in humans is demonstrated. In this specific instance the results of the study demonstrate that, although the products tested contain analytically the same quantity of chloramphenicol, the rate of release of the antibiotic, and hence its availability from the capsules differs significantly. The commonly accepted scheme which defines drug absorption from dosage forms is modified to include the deaggregation rate.

THE IMPORTANCE of physical properties of drugs in relation to their pharmacological availability and activity has been demonstrated and emphasized repeatedly in the past. Until recently, proportionally little attention was paid to studies related to drug release from pharmaceutical dosage forms. For example, there has been a vast amount of research done to evaluate dissolution rates of drugs from tablets where the geometry of the dissolving surface is kept constant during the dissolution process. However, most tablets and capsules of drugs used in clinical therapy are intended to disintegrate in the gastrointestinal environment after oral administration.

If the tablets or capsules disintegrate, the surface area and the geometry of the dissolving surface will change continuously with time. The dissolution rate which is dependent on the surface area will also change. Therefore, studies in which the dispersion (or deaggregation) as well as the dissolution rates are measured are of greater value in assessing the optimum formula for a capsule or tablet of a drug than simple dissolution measurements. The studies would also allow a closer correlation of drug availability from the dosage forms with plasma levels or other pharmacological criteria of drug efficacy.

The present work is an evaluation of the availability of chloramphenicol from four commercial lots of chloramphenicol capsules. Specifically the study is an attempt to relate pharmaceutical and physical factors with the differences in plasma levels observed when the four capsules were tested in adult human volunteers. The human absorption studies are reported elsewhere (1, 2).

Received April 22, 1968, from the Research and Quality Control Divisions, Parke, Davis & Co., Detroit, MI 48232
Accepted for publication June 11, 1968.
This paper is the second of a series on Clinical Equivalency. See *References 1* and *2* for the first and third papers.
The authors thank Dr. A. W. Kinkel and Dr. E. Holmes for the human absorption data shown in Fig. 5; Miss B. Shawler for assistance in the dissolution measurements; and Dr. J. F. Sadusk and Dr. L. M. Lueck for their constructive suggestions during the course of this work.

EXPERIMENTAL

The capsules tested were four commercial lots of chloramphenicol capsules produced by different manufacturers and were purchased from a local

pharmacy. For purposes of these studies, they were labeled as A,[1] B, C, and D.

The study was carried out in three parts: (*a*) deaggregation or dispersion determinations, (*b*) dissolution studies, and (*c*) *in vitro* gut permeation using an everted sac technique.

The method employed to measure the deaggregation rates was essentially the one reported earlier (3). In the present study the simulated gastric fluid T.S. (USP XVI) was presaturated with chloramphenicol to minimize the dissolution of the drug particles as they appeared in the medium.

Five hundred milliliters of simulated gastric fluid T.S. presaturated with chloramphenicol was added to an 800-ml. beaker placed in a constant-temperature bath set at 30 ± 0.5°. The solution was stirred at 100 r.p.m. and allowed to come to temperature. The design of the all-glass stirrer used was such that it provided random agitation throughout the depth of the liquid without creating a vortex.

Two capsules from each lot were added to the gastric fluid for each determination. A stopwatch was started as soon as the gelatin shell of the capsule dissolved and first traces of the drug appeared, generally in about 2 min.

The percent transmittance (from which the absorbance was calculated) was measured at 650 mμ using a Coleman Junior spectrophotometer. A pump circulated the fluid *via* a 3-mm. diameter Tygon tubing from the beaker through a flow-through cell located in the spectrophotometer. The force necessary to deaggregate the drug was provided by the shearing action of the stirred liquid.

The dissolution rates of the capsules were measured by two different methods. In the first, a USP tablet disintegration apparatus of the Vanderkamp[2] design was employed, and the dissolution was carried out in simulated gastric fluid T.S. (USP XVI). One capsule was placed in each of the six tubes of the basket-rack assembly, omitting the plastic disks. The assembly was immersed in 800 ml. of simulated gastric fluid T.S. contained in a 1,000-ml. beaker, and held at 37° ± 0.5°. The assembly was put in operation at a constant frequency rate of 30 c.p.m. and 5-ml. aliquots of the solution were withdrawn after 10, 20, and 30 min. Each aliquot was centrifuged at 2,000 r.p.m. for 2 min., and the supernatant diluted and assayed spectrophotometrically for chloramphenicol at 278 mμ.

The other method used for measuring the dissolution rates was as follows. One capsule was dropped into 400 ml. of simulated gastric fluid T.S. (without pepsin) held in a beaker maintained at 30 ± 0.5°. The solution was stirred at 11 r.p.m. using an electronic-controlled laboratory stirrer[3] designed with three parallel prongs facing down. Five-milliliter samples were removed at 2-min. intervals initially and 5-min. intervals after the first hour. The samples were filtered through a 0.45-μ Millipore filter disk using a Swinney syringe adapter and assayed spectrophotometricly at 278 mμ after appropriate dilution with the gastric fluid.

The *in vitro* gut permeation of chloramphenicol was studied using a modified Crane and Wilson everted sac technique (4).

The excised small intestine from Holtzman rats weighing 120–150 g. was everted so that the mucosa faced outward. A 10-cm. segment taken 1 cm. from the stomach was tied tightly at the lower end, 1 cm. from the tip, and trimmed. The upper portion was attached 1 cm. from the top to a blunt cannula supported by a rubber stopper, leaving 8 cm. of the everted sac free and available for absorption. The segment supported by the cannula and rubber stopper was suspended in an 80-ml. glass tube containing 70 ml. of buffer–chloramphenicol mixture maintained at 37 ± 0.5° in a constant-temperature bath. One and six-tenths milliliters (1.6) of buffer without drug at 37° was introduced into the sac through the cannula.

A mixture of 95% oxygen and 5% carbon dioxide was bubbled through the solution through a longer cannula extending from the rubber stopper to the bottom of the glass tube.

At predetermined time intervals the solution inside the sac was removed, the sac was rinsed with a further 1.6 ml. of buffer which was also removed, and the combined solutions were assayed for chloramphenicol. The rinse solution was replaced with 1.6 ml. of fresh buffer.

The buffer solution used was Krebs-Ringer bicarbonate solution (5) (pH 7.2) with 0.3% glucose. The solutions were prepared in distilled water, 0.3% glucose was added, and the final solution was gassed for 10 min. with 95% oxygen–5% carbon dioxode. The gut segment was considered to be viable for 2 hr.

In order to measure the chloramphenicol bound by the gut tissue, the everted sac was removed from the apparatus at the end of the run, washed on the inner and outer surfaces with distilled water, and dried at 65°. The dried material remaining was extracted with ethanol and assayed.

The solutions were assayed spectrophotometrically for chloramphenicol at 278 mμ.

RESULTS AND DISCUSSION

The purchased capsules were subjected to various analytical tests, and the results are summarized in Table I.

Deaggregation Studies—The deaggregation profiles of the four commercial lots of chloramphenicol capsules tested are shown in Fig. 1. This plot of percent transmittance *versus* time provides a convenient comparison of the rates. It is readily apparent from the plot that Capsule A has the fastest deaggregation rate, and reaches the steady state in approximately 10 min. On the other hand, Capsule D has a very poor deaggregation rate. In fact, after 3 hr., Product D maintains its capsular appearance (Fig. 2). The initial increase in turbidity with Capsule D can perhaps be partly ascribed to the presence of additives in the capsule.

Capsules B and C have faster deaggregation rates than Capsule D, but the rates were slower than that of Capsule A.

The theoretical concepts developed earlier (3) to quantify the deaggregation rates were based on the premises that the deaggregation rate followed a first-order process and that the species present in the media were either the large aggregates and/or small aggregates. This implied that the large aggregates were broken down into small aggregates without

[1] Product A Chloromycetin Kapseals, Lot P 13542A, Parke, Davis & Co.
[2] Van-Kel Industries, Livingston, N. J.
[3] Gerald K. Heller Co., Las Vegas, Nev.

TABLE I—ANALYSIS AND COMPARISON OF FOUR COMMERCIAL LOTS OF CHLORAMPHENICOL CAPSULES

Analytical Test	Capsule A	Capsule B	Capsule C	Capsule D
Uniformity of capsule fill				
Average capsule fill, mg.	351.3	462.8	343.4	355.1
Range of capsule fill, mg.	339.6–369.8	379.9–504.8	333.0–352.1	318.6–371.7
Assay Uniformity				
Average assay, mg. chloramphenicol/capsule	248.3	261.9	249.1	244.6
Average percent of label claim	99.3	104.8	99.7	97.8
Range of assays in percent of label claim	96.2–104.8	86.6–114.4	96.6–101.6	88.1–102.0

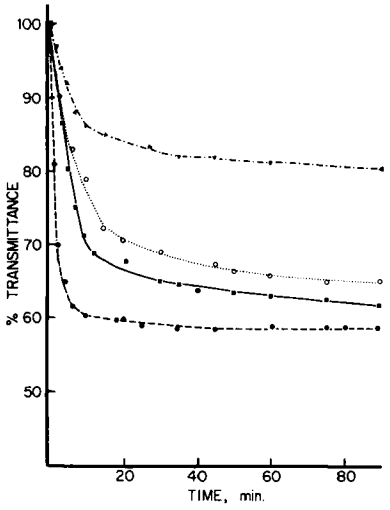

Fig. 1—Plot of percent transmittance versus *time showing the relative deaggregation rates of the four capsules in simulated gastric fluid T.S. Key: ●, Capsule A; ■, Capsule B; ○, Capsule C; ▲, Capsule D.*

going through the intermediate states, or if these were formed they were extremely short lived.

Based on these premises, equations were developed which described the deaggregation process. In the final form the equation was written as:

$$\log \frac{A_s}{A_s - A_t} = \frac{k}{2.303} t \qquad \text{(Eq. 1)}$$

Fig. 2—Chloramphenicol Capsule D after 3 hr. in simulated gastric fluid (deaggregation rates determination).

where A_s is the absorbance at the final state and A_t the absorbance at time t.

If the deaggregation process follows Eq. 1, a plot of $\log A_s/(A_s - A_t)$ *versus* t should be linear. From the slopes, the relative deaggregation rates can be evaluated.

In Figs. 3 and 4, the deaggregation rates of the four commercial lots of chloramphenicol capsules are plotted in accordance with Eq. 1. It is readily evident that the deaggregation rate of Capsule A follows Eq. 1. The deaggregation rates of the other three capsules are more complex.

In the case of Capsule D (Fig. 4), during the first 60 min. the deaggregation rate is indeterminate, possibly due to the appearance of additives in the medium. After the first hour, the rate appears to follow a first-order process, but it is extremely slow.

The deaggregation rates of Capsules B and C are intermediate between A and D and are nonlinear. The nonlinearity is possibly due to the reaggregation of the particles after they appear in the test medium. If the dispersed particles reaggregate, then the mechanism for the reverse reaction must also be taken into account, and the equation describing the phenomena would be much more complex than that shown in Eq. 1.

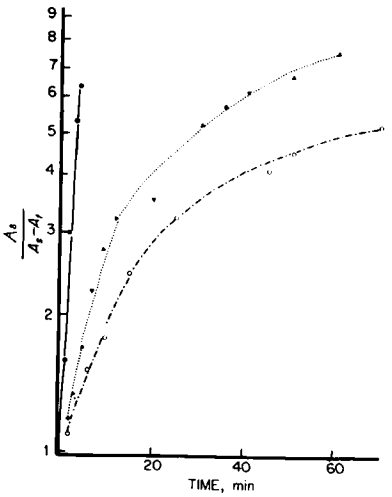

Fig. 3—Deaggregation rates of Capsules A, B, and C in simulated gastric fluid T.S. plotted according to Eq. 1. Key: ●, Capsule A; ▲, Capsule B; ○, Capsule C.

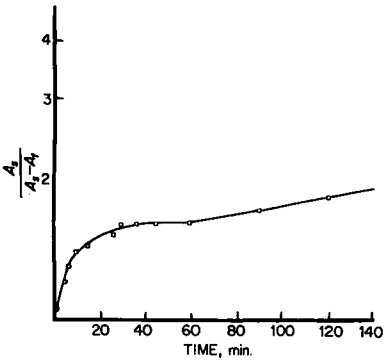

Fig. 4—Deaggregation rate of Capsule D in simulated gastric fluid T.S. plotted according to Eq. 1.

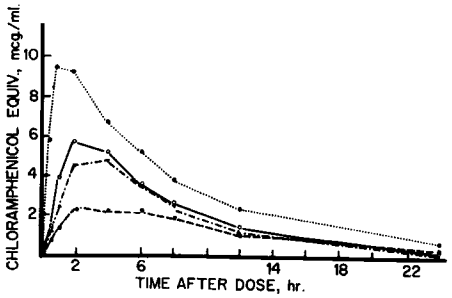

Fig. 5—Mean plasma levels in human volunteers of nitro compounds (chloramphenicol equivalents) following single oral doses of chloramphenicol capsules. [Dose 0.5 g. (2 capsules), 10 subjects] (see Reference 2*). Key: ●, Capsule A; △, Capsule B; ○, Capsule C; ■, Capsule D.*

Furthermore, the turbidity of the deaggregation medium is dependent on the particle size of the drug and in the present case also on the additives in the capsule such as fillers and lubricants. Equation 1 describes a situation (3) in which the particle size of the drug tested was uniform. If the particle size of the chloramphenicol in the capsules is different, or if the nature and amount of additives are dissimilar, the application of Eq. 1 in the present study would serve only as a qualitative comparison of the deaggregation rate rather than a quantitative measurement.

Figure 3 shows that there is at least a fivefold difference in the initial slopes of the lines between Capsules A and B and at least a sevenfold difference between A and C. The differences in the initial slopes of the lines emphasize the earlier and greater release of chloramphenicol from Capsule A in contrast to the other three capsules.

A qualitative correlation exists between the deaggregation rates of the four commercial lots of chloramphenicol capsules and the plasma levels shown in Fig. 5.[4] Capsule A shows a higher and earlier mean chloramphenicol peak plasma level in comparison with the others, and the deaggregation rate of Capsule A is also much faster. The mean peak plasma level observed with Capsule D is much lower and delayed while the deaggregation rate of the capsule is also very slow. Capsules B and C show intermediate deaggregation rates and intermediate mean plasma levels, although the order is reversed.

The apparent faster deaggregation rate of Capsule B in comparison to C can possibly be ascribed to the larger amount of additives present in B, as the turbidity is a function of the total quantity of particles present in the medium. Since the capsules were purchased it was not possible to ascertain definitively the composition and quantity of the additives present in Capsules B, C, and D.

A few of the D capsules were then emptied, and an equivalent weight of the powder was packed into a larger (No. 1) capsule to determine if the packing force would affect the rate. Packing into a larger capsule increased the initial dispersion. However, the particles quickly formed two or three large aggregates and further deaggregation was very slow indicating that the nature of the adjuvants was more important than the packing density or force.

Dissolution Studies—The results of the dissolution studies carried out with the Vanderkamp disintegration apparatus according to USP specifications are summarized in Table II.

The data presented in Table II show that about 90% of the chloramphenicol in Capsule A is in solution within the first 10 min. and emphasizes the rapidity of the dissolution of the product. Capsule C shows only about 50% of the drug in solution within this period while Capsules B and D have less than 10% in solution.

The rapid dissolution of Capsule A correlates well the rapid absorption and high plasma levels observed with the product.

The data presented in Table II also show that Capsules B and D have the lowest rates of solution. The mean plasma level obtained with Capsule D is also the lowest. The mean plasma level observed for Capsule B is higher than that with Capsule D, although their dissolution rates appear to be the same.

The results of the dissolution studies carried out with the electronic stirrer shown in Fig. 6 indicate the rapid dissolution rate of Capsule A in comparison with the others. With the experimental procedure that was used, there appears to be no significant difference between the rates of solution of the other three capsules.

The rapid dissolution of Capsule A again correlates well with the rapid absorption and high chloramphenicol plasma level shown in Fig. 5. The dissolution rates (as determined) do not differentiate between the blood levels of the three other capsules tested.

The particle-size distribution of chloramphenicol in the four capsules was approximated by sieve analysis. About 10 g. of the powder blend obtained by emptying 30 capsules from each lot was passed through a series of screens with progressively decreasing mesh size. The powder retained on the sieve was weighed, and an aliquot assayed spectrophotometrically for chloramphenicol content. The percent chloramphenicol retained on each sieve was computed and the data are shown in Fig. 7.

Admittedly there are some errors involved in determining the particle-size distribution by screen-

[4] Figure 5 is presented to facilitate the discussions and comparisons in this paper. For details and other information, see *References 1* and *2*.

TABLE II—RESULTS OF DISSOLUTION STUDIES OF COMMERCIAL LOTS OF CHLORAMPHENICOL CAPSULES[a]

Time, min.	% Total Drug Dissolved/Capsule						
	A	B Trial 1	B Trial 2	C	D Trial 1	D Trial 2	D Trial 3
10	91.8	9.9	8.3	48.7	7.2	9.8	9.3
20	97.5	16.9	17.5	88.3	13.4	21.7	16.8
30	98.6	21.3	28.2	94.3	19.1	35.9	20.4

[a] USP disintegration apparatus and artificial gastric fluid T.S. at 37 ± 0.5°.

ing techniques. However, qualitatively it is evident from Fig. 7 that the particle-size distributions of chloramphenicol in Capsules A, B, and D are not significantly different. The distribution of the drug particles in Capsule C is slightly different, showing a larger amount of fines which could be due to the presence of smaller chloramphenicol particles or alternatively due to the lubrication of the drug particles engendered during the sieve analysis by the additives in the capsule.

If the particle-size data presented in Fig. 7 are indeed representative of the distribution of chloramphenicol in the capsules, one would expect that the mean plasma level obtained with Capsule C would be higher than that obtained with the other capsules.

From the evidence presented, it appears that the deaggregation or dispersion rate is of importance in determining the availability of chloramphenicol (from the capsules) for dissolution and absorption.

Permeation Studies—The permeation studies using the everted sac technique were carried out to determine whether the additives in the chloramphenicol capsules interfered with the absorption of the drug by binding, *etc.*

The total quantities of drug permeating through the everted sac at various time intervals from the four lots of chloramphenicol capsules are given in Table III.

The profiles demonstrating the relative rates of permeation are shown in Fig. 8.

It is evident that the rates of permeation of Capsules A and C are similar, followed closely by Capsule B. The rate of permeation of Capsule D is approximately one-half of Capsule A, as is shown by the slope of the line.

It was noted that Capsule D contained a large quantity of an insoluble compound which coated the gut tenaciously during the permeation study. To determine whether the coating interfered with permeation of the drug, a second run was made with the capsule contents filtered before placing in the apparatus. Removal of the insoluble material produced a marked increase in both the rate and level of the drug. All four commercial chloramphenicol capsules were then treated similarly and resubmitted to permeation testing.

The quantities of drug permeating through the gut over the various time intervals for the filtered preparations are given in Table IV.

The profiles showing the relative rates of permeation after filtration are plotted in Fig. 9.

The increased permeation rates shown by the filtered solutions of the Capsules A, B, and C may be due to removal of the gelatin shell from the solution. The shaking time of 15 min. for the buffer solution containing the opened capsule was sufficient to saturate the solution with chloramphenicol, but was not sufficient to dissolve all of the gelatin capsule shell. The remainder of the gelatin shell dissolved during the 2 hr. of the experiment. Filtration of the buffer-capsule mixture before placing in the apparatus removed the undissolved gelatin material. Filtering the mixture from Capsule D removed the white insoluble material, thus increasing the permeability of the gut.

Assays of the ethanol extract of the gut, dried after use, showed less than 1% (of the dry weight) of chloramphenicol bound to the gut membrane.

If the permeation of the drug through the everted sac is related to the absorption process through the human intestine, one could postulate from the data that the permeation rate of chloramphenicol is not the rate-determining step in the absorption process.

It is difficult to assess the significance of the coating of the gut membrane observed with Capsule D. As indicated earlier, the coating reduced significantly the absorption through the everted sac of chloram-

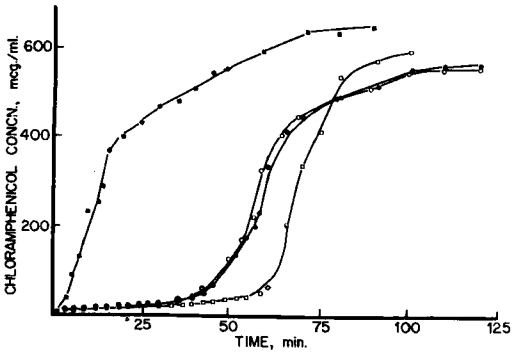

Fig. 6—Dissolution rates of chloramphenicol capsules in simulated gastric fluid T.S. Key: ■, Capsule A; □, Capsule B; ○, Capsule C; ●, Capsule D.

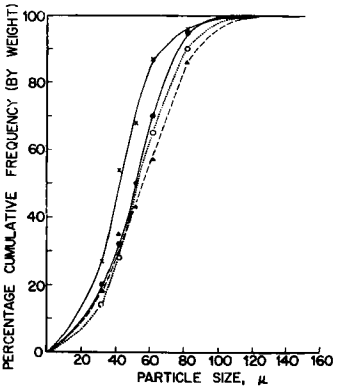

Fig. 7—Plot showing the particle-size distribution of chloramphenicol in Capsule A, ●; Capsule B, △; Capsule C, ×; Capsule D, ○.

TABLE III—COMPARATIVE PERMEATION OF CHLORAMPHENICOL FROM THE CAPSULES THROUGH THE EVERTED SAC (CUMULATIVE AMOUNTS IN mcg.)

Time, min.	Capsule A	B	C	D
5	82	38	50	81
15	183	122	152	177
25	412	308	323	294
35	573	523	515	414
45	862	667	707	557
60	1,052	896	1,030	709
75	1,464	1,189	1,422	889
90	1,752	1,560	1,750	1,057
105	1,976	1,873	2,046	1,211
120	2,340	2,414	2,376	1,414

TABLE IV—COMPARATIVE PERMEATION OF CHLORAMPHENICOL FROM THE CAPSULES THROUGH THE EVERTED SAC[a]

Time, min.	Capsule A	B	C	D
5	49	54	50	106
15	193	165	157	274
25	408	297	358	494
35	656	468	552	752
45	978	676	789	1,013
60	1,333	949	1,112	1,237
75	1,788	1,191	1,452	1,507
90	2,284	1,445	1,764	1,952
105	2,781	1,722	2,082	2,292
120	3,375	1,983		2,791
125			2,694	

[a] Solution of capsule contents filtered. (Cumulative amounts in mcg.)

phenicol from Capsule D. Since the total area present for absorption in the human intestine is very much larger, the quantity of the additives in the capsule may be too small to significantly affect the absorption in man.

GENERAL DISCUSSION

When a drug is administered in a solid particulate state, it is not unreasonable to assume that the absorption of the drug from the gastrointestinal media follows the sequence:

$$\underset{\text{drug}}{\text{solid particulate}} \rightarrow \underset{\text{drug}}{\text{dissolved}} \rightarrow \underset{\text{drug}}{\text{absorbed}}$$

If the intrinsic solubility of the drug is high, the permeation rate through the gastrointestinal barrier is assumed to be the rate-determining step in the absorption sequence. On the other hand, when a drug has a relatively low solubility, the build-up of an effective concentration at the absorption site will probably govern the rate of absorption.

Although the scheme outlined above has been shown to be true in many cases, it appears to be inadequate to describe the absorption of drugs from dosage forms, particularly from tablets and capsules.

It is proposed that a modified version such as:

$$\underset{\text{(tablet, capsule)}}{\text{drug in dosage form}} \xrightarrow{\text{deaggregation}} \underset{\text{solid state}}{\text{drug in particulate}} \xrightarrow{\text{dissolution}} \text{drug in solution} \xrightarrow{\text{absorption}} \underset{\text{drug}}{\text{absorbed}}$$

is a more realistic way of describing the absorption sequence.

Evidence gathered in the study emphasizes the importance of pharmaceutical factors in controlling the availability of chloramphenicol from the capsules for gastric absorption. As the solubility of chloramphenicol is approximately 5 mg./ml. at 37°, it would appear that the drug would saturate the gastrointestinal media relatively fast if it were released rapidly from the capsule. The higher and earlier chloramphenicol plasma level seen in humans receiving Capsule A correlates well with its faster rates of deaggregation and dissolution. The results of the study show that different chloramphenicol products containing analytically the same quantity of chloramphenicol are not equivalent in terms of drug release or drug availability.

The study also suggests *in vitro* methods of defining and establishing factors which control the absorption of a drug from dosage forms, particularly where a rapid plasma level is desirable. By determining the deaggregation and dissolution rates and using the everted sac technique to gauge any binding of the drug, one is able to define more clearly and obtain a better correlation of drug release and availability.

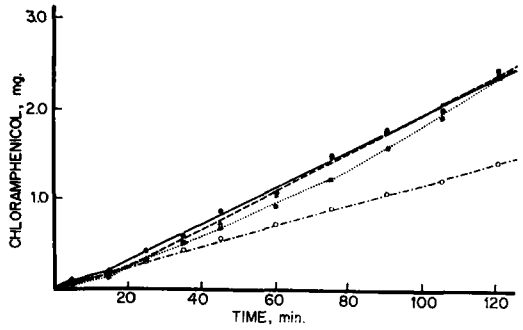

Fig. 8—Permeation rates of chloramphenicol from capsules through the everted sac. Solutions not filtered. Key: ●, Capsule A; ■, Capsule B; △, Capsule C; ○, Capsule D.

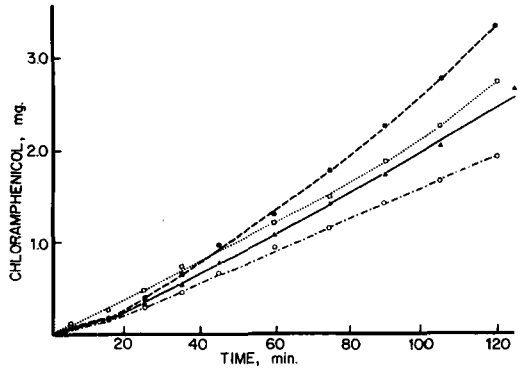

Fig. 9—Permeation rates of chloramphenicol through the everted sac, of filtered solutions from capsules. Key: ●, Capsule A; ○, Capsule B; ▲, Capsule C; □, Capsule D.

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Keyphrases

Chloramphenicol capsules
Release, availability—chloramphenicol capsules
Deaggregation rates, capsules—turbidity measurements
Dissolution, capsules—*in vitro*
Intestinal sac, everted—drug permeation

Significance of Salicylic Acid Sublimation in Stability Testing of Aspirin-Containing Solids

By A. Y. GORE*, K. B. NAIK, D. O. KILDSIG, G. E. PECK, V. F. SMOLEN, and G. S. BANKER

The salicylic acid content, formed from the decomposition of aspirin, was found to be an unreliable basis for judging the stability of aspirin tablets. Under conditions of accelerated stability testing, the loss of salicylic acid from the system by sublimation can incur appreciable errors in the direction of overestimating aspirin stability. Since aspirin was not detected to sublime under these same conditions, its residual content is an improved indication of its stability. A method for its simultaneous determination with salicylic acid is presented.

SALICYLIC ACID was observed to be lost from aspirin tablets undergoing accelerated stability testing in this laboratory. This is illustrated in Fig. 1 where deposits, analytically identified as salicylic acid, are shown on the surface of aspirin tablets coated with a cellulosic film.

Considering that salicylic acid is a hydrolytic decomposition product of aspirin and sublimation is a commercial method for its purification (1), its volatilization under the elevated temperature and humidity conditions employed in accelerated stability testing could be anticipated. However, this phenomenon apparently has not been previously studied. An earlier investigation (2) either overlooked it or treated it as being unappreciable.

A more than negligible loss of the salicylic acid formed from the decomposition of aspirin would preclude the common practice of analytically determining changes in salicylic acid content in solid dosage forms of aspirin as a measure of degrading aspirin. The salicylic acid method could obviously underestimate the extent of decomposition of aspirin and therefore provide

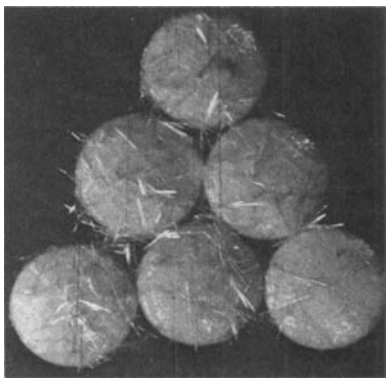

Fig. 1—Aspirin tablets coated with a cellulosic film and stored at 81.2% relative humidity and 50° for 98 days. The crystalline deposits on the surface were identified as salicylic acid formed from the decomposition of aspirin.

Received May 29, 1968, from the Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907

Accepted for publication July 18, 1968.

Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

This research was supported by a grant from the Robertshaw Chemical Co., Phoenix, Ariz.

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Keyphrases

Chloramphenicol capsules
Release, availability—chloramphenicol capsules
Deaggregation rates, capsules—turbidity measurements
Dissolution, capsules—*in vitro*
Intestinal sac, everted—drug permeation

Significance of Salicylic Acid Sublimation in Stability Testing of Aspirin-Containing Solids

By A. Y. GORE*, K. B. NAIK, D. O. KILDSIG, G. E. PECK, V. F. SMOLEN, and G. S. BANKER

The salicylic acid content, formed from the decomposition of aspirin, was found to be an unreliable basis for judging the stability of aspirin tablets. Under conditions of accelerated stability testing, the loss of salicylic acid from the system by sublimation can incur appreciable errors in the direction of overestimating aspirin stability. Since aspirin was not detected to sublime under these same conditions, its residual content is an improved indication of its stability. A method for its simultaneous determination with salicylic acid is presented.

SALICYLIC ACID was observed to be lost from aspirin tablets undergoing accelerated stability testing in this laboratory. This is illustrated in Fig. 1 where deposits, analytically identified as salicylic acid, are shown on the surface of aspirin tablets coated with a cellulosic film.

Considering that salicylic acid is a hydrolytic decomposition product of aspirin and sublimation is a commercial method for its purification (1), its volatilization under the elevated temperature and humidity conditions employed in accelerated stability testing could be anticipated. However, this phenomenon apparently has not been previously studied. An earlier investigation (2) either overlooked it or treated it as being unappreciable.

A more than negligible loss of the salicylic acid formed from the decomposition of aspirin would preclude the common practice of analytically determining changes in salicylic acid content in solid dosage forms of aspirin as a measure of degrading aspirin. The salicylic acid method could obviously underestimate the extent of decomposition of aspirin and therefore provide

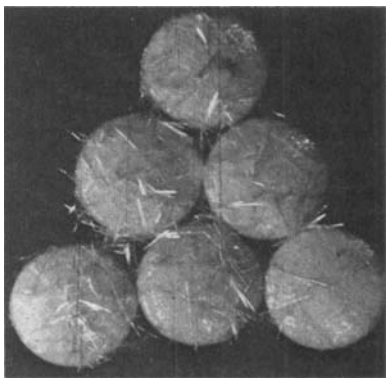

Fig. 1—Aspirin tablets coated with a cellulosic film and stored at 81.2% relative humidity and 50° for 98 days. The crystalline deposits on the surface were identified as salicylic acid formed from the decomposition of aspirin.

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false confidence in the stability of the tested products.

The purpose of the present study was to determine whether the loss of salicylic acid by sublimation could introduce an appreciable error into stability tests based on its content within aspirin-containing solids. To achieve this objective it was first necessary to develop a method of gauging aspirin stability in solids which would be unaffected by any loss of salicylic acid.

MATERIALS AND METHODS

Reagents—All reagents were of analytical grade with the exception of aspirin USP. The aspirin used in the determination of Beer's law plots and spectral absorbance curves was purified by four successive recrystallizations. Each recrystallization involved dissolving 25 g. of aspirin in 25 ml. of anhydrous ether, filtration, addition of 20 ml. of petroleum ether, cooling to 0°, collection of crystals, washing with petroleum ether, and final drying in air. The purified crystals were stored in a vacuum desiccator. The recrystallized aspirin was found to be 100% pure as judged from the USP titration method for aspirin.

Determination of Salicylic Acid and Aspirin Sublimation—Samples of salicylic acid, aspirin, or mixtures of salicylic acid and aspirin were placed on the pan of a Cahn model RG electrobalance. The samples were maintained at constant temperatures of 40, 50, and 70 ± 1°, by placing the weighing chamber assembly within an oven. The initial sample weights were the same at each temperature studied. The powdered samples were spread evenly onto the balance pan so as to cover the entire pan thereby exposing a constant surface area. The loss of weight of the samples was continuously followed as a function of time with the aid of a Sargent SR recorder used in conjunction with the Cahn balance.

Development of a Simultaneous Assay Method for Aspirin and Salicylic Acid—A UV spectrophotometric method of analysis employing a pH 7.4 Clark and Lubs buffer as the solvent was developed. In comparison with the use of spectral grade chloroform as the solvent, this method provided improved precision. The method was adopted from Edward's study of aspirin hydrolysis (3). The pH of the buffer was chosen as 7.4. This value is sufficiently above the acidic pK's of salicylic acid and aspirin (3.49 and 2.97, respectively) that slight variations in the pH of the medium would not introduce an error into the determinations as a consequence of the differential absorption of ionized and unionized species (3). Furthermore, the slightly alkaline medium affords relatively rapid solution of solid aspirin and salicylic acid samples. The dissolution must be rapid in order to minimize aspirin hydrolysis during sample preparation and analysis. Edwards (3) has reported the apparent first-order hydrolysis of aspirin at 17° to be independent of pH in the range of 4–8.

In order to estimate the magnitude of the error resulting from aspirin hydrolysis in the pH 7.4 buffer prior to spectrophotometrically reading the samples, the hydrolysis rates were determined at temperatures ranging from 17.2 to 30.2 ± 0.1°. The aspirin hydrolysis was continuously followed with time using a Beckman DB spectrophotometer in conjunction with a Sargent SR recorder. The temperature of the reaction mixture was controlled by the use of a jacketed sample compartment through which water of the desired temperature was circulated. The reactions were followed for 1-hr. periods by recording the change in absorbance at 296.5 mμ (3). The results are summarized in Table I and by the Arrhenius plot of the apparent first-order rate constants in Fig. 2. The energy of activation was found to be 17.060 kcal./mole. This value is in fair agreement with the value of 17.40 kcal./mole reported by Garrett (4) for aspirin hydrolysis at pH 5.05 in 0.5% ethanol solution.

Table I—Rate Constants for the Hydrolysis of Aspirin in pH 7.40 Buffer Solution

Temp., °C.	k (Rate Constant), Day^{-1}	$\bar{k}$ (Average Rate Constant)
17.2	i, 0.08958 ii, 0.09786	0.09372
21.3	i, 0.1418 ii, 0.1595	0.1506
25.5	i, 0.1914 ii, 0.2221	0.2067
30.2	i, 0.3434 ii, 0.3425	0.3429

In accordance with the hydrolysis results, a delay of 13 min., at 25.5°, between the time of sample preparation and reading on the spectrophotometer would cause an error of approximately 1%. The actual error was reduced to below 0.1% by maintaining the solutions below 15°, usually at 0°, and reading them within 5–10 min. of their preparation.

The selection of wavelengths for the spectrophotometric determination of aspirin and salicylic acid was made on the basis of the spectral absorption curves shown in Fig. 3. These curves were obtained using a Bausch and Lomb Spectronic 505 spectrophotometer and the pH 7.4 buffer as the solvent. Based on these curves in Fig. 3, wave-

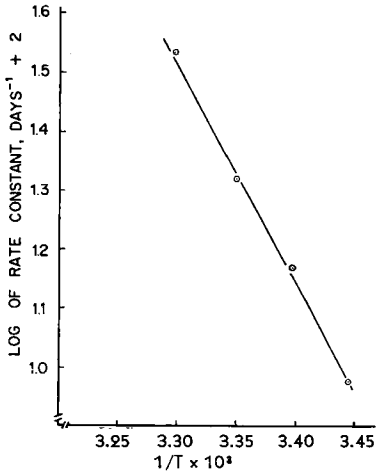

Fig. 2—Arrhenius plot for aspirin hydrolysis in pH 7.40 buffer solutions.

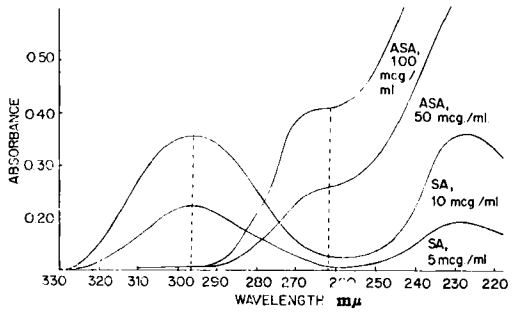

Fig. 3—Wavelength-absorbance spectra for aspirin and salicylic acid in pH 7.40 buffer solution.

lengths of 296.5 and 262 mμ were selected to be routinely employed for the assay. It may be noted that at 296.5 mμ aspirin absorbance is independent of concentration. The small absorbance noted in this region for aspirin may be merely due to the base line of the instrument. The absorbance of a mixture of aspirin and salicylic acid at 296.5 mμ is therefore attributable to that of salicylic acid plus a constant independent of the amount of aspirin present. The data pertinent to the construction of the required Beer's law calibration curves is presented in Table II. Variations from

TABLE II—CONSTANTS FOR ASPIRIN AND SALICYLIC ACID ULTRAVIOLET SPECTROPHOTOMETRIC ASSAY

Drug	Concentration Range, Mcg./ml.	Absorptivity —Abs./mg./ml.— 262 mμ	296.5 mμ
Aspirin	0–160	3.2	0
Salicylic acid	0–10	3.3	—
	0–30	—	26.0

the means of triplicate absorbance values were found to be within 0.002 absorbance units. A Beckman DU-2 spectrophotometer was employed. In order to correct for any variations in instrument response the apparently constant, concentration independent, absorbance of aspirin at 296.5 mμ was determined each experimental day. At 262 mμ the calibration curve for salicylic acid deviates from Beer's law below a concentration of 2 mcg./ml. Absorbance values of 0.006–0.007 were commonly observed below this concentration. When this condition arose, a known amount of salicylic acid was added to the sample solution to raise its concentration to approximately 6 mcg./ml. and therefore into a region where Beer's law is followed. Raising the concentration of salicylic acid in this manner allows the absorbance of aspirin at this wavelength to be more precisely determined.

Assay Procedure—A solid sample was comminuted and 10–50 mg. of the powder was dissolved in 25 ml. of pH 7.4 buffer maintained at 0°. Following filtration, 3.0 ml. of the solution was diluted to 50 ml. and the absorbance determined as described earlier. The entire procedure was completed within a period of 5–10 min. The absorbance measured at the 2 wavelengths and the constant absorbance for aspirin at 296.5 allowed the concentrations of aspirin and salicylic acid to be readily calculated (5).

Preparation and Stability Testing of Aspirin Tablets—Aspirin tablets containing 10% starch and 3% talc, having a total weight of 624 ± 5.71 mg. (*SD*) were prepared using a 1.11 cm. (7/16 in.) diameter punch. Tablets having a thickness of 0.088 cm. (0.2236 in.) ± 2.09 × 10^{-3} and a hardness of 32.95 ± 1.27 lb. were produced. Prior to stability testing the tablets were stored in a vacuum desiccator. For stability testing the tablets were placed on a fiberglass screen within a desiccator containing a saturated potassium chloride solution which maintained the humidity at 81.29% relative humidity (74.20 mm. Hg) at 50°. The desiccator was placed within an oven maintained at 50 ± 1°. At selected time intervals four tablets were removed for analysis. Under these test conditions, no moisture condensation was observed in the storage desiccators.

RESULTS

Verity and Error in the Method of Analysis—The developed assay procedure was applied to solutions of known salicylic acid and aspirin concentration. The agreement between known and determined concentrations of aspirin and salicylic acid was exact within the number of figures significant in the experiment. The data are presented in Table III.

The assay procedure was developed for the determination of aspirin in solids. In this application an error of 0.003 absorbance units at 262 mμ can contribute an error of approximately 1% in the determination of the aspirin content of a solid consisting of 80% aspirin using a 50-mg. aliquot for analysis. A single analysis of a 9:1 powdered mixture of aspirin and salicylic acid revealed an error of 1.09% and 1.52% in its aspirin and salicylic acid content, respectively. These results are shown in Table IV.

TABLE III—VERIFICATION OF THE ASSAY OF ASPIRIN IN MIXTURES OF SALICYLIC ACID AND ASPIRIN

Buffered Solution, pH 7.40	Known Concentration, mcg./ml.	Absorbance, 262 mμ	296.5 mμ	Concentration Determined, mcg./ml.
Salicylic acid (SA)	10	0.034	0.260	10
Aspirin (ASA)	104	0.336	0.012	104
Mixture equal vol. SA and ASA	5(SA) 52(ASA)	0.184	0.138	5(SA) 52(ASA)

TABLE IV—MASS BALANCE AND RESULTS OF ASSAYING A KNOWN 9:1 MIXTURE OF ASPIRIN AND SALICYLIC ACID BEFORE AND AFTER STORAGE FOR 12 HR. AT 70°C.

	Zero Time	After Storage at 70°C. for 12 hr.
Actual weight of sample[a]	55.35	51.90[b]
Assayed aspirin content	50.42	50.60
Assayed salicylic acid content	4.69	1.33
Total sample weight on basis of assay	55.11	51.93
Aspirin, %	91.09	91.42[b]
Salicylic acid, %	8.48	2.40[b]

[a] Weights are in mg. determined with a Cahn model RG electrobalance. [b] The loss of sample weight was 3.35 mg. Based on the assay results 3.18 mg. is attributable to salicylic acid and a weight gain of 0.18 mg. occurring for aspirin.

TABLE V—SUBLIMATION RATES OF SALICYLIC ACID AT 40, 50, AND 70°C.

Temp., °C.	Rate of Sublimation, mg./hr.
40° ± 0.05	0.026
50° ± 0.20	0.062
70° ± 0.20	0.372

Sublimation of Salicylic Acid and Aspirin—Sublimation rates of salicylic acid were studied at 40, 50, and 70°. The rates, under conditions of constant exposed surface area, were independent of time for the 12-hr. period in which they were recorded. The values are presented in Table V. An Arrhenius type plot of the apparent zero-order sublimation rates is presented in Fig. 4. The slope of the curve in Fig. 4 is likely predominately determined by the enthalpy of sublimation of salicylic acid. The observed rates of sublimation may be expected to directly depend upon the area

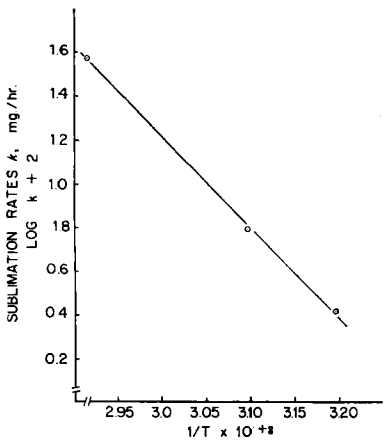

Fig. 4—Arrhenius type plot for sublimation of salicylic acid.

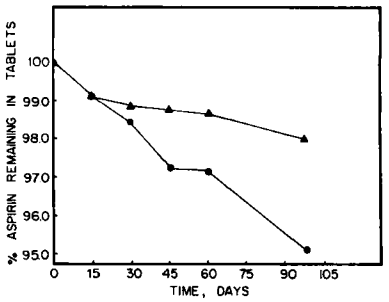

Fig. 5—Hydrolysis of aspirin in tablets at 50° and 81.20% relative humidity (74.20 mm. Hg water vapor pressure). Key: ▲, calculated on basis of salicylic acid content; ●, calculated on basis of aspirin content.

through which the mass transfer occurred. These results therefore are not intended to be quantitatively indicative of sublimative loss of salicylic acid during stability testing, but merely substantiate that such loss can occur even at moderately elevated temperatures.

A similar sublimation study of purified aspirin revealed no significant loss of weight up to 70°. The sensitivity of the Cahn electrobalance is 1×10^{-4} mg. It was therefore concluded that aspirin does not appreciably sublime under the conditions of the experiment.

The observed lack of sublimation of aspirin and appreciable loss of salicylic acid was further confirmed in mixtures. The loss of weight of a mixture of 9 parts aspirin to 1 part salicylic acid was followed at 70° for 12 hr. The mixture was assayed for aspirin and salicylic acid before and after storage. The results presented in Table IV indicate that only salicylic acid was lost from the sample.

If the 9:1 mixture of aspirin and salicylic acid had been a solid dosage form of aspirin, which had decomposed to the extent of 10%, a serious underestimation of its extent of decomposition would occur if it was based upon salicylic acid content rather than residual aspirin content. On the basis of residual aspirin content, following 12 hr. storage, the extent of decomposition would be recorded as 8.58%.[1] Under similar conditions the extent of decomposition based on salicylic acid content would be determined as only 3.12%.[1]

Sublimation of Salicylic Acid from Aspirin Tablets Undergoing Stability Testing—The average percentages of original aspirin content, present at zero time, remaining in tablets stored at 81.2% relative humidity and 50° are presented in Fig. 5. The upper plot is based on the salicylic acid content of the tablets. The lower curve is based upon determinations of the aspirin content of the tablets. The disparity between the two curves is noted to increase with time. The vertical difference between the two curves represents the percent error involved in assuming the salicylic acid content of the tablets is representative of the extent of aspirin decomposition. This same vertical distance between the curves also represents the salicylic acid lost by sublimation from the tablets. The upper curve in Fig. 5 appears to possess some tendency to approach constancy. This observation could

[1] These values are the result of a single determination.

TABLE VI—COMPARISON OF ASPIRIN TABLET STABILITY TESTING RESULTS AT 50°C. AND 81.2% RELATIVE HUMIDITY, BASED ON THE DETERMINATION OF ASPIRIN AND SALICYLIC ACID CONTENT OF THE TABLETS[a]

Time, Days	Aspirin Content Based on Analysis of Aspirin, %	Aspirin Content Based on Analysis of Salicylic Acid, %	Error Due to Sublimation of Salicylic Acid, %
0	100	100	0
15	99.1 ± 0.038	99.1 ± 0.028	0
30	98.4 ± 0.042	98.8 ± 0.215	0.4
45	97.2 ± 0.123	98.7 ± 0.075	1.5
60	97.1 ± 0.178	98.6 ± 0.023	1.5
98	95.0 ± 0.288	97.9 ± 0.311	2.9

[a] Each value is the average of 4 determinations recorded with ±1 standard deviation.

be interpreted as a trend toward equilibrium within the tablet (2). Judging from the lower curve, no such trend toward equilibrium is apparent. The data pertaining to Fig. 5 is summarized in Table VI.

SUMMARY AND CONCLUSIONS

Previously reported studies of the stability of aspirin-containing solids have utilized the salicylic acid content of the solids as a measure of aspirin decomposition. The underestimation in the extent of decomposition of the aspirin which can result from such a practice is clearly demonstrated by the results of the present work. The error can be expected to become increasingly serious with storage time and the severity of the temperature and humidity conditions under which the solids are stored. This error can be circumvented by employing a method of determining decomposed aspirin which is independent of the loss of salicylic acid from the solid due to sublimation. Since aspirin was found not to sublime, the determination of its residual content in a solid is a valid means of gauging the stability of the formulation. A method of analysis for aspirin with an accuracy of at least 1.5% was developed for application to testing of aspirin stability in solid dosage forms.

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Keyphrases

Aspirin stability—salicylic acid sublimation
Salicylic acid sublimation—aspirin tablets
Temperature effect—aspirin tablets stability
UV spectrophotometry—analysis

Stability of Cyanocobalamin in Film-Coated Multivitamin Tablets

By JAMES T. JACOB, ROBERT J. NESSEL, and JACK BLODINGER

Multivitamin tablets containing each of two commercially available protected forms of cyanocobalamin showed considerable loss of vitamin B_{12} after exposure to methanol vapor for 1 month at room temperature. Other solvents commonly used in film coating such as acetone, *n*-butanol, butyl acetate, isopropanol, and methylene chloride did not affect the stability of vitamin B_{12}. Tablets containing vitamins B_1, B_{12}, ascorbic acid, and niacinamide, alone and in combination with one another after exposure to methanol vapor at room temperature showed considerable loss of vitamin B_{12} only in presence of ascorbic acid and/or niacinamide.

THERE ARE SEVERAL reports in the literature on the stability of cyanocobalamin (vitamin B_{12}) in liquid multivitamin preparations. Decomposition products of other vitamins, pH, heat, and light can in most cases contribute to the degradation of vitamin B_{12}. Feller and Macek (1) reported that decomposition of vitamin B_{12} occurs at elevated temperatures in the presence

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of thiamine decomposition products. Doerge, *et al.* (2) found that a thiol-containing degradation product of thiamine hydrochloride may be responsible for the losses of vitamin B_{12} potency during storage. Bartilucci *et al.* (3) showed that in liquid preparations, dehydroascorbic acid, the first decomposition product of ascorbic acid, caused greater instability of vitamin B_{12} than ascorbic acid.

Cyanocobalamin has been found to be stabilized in solid dosage forms, such as multivitamin tablets by the incorporation of the vitamin in the sugar coating, thus separating it from the other active components. However, with the increasing use of film-coating, this method is not applicable. For this reason, the use of a protected form of the vitamin is a necessity. Two such forms commercially available are cyanocobalamin dispersed in gelatin[1] and cyanocobalamin adsorbed on resin.[2]

The process of film-coating involves spraying a solution of a film-forming polymer in single or mixed organic solvents onto the moving bed of tablets or within an air suspension column. The solvents are then removed by intermittent drying of the tablets. The total time for film-coating varies, depending upon the type of film-forming polymers and solvents used, thickness of the film, number of tablets coated, and the process used. It is quite possible for the solvent(s) to penetrate into the tablet core during the long exposure. Holl *et al.* (4) reported that tablets that were film-coated with a solution of cellulose acetate phthalate, even after considerable drying, retained residual amounts of acetone. Other solvents commonly used in film-coating are methanol, methylene chloride, chloroform, butanol, and isopropanol.

Vitamin B_{12} dispersed in gelatin has shown very good stability in uncoated multivitamin tablets. However, because of an unexpected loss in potency observed when incorporated in tablets that were film-coated, this investigation was undertaken. The purpose of this investigation was to determine whether the solvents used in film-coating may be the cause of the aforementioned instability. For this study, solvent effects were determined on two commercially available protected cyanocobalamin forms.

EXPERIMENTAL

Materials—Acetone,[3] ascorbic acid, USP, butyl acetate,[4] *n*-butyl alcohol,[3] chloroform,[3] 1% cyanocobalamin dispersed in gelatin, 1% cyanocobalamin adsorbed on resin, isopropyl alcohol,[3] anhydrous methanol,[3] methylene chloride,[3] niacinamide, USP, thiamine mononitrate, USP.

TABLE I—COMPOSITION OF MULTIVITAMIN TABLETS

Ingredient	Potency per Tablet
Vitamin A acetate	5000 units
Vitamin D_2	400 units
Thiamine mononitrate, USP	1.4 mg.
Riboflavin, USP	2.0 mg.
Ascorbic acid[a]	70.0 mg.
Niacinamide[a]	22.0 mg.
d-Calcium pantothenate, USP	10.0 mg.
Cyanocobalamin[b]	4.0 mcg.
Vitamin E succinate	15 units
Folic acid	0.1 mg.
Dicalcium phosphate	20.0 mg.
Microcrystalline cellulose[c]	30.0 mg.
Magnesium stearate	2.5 mg.
Stearic acid	5.0 mg.
Talc	10.0 mg.

[a] In the form of Merpress—registered trademark for niacinamide ascorbate, Merck & Co., Inc. Rahway, N. J. [b] Added as 1% cyanocobalamin dispersed in gelatin; second batch contained the same quantity in the form of 1% cyanocobalamin adsorbed on resin. [c] Marketed as Avicel by American Viscose Co., Marcus Hook, Pa.

Preparation of Tablets—Two 1,000-tablet batches of a full formula multivitamin were prepared by a direct compression method using 0.79-cm. ($^5/_{16}$-in.) dies and standard concave punches on a Manesty single-punch machine. One batch contained vitamin B_{12} in the form of cyanocobalamin dispersed in gelatin and the other, in the form of cyanocobalamin adsorbed on resin. The quantitative formula is shown in Table I.

Immersion in Solvents—Ten tablets were immersed for exactly 60 sec. in a beaker containing each of the following solvents: acetone, butyl acetate, *n*-butyl alcohol, chloroform, isopropyl alcohol, methyl alcohol and methylene chloride. The solvent was then poured off, the tablets were air dried for 30 sec. and one-half of the tablets were stored at room temperature and the rest at 50°. After 1 month, the tablets were removed and were assayed for vitamin B_{12} content.

Exposure to Solvent Vapors—Twenty multivitamin tablets were placed in loosely capped 20-ml. amber bottles, and stored in desiccators containing each of the solvents used in the immersion test at room temperature. After storage for 1 month, the tablets were assayed for vitamin B_{12} content.

Solubility Test—The dissolution of cyanocobalamin from the two protected forms in methanol was determined as follows: 200 mg. of each powder was weighed into 125-ml. amber colored conical flasks. Fifty milliliters of anhydrous methanol was added and the flasks shaken on a mechanical shaker[5] for 48 hr. at room temperature. The solution was filtered and the absorbance of the filtrate was measured at 361 mμ on a Cary II spectrophotometer using methanol as the reference solvent. The quantity of dissolved cyanocobalamin was calculated from a standard curve of cyanocobalamin solution in methanol.

Assay Methods—The tablets were assayed for vitamin contents using standard assay methods.

[1] Marketed as Stabicote by Merck & Co., Inc., Rahway, N. J.
[2] Marketed as Stablets by Chas. Pfizer & Co., Inc., New York, N. Y.
[3] Reagent Grade, Merck & Co., Inc., Rahway, N. J.
[4] Matheson, Coleman and Bell, Norwood, Ohio.
[5] Burrel Wrist Action shaker, Pittsburgh, Pa.

TABLE II—% LOSS OF VITAMIN B_{12} FROM MULTIVITAMIN TABLETS EXPOSED TO VARIOUS SOLVENT VAPORS FOR 1 MONTH AT ROOM TEMPERATURE

Batch No.	Acetone	*n*-Butanol	Butyl Acetate	Chloroform	Isopropanol	Methylene Chloride	Methanol
1[a]	No loss	No loss	1.2	No loss	No loss	No loss	24.5
2[b]	No loss	0.5	5.7	0.5	No loss	No loss	26.5

[a] Contained B_{12} as 1% cyanocobalamin dispersed in gelatin. [b] Contained B_{12} as 1% cyanocobalamin adsorbed on resin.

RESULTS AND DISCUSSION

Immersion in Solvents—The time for immersion of the tablets in the solvents was limited to 1 min. so that the tablets would not be soaked with the solvents. The assay results showed little or no loss of vitamin B_{12} potency after storage either at room temperature or at 50° for 1 month. The concentration of the solvents retained in the tablets may not have been quite sufficient to cause any stability problem for vitamin B_{12}.

Exposure to Solvent Vapors—The tablets stored in desiccators containing *n*-butyl alcohol, butyl acetate, chloroform, isopropanol, and methylene chloride showed no loss of vitamin B_{12} potency, whereas tablets similarly exposed to methanol vapors showed considerable loss (Table II). Because of this loss, the dissolution of cyanocobalamin in methanol from its two protected forms was determined in order to consider the possibility of extraction of vitamin B_{12} from the protective matrix into the tablet. Twenty-five percent of vitamin B_{12} dissolved in methanol from the gelatin-dispersed form, while 55% of the vitamin leached from the resin adsorbate. Exposure of the two protected forms of vitamin B_{12} *per se* to methanol vapor for 1 month showed no loss from the gelatin-dispersed form, whereas 20% loss was observed from the resin adsorbate. This loss of vitamin B_{12} may be attributed to the fact that methanol vapor easily penetrated the resin adsorbate, which remained as a powder, whereas the gelatin-dispersed powder solidified in the bottle thereby preventing any penetration of methanol vapor. Since in aqueous solutions, degradation products of ascorbic acid, especially dehydroascorbic acid, are known to adversely affect the stability of vitamin B_{12} (4), it was necessary to determine whether this or any other vitamin in presence of methanol vapor may have any deleterious effect on the stability of vitamin B_{12}. For this purpose, tablets were prepared with ascorbic acid, niacinamide, thiamine mononitrate, cyanocobalamin dispersed in gelatin, and cyanocobalamin adsorbed on resin, alone and in combination with one another. The potency of each vitamin and the excipient quantities were maintained at the same level as in the multivitamin tablets except that spray-dried lactose was used to adjust the weight to that of the multivitamin tablets. All tablets were formulated so as to be of same size, weight, and hardness. These tablets were then exposed to methanol vapor by storage in desiccators for 1 month at room temperature.

TABLE III—COMPOSITION[a] AND POTENCY LOSS OF INDIVIDUAL VITAMINS FROM TABLETS EXPOSED TO METHANOL VAPOR FOR 1 MONTH AT ROOM TEMPERATURE

Batch No.	Potency of Each Tablet: B_{12}, mcg.	B_1, mg.	Ascorbic Acid, mg.	Niacinamide, mg.	% Loss
3	4.0[b]	—	—	—	0.0
4	4.0[c]	—	—	—	5.0
5	—	1.4	—	—	18.0
6	—	—	70.0	—	4.4
7	—	—	—	22.0	0.0

[a] In addition to the vitamin, each tablet contained the same quantities of excipients as in the multivitamin tablet (Table I) and sufficient quantity of spray-dried lactose to adjust the weight to that of the multivitamin tablet. [b] As 1% cyanocobalamin dispersed in gelatin. [c] As 1% cyanocobalamin adsorbed on resin.

A study of Table III shows that methanol vapor did not cause an appreciable decrease in the potencies of protected vitamin B_{12} (both forms), ascorbic acid, and niacinamide when these vitamins were present alone in the tablets. The effect was more pronounced on vitamin B_1 tablets which lost 18% of the vitamin (see Batch No. 5). Table IV shows that there was only a small loss of ascorbic acid, but its presence in the tablet caused considerable loss of vitamin B_{12}. There was also a significant loss of vitamin B_1 from the tablets (see Batch Nos. 10 and 11). Table V shows that tablets containing vitamins B_1-B_{12}-niacinamide combinations had little or no loss of either vitamin B_1 or niacinamide in presence of methanol vapor. Only one batch (Batch No. 16) lost a substantial amount of vitamin B_{12}. The large loss of vitamin B_1 did not cause any stability problem for vitamin B_{12} when these two vitamins were present together in the tablet (see Batch Nos. 12 and 13). Since this study was limited only to the assay of vitamin contents after exposure to methanol vapors, no attempt was made to isolate the degradative products of the vitamins, especially those of ascorbic

TABLE IV—COMPOSITION[a] AND POTENCY LOSS OF VITAMINS FROM B_1-B_{12}-ASCORBIC ACID TABLETS EXPOSED TO METHANOL VAPOR FOR 1 MONTH AT ROOM TEMPERATURE

Batch No.	Potency of Each Tablet: B_1, mg.	B_{12}, mcg.	Ascorbic Acid, mg.	% Loss: B_1	B_{12}	Ascorbic Acid
8	—	4.0[b]	70.0	—	14.5	3.7
9	—	4.0[c]	70.0	—	19.4	8.1
10	1.4	4.0[b]	70.0	17.6	7.3	5.2
11	1.4	4.0[c]	70.0	13.0	17.0	3.0

[a] In addition to the vitamins, each tablet contained the same amount of excipients as in the multivitamin tablets (Table I) and sufficient quantity of spray-dried lactose to adjust the weight to that of the multivitamin tablet. [b] As 1% cyanocobalamin dispersed in gelatin. [c] As 1% cyanocobalamin adsorbed on resin.

TABLE V—COMPOSITION[a] AND POTENCY LOSS OF VITAMINS FROM B_1-B_{12}-NIACINAMIDE TABLETS EXPOSED TO METHANOL VAPOR FOR 1 MONTH AT ROOM TEMPERATURE

Batch No.	Potency of Each Tablet			% Loss		
	B_1, mg.	B_{12}, mcg.	Niacinamide, mg.	B_1	B_{12}	Niacinamide
12	1.4	4.0[b]	—	12.6	2.4	—
13	1.4	4.0[c]	—	14.2	4.9	—
14	1.4	—	22.0	0.0	—	0.0
15	1.4	4.0[b]	22.0	1.1	4.9	3.8
16	1.4	4.0[c]	22.0	1.1	14.6	4.0

[a] In addition to the vitamin, each tablet contained the same quantities of excipients as in the multivitamin tablet (Table I) and sufficient quantity of spray-dried lactose to adjust the weight to that of the multivitamin tablet. [b] As 1% cyanocobalamin dispersed in gelatin. [c] As 1% cyanocobalamin adsorbed on resin.

acid and vitamin B_1. No quantitative relationship between loss of vitamin B_{12} and loss of other vitamins could be observed. Nevertheless, it is quite apparent that methanol vapor when allowed to penetrate multivitamin tablets can cause loss of potency of individual vitamins, especially vitamins B_1 and B_{12}. It can be postulated that methanol vapor causes degradation of either ascorbic acid or vitamin B_1, or both, the degradation products of which in turn influence stability of vitamin B_{12}. This fact should be taken into consideration when methanol is used as a solvent in film coating of multivitamin tablets.

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Keyphrases

Cyanocobalamin (vitamin B_{12})—stability

Film-coated multivitamin tablets—vitamin B_{12} stability

Methanol vapor effect—vitamins B_1, B_{12} stability

Ascorbic acid effect—vitamins B_1, B_{12} stability

Fluorometric Determination of Norgestrel and Structurally Related Steroids

By L. F. CULLEN, J. G. RUTGERS, P. A. LUCCHESI, and G. J. PAPARIELLO

A sensitive procedure, based on sulfuric acid-induced fluorescence, has been developed for the analysis of norgestrel (*dl*-13-ethyl-17α-ethinyl-17-hydroxygon-4-en-3-one) in tablets of low dosage, *i.e.*, 15–75 mcg. Optimum conditions for fluorescence have been established and the fluorescent properties of structurally related steroids studied to determine the selectivity of the reaction and mechanism of fluorescence formation. The reaction is specific for Δ^4-3-ketosteroids which have both a 17β-hydroxyl and 17α-alkyl or alkyne substitution and $\Delta^{1,3,5(10)}$-triene-3-ol steroids. A two-step mechanism is tentatively explained on the basis of the effects of temperature, time, initial acid concentration, and subsequent dilution with water on fluorogen development. Specificity of the method with respect to the analysis of intact norgestrel in the presence of its photochemical and thermal degradation products was demonstrated by comparison to quantitative thin-layer chromatography values. This procedure has been automated to permit unit dose analysis. This automated procedure is capable of analyzing 15 samples per hour with a relative standard deviation of ±1.4 percent at the 50-mcg. level.

THE SYNTHESIS of a new, extremely potent progestational steroid, norgestrel (*dl*-13-ethyl-17α-ethinyl-17-hydroxygon-4-en-3-one), and subsequent formulation of this steroid in tablets of low dosage, *i.e.*, 15–75 mcg. per tablet, had presented a challenging analytical problem. A sensitive, accurate, and rapid procedure was desired for content uniformity testing of the dosage form.

Steroids structurally related to norgestrel, having a characteristic Δ^4-3-keto group in the

Received May 22, 1968, from Wyeth Laboratories, Philadelphia, PA 19101

Accepted for publication August 5, 1968.

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Batch No.	Potency of Each Tablet			% Loss		
	B_1, mg.	B_{12}, mcg.	Niacinamide, mg.	B_1	B_{12}	Niacinamide
12	1.4	4.0[b]	—	12.6	2.4	—
13	1.4	4.0[c]	—	14.2	4.9	—
14	1.4	—	22.0	0.0	—	0.0
15	1.4	4.0[b]	22.0	1.1	4.9	3.8
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A-ring, have been quantitatively assayed utilizing the well-known 2,4-dinitrophenylhydrazine (1) and isonicotinic acid hydrazide (2) colorimetric methods, but these procedures lack the necessary sensitivity for single-tablet analyses at the dosage levels considered. A direct UV spectrophotometric procedure as well as the UV measurement of the salicyloyl hydrazone (3) or thiosemicarbazone (4) derivatives of the α,β-unsaturated carbonyl group did not exhibit the sensitivity or specificity required in the presence of tablet excipients for an accurate analytical method. Quantitative paper chromatographic (5) and thin-layer chromatographic (6) methods have been applied with some success but are limited, due to the lack of precision and accuracy characteristic of such procedures. Gas chromatographic (7) and polarographic (8, 9) procedures are available for several Δ^4-3-ketosteroids, but these procedures did not lend themselves to the analysis of large numbers of samples.

A fluorometric approach was considered to have the greatest potential and was studied further. Since the appearance of fluorescence in acid or base solution is a property of almost all steroids, there have appeared countless papers relating it to the identification, characterization, and analysis of steroids (10–13). However, there have been relatively few reports of fluorometric methods devised for Δ^4-3-ketosteroids at the sensitivity level required for unit-to-unit variation of formulations.

Bush and Sandberg (14) noted that paper chromatograms sprayed with sodium hydroxide developed an orange-yellow fluorescence specific for Δ^4-3-ketosteroids under UV irradiation. Subsequently, Abelson and Bondy (15) found that potassium *tert*-butoxide could produce the alkaline fluorescence reaction in solution in a satisfactory manner. However, the procedure requires meticulous care for satisfactory results, and it is insufficiently rapid and precise for the analysis of single tablets. Smith and Foell (5) demonstrated that Δ^4-3-ketones and $\Delta^{1,4}$-3-ketones, on paper chromatograms, exhibit a fluorescence under UV light when sprayed with isonicotinic acid hydrazide in acid alcohol. In attempting to apply this reaction to the problem at hand it was found that the procedure lacked the necessary sensitivity.

A sulfuric acid-induced fluorescence procedure used in the USP as an identification reaction for hydrocortisone (16) suggested a promising approach. A study of this reaction led to the development of a sulfuric acid fluorogenic reagent of definite and critical proportions which provided a stable and intense fluorescence suitable for quantitative measurement of microgram amounts of norgestrel in tablets of low dosage.

Although this procedure can be carried out by conventional techniques, adaptation of the method to an automated system offered advantages of accuracy, precision, and economy. Optimum conditions for the acid-induced fluorescence were established prior to the development of the automatic analyzer system. The results of a systematic study of the excitation and emission properties of norgestrel after treatment with varying concentrations of sulfuric acid and the influence of time and temperature are described. Fluorescent properties of structurally related steroids were studied to determine the selectivity of this reaction, possible applications of the procedure, and the mechanism of fluorescence formation.

EXPERIMENTAL

Apparatus—Spectral and optimum condition measurements were made on a Farrand spectrofluorometer with a fused quartz cell (10 × 10 × 45 mm. internal dimensions), using four 20-mμ slits and an RCA 1-P-28 photomultiplier tube. The excitation and fluorescent spectra are obtained on a Honeywell Electronik 15-strip-chart recorder.

A standard Technicon automatic analyzer (Auto-Analyzer) system consisting of the following modules: (*a*) Solid-prep sampler, programmed at 15 samples/hr. (*b*) Proportioning pump. (*c*) Continuous filter; speed 2. (*d*) Fluorometer; equipped with fluorescent lamp (Turner 110-853) and Corning filters: primary, No. 7-59 and secondary, No. 3-68. (*e*) Linear recorder. (*f*) Transmission lines and proportioning tubes carrying alcohol, sulfuric acid, and water consist of Acidflex tubing, while those carrying air are Tygon.

Reagents and Solutions—Eighty-five percent SDA No. 30 alcohol (ethanol–methanol, 10:1) in water (v/v); 85% sulfuric acid in water (v/v); all steroids used were of the purest grade available commercially.

Standard Norgestrel Solutions—Prepare a 0.5 mg./ml. solution of norgestrel in 85% denatured alcohol. The standard calibration curve is derived from a series of diluted solutions ranging in concentration from 15–85 mcg./ml.

Thin-Layer Chromatographic System—MN Silica Gel G-HR/UV precoated glass plates (10 × 20 cm. with a 250-μ adsorbent layer), obtainable from Analtech, Inc., were activated by heating at 105° for 0.5 hr. before use. The chromatogram was initially developed with a chloroform system. When the solvent front ascended approximately 15 cm. from the origin, the plate was removed, air-dried, and redeveloped with a benzene–chloroform (8:2 v/v) solvent system. Visualization was effected by: (*a*) inspecting the plate under short-wave (253.7 mμ) and long-wave (366.0 mμ) UV light; (*b*) spraying the plate with 85% ethanolic sulfuric acid and heating at approximately 105° for 5 min.

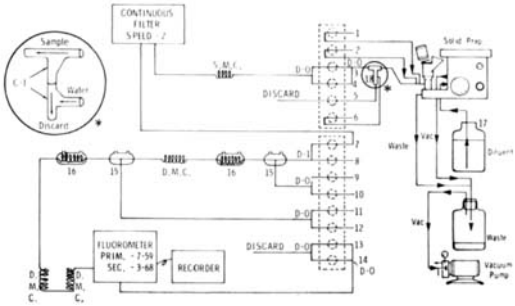


Fig. 1—Flow diagram for norgestrel. Key: 1, *0.8 ml./min. air;* 2, *5.52 ml./min. 85% SDA No. 30 alcohol;* 3,4, *2.03 ml./min. sample;* 5, *0.6 ml./min.;* 6, *0.42 ml./min. water;* 7, *2.76 ml./min. sample;* 8, *2.90 ml./min. air;* 9,10, *2.76 ml./min. 85% H_2SO_4;* 11,12, *2.03 ml./min. water;* 13,14, *2.03 ml./min. debubbler;* 15, *A-7 water-cooled TEE;* 16, *water-cooled mixing coil (No. 114–209);* 17, *reservoir 85% SDA No. 30 alcohol;* 18, *decantation trap.*

Automated Methodology—Figure 1 is the flow diagram indicating the automated equipment arrangement for this analytical procedure. In operation, four standards of the appropriate levels of norgestrel are placed on the sample plate, followed by samples of the intact or disintegrated tablets. Standardization is repeated at the end of a series of 15 samples in order to minimize the effects of reagent changes and instrumental variations. Samples are introduced into the Solid-prep sampler, programmed at 15 samples/hr., and homogenized in the 85% denatured alcohol. During the period of homogenization the sample is diluted and dissolution of norgestrel occurs. A small portion of the the mixture is aspirated into the flow system and is automatically filtered to remove insolubles. To prevent mechanical obstructions from the insoluble excipient materials at the tubing connections, a decantation trap between the Solid-prep unit and the proportioning pump removes the majority of the solid matter. The residual insolubles are removed by the continuous filter module. After passage through the filter module, the filtrate is segmented with air and then combined with a stream of the 85% sulfuric acid. The sample stream is immediately cooled in a water-jacketed coil and passed through a double mixing coil (D.M.C.) to permit a thorough mixing. After the stream is diluted with water, again cooled, and passed through double mixing coils, the resultant fluorometric development is measured. Calculations are made using corresponding fluorescent intensities of standards and solid dosage formulation samples.

RESULTS AND DISCUSSION

Optimum Conditions—Denatured alcohol was considered the ideal solvent for dissolution of the norgestrel during the automated extraction procedure because of the steroid's solubility in this solvent and the relatively low cost of large volumes of this solvent. In investigating the influence of alcohol concentration on acid-induced fluorescence, it was demonstrated that a significant level of alcohol could be tolerated without a fluorescent quenching effect. With a final sulfuric acid to alcohol volume ratio of 2:1 and a reaction time of 5 min., a series of acid concentrations from 25 to 100% (v/v) was evaluated. Each sample was measured against an acid–alcohol reagent blank of the appropriate composition. As illustrated in Fig. 2, maximum response is produced with 85% sulfuric acid.

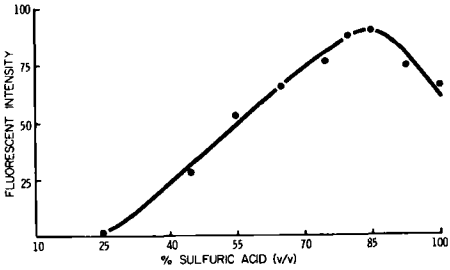


Fig. 2—Effect of initial sulfuric acid concentration on fluorescent intensity.

Considering the maximum volume of alcohol that could be aspirated into the automated flow system from the Solid-prep unit and the volume of 85% sulfuric acid required for sufficient fluorescence, it was realized that the final solution could not satisfy the essential flow dynamics of the fluorometer flow cell. As a result a series of experiments was performed to determine the effect of dilutions with water, after initial sulfuric acid-induced fluorescence. Diluting with water did not quench fluorescence, while the blank fluorescence produced by the reagents was significantly reduced. Thus, the flow difficulties could be overcome without a critical loss in sensitivity by the addition of water into the system, after initial fluorogen development with 85% sulfuric acid.

The excitation and fluorescence spectra for norgestrel after the addition of 85% sulfuric acid and on subsequent dilution with water appear in Fig. 3. Dilution with 3 ml. of water after fluorogen development corresponds to a final sulfuric acid concentration of 38%. This represents the optimum water dilution level with respect to both flow characteristics of the automated procedure and sensitivity requirements. Any further addition of water simply produced a proportional decrease in intensity. Furthermore, the addition of water to the system produced an unexpected shift in the excitation

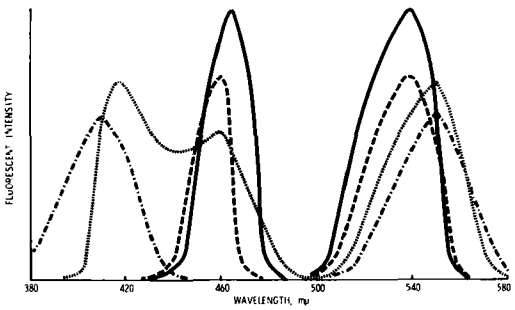


Fig. 3—Excitation and emission spectra of norgestrel. Key: ——, initial addition of 85% H_2SO_4; ---, dilution with 1 ml. H_2O; · · ·, dilution with 3 ml. H_2O; -·-, dilution with 5 ml. H_2O.

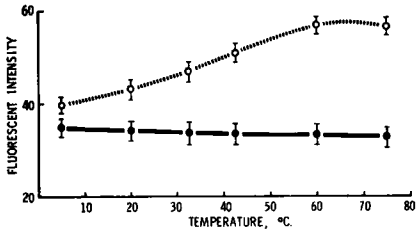


Fig. 4—Relationship between fluorescence development and temperature. Key: ---, initial addition of 85% H_2SO_4; ——, dilution with 3 ml. H_2O.

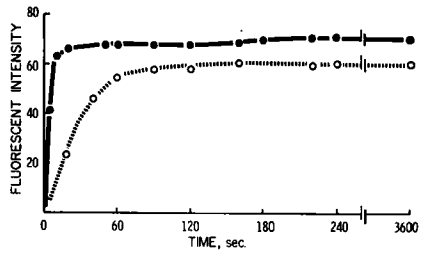


Fig. 5—Relationship between fluorescence development and time. Key: ---, initial addition of 85% H_2SO_4; ——, dilution with 3 ml. H_2O.

maxima. This unique spectral characteristic of norgestrel at the 38% sulfuric acid level suggested a basis for a more selective assay in the presence of other structurally related 3-keto-17-hydroxyl or 3,17-diketo-type steroids which have reported excitation maxima in sulfuric acid in the 460–470-mμ range (17). This will be discussed in greater detail in the section on specificity.

The fluorescence after the addition of 85% sulfuric acid and the fluorescence of the water-diluted final solution as a function of temperature and time were measured. Fluorometric measurements were made against reagent blanks treated in a similar manner. In the temperature study the intensities were recorded after exactly 5 min. The time study was run at 25°. From Figs. 4 and 5 it is evident that the fluorescence development with 85% sulfuric acid is more sensitive to time and temperature than the fluorescence of the water-diluted, final solution.

Data obtained under the above experimental conditions indicate that the maximum fluorescent yield of the initial fluorogen formation occurs above 60°. However, with respect to automated analysis adaptation, the small loss in sensitivity by maintaining the entire system at ambient room temperature overbalances the physical difficulties introduced by proportioning 85% sulfuric acid at 60°.

Specificity—In order to determine the selectivity of the reaction and the effect of group substitution on the molecule, a number of structurally related steroids were studied. The fluorescent intensities with respect to norgestrel were obtained under the exact analytical conditions described under *Automated Methodology*. The remarkable selectivity of the fluorogenic reaction is illustrated in Table I. Of the various steroids examined, only the particular group of Δ^4-3-ketosteroids which have both a 17β-hydroxyl and 17α-alkyl or alkyne substitution and $\Delta^{1,3,5(10)}$-triene-3-ol steroids produced a significant level of fluorescence. Δ^4-Steroids, $\Delta^{1,4}$-diene-3-ketosteroids, and $\Delta^{2,5(10)}$-diene-3-methoxy-steroids did not fluoresce under the prescribed conditions.

From these experiments it would appear that fluorescent intensity is strongly affected by the alkyl group at the 13-position. The replacement of a methyl group for an ethyl group as illustrated by pairs 19-norethisterone and norgestrel, nortestosterone and 13-ethyl-17β-hydroxygon-4-ene-3-one produced a decrease in the fluorescence to approximately one-half the ethyl substituted compounds.

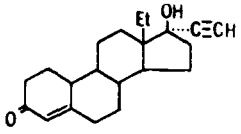
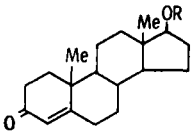
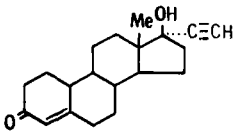
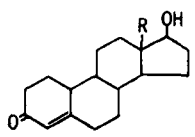
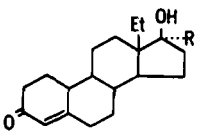
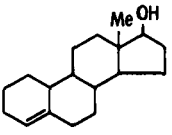
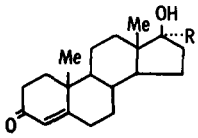
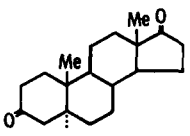
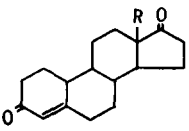
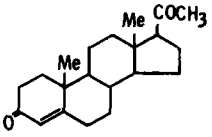
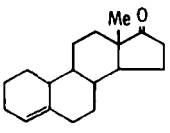
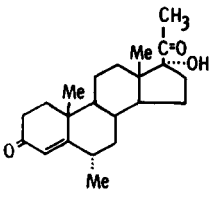
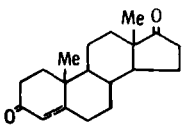
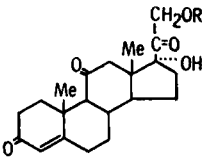
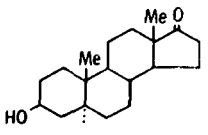
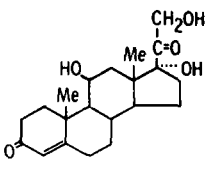
The replacement of a hydroxyl group by a ketone group at the 17-position in steroids which differ only in this single structural characteristic almost completely removes its capability to develop fluorescence.

In order to study the chemistry of the fluorogen formation and the influence of the 13-position alkyl group on the spectral characteristics, the effect of dilution with water upon the excitation spectra of all steroids which produced a fluorescent yield in the automated system were observed. As illustrated in Table II, progressive dilution produced unique sequences of change in the excitation spectra of Δ^4-3-ketosteroids with ethinyl and hydroxyl groups at the 17-position, quite comparable with those observed for norgestrel. It should be noted that further dilution beyond 31% sulfuric acid did not cause a further shift in the excitation spectra. However, Δ^4-3-ketosteroids which have a hydroxyl, keto, acetyl, ketol or combination ketol and hydroxyl functional groups at the 17-position demonstrated excitation wavelength maxima at the characteristic 460–470-mμ range and are not affected by the final acid concentration. The absence of an excitation maximum shift for steroids IV, X, and XI emphasizes the necessity for the presence of both the ethinyl group at the 17-position and α,β-unsaturated ketone in the A-ring. Furthermore, emission spectra and the data presented in Table II demonstrate that the increased intensity produced by an ethyl substitution at the 13-position is not a result of an alteration in the characteristic spectral curves of compounds containing this group, for example, compare steroid pairs I and II.

Chemistry of Reaction—Evaluation of the data suggested that under the experimental conditions used in this study the sulfuric acid-induced fluorescence of norgestrel is a two-step reaction. Eighty-five percent sulfuric acid developed a fluorescent intensity fourfold greater than 38% sulfuric acid (Fig. 2). However, after initial fluorogen formation with 85% sulfuric acid and subsequent dilution to a final sulfuric acid concentration of 38%, the relative fluorescent intensities were essentially equivalent and the unique hypsochromic shift in excitation maxima from 463 to 418 mμ was introduced (Fig. 3). The time and temperature effects (see Figs. 4 and 5) on the two-step fluorogen formation, *i.e.*, (*a*) addition of 85% sulfuric acid, (*b*) dilution with water, further support a two-stage reaction mechanism.

Fluorescence spectral data and thin-layer chromatography (TLC) data were collected to determine whether or not denatured alcohol participated in the fluorogen formation. Samples with and without alcohol showed similar excitation and fluorescence spectra with equivalent responses when treated with

TABLE I—FLUORESCENCE OF NORGESTREL AND STRUCTURALLY RELATED STEROIDS

Structure	Compound	Relative Fluorescent Intensity	Structure	Compound	Relative Fluorescent Intensity
	*Norgestrel*	100		Testosterone (R = H) Testosterone acetate (R = acetyl)	Nil Nil
	19-Norethisterone	58		13-Ethyl-17β-hydroxygon-4-ene-3-one (R = ethyl) Nortestosterone (R = methyl)	13 6
	17α-Chloroethinyl-13-ethyl-17-hydroxygon-4-ene-3-one (R = chloroethinyl) 13, 17α-Diethyl-17-hydroxygon-4-ene-3-one (R = ethyl)	23 21		Estrenol	Nil
	Ethisterone (R = ethinyl) Methyltestosterone (R = methyl)	28 166		5α-Androstane-3, 17-dione	Nil
	13-Ethylgon-4-ene-3, 17-dione (R = ethyl) Norandrostenedione (R = methyl)	3 Nil		Pogesterone	Nil
	Estrenone	Nil		Medroxyprogesterone	Nil
	Δ⁴-Androstene-3,-17-dione	Nil		Cortisone (R = H) Cortisone Acetate (R = acetyl)	Nil Nil
	Epiandrosterone	Nil		Hydrocortisone	5

(*Continued on next page.*)

TABLE I—(*Continued.*)

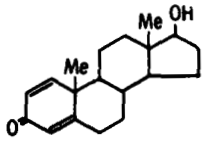
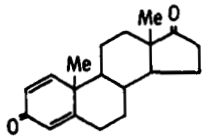
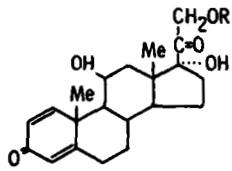
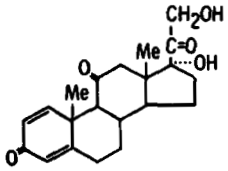
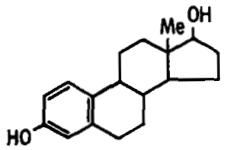
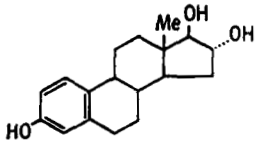
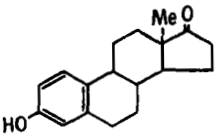
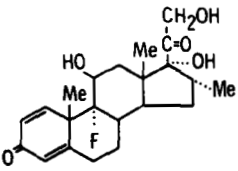
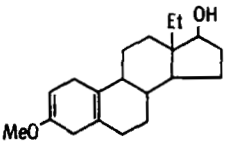
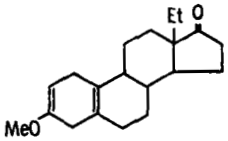
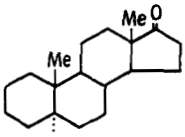
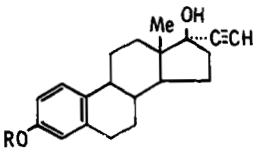
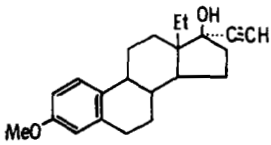
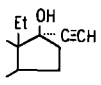
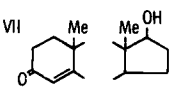
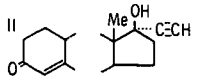
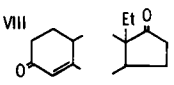
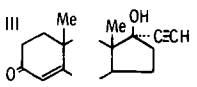
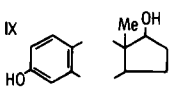
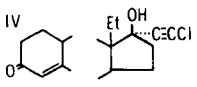
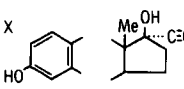
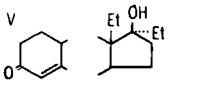
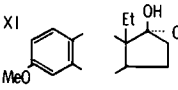
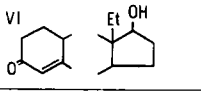
Structure	Compound	Relative Fluorescent Intensity
	$\Delta^{1,4}$-Androstadiene-17β-ol-3-one	Nil
	$\Delta^{1,4}$-Androstadiene-3,17-dione	Nil
	Prednisolone (R = H) Prednisolone acetate (R = acetyl)	Nil Nil
	Prednisone	Nil
	β-Estradiol	46
	Estriol	Nil
	Estrone	8
	Dexamethasone	Nil
	13-Ethyl-3-methoxygona-2,5(10)-diene-17β-ol	Nil
	13-Ethyl-3-methoxygona-2,5(10)-diene-17-one	Nil
	5α-Androstane-17-one	Nil
	Ethinyl estradiol (R = H) *mestranol* (R = methyl)	660 880
	13-Ethyl-17-ethinyl-3-methoxygona-1,3,5(10)-triene-17β-ol	1,020

TABLE II—EFFECT OF FINAL SULFURIC ACID CONCENTRATION ON EXCITATION PROPERTIES

Structure		Final Sulfuric Acid Concn.			Structure		Final Sulfuric Acid Concn.		
A-Ring	D-Ring	49%	38%	31%	A-Ring	D-Ring	49%	38%	31%
I		460	418	408	VII		465	465	465
II		460	412	406	VIII		462	462	462
III		465	425	415	IX		460	460	460
IV		461	461	460	X		535	535	535
V		473	472	465	XI		545	543	540
VI		470	470	465					

concentrated or diluted sulfuric acid, indicating that alcohol did not enter into the reactions. TLC confirmed these data by finding the same number of fluorophors with identical R_f values in the alcohol and alcohol-free systems.

Any hypothesis concerning the reaction mechanism must consider the reversibility or irreversibility of the individual reaction step in the fluorogen formation. TLC demonstrated that the products formed by the action of 85% sulfuric acid on norgestrel did not revert back to the intact steroid on progressive dilution of the reaction mixture with water, thereby indicating induced irreversible changes. The multiplicity of products obtained chromatographically from extracts of the water-diluted reaction mixtures was additional evidence of this irreversible reaction.

Spectral data demonstrated that in the second stage of fluorogen formation the intensities of the principal excitation maxima of norgestrel at 408 and 460 mμ are dependent on acid concentration and are reversible.

From these findings it would appear that the initial steroid-acid interaction is an extremely complex phenomenon, involving the possibility of dehydration, sulfonation, or isomerization. The second part of the mechanism is an immediate reversible reaction which suggests an ionic species interaction.

With the aid of preparative TLC, studies are now being carried out to isolate the products formed during the reaction. Based upon the identification of these fluorogens, a definitive mechanism will be presented.

Suitability as Stability Method—Specificity of the method for analysis of intact norgestrel in the presence of its photochemical and thermal degradation products was demonstrated by comparing analytical values of intentionally degraded samples to those obtained by quantitative TLC. In the TLC procedure, the norgestrel was separated from its degradation products on a TLC plate. The intact norgestrel was removed from the plate, eluted, then assayed to obtain a quantitative value. Examples of the data obtained are summarized in Table III. Since there is good agreement between the values by the two techniques it is concluded that the fluorometric procedure is stability indicating.

Linearity—An actual working curve which was obtained by analyzing standards of known concentration is demonstrated in Fig. 6. A linear relationship exists for fluorescent intensity and norgestrel concentration in the range which was studied. A recording of the steady state, during which time an 85-mcg. standard was continuously sampled, is also shown.

Precision—Repeatability of the procedure was demonstrated by assaying equal aliquots of norgestrel standard solutions. Relative standard deviations of 0.9, 1.4, 2.2, and 3.1% were determined at the 75, 50, 25, and 15-mcg. levels, respectively,

TABLE III—COMPARISON OF FLUOROMETRIC AND QUANTITATIVE TLC ANALYSIS OF INTENTIONALLY DEGRADED NORGESTREL SAMPLES

Sample Treatment	% Initial	
	Fluorometric Method	TLC Method
Suspension of the norgestrel heated in 0.1 *N* NaOH for 2 hr. at 70°	68	60
Norgestrel stored at 210° for 30 min.	90	87
Norgestrel exposed to UV light for 6 months	79	80
Synthetic mixture containing 0.5 mg. of norgestrel in 100-mg. tablet excipients stored at 100° for 49 days	42	50

Table IV—Precision of Fluorometric Method on Analysis of Composite Tablet Sample

Norgestrel, mcg. per Equivalent Tablet Weight	
49.4	50.3
50.9	50.8
48.7	50.6
49.7	49.2
50.3	50.9
51.3	49.1
50.6	49.6
50.3	50.6
49.1	49.4
50.6	49.4
Average: 50.0 mcg.	
Relative standard deviation: ±1.5%	

by performing 30 replicate assays. A series of 20 replicate assays was run on a composite sample of a tablet formulation at the 50-mcg. norgestrel per tablet level. The resulting precision data in Table IV indicate a relative standard deviation of 1.5%.

Sensitivity—The flow diagram of Fig. 1 is applicable to the analysis of samples which initially contain 15 to 200 mcg. of norgestrel. At the 15-mcg. level, the final concentration of norgestrel is approximately 0.06 mcg./ml. However, the method has been shown to be sensitive enough so that it can easily detect less than 0.02 mcg. of norgestrel/ml. Consequently, the method offers greater sensitivity than previously reported methods for the Δ^4-3-ketosteroids.

Accuracy—The accuracy of the method was determined by adding 25.0 mcg. of norgestrel to a tablet excipient mixture and measuring the percentage recovery. The data collected indicate recoveries of 97 to 103% of the theoretical amount present with a relative standard deviation of 2.1%.

A further evaluation of the accuracy of the procedure was ascertained by studying the effect of common tablet excipients and lubricants on fluorescent development. The inactive components evaluated were lactose, sucrose, magnesium stearate, stearic acid, microcrystalline cellulose (Avicel), alginic acid, an ion-exchange resin (Amberlite), methylcellulose (Methocel), and starch. No interference was experienced from these materials.

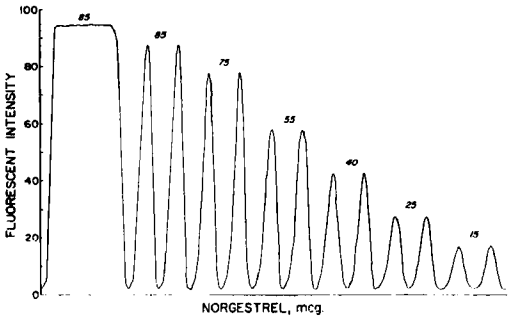


Fig. 6—Typical recording of automated system for norgestrel standards at the rate of 15 samples/hr.

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Keyphrases

Norgestrel tablets—analysis
Fluorometry—analysis
TLC—separation
UV light—spot visualization
Automated procedure—norgestrel analysis
Diagram—automated system

Determination of Molecular Binding in Aqueous Solution from Optical Activity Measurements

Interaction of Tryptophan with Alkylxanthines

By MASAHIRO NAKANO* and TAKERU HIGUCHI†

Although the feasibility of using ORD for determining stability constants of molecular complexes in organic solvent has already been shown, the present investigation appears to be the first attempt to apply it to an aqueous system. Measurements at 330 mμ show distinct changes in the apparent optical activity of the zwitter ionic form of the amino acid when alkylxanthines were added to its solutions. These readings when analyzed by the iterative procedure previously developed for PMR data have yielded self-consistent stability constant values. Results are given for interaction with caffeine, theophylline, 8-methoxycaffeine, 1,3-dimethyluracil, and sarcosine anhydride. From measurements at several temperatures the heat of binding of tryptophan with caffeine was shown to be −4.0 kcal./mole and that with theophylline to be −4.2 kcal./mole. These results appear to be consistent with results obtained previously on related systems.

ALTHOUGH IT HAS BEEN demonstrated that optical activity measurements can be effectively used to determine stability constants of bound species in essentially nonpolar solvents (1), no attempt appears to have been made to utilize these readings for estimating binding tendencies in aqueous solutions. In nonpolar solutions the binding behaviors arise primarily through hydrogen bonding of the interactants. In aqueous systems the source or sources of the associative tendencies of organic species are still not clearly elucidated (2). The present investigation was concerned with evaluation of the optical activity approach to estimation of stability constants in water based on tryptophan as the primary optically active species. It would appear reasonable to expect that intimate bonding of another organic species on the amino acid will so affect the electrical field polarization in the immediate vicinity of the asymmetric center that significant change in the ORD spectrum may result.

That tryptophan binds with xanthines has not been definitely reported previously but interactions of bovine serum albumin with xanthine molecules have been reported by Guttman *et al.* (3–5). A modification in pharmacological effect of ergotamine tartrate in the presence of caffeine has been suggested to be due to complex formation (6). Since these molecules have an indole group in them, the interactions are likely to be similar to that of tryptophan with caffeine.

Received May 22, 1968, from the †Department of Pharmaceutical Chemistry, School of Pharmacy, University of Kansas, Lawrence, KS 66044 and School of Pharmacy, University of Wisconsin, Madison, WI 53706

Accepted for publication July 16, 1968.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

This study was supported in part by a grant from the Institute of General Medical Sciences, NIH, Bethesda, Md. (GM-05830).

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EXPERIMENTAL

Reagents—L-Tryptophan, as supplied by Sankyo Kasei (Tokyo), was recrystallized from ethanol–water before use. Caffeine and theophylline were of USP and NF grades, respectively. L-Tyrosine and L-phenylalanine were from Nutritional Biochemicals and were used without further purification. 8-Methoxycaffeine (Eastman), 1,3-dimethyluracil (Nutritional Biochemicals), sarcosine anhydride (K&K), and tetramethylsuccinamide (C. P. Hall) were all recrystallized from methanol–carbon tetrachloride.

Instrumentation—A Cary 60 spectropolarimeter equipped with a thermostated cell compartment maintained at 25° with a 10-cm. cell was used for spectropolarimetry. Samples were equilibrated in a 25° water bath prior to use. For variable temperature measurements, a 10-cm. Cary 60 thermostatable cell connected to a constant-temperature water bath was used.

Determination of Stoichiometry—To establish the stoichiometry of the complexes under investigation the method of continuous variations (Job's method) (7) was employed. Although this method was developed especially for use with spectrophotometric measurements, it was readily modifiable for use with spectropolarimetric measurements. It was assumed that only a single complex AB_n was formed from A and B, the complexation reaction being written,

$$A + nB = AB_n \qquad \text{(Eq. 1)}$$

Solutions were prepared in which the sum of the number of moles A and B present was maintained constant at S M. These were prepared by making solutions which contained $(1 - x)S$ M of A and xS M of B. It was evident that

$$n = \frac{x}{1 - x} \qquad \text{(Eq. 2)}$$

Molar rotations[1] of A, B, and AB_n in 1-cm. pathlength cells were set equal to r_a, r_b, and r_c, respectively; the difference, R, between the measured rotation and that expected if no interaction took place was then

$$R = l[r_a(C_a - C_c) + r_b(C_b - nC_c) + r_cC_c - r_aC_a - r_bC_f]$$
$$= l(r_c - r_a - nr_b)\ C_c \qquad \text{(Eq. 3)}$$
$$= lr\ C_c$$

where l was the pathlength of the cell in the study in cm. and $C_a - C_c$, $C_b - nC_c$, and C_c, were the concentrations of species A, B, and C, respectively, at equilibrium and $r = r_c - r_a - nr_b$. When C_c was a maximum $(dC_c)/dx = 0$, then $(dR)/(dx) = 0$. Thus R passed through an extremum when C_c was at maximum. In this study total concentration, S, was kept at 3×10^{-2} M, measurements being made at 330 mμ.

Computation of Stability Constants—For the computation of stability constants from ORD measurements, a new iterative method was developed and employed. This method is analogous to that described earlier for the NMR technique (8).

If two species, A and B, interact reversibly in one-to-one stoichiometry,

$$A + B = C \qquad \text{(Eq. 4)}$$

at equilibrium,

$$K = \frac{C_c}{(C_a - C_c)(C_b - C_c)} \qquad \text{(Eq. 5)}$$

Eq. 5 can be rearranged to give

$$KC_aC_b - KC_c(C_a + C_b - C_c) = C_c \qquad \text{(Eq. 6)}$$

When $n = 1$, Eq. 3 may be written

$$R = l(r_c - r_a - r_b)C_c = lrC_c \qquad \text{(Eq. 7)}$$

then

$$C_c = \frac{R}{rl} \qquad \text{(Eq. 8)}$$

Eq. 6 and Eq. 8 give

$$\frac{C_aC_b}{R} = \frac{1}{Krl} + \frac{C_a + C_b - C_c}{rl} \qquad \text{(Eq. 9)}$$

K and r_c were computed from the difference in measured rotation, R, and the initial concentrations of A and B, C_a and C_b, as follows.

Step 1—$(C_aC_b)/R$ was plotted against $(C_c + C_b)$ and the slope of the line, slope_0 was obtained.

Step 2—C_{c1}, the first approximate values of C_c, was calculated employing slope_0 in Eq. 8, *i.e.*, $C_{c1} = \text{slope}_0 \cdot R$.

Step 3—$(C_aC_b)/R$ was plotted against $(C_a + C_b - C_{c1})$ and the slope, slope_1 was obtained.

Step 4—C_{c2} was calculated as in Step 2, *i.e.*, $C_{c2} = \text{slope}_1 \cdot R$.

Step 5—Steps 3 and 4 were repeated until the convergent slope was obtained.

Step 6—K was calculated from the slope and the intercept of the last cycle, and r_c from the final slope, K = slope/intercept, $r_c = r_a + r_b + 1/(\text{slope} \cdot l)$.

[1] Molar rotation stands for optical rotation which will be observed when 1 M solution is placed in 1-cm. pathlength cell, whereas molecular rotation is defined by $[\alpha]^{\lambda}\ M/100$, where $[\alpha]^{\lambda}$ = specific rotation and M = molecular weight.

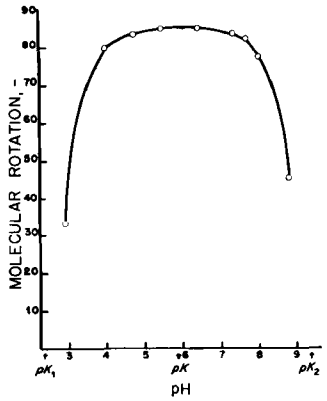

Fig. 1—pH profile of rotations of tryptophan at 330 mμ. Concentration of tryptophan = 4×10^{-3} M.

Optical rotations of tryptophan solutions with varying concentrations of amides were measured at 330 mμ. The concentration of tryptophan was kept constant at 4×10^{-3} M whereas concentrations of caffeine and theophylline were varied between 4×10^{-3} M and 32×10^{-3} M

RESULTS AND DISCUSSION

The observed pH profile of the rotatory activity of tryptophan at 330 mμ is shown in Fig. 1. The rotation was negative and constant in the neighborhood of isoelectric point (pKI), namely between pH 5 and 7. Because of the independence with respect to pH, rotation measurements for subsequent studies were made in this region. The observed optical rotatory dispersion curve for tryptophan alone and those in the presence of alkylxanthines are shown in Fig. 2. As is evident from Fig. 2 the relatively low concentrations of the added solutes produced significant changes in the ORD spectrum, the rotatory activity becoming even more strongly negative and the trough shifting toward shorter wavelength. If the observed shifts can be entirely attributed to formation of a complex, stability constants can be readily calculated from these data.

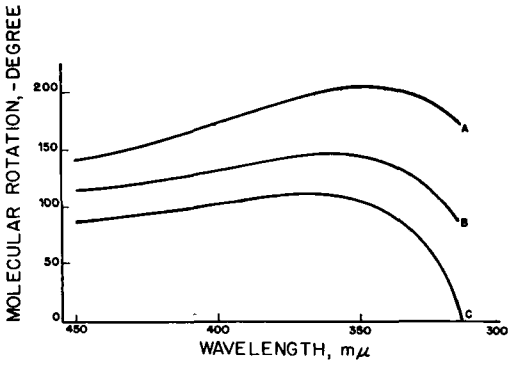

Fig. 2—Optical rotatory dispersion spectra of tryptophan in water (4×10^{-3} M). Key: A, with 3.2×10^{-2} M caffeine; B, with 3.2×10^{-2} M theophylline; C, without alkylxanthines.

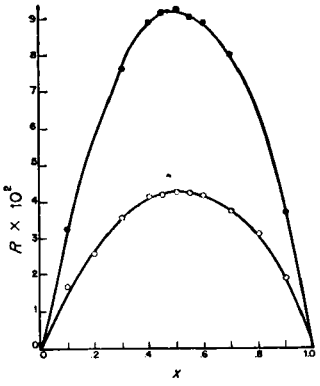

Fig. 3—The difference between the measured rotation and that predicted upon the assumption of no interaction, R, against mole fraction of tryptophan, x, measured at 330 mμ, total concentration S = 3 × 10^{-2} M. Key: ●, tryptophan-caffeine; ○, tryptophan-theophylline.

Optical rotation measurements, moreover, can be used to establish the stoichiometry of the complex species by the method of continuous variations. Plots of the difference between the measured rotation and that expected if no interaction took place, R, against x for tryptophan-caffeine and tryptophan-theophylline at 25° are shown in Fig. 3. For both interactions, $x = 0.5$ appear to correspond to the maximum values of R. The indicated stoichiometric ratio was, therefore, one-to-one.

The stability constant for the tryptophan-caffeine system calculated from ORD measurements in water at 25° by the iterative procedure was 30.1 M In Fig. 4 the initial plot required by the method is shown to illustrate the adherence of the data to the expected relationship. The stability constants for tryptophan complexes with some other amides were computed in the same manner and are listed in Table I. It is of interest to note that 8-methoxycaffeine showed a much stronger binding tendency than caffeine which has been generally considered to be among the stronger complexing agents. 1,3-Dimethyluracil was a much weaker binder and sarcosine anhydride interacted little, possibly because of its unfavorable nonplanar structure and nonaromatic character.

TABLE I—STABILITY CONSTANTS OF TRYPTOPHAN COMPLEX WITH SOME AMIDES AT 25° IN WATER

Compound	Stability Constant, M^{-1}
Caffeine	30
Theophylline	35
8-Methoxycaffeine	50
1,3-Dimethyluracil	5.8
Sarcosine anhydride	≃0
Tetramethyl succinamide	≃0

The higher complexing behavior with theophylline than with caffeine was, however, somewhat unexpected (2). Guttman *et al.* (3) had also found stronger binding tendency of theophylline to bovine serum albumin than caffeine. Since the abnormality was also observed in 8-chlorotheophylline-tryptophan system and was absent in theophylline-indole system [based on ORD studies in methanol and on phase-solubility studies (9)], it may be suspected that 7-proton of theophylline may be interacting with the zwitter ion part of tryptophan. Although the extent of the interaction with the zwitter ion part may be small, it may modify the optical rotation to a certain extent. The observed increase in binding thus may be the sum of interactions of the indole ring and the zwitter ion part of the molecule with theophylline.

The temperature effect on stability constants was studied using a Cary 60 thermostatable sample cell connected with a water bath. Logarithms of the observed stability constants plotted against the reciprocal of the absolute temperature are shown in Fig. 5. $\Delta H°$ calculated from the slope of the straight line was found to be −4.0 kcal./mole for the tryptophan-caffeine system and −4.2 kcal./mole for the tryptophan-theophylline system. $\Delta S°$ was also negative in both instances (−6.7 cal. $mole^{-1}$ and −7.0 cal. $mole^{-1}$ $deg.^{-1}$, respectively).

These values including those in Table I are in agreement with those which may be expected from earlier studies (2, 10). In general it has been shown that largely nonaromatic structures such as in the case of sarcosine anhydride bind very little. 8-

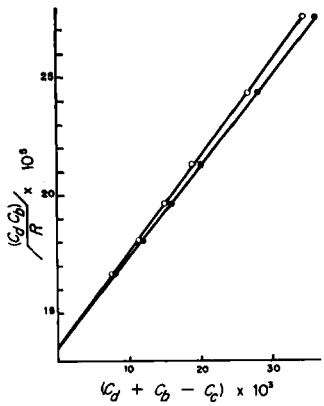

Fig. 4—Plots based on Eq. 9 for tryptophan (4 × 10^{-3} M) — caffeine (4 — 32 × 10^{-3} M) system. Key: ●, 1st approximation; ○, 3rd and final approximation.

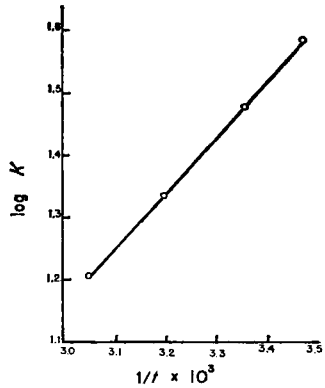

Fig. 5—Temperature dependence of stability constants in tryptophan-caffeine system in water. Tryptophan concentration = 4 × 10^{-3} M; caffeine concentration = 4 — 32 × 10^{-3} M.

Methoxycaffeine, on the other hand, appears to be one of the strongest binders that the authors have found to date.

Partition studies between water and cyclohexane–chloroform (1:1 by volume) have shown that $K = 32\ M^{-1}$ for tryptophan–theophylline complex and $K = 26\ M^{-1}$ for tryptophan–caffeine complex. Although these values are somewhat smaller than those determined by ORD method (35 and 30, respectively), the agreement appears reasonable if one considers the difference in concentrations employed (for partition work original concentration of each species was $1 \times 10^{-2}\ M$). It is believed that phase-solubility method will give only approximate K values for these systems because of the great solubility of tryptophan in water (soly. $= 6 \times 10^{-2}\ M$), yielding a rather complex overall system at saturation.

Experiments based on the ORD method have shown that caffeine interacts with L-tyrosine ($K = 13.4\ M^{-1}$ at 25° in water measured at 315 mμ with tyrosine concentration $= 8 \times 10^{-4}\ M$ and caffeine concentration $= 1 - 5 \times 10^{-2}\ M$). The extent of interaction measured in the same manner between caffeine and L-phenylalanine was much less ($K = 6.3\ M^{-1}$ at 25° in water measured at 320 mμ with phenylalanine concentration $= 3 \times 10^{-3}\ M$ and caffeine concentration $= 1 - 6 \times 10^{-2}\ M$). Preliminary experiments on optical rotatory dispersion studies of tryptophan indicated that tryptophan also interacted with pyrimidines and purines of biological importance.

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Molecular binding determinations—optical activity measurements
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Complex formation—tryptophan-alkylxanthines
Stability constants—tryptophan-alkylxanthine complexes
Optical rotatory dispersion—analysis

Differential Scanning Calorimetry

Advantages and Limitations for Absolute Purity Determinations

By N. J. DeANGELIS and G. J. PAPARIELLO

A critical evaluation of the effectiveness of absolute purity determination by differential scanning calorimetry (DSC) is presented. Several pure drugs of widely differing structure have been investigated with respect to the detection of known amounts of added impurities of all types. The accuracy of purity values obtained by the DSC technique is shown to fall off rapidly below purities of 99 mole %. Experiments are described which demonstrate that impurities present in solid solution are not detected. Independent purity determinations by phase solubility and quantitative thin-layer chromatography are shown to be in good agreement with DSC values for samples that are 99% pure or better. A technique for extending the useful range of DSC purity determination to about 95% is given.

CALORIMETRIC METHODS of absolute purity determination by measurements related to melting and freezing point depressions have been successfully applied to the analysis of organic compounds that melt without decomposition for many years. However, prior to the availability of the Perkin-Elmer differential scanning calorimeter (DSC), these methods had not been widely used in pharmaceutical laboratories. With the introduction of this type of instrumentation, there has been increased interest and application of purity measurements of this type (1).

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The theoretical justification for classical calorimetric purity measurements imposes as one of its restrictions that an equilibrium exist between the solid and liquid phases (2). Hence, in measurements of this kind it has been most common to employ heating rates of 0.1° C. per hour or less in the vicinity of the melting point of the compound. This condition assures that equilibrium is established but results in a long analysis time. Unlike the above static methods, DSC utilizes a minimum rate of temperature rise of 0.625° C. per minute. In this dynamic technique one benefits from much shorter analysis time (less than 2 hr.), but under these circumstances it cannot be assumed that true solid-liquid equilibrium is always attained. The authors feel that it is necessary to investigate this latter aspect because sufficient experimental data have not been presented in the scientific literature to indicate the incidence of this occurrence and the resulting consequences. Thus, the authors have performed a comprehensive series of experiments to evaluate the DSC technique for purity measurements and to reveal its advantages and limitations.

Among the areas considered are the following: (*a*) the detection of contaminants that are either structurally similar or structurally dissimilar to the major component, (*b*) the accuracy of DSC purity determinations for various levels of impurity content, (*c*) comparison of results with other established purity techniques, (*d*) a method for extending the useful range of DSC purity determinations.

DSC TECHNIQUE

Detailed discussions of the theory of calorimetric purity determinations have already been presented (3, 4). A brief review of the theory and the details of how it is applied to the DSC method was given by Gray (5) and Reubke and Mollica (1). In this paper, the procedure will merely be outlined.

The DSC technique centers around an analysis of the shape of the melting curve of the compound. Measurements of the amount of sample melted at several temperatures are made and their inverse is plotted against the temperature at which the individual fractions were measured. Theoretically, for systems that behave as ideal solutions this should represent a straight line since the following relationship can be derived (5):

$$T_s = T_0 - \frac{RT_0^2x_2}{\Delta H_f}\left(\frac{1}{F}\right) \qquad \text{(Eq. 1)}$$

where

T_s = instantaneous temperature of sample in °K.
T_0 = melting point of pure solvent in °K.
R = gas constant in calories/mole-degree
x_2 = mole fraction of solute
ΔH_f = heat of fusion of solvent in cal./mole
F = mole fraction melted at T_s

ΔH_f is directly related to the total area under the curve, T_0 is the corrected temperature of the peak of the melting curve, and F is determined by dividing the area under the curve up to the temperature T_s by the total area.

When the raw data are plotted one rarely obtains a linear relationship, and the area measurements must be mathematically adjusted to make them fit a straight line. In the absence of solid solutions, the reason for this behavior has been attributed to sensitivity limitations of the instrumentation which causes the early stages of the melt to go undetected (6).

Another approach has recently been presented by Driscoll, Duling, and Magnotta (7). In it, all samples are treated as though they form solid solutions and the equation of Randall and Lewis for such systems is applied (8). The authors have incorporated this procedure into a computer program which shortens the data reduction time and provides better accuracy.

EXPERIMENTAL

The Perkin-Elmer DSC-1B was used in all the experiments. A heating rate of 0.625° C. per minute was employed and all samples were run in a nitrogen atmosphere. Area measurements were made with a K & E compensating polar planimeter and all weighings were made with a Cahn gram electrobalance (precision ±0.1 mcg.). Sample sizes were typically in the range of 1–4 mg. The nature of the samples did not necessitate the use of the volatile sample pans and calculations were made according to the procedure outlined in the literature (5, 6).

When the dry mixing technique was used for sample preparation both the pure component and the contaminant were separately weighed into a tared sample pan and the weights of each were obtained by difference. This eliminated the possibility of sampling errors that could have been present if both components were premixed and a single aliquot taken. For preparation of samples which were mixed in solution, an organic solvent which did not adversely affect either component and yielded good crystalline material was employed.

Materials used for the major component were of analytical reference standard quality, *i.e.*, 99.8 mole % pure or greater as established by independent purity measurements. The methods employed were phase solubility and quantitative thin-layer chromatography. Chromatographically, the amount of impurity present was determined by visual comparison of the contaminant spot intensity with actual samples of the isolated impurities.

RESULTS AND DISCUSSION

Structurally Similar Impurities—Since the contaminants encountered in synthesized raw material are usually closely related to the major component,

TABLE I—DSC PURITY VALUES FOR STRUCTURALLY SIMILAR IMPURITIES

Pure Compound	Impurity	Actual Purity, Mole %	DSC Purity, Mole %
dl-13-Ethyl-17α-ethynyl-17-hydroxygon-4-en-3-one	*dl*-13-Ethyl-17-ethynyl-3-methoxygona-2,5(10)-dien-17β-ol	99.5	99.6
		99.2	99.2
	d-Form of pure compound	99.4	99.4
		99.0	99.1
	dl-13-Ethyl-17α-ethynyl-17-hydroxygon-5(10)-en-3-one	99.5	99.5
		99.1	99.1
7-Chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one	7-Chloro-1,3-dihydro-3-hydroxy-5-phenyl-2*H*-1,4-benzodiazepin-2-one	99.4	99.5
		99.2	99.2
	Polymorph of pure compound	99.4	99.5
		99.0	99.0
p-Ethoxyacetanilide	Acetanilide	99.5	99.4
		99.2	99.1
	p-Chloroacetanilide	99.6	99.6
		99.1	99.1
An investigational substituted carbamate	A position isomer of the pure compound	99.2	99.2
		98.8	98.9
dl-Malic acid	*l*-Form of pure compound	99.3	99.4
		98.9	99.0

the presence of this type of impurity in several different classes of compounds was investigated. Examples of position and optical isomerism, polymorphism, and common synthetic reaction contaminants were considered. These impurities were added to the drug by dry mixing and the final purity determined by the DSC method. In all cases, the purity of the starting major component was independently determined and the DSC value was in good agreement with it. The results of this study are given in Table I. The actual purity values listed in the table represent the sum of the impurities in the starting major component and the amounts of contaminant added. It is apparent that there is excellent agreement in the range considered, independent of the system investigated. This is especially significant because the nature of the impurities is such that they would be very difficult to quantitate by conventional analytical techniques.

Structurally Dissimilar Impurities—To further test the "absoluteness" of the DSC purity method, it was necessary to determine if impurities structurally different from the major component could be detected. To investigate this aspect, contaminants were dry mixed with nonrelated pure compounds and the DSC purity was determined. The results are given in Table II. With the exception of sodium chloride, lactose, and sodium acetate, the contaminants present were accurately determined.

Since the theory of calorimetric purity measurements predicts inaccuracies for systems that contain salts, the authors did not expect perfect agreement for those type samples. However, it is interesting to note that they were not detected even though present in large quantities. The behavior of lactose was not expected and indicates that either equilibrium is not rapidly established or that the two components do not form a solution. The latter point is very often neglected, but it is an important consideration since it is required that the impurity form a solution with the major component in order to be detected. It is reasonable to expect that as the major and minor component become more and more dissimilar the incidence of this effect will become more prevalent.

TABLE II—DSC PURITY VALUES FOR STRUCTURALLY DISSIMILAR IMPURITIES

Pure Compound	Impurity[a]	Actual Purity, Mole %	DSC Purity, Mole %
dl-13-Ethyl-17α-ethynyl-17-hydroxygon-4-en-3-one	7-Chloro-1,3-dihydro-3-hydroxy-5-phenyl-2*H*-1,4-benzodiazepin-2-one	99.1	99.1
p-Ethoxyacetanilide	An investigational substituted carbamate	99.3	99.3
7-Chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one	*N*-Acetyl-*p*-aminophenol	99.4	99.3
N-Acetyl-*p*-aminophenol	2-Methyl-2-*N*-propyl-1,3-propanediol dicarbamate	99.0	99.1
dl-13-Ethyl-17α-ethynyl-17-hydroxygon-4-en-3-one	Lactose	98.0	99.6
	Sodium acetate	95.0	99.6
	Sodium chloride	95.0	99.8

[a] Impurity added by dry mixing.

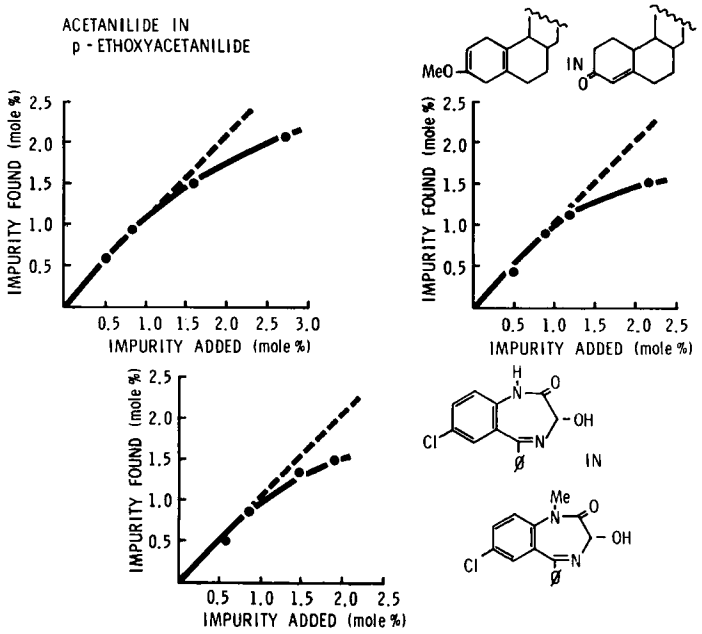

Fig. 1—Limits of accuracy. Key: —, amount of impurity as determined by DSC; - - -, actual amount of impurity present.

Accuracy Range—The range of impurity levels which can be accurately quantitated by the DSC technique (±0.1%) was also investigated by dry mixing known amounts of impurity with a pure drug. Structurally similar impurities representing 1 mole % or less of the total sample were precisely determined. In some cases, this range was increased slightly but not beyond a limit of approximately 1.5 mole %. Representative results are given in Fig. 1. Typically, the analysis of a sample containing 2 mole % impurity yielded values of near 1.5%, and approximately 2.5% for those that were actually 5% impure. It must be understood that the above results may not hold true for every system considered, because the range of applicability will primarily be determined by the ideality of the system and the rate at which equilibrium between the solid and liquid phases is attained. However, the authors have not yet encountered any system in which accurate results were obtained beyond 1.5 mole % impurity and it is indicated that DSC purity values of less than 99% are likely to be in error.

Solid Solution Effect—The use of Eq. 1 assumes the absence of solid solutions. To demonstrate the effect that their presence will have on purity measurements by the DSC method the following experiments were performed.

(*a*) Varying concentrations of different types of impurities were mixed with *p*-ethoxyacetanilide in chloroform solution. Aliquots of these solutions where pipeted into the DSC sample pan with a microliter syringe and then recrystallized rapidly by evaporation. This same procedure was used

TABLE III—EFFECT OF SOLID SOLUTIONS

		Method of Recrystallization			
		Fast Evaporation		Controlled Process	
Pure Compound	Impurity	Actual Purity, Mole %	DSC Purity, Mole %	Actual Purity, Mole %	DSC Purity, Mole %
p-Ethoxyacetanilide	*p*-Chloroacetanilide	99.6	99.9	99.5	99.5
		98.9	99.8	99.3	99.2
		98.4	99.5	98.9	98.9
	Acetanilide	99.4	99.7	99.7	99.7
		98.9	99.5	99.4	99.4
		98.4	99.3	99.0	99.1
	An investigational substituted carbamate	99.5	99.6	—	—
		99.0	99.1	—	—
		98.5	98.7	—	—
dl-13-Ethyl-17α-ethynyl-17-hydroxygon-4-en-3-one	*dl*-13-Ethyl-17-ethynyl-3-methoxygona-2,5(10)-dien-17β-ol	99.5	99.8	99.5	99.5
		99.0	99.6	99.2	99.2
		98.5	99.3	98.8	98.9
	7-Chloro-1,3-dihydro-3-hydroxy-5-phenyl-2*H*-1,4-benzodiazepin-2-one	99.6	99.7	—	—
		99.2	99.2	—	—
		98.8	99.0	—	—

TABLE IV—COMPARATIVE PURITY[a] RESULTS BY OTHER METHODS

Sample	DSC	Phase Solubility	TLC
dl-13-Ethyl-17α-ethynyl-17-hydroxygon-4-en-3-one			
1	99.4	99.3	99.2
2	99.6	99.5	99.5
3	99.8	99.6	99.7
4	99.8	100	99.8
5	99.1	99.2	99.2
An Investigational Substituted Carbamate			
Sample	DSC	NMR[b]	TLC
1	99.7	>99	>99.5
2	99.5	>99	99.5
3	98.6	98.2	98
4	97.4	95.4	95–96
5	96.5	94.4	<95

[a] All purity values in mole %. [b] Impurity not detectable by NMR if it does not exceed 1%.

for a system whose major component was *dl*-13-ethyl-17α-ethynyl-17-hydroxygon-4-en-3-one. These samples, when analyzed by the DSC method, gave poor results if the contaminants were closely related in size and structure to the major component. For impurities that were dissimilar, the agreement was equivalent to that obtained by dry mixing. The results are summarized in Table III.

The above results are not surprising, because where the compounds were closely related and then rapidly recrystallized, migration of one component into the crystal lattice of the other is likely to occur. In the resulting solid solution, the contaminant is either in part or in whole dissolved in the solid phase of the major component. Hence Eq. 1 must be modified by the addition of a distribution coefficient to take this into account. Mastrangelo and Dornte (9) have derived a method for the analysis of systems which form solid solutions but Driscoll *et al.* (7) have shown that this treatment is not applicable to the DSC technique when they actually occur. The results verify this conclusion. The reason for this behavior is most likely due to the nonequilibrium operating condition. Unfortunately, one does not know *a priori* if a solid solution exists, and this information is not obtainable from the DSC curve.

(*b*) The systems described above for which inaccurate results were obtained were recrystallized under controlled conditions to simulate actual synthetic reaction processes. This was done to determine if the solid solution limitation would be prevalent in production-recrystallized material. The combination of *dl*-13-ethyl-17α-ethynyl-17-hydroxygon-4-en-3-one and *dl*-13-ethyl-17-ethynyl-3-methoxygona-2,5(10)-dien-17β-ol was slowly recrystallized from ethyl acetate by the addition of isooctane. The *p*-ethoxyacetanilide systems were dissolved in ethyl alcohol and precipitated by dilution with water. DSC purity determinations on the above samples were in good agreement with theory as seen in Table III.

It is true that there will always be uncertainty in the purity values obtained on samples for which the nature of the contaminants and the history of the sample is not known. However, it has been demonstrated in this laboratory, at least for two systems, that DSC analysis of production material is not limited by solid solutions. The justification for this statement is based on the excellent agreement obtained with independent purity determinations as will be shown below.

TABLE V—DSC PURITY BY DILUTION TECHNIQUE

Compound	Actual Purity, Mole %	DSC Purity— Before Dilution, Mole %	DSC Purity— After Dilution, Mole %
dl-13-Ethyl-17α-ethynyl-17-hydroxygon-4-en-3-one	95.5	97.4	95.5
p-Ethoxyacetanilide	96.0	97.6	96.2
2-Methyl-2-propyl-1,3-propanediol dicarbamate	97.0	97.8	97.2
7-Chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one	96.5	97.6	96.4

Comparative Results—Comparative purity results by the phase solubility technique, which has been generally accepted as the standard method for determining absolute purity, and quantitative TLC were found to be in good agreement with DSC purity values on production material which was 99 mole % pure or better. This is shown in Table IV. At the same time it is apparent from the NMR and TLC results on the carbamate that impurities of 2 mole % or more are not accurately determined. This is in agreement with findings on the dry mixed samples.

Extending Accuracy Range—To circumvent this problem of inaccurate DSC results for samples more than 1 mole % impure, the following method is applicable.

Mix in known proportions the sample whose purity is to be determined with a known pure sample (>99.5%) of the same compound. Adjust the amounts so that the final purity of the mixture is between 99% and 99.5% pure. The ratios to be used can be estimated from the initial DSC results of the nondiluted sample. Determine the DSC purity of the mixture and from this information, along with the known weights and purity of the diluent, algebraically calculate the values for the sample in question.

Using this technique, the results shown in Table V are obtained.

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Keyphrases

Differential scanning calorimetry—analysis
Purity determination, drugs—differential scanning calorimetry
Solid solution effect—purity determination
TLC—analysis confirmation
NMR spectroscopy—analysis confirmation

Pharmaceutical Heterogeneous Systems II

Study of Hydrolysis of Aspirin in Combination with Fatty Acid Tablet Lubricants

By H. V. MAULDING, M. A. ZOGLIO, and E. J. JOHNSTON

Stearic acid USP, in combination with aspirin in tablets, capsules, and powder mixes increases the rate of salicylic acid formation over pure aspirin controls. The breakdown of aspirin in combination with stearic acid USP and reagent grade stearic and palmitic acids has been studied with regard to the relative suitability of the fatty acids in question as tablet lubricants. Commercial stearic acid was found to have a marked effect on acceleration of salicylic acid formation as compared to reagent palmitic and stearic acids which were 95% + pure (gas chromatography). In powders and tablets with a constant aspirin:fatty acid ratio (20:1) the degree of aspirin degradation reaches a maximum when the two reagent acids are mixed together at approximately the same molar ratio as found in stearic acid USP. This maximum closely parallels the minimum on the melting point curve for mixtures of stearic and palmitic acids.

IT HAS BEEN observed that the commonly used tablet lubricant, stearic acid USP, accelerates aspirin decomposition and salicylic acid formation upon combination with pure aspirin in tablets, powders, and capsules (1). This deleterious effect has been well documented in this laboratory (2). It was thought that by varying the purity of the stearic acids used, one might note a difference in the degree of acetylsalicylic acid degradation as a result of either chemical and/or physical effects of the fatty acid lubricants. For this comparison aspirin powder mixes and tablets containing food grade stearic acid[1] USP and reagent grade stearic and palmitic acids (Fisher Chemical Co.) were compared.

EXPERIMENTAL

Free Salicylic Acid Determination (3)—A sample of powder or tablet (crushed in a mortar) equivalent to 200 mg. of aspirin was dissolved in 10 ml. of water-saturated chloroform with agitation (1 min.). This solution was then poured onto a column containing 8 g. of acid-washed diatomaceous earth[2] previously mixed with 8 ml. of 2% ferric chloride. The column was eluted with water-saturated chloroform (about 50 ml.) to remove the aspirin. The purple complex was eluted into a volumetric flask with 10% acetic acid in chloroform (10 ml.) followed by 1% acetic acid in water-saturated chloroform to remove the complex. The concentration of salicylic acid was determined by measuring the absorbance of the solution at 310 mμ.

Melting Point Determination—Exactly weighed quantities of reagent steric and palmitic acids (Fisher) were mixed and heated to melting on a water bath. The mixtures were stirred and allowed to cool overnight. The congealed mixtures were crushed, mixed intimately, and melting points run on a Thomas-Hoover melting point apparatus with a temperature increase of 1°/min.[3]

Gas Chromatography—*Esterification Procedure*—One gram of fatty acid was transferred to a 125-ml. conical flask, and 30 ml. of BF_3-methanol reagent, 14% w/v (Applied Science Laboratories, Inc., State College, Pa.) added. The flask was fitted with an air condenser and the reactants refluxed on a steam bath for at least 0.5 hr. The reactants were cooled at room temperature, 20 ml. of petroleum ether added, the flask swirled, and the mixture

Received March 20, 1968, from the Pharmacy Research and Development and Analytical Department, Sandoz Pharmaceuticals, Hanover, NJ 07936
Accepted for publication July 17, 1968.
Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
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quantitatively transferred to a 125-ml. separator fitted with a Teflon stopcock. Twenty milliliters of water was added to the mixture, extracted with petroleum ether, and two additional extractions performed with 20-ml. portions of petroleum ether. The extract was filtered through anhydrous sodium sulfate into a tared 100-ml. beaker. The petroleum ether was evaporated and the residue weighed, then redissolved in 50 ml. of chloroform. The solution was chromatographed and the fatty acid distribution calculated.

Conditions for Gas Chromatography—Chromatography was performed with an Aerograph model 1520 gas chromatograph equipped with a thermal conductivity detector, a Leeds & Northrup Speedmax "W" recorder, and a 1.5-m. stainless steel column, packed with 11.6% SE-30 on Anakrom ABS, 70/80 mesh (Analabs Inc., Hamden, Conn.). The temperatures were: column 215°, injector, 265°, and detector, 250°. The carrier gas was helium with a flow rate of 30 ml./min. The detector was calibrated with C_{14}, C_{16}, and C_{18} methyl ester standards (Mann Research Laboratories, New York, N. Y.). Peak areas were calculated with a Disc integrator.

Tablet Preparation—The reagent stearic and palmitic acids were chilled to −10° and passed through a 35-mesh screen while still cold. The commercial stearic acid was passed through a bolting cloth. The powders were then mixed in a Hobart mixer for 20 min. followed by compression of the tablets on a Stokes E machine to give 10-mm., 320-mg. tablets. The powders used were taken from these mixtures. Tablet hardness varied from 3 to 5 on Strong Cobb Arner Inc. tablet hardness tester.

Kinetic Studies—Aspirin (100 mg.) and the fatty acid (8 g.) were placed in a sealed ampul in an oil bath at 70° (±0.01°) and rotated at 60 r.p.m. Samples were taken out at intervals and quickly frozen. One gram of the mixture was crushed in a mortar and analyzed for free salicylic acid, as previously mentioned.

The lower concentrations of aspirin (30 mg.)/8 g. of fatty acid were analyzed in the same manner except a 2-g. sample was used.

The salicylic acid solution was eluted into a volumetric flask and the concentration determined by measuring the absorbance at 310 mμ.

The mineral oil study was carried out by diluting a sample containing 30 mg. aspirin in 10 ml. heavy mineral oil to 100 ml. with chloroform and reading in the UV for salicylic acid with correction for unreacted aspirin.

Table I—Salicylic Acid Formed from Aspirin in Powders and Capsules

Aspirin: Stearic Acid[a]	Final Salicylic Acid[b]	Initial Salicylic Acid[b]	Salicylic Acid Formed[b]
Aspirin-Stearic Acid Powders[c]			
1.0:0.0	0.25	0.23	0.02
0.9:0.1[d]	1.00	0.24	0.76
0.5:0.5[d]	3.80	0.25	3.55
0.9:0.1[e]	0.42	0.23	0.19
0.5:0.5[e]	0.93	0.23	0.70
Aspirin-Stearic Acid Tablets[c]			
1.0:0.0	0.28	0.23	0.05
0.9:0.1[d]	0.53	0.25	0.28
0.8:0.2[d]	1.13	0.23	0.90
0.5:0.5[d]	3.13	0.25	2.88
0.9:0.1[e]	0.30	0.23	0.07
0.8:0.2[e]	0.41	0.25	0.16
0.5:0.5[e]	0.59	0.25	0.34

[a] Ratios on mole fraction basis; time, 36 days, temperature, 50° (±0.25°). [b] Salicylic acid calculated in mg./200 mg. aspirin. [c] Stored in closed amber glass bottles. [d] Commercial triple pressed stearic acid (Emery Ind.). [e] Fisher reagent grade stearic acid.

Table II—Gas Chromatographic Analysis of Acids

	Mole, % C_{14}	C_{16}	C_{18}
Stearic acid USP	3.2	52.7	44.1
Reagent stearic acid	—	2.8	97.2
Reagent palmitic acid	—	97.0	3.0

RESULTS AND DISCUSSION

Powders and tablets composed of variable quantities of aspirin with the two grades of stearic acid were prepared and the following results were obtained (Table I) after allowing them to stand at 50° (±0.25°) for 36 days.

From the preceding data it can be seen that although both reagent and practical grades of acid increase salicylic acid formation over pure aspirin controls, the commercial product produces considerably greater decomposition than the reagent grade.

Stearic acid USP (4), is a mixture of acids consisting principally of palmitic and stearic. Upon gas chromatographic analysis of reagent palmitic and stearic as well as stearic acid USP, the data as shown in Table II were obtained.

As the possibility of a liquid phase serving as a reaction media was suspected, a melting point curve was run on a binary system of reagent stearic and palmitic acids to determine the lowest temperature at which liquefaction might occur (Fig. 1). The curve shows a minimum at approximately 70 mole % of palmitic acid. This low point corresponds closely to that previously shown for mixtures of pure stearic and palmitic acids (5). It may also be noted that stearic acid USP falls quite close to this minimum (see Fig. 1).

Aspirin tablets and powders containing realistic ratios (20:1) of acetylsalicylic acid to lubricant were prepared. Various concentrations of reagent stearic and palmitic acids mixed together were used along with stearic acid USP and aspirin controls and their stability studied at room temperature, 20° (±0.5°), 40° (±0.25°), and 50° (±0.25°) (Figs. 2 and 3). Moisture determinations were run on the tablets and powders by the Karl Fischer method and all were found to be lower than 0.066% which is below the confidence limits of this procedure. Too large a weight of sample is necessary (not dissolvable in the medium used for the determination) to obtain meaningful data.

The results (Figs. 2 and 3) indicate maximum aspirin degradation occurs around the range of composition (with regard to stearic and palmitic acid content) of the commercial stearic acid.

The reagent palmitic and stearic acids were chilled to −10° prior to reduction of their particle size.

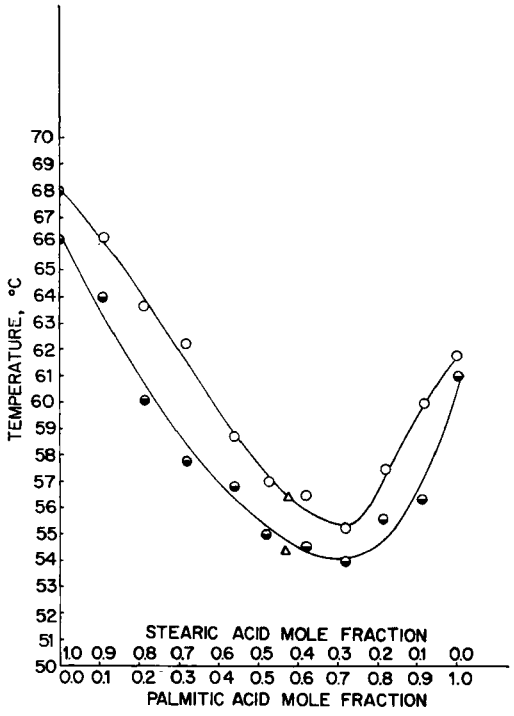

Fig. 1—Melting point curve for mixtures of reagent grade stearic and palmitic acids. Key: ○, complete melting; ◒, melting begins; △, stearic acid USP.

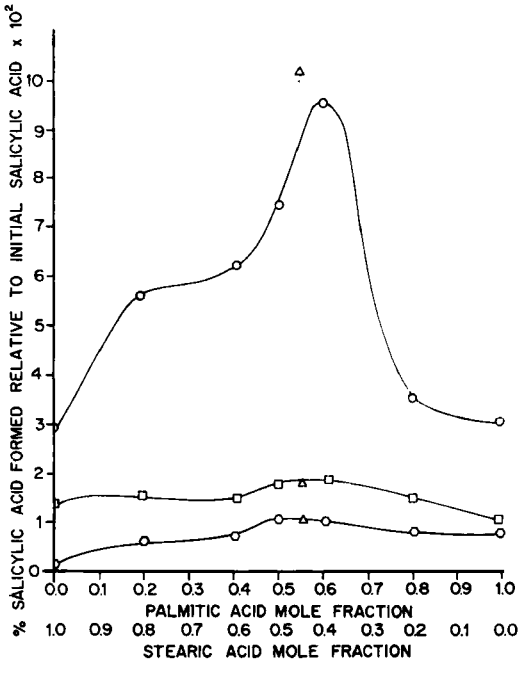

Fig. 2—Aspirin degradation in powder mixes (320 mg.) containing aspirin plus fatty acid lubricant (20:1 w/w ratio, time = 60 days). Pure aspirin controls formed less salicylic acid at all temperatures than aspirin lubricant combinations. Key: ○, 50° (±0.25°); □, 40° (±0.25°); ○, R.T. (±0.50°); △, stearic acid USP.

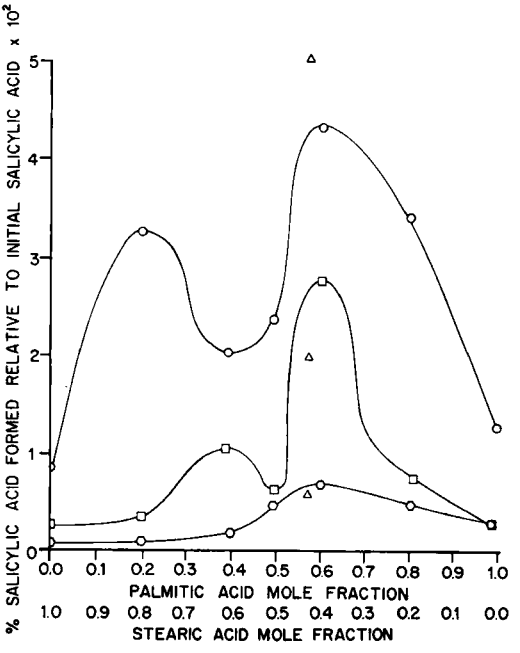

Fig. 3—Aspirin degradation in tablets containing aspirin plus fatty acid lubricant (20:1 w/w ratio, time = 60 days). (Pure aspirin controls formed less salicylic acid at all temperatures than aspirin lubricant combinations.) Key: ○, 50° (±0.25°); □, 40° (±0.25°); ○, R.T. (±0.50°); △, stearic acid USP.

This was necessary because of their consistency which caused difficulty in both their passage through a screen or a bolting cloth. After screening the material was allowed to warm up to room temperature.

It is apparent that in most cases there is a significant increase in aspirin degradation in powders over tablets of identical lubricant concentration.

Kinetic studies were run at 70° on solutions of aspirin in each of the 3 acids as well as in mineral oil to determine their role in increasing salicylic acid formation (Fig. 4). The graph indicates that stearic acid USP, shows the most aspirin decomposition at any given time interval up to 96 hr. Reagent grade palmitic acid gives less degradation with reagent stearic acid giving the least for the 96 hr.

Mineral oil shows little salicylic acid formation after the initial 12-hr. assay. The leveling effect is probably caused by consumption of the total water present in the system.

Kinetic studies on lower concentrations of aspirin (30 mg.) in stearic acid USP (8 g.) give the predictable first-order picture at 70° for 96 hr. (Fig. 5). This may be a result of the lesser concentration of aspirin relative to the water in the system.

Moisture determinations (Karl Fischer) again failed to produce any conclusive evidence of the amount of water present in the aspirin-fatty acid system due to the same reasons previously stated.

Although the concentration of water in any of these fatty acids is exceptionally small, one must bear in mind that only 10 mg. of water is required

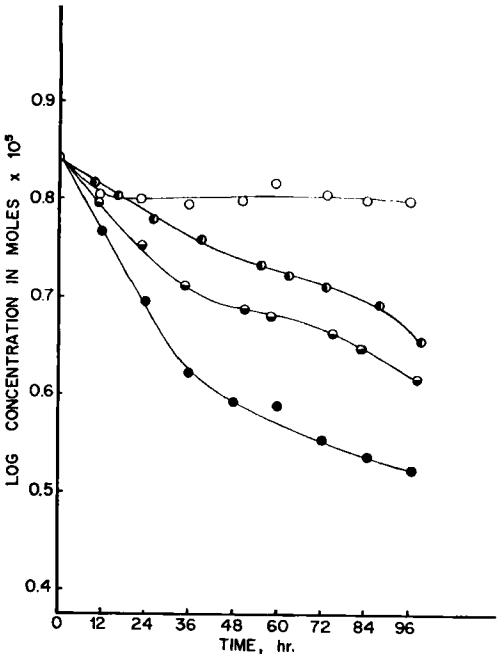

Fig. 4—Kinetic studies at 70° (±0.01°) of aspirin degradation in fatty acid lubricants and mineral oil (100 mg. aspirin/8.100 g. sample). Key: ○, mineral oil; ◐, reagent stearic acid; ◒, reagent palmitic acid; ●, stearic acid USP.

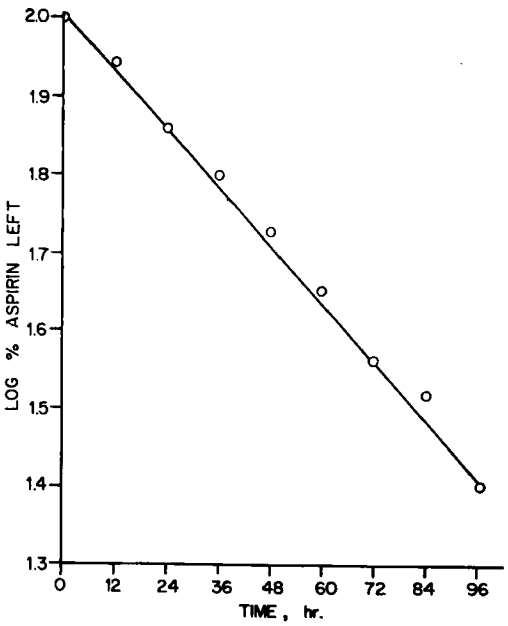

Fig. 5—Kinetic studies at 70° (±0.01°) of aspirin degradation in stearic acid USP (30 mg. aspirin/8.000 g. stearic acid).

to hydrolyze 100 mg. of aspirin. This would represent only a 0.12% moisture content/8 g. of acid.

From a practicality viewpoint, the reagent palmitic and stearic acids appear to be efficient lubricants, however, the cost differential is appreciable between them and stearic acid USP.

CONCLUSIONS AND DISCUSSION

Stearic acid USP, in combination with aspirin tablets and powders increases salicylic acid formation over pure aspirin controls.

Reagent grade stearic and palmitic acids (Fisher Chemical Co.) are found to retard aspirin degradation relative to the commercial "triple pressed stearic acid," stearic acid USP.

The effect of myristic acid was not evaluated due to its low concentration in the two fatty acids examined although the same basic situation with regard to aspirin stability would be expected to exist.

From the results presented it appears there are better fatty acid lubricants from a stability standpoint for use with aspirin than the standard commercial stearic acid.

Powders and tablets of the same composition (20:1, aspirin:fatty acid) show a significant difference in decomposition for a given period of time. As moisture content of the two should be equal, this anomaly could be partially due to the greater surface area and looser consistency of a given weight of powder when compared to tablets. It may be that the driving force of the reaction is sublimation and removal of salicylic acid from the system which should be much easier in the case of the loose powder than in the relatively hard-surfaced tablet. There will be a later report on this phenomenon.

The decomposition of aspirin powders and tablets (20:1) made of different ratios of reagent stearic and palmitic acids reach a maximum near the same point where the two acids reach a minimum in the melting point curve with stearic acid USP, being the worst of the group.

The kinetics of aspirin (100 mg.) solutions in the fatty acids (8 g.) show no definite order of reaction at 70° although, if the total amount of water in the system were known, the reaction would probably prove to be second order. In the lower aspirin concentrations (30 mg.)/8 g. acid a first-order plot is demonstrable, probably due to excess water in the system strengthening the case for a second-order mechanism.

The kinetic results with mineral oil indicate that little thermal degradation of aspirin takes place at 70° and thus is not a factor with the principal reaction in all cases being simple hydrolysis by the water present in the system.

The possibility exists of liquid or semiliquid being present in the system as a result of the low melting stearic acid which serves as the media for aspirin hydrolysis.

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Keyphrases

Aspirin hydrolysis—tablets
Fatty acids, effect—aspirin hydrolysis
Column chromatography—separation
UV spectrophotometry—analysis
GLC—analysis

Pharmaceutical Heterogeneous Systems III

Inhibition of Stearate Lubricant Induced Degradation of Aspirin by the Use of Certain Organic Acids

By M. A. ZOGLIO, H. V. MAULDING, R. M. HALLER, and S. BRIGGEN

The acceleration of aspirin degradation in capsule formulations where an alkali stearate is employed as a lubricant can be inhibited by the inclusion of malic, hexamic, or maleic acid. The acids when included at a level of 20 percent by weight of the complete mixture achieve a level of inhibition at which it could be said that the preparations are stable with respect to salicylic acid content. The mechanisms operative in these systems and the factors contributing to successful inhibition are described. Moisture content of the capsule mix and in the gelatin capsule shell is studied in regard to effects on the stability of aspirin and the dissolution rate of aspirin from the capsule formulations considered.

THE ASPIRIN MOLECULE is subject to instability whenever moisture is present in appreciable amounts in an aspirin formulation (1). Excipients as well as physiologically active substances which influence the pH of the moisture in the solid dosage form can influence the rate of degradation. Aspirin hydrolysis is accelerated at both low and moderately high pH values (2). Substances, such as antacids, have been cited as being detrimental to aspirin stability (3). In a recent study the acceleration of aspirin hydrolysis by alkali stearate lubricants was demonstrated (4). The physicochemical mechanism leading to this effect was explained on the basis of a reaction between the lubricant and aspirin. The reaction leads to formation of a soluble alkali salt of aspirin which maintains the moisture in the formulations at a hydroxyl ion concentration and greatly accelerates the breakdown of aspirin. The object of the present study was to investigate the feasibility of including organic acids of comparable or lower pKa values and greater solubility than aspirin to compete for the magnesium cation creating an environment buffered close to the optimum pH for aspirin stability. Factors influencing the amount of acid and/or alkali salt present in the moisture in the system would, of course, determine the effectiveness of the individual acids. The study was carried out using capsule formulations. A second phase of the study was concerned with the humid microatmosphere very often existing within the capsule shell. The effect of reducing the moisture content in the gelatin shell on aspirin stability and the dissolution rate of aspirin from aspirin-stearate lubricant-organic acid capsule formulations is demonstrated.

EXPERIMENTAL

Capsules[1] containing a mixture of 20 parts aspirin USP (40 mesh), 1 part magnesium stearate USP, and 1, 2, 5, 10, and 20 parts, respectively, of organic acid by weight were prepared. Control capsules containing aspirin alone and aspirin plus magnesium stearate (20:1) were also prepared. The acids chosen for the study were hexamic,[2] maleic,[3] malic,[4] and tartaric acid NF. Maleic anhydride[5]

Received May 22, 1968, from the Pharmacy Research and Development Departments, Sandoz Pharmaceuticals, Hanover, NJ 07936
Accepted for publication July 9, 1968.

[1] Parke Davis No. 3, clear gelatin capsules, Parke Davis and Co., Detroit, Mich.
[2] Abbott Laboratories, North Chicago, Ill.
[3] Practical Grade, Matheson Coleman & Bell, East Rutherford, N. J.
[4] Practical Grade, Eastman Organic Chemicals, Distillation Products Inc., Rochester, N. Y.
[5] Monsanto EMA Grade 31, Monsanto Co., St. Louis, Mo.

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was also included in the study and employed in the same parts by weight as the acids.

Two sets of capsules and corresponding capsule mixes, independently prepared, were stored at 22° (±0.5°), 40° (±0.25°), and 50° (±0.25°) for 30 days at which time salicylic acid analysis, dissolution rate, and moisture determinations, as previously described, were performed.

A third and fourth set of capsules identical with the first two, except that tartaric acid and maleic anhydride were eliminated from this phase of the study, were prepared and subjected to vacuum drying for 24 hr. at 40° (±0.1°) and 0.1 mm. Dissolution rates, moisture, and salicylic acid determinations were performed initially (after drying) and after 1 month's storage, as described for the first sets of samples. Nondried capsules and powder mixes were run as controls with the dried samples. The results reported in Tables I and II, and Figs. 1–6 are average results from two separate experiments. The error of reproducibility between experiments was less than 5%. To determine loss of moisture from the gelatin on drying, 10 g. (weighed exactly) of empty gelatin capsules[1] were dried under the conditions described for the filled capsules and moisture loss determined by weight loss. In addition, a Karl Fisher moisture analysis was performed on the same batch of empty gelatin capsules before and after drying. The gelatin was ground to a fine particle size in an electric blender (Waring) before analysis. Samples equivalent to 200 mg. of aspirin were analyzed according to the method of Levine (5). Moisture determinations were performed on all capsules and powder mixes immediately after preparation by Karl Fischer titration.

Dissolution rates of aspirin from the capsules were in all instances performed in duplicate. In some cases a third experiment was added to fortify data. The experiments were carried out in the following manner.

One capsule was placed in a bottle containing 100 ml. of distilled water, the bottle sealed, and rotated at 60 r.p.m. in a water bath at 37° (±0.1°). Two-milliliter samples were taken at 5, 10, 15, 20, 45, and 60 min. using pipets with filters attached. Two milliliters of distilled water was added to the bottle after sampling to maintain volume. One milliliter of the sample was quickly (within 2 min.) brought to 50 ml. volume with water and a reading taken at 280 mμ on a spectrophotometer (Perkin-Elmer 202) to determine aspirin in solution.

TABLE I—HYDROLYSIS OF ASPIRIN IN CAPSULES CONTAINING ASPIRIN PLUS MAGNESIUM STEARATE (20:1 WEIGHT TO WEIGHT RATIO)

	Salicylic Acid Formation/200 mg. Aspirin[a]		
	R.T. (22°C. ±0.5°), mg.	40°C. (±0.25°), mg.	50°C. (±0.25°), mg.
Aspirin-magnesium stearate capsules[b]	1.02	3.22	15.81
Aspirin-magnesium stearate powder mix[b]	1.00	2.68	5.56
Aspirin capsules, control[b]	0.0429	0.262	0.348
Aspirin USP, control[b]	0.0362	0.0719	0.2623

[a] Storage time = 30 days. [b] All samples contained less than 0.1% moisture.

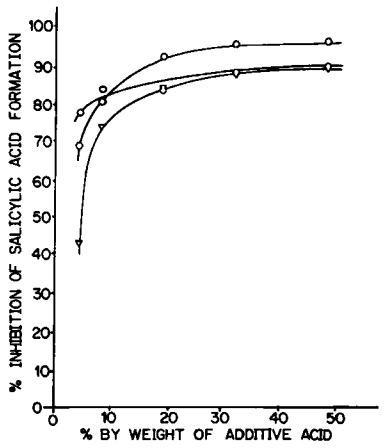

Fig. 1—Inhibition of salicylic acid formation in aspirin-magnesium stearate-maleic acid capsules. Percentage inhibition is compared to aspirin-magnesium stearate capsules (Table I). Time of storage is 30 days. Key: ○, R.T. (22 ± 0.5°); ▽, 40° (± 0.25°); ○, 50° (± 0.25°).

RESULTS AND DISCUSSION

The acceleration of the hydrolysis of aspirin by inclusion of magnesium stearate as a lubricant in a capsule formulation is shown in Table I.

Figures 1, 2, and 3 indicate that malic, maleic, and hexamic acids successfully inhibit the stearate-induced degradation of aspirin at the three storage conditions. A minimum of 20% by weight of these acids is needed for successful inhibition. The 40° curves for these acids show less effect than the room temperature and 50° curves. This could possibly be due to the following explanation. At lower temperatures the additive acid is most likely operating through pH effects. The amount of solubilized

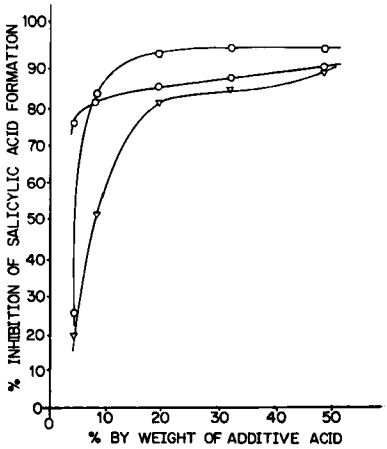

Fig. 2—Inhibition of salicylic acid formation in aspirin-magnesium stearate-malic acid capsules. Percentage inhibition compared to aspirin-magnesium stearate capsules (Table I). Time of storage is 30 days. Key: ○, R.T. (22 ± 0.5°); ▽, 40° (±0.25°); ○, 50° (± 0.25°).

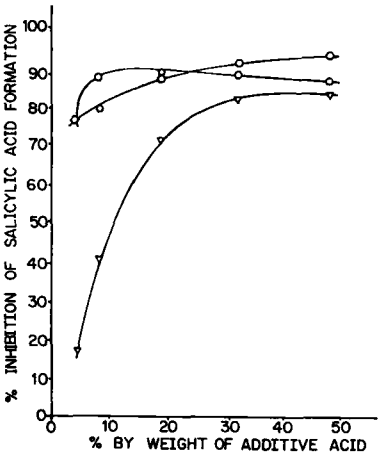

Fig. 3—Inhibition of salicylic acid formation in aspirin-magnesium stearate-hexamic acid capsules. Percentage inhibition is compared to aspirin-magnesium stearate capsules (Table I). Time of storage is 30 days. Key: ○, R.T. (22° ± 0.5°); ▽, 40° (± 0.25°); ○, 50° (±0.25°).

aspirin is decreased by the lower pH. The small amount in solution should not be degraded rapidly unless the pH of the moisture in the system is below 2.0 (2). At moderately high temperatures (40°) the additive acid concentration is apparently increased to a point where the pH slightly accelerates degradation. At 50° the salt-forming reaction between the additive acid and magnesium ion becomes quite significant and a buffer system composed of the additive acid and its magnesium salt raises the pH to a safe level for aspirin stability. All three effects, that is, inherent pH, solubility, and the salt-forming reaction are most likely operating to some degree at all of the conditions of storage. Malic, hexamic, and maleic acids appear to possess a good balance with regard to their ability to dominate the pH picture through solubility and reactivity

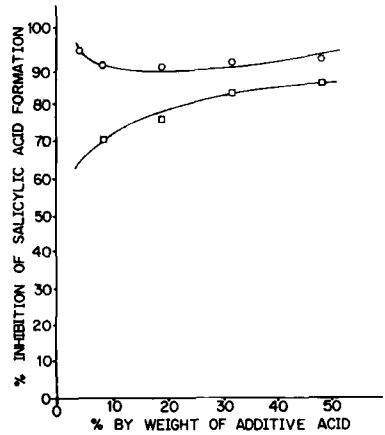

Fig. 4—Inhibition of salicylic acid formation in aspirin-magnesium stearate-tartaric acid and aspirin magnesium stearate-maleic anhydride capsules. Percentage inhibition is compared to aspirin-magnesium stearate capsules control (Table I). Storage condition is 22° (±0.5°). Time of storage is 30 days. Key: ○, tartaric acid formulations; □, maleic anhydride formulations.

with magnesium stearate. The formation of the relatively alkaline aspirin:magnesium-aspirin buffer system described in a previous report (4) is apparently successfully inhibited by these acids.

Figure 4 demonstrates the inhibition of aspirin-stearate lubricant degradation employing tartaric acid and maleic anhydride. The effects at room temperature are shown only since at 40° and 50° there was no significant inhibition by tartaric acid and maleic anhydride accelerated salicylic acid formation above the magnesium stearate-aspirin control. Maleic anhydride was apparently effective at room temperature through a pH effect, but introduced an additional 1.3% (average) moisture into the samples which supported enough hydrolysis to offset any pH or buffer system effects at the higher temperatures. (In general, samples in this study ranged from 0.1 to 0.5% moisture content, whereas the maleic anhydride formulations averaged 1.7%.) The ineffectiveness of tartaric acid at the higher temperatures was due to a relatively high pKa and the poor solubility of its magnesium salt.

TABLE II—HYDROLYSIS OF ASPIRIN IN CAPSULES CONTAINING ASPIRIN ALONE AND ASPIRIN PLUS MAGNESIUM STEARATE (20:1 WEIGHT TO WEIGHT RATIO)[a]

	Salicylic Acid Formation/200 mg. Aspirin[b]		
	R.T. (22° ± 0.5°C.), mg.	40° (± 0.25°C.), mg.	50° (±0.25°C.) mg.
Aspirin capsules control[c]	0.036	0.053	0.091
Aspirin-magnesium stearate capsules, control[c]	0.10	1.68	4.5

[a] The filled capsules were dried at 40°, 0.1 mm. pressure for 24 hr. [b] Storage time = 30 days. [c] Capsule ingredients contained less than 0.1% moisture after drying.

Table I illustrates an important point with regard to the role of the gelatin capsule in aspirin breakdown. Moisture present within the gelatin appears to set up a humid microatmosphere which accelerates aspirin breakdown in both plain aspirin and aspirin-magnesium stearate capsules. Moisture was removed from the gelatin by vacuum drying the filled capsules, and its effect on aspirin stability, moisture content of the capsule mix, and dissolution rate studied. The moisture loss from the gelatin was calculated as 10.1% by the two methods described. Malic, maleic, and hexamic were used in this phase of the study, since they presented the most successful inhibition in the initial study. Table II demonstrates the effect on aspirin stability with and without stearate lubricant.

Table II illustrates that decreasing moisture content of gelatin has a stabilizing effect on the aspirin. This effect was not so dramatic in the capsules containing aspirin-magnesium stearate-organic acid. Figures 5 and 6 demonstrate an inhibition of degradation, but not significantly greater on a percentage basis than had been observed with the nondried capsules. Maleic acid capsules showed no inhibition of degradation. The removal of the moisture from the system resulted in complete saturation of available moisture by the magnesium maleate-maleic acid buffer. This leads to pH of

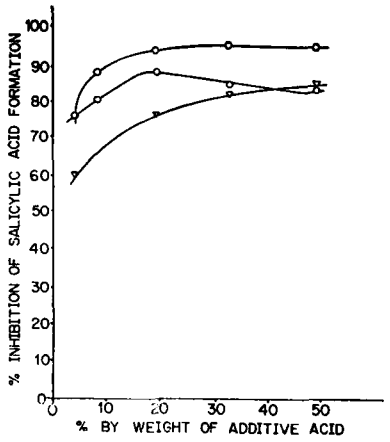

Fig. 5—Inhibition of salicylic acid formation in aspirin-magnesium stearate-hexamic acid capsules (dried in vacuo). *Percentage inhibition is compared to aspirin-magnesium stearate capsules (dried* in vacuo, *Table II), Time of storage is 30 days. Key:* ○, *R.T. (22 ± 0.50°);* ▽, *40° (±0.25°);* ○, *50° (±0.25°).*

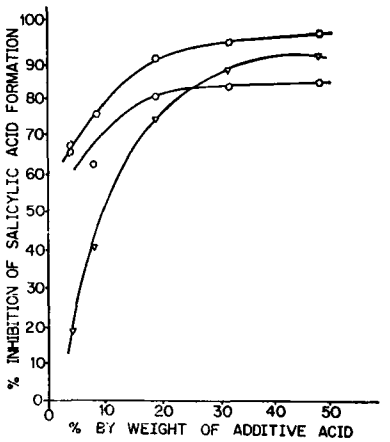

Fig. 6—Inhibition of salicylic acid formation in aspirin-magnesium stearate-malic acid capsules (dried in vacuo). *Percentage inhibition is compared to aspirin-magnesium stearate capsules (dried* in vacuo, *Table II). Time of storage is 30 days. Key:* ○, *R.T. (22 ± 0.5°);* ▽, *40° (±0.25°);* ○, *50° (±0.25°).*

approximately 1.3 of the saturated aqueous suspension, magnesium maleate, and maleic acid.

The decrease in moisture in this system was not only due to the decrease in the gelatin moisture, but also to a significant decrease in the capsule mix after drying. The initial moisture content of the samples was 0.4% (average) and was reduced to 0.2% (average) after drying. All other capsules showed insignificant loss of moisture from the ingredients after the drying process. In general, the drying effect on dissolution rate was negligible when measured immediately after drying. If an appreciable amount of salicylic acid was formed after storage, the dissolution rate appeared to be slightly decreased. The capsule ingredients set up when a significant amount of salicylic acid was formed causing the decreased dissolution rates.

The acceleration of aspirin degradation in capsule formulations where an alkali stearate is employed as a lubricant can be inhibited by the inclusion of malic, maleic, and hexamic acid. To achieve a level of inhibition at which it could be said that the preparation is stable with respect to salicylic acid content, approximately 20% by weight of the acid must be included in the formulation. In addition to pH effects contributed by the acids the mechanism operative in inhibiting degradation involves a competition for the lubricant cation between aspirin and the additive acid. The important factors when considering this type of inhibition are as follows.

(*a*) The pKa of the additive acid should be in the range of aspirin or lower.

(*b*) The solubility of the additive acid and alkali salt formed should be sufficient so as not to fix the acid–acid salt ratio through saturation at a pH value detrimental to aspirin stability.

(*c*) The moisture content of the capsule mix should be between 0.4 and 0.6%. If maleic acid is used, the lower limit is critical since very low moisture shifts the acid–acid salt ratio to the acid side accelerating degradation.

Decreasing the amount of moisture in the gelatin capsule shell was successful in decreasing degradation in capsules containing aspirin and aspirin plus magnesium stearate. The extent of aspirin hydrolysis inhibited in dried aspirin-lubricant-additive acid capsules was improved only in the hexamic acid formulation. The removal of moisture from the gelatin improves aspirin stability except when this lowering of available moisture creates a high concentration of substance detrimental to stability through a pH effect. This was apparently the case with maleic acid. The dissolution rates were not affected to any great extent by the drying process. A slight decrease was noted after storage with samples containing higher levels of salicylic acid.

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Keyphases

Heterogeneous systems—pharmaceutical
Aspirin degradation, stearate-induced—inhibition
Organic acids effect—stearate degradation, aspirin
Moisture effect—aspirin degradation
UV spectrophotometry—analysis

Comparative Pharmacokinetics of Coumarin Anticoagulants III

Factors Affecting the Distribution and Elimination of Bishydroxycoumarin (BHC) in Isolated Liver Perfusion Studies

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The kinetics of elimination of BHC in man is unusual in that the apparent first-order rate constant for elimination appears to decrease with increasing dose. No such dose dependency is apparent in rats and certain other species of animals. The purpose of this study was to determine if this difference in BHC elimination kinetics is due to a difference in the interaction of BHC with plasma proteins of rats and man. Isolated rat livers were perfused with a mixture of red blood cells from rats and plasma from donor rats or man (two concentrations) which contained various concentrations of BHC. The concentration of BHC was determined periodically in the perfusate and terminally in the liver. It was found that the apparent rate constant for the metabolism of BHC in isolated perfused rat livers is affected by the initial concentration of drug and by the source and concentration of plasma proteins, and that it is a function mainly of the distribution of BHC between liver and plasma. There is a significant difference in the affinity of BHC to plasma proteins from man and rat, respectively, and there appears to be also a difference in the relative importance of the factors which affect BHC elimination in man and rats.

THE PHARMACOKINETIC characteristics of the anticoagulant drug bishydroxycoumarin (BHC) in man are unusual in that drug levels in the plasma decline exponentially following absorption and distribution, but with half-lives which increase with increasing dose (1, 2). Similar dose-dependent characteristics have been noted in the elimination of several other drugs (3) but the basis for this unusual phenomenon has not as yet been elucidated. In addition, there is some indication that the apparent volume of distribution of BHC in man decreases with increasing dose (2). This type of effect has apparently not been observed with any other drug.

The mechanism of the unusual pharmacokinetic characteristics of BHC has been the subject of continuing inquiry in this laboratory. The unique concentration-dependent plasma protein binding characteristics of BHC described in the initial report in this series (4), and the unexpected type of dose dependence of the apparent volume of distribution of this drug in man as reported by O'Reilly *et al.* (2), suggested that the unusual pharmacokinetic characteristics of BHC may be due to protein binding. On the other hand, it has been suggested that a dose dependence in the biologic half-life of a drug may be the result of substrate inhibition of the enzyme responsible for the biotransformation of that drug (3). A recent study in this laboratory (5) has shown that there is no apparent dose dependence in the distribution and elimination kinetics of BHC in rats in the dose range of 2 mg./kg. to 20 mg./kg. (which is equivalent to an initial plasma concentration of about 20 mcg./ml. and 200 mcg./ml.). It was also found in initial experiments that the affinity of BHC to human plasma proteins is much greater than to rat plasma proteins. It was therefore thought that a study of the effect of human plasma and rat plasma, respectively, on the elimination of BHC by isolated perfused livers would establish the role, if any, of plasma protein binding in the elimination of this drug. Furthermore, this technique permits an assessment of the effect of drug "dose" (*i.e.*, concentration) on the elimination kinetics under conditions where distribution factors can be accounted and corrected for.

In the study described here, the kinetics of BHC at various concentrations was investigated in isolated rat liver perfusion systems, using rat blood as well as mixtures of human blood plasma and blood cells from rats. (Plasma containing human blood cells does not flow adequately through rat livers.) The kinetic constants thus obtained were compared with similar constants obtained from studies on intact animals. The results of this investigation demonstrate the relative importance of distribution and concentration in the elimination of BHC in both isolated livers and intact animals. They demonstrate also that

Received May 6, 1968, from the Biopharmaceutics Laboratory, Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication July 24, 1968.
Supported in part by a grant from the Heart Association of Erie County, Inc.
Previous paper: Nagashima, R., Levy, G., and Back, N., *J. Pharm. Sci.*, **57**, 68(1968).
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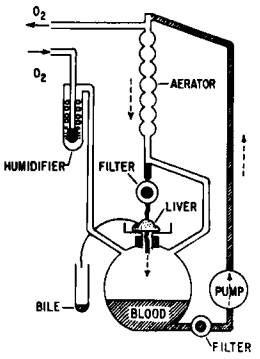

Fig. 1—Schematic representation of isolated rat liver perfusion apparatus. The filter is a sheet of Nylon (100 mesh), and the perfusion pump is of peristaltic type (model T-8, Sigmamotor, Inc., Middleport, N.Y.). The whole system is enclosed in a Plexiglas chamber in which the temperature is thermostatically maintained at 37°. The liver is covered with gauze pads saturated with Ringer solution. The blood is oxygenated with a humidified O_2–CO_2 mixture (95:5 by volume).

a rigorous pharmacokinetic analysis, incorporating all pertinent variables, permits a quantitative correlation of data obtained *in vitro* and *in vivo*.

EXPERIMENTAL

Liver Perfusion Techniques and Apparatus—The techniques and apparatus for the isolated rat liver perfusion studies (Fig. 1) were similar to those described by Miller *et al.* (6) and Sokal *et al.* (7, 8). Liver donors were normal, fed, male Sprague-Dawley rats weighing 300–500 g. Their livers were excised and perfused with 100 ml. oxygenated perfusing fluid consisting of diluted rat blood or mixtures of diluted human plasma and rat blood cells. The perfusing fluid entered the liver by free flow through a cannula in the portal vein, at a hydrostatic pressure of 13–15 cm. The fluid returned to the reservoir through a cannula in the inferior vena cava. It could then recirculate through the liver. The bile was collected every 0.5 hr. The viability of the livers was monitored by the appearance of the organ, flow rate of the perfusing fluid, bile production, and glucose levels in the perfusing fluid.[1]

Surgical Procedures—Surgical procedures were performed under light ether anesthesia. The abdomen was opened, the gastrohepatic and gastroduodenal ligaments were cut, and the bile duct was cannulated with a PE-10 polyethylene tube (Clay Adams, Inc., New York, N.Y.). The hepatic artery was ligated above the branch running toward the duodenum, and two loose ligatures were placed around the portal vein. The inferior vena cava was ligated and the ligature previously placed around the portal vein distal to the liver was tied. A glass cannula (o.d. 3 mm.) was firmly placed through an incision in the portal vein. One and one-half milliliters sodium heparin solution (250 USP units/ml.) was injected through this cannula. The chest was then opened and reflected, and a cannula was inserted through the inferior vena cava above the diaphragm. One-half milliliter sodium heparin solution was injected into the inferior vena cava through the second cannula. Finally, the liver was carefully dissected out and removed from the animal together with the attached diaphragm, the two cannulas, and the catheter attached to the bile duct. The total time for the procedure from opening the abdomen to starting the perfusion was approximately 15 min.

Perfusion Fluid—Heparinized blood was obtained from normal, fed, male Sprague-Dawley rats by cardiac puncture. Generally, the blood from 10–15 donor rats was pooled for any one perfusion experiment. It was diluted with Ringer solution such that for each part by volume of plasma, there was added 0.7 part by volume of Ringer solution. The actual volume of Ringer solution added to the blood was determined on the basis of hematocrit values, which ranged from 43–46% in the pooled blood. One milliliter of BHC solution (various concentrations),[2] 0.25 ml. sodium heparin solution (1,000 USP units/ml.), and 0.5 ml. antibiotics solution[3] were added to 100 ml. of the diluted rat blood.

Heparinized human plasma was prepared by centrifugation at $1,800 \times g$ for 20 min. of blood obtained by venipuncture from a healthy adult male volunteer. The plasma was then diluted by adding either 0.7 or 1.7 parts of Ringer solution to 1 part by volume of plasma. The rat blood cells were obtained by centrifugation at $1,800 \times g$ for 20 min. of heparinized blood obtained from 10 to 15 donor rats. The cells were washed with Ringer solution. Thirty-two milliliters of packed rat blood cells was mixed with 68 ml. of diluted human plasma, to which 1 ml. of BHC solution (various concentrations), 0.25 ml. sodium heparin solution (1,000 USP units/ml.), and 0.5 ml. antibiotics solution were added. The blood was collected and the perfusing fluid was prepared from it no longer than 3 hr. before a perfusion experiment. During the experiment, 0.4 ml. each of sodium heparin solution and antibiotics solution were added to the perfusing fluid in the reservoir every hour.

Collection of Perfusion Fluid Samples—Two and two-tenths-milliliter aliquots of perfusing fluid were withdrawn from the reservoir every 10 min. for the initial 0.5 hr. and every 0.5 hr. thereafter for a total of 4 hr. Part of each sample was used to determine glucose levels, the remainder was centrifuged to obtain "plasma phase" for the determination of BHC.

Assay Method for BHC—BHC in the plasma phase was determined by a modification of the method of Axelrod *et al.* (10). The assay consists essentially of adjustment of plasma to pH 3.0, extraction with heptane, transfer of the drug into 2.5 *N* NaOH, and spectrophotometry at 314 mμ (4). BHC metabolites and the antibiotics added to the plasma phase did not interfere with the assay.

BHC concentration in the liver was determined by the paper chromatographic method of Christensen (11) except that the liver homogenate was adjusted to pH 4.3 using 1.5 *M* citrate–phosphate buffer prior to the extraction of the drug with ethylene dichloride. The average recovery of BHC from liver

[1] Glucose levels were determined by an AutoAnalyzer (Technicon Instruments Co., Chauncey, N. Y.) using a ferricyanide reduction method (9).

[2] Prepared by dissolving one part by weight of bishydroxycoumarin (Nutritional Biochemicals Co., Cleveland, Ohio) and 10 parts by weight of tris(hydroxymethylamino)methane (Nutritional Biochemicals Co.) in freshly distilled water.

[3] The antibiotics solution contained 2.46 mg. penicillin G potassium, 0.6 mg. polymyxin, and 0.48 mg. streptomycin/ml.

homogenates to which 3.3 to 100 mcg. of the drug/g. wet tissue were added was 87%. Triplicate determinations were made.

Stability of BHC in Aqueous Solution Under Oxygenated Condition—An aqueous solution of 25 mcg. BHC/ml. in 0.05 *M* phosphate buffer (pH 7.45) was maintained at 38° and gassed continuously with a mixture of 95% oxygen and 5% carbon dioxide. One-half-milliliter aliquots were withdrawn at zero time and after 3 hr. of oxygenation. The concentrations of BHC were determined as described previously for plasma samples.

Distribution of BHC in the Red Blood Cells (RBC)—Exactly 1 ml. of an aqueous solution containing 0.2 mcg. BHC/ml. was added to a 10-ml. mixture consisting of 2.75 ml. rat RBC and 7.25 ml. heparinized diluted rat plasma (1.7-fold dilution). This was mixed thoroughly and maintained at 37°. One-milliliter aliquots of the fluid were withdrawn at appropriate intervals and centrifuged to obtain plasma phase for the determination of BHC. The fluid was mixed before each withdrawal by repeated inversion of the tube to prevent sedimentation of the cells.

Equilibration of BHC Between Human Plasma and Rat Plasma—Cellophane tubing [2.54 cm. (1 in.) width when flat, Visking Co., Chicago, Ill.] was soaked in $^1/_{15}$ *M* phosphate buffer (pH 7.4) for 4 days. The tubing was then sleeved onto a glass rod and half-everted to make a double cellophane wall with only one end open. Three milliliters of heparinized plasma (either rat or human), 0.5 ml. BHC solution of specified concentration, and 1.5 ml. $^1/_{15}$ *M* phosphate buffer (pH 7.4) were pipeted into the space between the two cellophane walls. The tubing was then placed in a 13-ml. round vial containing 3 ml. plasma from the other source, 0.5 ml. BHC solution, and 1.5 ml. of $^1/_{15}$ *M* phosphate buffer (pH 7.4). The tubing in the vial was adjusted so that the two fluids were at the same level. This was done by placing one end of the tubing between the screw cap and the thread of the vial. The vials were agitated in an upright position on a reciprocating shaker in a cold room maintained at 2°. One-half-milliliter aliquots were withdrawn from both plasma phases at 4-day intervals and were assayed for BHC as described previously.

The same experiment was carried out also with more diluted plasmas (10-fold dilution). Here, each of the fluids consisted of 0.5 ml. plasma, 0.5 ml. BHC solution of specified concentration, and 4 ml. $^1/_{15}$ *M* phosphate buffer.

Determination of Elimination Rate Constants and Distribution Ratios—The apparent rate constant for drug elimination in a liver perfusion system is inversely proportional to the volume of the perfusing fluid (12). The drug concentrations in aliquots of the perfusing fluid obtained during a liver perfusion experiment had to be corrected therefore for the decreasing volume of this fluid with time due to repetitive withdrawal of samples. This correction was made by means of the relationship:

$$C_2^c = C_1^c - \frac{V}{V_0}(C_1 - C_2) \qquad \text{(Eq. 1)}$$

where C_2^c is the corrected plasma concentration of BHC in a given sample of perfusing fluid, C_2 is the actual concentration of BHC in that sample, C_1 is the actual concentration of BHC in the preceding sample,

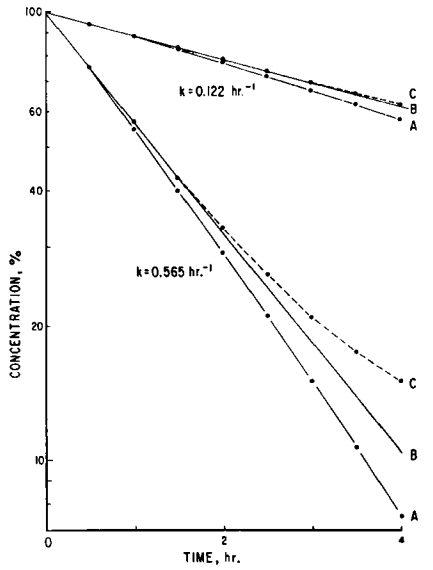

Fig. 2—The effect of a decrease in the volume of the perfusing fluid (due to repeated withdrawal of samples) on the time course of the drug concentration decline in an isolated perfused liver system. Shown are the expected concentration changes for a rapid and a slow apparent first-order elimination process if the volume of the perfusing fluid remains constant with time (B); results which would be obtained if the volume of the perfusing fluid (initially 100 ml.) decreases with time due to removal of 2.2-ml. samples every 10 min. for the first half hour and every 30 min. thereafter (A); results obtained by correcting the data in A by the procedure described in the text (C).

C_1^c is the corrected plasma concentration of BHC in the preceding sample (or the actual concentration if the preceding sample is the first sample), V is the volume of perfusing fluid prior to withdrawal of the sample containing BHC = C_2, and V_0 is the initial volume of the perfusing fluid. Equation 1 applies exactly if drug elimination proceeds at a constant rate and can be used also if the rate of drug elimination is proportional to concentration, provided that the concentration change in the sampling interval is small. Figure 2 shows the effect of a continuous decrease in the volume of perfusing fluid, due to withdrawal of 2.2-ml. samples every 30 min. from an original volume of 100 ml., on the observed concentrations of a drug. The examples in the figure refer to a rapid and a slow hypothetical first-order process, respectively. The line labeled B shows the concentration decline if the volume of the perfusing fluid had remained constant; Line A shows the expected actual concentrations when samples are withdrawn at intervals (note that this line curves downward and may suggest a saturation phenomenon if the need for data correction is not recognized); Line C shows the expected data obtained by applying the correction procedure described in Eq. 1. The deviation from the "true" line becomes significant only after about two half-lives; no such deviation occurs if the drug is eliminated at a constant rate. The rate constants of the hypothetical examples presented in Fig. 2 are identical with the lowest and the highest rate constant, respectively, encountered in this study.

TABLE I—PHYSIOLOGIC STATUS OF ISOLATED RAT LIVERS DURING PERFUSION[a]

Plasma Source	Amount of BHC in System,[b] mg.	No. of Experiments	Flow Rate of Perfusing Fluid, ml./min.[c]	Volume of Bile Excreted, ml.[c]	Glucose Concn. in Perfusing Fluid, mg./100 ml.[d]
Rat	None	1	15	1.8	225
Rat	1–6	8	19 ± 4	1.6 ± 0.2	220 ± 46
Man	1–6	6	19 ± 3	1.3 ± 0.3	246 ± 51
Man, more diluted[e]	2	2	18, 19	1.5, 1.9	298, —

[a] The data are presented as mean values ± one standard deviation, or as individual values. [b] The system consisted of 32 ml. (packed volume) rat blood cells and 68 ml. diluted plasma (58% unless indicated otherwise). BHC = bishydroxycoumarin, [c] Time, 4 hr. [d] Time, 2 hr. [e] The more diluted plasma consisted of 1 part human plasma and 1.7 p arts Ringer solution, *i.e.* dialysis plasma.

The reported apparent first-order elimination constants ($k_{app.}$) were not obtained from the slopes of the log plasma phase concentration *versus* time plots, but rather by successive calculations based on the relationship:

$$k_{app.} = \frac{\Delta C}{C_m} \cdot \frac{V}{V_0} \qquad \text{(Eq. 2)}$$

where ΔC is equal to the observed drug concentration change in a sampling interval, C_m is the observed drug concentration at the midpoint of that sampling interval, V is the volume of the perfusing fluid during the sampling interval, and V_0 is the original volume of the perfusing fluid. Thus, each 4-hr. experiment yielded seven values of $k_{app.}$ (data for the first 30-min. sampling interval were affected by the initial distribution of the drug and were not used in the calculations). These values were quite constant with time and the average $k_{app.}$ for any one experiment usually did not differ by more than 5% from the $k_{app.}$ obtained graphically.

The initial distribution ratio of BHC between liver and plasma phase (C_L^0/C_P^0) was calculated from the extrapolated zero time concentration of BHC in the plasma phase (C_P^0) and add the initial concentration of BHC in the liver (C_L^0). The latter was determined on the basis of the relationship:

$$C_L^0 = (A - V_P C_P^0)/W \qquad \text{(Eq. 3)}$$

where A is the amount of drug introduced into the system, V_P is the volume of the plasma phase,[4] and W is the wet weight of the liver in grams. Equation 3 is based on the observation by Christensen (13), which was verified in this laboratory, that no measurable amounts of BHC are present in or on red blood cells.

RESULTS

Viability of the Isolated Liver Preparation—The flow rate of perfusing fluid at the end of the experiments, the cumulative volume of bile produced during the experiments, and the glucose concentration in the perfusing fluid at the midpoint of the experiments are listed in Table I. These data are representative only since the listed functions were monitored continuously throughout the experiments. No discontinuities were observed. The continuing viability of the liver preparations during the experimental period is evident also from the drug elimination data described in subsequent paragraphs. The flow rates of perfusing fluid observed in this study were similar to those reported for a similar perfusion system (6) and were in the range of hepatic blood flow rates in intact rats (14). They were considerably in excess of the minimal flow rate necesssary to supply fully the oxygen requirement of the liver (15). The time course and rates of bile production observed in this study compare well with those described by other investigators (16, 17), the rates being also in the range obtained with intact rats (18). Glucose levels were also similar to those reported by others (8, 17, 19, 20). The single experiment without drug reported in Table I was not for the purpose of establishing physiologic controls; the listed data were obtained incidentally to a determination of BHC blank values in the isolated perfused liver system.

Stability of BHC—The concentration of BHC in 0.05 *M* phosphate buffer (pH 7.45) at 38° decreased only by 2% after 3 hr. of oxygenation with a mixture of 95% oxygen and 5% carbon dioxide. The concentration of BHC in perfusing fluid containing human plasma, when circulated under oxygenated conditions at 37° in the perfusion apparatus without liver, did not change measurably during 4 hr. Similar control experiments with rat plasma were complicated by destruction of some of the red blood cells due to the mechanical action of the peristaltic pump. For unknown reasons, this did not occur in experiments with livers. The destruction of the red blood cells in the rat plasma control experiments was accompanied by a decrease in BHC concentration in the plasma, apparently due to adsorption of this drug on the cell debris. This decrease, which was about 15%, occurred suddenly after some time and seemed to coincide with red cell destruction. Since no such destruction occurred in experiments with livers, it was assumed that this effect could be neglected. This assumption is justified also by the fact that in none of the actual experiments did there occur a sudden decrease in BHC concentration in the plasma phase.

Distribution of BHC in the Blood—Addition of BHC to diluted rat blood (the same composition as used in the perfusion experiments) yielded constant plasma levels of BHC during half-hourly sampling for 4 hr. The observed concentration of BHC in the plasma phase was identical to the expected concentration if it is assumed that BHC does not enter or bind on blood cells. These results are consistent with those reported by Christensen (13). They show also that BHC is stable in rat plasma.

Relative Affinity of BHC for Plasma Proteins from Rat and Man—Equilibration of human blood plasma containing BHC against rat blood plasma containing BHC, with the two plasma phases separated by a dialysis membrane, showed that BHC concentration in the human plasma increased with time (Table II).

[4] The volume of the plasma phase was corrected for the volume of the liquid trapped between the packed cells (4% of packed cell volume) according to Chien *et al.* (21).

TABLE II—THE RATIO OF BHC CONCENTRATIONS AND AMOUNTS IN HUMAN PLASMA AND IN RAT PLASMA SEPARATED BY A DIALYSIS MEMBRANE

Dilution of Plasma[a]	Initial BHC Concentration, mcg./ml. Human Plasma	Initial BHC Concentration, mcg./ml. Rat Plasma	BHC Concentration Ratio, Human:Rat[b] Time, days 0	4	8	12	16	Ratio of Amounts of BHC in Plasma, Human:Rat, 16 days[b]
1.7-fold	20	20	1.0	1.1	1.1	1.4	1.7	2.5
1.7-fold	20	16.7	1.2	1.1	1.2	1.6	2.0	3.0
1.7-fold	20	13.3	1.5	1.4	1.4	1.6	1.9	2.6
10-fold	3.3	3.3	1.0	1.2	1.8	2.5	2.9	3.1

[a] Diluted with 1/15 *M* phosphate buffer (pH 7.45). [b] Average of two experiments each.

The initially observed decrease was due to water movement to the human plasma phase, resulting from the higher colloid osmotic pressure of human plasma as compared with rat plasma.[5] The very slow rate of equilibration of BHC is due to its extensive plasma protein binding and the resulting low concentration gradient of free drug across the dialysis membrane. Regardless of the criterion used (concentration ratio or ratio of amounts), and despite the fact that equilibration was not complete after 16 days, it is evident that the affinity of BHC to human plasma proteins is much greater than to rat plasma proteins.

Distribution of BHC Between Liver and Plasma Phase—The distribution of BHC between liver and plasma phase was determined by direct assay in five experiments. Despite the difficult and time-consuming assay procedure, the results thus obtained agreed well with distribution ratios obtained by calculations based on liver weight, plasma volume, amount of drug introduced into the system, and extrapolated zero time concentration in the plasma phase (Table III). All other distribution ratios were therefore determined by these calculations.

TABLE III—COMPARISON OF CALCULATED AND DIRECTLY DETERMINED LIVER:PLASMA CONCENTRATION RATIOS OF BISHYDROXYCOUMARIN (BHC) IN RAT LIVER PERFUSION SYSTEMS

Expt. No.	Concentration Ratio, ml./g. By Calculation[a]	By Assay[b]
6	2.2	2.7
12	0.8	0.9
13	1.8	1.8
16	0.6	1.0
17	1.7	1.8

[a] Based on the amount of BHC added to the perfusion system and the extrapolated zero time concentration of BHC in the plasma phase of the perfusing fluid. [b] Determined by assay of BHC in the plasma phase of the perfusing fluid and in rat livers after 4 hr. of perfusion.

Pharmacokinetics of BHC Elimination in Livers Perfused with Rat Plasma—The elimination of BHC proceeded by apparent first-order kinetics in the dose range of 1–6 mg. Figure 3 shows the data obtained in the experiment which yielded the lowest $k_{app.}$ value at each dose level. The other $k_{app.}$ values as well as distribution data are listed in Table IV. It is evident that $k_{app.}$ decreased with increasing dose and that the concentration of BHC in the liver was higher than in the plasma.

[5] The colloid osmotic pressure of undiluted plasma is 330 (280–480) mm. H_2O in man and 260 (220–290) mm. H_2O in rats (22). This difference could cause a movement of 1.1 ml. water from the rat plasma to the human plasma phase under the conditions of this study. The observed value was about 0.8 ml. at 16 days.

Pharmacokinetics of BHC Elimination in Livers Perfused with Human Plasma—The kinetics of BHC elimination from the human plasma phase also followed apparent first-order kinetics. The values of $k_{app.}$ were generally lower than with rat plasma (Table IV). There was no distinct dose dependence of $k_{app.}$ Two experiments with more diluted plasma (2.7-fold rather than 1.7-fold) yielded higher $k_{app.}$ values than the other experiments. Five out of the eight experiments showed liver:plasma concentration ratios of BHC less than unity and therefore considerably lower than those obtained with rat plasma.

DISCUSSION

The apparent first-order rate constant for BHC elimination in an isolated perfused rat liver system should reasonably be a function of the concentration of BHC at the site of the biotransformation (the liver) and of the size of the liver. Thus, it is evident that distribution factors should profoundly affect the elimination of BHC. The "true" rate constant for elimination may be determined by correcting for variable drug distribution between liver and plasma, and for the weight of the liver. This may be done by calculating the fraction, F, of the total amount of drug in the system which is actually in the liver. Thus,

$$k_{app.} = k \cdot F \qquad \text{(Eq. 4)}$$

where k is the "true" rate constant for BHC elimination. F and $k_{app.}/F$ values for the experiments with rat plasma are shown in Table V. Listed also are comparable data obtained from intact rats. It may be noted that $k_{app.}$ values determined *in vitro* were

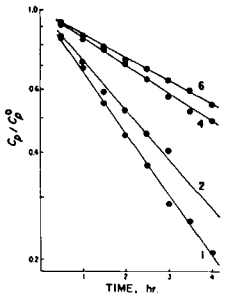

Fig. 3—Concentration of bishydroxycoumarin (BHC) in plasma phase of an isolated rat liver perfusion system as a function of time. The perfusing fluid consisted of diluted rat blood and the amount of BHC in the system ranged from 1–6 mg. (indicated by figures in the graph). The BHC concentrations are expressed as a fraction of the extrapolated zero time concentration.

TABLE IV—DISTRIBUTION AND ELIMINATION OF BHC IN ISOLATED PERFUSED RAT LIVER SYSTEMS

Expt. No.[a]	Liver Wt.,[b] g.	Plasma Source[c]	Amt. of BHC in System, mg.[c]	Calculated Concn. Ratio[d] (Liver:Plasma), ml./g.	$k_{app.}$, hr.$^{-1}$	$t_{0.5}$, hr.
3	11.5	Rat	1	2.5	0.45	1.5
4	11.4	Rat	1	2.9	0.57	1.2
5[e]	11.1	Rat	2	2.5	0.31	2.2
6	14.3	Rat	2	2.2	0.38	1.8
20	15.6	Rat	4	1.5	0.31	2.3
21	17.7	Rat	4	1.8	0.19	3.7
7	14.9	Rat	6	2.0	0.16	4.4
8	13.2	Rat	6	1.8	0.25	2.8
10	12.9	Man	1	0.8	0.21	3.2
11	12.6	Man	1	1.7	0.17	4.1
12	12.2	Man	2	0.8	0.18	3.8
13	15.7	Man	2	1.8	0.19	3.7
14	13.6	Man	6	0.7	0.19	3.6
15	13.1	Man	6	0.8	0.12	5.7
16	13.0	Man, more diluted	2	0.6	0.23	3.0
17	14.9	Man, more diluted	2	1.7	0.29	2.4

[a] Experiments 1, 2, 9, 18, and 19 were control experiments (no drug or no liver) and therefore are not listed. [b] Wet weight. [c] See footnotes *b* and *e* in Table I. [d] See footnote *a* in Table III. [e] This experiment was the first one in the series and was terminated at 3 hr.

TABLE V—EFFECT OF DISTRIBUTION AND DOSE ON THE ELIMINATION KINETICS OF BHC IN PERFUSED RAT LIVER SYSTEMS AND INTACT RATS

Expt. No.	Dose, mg./kg. Body Wt.[a]	C_P^0, mcg./ml.	Initial Concn. in Liver,[b] mg./g. Liver	Fraction of Dose in Liver: Total F	Fraction of Dose in Liver: Per g.	$k_{app.}$, hr.$^{-1}$	$k_{app.}/F$, hr.$^{-1}$
Isolated Liver Perfusion Systems							
3	2.9	10.2	0.026	0.30	0.026	0.45	1.5
4	2.9	9.8	0.028	0.32	0.028	0.57	1.8
6	4.7	19.8	0.044	0.32	0.022	0.38	1.2
5	6.0	20.7	0.051	0.29	0.026	0.31	1.1
20	8.6	43.5	0.063	0.25	0.016	0.31	1.2
21	7.6	39.6	0.071	0.32	0.018	0.19	0.6
8	15.2	64.1	0.118	0.26	0.020	0.25	1.0
7	13.5	61.0	0.119	0.30	0.020	0.16	0.5
Intact Animals							
From Ref. 5	2	23	0.006[c]	0.11	0.0031	0.12	1.1
From Ref. 23	5	47	0.011[c]	0.079	0.0025	0.11	1.4
From Ref. 13	27	160	0.070[c]	0.087	0.0026	0.075	0.9
From Ref. 5	20	200	0.088[c]	0.15	0.0044	0.14	1.0

[a] In the case of perfused livers, the weight of the donor rat. [b] $C_P^0 \cdot$ (concn. ratio, liver:plasma)/1,000. [c] Based on 33.5 g. liver/kg. body weight (26). Concentration ratios, liver:plasma, in intact rats from *Reference 23*.

generally two to three times higher than those obtained *in vivo*, but that $k_{app.}/F$ values were comparable. This demonstrates the marked effect of distribution on the elimination kinetics of BHC and

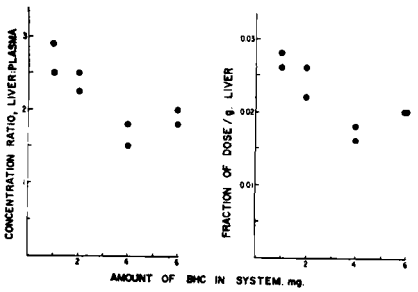

Fig. 4—Effect of the amount of BHC on the distribution of this drug between liver and plasma phase in an isolated rat liver perfusion system with diluted rat blood as the perfusing fluid.

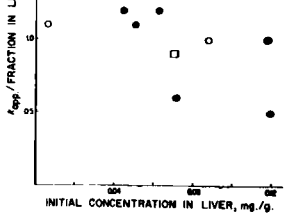

Fig. 5—The relationship between the rate constant for BHC elimination (corrected for variable distribution of BHC between liver and plasma due to variable liver weight, drug binding, etc.*) and the initial concentration of this drug in the liver. Key: ●, isolated perfused livers; ○, intact rats; □, intact rats based on data from* Reference 13.

TABLE VI—EFFECT OF DISTRIBUTION AND DOSE ON ELIMINATION KINETICS OF BHC IN ISOLATED RAT LIVERS PERFUSED WITH A MIXTURE OF HUMAN PLASMA AND RAT BLOOD CELLS

Expt. No.	Dose mg./Kg., Body Wt.[a]	C_{P^0}, mcg./ml.	Initial Concn. in Liver,[a] mg./g. Liver	Fraction of Dose in Liver, Total F	Fraction of Dose in Liver, Per g.	$k_{app.}$, hr.$^{-1}$	$k_{app.}/F$, hr.$^{-1}$
10	2.6	12.5	0.010	0.13	0.010	0.21	1.6
11	2.7	11.0	0.019	0.24	0.019	0.17	0.71
12	5.5	25.2	0.019	0.11	0.009	0.18	1.6
13	4.3	20.4	0.037	0.29	0.018	0.19	0.66
14	14.8	75.6	0.056	0.13	0.010	0.19	1.5
15	15.3	74.9	0.055	0.12	0.009	0.12	1.0

[a] As in Table V.

explains why the half-life of BHC was so much shorter in the perfused liver system than in the intact animals. The dilution of the plasma, which is necessary to obtain adequate flow rates in the *in vitro* system, results in greater partitioning of BHC into the liver than is the case in intact rats, and therefore results in greater $k_{app.}$ values.

The results of the perfusion studies with rat plasma show two dose (concentration)-dependent effects. One of these is the liver:plasma concentration ratio of BHC. It first decreases with an increasing amount of BHC in the system and then increases (Fig. 4). This effect is evident also in a plot of the fraction of the total dose/g. of liver *versus* the amount of BHC in the system (Fig. 4). The same phenomenon has been found in intact rats and in *in vitro* extraction studies (23). It is due apparently to the unusual concentration dependency in the plasma protein binding of BHC as described and discussed in a previous report (4). The other dose-dependent effect shown by the results of the perfusion experiments with rat plasma is the decrease in the "true" rate constant for BHC elimination with increasing "dose." In this context, "dose" or "concentration" is more appropriately the concentration of BHC in the liver rather than in the plasma phase. A plot of $k_{app.}/F$ *versus* BHC concentration in the liver is shown in Fig. 5. It is readily apparent that the *in vitro* $k_{app.}/F$ value decreases with increasing BHC concentration in the liver and that the *in vivo* data fit very well into the pattern. Therefore, there are two dose-dependent factors which affect the kinetics of BHC elimination, often in opposite directions: drug distribution between liver and plasma, and the apparent self-inhibition in the metabolism of BHC at higher doses. The mechanism of this latter effect is not yet understood, but there are several other drugs which exhibit the same phenomenon (3).

The liver:plasma concentration ratio of BHC in isolated rat liver systems perfused with human plasma was generally lower than in systems perfused with rat plasma, and the $k_{app.}$ values were consequently generally lower (Table IV). No such difference between the data obtained with rat plasma and human plasma, respectively, is evident in $k_{app.}/F$ values, *i.e.*, in the rate constants corrected for distribution effects (Tables V and VI). The differences in the distribution of BHC are consistent with the greater affinity of this drug to human plasma proteins (Table II). The distribution data obtained with human plasma were much more variable than those obtained with rat plasma. The rat plasma used in any one perfusion experiment was pooled from 10 to 15 animals while the human plasma was always obtained from one and the same individual. The latter procedure was expected to yield a plasma with relatively constant characteristics but it is apparent retrospectively that the use of pooled human plasma would have been more appropriate. Differences from one day to the other in the fatty acid content of the plasma obtained from a single human subject, related to the time and type of meals prior to collection of blood, are likely to have resulted in differences in the BHC binding characteristics of each plasma sample (24).

It is evident from the results of this study that the pharmacokinetics of BHC distribution and elimination are quite complex. There are indications that the relative importance of the dose-dependent distribution and apparent self-inhibition effects differs with each animal species. The biologic half-life of very high doses of BHC in rats is actually much shorter than the half-life of small doses (23), suggesting that the contribution of the distribution effect is greater than that of the apparent self-inhibition effect. On the other hand, the biologic half-life of BHC increases with increasing dose in man (1, 2, 25), indicating that the apparent self-inhibitory effect is more important than the distribution effect in the elimination of BHC in man in the dose range studied.

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Biologic half-life—dose dependency
Liver perfusion, isolated—experimental technique
Pharmacokinetics—BHC distribution, elimination

Comparative Pharmacokinetics of Coumarin Anticoagulants IV

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THERE ARE NOW several examples of drugs which are eliminated exponentially from the plasma (following absorption and initial distribution) but where the apparent first-order rate constant for drug elimination decreases with increasing dose (1). One of the earliest and best documented examples is the elimination of bishydroxycoumarin (BHC) in man (2, 3) and monkeys (4). Recent investigations with isolated perfused rat livers have shown that the distribution of BHC into the liver (*i.e.*, the site of biotransformation) increases with increasing drug concentration but that BHC apparently inhibits its own biotransformation when high concentrations of the drug are present in the liver (5). Physical-chemical studies of the interaction between BHC and plasma proteins

Received May 22, 1968 from the Biopharmaceutics Laboratory, Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214
Accepted for publication July 3, 1968.
Supported in part by U. S. Public Health Service grant HE 8058-06.
The authors express their appreciation to Dr. M. Gibaldi, and Mr. Wm. T. Tusko, Department of Pharmaceutics, and Dr. S. Ohki, Department of Biophysics, State University of New York at Buffalo, for stimulating discussions and helpful review of the paper.
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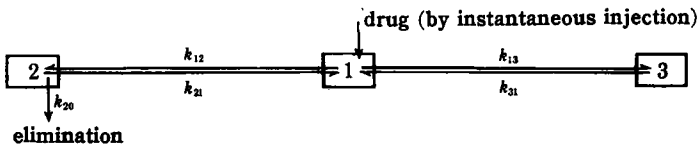
have shown also that the protein binding of BHC *increases* with increasing drug concentrations up to a point (6). Accordingly, it appears that the dose dependency of BHC elimination in man may be due to both distribution effects and apparent self-inhibition of biotransformation. Plasma concentration data for BHC following intravenous administration of this drug at three different doses, utilizing a very intensive blood sampling schedule, have been obtained so that a detailed pharmacokinetic analysis of BHC elimination in man could be carried out. For this purpose, a three-compartmental open pharmacokinetic model has been developed. This model has the unique but realistic feature of representing the actual plasma volume as the first compartment. As such, it overcomes the definite limitations of the single- and two-compartmental models which are apparent in the analysis of the experimental data obtained following BHC administration.

THEORETICAL

When a drug is administered intravenously it is first distributed in the plasma and from there to other tissues. These other tissues can be classified conveniently as well perfused and poorly perfused, respectively (see *Reference 8* for a review of the literature on this subject). The usual two-compartmental pharmacokinetic models combine the plasma and the well perfused tissues into a single compartment. The basis of the three-compartmental model to be presented here is the concept that drug distribution between plasma and well perfused tissues is not instantaneous, and that it is realistic therefore to treat these as two separate compartments. This is particularly appropriate in the case of certain highly plasma protein bound or macromolecular substances. The concept of the plasma as the first compartment is justified when values of dose/$(P + A + B)$ are essentially equal to the actual plasma volume. A pharmacokinetic analysis of this type usually requires very intensive blood sampling; where technical difficulties make this impossible, the use of simpler mathematical models becomes appropriate.

The three-compartmental open model may be depicted by Scheme I.



Scheme I

METHODS

Experimental Details—BHC was administered intravenously to a normal male subject (age 51 years, body weight 70 kg.) as the disodium salt in a concentration of 5 mg./ml. of solution. Doses of 150, 286, and 600 mg. BHC were administered in separate experiments. The drug was injected slowly over a period of 5 to 30 min., depending on the size of the dose. The instant when the injection had been completed was taken as zero time. Blood samples were collected at the times indicated in the figures. The concentration of BHC in the plasma was determined by the method of Axelrod *et al.* (7). Additional details have been described in a previous report (2).

Data Analysis—The plasma concentrations (C_p) of BHC were plotted as a function of time on semilogarithmic paper. Using back-extrapolation procedures, each of the curves was resolved into three exponential components. The data could therefore be described by the equation:

$$C_p = P \exp(-\pi t) + A \exp(-\alpha t) + B \exp(-\beta t) \quad \text{(Eq. 1)}$$

where P, A, and B represent intercepts on the ordinate at zero time and $-\pi/2.3$, $-\alpha/2.3$, and $-\beta/2.3$ are the slopes of the respective exponential segments. By definition, $\pi > \alpha > \beta$.

Compartment 1 represents the plasma volume Compartment 2 is designated as the rapidly accessible compartment [*i.e.*, the well perfused tissues such as the liver, kidneys, and viscera (9)], and Compartment 3 is designated as the slowly accessible compartment (*i.e.*, the poorly perfused tissues such as muscle, bone, and fat depots). Drug elimination is assumed to take place in Compartment 2. The processes designated by arrows and rate constants are conceived to be apparent first-order, the rate constants being defined with respect to the amount (rather than concentration) of the drug in the respective compartment. The classification of body tissues on the basis of rate of accessibility or perfusion does not imply that prefusion rate is necessarily a limiting step in the transfer of the drug from one real or hypothetical compartment to the other. It should be noted that the model presented here applies only to drugs such as bishydroxycoumarin which are eliminated by biotransformation and not by renal excretion from Compartment 1.

Evaluation of Rate Constants—As shown in *Appendix I*, the fraction of the administered dose of a drug present in each of the three compartments at time t is given by:

$$X_1/X_0 = C_1 \exp(-b_1 t) + C_2 \exp(-b_2 t) + C_3 \exp(-b_3 t) \quad \text{(Eq. 2)}$$

$$X_2/X_0 = C_1' \exp(-b_1 t) + C_2' \exp(-b_2 t) + C_3' \exp(-b_3 t) \quad \text{(Eq. 3)}$$

$$X_3/X_0 = C_1'' \exp(-b_1 t) + C_2'' \exp(-b_2 t) + C_3'' \exp(-b_3 t) \quad \text{(Eq. 4)}$$

where X_1, X_2, and X_3 are the amounts of the drug in Compartments 1, 2, and 3, respectively, X_0 is the dose (the initial amount of drug in Compartment 1) and C's and b's are time-independent constants. It is important to note that the right sides of the three equations differ only with respect to the C's. In particular, Eq. 2, which represents the fraction of the dose in the plasma, is equivalent to Eq. 1 which describes plasma concentration of the drug as a function of time. This is so, since

$$\frac{X_1}{X_0} = \frac{C_p}{C_p^0}$$

and, by definition,

$$C_p^0 = P + A + B$$

Therefore, the following relationships apply:

$$\left.\begin{aligned} b_1 &= \pi & C_1 &= P/C_p^0 \\ b_2 &= \alpha & C_2 &= A/C_p^0 \\ b_3 &= \beta & C_3 &= B/C_p^0 \end{aligned}\right\} \quad \text{(Step I)}$$

π, α, and β, as well as P, A, and B are obtained directly from the plasma concentration data.

Let

$$K_1 = k_{12} + k_{13}$$
$$K_2 = k_{21} + k_{20}$$
$$K_3 = k_{31}$$

Then, as shown in *Appendix I*,

$$\left.\begin{aligned} K_2 &= (R + Q)/2 \\ K_3 &= (R - Q)/2 \end{aligned}\right\} \quad \text{(Step III)}$$

and

where

$$\left.\begin{aligned} R &= C_1(b_3 - b_1) + C_2(b_3 - b_2) + b_1 + b_2 \\ Q &= [\{C_1(b_3 - b_1) + C_2(b_3 - b_2) + b_1 - b_2\}^2 + 4C_2(b_2 - b_1)(b_3 - b_2)]^{1/2} \end{aligned}\right\} \quad \text{(Step II)}$$

Let

$$\left.\begin{aligned} G_1 &= b_1 + b_2 + b_3 \\ G_2 &= b_1 b_2 + b_2 b_3 + b_3 b_1 \\ G_3 &= b_1 b_2 b_3 \end{aligned}\right\} \quad \text{(Step IV)}$$

Then,

$$K_1 = G_1 - K_2 - K_3 \quad \text{(Step V)}$$

Knowing the values for K_1, K_2, and K_3, the individual rate constants k_{12}, k_{21}, k_{20}, and k_{13} can be determined from:

$$\left.\begin{aligned} k_{13} &= \frac{G_3 - K_3(G_2 - K_1K_3 - K_2K_3)}{K_3(K_3 - K_2)} \\ k_{12} &= K_1 - k_{13} \\ k_{21} &= (K_1K_2 + K_2K_3 + K_3K_1 - G_2 - k_{13}K_3)/k_{12} \\ k_{20} &= K_2 - k_{21} \end{aligned}\right\} \quad \text{(Step VI)}$$

Recall that $k_{31} = K_3$. It should be noted that k_{20} represents the intrinsic drug-metabolizing activity of the body and excludes factors which affect the distribution of the drug in the three compartments. The latter factors are represented by k_{12}, k_{21}, k_{13}, and k_{31}.

The sequence of calculations by which the rate constants are determined have been outlined for the convenience of the reader in terms of 6 separate steps. In Step I, b and C values are calculated. These are then used in Step II to calculate R and Q. This permits determination of K_2 and K_3 in Step III. Determination of G values in Step IV permits the determination of K_1 in Step V. All terms necessary for the determination of k values in Step VI are now available. While these calculations can be carried out manually, the use of a computer will result in considerable saving of time.

An Analysis of the Relative Effect of Distribution and Biotransformation on the Slope of the Terminal Linear Portion (β-Phase) of a Log Plasma Concentration versus Time Plot—In the course of an experiment, the curves expressed by Eqs. 2, 3, and 4 eventually reduce to:

$$X_1/X_0 = C_3 \exp(-\beta t) \quad \text{(Eq. 5)}$$

$$X_2/X_0 = C_3' \exp(-\beta t) \quad \text{(Eq. 6)}$$

$$X_3/X_0 = C_3'' \exp(-\beta t) \quad \text{(Eq. 7)}$$

where $\beta = b_3$ as defined previously. Therefore, if the fractions of the dose in each of the compartments are plotted semilogarithmically against time, curves are obtained which become linear in the terminal portion (β-phase). Let the total amount of the drug in the body at any given time be X_B so that

$$X_B = X_1 + X_2 + X_3$$

Also let

$$f_1 = X_1/X_B$$
$$f_2 = X_2/X_B$$
$$f_3 = X_3/X_B$$

The fractions f_1, f_2, and f_3, therefore, become constant during the β-phase and may be expressed on the basis of Eqs. 5, 6, and 7 as:

$$f_1 = C_3/(C_3 + C_3' + C_3'')$$
$$f_2 = C_3'/(C_3 + C_3' + C_3'')$$
$$f_3 = C_3''/(C_3 + C_3' + C_3'')$$

Substituting for C_3, C_3', and C_3'' the equations presented in *Appendix I* yield:

$$f_1 = (K_2 - b_3)(K_3 - b_3)/Z \quad \text{(Eq. 8)}$$

$$f_2 = k_{12}(K_3 - b_3)/Z \quad \text{(Eq. 9)}$$

$$f_3 = k_{13}(K_2 - b_3)/Z \quad \text{(Eq. 10)}$$

where

$$Z = (K_2 - b_3)(K_3 - b_3) + k_{12}(K_3 - b_3) + k_{13}(K_2 - b_3).$$

The total amount of the drug in the body at a given time, X_B, may be expressed as:

$$X_B = X_1/f_1 \quad \text{(Eq. 11)}$$

and

$$X_1 = V_p \cdot C_p \quad \text{(Eq. 12)}$$

where V_p is the actual plasma volume and C_p is drug concentration in the plasma. Since during the β-phase,

$$C_p = B \exp(-\beta t) \quad \text{(Eq. 13)}$$

Eqs. 11, 12, and 13 may be combined to yield:

$$X_B = \frac{V_p}{f_1} B \exp(-\beta t) \quad \text{(Eq. 14).}$$

At $t = 0$, Eq. 14 reduces to:

$$X_B^0 = \frac{V_p}{f_1} \cdot B \quad \text{(Eq. 15)}$$

which upon substitution into Eq. 14 yields:

$$X_B = X_B^0 \exp(-\beta t) \quad \text{(Eq. 16)}$$

It should be noted that X_B^0 is not the dose X_0 but a quantity defined by Eq. 15. Equation 16 shows that the apparent first-order rate constant for the elimination of drug from the body as a whole is identical to the apparent rate constant obtained from plasma concentration data in the terminal phase of the elimination process. Therefore, the value $0.693/\beta$ is justifiably called "body half-life" or "biologic half-life." This reasoning leads to the following expression for drug elimination from the body during the β-phase:

$$\frac{dX_B}{dt} = -\beta X_B \quad \text{(Eq. 17)}$$

In terms of the three-compartmental model, where drug elimination takes place only in Compartment 2,

$$\frac{dX_B}{dt} = -k_{20} \cdot X_2$$

and, as shown previously,

$$X_2 = f_2 \cdot X_B.$$

Hence,

$$\frac{dX_B}{dt} = -k_{20} \cdot f_2 \cdot X_B \quad \text{(Eq. 18)}$$

Equating Eqs. 17 and 18, yields:

$$\beta = f_2 \cdot k_{20} \quad \text{(Eq. 19)}$$

Equation 19 has important implications in that it shows that β is a function of the distribution of the drug in the body (specifically, the fraction f_2 of the total amount of the drug which is in the drug-metabolizing compartment) and of the intrinsic drug metabolizing activity as represented by the rate constant k_{20}. This conclusion has been verified experimentally in this laboratory (5).

The derivations presented here lead to a concept of an apparent volume of distribution which has considerable practical advantages. Since, during the β-phase of drug elimination, f_1 is a time-independent constant, the following definition may be introduced

$$V_d^* = \frac{V_p}{f_1} \quad \text{(Eq. 20)}$$

The amount of drug in the body at any time during the β-phase, can therefore be calculated from the expression:

$$X_B = V_d^* \cdot B \exp(-\beta t) \quad \text{(Eq. 21)}$$

which is based on Eq. 14. This volume of distribution is totally different from that defined by Riggs (10) and adopted by Wagner and Northam (11), and Riegelman *et al.* (12). Their volume of distribution multiplied by plasma concentration equals the amount of drug in the body only at the one instant when the amount of the drug in the "peripheral" compartment reaches its maximum and cannot be used to determine the amount of drug in the body from drug concentrations in the plasma at any other time (15).

To calculate V_d^* from Eq. 20, V_p may be obtained from the body weight [plasma volume is about 4.3% of body weight (13)], and f_1 is calculated from Eq. 8 which consists of terms the determination of which is described in the first part of this paper. The use of a digital computer for these calculations is desirable since any rounding-off of values during the large number of individual calculations can lead to large errors in the final results. In the present study, the original data introduced into the computer program were expressed in two or three significant figures, and the computer carried out all subsequent calculations to a maximum of nine significant figures. The final answers printed out by the computer were then rounded off to three figures.

Determination of the Apparent Volumes of the Rapidly and Slowly Accessible Hypothetical Compartments—Defining

$$V_d^* = V_p + V_2 + V_3$$

where V_2 and V_3 are the apparent volumes of Compartment 2 and Compartment 3 with respect to a specific drug, and

$$C_p V_d^* = \frac{C_p V_p}{f_1} = \frac{C_p V_2}{f_2} = \frac{C_p V_3}{f_3} \quad \text{(Eq. 22)}$$

then

$$\frac{V_p}{f_1} = \frac{V_2}{f_2}$$

and

$$V_2 = V_p \cdot \frac{f_2}{f_1}$$

Similarly,

$$V_3 = V_p \cdot \frac{f_3}{f_1}$$

The values of V_2 and V_3 can therefore be determined from V_p and Eqs. 8 to 10.

RESULTS AND DISCUSSION

Primary Analysis of the BHC Plasma Concentration Data—The plasma concentrations of BHC following intravenous administration of 150, 286, and 600 mg. are listed in Table I and plotted in Fig. 1. Secondary concentration maxima were noted 20 to 24 hr. following injection of the two higher doses. The reason for the occurrence of these maxima, which reflect apparently a redistribution of the drug, is not clear at present. The pharmacokinetic analysis of the data was limited

TABLE I—CONCENTRATIONS OF BISHYDROXYCOUMARIN (BHC) IN THE PLASMA FOLLOWING INTRAVENOUS ADMINISTRATION OF DIFFERENT DOSES OF BHC TO A NORMAL HUMAN SUBJECT[a]

Dose, mg.					
150		286		600	
Time, hr.	Concn., mg./l.	Time, hr.	Concn., mg/l.	Time, hr.	Concn., mg./l.
0.17	36.2	0.17	60.5	0.17	120
0.33	34.0	0.51	49.8	0.33	111
0.50	27.0	1	37.2	0.50	104
0.67	23.2	2.83	27.8	0.67	96
1	20.8	5	22.2	1	93
1.5	17.8	7.25	18.1	1.5	86
2	16.5	11	14.1	2	86
3	13.9	13	12.6	3	74
4	12.0	21	8.4	5	70
6	8.7	24.3	8.3	7.5	65
7.7	7.7	36	5.6	18	44
18	3.2	48	2.6	21	39
23.3	2.4			27	34
				32	38
				42	29
				48	27
				68.5	17
				92	9
				121	4

[a] Male, 70 kg. body weight, 51 years old.

therefore to the first 20 to 24-hr. portion of the curves and these data are presented in a more expanded form in Fig. 2. The results of the resolution of these data into three exponential components are shown in Figs. 2 and 3. The values of the intercepts P, A, and B, and of π, α, and β for the three segments are listed in Table II. It is evident that π and α are dose independent while β decreases with increasing dose. The values for dose/$(P + A + B)$ listed in Table II, and particularly the value for the lowest dose, are in good agreement with the plasma volume, *i.e.*, about 3 l. for a 70-kg. man (13). It is to be expected that the long injection times for the two higher doses will have had a pronounced distorting effect on P and a somewhat smaller effect on A. Since π is equivalent to a $t_{1/2}$ of about 20 min. while the time required for the injection of the 600-mg. dose was 30 min., a negative time shift was carried out in the case of the 286-mg. and 600-mg. data, using the π, α, and β values listed in Table II but setting dose/$(P + A + B)$ = 3.2 l. The corrected P, A, and B values obtained by this procedure are listed in Table III. The zero time shift required for these corrections was −19 min. in each instance.

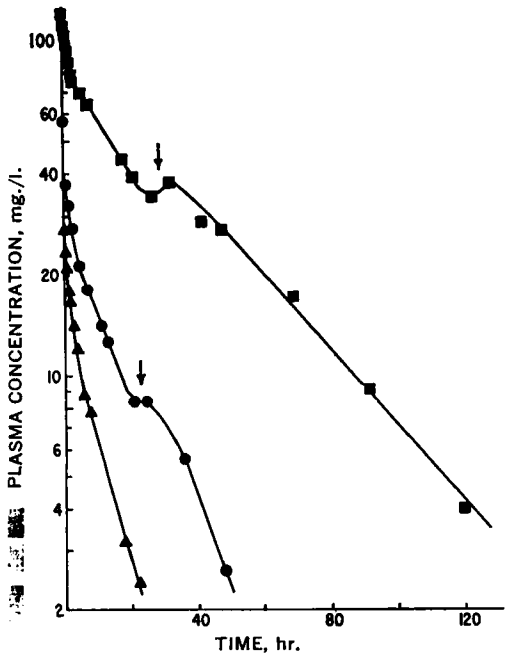


Fig. 1—Concentration of bishydroxycoumarin in the plasma as a function of time after intravenous administration of 150 mg., ▲; 286 mg., ●; and 600 mg., ■, respectively, to a normal human subject. The arrows indicate secondary concentration maxima observed 1 day after drug administration.

Determination of Pharmacokinetic Constants Based on a Three-Compartmental Open System—Using the mathematical procedures described in the Theoretical section, the apparent first-order rate constants k_{20}, k_{12}, k_{21}, k_{13}, and k_{31} were computed from the values for π, α, and β (Table II) and from the corrected values for P, A, and B (Table III) by means of an IBM 7044 digital computer. The results of these computations are listed in Table IV. Essentially similar values for all rate constants except for k_{20} were obtained at the three doses, but k_{20} decreased with increasing dose. The distribution of BHC in the three compartments as a function of dose is listed in Table V which shows the fraction of the total amount of the drug in each of the three compartments. It can be noted that f_2, the fraction of the total amount of drug present in the compartment in which the drug is metabolized, shows no consistent change with dose. This leads to the significant conclusion, based on the relationship represented by Eq. 19, that the dose dependence in the elimination of BHC as reflected by a decrease in β with increasing dose is due to a decrease in the intrinsic drug-metabolizing activity and not to distribution effects.

Finally, a comparison of the product of f_2 and k_{20} as obtained from the lengthy and complex calculations described in the *Theoretical* section with

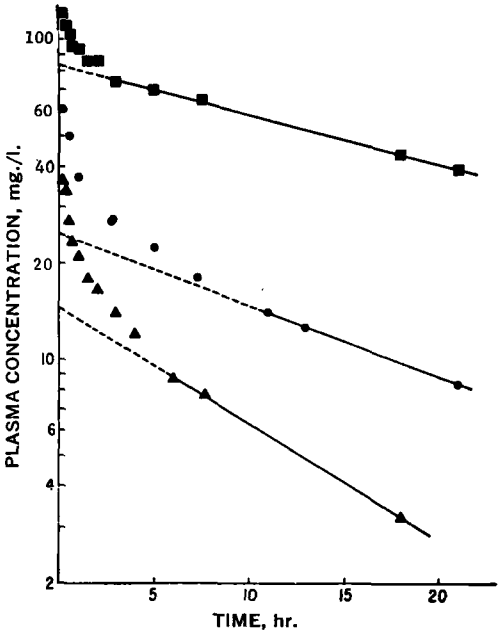


Fig. 2—The data from Fig. 1 presented on an expanded scale over 24 hr. after drug administration. The slope of the linear segment of each curve is designated as $-\beta/2.3$ and the intercept on the ordinate at zero time is designated as B. *Symbols as in Fig. 1.*

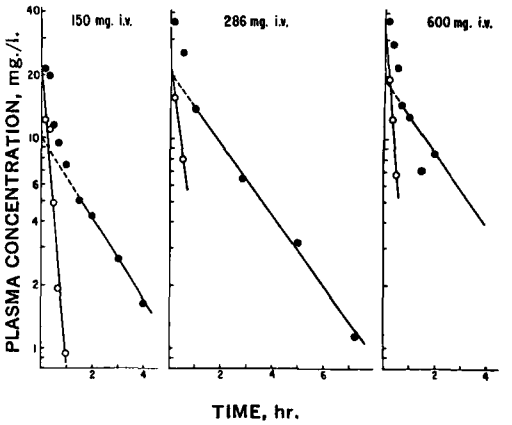


Fig. 3—Plots of residuals obtained by subtracting from the initial plasma concentrations of bishydroxycoumarin shown in Fig. 2, the respective theoretical concentration values represented by the stippled lines (solid symbols), and by subtracting from the initial data points represented by the solid symbols in this figure the theoretical values represented by the stippled lines (open symbols). The slope of the line fitted to the solid symbols is designated as $-\alpha/2.3$ and the intercept at zero time is designated as A. *The slope of the line fitted to the open symbols is designated as $-\pi/2.3$ and the intercept at zero time is* P.

TABLE II—PHARMACOKINETIC PARAMETERS OBTAINED BY RESOLVING THE BHC PLASMA CONCENTRATION DATA INTO THREE COMPONENTS

Parameter[a]	Dose, mg. 150	286	600	Units
P	22	22	34	mg./l.
A	10	22	19	mg./l.
B	14.5	24.5	83	mg./l.
Dose/$(P + A + B)$	3.2	4.2	4.4	l.
π	3.5	2.3[b]	3.5	hr.$^{-1}$
α	0.46	0.41	0.41	hr.$^{-1}$
β	0.085	0.051	0.036	hr.$^{-1}$

[a] $C_p = P \exp(-\pi t) + A \exp(-\alpha t) + B \exp(-\beta t)$ where C_p is the plasma concentration of BHC at time t. The other symbols are defined in the text and in Figs. 2 and 3. [b] Based on 2 data points only.

β values obtained by simply fitting a straight line to the terminal log plasma concentration values provides a direct check on the mathematical derivations and the accuracy of the calculations. The $f_2 \cdot k_{20}$ values calculated from the data in Tables IV and V are 0.085, 0.052, and 0.036 hr.$^{-1}$ for the 150-, 286-, and 600-mg. doses, respectively. These agree perfectly with the β values listed in Table II.

TABLE III—THE VALUES FOR P, A, AND B WHEN CORRECTED FOR VARIABLE DURATION OF THE INTRAVENOUS INJECTION[a]

	Dose, mg. 150	286	600
P	22	40	81
A	10	23	22
B	14.5	25	84
Zero time shift, min.	−0	−19	−19

[a] Expressed as mg./l. The corrections are based on an initial value for dose/$(P + A + B)$ of 3.2 l.

APPENDIX I

The transfer and elimination processes in the three-compartmental open system presented in the *Theoretical* section may be described as follows:

$$\frac{dX_1}{dt} = -K_1X_1 + k_{21}X_2 + k_{31}X_3$$

$$\frac{dX_2}{dt} = -K_2X_2 + k_{12}X_1$$

$$\frac{dX_3}{dt} = -K_3X_3 + k_{13}X_1$$

In these expressions, X is the amount of drug in the designated compartment, the numerical subscripts for X define the compartment, the respective k's are defined in the model presented in the *Theoretical* section, and

$$K_1 = k_{12} + k_{13} \qquad \text{(Eq. 1}a\text{)}$$

TABLE IV—APPARENT FIRST-ORDER RATE CONSTANTS CHARACTERIZING THE THREE-COMPARTMENTAL OPEN SYSTEM FOR THE DISTRIBUTION AND ELIMINATION OF BHC[a]

Rate Constants, hr.$^{-1}$	Dose, mg. 150	286	600
k_{20}	0.295	0.235	0.11
k_{12}	1.56	0.91	1.44
k_{21}	1.67	1.13	1.93
k_{13}	0.22	0.26	0.14
k_{31}	0.30	0.23	0.32

[a] See Scheme I in the *Theoretical* section.

TABLE V—DISTRIBUTION PATTERN OF BHC IN THE TERMINAL ELIMINATION PHASE (β-PHASE)

Compartment[a]	Dose, mg. 150	286	600
1, plasma (f_1)	0.35	0.31	0.455
2, rapidly accessible, drug eliminating (f_2)	0.29	0.22	0.33
3, slowly accessible (f_3)	0.36	0.47	0.215

[a] f_1, f_2, and f_3 are the fractions of the total amount of drug in the body present in Compartments 1, 2, and 3, respectively, and were calculated from Eqs. 8, 9, and 10.

$$K_2 = k_{20} + k_{21} \qquad \text{(Eq. 2}a\text{)}$$

$$K_3 = k_{31} \qquad \text{(Eq. 3}a\text{)}$$

In order to solve the above simultaneous differential equations by using Laplace transforms, it is convenient to set up the following determinant:[1]

$$\Delta = \begin{vmatrix} s + K_1 & -k_{21} & -k_{31} \\ -k_{12} & s + K_2 & 0 \\ -k_{13} & 0 & s + K_3 \end{vmatrix} = (s + b_1)(s + b_2)(s + b_3) \qquad \text{(Eq. 4}a\text{)}$$

where $-b_1$, $-b_2$, and $-b_3$ are the real roots of the equation, $\Delta = 0$. With the initial condition that $X_1 = X_0 =$ dose when $t = 0$, the fractions of the dose in each of the three compartments are:

$$X_1/X_0 = C_1 \exp(-b_1 t) + C_2 \exp(-b_2 t) + C_3 \exp(-b_3 t)$$

$$X_2/X_0 = C_1' \exp(-b_1 t) + C_2' \exp(-b_2 t) + C_3' \exp(-b_3 t)$$

$$X_3/X_0 = C_1'' \exp(-b_1 t) + C_2'' \exp(-b_2 t) + C_3'' \exp(-b_3 t)$$

where the C values (with various superscripts and subscripts) are constants. These may be evaluated on the basis of the following relationships:

$$C_j = \left[\frac{\Delta_{1,1}}{\Delta'}\right] s = -b_j$$

$$C_j' = \left[\frac{-\Delta_{1,2}}{\Delta'}\right] s = -b_j$$

$$C_j'' = \left[\frac{\Delta_{1,3}}{\Delta'}\right] s = -b_j$$

where the subscript j represents either subscript 1, 2, or 3, $\Delta_{1,i}$ is the determinant obtained from Δ by suppressing the first row and the ith column,[2] and Δ' is $d\Delta/ds$ of the right hand of Eq. 4a.[3]

$$C_1 = \frac{(K_2 - b_1)(K_3 - b_1)}{(b_2 - b_1)(b_3 - b_1)};$$

$$C_2 = \frac{(K_2 - b_2)(K_3 - b_2)}{(b_1 - b_2)(b_3 - b_2)};$$

$$C_3 = \frac{(K_2 - b_3)(K_3 - b_3)}{(b_1 - b_3)(b_2 - b_3)}$$

$$C_1' = \frac{k_{12}(K_3 - b_1)}{(b_2 - b_1)(b_3 - b_1)};$$

$$C_2' = \frac{k_{12}(K_3 - b_2)}{(b_1 - b_2)(b_3 - b_2)};$$

$$C_3' = \frac{k_{12}(K_2 - b_3)}{(b_1 - b_3)(b_2 - b_3)}$$

$$C_1'' = \frac{k_{13}(K_2 - b_1)}{(b_2 - b_1)(b_3 - b_1)};$$

$$C_2'' = \frac{k_{13}(K_2 - b_2)}{(b_1 - b_2)(b_3 - b_2)};$$

$$C_3'' = \frac{k_{13}(K_2 - b_3)}{(b_1 - b_3)(b_2 - b_3)}$$

The next step is to develop equations from which the values for k_{20}, k_{12}, k_{21}, k_{13}, and k_{31} may be determined from the known values for b_1, b_2, b_3, C_1, C_2, and C_3. Rearrangement and expansion of the equation for C_1 yields:

$$C_1(b_2 - b_1)(b_3 - b_1) = K_2K_3 - b_1(K_2 + K_3) + b_1^2 \qquad \text{(Eq. 5}a\text{)}$$

Similarly for C_2:

$$C_2(b_1 - b_2)(b_3 - b_2) = K_2K_3 - b_2(K_2 + K_3) + b_2^2 \qquad \text{(Eq. 6}a\text{)}$$

Defining

$$R = K_2 + K_3 \qquad \text{(Eq. 7}a\text{)}$$

$$q = K_2K_3 \qquad \text{(Eq. 8}a\text{)}$$

and solving for R and q from the simultaneous Eqs. 5a and 6a, yields:

$$R = C_1(b_3 - b_1) + C_2(b_3 - b_2) + b_1 + b_2 \qquad \text{(Eq. 9}a\text{)}$$

$$q = b_2[C_1(b_3 - b_1) + C_2(b_3 - b_2) + b_1] + C_2(b_1 - b_2)(b_3 - b_2) \qquad \text{(Eq. 10}a\text{)}$$

From Eqs. 7a and 8a, and on the basis of $K_2 > K_3$ (see *Appendix II*),

$$K_2 = (R + \sqrt{R^2 - 4q})/2 \qquad \text{(Eq. 11}a\text{)}$$

$$K_3 = (R - \sqrt{R^2 - 4q})/2 \qquad \text{(Eq. 12}a\text{)}$$

Substituting Eqs. 9a and 10a for R and q in Eqs. 11a and 12a, and simplifying, gives the following

[1] The reader may find it helpful to refer to *Reference 14* for examples of similar derivations based on other models.

[2] For example,

$$\Delta_{1,2} = \begin{vmatrix} -k_{12} & 0 \\ -k_{13} & s + K_3 \end{vmatrix} = -k_{12}(s + K_3).$$

[3] Specifically,

$$\Delta' = (s + b_2)(s + b_3) + (s + b_1)(s + b_3) + (s + b_1)(s + b_2).$$

TABLE VI—CONSTANTS DESCRIBING THE DISTRIBUTION AND ELIMINATION OF BHC ASSUMING THAT $K_3 > K_2$ (SEE *Appendix II*)

Constants[a]	Dose, mg. 150	286	600
k_{20}	0.152	0.0845	0.118
k_{12}	0.457	0.416	0.215
k_{21}	0.145	0.140	0.209
k_{13}	1.32	0.751	1.37
k_{31}	1.97	1.37	2.04
f_1	0.26	0.25	0.41
f_2	0.56	0.60	0.31
f_3	0.18	0.14	0.28

[a] Units of k = hr.$^{-1}$; f has no units.

equations for K_2 and K_3:

$$K_2 = (R + Q)/2$$

$$K_3 = (R - Q)/2$$

where $Q = \sqrt{R^2 - 4q}$. These are the equations for K_2 and K_3 presented in the Theoretical section.

Expanding both sides of Eq. 4*a* and identification of equivalent terms yields:

$$b_1 + b_2 + b_3 = K_1 + K_2 + K_3 \quad \text{(Eq. 13}a\text{)}$$

$$b_1b_2 + b_2b_3 + b_3b_1 = K_1K_2 + K_2K_3 + K_3K_1 - k_{12}k_{21} - k_{13}k_{31} \quad \text{(Eq. 14}a\text{)}$$

$$b_1b_2b_3 = K_1K_2K_3 - k_{12}k_{21}K_3 - k_{13}k_{21}K_2 \quad \text{(Eq. 15}a\text{)}$$

K_1 can now be evaluated from Eq. 13*a*, since K_2 and K_3 are already known. The values for $k_{12}k_{21}$ and $k_{13}k_{31}$ are obtained from the two simultaneous equations 14*a* and 15*a*. Since $k_{31} = K_3$ and has already been determined from Eq. 12*a*, k_{13} can be determined from the value of $k_{13}k_{31}$. The value of k_{12}, obtained from Eq. 1*a*, is used to solve for k_{21} from $k_{12}k_{21}$. Finally, k_{20} is obtained from Eq. 2*a*, since k_{21} and K_2 are now known.

APPENDIX II

The data treatment leading to Eqs. 11*a* and 12*a* assumes that $K_2 > K_3$ (*i.e.*, $k_{20} + k_{21} > k_{31}$). The opposite assumption ($K_3 > K_2$) resulted in $k_{13} > k_{12}$, which is inconsistent with the proposed model. However, this latter possibility (which means that drug metabolism occurs in the slowly accessible compartment) cannot be excluded solely on the basis of the plasma concentration data although it is unlikely on the basis of physiologic considerations. A similar uncertainty occurs in the use of two-compartment models although this has apparently been disregarded so far in the pharmacokinetic literature.

When the experimental data were analyzed on the basis of the assumption that $K_3 > K_2$, k_{20} did not change consistently with dose but k_{12} decreased with increasing dose (Table VI). It is interesting that the k_{12} values are quite similar to the k_{21} values, and k_{13} values to k_{31} values, in the model assuming drug metabolism in the rapidly accessible compartment (Table V). This is not the case where $K_3 > K_2$ (Table VI). Preliminary evidence based on the relationship between the plasma concentration and pharmacologic activity of BHC in man is consistent with the assumption that $K_2 > K_3$. However, a definitive conclusion concerning the two possibilities will require determinations of BHC biotransformation rates at various drug concentrations in human liver preparations.

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Keyphrases

Coumarin anticoagulants

Pharmacokinetics—bishydroxycoumarin elimination

Dos-dependence—bishydroxycoumarin elimination

Plasma concentration—bishydroxycoumarin

Kinetic equations—three-compartmental model

Rate Studies on the Binding of Bilirubin by Ion-Exchange Resins

By R. J. SAWCHUK and J. G. NAIRN

The rate of binding of bilirubin by strongly basic anion-exchange resins was determined by the batch method. The rate of uptake of bilirubin from solution was found to follow a second-order rate expression, and the rate constant was dependent upon the size of the resin beads and the degree of crosslinking of the resin. It was concluded that the rate of binding, which was influenced by the fractional pore volume of the resin phase, was controlled mainly by a particle diffusion mechanism. Aqueous alkaline solutions of bilirubin of improved stability were prepared in order to study the binding phenomena.

A FEW CASES have been reported in which abnormally high serum levels of bilirubin in humans and animals have been decreased by the oral administration of an ion-exchange resin. Lester *et al.* (1) having investigated both the absorption and transport of unconjugated bilirubin across the intestinal mucosa, made the following postulation, "If it were possible to achieve irreversible binding of intestinal bilirubin, its reabsorption would be prevented, the efficiency of pigment disposition would be increased, and the plasma level (and, consequently, the level in the total miscible pool) would be reduced to a new, lower plateau." They demonstrated that high serum levels of bilirubin in rats, due to administration of labeled bilirubin, could be effectively reduced by adding an ion-exchange resin to the rat's diet. A report (2) of a case of xanthomatous biliary cirrhosis in a patient showing a slowly progressive jaundice described the reduction of bilirubin levels after administration of an ion-exchange resin. A reduction in serum levels of bilirubin has been obtained using an ion-exchange resin in a case of intrahepatic biliary atresia (3). A strongly basic quaternary ammonium ion-exchange resin has also been used to bind bile acids (4, 5).

Some work has been published on the *in vitro* uptake of bilirubin by ion-exchange resins. The pigment has been removed from solutions by a resin in a column (3). The transfer of bilirubin from albumin to resin has also been achieved (6). Other papers (7, 8) describe the problems of eluting bilirubin from ion-exchange resins. Preliminary work for the present research involved several attempts to elute bilirubin from ion-exchange resins, which had previously been in contact with a solution of bilirubin. These attempts included both column and batch methods. Solvents such as dioxane, tetrahydrofuran, and aqueous solutions of sodium hydroxide, sodium acetate, or hydrochloric acid were employed; however, satisfactory removal of bilirubin from ion-exchange resins was not achieved.

The purpose of this research was to investigate the rate of binding of bilirubin to ion-exchange resins *in vitro*. The rate of binding of bilirubin as a function of crosslinking and particle size of the resin was studied in detail so that properties of the resin which control the rate could be elucidated. This was achieved by allowing solutions of bilirubin to come in contact with resin beads using the batch technique.

The lack of stability of bilirubin in both organic and aqueous solvents has posed a serious problem to researchers and clinicians. Several factors, such as light, oxygen, pH, metals, solvent, and temperature cause decomposition (9–14). The kinetics of decomposition have also been reported (15, 16). Many agents and techniques have been employed to stabilize solutions of bilirubin, for example: serum (14, 17), temperature (18), chemicals (19–21), a complexing agent (14), and nitrogen (22). Unfortunately, as the solubility of bilirubin increases with pH (23) so also does the rate of decomposition (16). In this study it was desirable to minimize the decomposition of bilirubin in solution. This was achieved after considerable preliminary investigation by using a weak base to achieve a suitable pH for solubility, employing a nitrogen atmosphere to reduce oxidation, maintaining the reaction vessel in the dark, and carrying out the experiments at a low temperature.

Another serious problem which had to be solved was the deposition of bilirubin from solution onto all surfaces except a clean smooth glass surface,

Received May 27, 1968 from the Faculty of Pharmacy, University of Toronto, Toronto 5, Ontario, Canada.
Accepted for publication July 19, 1968.
Abstracted in part from the thesis submitted by R. J. Sawchuk to the School of Graduate Studies, University of Toronto, in partial fulfillment of Master of Science degree requirements.
The authors gratefully acknowledge the financial assistance of the Canadian Foundation for the Advancement of Pharmacy, the National Research Council of Canada, the University of Toronto, and the Warner-Lambert Company. The authors appreciate the donation of several ion-exchange resins by the Dow Chemical Company.

TABLE I—SUMMARY OF DATA OBTAINED FOR THE BINDING OF BILIRUBIN TO ION-EXCHANGE RESINS

Resin	Mesh Size	Average Wet Bead Radius, r, mm.[a]	Capacity, b, meq./g. dry resin[b]	Moisture Content, % w/w[b,c]	Rate Constant, k, l./(meq. hr.)[b]
1-X1	50–100	0.213	4.72	86.8	0.99
1-X2	50–100	0.179	4.42	72.0	0.65
1-X4	20–50	0.285	4.21	52.8	0.061
1-X4	50–100	0.151	4.20	53.8	0.19
1-X4	100–200	0.076	4.14	64.8	2.4
1-X4	200–400	0.038	4.08	61.3	2.2
2-X4	20–50	0.285	4.02	47.7	0.029
1-X8	50–100	0.137	3.77	45.5	0.045
2-X8	50–100	0.137	3.58	41.0	0.054

[a] The average wet bead radius was determined from data in *Reference 36*. [b] The values given are the average of duplicate determinations. [c] Fractional pore volume (ϵ) = % moisture content/100.

and consequently a special stirring apparatus was used (24).

EXPERIMENTAL

Materials—The strongly basic resins[1] were washed, and conditioned by conversion to the hydroxide, the chloride, and back to the hydroxide form. The resin was then converted to the chloride form and washed thoroughly with freshly boiled, deionized water and stored under deionized water. Samples of the conditioned resin for experiments and characterization were centrifuged at 1,300 r.p.m. for 30 min. in a sintered-glass filter tube of medium porosity to remove interstitial water. The moisture contents of the resins were determined by heating the resin in an oven at 110° for 12 hr. The scientific or equivalent weight capacities of the resins were determined by converting the resin to the hydroxide form by the column method, rinsing the resin with boiled deionized water, eluting the hydroxide with sodium chloride solution, and titrating the effluent with hydrochloric acid. The results of these determinations are given in Table I. The bilirubin used was Fisher Certified reagent grade, lot No. 740759. The molar absorptivity was within the range of proposed standards for pure bilirubin (25). IR analysis of bilirubin by the potassium bromide disk method agreed with the literature (22).

Reaction Mixtures—A blank for spectrophotometric analyses and a suitable solvent for bilirubin was prepared by adding 1.2 ml. of a 5% v/v aqueous solution of freshly distilled *n*-propylamine to 650 ml. of boiled, deionized water which had been previously treated by bubbling with high purity nitrogen for 45 min. Bilirubin, approximately 4 mg., was added in the dark to 580 ml. of the above solvent. The mixture, kept under a nitrogen atmosphere, was protected from light and shaken at room temperature for 1.5 hr. and at 3° for 0.5 hr. in order to effect solution.

An accurately weighed quantity of centrifuged resin in the chloride form, equivalent to approximately 12 mg. of dry resin was added to a conical flask onto the shoulder of which was fused a square spectrophotometric cell. The flask was covered with aluminum foil and was equipped with a stirring apparatus described previously (24). A second flask was prepared in the same manner for the duplicate experiment. A third flask without resin was used as a control. The cold bilirubin solution (190 ml.) was added to each flask in the dark, nitrogen was swept over the surface, and the flasks were stoppered.

Analyses—The percentage transmittance of the bilirubin solutions was determined (and converted to absorbance values) at 432 mμ, the wavelength of maximum absorbance, on a Bausch and Lomb Spectronic 20 spectrophotometer fitted with a square-cell adapter. The concentration of bilirubin remaining in solution was determined from a Beer-Lambert plot of bilirubin in the water–*n*-propylamine solvent.

Kinetic Studies—Spectrophotometric analyses were carried out as soon as practical, and the solution in the cell was returned to the body of the flask. The flasks were positioned in a water bath (5.3 ± 2.0°), over magnetic mixers, which had been adjusted to 360 r.p.m. The experiment was protected from light at all times. Analyses of the mixtures were made at suitable intervals, by removing the flasks from the bath, allowing the resin beads to settle, and then pouring some of the solution into the attached cell.

Ion-exchange resin beads of 100–200 and 200–400 mesh sizes were found to adhere to the flask wall, and occasionally when they entered the spectrophotometric cell, they could not be returned to the flask after the analysis had been completed. This problem of beads adhering to the glass was solved by coating the inside of the vessels with Dow Corning 200 Fluid (350 CS.) using the method recommended by the manufacturer, thereby effecting agitation of all beads by the magnetic stirrer.

RESULTS AND DISCUSSION

The capacities and moisture contents which were determined in this study are given in Table I.

The stability of the bilirubin in solution was determined by noting the change in the absorbance in the control flasks. The maximum change of absorbance in the control flasks for the data presented in Fig. 1 (*i.e.*, for the first 14 hr.) was never greater than 1%. A few of the solutions were observed for longer periods of time (about 50 hr.) when the rate of binding was very low, and in some of these experiments the change of absorbance was as high as 2.6%.

Since the binding of ions to a resin is a diffusion phenomenon (26) the rates of binding can best be compared by evaluating diffusion coefficients, which

[1] Dowex 1 and Dowex 2, Dow Chemical Co., Midland, Mich.

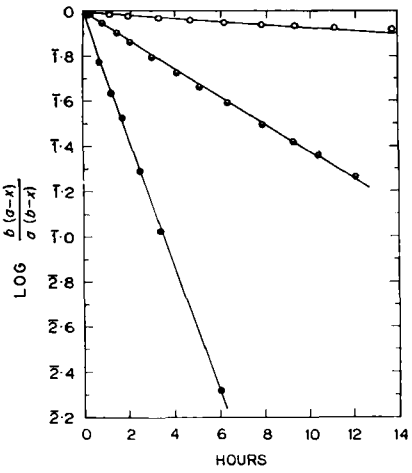

Fig. 1—Plots illustrating the removal of bilirubin from solution by ion-exchange resins. Key: ○, *1-X4 (20–50 mesh);* ◒, *1-X2 (50–100 mesh);* ●, *1-X4 (200–400 mesh).*

are obtained by considering the fractional attainment of equilibrium as a function of time. Although bilirubin was reasonably stable during the binding studies, attempts to achieve equilibrium would probably have resulted in the eventual disappearance of bilirubin from the solution as a consequence of binding to the resin and the decomposition of bilirubin in solution. For the latter reason, and since long equilibrium times would be involved where the binding rates were very low (27), and also because of the complexity of the system, an empirical rate expression was sought for the binding process. A second-order rate expression was found to be satisfactory for the present studies (28, 29) as can be seen in Fig. 1. The mathematical expression which suitably characterizes the rate of binding to resins for fast processes, and initial stages of slow processes is:

$$\ln \frac{b\,(a - x)}{a\,(b - x)} = (a - b)\,kt$$

where

a = the initial number of meq. of bilirubin in solution,

b = the initial number of meq. of ion-exchange resin in the reaction flask based on the weight of wet resin used, the moisture content of the resin, and the experimentally determined weight capacity,

$a - x$ = the number of meq. of bilirubin remaining in solution at time t,

$b - x$ = the number of meq. of ion-exchange resin remaining in the chloride form at time t,

k = second-order rate constant, 1./(meq. hr.),

t = time in hr.

It was assumed that bilirubin was bound by ion exchange and that it existed as a divalent anion under the conditions employed with an equivalent weight of 292.

The second-order rate constants were calculated from the initial slope of the lines (0 to approx. 12 hr.) obtained by plotting log $[b(a - x)]/[a(b - x)]$ against t as shown in Fig. 1. In order to obtain k in the proper units it is necessary to multiply the slope by $[2.303/(a - b)]$ [190/1,000] to change from decadic to natural logarithms, and to account for the experimental volume of 190 ml. The averages of the rate constants determined in duplicate for each resin are given in Table I.

While the binding of bilirubin obeys a second-order rate law as mentioned previously, plots over longer periods of time for slow processes, *i.e.*, binding of bilirubin to resins with a large mesh size and a high degree of crosslinking, show that the experimental points deviate from linearity with decreasing slopes as time increases. This can be explained if it is assumed that the capacity of the resin for bilirubin is less than that for the chloride ion. The reduction in capacity could be due to insufficient space within the resin structure for the accommodation of the bilirubin ion in a concentration equivalent to the total exchange capacity of the resin, even if each exchange site is accessible to the organic ion at low concentrations of bilirubin ion in the resin phase. Another explanation which has been suggested is that some regions of the resin phase are completely inaccessible to the bilirubin ion even at low concentration of the organic ion in the resin (30).

If the uptake of bilirubin by an ion-exchange resin were to be considered strictly as a chemical reaction, two ion exchangers differing only in particle size should bind the pigment at the same rate. It can be seen from Table I that this is not the case for the ion-exchange[2] resins of different mesh sizes. Particle size has a marked effect on the rate constant indicating that chemical reaction at the exchange sites is not the rate-controlling step. If the rate is controlled by diffusion into the particle it should vary as $1/r^2$. If, however, film diffusion is the controlling factor the rate should vary as $1/r$ (31). It can be seen from Table I that the rate constant k generally increases with decreasing bead radius for resins with the same crosslinking. A consideration of the 1-X4 resins indicates that the rate is primarily particle-diffusion controlled because the product kr^2 has less variation than the product kr.

The other variable studied in this research was the influence of the degree of crosslinking of the resin on the rate of binding. The crosslinking of the resin determines the swelling and the moisture content of the resin beads. Table I shows that the rate constant k increases with decreasing crosslinking for resins of the same mesh size. Resins with low crosslinking swell more in an aqueous medium and thus contain a larger proportion of water. As a result more exchange sites are available to the large organic ion. Furthermore, the fractional pore volume of the ion exchanger is increased; hence retardation of the framework will be less. Resins with a high degree of crosslinking were found to have low moisture contents and low rates of binding (Table I). A comparison of resins of the same particle size and crosslinking shows that the moisture contents of Type 1 resins are higher than those determined for Type 2 resins, however only the resins with 4% divinylbenzene content have rates of binding which are in agreement with the previous

[2] Dowex 1-X4.

statement. The effect of crosslinking on the rate of binding can be interpreted by considering that the fractional moisture content equals the fractional pore volume of the ion exchanger. The effect of the resin matrix is to retard diffusion and thus decrease the rate of binding. The reasons given by Helfferich (32) for this phenomenon are: (*a*) part of the cross-section in the ion exchanger is occupied by the polymer chains and is not available for diffusion, (*b*) the diffusion paths in the resin are more tortuous and therefore longer, (*c*) large ions or molecules in the ion exchanger may be impeded in their mobility by the framework, (*d*) interaction with fixed ionic groups may retard counterion diffusion. A quasi-homogeneous model has been described which relates diffusion coefficients in ion exchangers to those in solutions, and the diffusion coefficients have been found to depend upon the fractional pore volume of the medium (33).

In order to obtain a correlation between the second-order rate constants and both the radius and the fractional pore volume (ϵ) of the resin beads, several relationships were considered. It was found that a linear relation could be obtained by plotting kr^2 against $[\epsilon/(2 - \epsilon)]^2$ as shown in Fig. 2.

This indicates that the binding of bilirubin by anion-exchange resins is controlled by particle diffusion in this experiment, since the rate is dependent on the square of the radius and the fractional pore volume of the ion-exchange resin. Because of the dependence of the rate on the term $[\epsilon/(2 - \epsilon)]^2$ rather than $\epsilon/2$, it can be assumed that the decrease in rate is caused not only by a reduction in cross-sectional area but also by diffusion paths which are more tortuous (34).

It is not possible at this time to give a satisfactory explanation for the behavior of the 1-X4 (200–400) resin. The rate-limiting step for binding may no longer be controlled mainly by particle diffusion, as

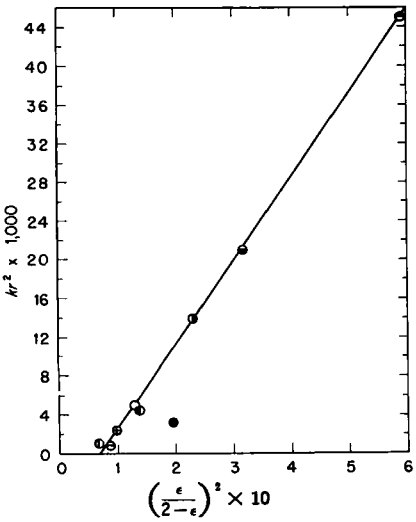

Fig. 2—Plot illustrating the relationship between the second-order rate constant, the radius, and the fractional pore volume. Key: ◓, *1-X1 (50–100 mesh);* ◒, *1-X2 (50–100 mesh);* ○, *1-X4 (20–50 mesh);* ◐, *1-X4 (50–100 mesh);* ◑, *1-X4 (100–200 mesh);* ●, *1-X4 (200–400 mesh);* ⊕, *2-X4 (20–50 mesh);* ⊖, *1-X8 (50–100 mesh);* ⊙, *2-X8 (50–100 mesh).*

the importance of film diffusion could increase as the particle size decreases (35).

Since oral administration of an ion exchanger would allow only a limited time of contact of the resin with intestinal contents, the rate of binding of bilirubin by the exchanger should be high if the resin is to be an effective therapeutic agent. On the assumption that there would be some correlation between the *in vitro* binding rates determined by this research and the rates that might occur under physiological conditions, the most effective anion-exchange resin for reducing high serum levels of bilirubin would be a Type 1 resin of low crosslinking and small bead diameter. It is to be expected that *in vivo* conditions, such as a low degree of agitation and the presence of competing ions, will alter pigment-binding rates. Nevertheless, it is quite conceivable that, as therapeutic agents, these resins would exhibit the same relative order of binding rates as was determined in this study.

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Keyphrases

Ion-exchange resin—binding
Bilirubin binding—ion-exchange resins
Kinetics—bilirubin-resin binding
Colorimetric analysis—spectrophotometer

Modified Confinement Motor Activity Test For Use in Mice

By GERALD MILNER

The modification of a confinement motor activity (CMA) unit, for use with mice, is described. Its use in quantitating the effect of drugs on the motor activity in mice is illustrated by experiments with amitriptyline and alcohol. Amitriptyline (10 mg./kg.), given orally, was found to significantly increase CMA when compared with control groups. Alcohol (25 ml./kg., 10% solution) depressed CMA in mice, but not to a statistically significant extent. When the amitriptyline and alcohol were given together, a significant depression of CMA was recorded. A positive joint action is suggested—amitriptyline, when taken by man, may add to the effects of alcohol.

MEASUREMENTS OF alterations in locomotor activity have proved valuable in testing for drug effects on animal behavior. In 1964 Tedeschi *et al.* described a photoelectric cell counting chamber (1) for measuring confinement motor activity (CMA) in rats. This consisted of a chamber so small as to prevent the rat moving from place to place, but high enough to permit the typical "up-and-down" exploratory behavior of the confined rodent. The rat's movements were counted by means of two photoelectric cells. The advantages of the CMA chamber over conventional locomotor activity units are that it is more compact, cheaper, and more sensitive—particularly when testing stimulant drugs such as caffeine, tranylcypromine, and amphetamine. Photoelectric cell activity chambers have been shown to be generally more satisfactory than mechanical tests, such as those involving rocking floors (2).

Mice are probably the most widely used and useful laboratory animals; they are small, easily maintained and bred, cheap, and can be used in large numbers for individual experiments. With many drugs, particularly alcohol, prediction of behavioral effects in man from their effects in mice have proved valid (3). The present paper describes the development of a confinement motor activity chamber for mice. Its use is illustrated by experiments involving amitriptyline and alcohol.

Amitriptyline is an iminodibenzyl drug (with a chemical formula closely related to chlorpromazine) and in doses of 30 and 50 mg./kg. body weight has been shown to significantly increase the length of loss of righting reflexes in mice given alcohol (4, 5). With alcohol levels of 25 ml./kg. of a 25% solution, the average length of loss of righting reflexes in an adult mouse is 1.2 hr., whereas a dose of 25 ml./kg. of 20% alcohol just fails to cause coma. The significant increase in the length of alcoholic coma brought about by amitriptyline might be due only to the sedation caused by each drug. A positive joint action of alcohol and amitriptyline is indicated in the experiments described in this paper, where low doses of the drugs were used.

EXPERIMENTAL

Development of a CMA Unit for Mice—Preliminary experiments enabled a rough estimate of the best size of chamber to be made. Tests were then

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performed using albino mice (Prince Henry strain, obtained from the Institute of Medical and Veterinary Science, Adelaide, South Australia). The laboratory temperature was held at 21.5 ± 1° and constant illumination was maintained. The experiments were performed at the same time each day. The mice used were matched for weight and sex; none of them had ever previously been dosed with a drug. The various factors were (*a*) weight: 20 ± 2 g., 25 ± 2 g., 30 ± 2 g.; (*b*) floor size, *i.e.*, the dimensions of the CMA chamber floor: 3.8 × 5 cm. (1.5 × 2 in.), 3.8 × 4.4 cm. (1.5 × 1.75 in.), 3.8 × 3.8 cm. (1.5 × 1.5 in.); (*c*) height, *i.e.*, the distance from the CMA chamber floor to the horizontal light beams [the two beams are parallel, 1.9 cm. (0.75 in.) apart, and each is focused on a separate photoelectric cell]: 5.6 cm. (2.25 in.), 5 cm. (2 in.) 4.4 cm. (1.75 in.); (*d*) replication, *i.e.*, with each combination of other factors a given group of mice was tested in the CMA chambers for 15 min. in each of 3 consecutive hr.: first replication, second replication, third replication; (*e*) 5-min. scores, during each replication, the score for each mouse was recorded as follows: 0–5 min., 6–10 min., 11–15 min.

In this series of preliminary experiments 2,430 different scores were collected (10 mice per group × 3 different weights × 3 different floor sizes × 3 heights × 3 replications at hourly intervals × three, 5-min. scores). A computer analysis of variance of these data was performed in order to discover which set of conditions combined sensitivity with minimum variability of scores. The optimal conditions proved to be: (*a*) weight = 25–30 g.; (*b*) floor size = 3.8 × 4.4 cm.; (*c*) height = 5 cm.; (*d*) second replication; (*e*) score at the end of 10 min.

Description of Apparatus—A 10-chamber CMA unit for mice was made—it measures only 61 × 20 × 12.7 cm. (24 × 8 × 5 in.); there are 10 mechanical counters (each connected to two photoelectric cells so that interruption of one or both light beams results in a single count); each chamber has opaque sides so that the individual mouse is isolated from, and not disturbed by, its fellows (an improvement over the original rat CMA chambers which had transparent sides); a single removable tray makes up the floor of all 10 chambers. The tray can be covered with blotting paper that is changed after each experiment to ensure uniform conditions. This compact equipment is illustrated in Figs. 1 and 2.

Subsequent trials have shown that variability can be further reduced by allowing the mice to become accustomed to the chamber by being put in it for 5 min. twice a day for 3 or more days. The mice can then be allocated to different treatment groups on the basis of their basal activity scores, so that each group contains an equal proportion of high and low responders.

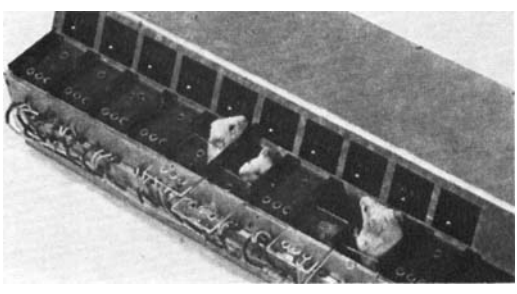

Fig. 1—Ten-chamber confinement motor activity unit for use with mice.

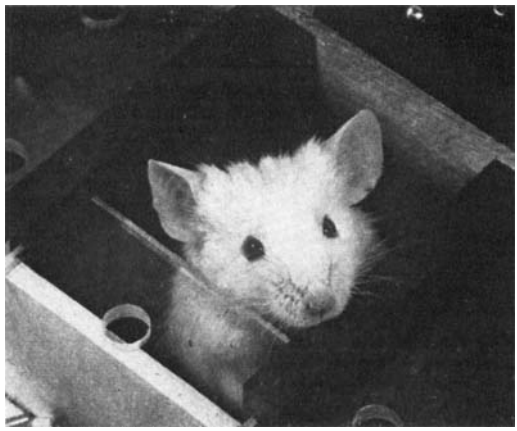

Fig. 2—Close-up of a mouse in one chamber of the confinement motor activity unit.

AMITRIPTYLINE AND ALCOHOL

In a series of three consecutive experiments a total of 120 male albino mice (26 ± 2 g.) were tested in the CMA chambers. The mice were fasted overnight, then dosed by stomach tube in four treatment groups: Group I received amitriptyline (10 mg./kg.) solution and ethyl alcohol (25 ml./kg., 10% solution); Group II received amitriptyline (10 mg./kg.) solution and placebo (5% glucose solution equal in volume to the alcohol); Group III received placebo (water equal in volume to the amitriptyline solution) and alcohol (25 ml./kg., 10%); Group IV received water and glucose as in Groups II and III (double placebo conditions).

The average total 5-min. score for the double placebo-treated groups was 2,858 when put in the CMA chambers 0.5 hr. after dosing. The alcohol and placebo group total scores averaged 2,514; this slight depression of activity was not statistically significant. The average total score for mice given amitriptyline plus placebo in place of alcohol was 4,968. This elevation of CMA was statistically significant at the 1% level of probability. The three groups of 10 mice given alcohol plus amitriptyline showed a significant depression of activity; the total score of each group averaged 719 ($p < 0.01$). Table I shows the scores obtained in the three experiments, and Table II the analysis of variance of the results. Figure 3 represents the results graphically.

The analysis of variance also revealed that only

TABLE I—TOTAL 5-MIN. CMA SCORES OF GROUPS OF 10 MICE GIVEN AMITRIPTYLINE (10 mg./kg.), ALCOHOL (25 ml./kg., 10%), AND PLACEBO SOLUTIONS

Expt.	Placebo	Alcohol Plus Placebo	Placebo and Drug	Alcohol Plus Amitriptyline
A	2,511	3,409	4,900	513
B	2,900	2,323	5,498	1,222
C	3,164	1,810	4,506	423
Average	2,858	2,514	4,968	719

TABLE II—ANALYSIS OF VARIANCE OF THE SCORES OF THREE EXPERIMENTS COMPARING THE EFFECT ON CMA PERFORMANCE OF FOUR CONDITIONS OF DRUG, ALCOHOL, AND PLACEBO

Source	*df*	*MS*	*F* Ratio
Conditions	3	911,595.32	11.30[a]
Replications	2	29,465.83	0.36[b]
Interaction	6	30,909.05	0.37[b]
Residual	108	83,440.39	
Total	119		

[a] $p < 0.01$. [b] Not significant.

the conditions effect was significant, *i.e.*, there was no "between replications" difference. The significance of the individual comparisons was ascertained by Duncan's multiple-range test. Amitriptyline plus alcohol resulted in lower CMA scores than any of the other three combinations. The difference between amitriptyline plus alcohol (Group I) and alcohol plus placebo (Group III) was significant at the 0.05 level of confidence.

DISCUSSION

The confinement motor activity unit has been shown by Tedeschi *et al.* to reveal the stimulant effects in rats of caffeine and tranylcypromine, although these drugs seem essentially inactive when tested by conventional photoelectric cell locomotor activity chambers. The present study has shown that the CMA unit, when modified for use with mice, enables the measurement of the excitation caused by low doses of the antidepressant drug, amitriptyline. Similar results were reported by Boissier *et al.* (6) who measured the rate of exploration of peg-board holes by mice. Higher doses of amitriptyline were shown to produce sedation.

When the stimulant dose of amitriptyline is combined with a low dose of alcohol (which by itself produced very little sedation), a reversal of drug effect is seen, the mice getting significantly low CMA scores, apparently a joint drug action.

Prediction of human response on the basis of animal experiments is possible when the laboratory tests are efficient, have high reproducibility, and adequacy of experimental design. Amitriptyline is of proven value in the relief of psychotic depression (7), but frequently causes sedation and drowsiness as side effects, especially in the first few days of administration. When prescribing amitriptyline, the possibility that it may add to the effects of alcohol should be considered.

SUMMARY

1. The confinement motor activity test, originally described for use in rats, has been appropriately modified for use in quantitating the motor activity of mice.

2. Amitriptyline in a dose of 10 mg./kg. body weight (orally) was shown to significantly increase the activity of mice.

3. Alcohol in a dose of 25 mg./kg. body weight of a 10% solution did not significantly depress the activity of mice in this test.

4. Amitriptyline (10 mg./kg.), plus alcohol (25 ml./kg., 10% solution) caused a significant depression of the activity of mice.

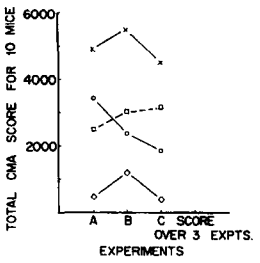

Fig. 3—Confinement motor activity scores in mice dosed with combinations of alcohol, placebo, and amitriptyline. Key: ×, amitriptyline, 10 mg./kg. + placebo (5% glucose); □, water + 5% glucose (double placebo); ○, alcohol 25 ml./kg., 10% + placebo (water); ◇, amitriptyline 10 mg./kg. + alcohol 25 ml./kg., 10%.

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Keyphrases

Motor activity test—mice
Confinement motor activity unit—modifications
Amitriptyline, alcohol, effects—motor activity
Alcohol effect—amitriptyline activity

Interaction of Salicylic Acid with Quaternary Ammonium Compounds

By LUCY S. C. WAN

A marked increase in viscosity is produced in solutions of dodecyl, tetradecyl pyridinium bromide, cetylpyridinium chloride, benzalkonium chloride, and benzethonium chloride containing salicylic acid. The viscosity increases with acid concentration to a maximum and then decreases. This viscosity effect is absent in quaternary ammonium compounds which are not surface active. Solutions of alkyl pyridinium and alkyl trimethyl ammonium compounds in the presence of salicylic acid form non-Newtonian systems which are dilatant while the alkyl aryl dimethyl compounds containing the same acid form Newtonian systems. The different rheological behavior is probably due to the number of substituents, R_1 R_2 ... present which are responsible for the surface activity of the molecule and which are attached to the nitrogen atom. The amount of salicylic acid required to produce maximum viscosity is directly proportional to the concentration of the quaternary ammonium compound which exhibits Newtonian flow.

QUATERNARY AMMONIUM compounds have been found to interact with various substances (1–4). In addition Rodgers (5) has demonstrated the interactions of hexylresorcinol and amaranth with cetylpyridinium chloride and cetyldimethylbenzylammonium chloride while Takruri and Ecanow (6) have studied the interactions of benzalkonium chloride and sodium lauryl sulfate with lipoproteins. In a previous study salicylic acid has been found to be the only one of several substituted benzoic acids to interact with cationic surfactants (7, 8). The interaction is highly specific since related compounds do not behave in like manner and therefore it seems desirable to collect more information about this interaction, in particular the type of quaternary ammonium compound that will exhibit this interaction and also in view of the possible practical applications in pharmaceutical formulations.

EXPERIMENTAL

Materials—Recrystallized salicylic acid m.p. 158.5–159° (British Drug House Ltd.), the quaternary ammonium compounds were benzethonium chloride monohydrate,[1] benzalkonium chloride 92–95% (Farbenfabriken Bayer Co.), dodecyl and tetradecyl pyridinium bromide,[2] cetylpyridinium chloride (E. Merck, Darmstadt), tetradecyl trimethyl ammonium bromide,[3] cetyltrimethylammonium bromide,[4] trimethylphenylammonium chloride, tetramethylammonium bromide, choline bitartrate, carbachol B.P. (E. Merck, Darmstadt), hexamethonium tartrate (May and Baker), decamethonium iodide, neostigmine methyl sulfate, hexamethonium bromide B.P.C. (L. Light Co.), and succinylcholine chloride (Burroughs Wellcome Co.). The critical micelle concentrations of the surfactants as obtained from surface tension measurements using the Du Nouy tensiometer are listed in Table I, those of tetradecyltrimethylammonium bromide and cetyltrimethylammonium bromide are 0.082 and 0.051%, respectively (7).

Apparatus—*Measurement of Viscosity*—Varying amounts of salicylic acid were weighed into a series of 100-ml. volumetric flasks containing the required concentration of the quaternary ammonium compound. The flasks were rotated in a thermostatically controlled water bath at 25 ± 0.1° for 24 hr. Their viscosities were measured using a portable viscometer (Ferranti) placed in a thermostatically controlled water bath at 25 ± 0.5°. The shear rates which ranged from 78.56 to 234.6 sec.$^{-1}$ were increased and then decreased for all viscosity measurements. An interval of 30 sec. was allowed between any two readings.

RESULTS AND DISCUSSION

Figures 1 and 2 show the effect of salicylic acid on the viscosities of solutions of benzalkonium chloride and benzethonium chloride, respectively. There is a rise in viscosity which increases with additions of the acid until a maximum is reached after which the viscosity then decreases. This fall in viscosity continues until an excess of the acid is present, except in the case of high concentrations of benzalkonium chloride where there is a rise in viscosity before a suspension is formed. The solutions are Newtonian systems, their viscosities being the same when measured at shear rates of 78.56, 117.35, 155.1, 195.9, and 234.6 sec.$^{-1}$ but the suspensions formed are

TABLE I—CRITICAL MICELLE CONCENTRATIONS OF SURFACTANTS AT 25°

Surfactant	C.M.C., %w/v
Dodecyl pyridinium bromide	0.039
Tetradecyl pyridinium bromide	0.074
Cetylpyridinium chloride	0.029
Benzalkonium chloride	0.049
Benzethonium chloride	0.063

Received May 28, 1968, from the School of Pharmacy, University of Singapore, Singapore, Malaysia.
Accepted for publication July 31, 1968.

[1] Marketed as Hyamine 1622 by Rohm and Haas Co., to whom the author is very grateful for the samples.

[2] Marketed as Morphan DPB and TPB, respectively, by Glovers Chemicals Ltd.

[3] Marketed as Morphan T by Glovers Chemicals Ltd., to whom the author is very grateful for the samples.

[4] Marketed as Cetrimide by Imperial Chemical Industries.

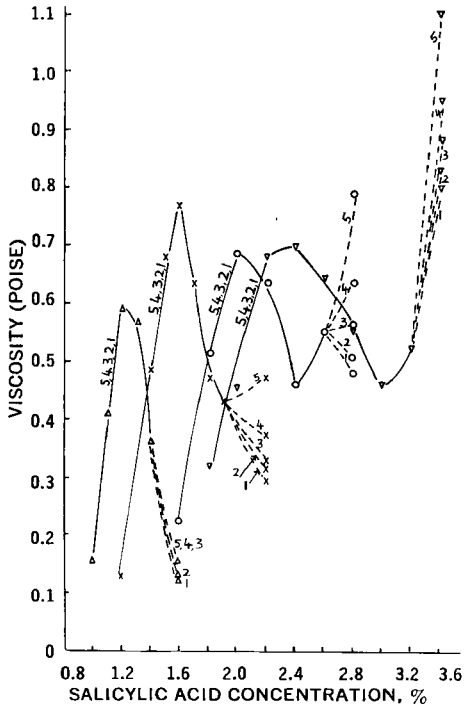


Fig. 1—Effect of salicylic acid on the viscosity of benzalkonium chloride solutions at 25°. Key: △, 4%; ×, 6%; ○, 8%; ▽, 10%; - - -, suspension. Shear rate: 1, 234.6 sec.$^{-1}$; 2, 195.9 sec.$^{-1}$; 3, 155.1 sec.$^{-1}$; 4, 117.35 sec.$^{-1}$; 5, 78.56 sec.$^{-1}$.

generally non-Newtonian systems. The amount of salicylic acid required to produce maximum viscosity is directly proportional to the concentration of the quaternary ammonium compound (Fig. 3). The ratios of the concentrations of the acid to the quaternary ammonium compound at which maximum viscosity occurs is 1:5 and 1:12.5 for benzalkonium chloride and benzethonium chloride, respectively. Once this ratio has been obtained further additions of the acid reduce the viscosity of the system.

The viscosity effect is also observed in solutions of alkyl pyridinium compounds containing salicylic acid. The increase in viscosity resulting from the interaction of the acid with dodecyl pyridinium bromide is not as marked as that produced by the interaction of the same acid with the corresponding tetradecyl derivative (Fig. 4). In this case the viscosity also increases with increase in acid concentration and then decreases, but when an excess of the acid is added the viscosity remains constant. Solutions of cetylpyridinium chloride gel readily when sufficient salicylic acid is added. If a small amount is present then gelling occurs; gelling is taken as the state in which the viscosity can no longer be determined by the Ferranti viscometer as the dial readings fluctuate during the rotation of the cylinder. These systems are dilatant and can easily be demonstrated by stirring vigorously a solution of cetylpyridinium chloride and salicylic acid which then becomes very viscous but when it is allowed to stand it reverts to a liquid of low viscosity.

In contrast to the alkyl aryl dimethyl compounds, the pyridinium compounds in the presence of salicylic acid form non-Newtonian systems, their viscosities vary with different shear rates (Fig. 4). This behavior is seen with other concentrations of tetradecyl pyridinium bromide solutions studied

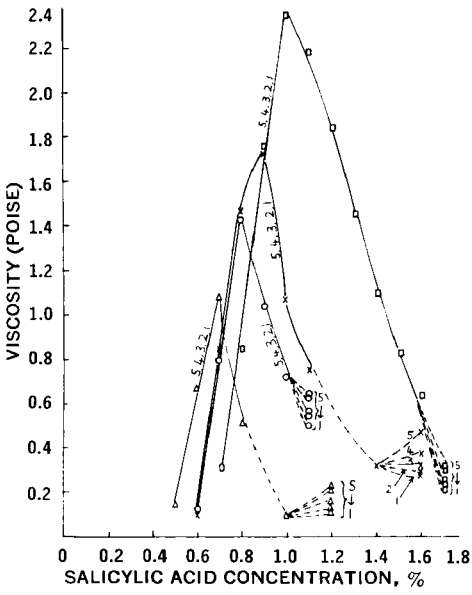


Fig. 2—Effect of salicylic acid on the viscosity of benzethonium chloride solutions at 25°. Key: △, 4%; ○, 5%; ×, 6%; □, 8%; - - -, suspension. Shear rate: 1, 234.6 sec.$^{-1}$; 2, 195.9 sec.$^{-1}$; 3, 155.1 sec.$^{-1}$; 4, 117.35 sec.$^{-1}$; 5, 78.56 sec.$^{-1}$.

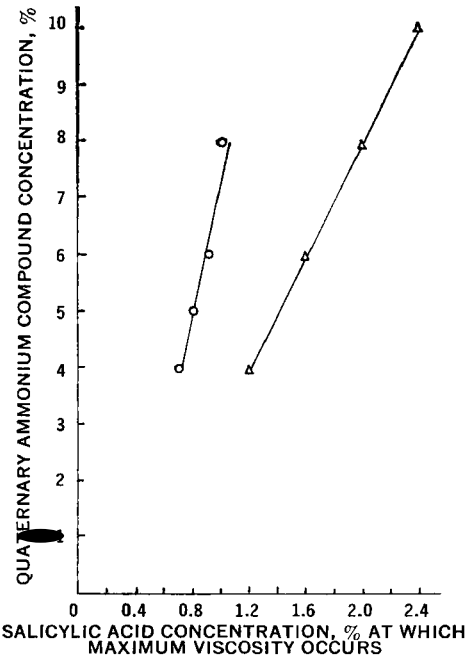


Fig. 3—Relationship between quaternary ammonium compound concentration and salicylic acid concentration required to produce maximum viscosity at 25°. Key: △, benzalkonium chloride; ○, benzethonium chloride.

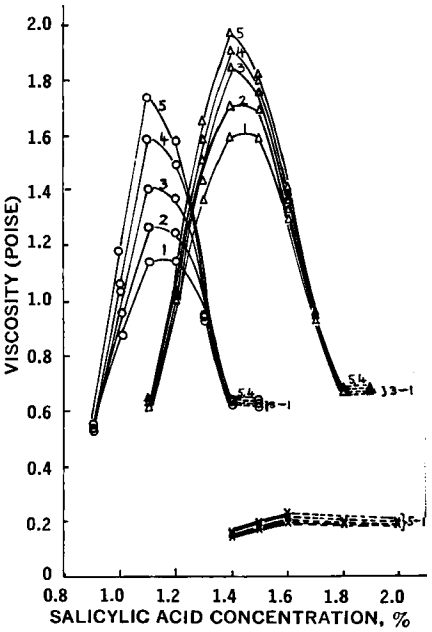


Fig. 4—Effect of salicylic acid on the viscosity of tetradecyl and dodecyl pyridinium bromide solutions at 25°. Key: ○, 3% TPB; △, 4% TPB; ×, 5% DPB; - - -, excess salicylic acid. TPB = tetradecyl pyridinium bromide, DPB = dodecyl pyridinium bromide.

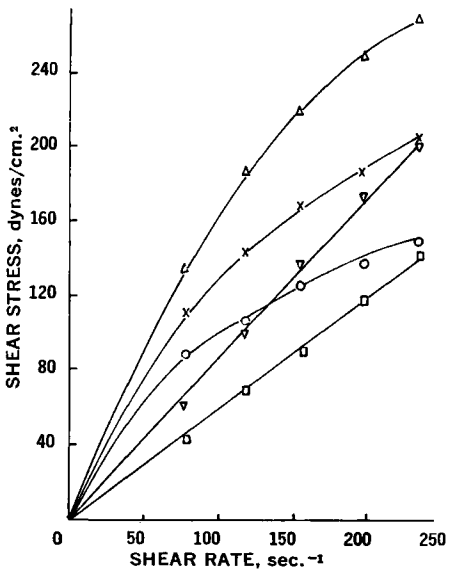
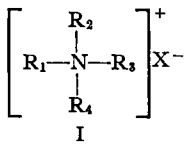


Fig. 5—Flow curves of quaternary ammonium compound solutions containing salicylic acid at 25°. Key: ×, 3% CTAB + 1.0% SA; ○, 3% TTAB + 0.8% SA; △, 3% TPB + 1.1% SA; □, 4% BZC + 1.2% SA; ▽, 6% H1622 + 0.7% SA. CTAB = cetyltrimethylammonium bromide. TTAB = tetradecyl trimethylammonium bromide. TPB = tetradecyl pyridinium bromide. BZC = benzalkonium chloride. H1622 = benzethonium chloride. SA = salicylic acid.

such as 2, 5, and 6%. Solutions of the alkyl aryl dimethyl compounds form Newtonian systems in the presence of salicylic acid (Figs. 1 and 2) except when the quantity of the acid present exceeds its solubility in the surfactant and then suspensions are formed which are non-Newtonian dispersions.

Figure 5 shows flow curves of solutions of tetradecyl pyridinium bromide, cetyl and tetradecyl trimethyl ammonium bromide containing salicylic acid. These systems are non-Newtonian and exhibit dilatancy. The flow curves of benzalkonium chloride and benzethonium chloride, however, demonstrate Newtonian flow. Similar behavior is observed with concentrations of the above-mentioned surfactants ranging from 3 to 10% in the presence of varying amounts of the acid. The difference in rheological behavior is probably due to the number of substituted organic groups which confer surface activity on the molecule and which are attached to the nitrogen atom. The quaternary ammonium compounds investigated including those studied previously (7, 8) can be considered to be analogs of the inorganic ammonium salts and their general structure (I) may be represented thus:



I

where R_1, R_2, R_3, and R_4 are organic groups and X is an inorganic radical which in this case is either a chloride or bromide. In one type, R_1 varies while R_2, R_3, and R_4 are kept constant such as dodecyl, tetradecyl, and cetyltrimethylammonium bromide; in a second type R_1 and R_3 are different while R_2 and R_4 are the same, such as benzalkonium chloride or benzethonium chloride. In a third type, a cyclic nitrogen compound replaces the single nitrogen of the former types thus making available in the configuration for R_1 only, such as dodecyl, tetradecyl pyridinium bromide, and cetylpyridinium chloride. Hence the interactions of salicylic acid with the alkyl trimethyl ammonium and alkyl pyridinium compounds represented as $R_1(CH_3)_3N^+Br^-$ and R_1N^+⬡ Br^-, respectively, lead to the formation of Newtonian solutions which may be attributed to the presence of a single substituent responsible for the surface activity of the molecule while the alkyl aryl dimethyl ammonium compounds represented as $R_1,R_3(CH_3)_2N^+$ Cl^- have two substituents and produce Newtonian solutions.

No increase in viscosity is observed when salicylic acid is added to solutions of trimethyl phenyl ammonium chloride, tetramethyl ammonium bromide, choline bitartrate, carbachol, hexamethonium tartrate and bromide, decamethonium iodide, neostigmine methyl sulfate, and succinylcholine chloride. Hence the quaternary ammonium compounds which are not surface active do not demonstrate this viscosity effect. The results obtained indicate that the interaction of salicylic acid with the quaternary ammonium compounds is associated with the presence of micelles as the surfactant concentrations used are well above the critical micelle concentration (C.M.C.) (Table I). When salicylic acid is added it is solubilized by the micelles, the placement of the solute in the micelles is possibly one of adsorption on the micellar surface. The negatively

charged salicylate ions will be attracted and adsorbed onto the positively charged micelles in such a manner of arrangement that gives rise to an increase in size and thereby to an increase in viscosity. As more acid is added more salicylate ions will be adsorbed on the micellar surface and this continues till a maximum viscosity is reached. At this stage the micellar surface may have adsorbed sufficient salicylate ions to attain an equilibrium state so that further additions of the acid will tend to upset the equilibrium resulting in a decrease in viscosity. When the amount of acid present exceeds that which can be solubilized by the micelles then the solution or gel becomes a suspension of salicylic acid in the surfactant solution. This change of nature of the system makes it no longer a simple liquid system and this gives rise to non-Newtonian flow which is not unusual with the flow properties of most suspensions. In the case of the alkyl pyridinium compounds the flow properties remain non-Newtonian in the presence of an excess amount of salicylic acid. This is also true for the alkyl trimethyl ammonium compounds studied previously (7).

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Keyphrases

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Viscosity—salicylic acid–quaternary ammonium compounds
Rheology—salicylic acid–quaternary ammonium compounds

Effect of Vagal Stimulation on Enterochromaffin Cell Granulation in the Guinea Pig Small Intestine

By MARTIN F. TANSY, ARTHUR S. MILLER, and ARTHUR STEIN

Experiments are presented which study the effect of vagal stimulation on the enterochromaffin cell granulation of the guinea pig duodenum. Concentration of granulated enterochromaffin cells in specially stained intestinal sections is used to determine the response. The results indicate the vagus nerves *per se* and not hydrochloric acid secretion, increased intraluminal pressure, or hyperperistalsis significantly alter the granulation of these cells. The data also suggest that vagal degranulation may occur *via* a noncholinergic mechanism.

THE IMPORTANCE of serotonin in the gastrointestinal tract has received considerable investigative attention. Most of the studies, however, have been concerned with its content, fate, and action; whereas, its release has received much less attention. Serotonin release has primarily been concerned with the observations that elevation of intraluminal pressure and/or increased intestinal motility augments the amount of 5-HT released into the intestinal lumen and venous blood (1). It has not, however, been shown that distension or hyperperistalsis of the intestine, kept within physiologic limits, produces in the intact animal a local or systemic discharge of serotonin from the enterochromaffin cells (2).

Argentaffin enterochromaffin cells are distributed throughout the mucosa of the gastrointestinal tract. They contain specific granules which are precipitated by formalin and will stain with silver salts (3). The complete chemistry of the granules is not known, but Barter and Pearse (4) suggest a fully conjugated β-carboline derivative of serotonin may precipitate the silver to give the argentaffin staining reaction. In any event, considerable experimental evidence (5–7) has shown that a decrease in the granularity of these cells parallels the release of serotonin from the gut. This phenomenon provides a convenient

Received May 22, 1968, from Temple University Schools of Dentistry and Pharmacy, Philadelphia, PA 19140
Accepted for publication July 22, 1968.
This investigation was supported by grant AM-11372-01 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.
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histologic method for studying gastrointestinal serotonin release.

The present study was designed to provide information on the effects of vagal stimulation on the granulation of the enterochromaffin cell system as a link to serotonin release in the bowel.

EXPERIMENTAL

Animals—Acute experiments were performed on 76 healthy male guinea pigs, weighing 350–450 g., aged about 6 months. The strain was random bred from closed colonies obtained from the Marland Farms, Wayne, N. J. The guinea pigs were kept in the laboratory for an acclimatization period of 3 days. Housing was in cages accomodating six animals. In addition to standard stock commercial pellet food, they were given "greens" and fresh tap water *ad libitum*. All animals were fasted but allowed water 24 hr. before operation.

Selection of the male guinea pig was for several reasons: (*a*) There is only one principal type of enterochromaffin cell in the alimentary tract of the guinea pig, the argentaffin cell (8). (*b*) The guinea pig has five to eight times as many argentaffin enterochromaffin cells in the proximal part of the duodenum as the rat (9, 10). (*c*) There are fewer mast cells in guinea pig intestine compared to the greater number found in rat and mouse bowel. (*d*) It is generally agreed the argentaffin reaction is the most convenient and suitable means for demonstration of guinea pig enterochromaffin cells (11–13). In this animal, these cells exhibit an intense argentaffin reaction compared to the weak reaction intensity of similar cells in other rodents. (*e*) Males were used because even in the same species, individual enterochromaffin cell counts vary depending on the animals' sex. A greater number of enterochromaffin cells has been found in the adult female guinea pig than in the male (14–17). It was pointed out, however, that the enterochromaffin cell count varies with the stage of the estral cycle (17).

Surgical and Recording Procedures—Under intraperitoneal urethan anesthesia (1.5 g./kg.), a midline abdominal incision was made and both branches of the subdiaphragmatic vagus isolated and secured to stimulating electrodes. In these laparotomized animals an opening was made in the stomach and jejunum, and in some animals the common bile duct was also ligated. These procedures were effected to minimize the effects of gastric juice acid, intraluminal pressure changes, and bile on the duodenal mucosa. In other animals the vagi were exposed, divided, and stimulated high in the neck. Peripheral vagal stimulation at the cervical level was carried out with the nerves either intact or cut. The external jugular vein was cannulated for administration of drugs. A tracheotomy was established in all animals for either recording intratracheal pressure with a volumetric transducer (Statham PT 5A) or maintaining artificial respiration. Right carotid arterial pressure was recorded continuously using a pressure transducer (Statham P23 AC). Scalar lead II of the electrocardiogram and tachograph was also recorded. All variables were charted on a polygraph (Grass model 7).

Electrical Stimulation—Bipolar electrodes were attached to the caudad portion of the vagus nerves and surrounded with liquid petrolatum to isolate the stimulating current. The vagi were stimulated with a stimulator (Grass model S8) *via* an isolation transformer. The stimulus was applied for a total of 15 min., 5 min. on, 5 min. off. This stimulus pattern delivered at the nerve site was monitored with an oscilloscope (Tektronix 564). The voltage was varied between 1 and 10 v. depending on response of the organ to nerve stimulation. Stimuli were applied at frequencies varying from 1–250 c.p.s./sec. Unless otherwise indicated, duration of the pulse was kept constant at 1 msec. throughout all experiments. In some cases, the duodenal musculature was stimulated directly using the same stimulus pattern. Control animals were similarly anesthetized, laparotomized, and remained on the operating table for the same period of time.

Histology—The guinea pigs were sacrificed by a Harvard small animal decapitator. The pyloric antrum, gall bladder with biliary tree, and duodenum were immediately excised, rinsed in Tyrodes, and fixed in 10% formalin. After 24 hr. fixation, sections of proximal, intermediate, and distal duodenum were selected and trimmed to about 4 mm. in length. Every attempt was made to consistently define from animal to animal, the precise anatomic tissue selection site for histologic evaluation. The proximal portion of duodenum was defined as that segment *juxta* the pylorus situated between the pyloric ring and entrance of the common bile duct. Selection of middle and distal portions of duodenum, however, tended to be more subjective due to absence of gross anatomical landmarks. From each specimen 2 to 3 transverse paraffin sections were cut at 6 μ. Sections were stained by the Fontana-Masson technique, counterstained with eosin, and mounted in a fixative (Permount).

Cell-Counting Technique—To give numerical results, argentaffin cells in each specimen were counted in adjacent fields at 430X using a light microscope. This method of determining the number of argentaffin cells in terms per visual field of stated size has been successfully employed in a number of studies (5, 18, 19). As a point of reference each section was adjusted so that musculature was always present in the microscopic field. The distribution of argentaffin cells is not uniform over the mucosa. The heaviest concentrations are found in the basal area, therefore, tangential sections were rejected as such fields contained a disproportionate amount of basal area. The number of cells in each tissue was expressed as cells per high-power field (H.P.F.). Up to 40 fields in each section were counted depending on circumference of tissue available. The mean number of cells/H.P.F. was calculated for the three specimens of duodenum in each animal. All sections were judged under blind conditions. The *t* test was used to determine the significance of differences between mean values of all reported data. *P* values are indicated where applicable.

Drugs—Each of 12 guinea pigs received an intravenous injection of atropine sulfate, six at a dose of 5 mg./kg., the remainder at 10 mg./kg. *via* the external jugular vein immediately before surgical exposure and mechanical stimulation of the cervical vagi. Four atropine controls were also employed. To ascertain if this method was sensitive enough to

detect changes in enterochromaffin cell granulation, reserpine[1] was administered to 6 animals. A single injection of reserpine (30 mg./kg.) was given intraperitoneally after the volume of the solution was adjusted to 4.0 ml. with warm saline. A comparable number of control animals received a similar volume of physiologic sodium chloride at 37°. Twenty-four hours postinjection, the reserpinized animals exhibited a 90% decrease in their mucosal granulated argentaffin cell count. This acknowledged chemical degranulator served as an effective technique control.

RESULTS

The results of these experiments indicate that the distribution of argentaffin enterochromaffin cells within the guinea pig duodenum varies considerably. The most consistent distribution as well as the greatest number of cells was found in the section of duodenum between pyloric ring and opening of the common bile duct. In some animals, however, peak levels of density occurred in the middle third of the duodenum. The lowest number of cells was always found in the distal segment.

Regardless of sectional origin, enterochromaffin cells were most numerous in the basal mucosa, especially in the crypts of Lieberkühn. Occasionally individual enterochromaffin cells were found in the villi and/or submucosa. No stained enterochromaffin cells were detected in musculature. The argentaffinity also varied considerably from animal to animal but no general difference was noticed between the villous and basal enterochromaffin cells in staining intensity. For the most part, enterochromaffin cells were easily distinguishable from other intestinal cells and hemosiderin-laden macrophages. The surgical procedure had no discernible effect on the enterochromaffin cell population.

Under the conditions of this experiment, peripheral vagal stimulation at either 10 v., 25 c.p.s., or 1 v., 250 c.p.s. for 1 msec. produced a significant reduction in argentaffin-positive enterochromaffin cell granularity in guinea pig duodena (Fig. 1). The percentage reduction of granulated argentaffin-positive cells with current of low intensity (1 v., 1 msec., pulse at 250/sec.) ranged from 60 to 91% with a mean reduction of 73% at the $p < 0.005$ level, compared with control animals. At increased current strength (10 v., 1 msec., pulse at 25/sec.), the percentage degranulation ranged from 22 to 82% with a mean reduction of 60% which proved highly significant. A 50% reduction of cell granularity was noted in those animals which were subjected to direct electrical stimulation at low voltage, high frequency stimulus parameters, to their duodenal musculature.

As previously reported (20), the administration of atropine sulfate (5–10 mg./kg.) by itself had no effect on the enterochromaffin cell granulation. The argentaffin degranulation which had been obtained in control animals upon peripheral vagal stimulation was not abolished by atropine at any dose, even though the cholinergic cardiovascular responses to cervical vagal stimulation were completely blocked. The results of vagal stimulation under these various conditions are summarized in Fig. 2.

[1] Serpasil, Ciba Pharmaceutical Co., Summit, N. J.

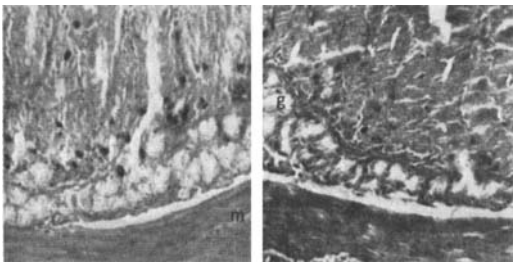

Fig. 1—Photomicrographs of a representative control section (left) and a section from a vagally-stimulated animal (right). Greater numbers of argentaffin cells are apparent in the control section adjacent to Brunner's glands (g). (m) *indicates the tunica muscularis (Fontana-Masson stain, ×100).*

DISCUSSION

Many of the observations on the distribution of enterochromaffin cells in this study are in agreement with earlier findings (9, 21–23). Hoeschen (9) also found guinea pig enterochromaffin cells were most numerous in that portion of duodenum adjacent to the pylorus and that the count markedly decreased in the distal part of this segment. This segmental gradient has too often been overlooked in interpreting changes in enterochromaffin cell granulation evoked by electric and pharmacologic stimuli.

Routine histologic examination of guinea pig duodena showed enterochromaffin cell granularity to be decreased following peripheral vagal stimulation. The effects of vagal stimulation on enterochromaffin cell density appear to depend on strength, duration, frequency, and mode of application of the stimulus. The degree of degranularity may be frequency-dependent. In any event, vagal degranulation was not attributable to changes in intraluminal pressure because the stomach and

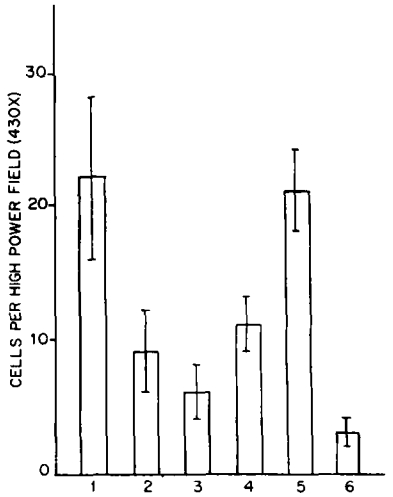

Fig. 2—Bar 1: controls; Bar 2: abdominal vagal stimulation with relatively high voltage, low frequency; Bar 3: abdominal vagal stimulation with low voltage, high frequency; Bar 4: direct electric stimulation to duodenal musculature; Bar 5: atropinized controls (5–10 mg./kg.); Bar 6: cervical vagal stimulation in the atropinized animal. Each bar represents the mean value ± standard deviation.

jejunum were opened at the time of stimulation, thereby negating any rise in pressure. The influence of any major endocrine system on enterochromaffin degranulation seemed unlikely because of the relatively short period and pattern of stimulation used.

Visual inspection of the atropinized animals' small intestine revealed an absence of spontaneous motor activities. In fact, during the stimulus interval, the small intestine remained atonic, flaccid, and completely quiescent. However, animals who had direct electric current applied to their duodenal musculature, exhibited increased intestinal motor activities, but the augmented motility was not as effective in degranulating enterochromaffin cells as was nerve stimulation. These observations led to the conclusion the vagus nerves *per se* and not hydrochloric acid secretion, increased intraluminal pressure, hyperperistalsis, or bile release altered the granularity of the enterochromaffin cells of the guinea pig small intestine.

Even more surprising was the fact that although atropine blocked the cardiovascular parameters to cholinergic stimulation, it failed to block the enterochromaffin cells from discharging their granules on cervical vagal stimulation. Straub and Stefansson (26) have shown convincingly in the guinea pig that stimulation of the vagus *in vivo* evokes small intestinal peristalsis which is easily blocked by atropine, even in doses as small as 10 mcg./kg. Assuming an even distribution of the drug, this would represent a concentration of 10^{-8}, which is in reasonable agreement of atropine required to block acetylcholine contractions on guinea pig ilea *in vitro* (27). In view of this, plus the high dosage level (10 mg./kg.), it seemed unlikely that atropine failed to block enterochromaffin degranulation to vagal stimulation due to acetylcholine release in an area where atropine was unable to penetrate. Furthermore, difficulty of access was not likely to be the answer here because the motor response of the bowel to peripheral vagal stimulation appeared to have been successfully blocked, suggesting that atropine had penetrated at least to the site of nervous action in the muscle. If, on the other hand, the neural pathway controlling enterochromaffin cell degranulation is acetylcholine mediated, it is certainly resistant to antagonism with atropine. Perhaps a more likely explanation is that vagal stimulation affects the enterochromaffin cell granulation in the guinea pig small intenstine *via* a noncholinergic mechanism.

In summary, even though it was found adequate electrical stimulation of the vagus nerve does alter granulation of guinea pig enterochromaffin cells, more work is needed to define the nature of the nerve fibers coursing in the vagus responsible for degranulation. Furthermore, even though the present experiments support this concept of serotonin release by vagal stimulation, at least as measured by enterochromaffin granulation, it remains to be seen if degranulation of enterochromaffin cells can be directly correlated with decreased tissue levels of serotonin under the conditions set forth in these experiments.

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Keyphrases

Enterochromaffin cell granulation—guinea pig duodenum
Vagal stimulation—enterochromaffin cell granulation
Atropine—enterochromaffin cell granulation
Reserpine—enterochromaffin cell granulation

Structure and Synthesis of 2-(4′-Substituted Phenyl)-Δ^1-pyrrolines from 4-Phthalimidobutyryl Chloride *via* Friedel-Crafts Acylation

By SHU-SING CHENG*, CLAUDE PIANTADOSI†, and J. LOGAN IRVIN

2-(4′-Substituted phenyl)-Δ^1-pyrrolines can be prepared conveniently from the hydrochlorides of 4′-substituted-4-aminobutyrophenones or their cyclization products, the corresponding Δ^1-pyrrolines hydrochlorides, by alkalinization. The hydrochlorides were prepared by the hydrolyses of 4′-substituted-4-phthalimidobutyrophenones, which were obtained from the Friedel-Crafts acylation reaction between the substituted benzenes and γ-phthalimidobutyryl chloride. Spectral evidence indicates that all the products obtained are Δ^1-pyrrolines rather than the Δ^2-isomers. This method should provide easy access to those 2-(4′-substituted phenyl)-Δ^1-pyrrolines unavailable by other routes due to the unavailability of the corresponding β-cyano and γ-nitro ketone intermediates or the corresponding Grignard reagents.

A GREAT NUMBER of 2-aryl-Δ^1-pyrrolidines have been prepared for a variety of pharmacological studies. For instance, 2-phenylpyrrolidine has been found to possess a weak antihypertensive effect against small doses of nicotine in the dog (1). These 2-arylpyrrolidines can be prepared from the low-pressure hydrogenation of the corresponding β-aroylpropionitrile or the intermediate 2-aryl-Δ^1-pyrrolines (2–5). Besides being employed as important intermediates for preparation of a variety of compounds, Δ^1-pyrroline has also been found to be a food flavor for fat or oil (6). However, the synthetic route mentioned above for the preparation of either aryl-Δ^1-pyrrolines or pyrrolidines is limited by the availability of the corresponding β-aroylpropionitriles and their precursors γ-amino ketones in the form of Mannich bases as indicated by Burckhalter and Short (1).

Other widely accepted methods for the synthesis of pyrrolines center on the reductive cyclization of γ-nitro ketones (7, 8) or the Grignard reaction between 4-chlorobutyronitrile and alkyl-, aryl-, or aralkylmagnesium halides (7, 9–16). All of the reductive cyclization reactions are based on the fact that the presumed intermediates, *N*-unsubstituted γ-amino ketones, are not stable and undergo dehydrative cyclization in the basic media. An early observation was made by Hielsher (17), who obtained 2-methyl-Δ^2-pyrroline from the reaction of 5-bromo-2-pentanone and alcoholic ammonia at 40–45°. Subsequently, Gabriel and Colman (18), reported the isolation of 2-phenyl-Δ^2-pyrroline hydrochloride from an attempted synthesis of 4-aminobutyrophenone hydrochloride, and that the corresponding pyrroline base was then obtained by alkalinization of the hydrochloride. Recent evidence has led to the concept that most of the Δ^2-pyrrolines reported in the earlier literature and those obtained from reaction between Grignard reagent and γ-chlorobutyronitrile are best represented by Δ^1-structures (7). In view of this fact and the observation made by Gabriel and Colman on the isolation of phenylpyrroline from the presumable cyclization of 4-aminobutyrophenone hydrochloride, it seemed feasible to prepare 2-(4′-substituted phenyl)-Δ^1-pyrrolines from substituted benzenes and γ-phthalimidobutyryl chloride *via* the Friedel-Crafts acylation reaction.

Since most of the substituted benzenes undergo Friedel-Crafts acylation with ease and give fair yields, this route should provide those 2-(4′-substituted phenyl)-Δ^1-pyrrolines which are difficult to obtain by other routes due to the unavailability of either the corresponding β-cyano and γ-nitro ketone intermediates, or the corresponding Grignard reagents.

In the work described herein, the condensations of γ-phthalimidobutyryl chloride with benzenes carrying *ortho* and *para* orienting groups afforded various substituted phenyl 4-phthalimidobutyrophenones. These can be alkyl, alk-

Received January 1, 1968, from the Departments of Medicinal Chemistry and Biochemistry, University of North Carolina, Chapel Hill, NC 27514

Accepted for publication July 18, 1968.

This investigation was supported by Public Health Service grants CA-06364-05 and CA-06364-06 from the National Cancer Institute, National Institutes of Health, Bethesda, Md. This work was also aided by an equipment grant GB-4577 to the Department of Biochemistry from the National Science Foundation, Washington, D. C.

The authors are grateful to Mr. James Sparrow for assistance in the NMR spectrometry, and to Mr. Chung Il Hong for aid in obtaining data for Figs. 5–7.

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oxyl, or halogens. However, iodobenzene failed to undergo the condensation under the influence of anhydrous aluminum chloride at elevated temperatures. Iodobenzene was decomposed with the liberation of iodine under these reaction conditions. The hydrolyses of these 4-phthalimidobutyrophenones in a mixture of glacial acetic acid and concentrated hydrochloric acid resulted in either the hydrochlorides of phenyl substituted 4-aminobutyrophenones or the 2-(4′-substituted phenyl)-Δ^1-pyrrolines. The latter are regarded as the cyclization products of the former and are readily obtainable by either raising the reaction temperature during the hydrolysis or by lengthening the period of acid treatment. The preparation of *para*-substituted 4-aminobutyrophenone hydrochlorides has recently been described in conjunction with the biological study of these compounds (19). By varying the reaction temperature and the length of acid treatment, both the open-chain *para*-substituted 4-aminobutyrophenone hydrochlorides and the corresponding 2-(4′-substituted phenyl)-Δ^1-pyrroline hydrochlorides can be obtained from the same reaction as will be described below. The open-chain *para*-substituted 4-aminobutyrophenone hydrochlorides are relatively unstable and tend to undergo dehydrative cyclization to form the corresponding 2-(4′-substituted phenyl)-Δ^1-pyrroline hydrochlorides, particularly during the melting process. For example, 4′-methyl-4-aminobutyrophenone hydrochloride first melted at 142–143° and solidified when the temperature reached approximately 160°, but after the material was cooled for several hours and reheated to determine the melting point, the melting did not occur until the temperature reached 201–202°, which corresponded to the melting point of the product of the dehydrative cyclization, 2-(4′-methylphenyl)-Δ^1-pyrroline hydrochloride. Thus, it is possible that the melting points that were reported for the other *para*-substituted 4-aminobutyrophenone hydrochlorides may represent the melting points of the corresponding 2-(4′-substituted phenyl)-Δ^1-pyrroline hydrochlorides, since the dehydration process occurs readily at elevated temperatures and the transition from one form to the other is not readily observable.

Since *N*-unsubstituted γ-amino ketones do not exist in the form of free base, but as pyrroline derivatives, it was conceivable that the 2-(4′-substituted phenyl)-pyrrolines could be obtained by the alkalinization of the corresponding 4′-substituted-4-aminobutyrophenone hydrochlorides. The experiments described below prove this is the case.

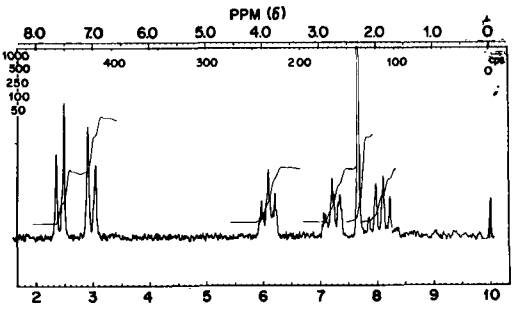


Fig. 1—The 60-Mc.p.s. proton NMR spectrum of 2-(4′-methylphenyl)-Δ^1-pyrroline in CCl_4 with tetramethylsilane internal standard (10τ).

All of the phthalimidobutyrophenones obtained from Friedel-Crafts condensations of 4-phthalimidobutyryl chloride with the monosubstituted benzenes are the *para*-substituted 4-phthalimidobutyrophenones as revealed by their IR spectra. The hydrolytic products are the corresponding *para*-disubstituted benzene derivatives (8.2–8.4 μ and 12.1–12.3 μ, *para*-disubstituted benzenes). The pyrroline bases obtained from the alkalinization of either the hydrochlorides of the γ-amino ketones or the corresponding pyrrolines are identical pairs, and are all Δ^1-pyrrolines, as can be shown by the lack of the chemical shift for olefinic proton in the 2.8–5.8 τ region of the 60 Mc. NMR spectra measured in carbon tetrachloride, using tetramethylsilane as internal standard (Figs. 1–3). In the spectrum of 2-(4′-methylphenyl)-Δ^1-pyrroline, the chemical shift of the methyl protons on the phenyl ring appears as a singlet at 7.68 τ, and the NMR pattern of the chemical shifts for phenyl protons agrees with that expected for *para*-disubstituted benzene with two different sets of protons. The two sharp bands at 2.35 and 2.5 τ may be assigned to the two phenyl protons at the *ortho* positions related to the carbon atom which is at the junction of the two rings and near the π-electron cloud of the —N=C<, whereas

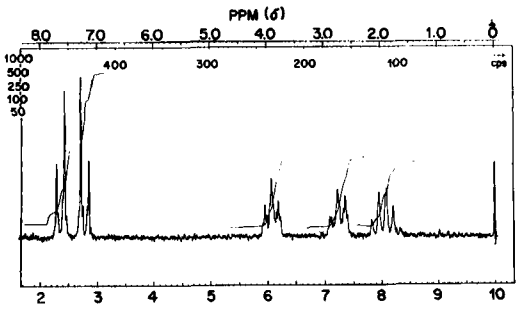


Fig. 2—The 60-Mc.p.s. proton NMR spectrum of 2-(4′-chlorophenyl)-Δ^1-pyrroline in CCl_4 with tetramethylsilane internal standard (10τ).

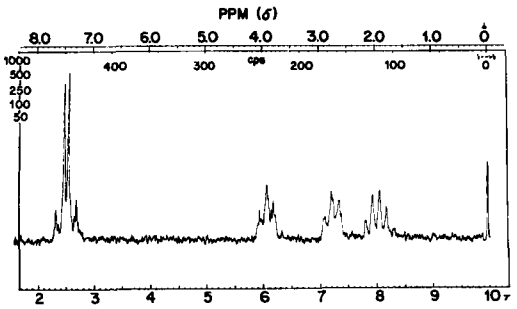


Fig. 3—The 60-Mc.p.s. proton NMR spectrum of 2-(4′-bromophenyl)-Δ¹-pyrroline in CCl_4 with tetramethylsilane internal standard (10τ).

the other two sharp bands at 2.9 and 3.05 τ might be the signals of the two other protons at the *meta* positions related to the carbon at the ring junction, and are far from the —N=C⟨.

All of the NMR spectra (Figs. 1–3) of the 2-(4′-substituted phenyl)-Δ¹-pyrroline bases studied herein display a quintet at 7.84, 7.95, 8.09, 8.20, 8.33 τ, and a triplet at 7.10, 7.23, 7.36 τ, and another low-field triplet at 5.95, 6.07, and 6.20 τ. The quintet may be due to the resonance of the two protons at C_4, split by two sets of two protons at C_3 and C_5. The high-field triplet may represent the chemical shift of two protons at C_3 split by the two protons at C_4, whereas the low-field triplet may be assigned to the two protons at C_5, which is in close proximity to the electron-rich nitrogen atom of the —N=C⟨. The spin-spin coupling constants are 7 c.p.s. for all the coupled protons mentioned above.

The UV spectra of 2-(4′-methylphenyl)-, 2-(4′-fluorophenyl)-, 2-(4′-chlorophenyl)-, and 2-(4′-bromophenyl)-Δ¹-pyrrolines in ethanol display an absorption maximum at 252 mμ. These absorption maxima were displaced to longer wavelengths when the ethanolic solutions were acidified with 0.1 *N* hydrochloric acid to a final concentration of approximately 0.001 *N*. The bathochromic shifts range from 13 to 23 mμ. The UV spectrum of 2-(4′-methoxyphenyl)-Δ¹-pyrroline has two absorption maxima, an intense end absorption occurs at 206 mμ with a molecular extinction coefficient 17,078, and a K-band maximum at 265 mμ (ε = 13,376), and on acidification, these maxima were displaced to 226 mμ (ε = 11,370) and 306 mμ (ε = 20,000), respectively (Fig. 4). The exceptionally long wavelength of these absorption maxima can be attributed to the auxochromic effect of the unshared electron pairs of the oxygen atom in the *para* methoxyl group. A similar effect also accounts for the longer wavelength of the absorption

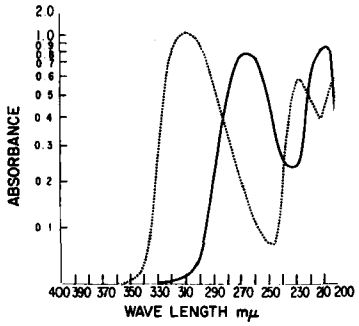


Fig. 4—Absorption spectrum of 2-(4′-methoxyphenyl)-Δ¹-pyrroline in ethanol (——) and in ethanol acidified with HCl (. . .).

maxima of the three *para*-halogeno-derivatives as compared with the absorption maximum of 2-phenyl-Δ¹-pyrroline ($\lambda_{max.}^{244 m\mu}$ $\epsilon_{max.}^{20,000}$). The relatively longer wavelength of the absorption maximum ($\lambda_{max.}^{255\ m\mu}$) in the spectrum of 2-(4′-methylphenyl)-Δ¹-pyrroline compared with that of 2-phenyl-Δ¹-pyrroline could be due to the effect of hyperconjugation. All of these UV spectral data are consistent with the observations made on the 2-aminoacetophenones and the 3-aminopropiophenones (20).

In the UV spectra, all of these Δ¹-pyrrolines display absorption maxima at the same wavelength in aqueous solution as in ethanol. Also, the same bathochromic shifts were observed for aqueous as for the alcoholic solutions. The absorption spectra of 2-(4′-chlorophenyl)-Δ¹-pyrroline in buffered aqueous solutions at various values of pH are presented in Fig. 5. From these data the apparent acid ionization exponent (pK′) of the 2-(4′-chlorophenyl)-pyrrolinium ion was calculated to be 6.46 at an ionic strength of 0.15 by use of the equation:

$$pH = pK' + \log \frac{\epsilon_{BH^+} - \epsilon}{\epsilon - \epsilon_B}$$

in which ε is the molar absorptivity at a certain pH value at which both the proton-donor species,

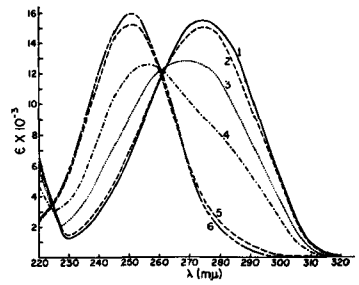


Fig. 5—Absorption spectra for aqueous solutions of 2-(4′-chlorophenyl)-Δ¹-pyrroline at several pH values. Key: 1, pH 1.0; 2, pH 4.58; 3, pH 5.9; 4, pH 6.53; 5, pH 7.99; 6, pH 12.2.

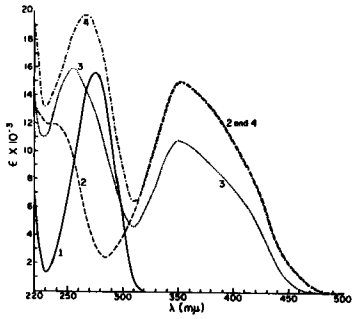


Fig. 6—Absorption spectra of aqueous solutions. Key: 1, 2-(4′-chlorophenyl)-Δ¹-pyrroline hydrochloride at pH 1; 2, picrate ion (picric acid in phosphate buffer at pH 7); 3, 2-(4′-chlorophenyl)-Δ¹-pyrroline picrate in distilled water; 4, the sum of Spectra 1 and 2.

BH^+ and the proton-acceptor species, B, are present in equilibrium; ϵ_{BH^+} is the molar absorptivity at a low pH at which the pyrroline is entirely in the form BH^+; ϵ_B is the molar absorptivity at a high pH at which the pyrroline is entirely in the form B. The average value of pK′ calculated from values of ϵ, ϵ_{BH^+}, and ϵ_B at $\lambda = 275$ mμ agreed closely with the average value calculated from absorptivity determined at 250 mμ.

In Fig. 6 are presented the absorption spectrum of the picrate of 2-(4′-chlorophenyl)-Δ¹-pyrroline (Spectrum 3) and for comparison, the spectra of the 2-(4′-chlorophenyl)-Δ¹-pyrrolinium ion (Spectrum 1), the picrate ion (Spectrum 2), and the sums of the latter two spectra (Spectrum 4). It can be seen that the absorption spectrum (Spectrum 3) of the picrate of 2-(4′-chlorophenyl)-Δ¹-pyrroline closely resembles that of the sum of the spectra of the picrate ion and the 2-(4′-chlorophenyl)-Δ¹-pyrrolinium ion, but the molar absorptivities are smaller at each wavelength than those of the combined spectrum (Spectrum 4). On the other hand, the absorption spectrum of the picrate complex also differs from that of the sum of the absorption spectra of free picric acid and the free base of 2-(4′-chlorophenyl)-Δ¹-pyrroline (Fig. 7). Consequently, it is concluded that the pyrroline in the picrate complex is incompletely protonated and exists as a mixture of the free base and the pyrrolinium ion.

The elemental analyses of the picrates of the various pyrrolines indicate a 1:1 ratio of picric acid to pyrroline. When an attempt (19) was made to prepare picrates of the 4′-substituted 4-aminobutyrophenones by addition of an excess of 4% picric acid in ethanol to an alcoholic solution of the hydrochlorides of the 4-aminobutyrophenones, the products isolated were identical in properties and analyses to the picrates of the corresponding 2-(4′-substituted phenyl)-Δ¹-pyrrolines, indicating that cyclization to the pyrrolines had occurred. A probable explanation may be that picric acid converted the water-soluble hydrochlorides into the difficultly soluble picrates, but did not completely protonate the *N*-unsubstituted γ-ketones; the latter underwent dehydrative cyclization to form the corresponding 5-membered ring Δ¹-pyrrolines, thus giving 2-(4′-substituted phenyl)-Δ¹-pyrroline picrates as products.

The IR absorption spectra of these 2-(4′-substituted phenyl)-Δ¹-pyrroline bases in mineral oil display a medium band in the region of 1613–1640 $cm.^{-1}$ (6.20–6.10 μ), characteristic of the —N=C< stretching. For the hydrochlorides, these bands shift to slightly higher frequencies.

All of the Δ¹-pyrroline bases studied herein gave 2,4-dinitrophenylhydrazones when dissolved in a saturated solution of 2,4-dinitrophenylhydrazine in 1 *N* hydrochloric acid. The onset of the precipitation started within 2–15 min., varying with the concentration of the base and the nature of the 2-substituent in the Δ¹-pyrroline ring. This illustrates the facility of the opening of the Δ¹-pyrroline ring, in the acidic medium when carbonyl reagent is present. The opening of the pyrroline ring under the influence of base and acyl halide to form the corresponding acylamido ketone derivatives of γ-amino ketones is documented in the literature (7, 21) and has also been observed in this laboratory. In view of the fact that these 2-(4′-substituted phenyl)-Δ¹-pyrrolines undergo ring opening with ease and behave chemically as open-chain γ-amino ketones, these pyrroline derivatives may be regarded as anhydro forms of γ-amino ketones and serve as a good source for the latter entities. Perhaps these two forms are in equilibrium in the solution, and any reagent capable of forming derivatives with either the amino or the carbonyl function will displace the equilibrium in favor of

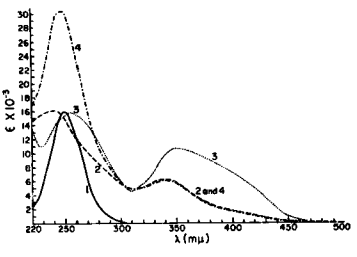
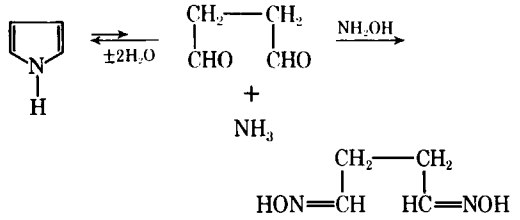


Fig. 7—Absorption spectra of aqueous solutions. Key: 1, 2-(4′-chlorophenyl)-Δ¹-pyrroline at pH 12; 2, picric acid in 2 N HCl; 3, 2-(4′-chlorophenyl)-Δ¹-pyrroline picrate in distilled water; 4, sum of Spectra 1 and 2.

the open-chain γ-amino ketone. Thus, with the reagent 2,4-dinitrophenylhydrazine in 1 *N* hydrochloric acid, the 2-(4′-chlorophenyl)-Δ¹-pyrroline base gave a 2,4-dinitrophenylhydrazone, m.p. 213–215°, in 2 min. This speculation seems analogous to the postulation made by Ciamician (22) on the reversible hydrolysis of pyrrole by bases (including the nearly neutral solution of hydroxylamine), in which the equilibrium, normally greatly in favor of the cyclic form, is displaced by fixing the succinaldehyde as the oxime:



The opening of the substituted pyrrole ring was also observed with hydrazines as the reagents (23), the products being the hydrazones of the diketones.

EXPERIMENTAL[1]

γ-Phthalimidobutyric Acid and γ-Phthalimidobutyryl Chloride—These compounds were prepared as described previously (19).

4′ - Methyl - 4 - phthalimidobutyrophenone—4-Phthalimidobutyryl chloride (40 g., 0.158 mole) was dissolved in 240 ml. of dry toluene. Forty grams (0.30 mole) of the anhydrous aluminum chloride (practical grade) was added in eight portions during a period of 40 min. while the solution was kept vigorously stirred and maintained at 90°. After 2 hr. of stirring, the mixture was poured with stirring into a mixture of 200 ml. of 1 *N* hydrochloric acid and 300 g. of crushed ice. The aqueous layer was extracted with four 30-ml. portions of toluene, and the extracts were combined with the toluene solution. The organic solution was washed with water, 5% sodium carbonate solution and dried over anhydrous sodium sulfate. The solid obtained from vacuum distillation of the solution was recrystallized from 95% alcohol. Twenty-nine grams of white crystals was obtained (60% yield), m.p. 115–116°.

4′-Methyl-4-aminobutyrophenone Hydrochloride and 2-(4′-Methylphenyl)-Δ¹-pyrroline Hydrochloride—The preparation of 4′-methyl-4-aminobutyrophenone hydrochloride has been reported (19). The procedure described below illustrates the isolation of both hydrochlorides of both 4′-methyl-4-amino-butyrophenone and 2-(4′-methylphenyl)-Δ¹-pyrroline from the same hydrolytic reaction by varying the reaction conditions as compared with those previously described (17).

4′-Methyl-4-phthalimidobutyrophenone (12 g., 0.052 mole) was dissolved in 36 ml. glacial acetic acid with the aid of heat. To this well stirred solution, 36 ml. of concentrated hydrochloric acid gradually was added, and the mixture was heated at the boiling point (*ca.* 107°) for 52 hr. A total of 60 ml. of concentrated hydrochloric acid was added during this period in order to compensate for the loss of hydrogen chloride due to refluxing. The mixture was allowed to cool to room temperature and filtered to remove the phthalic acid. The filtrate was evaporated under vacuum to remove hydrogen chloride and the resulting residue dissolved in water, extracted with diethyl ether to remove the remaining phthalic acid and the aqueous solution was then treated with decolorizing carbon.[2] The mixture was filtered and the filtrate evaporated *in vacuo* to a yield a syrupy liquid which was dissolved in ethanol, and upon the addition of ether, 6 g. of white crystals as the hydrochloride were obtained. Recrystallization from 20 ml. of ethanol gave 2.2 g. of crystals, m.p. 202–203°. The addition of acetone to the mother liquid gave a 2.6 g. yield of needle crystals, m.p. 142–143° after recrystallization from ethanol. From all the combined mother liquids, 0.5 g. of a mixture of these two hydrochlorides was obtained.

Both hydrochlorides gave a 2,4-dinitrophenylhydrazone when a saturated solution of 2,4-dinitrophenylhydrazine in 1 *N* hydrochloric acid was added to their aqueous solutions. Both of the 2,4-dinitrophenylhydrazones are identical and melt at 205–208° dec. The elemental analyses for the hydrochloride having a melting point of 142–143° correspond to the values calculated for 4′-methyl-4-aminobutyrophenone hydrochloride as previously described, whereas the elemental analyses of the hydrochloride having a m.p. of 202–203° agree with the structure for the dehydrated form, 2-(4′-methylphenyl)-Δ¹-pyrroline hydrochloride.

Anal.—Calcd. for $C_{11}H_{14}ClN$: C, 67.50; H, 7.21; Cl, 18.14; N, 7.17. Found: C, 66.84; H, 7.41; Cl, 18.05; N, 7.15. The UV spectra of both these hydrochlorides display an absorption maximum at 278 mμ. The $\epsilon_{max.}$ of 4′-methyl-4-aminobutyrophenone hydrochloride is 19,580, whereas that of 2-(4′-methylphenyl)-Δ¹-pyrroline hydrochloride is 16,904. When the solutions of these two compounds were buffered with sodium acetate, the maxima in both spectra were displaced to 252 mμ, the $\lambda_{max.}$ of the free 2-(4′-methylphenyl)-Δ¹-pyrroline.

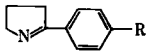
2-(4′-Methylphenyl)-Δ¹-pyrroline—4′-Methyl-4-aminobutyrophenone hydrochloride (1.65 g., 0.0077 mole) was dissolved in 15 ml. of water, and to this solution was added dropwise a solution of 5% sodium carbonate until the pH of the mixture was approximately equal to 8. The white precipitate was filtered and thoroughly washed with water to obtain 1 g. (81%) of white crystals, m.p. 58–58.5°.

Anal.—Calcd. for $C_{11}H_{13}N$: C, 82.96; H, 8.23; N, 8.80. Found: C, 83.08; H, 8.26; N, 8.80.

[1] All elemental analyses were performed by Alfred Bernhardt Microanalytical Laboratories, Ruhr, Germany. All melting points were taken in a Fisher-Johns melting point apparatus and have been corrected. The IR spectra were determined for mineral oil mulls, using the Perkin-Elmer Infracord spectrophotometer. The UV spectra for aqueous or ethanolic solutions were determined manually by using a Beckman DU spectrophotometer, and automatically by using a Beckman DB and DK-1 spectrophotometer with automatic recorders. NMR spectra were determined for carbon tetrachloride solutions, using the Varian A-60 high-resolution NMR spectrometer.

[2] Norit A, American Norit Co., Jacksonville, Fla.

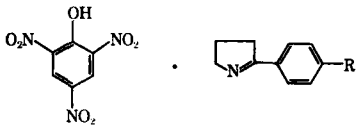
TABLE I—2-(4′-SUBSTITUTED PHENYL)-Δ^1-PYRROLINES



R	M.p., °C.	Yield, %	Molecular Formula	Anal., % Calcd.	Found
OCH_3	72–73[a]	90	$C_{11}H_{13}NO$	C, 75.38	C, 75.33
				H, 7.48	H, 7.62
				N, 7.80	N, 8.15
Br	87–88	80	$C_{10}H_{10}BrN$	C, 53.70	C, 53.60
				H, 4.52	H, 4.58
				N, 6.22	N, 6.23
				Br, 35.60	Br, 35.77
Cl	68–69	81	$C_{10}H_{10}ClN$	C, 66.83	C, 66.09
				H, 6.12	H, 5.68
				N, 7.09	N, 6.84
				Cl, 19.74	Cl, 19.80
F	42.5–43	84	$C_{10}H_{10}FN$	C, 73.57	C, 74.23
				H, 6.19	H, 6.02
				N, 8.58	N, 8.60
				F, 11.65	F, 11.57

[a] Lit. (24), m.p. 73–73.5°.

TABLE II—2-(4′-SUBSTITUTED PHENYL)-Δ^1-PYRROLINE PICRATES



R	M.p., °C	Yield, %	Molecular Formula	Anal., % Calcd.	Found
OCH_3	174–177	90	$C_{17}H_{16}N_4O_8$	C, 50.48	C, 50.77
				H, 3.98	H, 4.20
				N, 13.86	N, 13.84
Br	225–230 dec.	90	$C_{16}H_{13}BrN_4O_7$	C, 42.38	C, 43.08
				H, 2.89	H, 3.10
				N, 12.36	N, 12.11
				Br, 17.64	Br, 17.40
Cl	215–218 dec.	90	$C_{16}H_{13}ClN_4O_7$	C, 46.99	C, 47.11
				H, 3.21	H, 3.43
				N, 13.71	N, 13.55
				Cl, 8.68	Cl, 8.68
F	190 dec.	85	$C_{16}H_{13}FN_4O_7$	C, 48.97	C, 49.01
				H, 3.34	H, 3.50
				N, 14.28	N, 14.08
				F, 4.84	F, 4.73

This product was identical with that prepared from the hydrochloride of 2-(4′-methylphenyl)-Δ^1-pyrroline. The other 2-(4′-substituted phenyl)-Δ^1-pyrroline bases prepared by a similar procedure are recorded in Table I.

2-(4′-Methylphenyl)-Δ^1-pyrroline Picrate—2-(4′-Methylphenyl)-Δ^1-pyrroline (0.2148 g., 0.0013 mole) was dissolved in 3 ml. of 95% alcohol, an excess of 4% picric acid in 95% ethanol was added dropwise to this solution to obtain a precipitate. After the mixture was allowed to stand at room temperature for 15 min., the precipitate was filtered and washed with 95% alcohol. The crude product was then recrystallized from 30 ml. of 95% boiling alcohol. The yellow shining crystals (0.445 g., 88% yield) melted at 187–189° with decomposition.

Anal.—Calcd. for $C_{17}H_{16}N_4O_7$: C, 52.56; H, 4.15; N, 14.43. Found: C, 52.58; H, 4.55; N, 14.49. Data for other picrates of 2-(4′-substituted phenyl)-Δ^1-pyrroline prepared by a similar procedure are recorded in Table II.

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Keyphrases

2-(4′-Substituted phenyl)-Δ^1-pyrrolines—synthesis
Friedel-Crafts acylation—synthesis method
NMR spectroscopy—structure
UV spectrophotometry—structure
IR spectrophotometry—structure

Model Catalysts Which Simulate Penicillinase III

Structure-Reactivity Relationship in Catalysis of Penicillin Hydrolysis by Morpholinomethyl Derivatives of Catechol and Pyrogallol

By RENAAT D. KINGET and MICHAEL A. SCHWARTZ*

Studies of catalysis of hydrolysis of benzylpenicillin by aminoalkylcatechols have been extended to a series of morpholinomethyl derivatives of catechol and pyrogallol. Catalytic rate constants for each of the active species are related to their basicity and presence or absence of electrophilic groups and a positively charged amine in proper position to assist nucleophilic attack by a phenolate ion. Ionization constants for these compounds determined spectrally were the same as those determined potentiometrically indicating that the catalytic species are hybrids between charged amine and phenolate ion. The structures of these species are correlated with their efficiency as catalysts on the basis of a mechanism involving participation by up to four functional groups on the catalyst.

PREVIOUS STUDIES in this laboratory (1, 2) have shown that 3,6-bis(dimethylaminomethyl)catechol (CDM) is an efficient catalyst for the hydrolysis of benzylpenicillin to benzylpenicilloic acid and that both metastable tetrahedral intermediates and a catechol monoester are probably involved in the reaction pathway. The nature of the reactive catalytic species (CDM^{+1}) was deduced on the basis of the pH-rate profile, which showed a maximum at pH 8, and the bathochromic shift in the UV absorption spectrum upon neutralization of one equivalent of the dihydrochloride.

In the present work the studies have been extended to include a series of morpholinomethyl derivatives of catechol and pyrogallol. Morpholine is much less basic than dimethylamine and, it was thought, this difference in basicity would affect the catalytic efficiency of the catecholamines. Additionally, spectrophotometric titrations were carried out in order to learn more about the nature of the various species produced in the stepwise ionization of both the morpholine and dimethylamine derivatives.

EXPERIMENTAL

Synthesis of Catalysts—The morpholinomethyl derivatives of catechol and pyrogallol were prepared by the method of Fields *et al.* (3). In the case of the pyrogallol derivative the reaction was carried out in a nitrogen atmosphere to minimize oxidation. The hydrochloride salts were prepared by admitting dry hydrogen chloride gas into a methanol solution of the base and crystallization was induced by addition of ethyl acetate or dry ether. Table I lists these

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Accepted for publication July 19, 1968.
Presented to Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
Supported by grant No. AI-06901 from the National Institute of Allergy and Infectious Disease, U. S. Public Health Service, Bethesda, Md.
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TABLE I—MORPHOLINOMETHYL DERIVATIVES

Compd.	Abbreviation	M.p., °C.	Anal.[a] Calcd.	Found
3-Morpholinomethylcatechol·HCl	CMo	161–163	C, 53.77 H, 6.56 N, 5.70 Cl, 14.43	C, 53.61 H, 6.63 N, 5.83 Cl, 14.40
3,6-Bis(morpholinomethyl)catechol·2HCl	CDMo	265 dec.	C, 50.40 H, 6.87 N, 7.35 Cl, 18.60	C, 50.50 H, 6.83 N, 7.39 Cl, 18.48
3,4,6-Tris(morpholinomethyl)catechol·3HCl	CTMo	170–176	C, 48.80 H, 7.00 N, 8.13 Cl, 20.58	C, 48.57 H, 7.20 N, 8.01 Cl, 20.30
4,6-Bis(morpholinomethyl)pyrogallol·2HCl	PDMo	209–210 dec.	C, 48.37 H, 6.60 N, 7.05 Cl, 17.85	C, 48.16 H, 6.49 N, 6.91 Cl, 17.67

[a] Analyses were done by Galbraith Laboratories, Knoxville, Tenn.

compounds. The preparation of the dimethylaminomethyl derivatives was previously described (1).

Reagents—Potassium benzylpenicillin was kindly supplied by Bristol Laboratories, Syracuse, N. Y. All other materials were Fisher certified reagents.

Potentiometric Titrations—The apparent acid dissociation constants (pKa′) were determined by potentiometric titration at 31.5°, ionic strength 0.20, using a Radiometer TTT-1 automatic titrator with PHA 630T scale expander. Catecholamine concentration was 0.008 *M* and 0.2 *M* NaOH was used as titrant. When successive pKa′ values were less than 3 units apart the method of Noyes (4) was used to calculate the result.

Spectrophotometric Titration—The change in absorption at a single wavelength in the UV region as a function of pH was measured as follows.

A solution of the compound, acidified to pH 2, was placed in a conical flask modified with exit and entry tubes and containing electrodes from a Radiometer M-26 pH meter, a titrant delivery tube, and tube for admission of nitrogen. The solution was circulated to a flow cell on a Hitachi-Perkin-Elmer model 139 spectrophotometer by means of a peristaltic pump at a rate of about 300 ml./min. Titrant (1.0 *M* NaOH) was added from an ultraburette model 200, the volume added during a titration being insignificant relative to the total volume of solution.

The wavelengths at which the titrations were conducted were selected after preliminary observation of the effect of pH on absorption spectrum of the compound. Generally, the wavelength chosen was that at which there was the greatest change in absorbance with pH. From the plots of absorbance *versus* pH, spectral pKa′ values were determined.

The spectral dissociation constants were determined at room temperature (about 27°). In addition to the morpholinomethyl compounds in Table I, two of the dimethylaminomethyl derivatives were also titrated. Table II lists all the compounds studied with the characteristics of their spectra and the wavelengths at which the titrations were conducted.

Hydrolysis Rates—Rate of hydrolysis of benzylpenicillin in presence of catalyst was carried out as previously described (1) by following the rate of acid production on a pH-stat. All rates were measured at 31.5° and ionic strength 0.20. First-order kinetics was observed at constant pH and the concentrations of materials used.

TABLE II—SPECTRAL CHARACTERISTICS OF COMPOUNDS STUDIED

Compd.	$\lambda_{max.}$ at pH 1.5(mμ)	Change with Increasing pH	Wavelength Titrated
CMo	285	Decrease at 285; shoulder at 298	285
CDMo	288	Decrease at 288; shoulder at 300	288
CTMo	295	Decrease at 295; shoulder at 312	295
PDMo	273	Increase at 273	273
CDM[a]	287	Shift of $\lambda_{max.}$ to 305	305
CTM[b]	293	Shift of $\lambda_{max.}$ to 307; new peak 253 mμ	253, 307

[a] 3,6-Bis(dimethylaminomethyl)catechol·2HCl. [b] 3,4,6-Tris(dimethylaminomethyl)catechol·3HCl.

RESULTS

Spectral Titrations—The effect of pH on the ultraviolet absorption spectra of CDMo and CTMo are shown in Figs. 1 and 2, respectively. In each case the change with increasing pH involves a decrease in absorbance at the maximum and the formation of a shoulder at a higher wavelength. The pattern for CMo is the same as that shown for CDMo and is not depicted. These curves may be contrasted with the pattern previously observed for CDM (1) which is typical of all the dimethylaminomethyl derivatives. The latter compounds show pronounced bathochromic shifts of the wavelength of maximum absorption with increasing pH.

Figure 3 shows the spectral titration curves for CDMo and CMo. The dissociation constants for the latter compound were determined from the pH at the midpoint of the descending and ascending arms of the curve. For CDMo the situation is more complex. There is an obvious curvature at about pH 6.5. Apparently two ionization constants are involved in this portion of the curve. These constants were taken as the pH values at the midpoints of each section.

In the curve for PDMo in Fig. 4 there are clearly three dissociation constants involved and these were

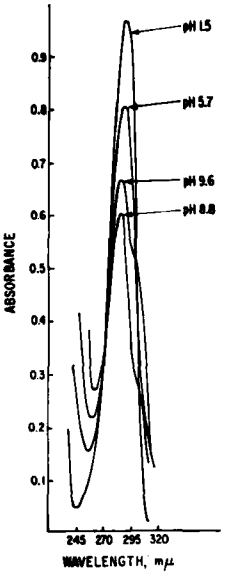


Fig. 1—UV absorption spectra of CDMo at various pH's.

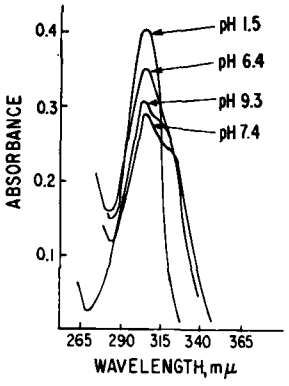


Fig. 2—UV absorption spectra of CTMo at various pH's.

estimated from the midpoints of the appropriate portions of the curve.

In Fig. 5 is shown the titration curve for CTMo in which are seen three distinct breaks in the downward portion corresponding to the three spectral pKa′ values taken as the midpoints of each section.

Figure 6 shows the titration curve for CDM with two unambiguous dissociations while in Fig. 7, showing the curve for CTM, there is a definite discontinuity in the ascending arm of the curve at both wavelengths studied. Again there are apparently two dissociations involved prior to the maximum at pH 9

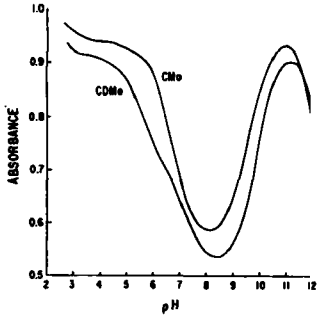


Fig. 3—Spectral titration curves for CDMo and CMo.

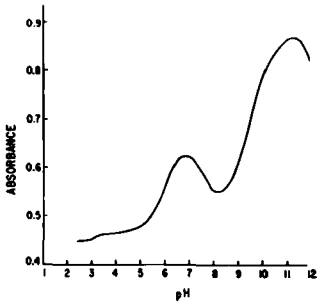


Fig. 4—Spectral titration curve for PDMo.

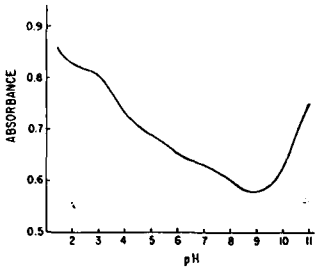


Fig. 5—Spectral titration curve for CTMo.

and the constants were determined as described above in the case of CDMo.

All of the pKa′ values determined both spectrally and potentiometrically are listed in Table III. It is apparent that in almost every case the values determined spectrally parallel those determined potentiometrically. The one apparent exception is pK_1' for PDMo where there is a difference of about 0.25 units between the two values. The value obtained by potentiometric titration is the more accurate of the two and the spectral pKa′ cannot be lower than the other on theoretical grounds. For this compound pK_1' and pK_2' are fairly close together and the overlap caused a change in direction of the spectral titration curve making it extremely difficult to separate pK_1' and pK_2' as was done in the potentiometric titration. The value for pK_1' used for purposes of this work was therefore 6.25.

Since the spectral and potentiometric ionization constants are the same, no microconstants are required and only one species is formed at each stage in the ionization scheme. This may be considered to be a hybrid between phenolate ion and free base of the amine.

Kinetic Studies—The observed first-order rate

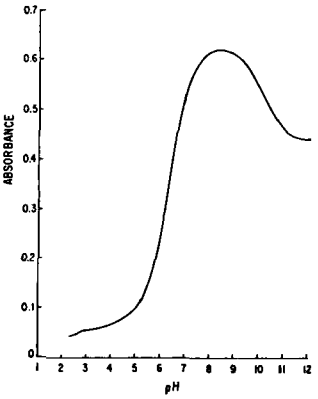


Fig. 6—Spectral titration curve for CDM.

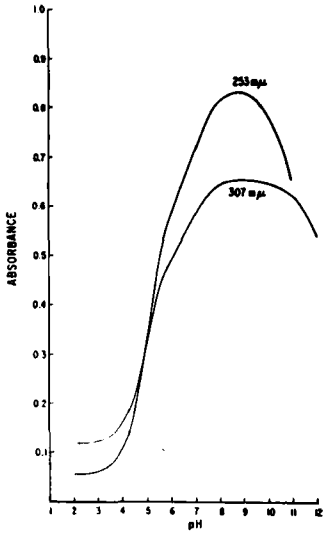


Fig. 7—Spectral titration curves for CTM at two wavelengths.

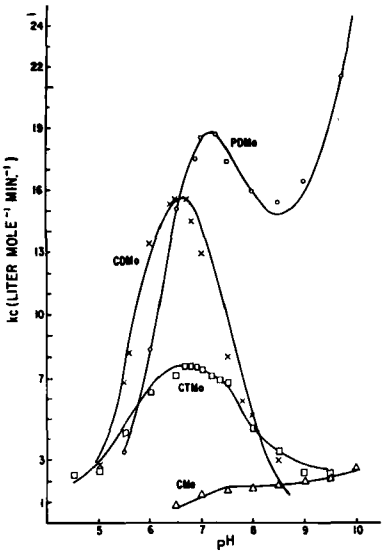


Fig. 8—pH-Rate profiles for morpholine derivatives. The curves were drawn from calculated constants while the points represent experimental data.

constants, after correction for alkaline hydrolysis where necessary, were divided by the molar concentration of catalyst to obtain the catalytic rate constants, k_c, at each pH. From the pH and pKa′ values the fraction of the total concentration of catalyst present in each ionic state was calculated. Specific rate constants for each species were calculated by assuming that the total rate constant, k_c, was the sum of the individual constants, *i.e.*,

$$k_c = k_{+2}f_{+2} + k_{+1}f_{+1} + k_0f_0 \ldots$$

where the subscripts represent the electrostatic charge on the particular species and the f's designate fraction. The curves shown in Fig. 8 were drawn from the calculated constants given in Table IV, while the points are the actual experimental data. Generally these follow a pattern similar to that of the corresponding dimethylamine derivatives (1) but the pH of maximum rate is different in each case. The species of CDMo with +1 charge has its maximum concentration at pH 6.5 where the maximum rate occurs, exactly as observed with CDM. The rate constant for $CDMo^{+1}$ is lower than that for CDM^{+1} (45 M^{-1} min.$^{-1}$) indicating the effect of basicity on catalytic efficiency. This effect is more explicitly depicted in the typical Brönsted plot of Fig. 9 which also includes data from the previous study (1). Statistical corrections of these data were made by the criteria of Benson (5). It can be seen that all of the species involved fall into three distinct groups. The lines drawn through two of the groups are essentially parallel while the right-hand line was drawn parallel to the other two through the points involved.

TABLE III—DISSOCIATION CONSTANTS

Compd.		pKa′ by Potentiometric Titration	pKa′ by Spectral Titration
CMo	pK_1'	6.75	6.7
	pK_2'	9.66	9.6
CDMo	pK_1'	5.65	5.6
	pK_2'	7.32	7.3
	pK_3'	9.86	9.8
CTMo	pK_1'	3.75	3.7
	pK_2'	5.80	5.7
	pK_3'	7.90	7.8
PDMo	pK_1'	6.25	6.0
	pK_2'	7.66	7.6
	pK_3'	9.65	9.6
CDM[a]	pK_1'	6.35	6.4
	pK_2'	9.65	9.7
CTM[a]	pK_1'	4.95	4.9
	pK_2'	7.10	7.2
	pK_2'	10.35	

[a] pKa′ by potentiometric titration was previously determined (1).

DISCUSSION

It is apparent from Fig. 9 that there is a consistent pattern in the relationship between the catalytic efficiency and structural features of the compounds studied. The most reactive group of species, taking into account basicity, is on the left-hand line and all have in common two electrophilic groups *ortho* and a positively charged amine *meta* to the nucleophilic phenolate. The next group (middle line in Fig. 9) lacks the charged amine in the *meta* position and as a result is 50-fold less reactive at the same pKa′ than the first group. The third group, consisting only of catecholate ion (C^{-1}), CMo^{-1}, and CMM^0, is 10-fold

TABLE IV—RATE CONSTANTS

Compd.	Net Charge on Species	Catalytic Rate Constant (M^{-1} min.$^{-1}$)
CMo	0	1.5
	−1	2.6
CDMo	+1	19.0
	0	2.0
	−1	—
CTMo	+2	1.5
	+1	8.5
	0	2.5
PDMo	+1	23.5
	0	10.0
	−1	30.0

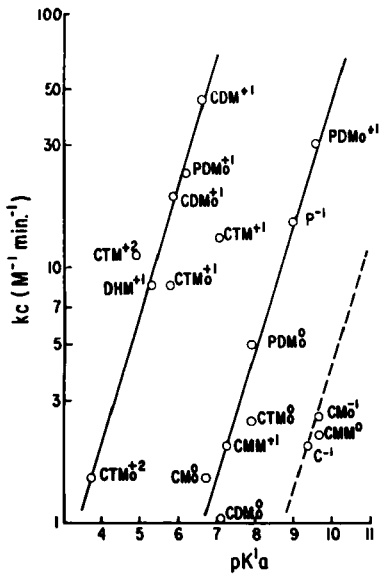


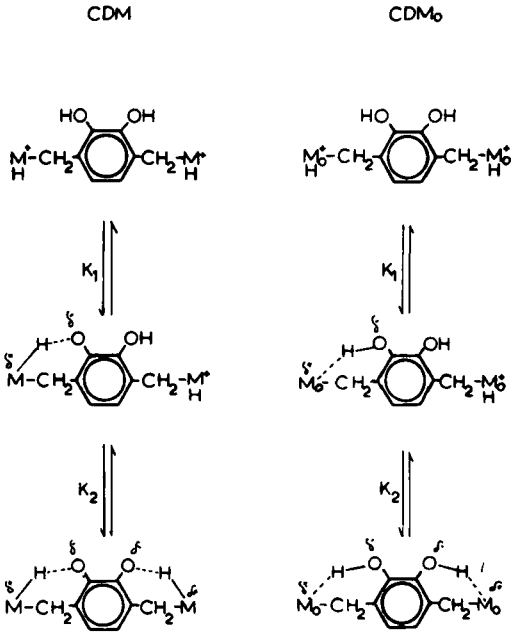
Fig. 9—Brönsted plot including data from the present study and Reference 1. *The symbols are all defined in the text.*

less reactive than the middle group and is missing one of the *ortho* electrophilic groups referred to above. These data provide a quantitative measure of the effect of each of the groups mentioned.

The results are in agreement with the previously proposed thesis that all four groups on species like CDM^{+1} are involved in the catalysis of penicillin hydrolysis. Study of rate of penicillin loss in addition to acid formation in the case of catechol (2) and CMo (6) has clearly demonstrated the presence of an intermediate, probably catechol monopenicilloate, in the reaction pathway. Thus the two electrophilic groups on the catalyst *ortho* to the phenolate may participate in either one or both steps, *i.e.*, ester formation and/or ester hydrolysis. Studies of hydrolysis of catechol monoesters (7, 8) have indicated participation by the phenolate ion and it seems likely that a positively charged amine on the other side of the ester would enhance the hydrolysis rate still further by general acid catalysis. The *ortho* phenolic hydroxyl group participates in the reaction of catecholate ion with isopropylmethylphosphonofluoridate (sarin) (9) and probably plays a similar role in formation of the ester intermediate in catechol-catalyzed penicillin hydrolysis. The role of the charged amine groups in the reaction with sarin is not clear. Epstein *et al.* (10) have referred to a "charge effect" by which these charged groups lower the basicity of the phenol without affecting its nucleophilicity toward the neutral substrate. This "charge effect" does not seem to be significant in the reaction of these compounds with penicillin. The species CMo° falls on the same line as pyrogallol in Fig. 9, in one case a phenolic hydroxyl and in the other a protonated amine being involved. If the charge effect were significant, one would expect CMo^{0} to show a somewhat greater rate enhancement than pyrogallol. Also CMM^{+1} [3,5-bis(dimethylaminomethyl)catechol] falls on the line in the Brönsted plot with pyrogallol (P^{-}), the charged amine in the 5 position having no apparent effect on catalysis, as it should according to the charge effect hypothesis.

One species which does not fit with any group is CTM^{+1}. This species if formed by neutralization of the dimethylamine group in the 4 position would retain all of the structural features of the species on the left-hand line in Fig. 9. While its basicity is lower than that of CTM^{+2}, its reactivity is changed only very slightly. It seems that neutralization in the 4 position has little effect on the reactivity of the phenolate ion in CTM^{+1} while in CTMo^{+1} there is a much larger effect. This result is consistent with the greater basicity of dimethylamine relative to morpholine.

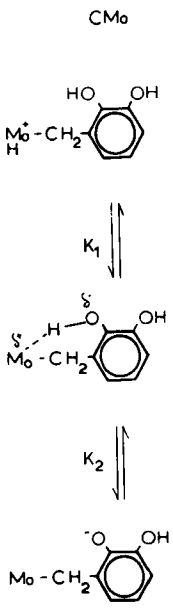
The kinetic data combined with the effects of pH change upon UV spectra provide a basis for consideration of the nature of each of the species of the catecholamines studied. In doing this, account must be taken of the fact that in each step some phenolic dissociation is involved and that only one species is produced. The spectral changes as a function of pH with the morpholine derivatives are much smaller than those observed with the dimethylamine compounds. This difference may be rationalized by the differences in basicity of the two amines. For comparison, pKb of *N*-methylmorpholine is 6.06 (11) and that of trimethylamine is 4.2 (12). The morpholine would therefore, have much less attraction for a proton than the dimethylamine and less influence on ionization of the phenol. Consider, for example, the comparison of CDM and CDMo in Scheme I. In each case neutralization of one acidic function leads to a hybrid species in which a proton is shared by the amine and phenolate ion. In CDM this proton is closer to the amine than in CDMo, consistent with the observed spectral behavior. While a rate constant for CDM° could not be determined accurately a value in the range 1–4 M^{-1} min.$^{-1}$ was estimated (1), placing this species in the



Scheme I

group with catecholate ion on the Brönsted plot. The similar species CDMo^0 however, fits better with the middle group containing pyrogallol anion. The rationale for this difference between the two species can be seen from the structures drawn in Scheme I. In CDMo^0 the protons are closer to the oxygens making them more available for the catalytic reaction than in CDM where they are closer to the amine groups.

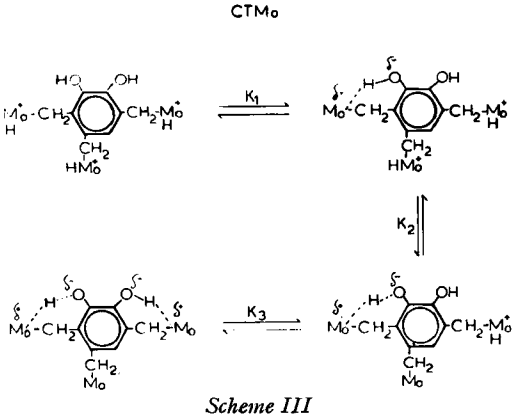
Scheme II depicts the proposed mode of ionization of CMo. It can be seen from Fig. 9 that the zero



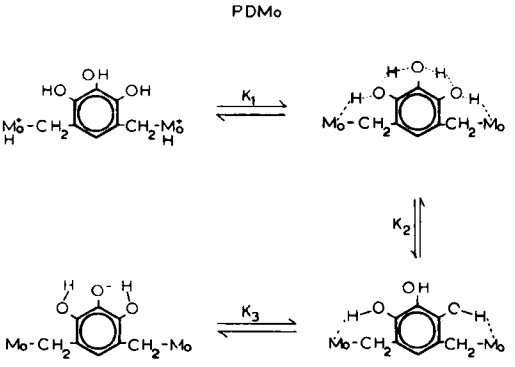
Scheme II

charged species behaves kinetically like pyrogallolate anion while CMo^{-1} resembles catechol in its catalytic activity. The structures drawn for these species reflect both the observed spectral changes and the rate data.

Of principal interest in Scheme III for CTMo is the fact that both CTMo^{+2} and CTMo^{+1} fall on the left-hand line of Fig. 9, and should resemble CDM^{+1} in arrangement of participating functional groups. Removal of a proton in the second step, from the



Scheme III



Scheme IV

morpholino in the 4 position leaves a structure with these features. Removal of the positive charge in the 4 position should enhance the attraction for the phenolic proton by the morpholine in the 3 position resulting in the observed further spectral shift. The structure shown for CTMo^0 is appropriate for a species resembling pyrogallolate ion in its catalytic activity.

In Scheme IV the dissociation process for PDMo is depicted. There are added complexities with this compound due to its symmetry and the fact that there are three hydroxyl groups which may influence spectral changes. Although there are probably other ways in which the kinetic and spectral data for this compound may be reconciled, that shown here does account for the observed results.

A point worthy of special note in this study is the effect of amino groups situated close to a phenol on the ionization of the latter. While phenols normally have pKa′ values in the range 9–10, the phenols in the present study have pKa′ values as low as 3.7. Even this weakly basic group shows a relatively high degree of nucleophilicity toward penicillin. Similar effects on phenolic groups in enzymes may occur if the environment surrounding the phenol is suitable. Therefore, tyrosine should not be excluded from consideration as a group in the active site of an enzyme on the basis of pKa′ measurements alone.

Perturbation of the pKa of a phenolic hydroxyl in a "reporter group" at the active site of chymotrypsin by a charged amine has been reported (13), further emphasizing the importance of local environment on the dissociation of an acidic group.

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Keyphrases

Catalysts, model—penicillinase simulation
Benzylpenicillin hydrolysis—catalyst structure-activity relationship
Catechol morpholinomethyl derivative—penicillin hydrolysis
Pyrogallol morpholinomethyl derivative—penicillin hydrolysis
Potentiometric titration—pKa determination
UV spectrophotometry—pKa determination

Synthesis of (±)-Norargemonine

By KUO-HSIUNG LEE and T. O. SOINE*

(±)-Norargemonine was synthesized in good yield by a multiple-step process. The procedure involved Bischler-Napieralski ring closure of *N*-4-benzyloxy-3-methoxy-phenethyl-2-(3,4-dimethoxyphenyl)acetamide, reduction of the ensuing 3,4-dihydro base to the tetrahydro form, and dehydrogenation of the latter to yield the isoquinoline which, however, had been debenzylated during dehydrogenation. Rebenzylation of the phenol, conversion to the methiodide, reduction to the corresponding *N*-methyl-1,2-dihydro base, and acid catalyzed cyclization accompanied by simultaneous debenzylation afforded (±)-norargemonine to complete the synthesis.

NORARGEMONINE WAS FIRST isolated by Soine and Gisvold (1) in 1944 from *Argemone hispida* (Gray). It was shown to be the monophenolic precursor of argemonine (I) by methylation with diazomethane (2–4) and argemonine, itself, has been shown to be (−)-*N*-methylpavine (2, 4, 5).

In a recent communication, the structure of norargemonine has been shown by Stermitz *et al.* (6) to be II by the reduction of protopapaverine methochloride with tin in hydrochloric acid according to the method of Späth and Epstein (7). An alternate reduction, not utilized but simply mentioned, would have been the catalytic method of Schöpf and Thierfelder (8). The Späth and Epstein method yielded the tetrahydro derivative [*i.e.*, (±)-codamine] together with a small amount of the objective compound (II). Since II is essentially a by-product of the above procedure and is obtained in poor yield it appeared that a more feasible synthetic route would be needed for other studies with II contemplated in these laboratories.

DISCUSSION

Benzyl vanillin (IV), prepared by benzylation of vanillin (III) according to Tomita *et al.* (9) was condensed with nitromethane, as described by Gairaud *et al.* (10) to furnish a good yield of 4-benzyloxy-3-methoxy-β-nitrostyrene (V). The latter was then subjected to lithium aluminum hydride reduction to give 4-benzyloxy-3-methoxyphenethylamine (VI), characterized as its formate salt. Condensation of VI and 3,4-dimethoxyphenylacetic acid (VII) was effected by application of the method reported by Shepard *et al.* (11) to yield the corresponding acid amide (VIII), *viz.*, *N*-(4-benzyloxy-3-methoxyphenethyl) - 2 - (3,4 - dimethoxyphenyl)acetamide (Scheme I).

Cyclization of VIII by the Bischler-Napieralski procedure (12) led to the expected 3,4-dihydroisoquinoline which was isolated as its hydrochloride (IX). Sodium borohydride reduction of IX furnished a quantitative yield of the corresponding 1,2,3,4-tetrahydroisoquinoline (X) which showed 3,300 cm.$^{-1}$ (ν NH) in the IR spectrum in mineral oil and was very unstable, decomposing within 24 hr. at room temperature. Characterization of this compound was carried out by preparing its stable hydrochloride salt (XI) which exhibited a group of relatively sharp bands over 2,440–2,700 cm.$^{-1}$ (ν $\overset{+}{N}H_2$) in the IR spectrum. It also showed a typical benzyltetrahydroisoquinoline UV absorption and no bathochromic shift was observed upon the addition of sodium hydroxide. The NMR (τ, in $CDCl_3$) of this compound showed signals at 6.26 (3H, s, C_3'-$OC_3\underline{H}$),[1] 6.18 (6H, s., C_4'-$OC\underline{H}_3$ and C_6-$OC\underline{H}_3$),[1] 5.20 (2H, s, C_7-$OC\underline{H}_2C_6H_5$), 3.83 (1H, s, C_8-$\underline{H}$),[1] 2.75 (5H, s, C_7-$OCH_2C_6\underline{H}_5$), and the remaining aromatic protons appeared at 3.34–3.45 (4H).

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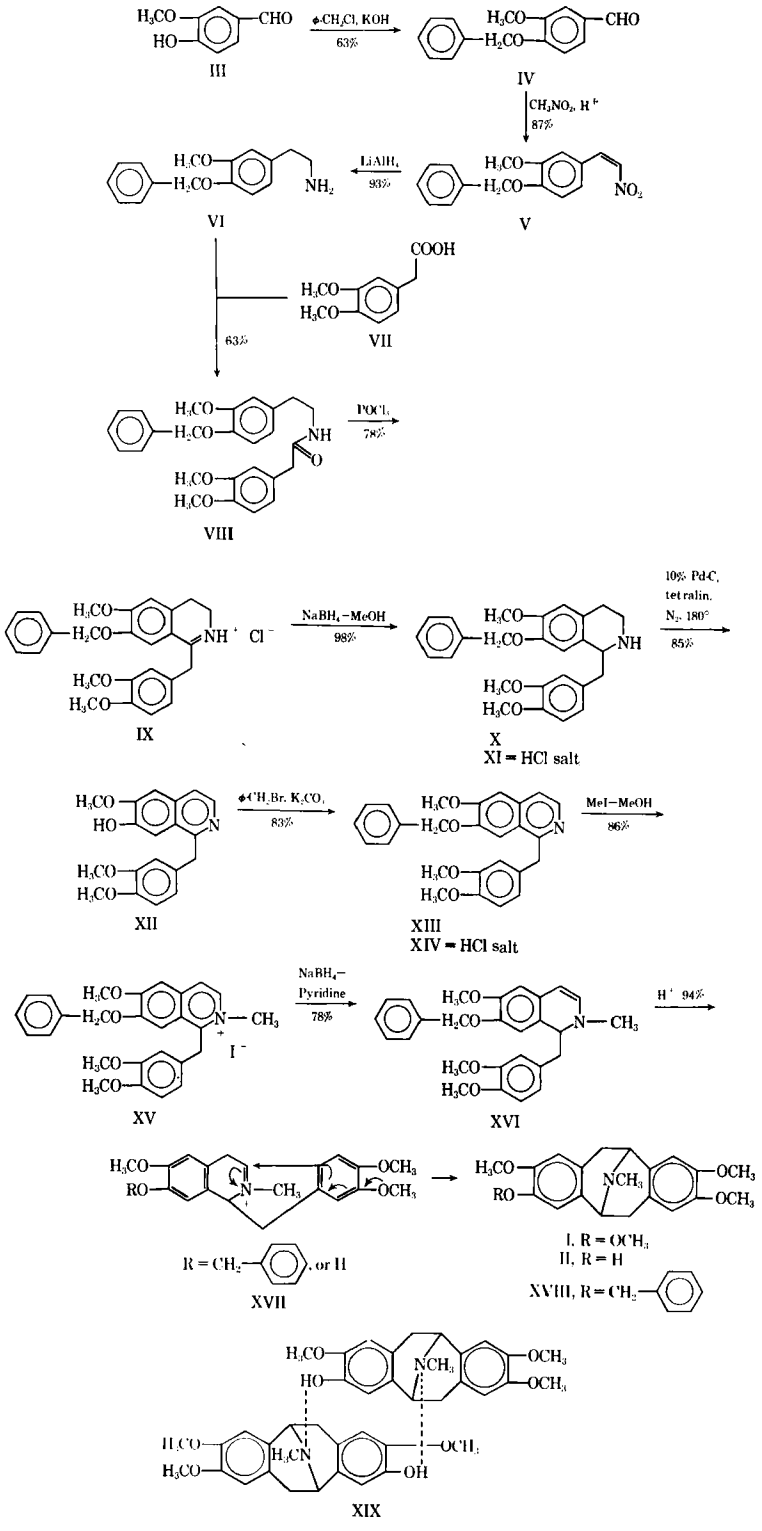

Scheme I

Catalytic dehydrogenation of X to the isoquinoline (XII) was readily achieved by the use of palladium-charcoal catalyst. The dehydrogenated product thus formed gave a negative ferric chloride test for phenol and failed to show hydroxy group absorption in the IR spectrum. However, the phenolic

character of this compound was indicated by its UV spectrum; *i.e.*, it gave a characteristic isoquinoline absorption which showed a bathochromic shift upon the addition of sodium hydroxide. The isoquinoline character together with the absence of the 7-benzyloxy group of XII was further indicated by its NMR (τ, in $CDCl_3$) spectrum which shows signals at 6.35 (3H, s, C_3'-OCH_3), 6.29 (3H, s, C_4'-OCH_3), 6.05 (3H, s, C_6-OCH_3), 5.62 (2H, s, C_1-CH_2), 2.64 (1H, d, C_4-H, $J_{3,4}$ = 6 c.p.s.), and 1.78 (1H, d, C_3-H, $J_{3,4}$ = 6 c.p.s.). From the above evidence, XII was considered to be identical with the compound prepared by Billek (14) by an alternate but less productive procedure. Debenzylation combined with dehydrogenation of X to yield XII in place of the expected 7-benzyloxyisoquinoline (XIII) appears to be a phenomenon thus far not reported, although the debenzylation can be readily rationalized in terms of hydrogenolysis by hydrogen transfer.

O-Benzylation of XII afforded the corresponding free base of the 7-benzyloxyisoquinoline (XIII) after liberation from its hydrochloride salt (XIV). The UV spectrum of XIII showed no bathochromic shift upon addition of sodium hydroxide and its NMR (τ, in $CDCl_3$) spectrum further indicated the presence of the methylene protons of the 7-benzyloxy group as a singlet at 4.88 τ. Conversion of XIII to the methiodide (XV) was readily achieved in good yield by conventional procedures. The NMR spectrum of XV showed a singlet at 5.02 τ (3H) ($\overset{+}{N}$—CH_3) in addition to other expected peaks. Reduction of XV to the 1,2-dihydroisoquinoline (XVI) was carried out smoothly and in excellent yield by using sodium borohydride in pyridine according to Barton *et al.* (15). XVI showed a characteristic 1,2-dihydroisoquinoline UV absorption almost identical with that of 1,2-dihydro-*N*-methyl papaverine and the singlet for protons of the *N*-methyl group in the NMR spectrum was shifted upfield from 5.02 τ in XV to 7.15 τ in XVI. Acid-catalyzed ring closure of XVI proceeds by way of the C-protonated intermediate (XVII) to the pavine (*i.e.*, argemonine) type skeleton according to Battersby *et al.* (16, 17) and was effected in almost quantitative yield. The cyclized product, m.p. 218–220°, gave every evidence of being the desired (±)-norargemonine based on its melting point and characteristic UV spectrum. The lack of a bathochromic shift on addition of sodium hydroxide and its failure to show significant hydroxyl absorption in the IR are similarly in keeping with the behavior of norargemonine from natural sources. However, because the starting material for cyclization was an *O*-benzyl derivative it seemed desirable to exclude the possibility that the product might be an *O*-benzyl norargemonine (XVIII). Thus, the compound obtained was further subjected to debenzylating conditions by refluxing it with concentrated hydrochloric acid in ethanol. No change in properties was observed under this treatment and, thus, it was concluded that debenzylation had occurred during the acidic cyclization process. This was not unexpected and is in keeping with the known lability of benzyloxy groups under these acidic hydrolytic conditions. The product, when compared with natural norargemonine, showed identical silica gel TLC behavior, UV and IR spectra and its NMR spectrum and melting point were in complete agreement with those of Stermitz *et al.* (6).

It is of interest to point out the absence or, at best, extremely weak absorption of the phenolic hydroxy group in the IR spectra of both synthetic and natural norargemonine in mineral oil. It is possible that this may be due to the existence of II in dimeric form through an intermolecular hydrogen bonding between the hydroxy and *N*-methyl group of two molecules as shown in XIX. Models certainly indicate the feasibility of such bonding. The behavior on addition of trace amounts of concentrated hydrochloric acid to the chloroform solutions of both synthetic and natural norargemonine supports this suggestion. Thus, identical IR spectra as well as a new free hydroxy absorption band were obtained and generated at $\nu_{max.}$ 3,640 cm.$^{-1}$ in both compounds, suggesting elimination of hydrogen bonding.

EXPERIMENTAL

Melting points were determined in capillary tubes in a Thomas-Hoover melting point apparatus checked for accuracy against a set of standard samples and are uncorrected. Microanalyses were determined by the Microanalytical Laboratory, School of Chemistry, University of Minnesota. UV spectra were determined on a Cary recording spectrophotometer, model 14. IR spectra were determined on a Perkin-Elmer 237 B grating infrared spectrophotometer. NMR spectra were determined on a Varian Associates A-60 instrument using tetramethylsilane (TMS) as the internal standard and s refers to singlet, d to doublet.

4-Benzyloxy-3-methoxybenzaldehyde [O-Benzy vanillin (IV)]—Prepared in 63% yield according to the method of Tomita *et al.* (9), m.p. 60.5–61.5°. Lit. (9) reported m.p. 64°.

4-Benzyloxy-3-methoxy-β-nitrostyrene (V)—This was prepared in 87% yield from IV by the method of Gairaud *et al.* (10), m.p. 118–119°. Lit. (18) reported m.p. 120–121°. IR $\nu_{max.}$ mineral oil cm.$^{-1}$: 1,510 and 1,330 (strong) (NO_2).

4-Benzyloxy-3-methoxyphenethylamine (VI)—A solution of V (30 g., 0.11 mole) in anhydrous tetrahydrofuran (240 ml.) was added dropwise to a cooled and stirred suspension of lithium aluminum hydride (15 g., 0.4 mole) in anhydrous ether (600 ml.) protected from moisture by means of a calcium chloride tube. After the addition (approximately 1 hr.), the mixture was further refluxed for 1.5 hr. The complex after cooling was decomposed by cautious successive addition of wet ether (20 ml.), water (50 ml.), 10% sodium hydroxide (10 ml.), and water (5 ml.). The resultant mixture was filtered and washed well with ether. The combined filtrates were dried over anhydrous magnesium sulfate, and evaporated under reduced pressure to yield 25 g. of orange-yellow oily amine (93%). Confirmation of the identity of this amine was made by preparing its formate salt which showed fine colorless scales, m.p. 142–144° [lit. (9) m.p. 146–149°], obtained by dissolving the above amine (2 drops) in a small amount of ethanol followed by adding 90% formic acid (1 drop) and then a large quantity of anhydrous ether. The free amine showed ν_{NH_2} at 3,330 cm.$^{-1}$ in its IR spectrum in liquid film.

N - (4 - Benzyloxy - 3 - methoxyphenethyl) - 2-(3,4-dimethoxyphenyl)acetamide (VIII)—A mixture of 54 g. (0.27 mole) of 3,4-dimethoxyphenylacetic acid (VII) and 70 g. (0.27 mole) of the foregoing 4-benzyloxy-3-methoxyphenethylamine (VI) was

condensed according to the method of Shepard *et al.* (11). The partially cooled mixture was poured into 300 ml. of absolute methanol. The suspension of crystals was held at 10° for 1 hr., filtered and redissolved in a mixture of absolute ethanol (800 ml.) and absolute methanol (200 ml.), decolorized with charcoal and allowed to cool. The crystals were filtered, washed with anhydrous ether to give colorless needles (74.7 g., 63%), m.p. 121–123°. A second recrystallization of this compound showed m.p. 124°. Lit. (18) reported m.p. 124°. IR $\nu_{max.}$ mineral oil cm.$^{-1}$: 3,290 (NH) and 1,630 (C=O).

1 - (3',4' - Dimethoxybenzyl) - 6 - methoxy - 7-benzyloxy - 3,4 - dihydroisoquinoline Hydrochloride (IX)—A mixture of VIII (2 g., 0.0046 mole), phosphorus oxychloride (2 ml., Mallinckrodt AR), and dry toluene (10 ml.) was heated for 2 hr. in a system protected by a calcium chloride tube with an oil bath temperature of 115°. The reaction mixture was evaporated *in vacuo* to dryness. The residue, after being washed three times with anhydrous skellysolve B, was then dissolved in absolute ethanol (10 ml.) and treated with 10% hydrochloric acid (10 ml.). Upon cooling in an ice bath, it crystallized easily to yield the imine hydrochloride salt which was filtered off, washed well with anhydrous ether, a mixture of absolute ethanol–anhydrous ether (1:50), and finally anhydrous ether again. This furnished, after drying, 1.55 g. (78%) of yellow needles, m.p. 219–220°. Lit. (18) reported m.p. 219–220°. IR $\nu_{max.}$ mineral oil cm.$^{-1}$: 2,540 (broad and strong), 1,850–1,925 (immonium band), and 1,640 (C=$\overset{+}{\text{N}}$H). The free base of this compound showed $\nu_{C=N}$ 1,653 cm.$^{-1}$ in liquid film.

1 - (3',4' - Dimethoxybenzyl) - 6 - methoxy - 7-benzyloxy - 1,2,3,4 - tetrahydroisoquinoline (X)—To a solution of IX (1 g.) in methanol (100 ml.) and water (5 ml.) was added, portionwise, sodium borohydride (1 g.) at room temperature. The mixture was allowed to stand for 10 min. and further refluxed on a steam bath for 1 hr. Removal of the solvent gave a residue which was admixed with water and extracted with ether. The ethereal extract was dried over anhydrous sodium sulfate and distilled to give a pale yellow glassy residue (900 mg., 98%) after drying in a desiccator overnight. Characterization of this base was effected by preparing its hydrochloride salt derivative using dry ethereal hydrogen chloride. The hydrochloride (XI) formed as colorless needles and had m.p. 220.5–222°, after two recrystallizations from methanol–ether.

Anal.—Calcd. for $C_{26}H_{30}ClNO_4$: C, 68.48; H, 6.63; N, 3.07. Found: C, 68.40; H, 6.82; N, 3.01. UV $\lambda_{max.}^{EtOH}$ mμ (log ε): 235 (4.23, shoulder), 282 (3.80); $\lambda_{min.}^{EtOH}$ mμ (log ε): 254(2.97).

1 - (3',4' - Dimethoxybenzyl) - 6 - methoxy - 7-hydroxyisoquinoline (XII)—The tetrahydro base (X) (320 mg.) obtained from the above procedure, without delay, was dissolved in tetralin (5 ml., Mallinckrodt OR) and combined with 10% palladium on charcoal (50 mg.). The air remaining in the reaction flask was first displaced by a current of dry nitrogen gas for 5 min., and the mixture was heated under dry nitrogen gas at 180° (oil bath temperature) for 50 min. Examination of the mixture by TLC (alumina with chloroform and visualized with iodine vapor) indicated that the slower moving tetrahydroisoquinoline spot had disappeared completely in favor of a much faster moving isoquinoline spot. The cooled mixture was diluted with absolute methanol and filtered to remove the catalyst and the filtrate, after removal of the methanol *in vacuo*, was treated with anhydrous ether (*ca.* 20 ml.). The resulting white crystals were filtered and washed well with anhydrous ether to yield 190 mg. (77%) of the aromatized isoquinoline, m.p. 164.5–166.5°. Lit. (14) reported m.p. 165–167°. The mother liquor was further digested with 10% hydrochloric acid and washed with ether several times. The acidic solution was rendered alkaline with 10% ammonium hydroxide and extracted with chloroform until the chloroform layer gave a negative test to Mayer's reagent. The extracts were then washed with water, dried over anhydrous potassium carbonate, and evaporated under reduced pressure to furnish a brown viscous oil which crystallized on rubbing with a small amount of absolute ethanol. Recrystallization from absolute ethanol afforded colorless prisms (20 mg.), m.p. 164–165°. Total yield: 85%. IR: absence of the secondary amine absorption in mineral oil. UV $\lambda_{max.}^{EtOH}$ mμ (log ε): 240 (4.43), 273 (3.46, shoulder), 281 (3.46), 321 (3.29, shoulder) and 332 (3.35), and upon the addition of sodium hydroxide, $\lambda_{max.}^{EtOH}$ mμ (log ε): 229 (4.02, shoulder), 259 (4.28), 282 (3.59), and 288 (3.59, shoulder).

1 - (3',4' - Dimethoxybenzyl) - 6 - methoxy - 7-benzyloxyisoquinoline (XIII)[2]—A stirred mixture of XII (100 mg., 0.3 mmole), anhydrous potassium carbonate (110 mg., 0.78 mmole, Baker AR granular), benzyl bromide (164 mg., 0.9 mmole), and dry methanol (4 ml.) was refluxed for 5.5 hr. The solvent was evaporated and the residue dissolved in water and extracted with ether. The extracts, after washing with 1% sodium hydroxide and water, were then extracted with 10% hydrochloric acid several times. The acidic solution was made alkaline with 20% ammonium hydroxide in the cold, and extracted with ether. The extract, after being washed with water, dried over anhydrous potassium carbonate, and evaporated *in vacuo*, gave a greenish oily substance (105 mg., 83%). This was dissolved in a small amount of absolute ethanol and treated with dry ethereal hydrogen chloride. The pale yellow precipitate was collected by filtration and recrystallized from chloroform–ethanol to yield the hydrochloride salt (XIV) as a white solid, m.p. 232–233.5°.

Anal.—Calcd. for $C_{26}H_{26}ClNO_4$: C, 69.09; H, 5.79; N, 3.09. Found: C, 68.58; H, 5.88; N, 2.96. IR $\nu_{max.}$ mineral oil cm.$^{-1}$: 2,440 (broad and strong), 1,925–1,975 (immonium band), and 1,635 (C=$\overset{+}{\text{N}}$H).

A suspension of 180 mg. of XIV in water was rendered alkaline with 10% ammonium hydroxide and extracted with ether until the ethereal extract gave a negative test to Mayer's reagent. The extract was washed with water, dried over anhydrous potassium carbonate, and evaporated to yield the free base as yellow prisms, m.p. 129–131°. Recrystallization from absolute ethanol–anhydrous ether gave white prisms with m.p. 131–132° (XIII).

Anal.—Calcd. for $C_{26}H_{25}NO_4$: C, 75.16; H, 6.07; N, 3.37. Found: C, 74.99; H, 6.26; N, 3.15. UV $\lambda_{max.}^{EtOH}$ mμ (log ε): 240 (4.65), 270 (3.69, shoulder), 279 (3.69), 314 (3.39), and 328 (3.49). NMR (τ, in $CDCl_3$): 6.28 (3H, s, $C_{3'}$-OC$\underline{H}_3$), 6.22

[2] An alternate synthesis for this compound has recently been published by Brochmann-Hanssen, E., and Hirai, K., *J. Pharm. Sci.*, **57**, 940(1968).

(3H, s, C_4'-OC$\underline{H}_3$), 6.05 (3H, s, C_6-OC$\underline{H}_3$), 5.62 (2H, s, C_1-C$\underline{H}_2$), and 4.88 (2H, s, C_7-OC$\underline{H}_2C_6H_5$).

1 - (3',4' - Dimethoxybenzyl) - 2 - methyl - 6-methoxy-7-benzyloxy-isoquinoline Iodide (XV)—To a solution of XIII (105 mg.) in absolute methanol (3 ml.) was added methyl iodide (1 ml.) and the mixture, after heating under reflux on a steam bath for 4 hr., was further treated with methyl iodide (1 ml.) and refluxed for an additional 4.5 hr. The mixture was allowed to stand in the refrigerator overnight, following which the solvent was removed by distillation to leave a yellowish brown glassy substance (148 mg.) which crystallized on rubbing with absolute ethanol and was collected by filtration. The residue was washed well with absolute ethanol to give 120 mg. (86%) of pale yellow solid, m.p. 202–204° (dec.) which upon recrystallization from absolute ethanol showed m.p. 203–205°.

Anal.—Calcd. for $C_{27}H_{28}INO_4$: C, 58.11; H, 5.02; N, 2.51. Found: C, 57.97; H, 5.26; N, 2.43. UV $\lambda_{max.}^{EtOH}$ mμ (log ε): 222 (4.67), 2.59 (4.83), 286 (3.92, shoulder), and 3.18 (4.06). NMR[3] (τ, in $CDCl_3$): 6.22 (6H, s), 5.89 (3H, s) (3 OC$\underline{H}_3$), 5.54 (2H, s, C_1-C$\underline{H}_2$), 4.72 (2H, s, 7-OC$\underline{H}_2C_6H_5$), and 5.02 (3H, s, $\overset{+}{N}$-C$\underline{H}_3$).

1 - (3',4' - Dimethoxybenzyl) - 2 - methyl - 6-methoxy-7-benzyloxy-1,2-dihydroisoquinoline (XVI)—The foregoing methiodide (XV) (500 mg.) was reduced with sodium borohydride-pyridine in exactly the same manner as described by Barton (15) for 1,2-dihydro-*N*-methyl papaverine from papaverine methiodide. A yellowish semicrystalline substance separated on addition of a small amount of anhydrous ether, m.p. *ca.* 120° (300 mg., 78%). Two recrystallizations from chloroform–absolute ethanol afforded fine colorless tufts, m.p. 120–122°.

Anal.[4]—Calcd. for $C_{27}H_{29}NO_4$: C, 75.15; H, 6.77; N, 3.25. Calcd. for $C_{27}H_{29}NO_4 \cdot 1/4\ H_2O$: C, 74.37; H, 6.70; N, 3.21. Found: C, 74.39; H, 7.05; N, 3.22. UV $\lambda_{max.}^{EtOH}$ mμ (log ε): 256 (3.81, shoulder), 283 (3.33), and 335 (3.67). NMR (τ, in $CDCl_3$): 6.29 (3H, s, C_3'-OC$\underline{H}_3$), 6.18 (6H, s, C_4'-OC$\underline{H}_3$ and C_6-OC$\underline{H}_3$), and 7.15 (3H, s, N-C$\underline{H}_3$).

Acid-Catalyzed Ring Closure of (XVI) to (±)-Norargemonine (II)—A solution of XVI (320 mg.) in a mixture of 85.6% phosphoric acid (1 ml.) and 90.8% formic acid (5 ml.) was heated at 100–105° for 2 hr. until the UV spectrum showed no further changes. The mixture was then left overnight at room temperature, diluted with water (10 ml.), and extracted three times with ether. The aqueous solution was made alkaline with cold 10% sodium hydroxide and extracted with chloroform until the chloroform extracts gave a negative test with Mayer's reagent. The combined chloroformic extracts were washed with water, filtered, and dried over anhydrous potassium carbonate. The dried extract was evaporated under reduced pressure to give a greenish brown residue (285 mg.) which crystallized on rubbing with anhydrous ether, m.p. 218–219° (sintered at 214°) (240 mg., 94%). Recrystallization from absolute ethanol provided fine white crystals, m.p. 218–219.5°. UV $\lambda_{max.}^{EtOH}$ mμ (log ε): 226 (4.18, shoulder), and a well-defined triplet at 283 (3.94, shoulder), 287 (3.98) and 293 (3.94, shoulder); $\lambda_{min.}^{EtOH}$ mμ (log ε): 254 (3.31), and upon addition of sodium hydroxide, $\lambda_{max.}^{EtOH}$ mμ: 283 (shoulder), 287, 293 (shoulder), and 310 (shoulder); $\lambda_{min.}^{EtOH}$ 262 mμ. Attempted debenzylation of this compound was carried out as follows: A solution of the foregoing product (141 mg.) in ethanol (5 ml.) and concentrated hydrochloric acid (8 ml.) was heated under reflux on a steam bath for 2 hr., and the solvent was evaporated *in vacuo* to leave a residue which was dissolved in water and extracted with ether to remove benzyl chloride. The aqueous solution was basified with 10% aqueous sodium hydroxide and extracted with ether. To the above ether-extracted alkaline solution was added an excess of crystalline ammonium chloride, and the ammoniacal solution was extracted three times with chloroform. The chloroformic extract was washed with water, dried over anhydrous potassium carbonate, and evaporated *in vacuo* to give a greenish brown syrup (96 mg.) which crystallized when rubbed with a small amount of absolute ethanol, m.p. 218–220° (sintered at 210°). One recrystallization from absolute ethanol yielded fine colorless needles, m.p. 218–220°. The UV and IR spectra, together with the TLC behavior of this compound, were superimposable on those of the above starting material (m.p. 218–219.5°) and natural norargemonine (II).

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Keyphrases

(±)-Norargemonine—synthesis
TLC—identity
IR spectrophotometry—structure
NMR spectroscopy—structure
UV spectrophotometry—structure

[3] The spectrum was taken without delay after XV was dissolved in $CDCl_3$ because of slow decomposition at room temperature.

[4] This analysis was the best obtainable in spite of several attempts. Its NMR, UV, and IR characteristics, however, are in accord with the suggested structure.

Rheology of Microcrystalline Cellulose-Carboxymethylcellulose Gels

By W. DOUGLAS WALKLING* and RALPH F. SHANGRAW

Rheological evaluations of MCC-CMC gels were employed to delineate their properties. It was concluded that the thixotropic nature of MCC-CMC gels depended upon the adsorption of the soluble CMC on the MCC. A certain degree of shear was required to unsheave the microcrystals. However, excessive shear reduced the degree of structuring. MCC-CMC in concentrations of 2.0 percent or higher produced thixotropic gels. CMC was found to be superior to HPMC as a linking polymer. At the concentration studied, alcohol and various glycols increased the viscosity of MCC-CMC gels independently of the viscosity of the adjuvant compound. Formulations containing acetaminophen and calamine in MCC-CMC systems were also evaluated.

ONE APPROACH to the preparation of pharmaceutically acceptable suspension systems involves the utilization of agents which possess different rheological properties. Samyn (1), in particular, has suggested that a combination of plastic and pseudoplastic suspending agents offers unique pharmaceutical advantages. The plastic agent would prevent low stress sedimentation or leakage while the pseudoplastic agent would assist high stress flow. Samyn investigated magnesium aluminum silicate-sodium carboxymethylcellulose systems. Simple pseudoplastic systems were found to settle with time while plain clay suspensions entrapped air and presented pouring problems. Combinations of the order of 0.6% magnesium aluminum silicate and 1.3% sodium carboxymethylcellulose were found to give superior results.

The insoluble magnesium aluminum silicate clay utilized by Samyn in his work actually imparted both plasticity and thixotropy to his suspension systems. Recently, a new suspending agent has become available which resembles this overall system but is unique in that the plasticity or thixotropy is imparted by microcrystals of organic cellulose. This agent, microcrystalline cellulose-carboxymethylcellulose[1] (MCC-CMC) is a specially prepared blend of 92 parts of microcrystalline cellulose and 8 parts of sodium carboxymethylcellulose with not more than 4% moisture at the time of manufacture. As a powder, 97% of the material will pass through a No. 325 mesh sieve, and, as a dispersion, the ultimate particle size varies around 0.15 μ (2). It is insoluble in water and organic solvents, but it disperses to form colloidal sols and opaque gels. Hydration is rapid and changes little if any on aging.

In order to postulate a mechanism for the structured formation on MCC gels, Hermans (3) forwarded the concept of primary reaction sites which lead to strong aggregation without branching and weaker secondary sites which cause branching. Consequently, when shear is introduced into a dispersion of MCC, linear aggregates formed by the primary reaction sites orient to the streamlines of flow and thus permit the secondary reaction sites to form branched networks as the microcrystals align themselves in a tighter, more parallel pattern. The thixotropy and high yield values observed in MCC-CMC gels are claimed to be due to the adsorption of the dissolved sodium CMC polymers onto the cellulose microcrystals (4). Consequently, the greater the number of surfaces for adsorption, the more extensive the structured network, the greater the thixotropy and yield value of the gel will be.

The purpose of this investigation is to explore and further define the rheological properties of MCC-CMC gels.

EXPERIMENTAL

All experiments were conducted using a Haake Rotovisco rotating viscometer (Polyscience Corp., Evanston, Ill., 60602). The MV system for medium viscosities and the type 50 and type 500 measuring heads were employed. A MV-1 plastic rotor with a 0.6-cm. height and a 2.004-cm. radius was employed along with a 2.10-cm. radius cup. These variables permitted a rate of shear range of 8.46 to 1,370 sec.$^{-1}$ and a shearing stress range of 7 to 3,540 dynes cm.$^{-2}$. The operating temperature for all experiments was 28°.

Preserved water containing 0.15% methylparaben and 0.02% propylparaben was employed in all formulations. All gels were prepared in terms of percent weight to weight.

Received May 22, 1968, from the School of Pharmacy, University of Maryland, Baltimore, MD 21201
Accepted for publication July 30, 1968.
Abstracted from a thesis submitted by W. Douglas Walkling to the Graduate School, University of Maryland, Baltimore, Md., in partial fulfillment of Doctor of Philosophy degree requirements.
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[1] Marketed as Avicel RC by American Viscose Div., FMC Corp., Marcus Hook, Pa.

Process Variables—Two percent gels of MCC-CMC were prepared in 500-g. quantities with various types of laboratory apparatus in order to determine which method produced the best gel.

Method I—Ten grams of MCC-CMC was added to 240 g. of water in a 1-qt. Waring blender, model PB-5A. The mixture was sheared at the highest speed for 9 min. Another 250 g. of water was added, and shearing was continued for another 11 min. The dispersion was then passed twice through an Eppenbach colloid mill, 1725 r.p.m. drive, adjusted to the "2" setting.

Method II—Ten grams of MCC-CMC was dispersed over a 5-min. period into 490 g. of water placed in a 1,000-ml. beaker. Dispersion was facilitated by means of a Talboy's mixer, model 101, operated at the "30" setting. The propeller was inserted midway into the liquid. After the powder was dispersed, mixing was continued for 30 min.

Method III—Same as Method II except that the final product was passed twice through the Eppenbach colloid mill.

Method IV—Same as Method III except that mixing was continued for 60 min.

Previous experience with these systems showed that at concentrations of 2% or less, 1 hr. of aging permitted the formation of an essentially static system. For higher concentrations, 24 hr. of aging was found to be sufficient.

Method III was selected as the method to be used in all other experiments. The basis for this selection will be discussed later.

Concentration—Gels of 1.0, 2.0, and 4.0% MCC-CMC were prepared. All preparations were aged 24 hr. prior to rheological evaluation.

Carboxymethylcellulose—Gels of 1.0, 2.0, and 4.0% of an 80/20 and a 50/50 blend of MCC-CMC and CMC were prepared. Solutions of the CMC, 300–500 cps. grade,[2] were added to fully dispersed gels of MCC-CMC. The combinations were blended by a Lightnin mixer, model V-7, adjusted to the "10" setting. After blending, each mixture was passed twice through the Eppenbach colloid mill.

Hydroxypropyl Methylcellulose—Using hydroxypropyl methylcellulose (HPMC), 3500–5600 cps. grade,[3] 80/20 blends of MCC-CMC and HPMC were prepared in the same manner as the MCC-CMC/CMC blends.

Alcohol and Glycols—The effects of common suspension adjuncts on 2.0% MCC-CMC gels were investigated. Blends containing 20.0% alcohol, glycerin, PEG 200, and propylene glycol were prepared. Solutions of the hydrophilic compounds were added to fully dispersed gels. The mixtures blended readily and additional shear through a colloid mill was not required.

Medicinal Agents—Samples of acetaminophen[4] and calamine were incorporated into gels so that the final concentration of the medicinal agent was 10.0% and that of the gel was 2.0%. A control suspension and a suspension utilizing 2.0% magnesium aluminum silicate[5] (MAS) as the suspending medium were also studied. All samples were aged 30 days at an ambient temperature of approximately 26°.

Structural Breakdown with Time—In order to evaluate thixotropy in greater detail, a time-dependence study was designed. Samples of gels containing 4.0% gelling agent with or without 10.0% acetaminophen or calamine were placed in the viscometer cup and allowed to remain there for 30 min. before commencing shear. The use of 4.0% agent permitted a more critical evaluation of the parameter. After the dwell period, the sample was sheared for 10 min. at one rate of shear. The sample was then replaced and the process repeated on another sample at another rate of shear. All samples were aged for 24 hr. except the MAS preparation which was aged for 30 days.

RESULTS AND DISCUSSION

MCC-CMC may be the first of a new class of pharmaceutical suspending agents. It is a blend of organic celluloses which develop gels having a high degree of thixotropy. The thixotropic structure rebuilds rapidly, and unlike other thixotropic pharmaceutical vehicles, its rheological properties do not change further upon aging. In addition, the effects of added substances on suspension properties are immediately reflected.

Most of the properties of MCC-CMC are detectable through simple laboratory techniques such as agitation, bottle inversion, and macroscopic observation. Though quantitative data are presented to define and measure these properties, a discussion of these data is desirable in terms of the mechanism of MCC-CMC gel structure formation, how this structure is altered by various substances, and how it can be applied to pharmaceutical formulations.

A unique and perplexing problem which was encountered in the preparation of MCC-CMC gels was their combined dependence upon and sensitivity to shear. A certain amount of shear was required to establish the maximum gel structure. This is believed to be due to the unsheaving of microcrystals which exposes more cellulose surface on which the CMC may be adsorbed. Microscopic examination of a weakly thixotropic dispersion and a highly

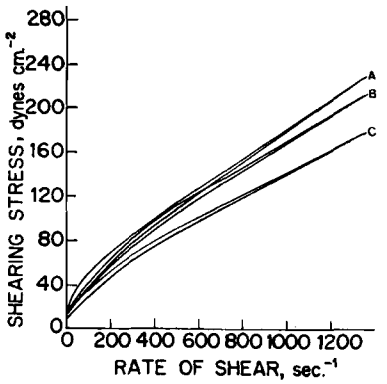

Fig. 1—Influence of processing variables on the rheology of 2% MCC-CMC gels. Key: A, Method III; B, Method IV; C, Method I. Method II would appear between A and B.

[2] Marketed as CMC-7MP by Hercules Powder Co., Inc., Wilmington, Del.
[3] Marketed as Methocel 90 HG Premium, 4000 cps., by Dow Chemical Co., Midland, Mich.
[4] Marketed as acetaminophen, NF, Micropowder, by S. B. Penick and Co., N. Y.
[5] Marketed as Veegum HV by R. T. Vanderbilt Co., Inc., Norwalk, Conn.

thixotropic gel made from the same concentration of MCC-CMC revealed that the weakly thixotropic dispersion contained many large bundles of microcrystals of cellulose while the other preparation contained only faintly visible microcrystals. As more cellulose surface became available, the possibility of adsorbing interconnecting or bridging soluble polymers increased. These interconnecting soluble polymers caused a structured, thixotropic network to be formed. The degree of thixotropy can be increased by either unsheaving the microcrystals or by increasing the length of the soluble polymer chains. Increasing the concentration of the soluble polymer was effective up to about a 20% increase. About that point, the pseudoplastic nature of the soluble polymer started to overwhelm the system.

As can be seen in Fig. 1, passing suspensions through the Eppenbach colloid mill subsequent to mixing increased the gel structure. Apparently, a certain percentage of the microcrystals was not unsheaved by mixing alone. With the colloid mill, a reasonably reproducible shear was obtained since all of the material was exposed to the same degree of shear by passage through a predetermined orifice. Such was not the case with the Waring blender or the simple mixer.

However, it can also be seen that too much shear resulted in a partially destructured system. The existence of such a phenomenon in MCC-CMC gels has been verified by Raynor (5). An explanation for this is disclosed in the work of Healy and LaMer (6). These investigators adsorbed polyacrylamide on calcium phosphate. The addition of a small amount of polymer formed unit flocs. The addition of more polymer caused bridging between the unit flocs to produce a three-dimensional macrofloc with a high degree of structure. Bridging occurred even though only half of the calculated adsorption sites on the calcium phosphate particles were occupied. At this point, agitation of the system destroyed the three-dimensional macrofloc as energy was imparted to the system. This destruction was not thixotropic breakdown as structure did not rebuild itself spontaneously. Further bridging could not occur now until all of the adsorption sites had reacted with the polymer and energy was subsequently reduced. Thus, shear had produced a system with a permanently lowered consistency.

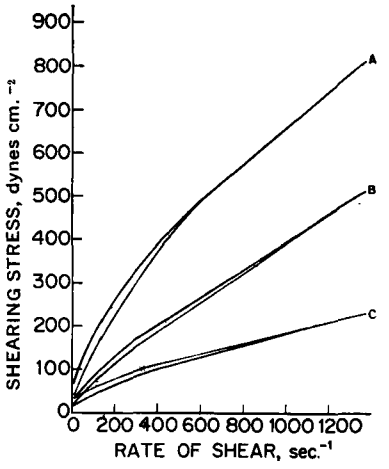

Fig. 3—Effect of CMC on the rheology of MCC-CMC gels. 2% combined polymer concn. employed. Key: A, MCC-CMC/CMC 50/50; B, MCC-CMC/CMC 80/20; C, MCC-CMC only.

Though the MCC-CMC gels are not the same as the calcium phosphate-polyacrylamide system, both systems involve the adsorption of a soluble polymer onto an insoluble particle, and both systems show evidence that, above a certain concentration of the respective materials, a structured network exists. Excessive shear during the unsheaving of the microcrystals may have decreased the structuring potential of this system by causing the soluble polymer to entwine around individual or groups of microcrystals and thus decrease the chance of intercrystal adsorption. The soluble cellulose polymer then serves as a protective colloid which inhibits rather than promotes interparticle structuring.

The thixotropy, yield values, and apparent viscosities of MCC-CMC gels increased with increasing concentration as evidenced in Fig. 2. At the 4.0% level, the rheogram exhibited definite evidence of a "foot." A "foot" occurs when the structure is

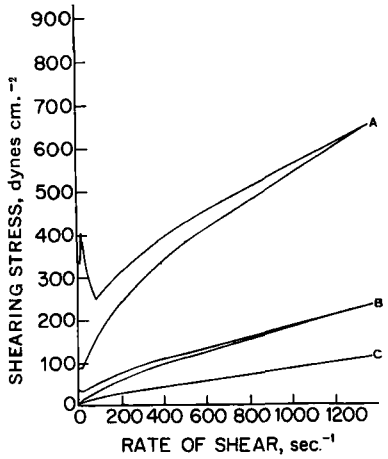

Fig. 2—Effect of concentration on the rheology of MCC-CMC gels. Key: A, 4%; B, 2%; C, 1%.

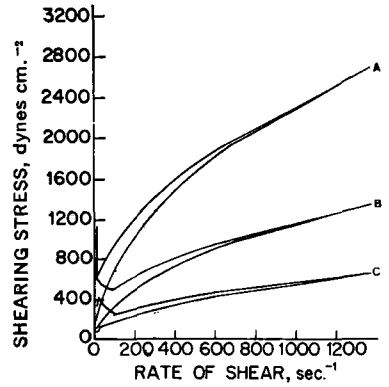

Fig. 4—Effect of CMC on the rheology of MCC-CMC gels. 4% combined polymer concn. employed. Key: A, MCC-CMC/CMC 50/50; B, MCC-CMC/CMC 80/20; C, MCC-CMC only.

TABLE I—EFFECT OF CONCENTRATION ON THE RHEOLOGY OF MCC-CMC/CMC AND MCC-CMC/HPMC 80/20 GELS

Rate of Shear, sec.$^{-1}$	Shearing Stress (dynes cm.$^{-2}$) CMC, 1%	CMC, 2%	CMC, 4%	HPMC, 1%	HPMC, 2%	HPMC, 4%
8.46	7	28	779	14	112	957
16.9	11	37	620	11	89	886
25.4	12	46	549	12	93	779
50.7	18	65	514	18	105	709
76.1	23	79	496	21	116	638
152	33	112	567	32	147	655
228	47	140	620	39	172	691
457	77	219	815	65	247	886
685	103	301	992	88	309	1,050
1,370	170	514	1,350	149	496	1,400
685	102	275	957	86	295	1,030
457	74	209	744	63	228	850
228	40	126	496	39	147	620
152	32	96	407	28	119	514
76.1	21	61	283	19	81	390
50.7	18	47	248	18	65	337
25.4	12	30	177	12	46	266
16.9	9	23	159	11	37	230
8.46	7	16	124	7	26	177

broken with a force far greater than that necessary to establish thixotropic equilibrium; hence, a very rapid and real decrease in the yield value takes place before equilibrium is attained (7). Naturally, the force imparted to the structure varies from instrument to instrument, and it is, therefore, more dependent upon the test conditions than anything else. Though difficult to reproduce quantitatively, the foot is real and must be given consideration.

Figures 3 and 4 show the effects of CMC on MCC-CMC gels. Table I shows that HPMC, 4,000 cps. grade, and CMC, medium viscosity (300–500 cps.) grade, had nearly identical influences upon MCC-CMC gels even though pure solutions of the former were much more viscous than the latter. The rheological similarity of the combined gel products was the result of the greater interaction between the MCC and the CMC as opposed to the interaction between the MCC and the nonionic HPMC.

The effects of pharmaceutical adjuvants can be seen in Table II. It is curious to note that alcohol, glycerin, PEG 200, and propylene glycol, materials of quite different viscosities, elicited the same effects on 2.0% MCC-CMC gels when employed in 20% concentrations. Apparently, the criteria for the structure of these mixtures were not the viscosity of the adjuvants themselves, but the presence of hydroxyl groups. Thixotropy and yield values went unchanged upon the addition to the adjuvants though apparent viscosity increased. At higher concentration levels, the effects of alcohols began to vary and the viscosity of the individual agent became a factor. Syrup and sorbitol solution had the same general effects as the other agents, *i.e.*, no effect on thixotropy and yield values but a marked increase in the apparent viscosity.

The rheological effects of the various suspending agents in combination with 10.0% acetaminophen

TABLE II—EFFECT OF 20% ALCOHOL AND GLYCOLS ON THE RHEOLOGY OF 2% MCC-CMC GELS

Rate of Shear, sec.$^{-1}$	Shearing Stress (dynes cm.$^{-2}$) Alcohol	Glycerin	PEG 200	Propylene Glycol
8.46	25	26	28	25
16.9	32	35	39	33
25.4	39	40	42	40
50.7	56	56	58	56
76.1	67	67	68	68
152	95	96	98	98
228	116	116	119	121
457	179	179	182	186
685	228	228	230	235
1,370	351	351	351	389
685	219	214	221	224
457	161	161	167	168
228	98	96	100	102
152	75	75	77	77
76.1	51	49	51	51
50.7	39	39	40	40
25.4	26	25	28	26
16.9	21	21	23	21
8.46	16	16	16	16

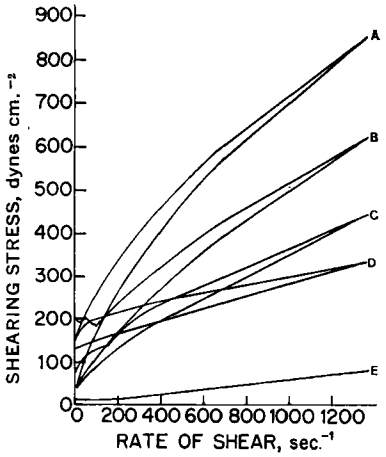

Fig. 5—Rheology of 10% acetaminophen suspensions. Key: A, MCC-CMC/CMC 80/20; B, MCC-CMC/HPMC 80/20; C, MCC-CMC; D, MAS; E, no susp. agent. 2% susp. agent and 30 days aging for all preparations.

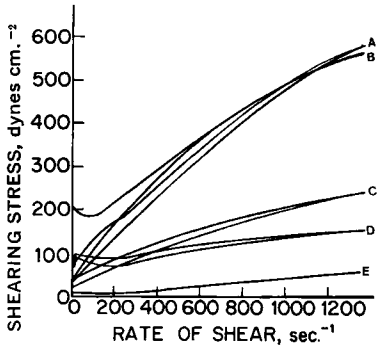

Fig. 6—Rheology of 10% calamine suspensions. Key: A, MCC-CMC/CMC 80/20; B, MCC-CMC/HPMC 80/20; C, MCC-CMC; D, MAS; E, no susp. agent. 2% susp. agent and 30 days aging for all preparations.

are presented in Fig. 5. In every case, thixotropy, yield value, and apparent viscosity were markedly increased. Although consistency effects in complete suspension formulations almost always exceed the sum of the effects of the individual components, acetaminophen systems exhibited exaggerated increases which could only result from drug-suspending agent interactions at a structural level. This can no doubt be attributed to the fine particle size of the acetaminophen and the presence of polar groups which provided additional adsorption sites for the soluble cellulose polymers allowing acetaminophen to play an active role in the structuring of the system. However, the fact that the consistency increases were particularly marked in the systems containing the higher quantities of CMC indicates that the anionic nature of that soluble polymer was particularly important in this interaction.

Calamine (Fig. 6) gave an excellent suspension with MCC-CMC. No unusual synergistic consistency increases were noted.

The effects of shear and time of shearing stress are shown in Tables III–V. In all cases, more structural breakdown occurred during the first minute than at any other time. Though not indicated by the data, the breakdown was most rapid during the first few seconds. When compared with MAS, MCC-CMC exhibited approximately the same yield stress at the lowest rate of shear. The clay exhibited a small foot at the lower rates of shear while the MCC-CMC produced a much larger foot. Breakdown was far more rapid and more complete in the MAS systems. Practical experience with suspensions containing 4.0% MCC-CMC showed that the amount of breakdown achieved with only moderate

Table III—Effect of Rate of Shear Per Unit Time on the Shearing Stress of MCC-CMC Gels

Rate of Shear, Sec.⁻¹	Shearing Stress (dynes cm.⁻²) 0 min.	1 min.	3 min.	10 min.
		4% MCC-CMC		
8.46	349	119	93	70
16.9	319	116	95	79
25.4	461	119	103	91
50.7	585	147	132	117
76.1	602	161	147	133
152	478	193	177	163
228	602	235	214	200
457	602	372	337	274
685	638	425	407	372
1,370	921	638	585	531
		4% MCC-CMC/CMC 80/20		
8.46	531	200	154	119
16.9	638	189	151	126
25.4	744	217	177	147
50.7	762	256	216	191
76.1	868	295	260	240
152	921	425	390	372
228	957	496	478	452
457	1,134	744	726	691
685	1,240	921	903	868
1,370	1,559	1,311	1,258	1,205

TABLE IV—EFFECT OF RATE OF SHEAR PER UNIT TIME ON THE SHEARING STRESS OF 4% MAS GEL (AGED 30 DAYS)

Rate of Shear, sec.$^{-1}$	Shearing Stress (dynes cm.$^{-2}$) 0 min.	1 min.	3 min.	10 min.
8.46	337	182	175	175
16.9	337	160	151	151
25.4	390	156	147	144
50.7	354	142	128	123
76.1	390	144	132	126
152	425	158	149	142
228	425	170	161	156
457	443	205	196	193
685	531	231	219	216
1,370	815	319	270	265

shaking was sufficient to allow rapid flow through a narrow-neck bottle. However, since a high degree of structure was still present after shaking, better retardation of sedimentation would have resulted during the static period immediately following the shear. The addition of CMC to the MCC-CMC gel increased the shearing stresses at all rates of shear and reduced thixotropy due to its inherent psuedoplastic nature.

Ten percent acetaminophen greatly increased the shearing stresses at all rates of shear. But what was most interesting was the enormous thixotropic breakdown which was produced during the first minute and probably during the first few seconds of shearing. In other words, the acetaminophen interacted with the polymers to produce a highly structured gel. However, since the molecular size was so small in relation to the polymers and since most of the drug was present as insoluble particles, the structural breakdown was very rapid. In a practical sense, a suspension of 10.0% acetaminophen in a 4.0% gel of MCC-CMC would remain suspended at rest, but would break down easily to a freely flowing liquid.

The experiences with 10.0% calamine in 4.0% MCC-CMC were similar to those just described for acetaminophen though the structure of the system was not as great.

Consequently, it appeared that a 4.0% concentration of MCC-CMC functioned effectively as a suspending agent for both a weakly soluble, acidic organic compound and an insoluble inorganic oxide.

CONCLUSIONS

Careful control over the preparation of MCC-CMC gels is important. A certain amount of shear is necessary to completely develop gel structure; however, excessive shear can interfere with the structuring of the system.

Gels are thixotropic when concentrations of 2% or higher of MCC-CMC are employed.

Gel structure is brought about by the adsorption of soluble cellulose polymers onto insoluble cellulose microcrystals. This system is unique in the realm of pharmaceutical suspending agents.

The anionic nature of CMC makes it superior to HPMC as a linking polymer.

TABLE V—EFFECT OF RATE OF SHEAR PER UNIT TIME ON THE SHEARING STRESS OF 4% MCC-CMC GELS

Rate of Shear, sec.$^{-1}$	Shearing Stress (dynes cm.$^{-2}$) 0 min.	1 min.	3 min.	10 min.
	10% Acetaminophen			
8.46	461	372	312	249
16.9	744	390	256	184
25.4	921	407	244	202
50.7	957	281	235	212
76.1	1,098	284	249	231
152	1,275	390	354	316
228	1,240	390	372	326
457	1,258	585	567	549
685	1,169	620	602	602
1,370	1,169	833	797	762
	10% Calamine			
8.46	461	283	210	130
16.9	602	249	154	130
25.4	762	207	156	137
50.7	921	200	172	154
76.1	939	214	191	179
152	939	261	242	224
228	886	425	390	354
457	1,205	496	452	425
685	1,346	531	478	461
1,370	1,134	726	673	638

At the concentration studied, alcohol and various glycols increase the viscosity of the gels to the same extent.

Compounds capable of interacting with CMC increase the viscosity of the gels.

Acetaminophen demonstrates extensive interaction with MCC-CMC.

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(1) Samyn, J. C., *J. Pharm. Sci.*, **50**, 517(1961).
(2) *Avicel RC Microcryst. Cellulose and Its Use in Pharm. Susp.*, Am. Viscose Div., FMC Corp., Marcus Hook, Pa., p. 1.
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Keyphrases

Microcrystalline cellulose (MCC)-carboxymethylcellulose (CMC)-gels
Rheology—MCC-CMC gels
Suspending adjuvants, effect—gels
Shear rate, stress range, effect—gels

Absorption, Metabolism, and Excretion of the Ephedrines in Man II

Pharmacokinetics

By G. R. WILKINSON* and A. H. BECKETT

The kinetics of absorption, metabolism, and excretion of (−)-norephedrine, (−)-ephedrine, and (−)-methylephedrine, after oral administration in aqueous solution, have been elucidated using analog computer analysis of urinary excretion data from three male subjects under constant acidic urine control. The kinetics of formation and elimination of norephedrine and ephedrine when present as metabolites have also been determined. The single body compartment mathematical models were based upon a catenary chain with parallel branch systems and all rate constants were assumed to be first order. Excellent agreement was obtained between the theoretical computer curves and the experimental excretion data for both unchanged drug and metabolite(s).

THE DEVELOPMENT of mathematical models to describe the absorption and fate of a drug and its metabolite(s) in the body, and the value of such models in the design of dosage forms and regimens has been treated by numerous authors including Teorell (1), Dominguez (2), Dost (3), Nelson (4), Wagner (5), Krüger-Thiemer (6) and references therein. For maximum exploitation of these techniques drug plasma levels are preferable, however, the distribution characteristics of many drugs, especially basic drugs (7), are such that this information is not readily available. In these cases it is possible to use urinary excretion data providing caution is taken in the interpretation of the results (8, 9).

In view of the long and extensive use of the ephedrines[1] it is surprising that relatively little is known about the kinetics of their absorption and elimination in man. The only published information concerns (−)-norephedrine, where the mean elimination half-life was reported to be 3.9 hr. (10). Recent publications (11, 12) have indicated that the relative metabolism and urinary excretion of the ephedrines are dependent upon the urinary pH and in certain circumstances the volume also. The validity of urinary excretion kinetics is based upon the assumption that the excretion rate reflects the plasma concentration of the drug. With drugs exhibiting non-

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Accepted for publication August 9, 1968.
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* Present address: College of Pharmacy, University of Kentucky, Lexington, KY 40506

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ionic reabsorption in the kidney tubule, this requirement will only be fulfilled when reabsorption of the drug back into the body is either absent or negligible. Such a condition exists for the ephedrines when the urine is acidic at about pH 5.0 (12).

This communication reports the kinetics of absorption, metabolism, and excretion of (−)-norephedrine, (−)-ephedrine, and (−)-methylephedrine after oral administration in aqueous solution. When norephedrine and ephedrine were present as metabolites the kinetics of their formation and elimination were also elucidated. The kinetic parameters were obtained by analog computer analysis of urinary excretion data obtained in a previous study, where the urinary pH was maintained acidic by the administration of ammonium chloride (12).

EXPERIMENTAL

The data for analysis consisted of 24-hr. cumulative urinary excretion and rate of excretion values for unchanged norephedrine, ephedrine, and methylephedrine after their oral administration to three healthy male volunteers. In the case of ephedrine and methylephedrine, metabolite excretion data were also available, *i.e.*, norephedrine and ephedrine and norephedrine formed from ephedrine and methylephedrine, respectively (12). These raw data allowed the development of simple models to describe the absorption, metabolism, and excretion of the three drugs when administered *per se*. Where norephedrine and ephedrine were present in the body as a consequence of metabolism, it was assumed that their fate was identical to that postulated after *per se* oral administration. The three models are shown in Fig. 1 while the assumptions and terminology used in their development are given in the *Appendix*.

The analog computer used was a Pace TR 20R (Electronics Associates Ltd.) along with an X-Y recorder (Advance Electronics Ltd.) and a digital voltmeter (Roband Ltd.). Standard methods were used to program the particular model under investigation and also to fit the generated curves to the experimental data (13). Both the cumulative and rate curves for excretion were used to obtain the best "goodness of fit." As a further aid to fitting, the absorption curve derived from the data by the method of Wagner and Nelson (14) was also used.

Lag time was estimated by extrapolating the derived curve for the amount of drug remaining to be absorbed, plotted semilogarithmically, to zero absorption, assuming absorption to be a single first-order process. This lag time was not programmed into the computer, instead the abscissa zero of the X-Y recorder was manually set at the lag time prior to any simulation.

In fitting the norephedrine excretion curves after the administration of (−)-ephedrine, it was assumed that although the value for the overall elimination constant *ky* might be different from that determined after *per se* administration, the ratio of the component rate constants, *kp* and km_3, would remain constant for each particular subject. A similar assumption was made for the overall elimination constant *kz* when fitting the ephedrine excretion curves after administration of (−)-methylephedrine. For subjects N.B. and G.R.W. the results for *ku*/*kz*, km_3/kz, and km_4/kz derived from the data obtained after administration of 20.48 and 25.00 mg. (−)-ephedrine were averaged and these values used in the (−)-methylephedrine simulation. For subject R.W.D., only the results obtained from the 20.48-mg. dose were used. In all cases the metab-

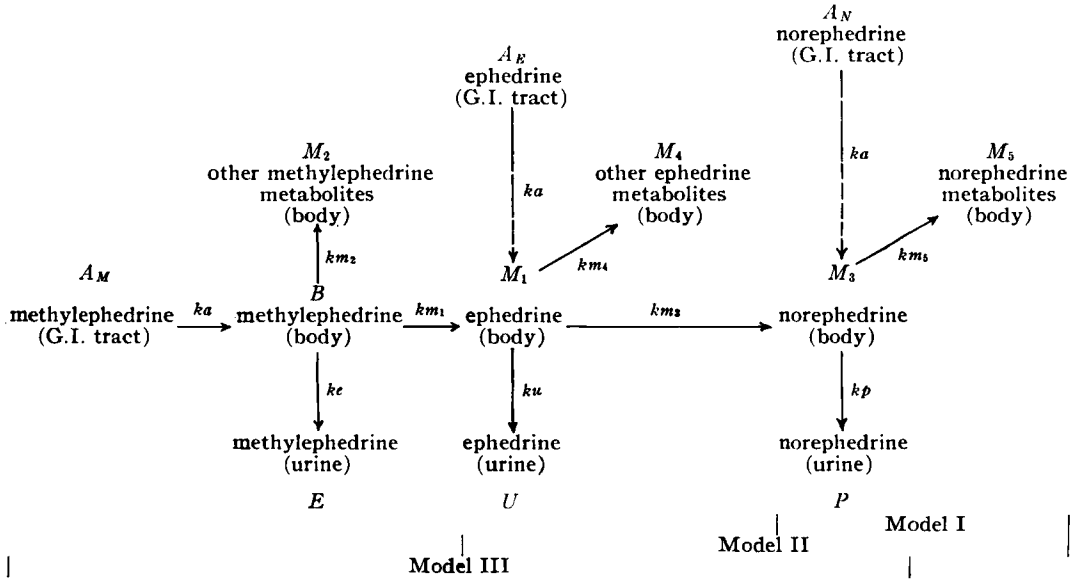

Fig 1—Pharmacokinetic models for the absorption, metabolism, and excretion of (−)-norephedrine (Model I), (−)-ephedrine (Model II), and (−)-methylephedrine (Model III) in man.

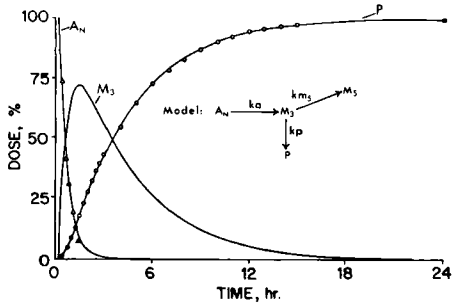

Fig. 2—Computer curves and experimental data points for the elimination, after oral administration, of (−)-norephedrine (subject, N.B.).

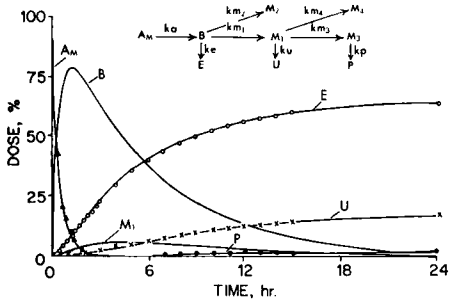

Fig. 4—Computer curves and experimental data points for the elimination, after oral administration, of (−)-methylephedrine (subject, N.B.).

olism of the norephedrine, formed after (−)-methylephedrine administration, was neglected and it was assumed that all of the drug was excreted unchanged in the urine, *i.e.*, *kp* becomes *ky*.

RESULTS AND DISCUSSION

Excellent agreement, for both unchanged drug and metabolite(s), was obtained between the fitted computer urinary excretion curves and the experimental data for all three amines, indicating the suitability of the postulated models to describe, within experimental error, the kinetics of absorption, metabolism, and excretion of these drugs. Typical fitted cumulative excretion curves along with various derived curves for some other compartments are shown in Figs. 2–4. The kinetic parameters for each subject are summarized in Tables I, II, and III.

Absorption of all three amines after oral administration could be described, with one exception, by a single first-order process following a lag period. Considerable intersubject and, in the case of (−)-ephedrine, intrasubject variation was observed in both the lag time and the rate constant for absorption. No correlation was apparent between these variations and either the drugs or the subjects. However, despite these variations all the ephedrines were rapidly absorbed within 3 hr. of administration. The absorption of (−)-norephedrine in subject G.R.W. appeared to be biphasic, a slow phase followed by a much faster phase, rather than mono-exponential. The computer curve was fitted only to the latter process accounting for the long lag time (67 min.). A number of physiological explanations may be proposed to describe these absorption profiles, however, until more detailed information than that presented herein is available, these would be purely speculative and of questionable significance.

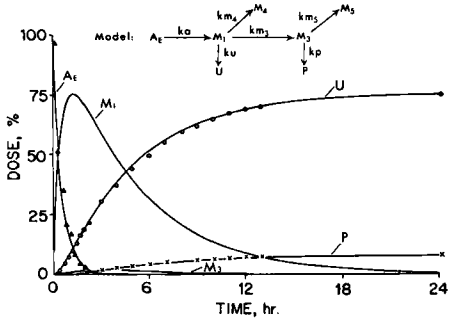

Fig. 3—Computer curves and experimental data points for the elimination; after oral administration, of (−)-ephedrine (subject, R.W.D.).

In the postabsorption phase, the elimination of unchanged drug, whether norephedrine, ephedrine, or methylephedrine, could be described by a series of simultaneous, competitive first-order processes—excretion and metabolism to one or more metabolite(s). As might be expected, intersubject and intrasubject variations occurred in the values of the rate constants for these processes. However, in the case of ephedrine, the ratio of the constants was remarkably constant within subjects N.B. and G.R.W. In all cases, the rate constants for urinary excretion of unchanged norephedrine (*kp*) and ephedrine (*ku*) were of the same magnitude, but the equivalent constant for methylephedrine (*ke*) was always significantly smaller. This was also apparent in the rate constants for overall elimination of the drug. Differences in the volume of distribution and/or urinary excretion behavior of methylephedrine compared to norephedrine and ephedrine may account for this finding. Despite these differences, under the experimental conditions used in the study, all three of the ephedrines and their measured metabolite(s) were rapidly eliminated from the body. For example, mean overall elimination $t_{1/2}$ values for unchanged drug were: (−)-norephedrine, 2.99 hr.; (−)-ephedrine, 3.03 hr.; and (−)-methylephedrine, 4.49 hr.

As stated above, the formation of the metabolites, ephedrine and norephedrine, after the administration of both (−)-ephedrine and (−)-methylephedrine, was described by first-order processes. The phar-

TABLE I—KINETIC PARAMETERS FOR THE ABSORPTION, METABOLISM, AND EXCRETION OF (−)-NOREPHEDRINE

Subject	Lag Time, min.	*ka*, hr.$^{-1}$	*kp*, hr.$^{-1}$	*km*$_5$, hr.$^{-1}$	*ky*, hr.$^{-1}$
N.B.	15	1.80	0.2581	0.0008	0.2589 (2.67)[a]
G.R.W.	67	2.00	0.2222	0.0210	0.2432 (2.85)
R.W.D.	8	9.24	0.1951	0.0063	0.2014 (3.44)

[a] Values in parentheses are the $t_{1/2}$ values, in hr., equivalent to the rate constant above.

TABLE II—KINETIC PARAMETERS FOR THE ABSORPTION, METABOLISM, AND EXCRETION OF (−)-EPHEDRINE

Subject	Dose Ephedrine, mg.	Lag Time, min.	ka, hr.$^{-1}$	ku, hr.$^{-1}$	km_2, hr.$^{-1}$	km_4, hr.$^{-1}$	kz, hr.$^{-1}$	kp, hr.$^{-1}$	km_3, hr.$^{-1}$	ky, hr.$^{-1}$
N.B.	20.48	15	1.75	0.2180	0.0220	0.0099	0.2499 (2.77)[a]	0.3588	0.0012	0.3600 (1.92)
	25.00	18	4.02	0.2172	0.0256	0.0096	0.2524 (2.74)	0.3400	0.0010	0.3410 (2.03)
G.R.W.	20.48	12	3.30	0.2566	0.0124	0.0060	0.2750 (2.52)	0.3290	0.0310	0.3600 (1.92)
	25.00	15	2.77	0.1780	0.0074	0.0056	0.1910 (3.63)	0.2741	0.0259	0.3000 (2.31)
R.W.D.	20.48	0	3.00	0.1933	0.0180	0.0012	0.2125 (3.26)	0.4068	0.0132	0.4200 (1.65)
	25.00	6	1.85	0.1596	0.0174	0.0337	0.2107 (3.29)	0.4940	0.0160	0.5100 (1.36)

[a] Values in parentheses are the $t_{1/2}$ values, in hr., equivalent to the rate constant above.

macokinetic models found to represent the elimination of these drugs after oral administration *per se* were also found suitable when the amines were present as metabolites. However, initial attempts to fit the theoretical urinary excretion curves of the metabolites using the same values for the rate constants as obtained from the *per se* administration data were unsuccessful. In all cases the rate of excretion was too small, even if urinary excretion was assumed to be the only elimination pathway. The only way of fitting the data was to increase, often substantially, each rate constant contributing to the overall elimination, assuming that the constants remained in the same ratio as after *per se* administration (the results from the two separate ephedrine trials would support the hypothesis of a constant ratio despite changes in the absolute values of the constants). For example, to fit the norephedrine excretion data after (−)-ephedrine administration the individual rate constants for elimination, ky, had to be increased as follows: N.B., 32 and 39%; G.R.W., 23 and 48%; and R.W.D., 109 and 153%. Similar increases for ephedrine (kz), based upon the mean value for the two *per se* trials, and norephedrine (ky) after (−)-methylephedrine administration were respectively: N.B., 12 and 34%; G.R.W., 33% (norephedrine not determined); and R.W.D., 155 and 60%.

Insufficient data prevent any statistical significance being placed upon these results, however, the authors consider increases of over 150% are well beyond the usual intrasubject variations found in individuals, particularly when an acidic urine is being maintained by ammonium chloride. This being the case, then it would appear that ephedrine and norephedrine, when present in the body as metabolites, are eliminated faster than when they are administered *per se*.

A possible explanation for this phenomenon is that the kinetics of elimination of the ephedrines are dose dependent; elimination is faster at low body drug levels than at high levels. Such a change should be apparent in the logarithmic excretion curve of unchanged drug if sampling is continued beyond the time that the critical drug level occurs. However, examination of the *per se* experimental data for norephedrine and ephedrine, over a time period greater than five orders of magnitude of the elimination half-life, does not show any deviation from a mono-exponential curve (12). In the present study, the values for the excretion rate of unchanged drug, whether norephedrine or ephedrine, did not completely extend into the range of values obtained when the drugs were excreted as metabolites; the 12–16 hr. values for unchanged drugs were approximately equal to the maximal rates found when the drug was a metabolite. Hence, it is possible that the critical body level of drug, after *per se* administration, had not been reached before hourly urine collection was terminated.

In the development of the present pharmacokinetic models it was necessary to assume that the body behaved as a single homogeneous compartment. Such a gross oversimplification may provide an alternative explanation for the elimination behavior of norephedrine and ephedrine when present in the body as metabolites. The shortcomings of conceiving the body to exhibit properties of a single compartment have recently been reemphasized by Riegelman *et al.* (15). These authors suggest that for many drugs it is more reasonable to consider the body as composed of a cen-

TABLE III—KINETIC PARAMETERS FOR THE ABSORPTION, METABOLISM, AND EXCRETION OF (−)-METHYLEPHEDRINE

Subject	Lag Time, min.	ka, hr.$^{-1}$	ke, hr.$^{-1}$	km_1, hr.$^{-1}$	km_2, hr.$^{-1}$	kd, hr.$^{-1}$	ku, hr.$^{-1}$	km_3, hr.$^{-1}$	km_4, hr.$^{-1}$	kz, hr.$^{-1}$	kp, hr.$^{-1}$
N.B.	6	2.00	0.1145	0.0345	0.0260	0.1750 (3.96)[a]	0.2426	0.0274	0.0100	0.2800 (2.47)	0.3462 (2.00)
G.R.W.	36	2.60	0.1112	0.0166	0.0122	0.1400 (4.95)	0.2862	0.0150	0.0088	0.3100 (2.23)	—
R.W.D.	15	4.60	0.1283	0.0237	0.0000	0.1520 (4.56)	0.4912	0.0457	0.0031	0.5400 (1.28)	0.2820 (2.45)

[a] Values in parentheses are the $t_{1/2}$ values, in hr., equivalent to the rate constant above.

tral compartment and at least one peripheral compartment. That such a system may be applied to the ephedrines is suggested by the data obtained after intravenous administration (12) and also by the fact that in some cases after oral administration a distribution nose is observed in the rate of excretion curve of unchanged drug.

In a two-compartmental open system, the identification of a particular exponential term with a particular process is erroneous, as all observed rate constants are hybrid constants each influenced by the factors controlling drug distribution and elimination. Hence, all of the rate constants determined in this study are apparent constants influenced not only by the intrinsic rate constants but also by the volumes of the central and peripheral compartments, and therefore the rate constants for distribution between these compartments. A change in the value of any of these factors will affect the observed rate constants. Such changes probably explain the observed intrasubject variations in the calculated constants and possibly the increased values obtained when a drug is present as a metabolite. This latter phenomenon and the mechanism by which the elimination of a metabolic ephedrine is influenced by the presence of a parent ephedrine is presently under further investigation.

Even within the small group of subjects used in this study, large inter- and intrasubject variations occurred in the various rate constants. However, it is considered that by using a small but well controlled number of subjects more meaningful and valuable information can be obtained compared to the use of larger groups, which although statistically more satisfying do not control the urinary pH.

It would be expected that the overall elimination of unchanged ephedrine would be faster when the urinary pH was maintained acidic than when no control was attempted, and this probably explains the difference between the present data for (−)-norephedrine and that of Heimlich *et al.* (10). However, the differences between the data are not large on account of the low pH-sensitivity of (−)-norephedrine (12, 16).

A kinetic study such as that presented in this paper is an oversimplification of complex physiological phenomena. Furthermore, kinetic parameters obtained under extreme urinary pH conditions are not applicable to normal conditions, especially if drug absorption, distribution, binding, *etc.*, are affected by acidification of the urine. However, if the limitations of the techniques are recognized, then they may be used advantageously for comparative drug metabolism studies and the determination of absorption profiles from both conventional and prolonged-release dosage forms, particularly for drugs which are extensively extravascularly distributed (17).

APPENDIX

Assumptions

1. Transfer from one compartment to another is irreversible, the rate constants for transfer being first order with the units of reciprocal time.

2. The compartments are uniform and homogeneous during the processes and equilibration is instantaneous.

3. There is no decomposition of drug at the absorption site, no enterohepatic recycling, no diffusion of drug from the blood into the stomach.

4. Drug is immediately and completely available at the absorption site at zero time and is 100% absorbed (12).

Definitions

Lag time: time interval between ingestion of the drug and zero time.

Zero time: time at which drug appearing in the body may be described by a single first-order process.

A_M, A_E, A_N: amount of methylephedrine, ephedrine, or norephedrine, respectively, present in the gastrointestinal tract.

B, M_1, M_3: amount of methylephedrine, ephedrine, and norephedrine, respectively, present in the body.

M_2: amount of metabolite(s) of methylephedrine other than ephedrine and/or methylephedrine eliminated by a pathway other than the urine.

M_4: amount of metabolite(s) of ephedrine other than norephedrine and/or ephedrine eliminated by a pathway other than the urine.

M_5: amount of metabolite(s) of norephedrine and/or norephedrine eliminated by a pathway other than the urine.

E, U, P: amount of methylephedrine, ephedrine, and norephedrine, respectively, in the urine.

ka: rate constant for the appearance of drug from the gastrointestinal tract into the body whether methylephedrine, ephedrine, or norephedrine.

ke, ku, kp: rate constant for excretion of methylephedrine, ephedrine, and norephedrine, respectively, from the body into the urine.

km_1: rate constant for the formation of ephedrine from methylephedrine.

km_2: rate constant for the formation of metabolite(s) of methylephedrine other than ephedrine and/or rate constant for elimination of methylephedrine by a pathway other than the urine.

km_3: rate constant for the formation of norephedrine from ephedrine.

km_4: rate constant for the formation of metabolite(s) of ephedrine other than norephedrine and/or rate constant for elimination of ephedrine by a pathway other than the urine.

km_5: rate constant for the formation of metabolite(s) of norephedrine and/or rate constant for elimination of norephedrine by a pathway other than the urine.

kd, kz, ky: rate constant for the overall elimination from the body by all pathways of methylephedrine, ephedrine, and norephedrine, respectively, *i.e.*, $kd = ke + km_1 + km_2$; $kz = ku + km_3 + km_4$; $ky = kp + km_5$.

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Keyphrases

Ephedrines—pharmacokinetics
Pharmacokinetics—ephedrines, absorption, metabolism, excretion
Models, single compartment—ephedrines, pharmacokinetics
Metabolites, ephedrines—formation, elimination

Stilbene Isothiocyanates as Potential Fluorescent Tagging Agents

By J. E. SINSHEIMER, J. T. STEWART*, and J. H. BURCKHALTER

Two groups of stilbene isothiocyanate derivatives, α-phenylcinnamic acids and α-phenylcinnamonitriles, have been synthesized. These compounds and their required intermediates were studied as to their structure-fluorescence relationships in an effort to develop a protein-tagging agent with blue fluorescence.

THE FLUORESCENT labeling of proteins as first developed by Coons *et al.* (1) and advanced by the introduction of fluorescent isothiocyanates by Burckhalter and co-workers (2, 3) has had extensive application to biological problems especially for the identification of pathogenic organisms. The agents and techniques employed together with their applications have been reviewed by Nairn (4). In varying degrees, all the presently available reagents can be improved upon in regard to purity, expense, fluorescent intensity, and stability to UV irradiation. It would also be highly desirable to have agents with contrasting fluorescent colors. For example, a blue fluorescent label would be valuable to supplement the green of fluorescein and orange to red fluorescence of rhodamine derivatives.

Limited studies with blue fluorescent tagging agents have employed stilbene optical brightening agents and a coumarin isocyanate (5) as well as β-anthryl isocyanate (6) with limited success.

Peck and Creech (7) synthesized 4′-isocyanato-4-dimethylaminostilbene for the purpose of combining it with proteins for UV analysis, but no mention was made of the possibility of utilizing the blue fluorescent properties of the stilbene and resulting protein conjugate. A blue fluorescent reagent, the disodium salt of 4′-acetamido-4-isothiocyanatostilbene-2,2′-disulfonic acid, has been described for the specific labeling of the outer components of the plasma membrane, but it has not been applied to antibody labeling (8).

Successful use of stilbene compounds as blue fluorescent optical brightening agents (9) of high stability encouraged the authors to seek improved fluorescent labeling agents among stilbene derivatives. The purpose of this investigation was to synthesize a stilbene isothiocyanate suitable for protein labeling as well as to study structure-fluorescence relationships in a series of model stilbene compounds. More specifically a stilbene isothiocyanate protein-tagging agent was postu-

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Accepted for publication August 8, 1968.
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lated to meet the needs for an agent of good stability when exposed to UV radiation and for a blue fluorescent label as a compliment to the existing red and green agents. A contrasting color was desired for such applications as multiple antigen identification and as an additional method of overcoming background color interference.

DISCUSSION

Primarily two groups of stilbene isothiocyanate derivatives, α-phenylcinnamic acids and α-phenylcinnamonitriles, were synthesized and evaluated as labeling agents. The inclusion of the carboxylic group in the case of the former series of compounds was to promote water solubility of these compounds as their sodium salts for ultimate use as protein labels in aqueous media. Even more extensive studies were conducted with the α-phenylcinnamonitriles. The literature indicated (10, 11), including personal experiences (12, 13), that these compounds possessed good fluorescent intensity in solution by visual detection. An added reason for choosing the nitriles is that the synthetic route to these compounds produces primarily *trans* stilbenes; it is noteworthy that *cis* stilbene possesses only 1% of the fluorescence of its *trans* isomer (14).

The fluorescent-enhancing effect of various constituents was of interest. Such groups, however, could not be reactive with isothiocyanates and, therefore, studies were restricted to the effects of methoxy, dimethylamino, and quaternary nitrogen substituents. The isothiocyanates were also reacted with benzylamine to form thioureas corresponding to the thiourea linkage of a conjugated protein and to observe the effect of this structure on fluorescence.

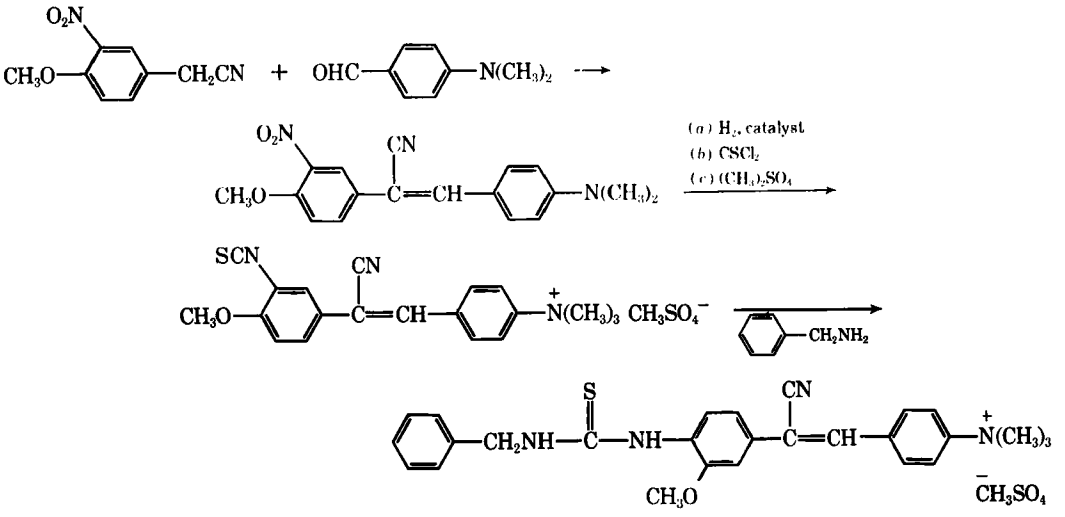
A typical synthetic route to a quaternary salt of an α-phenylcinnamonitrile and conversion to the thiourea is shown in Scheme I.

The α-phenylcinnamic acids were synthesized *via* base-catalyzed condensations of substituted phenylacetic acids and benzaldehydes (15, 16). Starting materials required for the synthesis were either available commercially or could be readily obtained in a few synthetic steps from commercial materials.

The isothiocyanates, thioureas, and new intermediates which were synthesized are shown together with their physical constants and fluorescence characteristics in Table I. This table also includes three stilbenes synthesized for purpose of comparison by methods analogous to known base condensation procedures (17–19).

Initially, measurement of the fluorescence of compounds with emphasis on isothiocyanate and thiourea compounds was by comparison to quinine sulfate by quinine reference unit (QRU) (20) with use of a filter fluorometer. Fluorescent values of aqueous solutions (ethanolic if required) were obtained when possible at a concentration where fluorescence was shown to be proportional to concentration. Compounds for which a linear concentration fluorescence relationship could not be obtained are indicated in Table I. For all compounds, fluorescence measurements were made as suggested by Hercules (21) at concentrations whose absorbance was less than 0.05 at the excitation wavelength. The fluorometer filters were selected for activation at 365 mμ (Corning 7-37 filter) and for detection of emitted light at wavelengths greater than 400 mμ (Kodak 2-A filter). These filters are similar to those commercially available in fluorescent microscopy and are suitable for the observation of blue fluorescence.

Structure-fluorescence relationships were obtained from the QRU data and comparisons of the data in Table I can be made to show the effect of different groups on the fluorescence of a molecule. In a limited study, α-phenylcinnamic acids were found to be less fluorescent than α-phenylcinnamonitriles, which possess a weaker fluorescence than stilbenes containing identical substitution. Methoxy and isothiocyanato substituents in α-phenylcinnamonitriles result in increased fluorescent intensity. The increase depended on the fluorescent species involved. Thiourea derivatives formed from isothiocyanates of the α-phenylcinnamonitrile series possess a weaker fluorescent intensity than corresponding isothiocyanates. This is in contrast



Scheme I

Table I—Stilbene Derivatives

Compd.	R_1	R_2	M.p., °C.	Method of Synthesis[a]	Yield, %	Anal. Calcd.	Anal. Found	UV $\lambda_{max.}$ mμ	IR $\nu_{max.}$ cm.$^{-1}$	Fluorescence Excit. $\lambda_{max.}$	Fluorescence Emiss. $\lambda_{max.}$	Relative Fluorescence QRU[r]
				A. α-Phenylcinnamic Acids								
1	*cis*-4-Nitrophenyl	4-Dimethylaminophenyl	268–269		83	C, 65.38 H, 5.16 N, 8.97	65.39 5.30 8.94	320 (ε 31,200)	1,660 (C=O) 1,600 (phenyl) 1,320 (NO_2)	—	—	—
2	*cis*-4-Aminophenyl	4-Dimethylaminophenyl	228–230	A	81	C, 72.32 H, 6.43	72.34 6.49	360 (ε 6,690) 280 (ε 9,080)	3,300 (NH) 1,650 (C=O) 1,600 (phenyl)	—	—	—
3	*cis*-4-Isothiocyanatophenyl	4-Dimethylaminophenyl	225–227	B	80	C, 66.64 H, 4.94 N, 8.64	66.73 5.05 8.56	360 (ε 33,000)	2,100 (NCS) 1,640 (C=O) 1,575 (phenyl)	—	—	0.001
4	*trans*-4-Aminophenyl	4-Dimethylaminophenyl	225–226	A[b]	85	C, 72.32 H, 6.43 N, 9.92	71.96 6.35 9.80	360 (ε 25,900) 240 (ε 10,600)	3,300 (NH) 1,670 (C=O) 1,600 (phenyl)	—	—	—
5	*trans*-4-Isothiocyanatophenyl	4-Dimethylaminophenyl	221–222	B	94	C, 66.64 H, 4.94 N, 8.64	66.73 4.90 8.58	353 (ε 24,100)	2,100 (NCS) 1,650 (C=O) 1,575 (phenyl)	—	—	0.000[s]
6	*cis*-4-Methoxyphenyl	4-Aminophenyl	223–226	A[c]	17	—	—[q]	—	1,625 (C=O) 1,550 (phenyl)	—	—	—
7	*cis*-4-Methoxyphenyl	4-Isothiocyanatophenyl	144–146	B	50	C, 65.58 H, 4.21 N, 4.50	65.30 4.44 4.46	333 (ε 17,000)	2,020 (NCS) 1,700 (C=O) 1,500 (phenyl) 1,240 (arom. ether)	—	—	0.000[s]
8	*trans*-4-Methoxyphenyl	4-Aminophenyl	205–208	A[c]	60	C, 71.36 H, 5.61 N, 5.20	71.21 5.61 5.30	330 (ε 10,500)	1,650 (C=O) 1,575 (phenyl) 1,250 (arom. ether)	—	—	—
9	*trans*-4-Methoxyphenyl	4-Isothiocyanatophenyl	156–158	B	80	C, 65.58 H, 4.21 N, 4.50	65.77 4.29 4.69	328 (ε 19,100)	2,100 (NCS) 1,675 (C=O) 1,600 (phenyl) 1,275 (arom. ether)	—	—	0.000
10	*cis*-4-Methoxyphenyl	3-Nitrophenyl	199–202	[c]	8.4	C, 64.21 H, 4.38 N, 4.68	64.28 4.48 4.71	305 (ε 25,500)	1,700 (C=O) 1,340; 1,520 (NO_2) 1,600 (phenyl)	—	—	—
11	*cis*-4-Methoxyphenyl	3-Aminophenyl	58–60(dec.)	A	51	—	—[q]	—	—	—	—	—
12	*cis*-4-Methoxyphenyl	3-Isothiocyanatophenyl	135–138	B	96	C, 65.58 H, 4.21 N, 4.50	65.52 4.33 4.50	218 (ε 33,200)	2,100 (NCS) 1,700 (C=O) 1,600; 1,520 (phenyl) 1,250 (arom. ether)	—	—	0.0004[t]
13	*trans*-4-Methoxyphenyl	3-Nitrophenyl	174–176	[c]	55	C, 64.21 H, 4.38 N, 4.68	64.31 4.42 4.70	225 (ε 21,000) 260 (ε 19,700)	1,650 (C=O) 1,600 (phenyl) 1,520; 1,340 (NO_2)	—	—	—
14	*trans*-4-Methoxyphenyl	3-Aminophenyl	222–224	A	70	C, 71.36 H, 5.61 N, 5.20	70.66 5.70 5.16	225 (ε 21,800) 280 (ε 13,100)	1,660 (C=O) 1,560 (phenyl) 1,225 (arom. ether)	—	—	—
15	*trans*-4-Methoxyphenyl	3-Isothiocyanatophenyl	148–151	B	90	C, 65.58 H, 4.21 N, 4.50	65.60 4.34 4.63	221 (ε 22,300)	2,100 (NCS) 1,660 (C=O) 1,560; 1,500 (phenyl) 1,250 (arom. ether)	—	—	0.0003[t]

B. α-Phenylcinnamonitriles

$$R_1\text{—}C(CN)\text{=}CH\text{—}R_2$$

	R_1	R_2	M.p., °C	Method	Yield, %	Calcd., %	Found, %	UV, λmax, mμ (ε)	IR, μ	NMR, τ	λmax, mμ	λmin, mμ	Ratio
16	4-Methoxyphenyl	3-Isothiocyanatophenyl	109–110	B[d]	89	C, 69.84 H, 4.14 N, 9.58	69.86 4.01 9.55	222 (ε 21,800)	2,200 (CN) 2,500 (NCS)		350	425	0.070
17	4-Methoxyphenyl	[3-(3-Benzylthioureido)-phenyl]	185–187	D	81	C, 72.15 H, 5.30 N, 10.52	71.89 5.38 10.52	240 (ε 30,000)	2,250 (CN) 1,550; 1,520 (phenyl)		365	490	0.020
18	4-Methoxyphenyl	2-Methoxy-5-nitrophenyl	150–152	[e,f]	93	C, 65.80 H, 4.55 N, 9.03	65.87 4.64 9.07	232 (ε 16,500) 305 (ε 22,000) 335 (ε 21,400)	2,200 (CN) 1,575; 1,560 (phenyl) 1,500; 1,325 (NO_2) 1,250 (arom. ether)		—	—	—
19	4-Methoxyphenyl	2-Methoxy-5-aminophenyl	146–149	A	62	C, 72.84 H, 5.75 N, 9.99	72.82 5.73 10.00	325 (ε 17,000)	2,200 (CN) 1,590 (phenyl) 1,225 (arom. ether)		400	570	0.007
20	4-Methoxyphenyl	2-Methoxy-5-trimethylammoniumphenyl iodide	183–185	C	71	C, 53.34 H, 5.15 N, 6.22	53.30 5.22 6.26	340 (ε 16,000)	2,275 (CN) 1,540; 1,520 (phenyl)		355	440	0.040
21	4-Methoxyphenyl	2-Methoxy-5-isothiocyanatophenyl	168–172	B	60	C, 67.06 H, 4.38 N, 8.69	67.16 4.43 8.67	225 (ε 29,300) 285 (ε 30,900)	2,200 (CN) 2,100 (NCS) 1,600; 1,500 (phenyl) 1,240 (arom. ether)		400	475	0.016
22	4-Methoxyphenyl	[2-Methoxy-5-(3-benzylthioureido)-phenyl]	157–158	D	70	C, 69.91 H, 5.40 N, 9.78	70.00 5.53 9.58	240 (ε 29,000) 320 (ε 11,100)	2,250 (CN) 1,625; 1,550 (phenyl)		370	500	0.013
23	4-Isothiocyanatophenyl	Styryl	128–130	B[g]	86	C, 74.97 H, 4.20 N, 9.72	74.84 4.20 9.56	285 (ε 21,600) 350 (ε 31,400)	2,200 (CN) 2,060 (NCS) 1,600 (phenyl)		400	465	0.010
24	[4-(3-Benzylthioureido)-phenyl]	Styryl	225–227	D	75	C, 75.92 H, 5.35 N, 10.62	75.66 5.41 10.75	245 (ε 24,500) 355 (ε 34,100)	2,250 (CN) 1,570 (phenyl)		400	465	0.050
25	4-Isothiocyanatophenyl	Phenyl	111–113	B[g]	90	C, 73.26 H, 3.84 N, 10.68	73.17 3.74 10.57	330 (ε 44,200)	2,275 (CN) 2,150 (NCS) 1,540 (phenyl)		380	570	0.003
26	[4-(3-Benzylthioureido)-phenyl]	Phenyl	202–204	D	83	C, 74.77 H, 5.18 N, 11.37	74.62 5.02 11.50	240 (ε 21,000) 340 (ε 24,900)	2,275 (CN) 1,610; 1,575 (phenyl)		360	510	0.0045[i]
27	Phenyl	3,4-Dimethoxyphenyl	87–88	[f]	80	C, 76.96 H, 5.70 N, 5.28	77.00 5.63 5.26	348 (ε 20,000)	2,200 (CN) 1,575; 1,500 (phenyl) 1,260 (arom. ether)		—	—	0.003[i]
28	4-Methoxyphenyl	2,3-Dimethoxyphenyl	90–93		90	C, 73.20 H, 5.80 N, 4.74	72.96 5.82 4.80	330 (ε 20,800) 225 (ε 17,800)	2,250 (CN) 1,615; 1,600 (phenyl) 1,275 (arom. ether)		—	—	0.003[i]
29	4-Hydroxyphenyl	4-Dimethylaminophenyl	228–232		50	C, 77.25 H, 6.10 N, 10.60	77.07 6.12 10.54	250 (ε 11,400) 375 (ε 22,400)	2,200 (CN) 1,590; 1,550 (phenyl)		365	450	0.005
30	4-Benzyloxyphenyl	4-Dimethylaminophenyl	134–136		80	C, 81.33 H, 6.26 N, 7.90	81.12 6.39 7.88	250 (ε 11,400) 380 (ε 21,000)	2,280 (CN) 1,640; 1,600 (phenyl)		365	440	0.009[i]
31	4-Benzyloxyphenyl	4-Trimethylammonium-phenyl iodide	166–168	C	90	C, 60.49 H, 5.08 N, 5.64	60.89 5.16 5.80	335 (ε 13,100)	2,250 (CN) 1,625; 1,525 (phenyl)		350	440	0.060
32	4-Butoxyphenyl	4-Dimethylaminophenyl	114–116		83	C, 78.71 H, 7.55 N, 8.74	78.59 7.45 8.78	375 (ε 36,400) 283 (ε 17,600) 252 (ε 24,400) 228 (ε 26,100)	2,175 (CN) 1,600 (phenyl)		270	450	0.002[i]

(Continued on next page.)

TABLE I (*Continued.*)

Compd.	R_1	R_2	M.p., °C.	Method of Synthesis[a]	Yield, %	Anal. Calcd.	Anal. Found	UV $\lambda_{max.}$ mμ	IR $\nu_{max.}$ cm.$^{-1}$	Fluorescence Excit. λ_{max}	Fluorescence Emiss. $\lambda_{max.}$	Relative Fluorescence QRU[r]
33	4-Butoxyphenyl	4-Trimethylammonium-phenyl iodide	155–157	C	80	C, 57.15 H, 5.89 N, 6.06	57.26 5.82 6.10	330 (ε 26,500)	2,275 (CN) 1,640; 1,600 (phenyl)	350	440	0.070
34	3,4-Dimethoxyphenyl	4-Dimethylaminophenyl	107–109	[f]	90	C, 74.00 H, 6.54 N, 9.08	73.94 6.53 9.17	380 (ε 30,000) 250 (ε 17,100)	2,200 (CN) 1,600 (phenyl)	280	450	0.011
35	3,4-Dimethoxyphenyl	4-Trimethylammonium-phenyl iodide	173–175	C	71	C, 53.34 H, 5.15 N, 6.22	53.30 5.23 6.21	350 (ε 11,700) 265 (ε 7,980) 255 (ε 7,670)	2,250 (CN) 1,660; 1,620 (phenyl)	365	490	1.750
36	3,4,5-Trimethoxyphenyl	4-Dimethylaminophenyl	169–170		88	C, 70.99 H, 6.55 N, 8.28	70.89 6.48 8.34	380 (ε 31,200) 255 (ε 15,600)	2,200 (CN) 1,600 (phenyl) 1,250 (arom. ether)	365	470	0.006[t]
37	3,4,5-Trimethoxyphenyl	4-Trimethylammonium-phenyl iodide	167–169	C	71	C, 52.51 H, 5.25 N, 5.83	52.29 5.36 5.84	340 (ε 18,000) 260 (ε 13,000)	2,275 (CN) 1,650; 1,610 (phenyl); 1,275 (arom. ether)	350	500	0.840
38	4-Methoxyphenyl	3-Trimethylammonium-phenyl iodide	164–168	C[d]	48	C, 54.30 H, 5.04 N, 6.67	54.36 5.11 6.62	330 (ε 15,200)	2,200 (CN) 1,590; 1,500 (phenyl) 1,235 (arom. ether)	350	440	0.200
39	4-Methoxyphenyl	4-Trimethylammonium-phenyl iodide	173–175	C[i]	60	C, 54.30 H, 5.04 N, 6.67	54.46 4.99 6.71	320 (ε 14,400) 230 (ε 27,600)	2,200 (CN) 1,590; 1,500 (phenyl) 1,235 (arom. ether)	350	425	0.150
40	3-Nitro-4-methoxy-phenyl	4-Dimethylaminophenyl	211–212	[k,l]	92	C, 66.86 H, 5.30 N, 13.00	67.10 5.41 12.90	395 (ε 26,100) 255 (ε 15,100)	2,200 (CN) 1,600; 1,560 (phenyl) 1,520; 1,340 (NO_2)	—	—	—
41	3-Amino-4-methoxy-phenyl	4-Dimethylaminophenyl	177–179	A	88	C, 73.69 H, 6.53 N, 14.32	73.63 6.48 14.40	375 (ε 28,000) 250 (ε 22,300)	3,500 (NH) 2,200 (CN) 1,590; 1,575 (phenyl)	365	440	0.007[t]
42	3-Isothiocyanato-4-methoxyphenyl	4-Dimethylaminophenyl	139–141	B	88	C, 68.03 H, 5.11 N, 12.53	68.00 5.28 12.41	385 (ε 35,600) 255 (ε 27,300)	2,200 (CN) 2,050 (NCS) 1,600; 1,575 (phenyl)	420	475	0.013[t]
43	3-Isothiocyanato-4-methoxyphenyl	4-Trimethylammonium-phenyl methosulfate	192–196	C	90	—	—[e]	275 (ε 19,100) 215 (ε 39,800)	2,200 (CN) 2,050 (NCS) 1,600 (phenyl) 1,250 (arom. ether)	350	450	0.300
44	3-(3-Benzylthioureido)-4-methoxyphenyl	4-Trimethylammonium-phenyl methosulfate	168–170	D	80	C, 59.13 H, 5.67 N, 9.85	59.03 5.61 9.75	250 (ε 25,100)	2,200 (CN) 1,600 (phenyl) 1,240 (arom ether)	365	460	0.020
45	4-Methoxyphenyl	2-Dimethylamino-5-nitrophenyl	174–175	[m,n]	94	C, 66.86 H, 5.30 N, 13.00	66.92 5.31 12.85	370 (ε 15,200) 325 (ε 16,900)	2,300 (CN) 1,650 (phenyl) 1,520; 1,350 (NO_2)	—	—	—
46	4-Methoxyphenyl	2-Dimethylamino-5-aminophenyl	109–111	A	83	C, 73.69 H, 6.53 N, 14.32	73.73 6.52 14.30	325 (ε 14,200) 240 (ε 13,600)	2,250 (CN) 1,515 (phenyl) 1,260 (arom. ether)	—	—	—
47	4-Methoxyphenyl	2-Dimethylamino-5-isothiocyanatophenyl	95–97	B	87	C, 68.03 H, 5.11 N, 12.53	68.17 4.96 12.49	305 (ε 27,400) 235 (ε 20,800)	2,250 (CN) 2,150 (NCS) 1,650 (phenyl)	335	550	0.013

48	4-Methoxyphenyl	[2-Dimethylamino-3-(3-benzylthioureido)-phenyl]	178–181	D	80	C, 70.56 H, 5.92 N, 12.66	70.36 5.79 12.56	285 (ε 21,800) 240 (ε 25,400)	2,250 (CN) 1,575; 1,515 (phenyl) 1,260 (arom. ether)	335	540	0.017
49	4-Aminophenyl	4-Dimethylaminophenyl	194–196	A[b]	56	C, 77.54 H, 6.51 N, 15.96	77.55 6.48 16.01	380 (ε 25,200) 250 (ε 17,500)	3,300 (NH) 2,200 (CN) 1,550 (phenyl)	420	480	—
50	4-Isothiocyanatophenyl	4-Dimethylaminophenyl	129–131	B	80	C, 70.78 H, 4.95 N, 13.75	70.83 5.03 13.72	292 (ε 17,900)	2,250 (CN) 2,150 (NCS) 1,640; 1,600 (phenyl)	365	460	0.008
51	[4-(3-Benzylthioureido)-phenyl]	4-Dimethylaminophenyl	190–192	D	60	C, 72.78 H, 5.86 N, 13.58	72.71 5.91 13.60	385 (ε 27,500) 250 (ε 24,000)	2,250 (CN) 1,640; 1,600 (phenyl)	390	460	0.005
				C. Stilbenes R_1—CH=CH—R_2								
52	4-Dimethylaminophenyl	4-Isothiocyanatophenyl	214–216	B[c,p]	90	C, 72.82 H, 5.75 N, 9.99	72.59 5.70 10.09	375 (ε 40,600) 285 (ε 14,500)	2,150 (NCS) 1,640; 1,550 (phenyl)	410	470	0.040
53	4-Dimethylaminophenyl	4-(3-Benzylthioureido)-phenyl	193–194	D	74	C, 74.38 H, 6.50 N, 10.84	74.51 6.50 10.78	355 (ε 30,900)	1,640; 1,550 (phenyl)	390	450	0.100

[a] Subscripts are for general literature procedures and key intermediates. Letters refer to the authors' experimental procedures. [b] The *cis* acid was prepared from the *cis* ethyl ester reported by Pfeiffer (23) with the same general procedure used by Pfeiffer for his preparation of the *trans* acid. [c] See Ref. 15 for general synthetic procedure. [d] See Ref. 13. [e] See Ref. 24. [f] Synthesized according to general procedure described in Ref. 25. [g] See Ref. 11. [h] Synthesized from Compd. 28 by demethylation with hydrobromic-acetic acids. [i] Synthesized from Compd. 29 by general procedure described in Ref. 26. [j] See Ref. 27. [k] See Ref. 28. [l] See Ref. 29. [m] See Ref. 30. [n] See Ref. 31. [o] See Ref. 7. [p] See Ref. 32. [q] This compd. was employed as an intermediate to yield a derivative whose structure was supported by analytical data. [r] Quinine reference unit, see Ref. 20. [s] No fluorescence even at a concentration with an absorbance of 0.05 at 365 mμ. [t] Compound for which a linear concentration-fluorescence relationship could not be obtained.

TABLE II—FLUORESCENCE OF SELECTED COMPOUNDS RELATIVE TO FLUORESCEIN ISOTHIOCYANATE

Compd.	Relative QRU[a]	Fluorescein Isothiocyanate Excitation at 365 mμ (with Visual Detection Filter)	490 mμ Visual Detection Filter Absent	490 mμ Visual Detection Filter Present
Fluorescein isothiocyanate	1.000	1.000	1.000	1.000
Quinine sulfate	2.100	0.240	0.490	0.0150
43	0.640	0.060	0.067	0.0037
44	0.040	0.003	0.002	0.0003
16	0.150	0.006	0.029	0.0004
17	0.040	0.005	0.017	0.0003
52	0.090	0.006	0.0047	0.0004
53	0.220	0.011	0.022	0.0007
39	0.320	0.014	0.049	0.0009
35	3.720	0.540	0.354	0.0350
37	1.800	0.600	0.417	0.0391
33	0.150	0.012	0.0306	0.0008
31	0.130	0.013	0.023	0.0009
20	0.090	0.004	0.063	0.0003

[a] Quinine reference unit, see Ref. 20.

to an example studied in the stilbene series in which the thiourea is more fluorescent than the isothiocyanate.

The intense fluorescence of the quaternary salts of the α-phenylcinnamonitriles is of special interest. It was shown that they were more fluorescent in absolute ethanol than water.

The three isothiocyanates, Compounds 16, 43, and 52, that possessed QRU greater than 0.04 were tested in the fluorescent antibody procedure.[1] A QRU of 0.04 represents an intensity of a tenth that of fluorescein isothiocyanate when it is excited at 365 mμ. Conjugation of isothiocyanate 43 to rabbit antiserum proceeded as established for the commercial isothiocyanates (4). The water-insoluble Compounds 16 and 52 were dissolved in a small amount of acetone and then added to the serum. Although different excitation and barrier filter combinations available in the microscope were employed, no usable fluorescence was observed.[1]

An important factor in reference to these results is that the antibody-labeling technique involves visual detection of emitted fluorescence and that the compounds under study fluoresce in the blue region of the spectrum where the sensitivity of the human eye is relatively poor. As previously described (22), a fluorometer employing a Kodak wratten filter No. 106 with a 1P28 phototube will approach the spectral response of the eye. Emission spectra of the isothiocyanates that had been tested in the fluorescent antibody procedure, along with their thiourea derivatives and other compounds of interest from the synthetic studies, were determined with an Aminco-Bowman spectrophotofluorometer with the 106 filter, 1P28 phototube system and are listed in Table II. Solvents, pH, and activation wavelength were selected to yield the maximum fluorescence for each compound studied.

The relative fluorescence data in Table II indicate why Compounds 16, 43, and 52, that appeared promising in the QRU procedure, did not give

[1] We are indebted to Dr. W. C. Eveland and his staff in the University of Michigan School of Public Health for evaluating these compounds.

satisfactory results in the fluorescent antibody technique. The relative fluorescence in reference to fluorescein isothiocyanate activated at 365 mμ and detected by a system comparable to the spectral response of the eye was only one-tenth of corresponding relative values obtained by the QRU procedure. Each of these blue fluorescent compounds was reduced even further to a small fraction of the fluorescence of fluorescein isothiocyanate activated at its maximum of 490 mμ. The comparison on this latter basis is also listed in Table II.

Thus, while the objective of obtaining a contrasting blue fluorescence was met, the intensity of the compounds was not great enough to overcome the disadvantage of emission in a region where the sensitivity of the eye is limited. This is particularly unfortunate because these compounds are excited by the intense mercury lines near 365 mμ and are reasonably stable to ultraviolet irradiation, advantages not found in the tagging agents presently employed.

EXPERIMENTAL[2]

Reduction of Nitro to Amino Compounds (Procedure A)—A suspension or solution of a nitro compound and 100 mg. of Adams' catalyst/10 g. of compound in absolute ethanol was allowed to consume a calculated amount of hydrogen in a Parr hydrogenator. The catalyst was removed by filtration through diatomaceous earth[3] and the ethanol was evaporated. The residue can be recrystallized from aqueous ethanol or another appropriate solvent.

Formation of an Isothiocyanate from an Amino Compound (Procedure B)—An acetone solution of the amino compound and an excess of thiophosgene (hood) was refluxed on a steam bath for 2 hr. Removal under reduced pressure at room temperature of the unreacted thiophosgene (hood) and acetone leaves a material, which can be recrystallized from glacial or aqueous acetic acid.

Quaternary Salt Formation (Procedure C)—A suspension of 0.01 mole of amino compound, 0.03 mole of dimethyl sulfate, 5 ml. of water, and 5 g. of potassium carbonate in 60 ml. of acetone was heated on a steam bath for 2 hr. Filtration of the hot suspension followed by air evaporation of the filtrate (hood) gave the crude methosulfate salt. Addition of potassium iodide to an aqueous solution of the methosulfate formed the iodide which was collected by filtration and was dried. The methosulfate salts were recrystallized from absolute ethanol and the iodide salts were recrystallized from absolute ethanol and/or water.

Thiourea Formation (Procedure D)—An equimolar solution of an isothiocyanate compound and amine in absolute ethanol was heated in a water bath for 15–30 min. When the reaction mixture cooled to room temperature, the precipitate was collected and dried. Recrystallizations from absolute ethanol gave a pure thiourea.

Quinine Reference Unit Procedure—Quinine reference units (QRU) (20) were obtained on an Aminco fluoro-microphotometer. The samples were excited by means of a G.E. No. F4T4/B1 4 w. UV lamp and a Corning 7-37 filter. The detection system consisted of a Kodak wratten 2-A filter and a RCA 931-A phototube.

One milliliter of stock solution of quinine sulfate (99.3×10^{-6} g./ml. 0.1 N H_2SO_4) was diluted to 100 ml. with 0.1 N sulfuric acid, and a reading was obtained on the fluorometer.

Solutions of the fluorescent compounds (50 to 100×10^{-6} g./ml. in their appropriate solvents) were diluted and readings of the dilutions were taken until a linear relationship was observed between fluorescence and concentration.

Values of dilutions equivalent to absorbance units of no greater than 0.05 at the exciting wavelength of 365 mμ were employed to calculate the QRU when a linear relationship of fluorescence to concentration was not observed.

[2] Catalytic hydrogenation reactions were carried out at room temperature and 60 p.s.i. by means of a Parr hydrogenator. Melting points were taken in open capillary tubes with a Mel-Temp electric block; they are corrected. UV spectra were determined in ethanol solution by means of a Beckman model DB spectrophotometer. IR spectra were obtained with either a Perkin-Elmer Infracord or model 337 grating spectrophotometer. Fluorescence spectra were determined with an Aminco-Bowman spectrophotofluorometer model No. 4-8106 equipped with a X-Y recorder; spectra were uncorrected. Microanalyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich.

[3] Celite 545, Johns-Manville Co., New York, N. Y.

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Keyphrases

Stilbene isothiocyanates—synthesis
Fluorescent tagging, antibodies—stilbene isothiocyanates
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Fluorometry—analysis

Dissolution Behavior and Solubility of Anhydrous and Trihydrate Forms of Ampicillin

By JOHN W. POOLE and CHANDER KANTA BAHAL

Anhydrous ampicillin and ampicillin trihydrate were compared for solubility and relative rates of dissolution in distilled water at temperatures ranging from 7.5 to 50°. Differences were noted in the physical-chemical properties of these two forms of ampicillin. The thermodynamic properties of these compounds have been experimentally evaluated. The properties noted for the two forms of the antibiotic are consistent with the observed differences in the biological utilization of the two forms after oral administration to laboratory animals and human subjects.

MANY ORGANIC medicinal compounds are capable of existing in more than one crystalline form having different physical-chemical properties. The resulting variation in the thermodynamic properties associated with differences in crystal form may be of considerable pharmaceutical importance as pointed out previously by Higuchi (1). The present report is concerned with studies conducted to determine the differences in some of the physical-chemical properties of two forms of ampicillin, a semisynthetic penicillin. Specifically, the solubilities and relative rates of dissolution in distilled water of anhydrous ampicillin and ampicillin trihydrate were determined and the thermodynamic properties of these crystal forms were experimentally evaluated.

Most of the past work reported on the physical-chemical properties of crystalline hydrates has been concerned with inorganic compounds. The studies of Taylor and Henderson (2) on the various hydrates of calcium nitrate and of Hill (3) on calcium sulfate are examples of such studies. More recently several investigations concerned with studies of organic molecules in the anhydrous and hydrated forms have been reported. An anhydrous form of phenobarbital and two of its hydrates were examined by Eriksson (4) for apparent solubility in water as a function of time. The relative dissolution rates of solvated and nonsolvated crystal forms of several types of compounds of pharmaceutical interest, including steroids and xanthines were reported by Shefter and Higuchi (5). These workers also determined the thermodynamic properties of several of these crystal systems.

EXPERIMENTAL

Apparatus—A constant-temperature water bath equipped with Unitherm Haake constant-temperature circulator[1] and a rotating-bottle apparatus,[2] Swinney hypodermic adaptor,[3] Millipore filters[3] (pore size 0.45 μ), amber bottles, 120 ml. with polyseal caps.[4]

Compounds—In all the experiments anhydrous ampicillin, (Wyeth Laboratories batch C-10575, m.p. 203–204°) was used. The trihydrate form of ampicillin was prepared from the anhydrous form by the method of Austin *et al.* (6). IR spectra and differential thermal analysis curves were obtained for this material.

Procedure—An excess of drug, 2 g., in the appropriate form was added to 100 ml. of distilled water previously equilibrated to the desired temperature.

Received June 26, 1968, from Pharmacy Reasearch & Development Division, Wyeth Laboratories, Philadelphia, PA 19101
Accepted for publication August 7, 1968.
Presented to Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

[1] Brinkmann Instruments, Westbury, N. Y.
[2] E. D. Menold Sheet Co., Lester, Pa.
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The bottles were rotated in a constant-temperature water bath maintained at the indicated temperature. Samples withdrawn at definite intervals were filtered through a Millipore filter and diluted immediately to avoid any precipitation of ampicillin in the filtered samples due to supersaturation. The penicillin content was determined by means of an iodometric titration procedure as described below. To 2.0-ml. aliquots containing 1 to 3 mg. of ampicillin, 2.0 ml. of 1 *N* sodium hydroxide was added and samples were allowed to stand at room temperature for 15 min. At the end of this time, 2.0 ml. of 1.2 *N* HCl was added followed by 10 ml. of 0.01 *N* iodine. After 15 min., the excess of iodine was titrated using 0.01 *N* sodium thiosulfate. For the blank determinations, to a 2.0-ml. sample, 10 ml. of 0.01 *N* iodine was added and titrated immediately.

RESULTS AND DISCUSSION

The solubility and dissolution behavior of the anhydrous and trihydrate forms of ampicillin at 7.5, 20, 30, and 40° are shown in Fig. 1. Similar data for the study conducted at 50° are shown in Fig. 2. These figures show the concentration of the antibiotic attained in solution as a function of time in the presence of an excess of the solid phase in the appropriate form and under essentially constant agitation. One interesting feature of these studies is the inverse relationship noted between temperature and solubility for the anhydrous form of the drug. The apparently greater dissolution rate observed for the anhydrous form at the lower temperatures cannot be attributed solely to the higher free energy content of this species since no serious attempt was made to maintain equal specific surface areas of the two forms. However, microscopic examination of the materials employed showed the anhydrous and trihydrate forms to be substantially the same with regard to particle size and shape and since the anhydrous form is significantly more soluble than the trihydrate form, the dissolution rate of the two forms is in the direction that would be expected on solubility considerations alone. In addition, the study at 50°, which is above the transition temperature of this system, shows the trihydrate to have an apparently greater dissolution rate than the anhydrous form. This supports the contention that solubility is the dominant factor in this system.

The dissolution behavior in water noted for the two forms of ampicillin suggest that the equilibrium

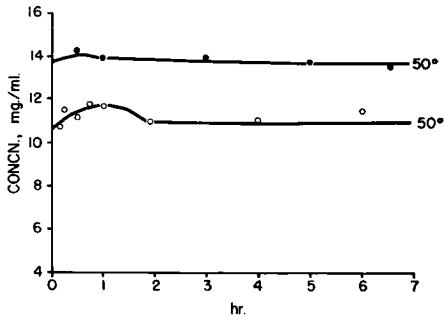

Fig. 2—The dissolution behavior of anhydrous and trihydrate crystalline forms of ampicillin in water at 50°. Key: ○, anhydrous; ●, trihydrate.

solubility observed are good approximations of the true solubility of these crystals. Consequently the measurements made at the several temperatures permit calculation of the thermodynamic quantities involved in the transition of the anhydrous form to the trihydrate. An extensive treatment of the thermodynamic relationship involving polymorphism and solubility is presented in reports by Shefter and Higuchi (5) and Higuchi *et al.* (7).

The apparent equilibrium solubilities observed over the temperature range 20 to 50° when plotted in the classical van't Hoff fashion gave a reasonably good linear relationship for both forms of the antibiotic as shown in Fig. 3.

The transition temperature for the trihydrate-anhydrous crystal system corresponds to the temperature at which the solubility of the two forms is equal. The transition temperature for this system is 42° as shown in Fig. 3. This plot also points up the fact noted earlier that the solubility of the anhydrous form decreases with an increase in temperature whereas that of the trihydrate exhibits the usual temperature-solubility relationship.

The values of the heat of solution for each of the crystal forms was calculated from the slopes of the van't Hoff-type plot (Fig. 4) and were determined to be −1000 and 5400 cal./mole for the anhydrous and trihydrate forms, respectively. The enthalpy of hydration ($\Delta H_{A,H}$), the heat of solution of the anhydrous form minus the heat of solution of the hydrated species, was determined to be −6400 cal./mole.

At constant temperature and pressure the free energy difference ΔF_T, between the anhydrous and

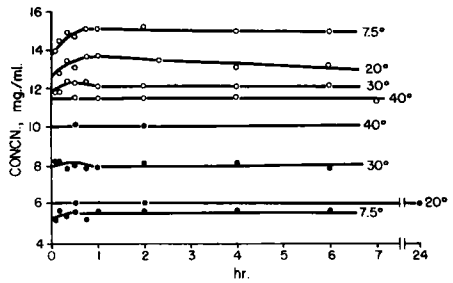

Fig. 1—The dissolution behavior of anhydrous and trihydrate crystalline forms of ampicillin in water at temperatures ranging from 7.5 to 40°. Key: ○, anhydrous; ●, trihydrate.

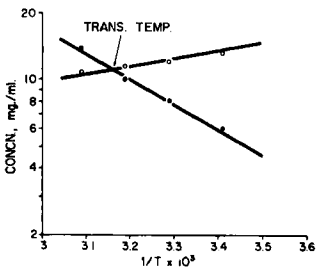

Fig. 3—The van't Hoff-type plot for the anhydrous and trihydrate forms of ampicillin in water. Key: ○, anhydrous; ●, trihydrate.

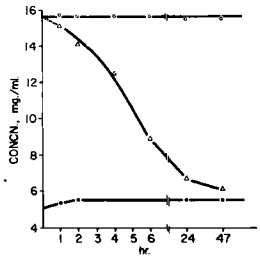

Fig. 4—The influence of seeding anhydrous ampicillin with 1% trihydrate crystals on the dissolution behavior in water at 10°. Key: ○, anhydrous; ●, trihydrate; Δ, anhydrous seeded with 1% trihydrate.

hydrated forms is determined by Eq. 1.

$$\Delta F_T = RT \ln \frac{C_s \text{ (anhydrous)}}{C_s \text{ (trihydrate)}} \qquad \text{(Eq. 1)}$$

where C_s is the solubility of the form under consideration at a particular temperature T, and R is the gas constant. This ΔF_T is a measure of the free energy change involved in conversion of the anhydrous crystal to the trihydrate crystal. The ΔF_T at 25 and 37° (corresponding to room and body temperatures) have been determined to be −430 and −140 cal./mole, respectively.

The entropy change, ΔS_T, for the reaction involved in hydrate formation can be calculated by Eq. 2.

$$\Delta S_T = \frac{\Delta H_{A,H} - \Delta F_T}{T} \qquad \text{(Eq. 2)}$$

The values computed for the hydration of the anhydrous to trihydrate ampicillin crystals at 25° and 37° were −20.0 e.u. and −20.2 e.u., respectively. At the transition temperature of the anhydrous-trihydrate crystal system ΔF is equal to zero and the entropy change can be calculated by Eq. 3.

$$\Delta S_{\text{trans.}} = \frac{\Delta H_{A,H}}{T_{\text{trans.}}} \qquad \text{(Eq. 3)}$$

For ampicillin $\Delta S_{\text{trans.}}$ was determined to be −20.3 e.u. The hydrated species in this system contains three molecules of water and the possible intramolecular hydrogen bond formation between these associated water molecules may account for the relatively large entropy change noted. It has been suggested that a hydrated ampicillin complex of this type may be responsible to some extent for the relative stability of this compound in acidic solution (8).

The thermodynamic values calculated for the anhydrous-trihydrate ampicillin system are summarized in Table I.

As noted earlier the equilibrium solubilities observed in these experiments apparently correspond to the solubilities of the anhydrous and trihydrate crystalline phases for the ampicillin molecule. At the temperatures utilized there was no evidence of conversion of the more soluble anhydrous form to the less soluble trihydrate species as would be expected from the thermodynamic considerations. This may be due in part to the steric factors involved in the association of three molecules of water in the crystal system.

However, at lower temperatures (10°) the seeding of the anhydrous form with trihydrate crystals resulted in a relatively rapid and complete conversion of the anhydrous to the trihydrate form, as shown by the decrease in solubility. These data are shown in Fig. 4.

TABLE I—THERMODYNAMIC VALUES CALCULATED FOR THE ANHYDROUS-TRIHYDRATE AMPICILLIN SYSTEM

Temp., °C.	ΔH, cal./mole Anhydrous	ΔH, cal./mole Trihydrate	ΔF_T, cal./mole[a]	ΔS_T, e.u.[a]
	(−1000)	(5400)		
25			−430	−20.0
37			−140	−20.2
42			0	−20.3

[a] Calculated for the conversion from the anhydrous to the trihydrate form.

The relative ease of conversion of anhydrous to trihydrate ampicillin at the lower temperatures is to be expected, since the rate of phase transformation in a given system depends on the solubilities of the forms at that temperature, the rates of solution, and the diffusion rates of the molecules in solution. The higher the solubility and the greater the difference in solubilities of the two phases the greater will be the rate of transformation. At the lower temperatures the anhydrous form exhibits an increased solubility due to its negative heat of solution. The trihydrate form shows a decrease in solubility at lower temperatures (positive heat of solution) which results in a relatively large difference in the solubilities for the two forms.

Higuchi (1) pointed out that the physiological activity and availability of a drug is often directly related to its thermodynamic activity in a system of this type. Recently Aguiar *et al.* (9) reported on the effect of polymorphism on the absorption of chloramphenicol from chloramphenicol palmitate. From the data presented in the present investigation it would be reasonable to expect an enhanced biological utilization of the anhydrous form of ampicillin compared to the trihydrate form of this agent. In addition, since the anhydrous system is apparently stable to conversion at room and body temperature, these differences should be maintained in the clinical situation. That this is the case was shown in a recent report by Poole *et al.* (10). In these investigations, various pharmaceutical formulations of the drug in each form were administered to laboratory animals (beagle dogs) and to human subjects in a series of crossover experiments. The formulations containing the anhydrous form of the penicillin resulted in blood serum levels of the antibiotic which were consistently earlier and significantly higher than those observed after administration of similar formulations containing the hydrated material. In every instance the area under the serum level-time curves was greater for the anhydrous form of the drug than for the hydrated substance indicating a more efficient biological utilization of this form of the medicinal agent. The results of the *in vivo* evaluation of the oral suspensions of the two forms of ampicillin are summarized in Table II.

SUMMARY

The solubility and relative rates of dissolution of anhydrous ampicillin and ampicillin trihydrate

TABLE II—PEAK SERUM LEVEL AND AREA UNDER THE BLOOD LEVEL-TIME CURVE AFTER ORAL ADMINISTRATION OF SUSPENSIONS OF ANHYDROUS AMPICILLIN AND AMPICILLIN TRIHYDRATE

Form of Ampicillin	Test Species	Peak Serum Level, mcg./ml.	Peak Time, min.	Area Under Curve (mcg./ml. × hr.)
Anhydrous[a]	Dog	20.6	45	36.6
Trihydrate[b]	Dog	11.0	90	22.8
Anhydrous[a]	Human	2.2	60	6.9
Trihydrate[b]	Human	1.7	120	5.7

[a] Administered as Omnipen for oral suspension, Wyeth Laboratories, Inc., Radnor, Pa. [b] Administered as Polycillin for oral suspension, Bristol Laboratories, Syracuse, N. Y.

in distilled water have been determined over a temperature range of 7.5 to 50°. Below the transition temperature, 42°, the anhydrous form was found to be significantly more water soluble than the trihydrate. In addition, the solubility of the anhydrous crystal was shown to be inversely related to temperature.

The thermodynamic values have been calculated for the anhydrous-trihydrate ampicillin system. The greater thermodynamic activity of the anhydrous form correlates with the observed enhanced biological availability noted with this crystal form of the antibiotic.

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Blood serum levels—ampicillin, anhydrous, trihydrate
Iodometric titration—analysis

Potential Antitumor Agents III

Sodium Salts of α-[*N*]-Heterocyclic Carboxaldehyde Thiosemicarbazones

By KRISHNA C. AGRAWAL and ALAN C. SARTORELLI

Sodium salts of four of the most active antineoplastic agents in a series of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones have been prepared as a means of solubilizing for parenteral administration these extremely insoluble compounds. The sodium salt of 1-formylisoquinoline thiosemicarbazone (II) is soluble in nonaqueous vehicles for injection such as propylene glycol, whereas the sodium salts of 5-hydroxy-1-formylisoquinoline thiosemicarbazone (III), 3-hydroxy-2-formylpyridine thiosemicarbazone (IV), and 5-hydroxy-2-formylpyridine thiosemicarbazone (V) are readily soluble in water. Compounds III and IV, at the optimum effective dosage regimens, caused a greater prolongation of the survival time of mice bearing the L1210 lymphoma than did the parent derivatives, while II and V produced antineoplastic activity against sarcoma 180 and the L1210 lymphoma, respectively, equivalent to that of the parent compounds.

A VARIETY OF thiosemicarbazones of α-(*N*)-heterocyclic carboxaldehydes has been prepared and tested for antineoplastic activity (1–7). Several of these derivatives, especially 1-formylisoquinoline thiosemicarbazone (2, 3), its 5-hydroxy derivative (4), and both 3-hydroxy-2-formylpyridine thiosemicarbazone and 5-hydroxy-2-formylpyridine thiosemicarbazone (5, 6), have demonstrated pronounced antineoplastic activity when tested against a relatively wide spectrum of transplanted rodent tumors. To

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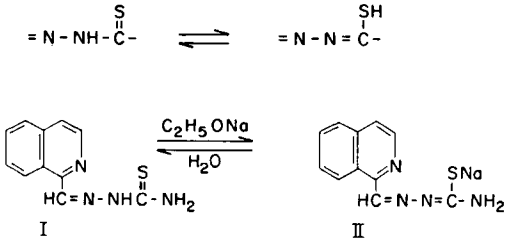
further elucidate the structural requirements for biological activity expounded by French and Blanz (2), the authors have synthesized and tested for tumor-inhibitory potency a series of 5-substituted 1-formylisoquinoline thiosemicarbazones (4), as well as a variety of derivatives with alterations in the side-chain of the molecule (7). These studies support the concept that an unsubstituted formylthiosemicarbazone group adjacent to a heteroaromatic ring nitrogen atom is required for optimum antineoplastic activity.

The biochemical basis for the growth-inhibitory activity of 1-formylisoquinoline thiosemicarbazone has also been studied in this laboratory (8–10); this agent caused marked inhibition of the synthesis of DNA by preventing the conversion of ribonucleotides to deoxyribonucleotide forms. Blockade of the formation of RNA and protein also occurred, but these were considerably less sensitive to drug-induced inhibition. A similar mechanism of action appeared to be operative with both 3-hydroxy-2-formylpyridine thiosemicarbazone and 5-hydroxy-2-formylpyridine thiosemicarbazone (11).

Although 1-formylisoquinoline thiosemicarbazone has been shown to possess activity against transplanted tumors in mice when administered orally (3), the expectation of variable gastrointestinal absorption of this relatively insoluble agent and related heterocyclic carboxaldehyde thiosemicarbazones would appear to preclude oral usage as the ideal route of administration. Thus, the ultimate utility of these heterocyclic carboxaldehyde thiosemicarbazones as antineoplastic agents in man appeared to be limited by an inability to formulate the compounds for parenteral administration because of their great insolubility. A number of solvents, both polar and nonpolar, have been investigated in an effort to solubilize these drugs; only dimethyl sulfoxide appeared to be capable of achieving the degree of solubilization necessary for concentrations required in tests of antineoplastic potency. However, the use of dimethyl sulfoxide in man at present is questionable. Neither salt formation, such as the hydrochloride of 5-amino-1-formylisoquinoline thiosemicarbazone (4), nor quaternization of the heterocyclic nitrogen atom as in 3-hydroxy-2-formyl-1-methylpyridinium iodide thiosemicarbazone (2) resulted in the attainment of effective tumor-inhibitory compounds with adequate solubility. Furthermore, initial efforts to synthesize water-soluble derivatives, such as 1-formylisoquinoline thiosemicarbazone-5-sulfonic acid, resulted in agents with decreased therapeutic potency (4).

DISCUSSION

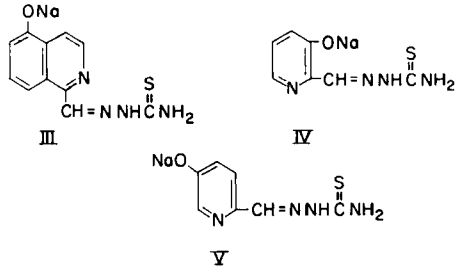
This report describes the preparation of sodium salts of four of the most active antitumor agents in this series. 1-Formylisoquinoline thiosemicarbazone (I), when treated with sodium ethoxide in alcoholic solution, reacts to form the monosodium derivative (II) of the resonance-hybrid anion as shown in Scheme I. Compound II



Scheme I

(sodium 1-amino-4-isoquinolyl-2,3-diazabuta-1,3-diene-1-thiol) is essentially a butadiene derivative in which the hydrogen atom of the —NH— group of the formyl thiosemicarbazone side-chain is apparently displaced in its lactim form by sodium. Compound II is, however, quantitatively hydrolyzed in the presence of water to give the original thiosemicarbazone. Compound I is very sparingly soluble in alcohols and glycols, whereas Compound II is very soluble in these solvents. Thus, solutions of Compound II in propylene glycol were found to be suitable for parenteral administration and were employed in biological tests for carcinostatic activity.

The sodium salts of the hydroxy derivatives shown in Scheme II were fabricated by dissolving



Scheme II

the thiosemicarbazones in equimolar aqueous NaOH solutions; excess water was removed under vacuum employing absolute ethanol. These compounds (III, IV, and V) are readily soluble in water. In accordance with the previous findings that the substitution of electron-donating groups increased antitumor activity, while electron-withdrawing groups lessened antineoplastic activity (4); it was anticipated that

TABLE I—EFFECT OF SODIUM SALTS OF HYDROXY DERIVATIVES OF α-(*N*)-HETEROCYCLIC CARBOXALDEHYDE THIOSEMICARBAZONES ON THE SURVIVAL TIME OF MICE BEARING L1210 LYMPHOMA

Drug	Dose, mg./kg.[a]	×	No. of Daily Doses	Av. Δ wt., %[b]	Av. Survival, days ± *S.E.*
None	—		—	+16.3	9.7 ± 1.6 (58)[c]
5-Hydroxy-1-formylisoquinoline thiosemicarbazone	30	×	2	+5.4	13.9 ± 0.9 (15)
	40	×	2	+10.2	15.5 ± 1.0 (15)
	50	×	2	+18.3	10.0 ± 0.9 (4)
	60	×	1	+8.9	13.6 ± 1.1 (10)
	80	×	1	+3.2	15.1 ± 1.9 (10)
	100	×	1	+18.7	12.4 ± 1.4 (5)
Sodium 1-formylisoquinoline-5-ol thiosemicarbazone (Compound III)	32.5	×	2	−7.4	24.7 ± 2.1 (13)
	43.5	×	2	−10.8	24.3 ± 1.7 (10)
	65	×	1	−16.0	13.5 ± 0.6 (10)
	87	×	1	−14.4	16.4 ± 1.6 (10)
	109	×	1	−16.7	9.6 ± 2.2 (5)
3-Hydroxy-2-formylpyridine thiosemicarbazone	10	×	4	−4.0	18.1 ± 1.2 (15)
	20	×	4	−5.3	19.2 ± 1.3 (5)
	20	×	2	−1.6	22.2 ± 1.9 (10)
	30	×	2	−2.7	21.1 ± 2.1 (10)
	40	×	1	−2.4	17.1 ± 0.7 (10)
	60	×	1	0.0	17.2 ± 0.7 (10)
	80	×	1	−2.4	16.8 ± 0.3 (10)
Sodium 2-formylpyridine-3-ol thiosemicarbazone (Compound IV)	11	×	4	−5.0	23.9 ± 1.6 (14)
	22	×	4	−10.0	22.2 ± 7.0 (5)
	22	×	2	−4.5	28.8 ± 2.9 (10)
	33	×	2	−6.9	21.6 ± 1.8 (9)
	44	×	1	+5.4	10.5 ± 0.2 (10)
	67	×	1	+6.2	10.8 ± 0.4 (10)
	89	×	1	0.0	13.9 ± 0.6 (10)
5-Hydroxy-2-formylpyridine thiosemicarbazone	10	×	2	+0.9	24.6 ± 2.0 (10)
	20	×	2	+1.0	21.5 ± 1.1 (10)
	30	×	2	0.0	27.6 ± 3.0 (10)
	40	×	2	−5.5	24.2 ± 2.1 (10)
Sodium 2-formylpyridine-5-ol thiosemicarbazone (Compound V)	11	×	2	+4.5	21.0 ± 1.1 (10)
	22	×	2	+4.0	23.4 ± 2.8 (10)
	33	×	2	−2.0	24.6 ± 1.8 (10)
	44	×	2	−8.1	21.2 ± 1.1 (10)

[a] Administered as indicated daily for 4 consecutive days, beginning 24 hr. after tumor implantation. [b] Average weight change from onset to termination of drug treatment. [c] The figure in parentheses indicates the number of mice employed.

the stronger electron-donating properties of the phenoxide anion of Compounds III, IV, and V might possibly serve to increase biological activity over the parent compounds.

Biological Tests—Experiments were performed on 21- to 24-g. male C57BL X DBA F_1 mice (Cumberland View Farms, Cumberland, Tenn.). Transplantation of lymphoma L1210 ascites cells was carried out by withdrawing peritoneal fluid from a donor mouse bearing a 7-day tumor growth. The suspension was centrifuged for 2 min. (1600×*g*), the supernatant peritoneal fluid was decanted, a 10-fold dilution with isotonic saline was made, and 0.1 ml. of the resulting cell suspension (approximately 8×10^6 cells) was injected intraperitoneally into each animal. For any one experiment, mice were distributed into groups of five to ten animals of comparable weight and maintained throughout the course of the experiment on Purina laboratory chow pellets and water *ad libitum*. Experiments with more than five mice per group were performed at least two times.

Drugs were administered by intraperitoneal injection as indicated for 4 consecutive days beginning 24 hr. after tumor implantation. The hydroxy derivatives of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones were given in fine suspension following homogenization in absolute ethanol (adjusted so that the final concentration of the drug solution was 5% with respect to ethanol) and 2–3 drops of a 20% aqueous solution of polysorbate 80[1] and then made up to volume with isotonic saline. Compounds III, IV, and V were injected in solution in distilled water. Control tumor-bearing animals given injections of comparable volumes of vehicle were included in each experiment. Mice were weighed during the course of the experiment, and the percentage change in body weight from onset to termination of therapy was used as an indication of drug toxicity.

The effects of molar equivalent doses of these agents on the survival time of mice bearing lymphoma L1210 was used as a measure of therapeutic efficacy; the results obtained are shown in Table I. In most instances the daily frequency of drug administration appeared to be of importance, with treatment twice daily being

[1] Tween 80, Atlas Chemical Co., Wilmington, Del.

optimum. The sodium salts of 3-hydroxy-2-formylpyridine thiosemicarbazone and 5-hydroxy-1-formylisoquinoline thiosemicarbazone, at the optimum effective dosage regimens, appeared to prolong the life-span of tumor-bearing animals to a greater extent than did the parent compounds; the most dramatic difference was obtained with the more insoluble compound 5-hydroxy-1-formylisoquinoline thiosemicarbazone, suggesting that the sodium salt of this agent was able to distribute itself more advantageously *in vivo*. The administration of the sodium salt of the most soluble of these α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones, 5-hydroxy-2-formylpyridine thiosemicarbazone, did not increase the tumor-inhibitory potency of this derivative; thus, only the advantage of complete solubilization was attained by formation of the sodium salt of this agent. Preliminary results indicate that the sodium salt of 1-formylisoquinoline thiosemicarbazone (Compound II) administered in propylene glycol solution to mice bearing sarcoma 180 ascites cells was essentially equal in growth-inhibitory activity to the parent thiosemicarbazone (Compound I) employed in suspension. The sodium salts of the hydroxy derivatives appeared to cause, in general, a greater loss in host body weight than did the parent compounds; however, no other observable toxicity, such as gross tissue damage at the injection site was produced by these compounds. It would appear, therefore, that the solubility and in some instances the increased antineoplastic activity of the sodium salts of these heterocyclic aldehyde thiosemicarbazones would make the sodium salts the drug form of choice in initial clinical trials of these agents.

EXPERIMENTAL

Melting points were determined using a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by the Schwarzkopf Microanalytical Laboratory, New York, N.Y. The analytical data are listed in Table II.

Sodium Salt of 1-Formylisoquinoline Thiosemicarbazone—1-Formylisoquinoline thiosemicarbazone (2.3 g., 0.01 mole) was added in small portions with stirring to a solution of sodium ethoxide (prepared by dissolving 0.01 mole of sodium in 25 ml. of absolute ethanol). The thiosemicarbazone slowly dissolved and the solution turned yellow. The reaction mixture was stirred for another 5 min. at room temperature and then excess solvent was removed under vacuum at 25° to leave an orange-colored thick syrup. This was triturated with 100 ml. of anhydrous ether to yield a crystalline yellow precipitate, which was filtered, washed with anhydrous ether, and dried at 80° to give 2.4 g. (95.2%), m.p. 214–216° dec. Compound II is sensitive to moisture and is best stored over anhydrous $CaCl_2$.

TABLE II—SODIUM SALTS OF α-(*N*)-HETEROCYCLIC CARBOXALDEHYDE THIOSEMICARBAZONES

Compound	Formula	Anal. Calcd.	Anal. Found
II	$C_{11}H_9N_4NaS$	C, 52.38 H, 3.57 S, 12.70	C, 52.03 H, 3.90 S, 12.92
III	$C_{11}H_9N_4NaOS \cdot H_2O$	C, 46.15 H, 3.85 S, 11.19	C, 46.00 H, 4.24 S, 10.71
IV	$C_7H_7N_4NaOS \cdot H_2O$	C, 35.59 H, 3.81 S, 13.56	C, 35.71 H, 4.17 S, 13.14
V	$C_7H_7N_4NaOS \cdot H_2O$	C, 35.59 H, 3.81 S, 13.56	C, 36.62 H, 4.20 S, 13.20

General Procedure for Sodium Salts of Hydroxy Derivatives of α-(N)-heterocyclic Carboxaldehyde Thiosemicarbazones—The hydroxy derivative of an α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazone (0.01 mole) was dissolved in a solution of sodium hydroxide (0.4 g., 0.01 mole) in 10 ml. of water. The mixture was filtered to remove suspended material and the water from the filtrate was removed under vacuum at 25° using absolute ethanol. The resultant syrupy liquid was dissolved in 5 ml. of absolute ethanol; addition of 100 ml. of anhydrous ether resulted in the precipitation of the sodium salt which was collected by filtration, washed with anhydrous ether, and dried under vacuum at 80° over anhydrous calcium chloride. These compounds have 1 mole of water of crystallization and are hygroscopic; therefore, they should be stored in closed containers over $CaCl_2$. Compound III melts at 213–216° with decomposition. Compounds IV and V initially collapse with frothing at 182–185° and 192–194°, respectively, and then decompose at 208–212° and 220–222°, respectively.

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Keyphrases

Antitumor agents
α-(*N*)-Heterocyclic carboxaldehyde thiosemicarbazones Na salts—synthesis
Antitumor activity—mice

Determination and Thin-Layer Chromatography of Phenylbutazone in the Presence of Decomposition Products

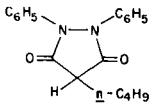
By H. D. BECKSTEAD, K. K. KAISTHA*, and S. J. SMITH

An assay procedure for phenylbutazone involving acid and base shake-out followed by UV spectrophotometric measurement at 264 and 232 mμ is described. The method enables potentially interfering substances such as other medicaments, excipients, and dyes to be removed, and the quantities of phenylbutazone and its decomposition products to be determined. A thin-layer chromatographic system involving cyclohexane, chloroform, methanol, and acetic acid (60:30:5:5) on Silica Gel GF is applied to screening phenylbutazone preparation and semiquantitative estimation of decomposition products present.

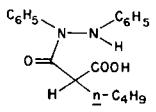
THE WIDE USAGE of the antiarthritic and analgesic compound phenylbutazone (4-*n*-butyl-1,2-diphenylpyrazolidine-3,5-dione) (I) has given rise to a large body of data on its *in vivo* properties (1). Parallel to this, a varied collection of analytical procedures for determining the drug in pharmaceutical and biological media has appeared.

Phenylbutazone was analyzed polarographically by Kalvoda and Zyka (2), while hydrolysis of the drug followed by color formation of the resulting products was employed by Pulver (3), Pemberton (4), Hoehlein (5), and recently by Maggiorelli *et al.* (6). Simple colorimetric (7, 8), halometric (9–11), and gravimetric (12) procedures have been utilized also. Direct UV analysis (13–16) including the automated procedure employed by Ahuja *et al.* (17), titrimetry against chloramines (18), and base (19, 20) also have been applied to phenylbutazone analysis.

Titration of phenylbutazone with sodium hydroxide has been adopted as the official method of analysis by the British Pharmacopoeia 1963 (21) and the National Formulary XII (22). The compendial methods suffer from some shortcomings.[1] The required initial extraction of the drug from tablets with acetone and/or ethanol as designated often does not give complete recoveries. Traces of tablet-coating dyes occasionally are extracted also so that the titration end point—phenolphthalein pink—is obscured. Moreover, nonofficial phenylbutazone–aluminum hydroxide combinations cannot be assayed by the official method. The BP and NF assays are inadequate too in that they are not specific for phenylbutazone. That is, common decomposition products, such as the carboxylic acid II and the α-hydroxycarboxylic acid III, are titratable and will afford higher than actual assay results if present. Current spectrophotometric analyses of phenylbutazone similarly are nonspecific. Herrmann recognized this in his work on phenylbutazone metabolism (23). In formulations, decomposition of the drug can give rise to 4-hydroxyphenylbutazone (IV) and *n*-caproylhydrazobenzene (V) in addition to Compounds II and III, all of which can interfere with a UV spectrophotometric assay.



I



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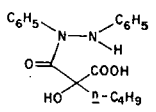

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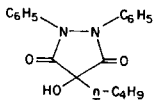
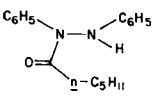


IV



V

To overcome the problems generated by the presence of decomposition products, other medicinal agents and interfering dyes, a specific, precise, and accurate method of analysis for phenylbutazone and its preparations, involving acid–base shake-out followed by UV spectrophotometric measurement was sought. In addition, a thin-layer chromatographic procedure suitable for semiquantitative estimation of any decomposition products present was investigated.

EXPERIMENTAL

Chloroform employed in the studies was analytical reagent grade used without further purification.

A Beckman model DU spectrophotometer was

Received June 4, 1968, from the Research Laboratories, Food and Drug Directorate, Ottawa, Ontario, Canada.

Accepted for publication August 19, 1968.

The authors wish to thank various members of the Pharmaceutical Chemistry Division for their suggestions and constructive criticisms, and Geigy Canada Ltd., Montreal, Canada, for generously supplying phenylbutazone and samples of the decomposition products employed in this study.

* To whom requests for reprints should be addressed.

[1] The NF assay procedure was revised in the First Interim Revision Announcement to NF XII, p. 4.

utilized for all analyses. When determining a (absorptivity) values for decomposition products at 264 mμ (11.07) and 232 mμ (48.4) the instrument was standardized against 1.439×10^{-4} M potassium dichromate in 0.01 N H_2SO_4 (0.04235 g. $K_2Cr_2O_7$/1. acid). Dichromate readings, when a values quoted in parentheses above were determined, were 0.522 at 232 mμ and 0.570 at 264 mμ.

Reference standard phenylbutazone was medicinal grade bulk drug which analyzed 100% by both the BP and NF procedures, and was shown by TLC to be free of both contaminants and decomposition products.

Analysis of Phenylbutazone—*Preparation of Samples*—Weigh 20 tablets, calculate the average weight per unit, and grind to 80 mesh. For capsules determine the weight of the contents of 20 capsules, calculate the average weight, and mix thoroughly. Analyze bulk drug and reference standard samples without further treatment.

Determination—*Formulations*—Accurately weigh a portion of powdered tablet or capsule fill equivalent to about 40 mg. phenylbutazone, and quantitatively transfer to a 250-ml. separator containing 20 ml. 0.1 N NaOH. Add 12 ml. $CHCl_3$ and rotate gently. Draw off the clear $CHCl_3$ layer and extract this with six 15-ml. portions of 0.1 N NaOH. Combine all NaOH solutions (discarding the $CHCl_3$). Acidify the aqueous alkaline phase with 1.5 ml. concentrated HCl. Extract the acidified solution with six 25-ml. portions of $CHCl_3$, combining the $CHCl_3$ solutions in a 250-ml. volumetric flask. Make to volume with $CHCl_3$ and mix thoroughly. Filter this solution through Whatman paper No. 30 or equivalent, discarding the first 10 ml. of filtrate. Evaporate an accurately measured 10-ml. aliquot of the filtrate to dryness with a rotary film evaporator. With the aid of 0.1 N NaOH quantitatively transfer the residue into a 200-ml. volumetric flask. Make up to volume with 0.1 N NaOH and mix.

Standard and Bulk Drug—Accurately weigh out about 40 mg. of standard phenylbutazone or bulk drug and proceed as above. Alternatively, dissolve the sample in 65 ml. $CHCl_3$ in a 250-ml. volumetric flask. Make up to volume with freshly prepared acid-washed $CHCl_3$ (200 ml. $CHCl_3$ treated with 1.5 ml. concentrated HCl) and mix. Proceed as above, starting at "Filter this solution...."

Measure the absorbance of both reference standard and sample solutions against a blank of 0.1 N NaOH at 232 and 264 mμ.

Calculation—Solve for X and Y

$$A \cdot X + 11.07\ Y = W \qquad \text{(Eq. 1)}$$

$$B \cdot X + 48.4\ Y = Z \qquad \text{(Eq. 2)}$$

where

A = a of phenylbutazone standard at 264 mμ
B = a of phenylbutazone standard at 232 mμ
W = absorbance of sample solution at 264 mμ
Z = absorbance of sample solution at 232 mμ
11.07 = a of decomposition product at 264 mμ
48.4 = a of decomposition product at 232 mμ

$$X \times \frac{250}{10} \times \frac{200}{1000} \times \frac{\text{av. wt. per unit}}{\text{wt. of sample taken}} = \text{g. phenylbutazone per unit} \qquad \text{(Eq. 3)}$$

$$Y \times \frac{250}{10} \times \frac{200}{1000} \times \frac{\text{av. wt. per unit}}{\text{wt. of sample taken}} = \text{g. decomposition product present} \qquad \text{(Eq. 4)}$$

Percent phenylbutazone on basis of total drug and decomposition products present at time of formulation is:

$$\frac{X}{X + Y} \times 100$$

Percent decomposition product on basis of total drug and decomposition products present at time of formulation is:

$$\frac{Y}{X + Y} \times 100$$

Thin-Layer Chromatography—*Preparation of Layers*—Prepare chromatoplates with standard equipment for TLC (24). To obtain five plates 20 × 20 cm., mix 25 g. Silica Gel GF (Merck) with 50 ml. water. Apply the slurry to plates (layer thickness *ca.* 250 μ), air dry, and then activate the chromatoplates at 110° for 20 min.

Solvent System—Cyclohexane–chloroform–methanol–glacial acetic acid (60:30:5:5).

Detection Reagents—1. Short-wavelength UV light.

2. Chlorine/*o*-toluidine (*a*) Chlorination: treat the developed dry chromatoplate with a Cl_2 atmosphere. When employing Cl_2 gas from a cylinder, chlorinate the plate for 5–10 min. If the gas is generated from equal volumes of 10% HCl and 1.5% $KMnO_4$, expose the plate for 15–20 min. (*b*) Spray reagent: dissolve 0.16 g. *o*-toluidine in 30 ml. glacial acetic acid, make up to 500 ml. with distilled water. Dissolve 1 g. KI in this solution. (*c*) Procedure: after chlorination allow the plate to stand in the air for 3–5 min. to remove excess Cl_2. Cautiously spray a corner of the chromatogram. If the background does not turn blue, complete the spraying process. Otherwise wait a few moments before proceeding.

3. Folin-Ciocalteu (FCR) (*a*) Stock solution: to a solution of 10 g. sodium tungstate and 2.5 g. sodium molybdate in 70 ml. water add successively 5 ml. 85% phosphoric acid and 10 ml. concentrated HCl. Reflux for 10 hr. then add 15 g. lithium sulfate, 5 ml. water, and 1 drop bromine. Boil this solution for 15 min. Cool and make up to 100 ml. in a volumetric flask. (*b*) Spray reagent I: 20% aqueous Na_2CO_3. Spray reagent II: dilute 1 volume stock solution with 3 volumes water. (*c*) Procedure: spray with I, air dry, and spray with II.

Preparation of Sample—Weigh 20 tablets or capsules and determine the average weight per unit. Powder five tablets or mix the contents of five capsules, and transfer a weighed portion equivalent to one unit to a 40-ml. centrifuge tube. Add 5 ml. 80% ethanol containing 1% concentrated HCl. Mix thoroughly for 1 min. and centrifuge.

Chromatographic Procedure for Decomposition Products—On duplicate plates, spot 10 μl. of solution to be examined adjacent to each of decomposition product standards equivalent to 3, 6, 9, 12, 15, 18, and 21% of label claim for phenylbutazone. Insert the plate in a suitable jar previously equilibrated with solvent, and allow the solvent to rise to a height of 15 cm. (time, about 40 min.). Air dry the plates and examine under UV light and/or treat each plate with one of the two spray reagents. Compare spot areas and color intensity.

TABLE I—ANALYSIS OF PHENYLBUTAZONE SAMPLES

	Label Claim, mg.	Label Claim Compendial Procedure, %		Label Claim Determined by Peak Height Measurement at 264 mμ		Determinations by Simultaneous Equation			
		As Stated	Modified[a]	Found, %	SD^b	Label Claim Found, %	SD^b	Decomposition Product Present Found, %	SD^b
Simulated phenylbutazone-alka	100	84–88	97–100	100.71	1.62	99.48	1.54	7.98[c]	1.06
Formulation A containing $Al(OH)_3$	100	87–93	94–97	100.86	0.70	100.24	0.62	3.88	0.92
Formulation B containing $Al(OH)_3$	50			100.34	0.32	99.24	0.32	6.72	0.62
Formulation B[d] containing $Al(OH)_3$	50			74.16	0.78	68.08	1.91	28.06	0.34
Formulation C containing $Al(OH)_3$	100			99.37	0.33	99.35	0.33	0.98	0.83
Formulation C[d] containing $Al(OH)_3$	100			94.00	0.56	93.52	0.57	2.95	0.29
Formulation D containing $Al(OH)_3$	100			97.64	0.73	96.48	0.79	7.51	0.58
Formulation D[d] containing $Al(OH)_3$	100			85.88	0.30	81.63	0.24	24.61	1.76
Formulation E containing $Al(OH)_3$	100	10–15	17–24	97.84	0.46	97.42	0.49	2.99	0.22
Formulation F[d] containing $Al(OH)_3$	100			88.70	0.30	87.06	0.37	10.20	0.54

[a] By increasing amount of solvent utilized in extracting phenylbutazone. [b] Four determinations. [c] Theoretical: 7.0%. [d] Sample at least 5 years old.

DISCUSSION

During routine analysis of both normal and alka[2] phenylbutazone formulations a number of difficulties were encountered and some doubts as to the reliability of official analytical methods and the suitability of published assays were raised. Both the BP (21) and the NF (22) procedures were applied and although simulated alka tablets gave satisfactory results (Table I) data for commercial formulations exhibited a broad analytical range. In addition, values often were low, but these could be elevated when volumes of solvent greatly in excess of those stipulated in the monographs were utilized.

The reliability of the compendial method could be questioned further when seven out of 16 brands of phenylbutazone, labeled BP or NF, which were obtained for analysis, could not be analyzed by the official methods.[3] This was due, apparently, to dyes extracted from tablet coatings interfering with the pink phenolphthalein end point of the compendial procedure. The dyes turned a dark blue color during titration. Skepticism regarding the pharmacopial procedure was heightened when examination of the two decomposition products (II and III) confirmed that these were extractable from the tablet mass of decomposed phenylbutazone (I) and, as expected, were titratable with sodium hydroxide. Thus, samples of drugs containing these would give higher than actual assay values.

The BP and NF procedures were found to be inapplicable to the analysis of alka phenylbutazone formulations. Application of the methods to these preparations afforded extremely low assay values—sometimes in the 20% range. This was probably due to the insolubility of the drug-antacid complex in the formulation.

Because of the shortcomings of the official method, the possibility of performing UV spectrophotometric analysis on phenylbutazone formulations in a manner similar to that of the other workers (16, 17, 20) was explored. However, this technique too appeared fraught with pitfalls. In addition to decomposition products II and III, which would interfere with the assay if present, two additional ones, IV and V, also could be present and would give rise to interference. In neutral ethanolic solutions, UV spectra of the four degradation products were found to exhibit $\lambda_{max.}$ 236 mμ while phenylbutazone showed $\lambda_{max.}$ 247 mμ. In basic solution the latter was found to undergo bathochromic and hyperchromic shifts to 264 mμ[4] while the decomposition products suffered hypsochromic and hypochromic shifts to 232 mμ (Table II). Nevertheless, these artifacts still showed absorbance at 264 mμ.

Assay of phenylbutazone in the presence of decomposition products can be expected to indicate slightly higher than actual values for the drug if only direct spectrophotometric measurement at 264 mμ is employed. Data in Table III indicate that near 100% phenylbutazone, the error introduced by decomposition products will be small, but as the concentration levels of the decomposition products increase the deviation becomes highly significant. For instance

[2] The term alka is used to designate those preparations containing phenylbutazone formulated with aluminum hydroxide and magnesium carbonate or magnesium oxide.

[3] See Footnote 1, p. 1952.

[4] This maximum also was observed with neutral aqueous solutions and the 255 mμ maximum reported by other workers (16) could not be reproduced.

TABLE II—MOLAR ABSORPTIVITY VALUES OF PHENYLBUTAZONE AND ITS DECOMPOSITION PRODUCTS

Compound	Neutral Solution $\lambda_{max.}$ mμ	$\epsilon_{max.}$	0.01 *N* NaOH $\lambda_{max.}$ mμ	$\epsilon_{max.}$	0.1 *N* NaOH • 264 mμ
I	247	15,360	264	20,925	20,925
II	236	18,810	232	15,425	3,550
III	236	19,000	232	16,550	3,915
IV	236	17,385	232	16,400	3,835
V	236	18,455	236	14,890	3,080

a decomposed sample of phenylbutazone assaying 92–93% active ingredient will appear to be at lower pharmacopeial limits but, in fact, will be subpotent. This discrepancy becomes still more significant when analysis of phenylbutazone by measuring the absorbance at 264 mμ is utilized in accelerated stability and shelf-life studies. Data based on highly decomposed samples analyzed in this way will indicate that the drug is much more stable than is actually the case.

Initial investigation indicated that the 264- and 232-mμ absorbances of both phenylbutazone and the decomposition products obeyed Beer's law. As expected, addition of known amounts of decomposition products to a standardized phenylbutazone solution exhibited a direct additive effect on absorbancy at these wavelengths. Thus it appeared that simultaneous equations could be applied to the calculation of phenylbutazone in the presence of decomposition products. Values for standard phenylbutazone and decomposition product would be constant in this, while the variables would be the observed absorbance value at 264 and 232 mμ for the sample solution. Variations in the standard would be compensated for by altering these constants in calculations.

Table II indicates the molar absorptivity (ϵ) of decomposition products II to IV. *n*-Caproylhydrazobenzene (V) has a lower value, but is eliminated during the analytical procedure. As this amide is a very minor decomposition product, no appreciable error is introduced in doing this. Variation in composition with accompanying changes in ϵ values at 264 mμ between the decomposition products could introduce differences of up to 0.05% at this wavelength, and of 0.2% at 232 mμ for a sample containing 97% phenylbutazone and 3% of only the highest (III) as compared to the lowest absorbing decomposition product (II). In a 10% decomposed sample this comparison would show up as 0.185 and 0.93% differences, respectively, for determinations at the two wavelengths. Since TLC work (see below) has shown that decomposed samples seldom contain only one decomposition product, the error introduced by variation in molecular extinction values will be considerably less than the figures quoted. To virtually eliminate the possibility of introducing such errors, an intermediate arbitrary value of ϵ that is 15,950 at 232mμ (a 48.4) and 3,700 at 264 mμ (a 11.07) for the contribution due to decomposition products was chosen. This reflects the fact that in decomposed phenylbutazone samples the carboxylic acid II is usually present in quantities comparable to the α-hydroxy analog III together with considerably smaller amounts of product IV.

Phenylbutazone solutions containing known amounts of decomposition products were assayed by UV absorptivity at 264 and 232 mμ. Table III tabulates single analysis data obtained by direct measurement at 264 mμ only, and results found by inserting spectrophotometer readings obtained at both wavelengths in the simultaneous equation and calculating the amount of phenylbutazone (see *Experimental*). The results indicate that values based on the one wavelength invariably are high.

One of the major shortcomings of the BP and NF methods was the problem of isolating all the phenylbutazone present in the sample. More than the specified volume of solvent often was required to effect complete isolation of the drug. Continuous extraction in a continuous extraction apparatus also was effective for regular phenylbutazone tablets. However, the drug could not be completely isolated from formulations containing aluminum hydroxide with this procedure (25). The solution appeared to lie in breaking up drug-excipient complexes. This could be accomplished by dissolving the sample in 0.1 *N* acid or base. Subsequent isolation of drug by solvent extraction indicated that the procedure afforded quantitative recovery.

Extraction was desirable from the point of view that it was a means of removing interfering dyes and other medicaments from the phenylbutazone formulation. By dissolving the specimen being assayed in 0.1 *N* base and extracting the resultant solution with chloroform, chloroform-soluble dyes, alkaloids, steroids, and other neutral and basic components could be removed from the formulation. The aqueous phase could then be made acidic and phenylbutazone and decomposition products recovered, by extracting the acidic solution with $CHCl_3$ followed by evaporation of the organic solvent. Water-

TABLE III—RECOVERIES OF PHENYLBUTAZONE IN PRESENCE OF DECOMPOSITION PRODUCTS[a]

Theoretical Content, mg.	Simultaneous Equation Found, mg.	Simultaneous Equation Recovery, %	264 mμ Peak Measurement Found, mg.	264 mμ Peak Measurement Recovery, %
100.00	100.00	100.00	100.00	100.00
88.88	88.37	99.42	90.23	101.52
87.50	88.34	100.96	90.52	103.45
85.71	85.58	99.85	87.44	102.02
80.00	78.27	97.84	81.52	101.90
77.77	76.61	98.51	79.96	102.81
72.72	70.91	97.51	75.21	103.42
70.00	68.55	97.93	73.03	104.33
66.66	65.75	98.63	71.04	106.57

[a] To demonstrate the validity of the simultaneous equation, Compound II was used as the decomposition product.

TABLE IV—COMPOUND R_f VALUES (×100) OF PHENYLBUTAZONE AND RELATED COMPOUNDS

Compound	R_f
I	49
II	18
III	13
IV	25
V	39
N-(α-Hydroxycaproyl)-hydrazobenzene	35
Oxyphenbutazone	20
Hydroazobenzene	75

soluble dyes and quaternary alkaloidal material remain in the aqueous phase. The thus-recovered drug and related compounds would then be determined by UV spectrophotometry in 0.1 *N* NaOH. Application of this procedure was found to give complete recovery of drug and decomposition products concomitant with removal of the interfering substances. The procedure, too, was effective in removing traces of the seldom-encountered *n*-caproylhydrazobenzene (V) decomposition product.

For routine use the initial (NaOH solution) stage of the extraction procedure often can be by-passed without affecting recovery, accuracy, or precision. If the sample contains no known neutral medicaments, is capsule material, or is tablet material whose dye is not $CHCl_3$ soluble (a small amount of powdered tablet in a test tube with $CHCl_3$ readily indicates this), simple sample dispersion in 0.1 *N* HCl followed by $CHCl_3$ extraction affords recovery of all phenylbutazone and decomposition products present. The final stage of the determination is then performed in a manner identical to the two-stage shake-out, as described in the *Experimental* section.

Alka formulations containing no medicaments other than phenylbutazone (and aluminum hydroxide gel) or alkali-soluble dyes can be assayed directly at 264 and 232 mμ after dissolving and diluting the samples in 0.1 *N* NaOH.

Table I gives the results obtained for commercial and simulated phenylbutazone formulations. Both peak height measurement and simultaneous equation values are given. The data show that although most preparations contain a "reasonable" amount of phenylbutazone (*i.e.*, at least 92.5% of the labeled amount) formulations may contain surprisingly large amounts of decomposition products.

Investigation showed that cyclohexane–chloroform–methanol–acetic acid (60:30:5:5) on Silica Gel GF was an ideal system for resolving and semiquantitatively estimating phenylbutazone (I), its decomposition products II to V, and hydrazobenzene, if present. Table IV lists the R_f values obtained for phenylbutazone (I) decomposition products II to V, and a fifth degradation product shown as *N*-(α-hydroxycaproyl)-hydrazobenzene. In addition, R_f values for hydrazobenzene, a potential oxidation product, oxyphenbutazone (4-*n*-butyl-2 - (4 - hydroxyphenyl) - 1 - phenylpyrazolidine-3,5-dione), and diethyl-*n*-butylamatonate, a synthesis intermediate, are included although none of these were detected in the samples examined.

Viewing plates under short-wavelength UV light indicates immediately the presence of phenylbutazone and most of the decomposition products in the sample. Chlorine/*o*-toluidine gives blue- to violet-colored spots with II, III, and V, with a sensitivity level well below 1 mcg. Phenylbutazone stains yellow while 4-hydroxyphenylbutazone (IV) gives only a weak color reaction even at very high concentrations. FCR affords colors with all of the compounds. Although it is not as sensitive as chlorine/*o*-toluidine for Compounds II and III, requires time for some spots to develop, and gives halos around spots often making quantitation difficult, FCR is useful in indicating the presence of components which could be overlooked by both UV light and chlorine/*o*-toluidine. Estimation of 4-hydroxyphenylbutazone (IV) is facilitated by FCR. This visualizer also shows that a seemingly minor (by UV and chlorine/*o*-toluidine) decomposition product appearing at R_f 0.35 in some samples is present in significant quantities.

Calibration experiments indicated that the level of decomposition products, II to V, could be determined with an accuracy of 3% by the semiquantitative procedures employed. To assure that decomposition was not occurring in solution during TLC analysis, duplicate solutions of phenylbutazone tablets, alka formulations, and bulk drug in the spotting solvent were stored in a refrigerator and at

TABLE V—DECOMPOSITION PRODUCT CONTENT OF PREPARATIONS CONTAINING $Al(OH)_3$

Formulation	TLC Method % Decomposition Product II	III	IV	V	[a]	Total	Simultaneous Equation Value for Total % Decomposition Present
A (contains alkaloids)	2	2	—	—	—	4	3.88
B (contains steroid and alkaloid)	2	2	—	—	+	4	6.70
B (contains steroid and alkaloid)[b]	15	6	—	—	+	21	28.06
C	1	1	<1	2	—	4	0.98
C[b]	1	1	<1	1	—	3	2.95
D (contains alkaloids)	< 1	<1	1	3	+	4	7.51
D (contains alkaloids)[b]	7	3	6	1	+	17	24.61
E	1	<1	2	—		3	2.99
F[b]	4	<1	1	7	—	11	10.20

[a] + indicates presence of *N*-(α-hydroxycaproyl)-hydrazobenzene. No quantitation performed on this compound due to lack of sufficient standard. [b] Sample at least 5 years old.

room temperature. Refrigerated solutions showed no decomposition of phenylbutazone after 24 hr. while those kept at room temperature showed some deterioration. Phenylbutazone samples spotted on TLC plates were found to be stable. "Half-developing" a chromatoplate, drying it in a warm air stream, and then rechromatographing produced no detectable shadow or decomposition spots.

Thirty-three brands of phenylbutazone tablets were examined with the TLC system. Thirty-two of these (labeled BP, NF, USP, and many without compendial designations) including 13 which were at least 5 years old exhibited only trace amounts (well under 0.5%) of decomposition products, II to V, relative to phenylbutazone. One of them contained about 6% decomposition products. In no case did the dyes present in tablet coatings interfere with the TLC method. Four samples of bulk drug, including one which was 10 years old, also were analyzed by TLC. One of them was completely free of decomposition products while the three others contained trace amounts totaling well under 0.5%.

All six phenylbutazone–aluminum hydroxide formulations examined (encompassing nine samples) contained decomposition products. In some cases the quantities could be described as high. Table V lists the levels of degradation products determined by semiquantitative TLC and compares these with the data found by the analytical method (based on the value of Y from the simultaneous equations). Agreement between the two methods is satisfactory. Where differences arise, these underline the specificity of the analytical method. Samples B, B^b, D, and D^b give lower results by TLC since the values quoted do not include the quantity of decomposition product appearing at R_f 0.35.

The presence of large amounts of decomposition products of phenylbutazone in some alka preparations together with acceptable levels of the drug appears to indicate that the manufacturers of these products include an overage at time of formulation, and that the drug in the formulation is unstable. Alternatively, the bulk drug utilized could have contained significant quantities of degradation products to begin with, or were generated during manufacturing processes. Since normal phenylbutazone formulations (*i.e.*, no alkaline compound ingredients) labeled as conforming to compendial monographs contain no significant amounts of decomposition products, one can assume that the former alternative explains the analyses of the so-called alka formulations.

The proposed method of phenylbutazone analysis has been found to be effective for the accurate analysis of the drug in the presence of decomposition products, other analgesics, antacid agents, steroids, alkaloids, and quaternary alkaloids. It also should be effective in analyzing the medicament when it is present in salt complexes containing *p*-chlorophenylether moieties (26). This procedure should be of value in more precise drug quality control and shelf-life testing.

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Keyphrases

Phenylbutazone tablets—analysis
Acid-base extraction—phenylbutazone
UV spectrophotometry—analysis
TLC—separation, identity
Decomposition products—phenylbutazone analysis

Tumor Localizing Agents VIII

Radioiodinated Phenylalanine Analogs

By R. E. COUNSELL, T. D. SMITH, W. DIGUILIO, and W. H. BEIERWALTES

Use of the currently available *l*-selenomethionine-^{75}Se for external pancreatic photoscanning is limited by the long biological half-life of the amino acid and the dense radiations arising from high liver concentrations of the compound. The present study was initiated in an effort to find superior pancreas-specific photoscanning agents. The approach presented here is based upon the previously established specificity of natural amino acids for pancreatic tissue. In the anticipation that this specificity might extend to other radiolabeled synthetic amino acids, *ortho-*, *meta-*, and *para*-iodophenylalanine-^{125}I and the corresponding acetylated derivatives were synthesized for tissue distribution analysis. All three iodophenylalanine-^{126}I isomers showed a specificity for pancreatic tissue in mice but not in dogs. Acetylation of the radioiodinated amino acids caused no qualitative changes in the tissue distribution results in either species.

FOR SEVERAL YEARS a research program has been directed toward the investigation of new radioscanning agents for selected tumors (1–5). A recognition of the current problems associated with presently available pancreatic radioscanning agents prompted the authors to synthesize and evaluate a series of radioiodinated phenylalanine derivatives for this purpose.

The lack of suitable diagnostic agents for pancreatic carcinoma was emphasized in a recent statistical, clinical, and pathological survey of the disease (6). It is the most difficult abdominal tumor to diagnose, and clinically correct diagnoses occur in only about 30% of autopsied cases. The prognosis of pancreatic carcinoma is poor mainly because existing diagnostic procedures confirm the pathological condition too late for proper treatment.

Radiolabeled zinc (^{65}Zn) compounds were used in previous attempts to design pancreas-specific radioscanning agents (7). Zinc-65 was expected to exhibit a predilection for pancreatic tissue because zinc is a common constituent of insulin. Pancreatic concentrations of the radionuclide, however, were insufficient to be of clinical value.

The demonstration that radiolabeled, natural amino acids concentrate in the pancreas shortly after intravenous administration (8, 9) initiated efforts to utilize this selectivity for the radiodiagnosis of pancreatic disorders. The elements (C, H, N, O, and S) present in natural amino acids, however, do not possess gamma-emitting radionuclides suitable for external *in vivo* scanning purposes. Consequently, it was necessary to examine synthetic amino acids for pancreatic selectivity. Reports have indicated that externally labeled synthetic amino acids such as 3-iodotyrosine-^{131}I (10, 11) are not selectively taken up by the pancreas, presumably because these agents take no part in protein synthesis. On the other hand, Blau and Manske (10) demonstrated that *l*-selenomethionine-^{75}Se possessed biological properties very close to those of methionine and that it did, indeed, show a predilection for the pancreas. Selenium-75 also possessed the necessary gamma emissions suitable for external photoscanning.

Subsequent clinical evaluations (11–26) of *l*-selenomethionine-^{75}Se resulted in mixed conclusions about its value as a pancreatic scanning agent. Certain problems such as high concentrations of radioactivity in the liver, patient variations in pancreas size and configuration, the distribution of radioactivity, and the long biological half-life of *l*-selenomethionine-^{75}Se were uniformly encountered.

Published data indicating the pancreatic specificity of other synthetic amino acids, such as 1-aminocyclopentane-^{14}C-carboxylic acid (27–29) and *p*-fluorophenylalanine-^{14}C (30, 31), suggested the feasibility of utilizing other radionuclides incorporated into synthetic amino acids. The suitability of iodine-125 and iodine-131 for external photoscanning and the reported pancreatic selectivity of phenylalanine-^{14}C (9) suggested the synthesis of a series of radioiodinated phenylalanines for tissue distribution studies.

The *ortho-*, *meta-*, and *para*-iodophenylalanines were prepared by the alkylation of diethyl acetamidomalonate and subsequent acid hydrolysis and decarboxylation according to the method of Redemann and Dunn (32) (Scheme I). All

Received May 22, 1968, from the Laboratory of Medicinal Chemistry, College of Pharmacy and the Department of Internal Medicine (Nuclear Medicine), University of Michigan, Ann Arbor, MI 48104

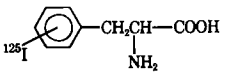
Accepted for publication August 1, 1968.

Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

This work was supported by training grant GM 1367-03 and grants CA-08349-02 and CA 08429-02 from the National Institutes of Health, Bethesda, Md. and award PRA-18 from the American Cancer Society, New York, N. Y.

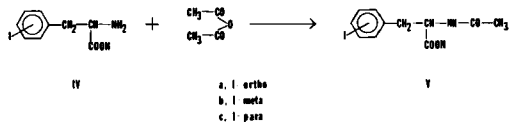
Part VII of this series, R. E. Counsell, P. Pocha, V. V. Ranade, J. Sterngold, and W. H. Beierwaltes, in preparation.

TABLE I—TISSUE DISTRIBUTION IN MICE 4 hr. AFTER INJECTION[a] (c.p.m./mg. TISSUE)



^{125}I	Pancreas	Liver	Kidney	Melanoma	Eyes
ortho	3823	411	622	807	646
meta	1192	196	396	244	501
para	496	91	205	132	114

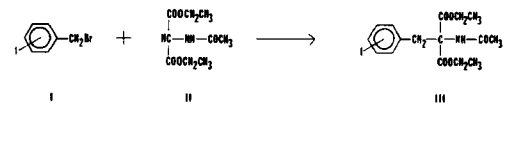
[a] Dose, 10 μc./mouse.



Scheme I—Syntheses of iodophenylalanines.

three iodophenylalanine isomers (33–36) have been reported previously but were inadequately characterized.

Radioiodinated derivatives were prepared by halogen exchange of the appropriate iodophenylalanine with $Na^{125}I$ in aqueous acetic acid. Initial attempts to carry out the exchange reaction in this solvent resulted in partial *N*-acetylation of the iodophenylalanines. Similar reactions of amino acids with acetic acid have been reported (37). The acetylated derivatives were isolated by ion-exchange chromatography during model exchange reactions and their structures elucidated. In addition, the *N*-acetyl iodophenylalanines were synthesized according to a U. S. patent (38) (Scheme II) and found to agree in all respects with the products isolated above.



Scheme II—Syntheses of N-acetyl iodophenylalanines

Tissue Distribution Studies—The preliminary studies to determine the biological distribution of *ortho-*, *meta-*, and *para*-iodophenylalanine-^{125}I in mice (Table I) indicated that all three isomers displayed a predilection for pancreatic tissue. Moreover, *para*-iodophenylalanine exhibited pancreatic specificity in mice when administered either intraperitoneally or intracardially. In contrast to these results, one previous study (39) reported an insufficient concentration of *para*-iodophenylalanine in the rat pancreas to be useful as a radiopaque media. In that report the presence of *para*-iodophenylalanine in pancreatic tissue was based upon a chemical analysis for protein-bound iodine. At the time of this writing, however, Ullberg and Blomquist (40) showed by autoradiography that mouse pancreas selectively concentrated *para*-iodophenylalanine-^{125}I in good agreement with the authors' own data. The radioiodinated *N*-acetyl amino acid analogs showed a similar predilection for pancreatic tissue at early time periods following administration.

In contrast to the pancreatic selectivity of the radioiodinated amino acids in mice, none of the three iodophenylalanine-^{125}I isomers showed any selective localization in the pancreas of dogs (Table II). Studies utilizing the *N*-acetyl derivative of *meta*-iodophenylalanine-^{125}I in dogs indicated that acetylation caused no significant change in tissue distribution.

Similar species differences between mice and dogs in the pancreatic localization of synthetic amino acids have been reported previously. For example *dl*-1-aminocyclopentane-^{14}C-carboxylic acid has been shown to exhibit a pancreatic selectivity in mice but not in dogs (29). Studies are now in progress to resolve the *dl*-amino acids prepared in this investigation to see if the *l*-isomers show a similar species variation in distribution.

EXPERIMENTAL[1]

Syntheses of Isomeric Iodophenylalanines—Sodium metal (*n* g. atoms) was dissolved in absolute ethanol[2] (8 *n* l.). Dry solid diethyl acetamidomalonate (*n* mole) was added and the stirred reaction mixture refluxed for 0.5 hr. The formation of the sodiomalonate was evidenced by turbidity. Addition of the appropriate iodobenzyl bromide[3] (*n* mole)

[1] Melting points were taken on a Fisher-Johns melting point apparatus and are corrected. NMR spectra were obtained with a Varian A-60A spectrometer using 10% solutions and tetramethylsilane as internal reference. Elemental analyses were performed by Spang Microanalytical Laboratories, Ann Arbor, Mich. Thin-layer chromatograms were run using 1-in. Eastman Chromatogram strips, type K301R with fluorescence indicator, and spots detected with UV light. Chromatograms of radioiodinated compounds were scanned with an Atomic Associates RCS-363 radiochromatogram scanner.

[2] Dry ethanol was prepared by the magnesium ethoxide method reported in Vogel, A. I., "Elementary Practical Organic Chemistry Part I: Small Scale Preparations," 2nd ed., Wiley, New York, N. Y., 1966, p. 158.

[3] Prepared according to Weizmann, M., and Patai, S., *J. Am. Chem. Soc.*, **68**, 150(1946).

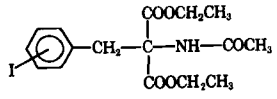
TABLE II—TISSUE DISTRIBUTION OF IODOPHENYLALANINES IN DOGS 1.5 hr. AFTER INTRAVENOUS ADMINISTRATION[a] (c.p.m./mg. TISSUE)

^{125}I-Compd.	Pancreas	Liver	Blood	Parathyroid	Thyroid	Adrenal	Kidney
o-Iodophenylalanine	1.5	1.5	7.2	0.8	1.0	1.5	2.7
m-Iodophenylalanine	1.9	3.1	10.7	1.7	3.4	1.9	4.3
p-Iodophenylalanine	3.4	4.6	11.1	1.6	2.9	3.5	11.1
N-Acetyl-*m*-iodophenylalanine	1.0	2.9	6.8	1.5	1.8	1.4	5.3

[a] Dose, 4 μc./kg.

was followed by a 4-hr. reflux period. Most of the alcohol was removed under reduced pressure and the reaction mixture diluted with water. Ether extraction afforded the crude alkylated diethyl-acetamidomalonate. A small fraction of the ether extract was dried ($MgSO_4$, charcoal) and the crude residue remaining after removal of the ether was recrystallized from ethanol–water[4] and characterized (see Table III).

TABLE III—INTERMEDIATE ACETAMIDOMALONATES



I	M.p., °C.	Found, %[a] C	H
ortho	93.8–94.5	44.40	4.57
meta	83–84.5	44.53	4.59
para	132–135	44.46	4.74

[a] *Anal.*—Calcd. for $C_{16}H_{20}INO_5$: C, 44.36; H, 4.65.

The NMR spectra were consistent in all cases with the assigned structures. The spectra of all three isomers were identical except for the benzylic protons and the aromatic region. The benzylic protons of the *ortho* isomer (3.82 δ) were shifted downfield by 14 c.p.s. with respect to the corresponding protons of the *meta* and *para* isomers (3.58 δ). Such downfield shifts are typical of *ortho*-substituted derivatives (3, 41). The aromatic resonance patterns were in good agreement with those reported for the iodotoluene isomers (41).

The remaining ether extract was evaporated to dryness yielding a crude oily residue. This residue, without further purification was refluxed with 48% HBr (3 *n* l.) for 8 hr. or until complete solution was effected. The solution was evaporated to dryness, diluted with a small amount of water, and brought to the isoelectric point[5] with 28% ammonium hydroxide. The flask was allowed to stand overnight; the solid iodophenylalanine was collected by filtration and recrystallized from ethanol–water (see Table IV). The NMR spectra of the pure iodophenylalanines taken as 10% solutions in trifluoroacetic acid were in good agreement with the reported spectra of phenylalanine taken in the same solvent (42).

Syntheses of Isomeric *N*-Acetyl Iodophenylalanines—The appropriate iodophenylalanine (200 mg.) was suspended in 25% acetic acid (2 ml.) and three 0.1-ml. portions of acetic anhydride added to the vigorously stirred suspension at 10-min. intervals. The reaction temperature was maintained at 40°. The subsequent addition of 10% HCl (2 ml.) was followed by continued stirring for 30 min. The white solid product which formed was collected by filtration and recrystallized from ethanol–water (see Table V).

^{125}I Exchange of Iodophenylalanines—The iodophenylalanine (100 mg.) was dissolved in acetic acid (0.5 ml.) and water (3 ml.) containing $Na^{125}I$ (~2 mc.). The mixture was magnetically stirred at 110° (external temperature) in a nitrogen atmosphere for 24 hr. The solvent was removed on a rotary evaporator and water (~2 ml.) was added to suspend the solid residue and wash out residual $Na^{125}I$. The solid was collected by filtration, washed with water (~2 ml.) and recrystallized from ethanol–water. Chemical purity of the exchanged product was verified by TLC (*n*-BuOH–H_2O–HOAc, 60:20:20) and IR spectra (KBr) comparisons with authentic material. Radiochemical purity was confirmed by scanning thin-layer radiochromatograms. The product was taken up in 0.1 *N* HCl for specific activity determinations[6] and animal administration.

Isomer	Recovery, mg.	Sp. Act. μc./mg.	% Exchange
ortho	36.2	16	31
meta	45.2	16.8	25
para	39.8	12.3	22

^{125}I Exchange of *N*-Acetyl Iodophenylalanines—The *N*-acetyl iodophenylalanine (100 mg.) was dissolved in acetic acid (2 ml.) and water (1 ml.) containing $Na^{125}I$ (~2 mc.). The solution was magnetically stirred and heated to 110° (external temperature) in a nitrogen atmosphere for 24 hr. At the end of the heating period, the solvent was removed *in vacuo*. The solid residue was collected by filtration, washed with water (~3 ml.), and recrystallized from ethanol–water. The final exchange product was identical with known material by melting point, IR spectra, and TLC analysis (*n*-BuOH–H_2O–HOAc, 60:20:20). Radiochromatogram scans of developed TLC strips indicated a radiochemically pure product. The final product was dissolved in ethanol (2 ml.) and the specific activity determined.[6]

Isomer[7]	Recovery, mg.	Sp. Act. μc./mg.	% Exchange
ortho	63.5	15.1	53
meta	57.5	15.4	49

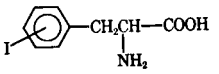
[4] The first crystallization was difficult to achieve. The products tend to form an initial oil which solidifies upon standing.

[5] The solution is titrated to the point of turbidity with NH_4OH (to about pH 7). If excess NH_4OH is accidentally added, the isoelectric point can be reached by evaporation of excess ammonia on a rotary evaporator.

[6] Samples used for specific activity determinations were counted on a Beckman ambient temperature liquid scintillation system model 200 in a dioxane cocktail composed of naphthalene, 100 g.; PPO (2,5-diphenyloxazole), 7 g.; and dioxane, a quantity sufficient to make 1,000 ml.

[7] The *para* isomer was not exchanged.

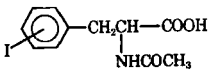
TABLE IV—IODOPHENYLALANINES



I	n	M.p.,[a] °C.	Yield, %[b]	Found,[c] % C	H
ortho	0.013	230–234	30	37.41	3.56
meta	0.009	213.5–216.5	55	37.43	3.58
para	0.017	223–226	68	37.27	3.66

[a] Melting points of amino acids are dependent upon the method of determination (43). No special precautions were used in these determinations. [b] Yields are reported as the overall yield of pure product based upon the appropriate iodobenzyl bromide. [c] *Anal.*—Calcd. for $C_9H_{10}INO_2$: C, 37.14; H, 3.46.

TABLE V—*N*-ACETYL IODOPHENYLALANINES



I	M.p., °C.	Yield, %	Found, %[a] C	H
ortho	185–187.5	31	39.97	3.71
meta	172–174.5	69	39.84	3.65
para	190–193.5	19	39.65	3.65

[a] *Anal.*—Calcd. for $C_{11}H_{12}IO_3$: C, 39.66; H, 3.63.

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Keyphrases

Tumor localizing agents
Phenylalanine analogs, radioiodinated—synthesis
Radioiodinated phenylalanines—tissue distribution
NMR spectroscopy—structure
Radiochromatography—purity

Androst-4-ene-3,17-dione-4-^{14}C Metabolism by the Homogenates of the Chicken Uropygial Gland I

Antibiotic Concentrations Necessary for Inhibition of Bacterial Growth

By EDWARD L. RONGONE and FRANK M. FERRARO

Supernatant fluid obtained from homogenates of chicken uropygial gland was incubated with androst-4-ene-3,17-dione-4-^{14}C in the presence of penicillin and streptomycin. Two metabolic products were isolated and identified by using preliminary thin-layer chromatography followed by gas chromatography of the free metabolites, trimethylsilyl ethers, *o*-methyloxime derivatives, and double derivatives on 1 percent SE-30 and 1 percent neopentyl glycol succinate (NGS) columns. Crystallization to constant specific activity was also used for the identification of the metabolites. The metabolites identified were 3α-hydroxy-5α-androstane-17-one and 5α-androstane-3α,17β-diol. The isolated organisms from the supernatant fluid were highly susceptible to the activity of the antibiotics added to the incubation mixture for metabolic activity. The minimal concentrations of the antibiotics required to inhibit bacterial growth under the experimental conditions of this investigation were determined.

THE PRESENCE of microorganisms in incubation preparations for metabolic activity is always of concern to investigators. Unless sterile conditions are used throughout the incubation procedure, the only alternative is to add excessive amounts of antibiotics. Since it has been previously demonstrated that the supernatant fluid obtained from homogenates of chicken uropygial gland is capable of oxidizing the C-17 hydroxyl group of testosterone (1), it is of interest to determine whether the supernatant fluid is also capable of reducing a C-17 carbonyl group. The present investigation concerns the demonstration of a 17β-hydrogenase in the supernatant fluid obtained from homogenates of chicken uropygial gland, isolation of bacteria from the incubating medium prior to the addition of antibiotics, and the determination of the minimal amounts of penicillin and streptomycin needed to prevent bacterial growth under the incubation conditions.

EXPERIMENTAL

Purification of Androst-4-ene-3,17-dione-4-^{14}C—Androst-4-ene-3,17-dione-4-^{14}C was purchased from Nuclear-Chicago Corp. and purified by TLC on Silica Gel G in a chloroform–ethanol system, 49:1. The resolved androst-4-ene-3,17-dione-4-^{14}C was eluted from the silica gel with absolute methanol and transferred to a 3-ml. centrifuge tube. One milliliter of benzene was added to the centrifuge tube and the radioactive compound then stored at 3° until used in the incubation procedures.

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Accepted for publication August 13, 1968.

This investigation was supported by grant 1-RO1-AM-09197 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md. One of the authors (E. L. R.) is a Research Career Development Awardee of the National Institute of Arthritis and Metabolic Diseases (K3-AM-25401).

Gas Chromatography—A Beckman model GC4 gas chromatograph equipped with a flame-ionization detector and glass columns 1.83 m. (6 ft.) long were used. The glass columns were packed with either 1% NGS (neopentyl glycol succinate) or 1% SE-30 (silicone rubber) on 100/120 mesh siliconized Gas-Chrom P (Applied Science Laboratories, State College, Pa.). Conditions employed were: column temperature, 210°; injector temperature, 240°; detector temperature, 230°; N_2 input flow, 40 ml./min.; O_2 output flow, 200 ml./min.; H_2 output flow, 50 ml./min. The recorder was a 25.4-cm. (10-in.) Beckman potentiometer set at 1 mv. and a speed of 0.5 in./min. Gas-Chrom P was prepared and coated with NGS and SE-30 according to the technique of Horning *et al.* (2).

Incubation Medium—The uropygial gland was removed immediately after decapitation (3 lb. cockerels or hens) and freed of fat, skin, connective tissue, sebum, and the uropygial duct. The remaining gland was homogenized in cold 0.10 *M* phosphate buffer, pH 7.2 (1 g. of trimmed gland/10 ml. of buffer) and the homogenate centrifuged at 5,000 r.p.m. at 0° for 10 min. After the centrifugation, the cellular debris and the fatty layer were carefully removed and the remaining supernatant fluid was decanted into a cold flask. This supernatant consisted of mitochondria, microsomal, and phosphate-soluble material of the chicken uropygial gland. It was used immediately for the metabolic investigations. The only aseptic conditions used during this investigation were during the removal of the uropygial gland and the use of sterile phosphate buffer for homogenization of the gland.

Incubation—Eleven milliliters of the supernatant fluid was used per flask. Thirty micrograms of androst-4-ene-3,17-dione-4-^{14}C, (200,000 c.p.m.) and 2 mg. each of triphosphopyridine nucleotide (TPN), citrate, and isocitrate were added to the supernatant fluid. The pH was readjusted to 7.2 and 1 ml. was removed and examined for bacteria. Penicillin (100,000 units) and streptomycin (166 mg.) were then added to the remainder and this mixture was incubated in a Warner-Chilcott

model 2156 water bath at 37° for 24 hr. Control mixtures lacking supernatant fluid or androst-4-ene-3,17-dione-4-^{14}C or antibiotics were prepared and incubated simultaneously. Experimental tubes were prepared either in duplicate or triplicate. After incubation, all tubes were checked for the presence of bacteria. Identification of the steroid metabolites was made only on tubes which were free of bacteria. The purity of the substrate, androst-4-ene-3,17-dione-4-^{14}C, was rechecked immediately before its addition to the incubation medium by TLC on silica gel as previously described. The radiochromatogram scan of the radioactive substrate demonstrated only one radioactive peak, thereby indicating that the substrate was chemically pure.

Extraction and Identification of Steroid Metabolites—The materials and methods for the extraction and identification of the steroid metabolites are standard procedures which have been previously reported (1, 3). Procedures used for the identification of the steroid metabolites were TLC, GLC, and crystallization to constant specific activity.

Isolation of Bacteria—An aliquot of the supernatant fluid, used as the enzyme source, was examined for the presence of bacteria by flooding 1 ml. on the surface of a blood agar plate. The plate was incubated at 37° for 24 hr. The large number of colonies present could be divided on the basis of colonial morphology into four distinct types. Samples of each different colony type were isolated and transferred to brain heart infusion for identification.

Enumeration of Bacterial Populations—A series of dilutions of the supernatant fluid, ranging from 10^1 to 10^7, were prepared through a 10-fold sequence. One-milliliter portions of the dilutions were flooded on the surface of agar plates prepared as described by Hentges (4) and incubated at 37° for 24 hr. Bacterial colonies that developed on the agar were counted with an electronic colony counter.

The population sizes of a 24-hr. broth suspension of each isolated organism were determined by the same technique. Since the suspensions presented counts considerably higher than those obtained from the supernatant fluid, each was diluted with sterile saline so that the number obtained by plate counts was comparable to that previously determined for the supernatant fluid. These concentrations of organisms were used for antibiotic sensitivity studies.

Determination of Survival Time of Isolated Bacteria in Antibiotic Broth—Ten milliliters of each suspension of organisms received 100,000 units of penicillin and 166 mg. of streptomycin. These concentrations correspond with the experimental conditions used in the initial work of Rongone *et al.* (1). The suspensions were incubated at 37° in a water bath shaker, and at 0.5-hr. intervals 1-ml. samples were removed and flooded on the surface of dried agar plates which were incubated at 37° for 24 hr.

Antibiotic Susceptibility of Isolated Bacteria—*Test Tube Serial Dilution Technique*—The technique as described in Bailey and Scott (5) was used with final antibiotic concentrations of 250, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.2, and <0.2 units or mcg./ml. of broth culture medium. Each tube was inoculated with the isolated bacteria and the series incubated at 37° for 18–24 hr. Tubes were examined macroscopically for evidence of turbidity and/or sediment. The lowest concentration of the antimicrobial agent which prevented growth was considered as the minimal inhibitory concentration (MIC) and expressed as units or mcg./ml.

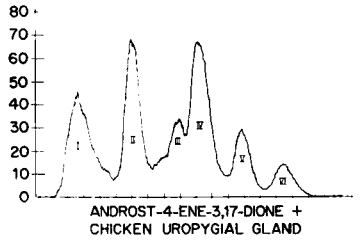

Fig. 1—A radiochromatogram scan of a residue obtained from an incubation mixture from cockerel glands including penicillin and streptomycin, phosphate buffer, and androst-4-ene-3,17-dione.

RESULTS AND DISCUSSION

Steroid Metabolism—Analysis of control mixtures, which were devoid of the enzyme preparation, by spectrophotometric procedures at 240 mμ, resulted in recovery of 96–98% of added androst-4-ene-3,17-dione-4-^{14}C. UV analysis at 240 mμ of the residue obtained from the incubation medium containing substrate, cofactors, and enzyme preparation demonstrated that 65% of the conjugated system in Ring A was destroyed during the incubation procedure. This compared favorably with the metabolism of testosterone (3) by the homogenates of the chicken uropygial gland suggesting that the compounds are metabolized at a similar rate. Although maximum metabolism of steroids by the supernatant fluid obtained from the homogenates of the chicken uropygial gland under the present conditions appeared to require from 6–8 hr., as indicated by spectrophotometric analysis, 24 hr. was chosen as a matter of convenience and to insure that metabolic reactions would proceed to their last product in the isolated pathways. Only the C-19 steroids identified which possessed radioactivity were considered to be metabolites. Figure 1 demonstrates a radiochromatogram scan of a residue obtained from an incubation mixture containing the supernatant fluid, penicillin, streptomycin, phosphate buffer, and androst-4-ene-3,17-dione-4-^{14}C on a thin-layer plate. The numbered peaks represent the different radioactive products present in the residue. The numbered peaks in this figure correspond to the same Roman numerals in Figs. 2–4. Table I shows the R_f values of the radioactive peaks

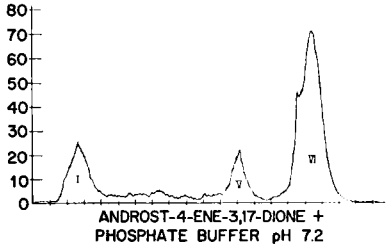

Fig. 2—A radiochromatogram scan of a residue obtained from an incubation mixture which contained phosphate buffer and androst-4-ene-3,17-dione only.

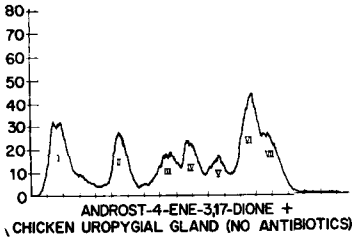

Fig. 3—A radiochromatogram scan of a residue obtained from an incubation mixture which contained cockerel glands, phosphate buffer, androst-4-ene-3,17-dione, but no antibiotics.

in Figs. 1–4. It can be seen that the R_f values of the corresponding numbered peaks are not identical in all instances. This may be readily explained in that thin-layer plate chromatography is not as reproducible as other methods of chromatography. Therefore, in order to determine whether the given numbered peaks were identical or similar to each other, the radioactive peaks were eluted from the thin-layer plates. Aliquots of corresponding numbers from each plate were mixed and rechromatographed as before. In all instances only one radioactive peak was detected demonstrating that the peaks with identical numbers were similar to each other. Figure 2 is a radiochromatogram of a control sample of androst-4-ene-3,17-dione-4-^{14}C without enzyme or antibiotics. It is obvious that there is a small amount of natural steroid breakdown which occurs during the incubation or extraction procedure since only Peak VI was present in the radiochromatogram scan of the substrate immediately prior to its addition to the incubation medium. Therefore, Peaks I and V were not considered to be enzymatic metabolites of pregnenolone. It is readily observed from Fig. 1 that three radioactive metabolites were partially resolved, two of which have been identified by gas chromatography procedures. Figure 3 demonstrates a radiochromatogram of a residue obtained from an incubation mixture from cockerels which contained no antibiotics. It is readily seen that the relative heights of the peaks in Fig. 1 differ from those of Fig. 3 indicating that bacterial enzymes or bacterial toxins may be the cause of this difference. The possibility also exists that the antibiotics used in this experiment may have been responsible for the slight difference between Figs. 1 and 3. Therefore, an incubation was performed in which the steroid substrate was incubated with phosphate buffer and

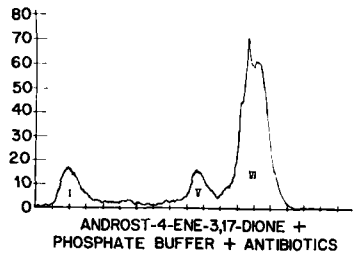

Fig. 4—A radiochromatogram scan of a residue obtained from an incubation mixture which contained androst-4-ene-3,17-dione, phosphate buffer, penicillin, and streptomycin.

TABLE I—THE R_f VALUES OF THE RADIOACTIVE PEAKS

Peak Number	Fig. 1, R_f	Fig. 2, R_f	Fig. 3, R_f	Fig. 4, R_f
I	0.15	0.10	0.11	0.11
II	0.47	—	0.35	—
III	0.66	—	0.47	—
IV	0.75	—	0.58	—
V	0.89	0.67	0.70	0.78
VI	0.97	0.91	0.90	0.95

penicillin and streptomycin (Fig. 4). Figure 4 demonstrates that under the experimental conditions, the antibiotics used *per se* had no effect on the metabolism of androst-4-ene-3,17-dione-4-^{14}C since Figs. 2 and 4 are almost identical. Fabian (6) has shown that strains of *Staphylococcus aureus* are capable of reducing the C-17 carbonyl of androst-4-ene-3,17-dione.

Table II shows the relative retention times to cholestane of authentic steroids and the radioactive metabolites which were isolated by gas chromatographic techniques on 1% NGS columns. The table demonstrates that the relative retention times of the isolated metabolites are identical or almost identical to those of the authentic steroids. Table III shows the steroid numbers of the authentic steroids and the isolated steroid metabolites calculated from data obtained by gas chromatographic techniques on 1% SE-30 columns. The table demonstrates that the steroid numbers of the isolated metabolites are identical or almost identical to those of the authentic steroids.

This table also demonstrates, without doubt, that the supernatant fluid obtained from homogenates of chicken uropygial gland is capable of converting androst-4-ene-3,17-dione into 3α-hydroxy-5α-androstane-17-one and 5α-androstane-3α, 17β-diol. Further confirmation of the identification of the two metabolites was obtained by crystallization to constant specific activity techniques.

It was previously demonstrated that the homogenates of the chicken uropygial gland are capable of oxidizing a C-17 hydroxyl to a C-17 carbonyl. This investigation demonstrated the presence of a 17β-hydrogenase. It also demonstrated that the homogenates of the uropygial gland from cockerels and hens destroyed equally well the conjugated system in Ring A of androst-4-ene-3,17-dione-4-^{14}C.

TABLE II—IDENTIFICATION OF PRODUCTS FROM REDUCTION STUDIES. RELATIVE RETENTION TIMES OF C-19 STEROIDS TO CHOLESTANE ON 1% NGS

Compd.	Androst-4-ene-3,17-dione-4-^{14}C	3α-Hydroxy-5α-androstane-17-one	5α-Androstane-3α,17β-diol
TMSi[a]			
Authentic	—	0.915	0.312
Isolated	—	0.915	0.312
O.M.O.[b]			
Authentic	2.88 3.26	2.75	—
Isolated	2.86 3.23	2.74	—
D.D.[c]			
Authentic	—	0.625	—
Isolated	—	0.625	—

[a] TMSi = Trimethylsilyl ether derivatives. [b] O.M.O. = o-methyloxime derivatives. [c] D.D. = Double derivatives of trimethylsilyl ether and o-methyloxime.

TABLE III—STEROID NUMBERS FOR COMPOUNDS IDENTIFIED FROM INCUBATION MIXTURES[a]

Compd.	Androst-4-ene-3,17-dione-4-^{14}C	3α-Hydroxy-5α-androstane-17-one	5α-Androstane-3α,17β-diol
Free			
Authentic	24.57	23.50	23.65
Isolated	24.54	23.47	23.64
TMSi			
Authentic	—	23.60	24.51
Isolated	—	23.62	24.50
O.M.O.			
Authentic	25.62	24.20	—
Isolated	25.59	24.20	—
D.D.			
Authentic	—	24.30	—
Isolated	—	24.31	—

[a] Steroid numbers are obtained from data resulting from chromatography on 1% SE-30 columns. Steroid numbers were determined by the method of Vanden Heuvel and Horning (7).

Again it is of interest to note that the only metabolites identified belong to the 5α-androstane family. This tends to suggest that the formation of the 5α-androstane series is the major route of metabolism for steroids, if not the only route, by the homogenates of the chicken uropygial gland.

Identification of Isolated Bacteria—By means of colonial morphology and microscopic examination the bacteria present in the supernatant fluid were identified as: (*a*) *Staphylococcus aureus*, (*b*) *Staphylococcus epidermidis*, (*c*) aerobic spore-forming bacilli, and (*d*) Gram-negative pleomorphic rods (morphology highly variable).

S. aureus and the spore-forming bacilli were β-hemolytic, whereas *S. epidermidis* and the Gram-negative rods were nonhemolytic. The coagulase test revealed *S. aureus* to be coagulase-positive and *S. epidermidis* coagulase-negative.

It should be noted, that aseptic conditions were used to remove the chicken uropygial gland to prepare it for metabolic investigations. The bacteria isolated could possibly have been laboratory or environmental contaminants rather than part of the normal flora of the supernatant fluid.

Survival Time—This portion of the project was concerned with the effect of antibiotics on the survival of isolated bacteria. Approximately 2,200/ml. of each of the species of isolated bacteria was used to inoculate brain heart infusion containing the antibiotics.

The 2,200 bacteria/ml. was chosen as the bacterial concentration for the survival time study because at the end of the incubation period the total bacterial count was 2,200/ml. It was observed that this bacterial contamination was found in approximately 80% of the incubating tubes. One-half hour after inoculation, no viable bacteria could be isolated from any of the samples. This indicates that the concentration of antibiotics used with these suspensions rapidly destroys any contaminating bacteria.

Antibiotic Susceptibility—*Test Tube Serial Dilution Technique*—This quantitative method was used to determine as accurately as possible susceptibilities of the cultures to measured amounts of penicillin and streptomycin. The data presented in Table IV are based on the minimum concentrations of the above-mentioned antibiotics inhibiting growth of the organisms under the experimental conditions used. It can be seen that a minimum concentration of 100 units/ml. of penicillin and 250 mcg./ml. of streptomycin were sufficient to destroy any organisms isolated.

TABLE IV—MINIMUM INHIBITORY CONCENTRATION OF ANTIBIOTICS AGAINST ISOLATED ORGANISMS

Organisms	Penicillin, units/ml.	Streptomycin, mcg./ml.
Staphylococcus aureus	0.20	3.12
Staphylococcus epidermidis	1.56	50.00
Aerobic spore-forming bacilli	6.25	6.25
Gram-negative rods	100.00	250.00

The addition of excessive amounts of antibiotic mixture to the supernatant fluid insured favorable conditions for metabolic investigations. Although the concentrations used were far in excess of the concentrations necessary for inhibition of all isolated bacteria, the large amounts of added antibiotics had no apparent metabolic effect under the experimental conditions of this investigation.

In prolonged incubations of biological materials, bacterial growth may significantly affect metabolic results. These bacterial effects may be avoided by determining and using the lowest amounts of antibiotics required to suppress the growth of likely contaminants.

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Keyphrases

Androst-4-ene-3,17-dione-4-^{14}C—*in vitro* metabolism
Uropygial gland homogenates, chicken—17β-hydrogenase
Bacterial contamination—uropygial gland homogenates
Antibiotic addition—uropygial gland homogenates
TLC—separation
GLC—separation, identity
Radiochromatography—identity
UV spectrophotometry—analysis

Serotonin Infusion in Rats with Experimental Renovascular Hypertension

By EDWIN R. FISHER and PAUL O. KEVERLINE

Simultaneous, constant infusion of serotonin (100 mcg./kg./min.) and angiotensin (2 mcg./kg./min.) for 2 hr. in normotensive rats resulted in a diminution of the pressor response noted with angiotensin alone. A more marked depression occurred following infusion of serotonin in rats with established renovascular hypertension and this agent failed to influence blood pressure in rats with unilateral renal artery constriction that did not develop hypertension. Serotonin and/or angiotensin in the doses employed did not affect the degree of granularity of renal juxtaglomerular cells in these situations. These findings fail to support the view relating some instances of hypertension in man to the combined effects of serotonin and subpressor amounts of angiotensin II.

SUBTOTAL OCCLUSION of the superior mesenteric artery in dogs has been observed to augment hypertension resulting from unilateral renal artery constriction (1). The former procedure alone has no effect on blood pressure (2). A high incidence of renal artery insufficiency or intrinsic renal disease has recently been reported in patients with occlusive disease of the superior mesenteric artery (3). Some normotensive individuals with known renal or renovascular alterations have been observed to develop arterial hypertension following onset of insufficiency of the superior mesenteric artery (3). It has been suggested that the hypertension in this situation may be induced by serotonin by increasing the pressor response to relatively small increases in angiotensin or by decreasing its inactivation by angiotensinase (3). Experimental occlusion of the superior mesenteric artery results in an increased release of intestinal serotonin into the portal circulation (4). However, no data are available concerning the pressor effect of simultaneous infusions of serotonin and angiotensin in normotensive rats or of serotonin in rats in which renovascular hypertension was successfully or unsuccessfully induced by unilateral renal artery constriction. Such information would appear pertinent to considerations relating some instances of renovascular hypertension to hyperserotonemia resulting from mesenteric insufficiency.

MATERIALS AND METHODS

Female Wistar rats weighing 200–250 g. were used in all experiments. These were housed in individual cages and allowed water and a standard ratio *ad libitum*.

All infusions were performed for 2 hr. using a Harvard pump adjusted to deliver 0.0247 ml./min. into a PE-50 polyethylene catheter whose tip was directly placed into the left femoral vein. Animals were lightly anesthetized with ether during the experiment and an airway was maintained with a small indwelling tracheal catheter. Groups of 10 rats received either isotonic saline; 2 mcg./kg./min. of angiotensin[1]; 100 mcg./kg./min. serotonin (5-hydroxytryptamine creatinine sulfate)[2]; or similar concentrations of both simultaneously. All solutions were prepared in isotonic saline. Preliminary studies revealed that smaller doses of angiotensin (1 mcg./kg./min.) failed to consistently evoke a pressor response in untreated rats even when perfused for as long as 5 hr. Infusions of larger doses of serotonin (200 mcg./kg./min.) result in prompt bronchospasm, severe vasodepression, episodes of apnea, and death in 90% of untreated rats, those simultaneously infused with 2 mcg./kg./min. of angiotensin, or those with established renovascular hypertension. Smaller doses of serotonin (50 mcg./kg./min.) failed to influence the blood pressure of untreated animals and did not alter the pressor responses of rats to simultaneous infusion of 2 mcg./kg./min. of angiotensin. Infusion of this dose of serotonin and/or angiotensin for 5 hr. resulted in subsequent decline in blood pressure, apnea, and death.

Rats subjected to unilateral renal artery constriction with a silver clip also received an infusion of isotonic saline or serotonin in the concentrations noted above, regardless of whether induction of hypertension was successful. Rats whose constricted kidneys exhibited infarction at the time of sacrifice were excluded from analysis.

Blood pressure was constantly monitored from the right femoral artery during the infusion. A PE-50 polyethylene catheter was inserted into the artery and attached to a pressure transducer in sequence with a transducer amplifier and Viso-Cardiette recorder.

Animals were exsanguinated at the conclusion of the infusion and blood urea nitrogen (BUN), hematocrit, and serum Na, K, and Cl determined by standard methods. Kidneys were fixed in Helly's fluid and sections stained for the estimation of juxtaglomerular cell granularity (JGI) (5).

Received July 1, 1968, from the Departments of Pathology, University of Pittsburgh and Veterans Administration Hospital, Pittsburgh, PA 15240
Accepted for publication July 31, 1968.
Supported by U. S. Public Health Service grant C-5195.

[1] Hypertensin, Ciba Pharmaceutical Co., Summit, N. J.
[2] Sigma Chemical Co., St. Louis, Mo.

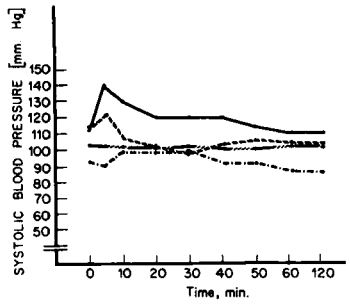

Fig. 1—Effect of constant infusion of serotonin and/or angiotensin on blood pressure of normotensive rats (average of 10 rats/group). Key: —···—, saline; ——, angiotensin; — · —, serotonin; - - -, angiotensin and serotonin.

RESULTS

As indicated in Fig. 1 slow infusion of isotonic saline failed to significantly alter blood pressure in normotensive controls. Administration of angiotensin resulted in an immediate pressor response of 30 ± 5 mm. Hg ($p < 0.01$) which lasted for approximately 5–10 min. Subsequent readings were slightly but not significantly ($p > 0.05$) elevated above control values for approximately 50 min., at which time pressure returned to normal. Administration of serotonin was followed by an immediate, insignificant ($p > 0.05$) depression of blood pressure followed by a slight (10 mm. Hg), statistically insignificant ($p > 0.05$) increase that was maintained for approximately 40 min., at which time pressures returned to control values (Fig. 1). Infusion of both angiotensin and serotonin together resulted in a prompt, slight (10 mm. Hg) insignificant ($p > 0.05$) increase in pressure followed within 5 min. by slight depression of blood pressure which was most marked (14 mm. Hg) at 0.5 hr. (Fig. 1).

The effect of serotonin on blood pressure of rats with established renal hypertension was characterized by an immediate, significant ($p < 0.01$) depression to normotensive levels followed by a significant ($p < 0.05$) rise reaching its maximum of 20–48 mm. Hg at 15 min. This elevation, which was subsequently sustained at this level, was never noted to exceed the initial hypertensive pressure (Fig. 2). Animals with unilateral renal artery constriction that did not develop hypertension exhibited a response to serotonin almost identical to that observed in unoperated, normotensive rats receiving this agent (Fig. 2). No change was noted in rats with unilateral renal artery constriction infused with saline.

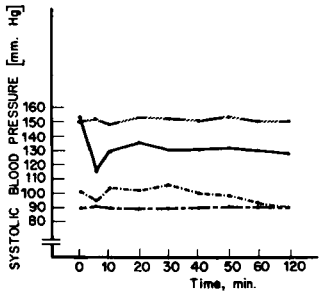

Fig. 2—Effect of constant infusion of serotonin on blood pressure of hypertensive and normotensive rats with unilateral renal artery constriction (average of 10 rats/group). Key: —···—, hypertensive-saline; ——, hypertensive-serotonin; — · —, normotensive-serotonin; — - —, normotensive-saline.

No significant differences in hematocrit, BUN, serum Na, K, or Cl were noted among the various groups of infused rats. All values were also comparable to those obtained from unoperated, normotensive controls in this laboratory. Similarly, no statistically significant difference ($p > 0.05$) in JGI was observed in rats infused with angiotensin and/or serotonin and those receiving only saline (Table I).

JGI of unclipped kidneys of rats with established renal hypertension were significantly ($p < 0.01$) less than those of clipped kidneys (Table I). These latter were significantly greater than control values ($p < 0.01$). No difference in JGI of clipped or unclipped kidneys from rats with unilateral renal artery constriction that did not develop renal hypertension was evident. Serotonin infusion in these latter animals failed to influence JGI.

DISCUSSION

Serotonin is generally regarded as a vasodepressor (6, 7). Such activity was observed only in preliminary studies with larger doses than that employed in the definitive investigations and was invariably accompanied by severe bronchospasm, apnea and death in 90% of the animals. The significance of dose-response relationships with this agent has been emphasized (8). Although pressor responses to serotonin vary in different species, the immediate, slight depression of blood pressure promptly followed by restoration to normal levels noted in this study coincides with the pattern observed by Page (9) in the rat. Development of compensatory cardiovascular reflexes or tachyphylaxis following constant infusion of angiotensin noted in rats in this study has also been noted previously in dogs (10). On the other hand, a depressor effect of serotonin was more perceptible when ad-

TABLE I—JUXTAGLOMERULAR INDEXES (JGI) FOLLOWING INFUSION OF SEROTONIN AND/OR ANGIOTENSIN IN RATS WITH AND WITHOUT UNILATERAL RENAL ARTERY CONSTRICTION

Infusion	No.	JGI	Clipped Kidney	Unclipped Kidney
Nonconstricted				
Saline	10	33 ± 12[a]		
Angiotensin	10	29 ± 10		
Serotonin	10	26 ± 14		
Angiotensin + serotonin	10	33 ± 11		
Constricted-hypertensive				
Saline	10		55 ± 19	10 ± 4
Serotonin	10		57 ± 17	7 ± 5
Constricted-normotensive				
Saline	10		32 ± 11	30 ± 6
Serotonin	10		29 ± 7	28 ± 4

[a] Standard deviation.

ministered simultaneously with angiotensin or to rats with established renovascular hypertension. These findings were consonant with evidence indicating a preferential inhibitory or vasodilating effect of serotonin on constricted arterioles, the net effect of which is vasodepression (8, 11). The slight, insignificant increase in blood pressure noted in normotensive rats infused with serotonin appears to be the result of the vasoconstriction of large vessels induced by this agent in the absence of arteriolar constriction (11).

This information, as well as the failure to observe significant elevation of blood pressure in rats with a degree of unilateral renal artery constriction that was insufficient to produce hypertension indicate that serotonin does not augment renovascular hypertension in the rat as has been suggested to occur in man (3). Indeed, the converse was obtained in this species. Although renal blood flow and glomerular filtration rate may be decreased following administration of serotonin (12), its lack of effect on JGI suggests that any action of serotonin on blood pressure is not mediated by renal juxtaglomerular cells, the purported sites of renal renin formation. The failure to observe any significant effect of angiotensin infusions on JGI is unlike the effect of daily subcutaneous injections of pressor doses of this agent. Katz *et al.* (13) observed increased granularity during the first week of treatment after which time return to control values was noted.

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Keyphrases

Serotonin infusions—rats
Renovascular hypertension—experimental
Angiotensin effect—serotonin activity
Renal artery constriction—serotonin activity

Toxicity of Emetine to Isolated Embryonic Chick-Heart Cells

By W. DAVID WATKINS* and WALLACE L. GUESS

Isolated beating embryonic chick-heart cells were cultured and observed *in vitro* to investigate the toxic specificity of emetine for the myocardium. The results of this investigation indicated that emetine exerts its toxic effects by at least two modes of action: at high concentrations, a physical disruption of the plasma membrane of the cell; and, at lower concentrations, an intracellular inhibition of the process of forming high-energy phosphate compounds. The site of this inhibition is thought to occur at the enzymatic oxidation of substrates, other than succinate, mediated by nicotinamide-adenine dinucleotide (NAD).

EMETINE HYDROCHLORIDE is a compound which has classically been used in the treatment of amebic dysentery. Although its use for this purpose has been largely superceded by more effective and less toxic drugs, it is still used to treat certain types of amebiasis which are refractory to treatment by the therapeutic alternatives.

The predominance of toxicities resulting from emetine involve the cardiovascular system, and most investigators today agree that the typical cardiovascular symptoms of emetine toxicity are due to a direct myocardial depression. Attention has been called to the apparent hypersensitivity of the heart toward emetine (1).

Wenzel (2) has recently presented a relatively comprehensive review of the literature concerning emetine cardiotoxicity. Significant toxic mani-

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Accepted for publication July 31, 1968.
Abstracted in part from a thesis presented to the Graduate School, University of Texas at Austin by W. David Watkins in partial fulfillment of the Master of Science degree requirements.
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festations of the drug include: electrocardiographic changes, ventricular fibrillation, diminished systolic force, tachycardia, and myocardial fatty degeneration and necrosis. Progressive cloudy swelling of the myocardium has also been reported (3) in conjunction with emetine administration.

A variety of investigations has been designed and undertaken to delineate the biochemical mechanism of emetine cardiotoxicity. Grollman (4) reported that emetine inhibits protein synthesis in a variety of cell types including mammalian L-cells and reticulocytes. It was demonstrated that emetine acts by inhibiting the aminoacyl-sRNA transfer reaction in protein biosynthesis. The suggested site of action of emetine is in the enzymatic formation of the peptide bond on the polyribosome.

Emetine has been shown (5) to inhibit the NAD—dependent oxidation of substrates by rat-heart homogenates, while the production of NAD has been shown to not be significantly altered.

Work done to date on emetine cardiotoxicity has generally been undertaken at three levels of biological organization: (*a*) the intact heart; (*b*) myocardial tissue; and (*c*) the heart homogenate. Certainly each of these approaches has offered valuable information necessary in the total toxicity evaluation of this compound.

There existed an apparent gap in the research efforts to date; none had reported the effects of emetine upon the heart cell *per se*. Since the cell is the functional "building-block" of organized life, it seemed reasonable that the explanation of toxicity should ultimately be found here also. Relatively recent advances in cell culture techniques have provided a unique means of further identifying and investigating such cellular toxicity.

MATERIALS AND METHODS

Culture Procedures—The routinely cultured contracting chick embryo heart cells were prepared from the hearts of 12–24 white leg-horn chick embryos 10–13 days *in ovo*. Aseptic techniques were used throughout all experimental procedures. The whole hearts were excised, placed in a phosphate-buffered saline solution (PBS 1×) (6), trimmed free of contiguous noncardiac tissue and membranes, and rinsed with PBS solution. The pooled hearts were placed in 10 ml. of freshly prepared 0.1% trypsin solution. Cell dispersion was initiated by gently forcing the cardiac tissue out of a 20-ml. syringe and into a flask. This flask and contents were incubated for 30 min. with frequent gentle agitation, then removed from the incubator. Gentle passage of the mince in and out of the syringe for about 5 min. was ordinarily sufficient time to complete the cell dispersion process. The resultant cell suspension was then centrifuged with a clinical centrifuge at three-fourths speed for 5 min., the trypsin solution decanted off, and the remaining packed cells washed twice by resuspension and centrifugation in PBS solution. Following the second washing, the cells were resuspended for the final time in Eagle's basal medium (BME 1×) (6) supplemented with 5% calf serum and 1% l-glutamine. The quantity of this basal medium used for the final suspension was 1.5 ml. of media per embryonic heart used. The approximate concentration of this suspension was 10^6 cells/ml. as determined by hemocytometer count.

A portion of the resulting heart-cell suspension was injected into sterile Sykes-Moore tissue culture chambers (Belco Glass, Inc., Vineland, N. J.) which, after cell settling and attachment to the glass coverslip, yielded a maximal working population of isolated cardiac cells. The remaining suspension was cultured in a sterile prescription bottle and incubated at 35° in a 5% CO_2 atmosphere. This culture provided the conditioned medium required for the cell-perfusion experiments described below.

Preparation of Isolated Atrial and Ventricular Cells—To develop the technique of visually distinguishing isolated beating atrial and ventricular cells, cultures were prepared from embryonic atrial and ventricular tissue, respectively, and characterized.

Cell Perfusion Procedure—A preliminary series of control cell-perfusion studies was performed to assure that any observed cellular effects following perfusion with emetine or other compounds could be related entirely to the particular compound, and not to certain environmental factors, such as changes in necessary nutrients, or tonicity effects. Preliminary experimentation and the work of others (7) indicated that various cellular alterations in response to fresh media could be prevented by perfusing with conditioned media. Such a conditioned medium was prepared by removing the cell-free fluid phase from the relatively large culture of heart cells. Multiple cell perfusions with this conditioned media produced no detectable variation in function or structure. As a result, conditioned medium was used to prepare all perfusion solutions used in this investigation.

To obtain useful and valid information from this study, it was necessary to prepare perfusion solutions which not only contained accurately determined quantities of perfusate compound, but which also closely approached the composition and tonicity of the original culture medium. Compounds to be perfused were accurately weighed and dissolved in PBS solution (1×). Serial dilutions of these solutions were prepared by taking a small, yet accurately measurable volume (0.1 ml.), and combining with this a relatively large volume (0.9 ml.) of conditioned media. The resulting perfusate fluid satisfied the above requisites.

It has been shown (8) that perfusion of 1 ml. through a 0.7-ml.-capacity Sykes-Moore chamber would result in 65% perfusate retention within the chamber. Perfusion of 2 ml. through the chamber results in essentially a complete medium change. In this report, all concentrations of perfused compound refer to the delivered concentration retained in the chamber following the perfusion process.

Perfusion of the cells contained within each cham-

ber could be initiated any time following cell attachment to the glass coverslip (15–20 hr.). The solutions and equipment to be used in the perfusion procedure were placed inside the microscope stage incubator for 15 to 20 min. so temperature equilibrium could be attained and thereby avoid physical perfusion alteration of the beating cells as much as possible. The inoculated chamber was firmly affixed to the microscope stage, and the cell(s) to be observed were located in the visual field. Perfusion of the cells was then performed using the procedures of Sykes and Moore (9).

Microscopy and Photography—All observations were made using a Nikon light-field inverted phase contrast microscope with stage encased in a thermostatically controlled incubator maintained at 35°. Photomicrographs were taken with a Nikormat FS 35-mm. camera using Kodak plus-X Panatomic film.

Drug Standardization—The quality and purity of emetine hydrochloride[1] used in this study were confirmed by IR and UV spectroscopy, melting point determinations, and differential thermal analysis.

RESULTS

The design of this experimental procedure was such that the following parameters associated with the functional status of the myocardial cells could be observed: (*a*) rate of beat (b.p.m.), (*b*) apparent strength of beat, (*c*) synchrony of beat, and (*d*) morphology.

Simply counting the b.p.m. of single cardiac cells represented a sensitive index of toxicity because, in essence, this analyzed the final effect of the drug on a delicate and highly organized enzyme system. Evidence indicating that the observed beat is an enzyme-regulated system stems from the observations (10) that in single cells, hydrogen ion concentration, temperature, salts, substrates, serum proteins, metabolic inhibitors, and a variety of chemical compounds affect the beating rate. Therefore, any alteration in beating rate was considered the initial indicator of toxicity, and cell lysis was taken to be the terminal manifestation of toxicity.

Rate of beating was recorded as b.p.m. and treated as strictly numerical data, and as such was relatively simple to collect. Recording cellular morphological changes also presented no significant problems in that photomicrographic techniques provided a means of recording at chosen intervals the condition of any cell in culture. Preliminary experience indicated that the apparent strength of beat and synchrony of beat, although purely qualitative parameters under these experimental conditions, were equally as vital to the measurement of cellular toxicity as rate of beat and morphology.

In most cultures, only a small proportion of the total population were observed to beat. This beating ranged from intermittent, irregular "twitches" to steady, deep rhythmic contractions at rates up to about 120 b.p.m. The majority of the beating cells, however, exhibited a beating range of about 30–80 b.p.m. It should be noted that each isolated beating cell maintained a rate independent of the other cells in the culture.

[1] Pfaltz and Bauer, Inc., Flushing, N. Y., and Ruger Chemcal Co., Inc., Long Island City, N. Y.

The characteristics of the untreated atrial and ventricular cell types used in the present study have been described by Mercer and Dower (11).

Observations of Emetine-Dosed Heart Cells—Emetine concentrations of 10^{-5} *M* or greater had severe and apparently equivalently toxic effects upon the beating atrial and ventricular cells. The inhibitory effects of emetine on beating were immediate and complete. In fact, the intended perfusate dosage could not be completely delivered before the cells terminated beating. After cessation of the converted contractions which formed the normal cellular beating pattern, weak irregular twitches were observed within the cytoplasmic regions. These occasionally continued up to the time of cell lysis. Within 10 min. after perfusion, the cells began retraction of the web-like pseudopodial projections and hyaloplasmic veil. Also, the entire cell, which under normal conditions was flattened and/or extended, slowly drew itself into a more compact, rounded form. Often the release of attachments of peripheral area during this rounding process were observable as rapid breaks, with subsequent withdrawal of the cellular component toward, and into the rounding cell. Cell lysis was almost always preceded by a rapid extrusion of the cytoplasmic contents into a portion of the plasma membrane. These large, spherical swellings are termed "superficial vacuoles." Normally, after the formation of these superficial vacuoles, only a short period of time elapsed prior to cell lysis, after which the superficial vacuoles were observed to remain intact and float freely in the media. In most cultures dosed with this level of emetine, a few cells of the population were observed to lyse within 30–45 min. Usually the first cells to undergo lysis were those which contained dense, heavy granules and were highly vacuolized. Progressive lysis of the cell population could be observed for 48 hr., after which no intact cells remained. Throughout this, and all other procedures, the pH was buffered between approximately 7.2 and 7.4. Most of the damage to the beating myoblasts occurred within the first hour after dosing. The cells which most effectively resisted the prolonged effects of emetine were those which were nonbeating and fibroblastic in nature. No further cell growth was observed in the period following drug perfusion.

Cells dosed with emetine concentrations less than 10^{-5} *M* responded differently than described above with the higher doses. Figure 1 illustrates the morphological effects 6 hr. after perfusion with 10^{-6} *M* emetine. Minutes after dosing, the portions of the plasma membrane normally engaged in pinocytotic activity, known as the "hyaloplasmic veil," were slowly retracted into the cell. The phenomenon known as "blebbing" was observed, but apparently this process could initiate at any time after cell perfusion. This process manifested itself as small swellings occurring on the plasma-membrane. These "blebs" would remain as such for intervals up to 2 or 3 min., after which they appeared to be resorbed back into the contour of the plasma membrane. While some blebs were being resorbed, others would spontaneously arise at apparently random sites of the plasma membrane which were exposed to the emetine. No superficial vacuolization or cell lysis was observed in the early portions of the observations. Figure 2 is a graphical

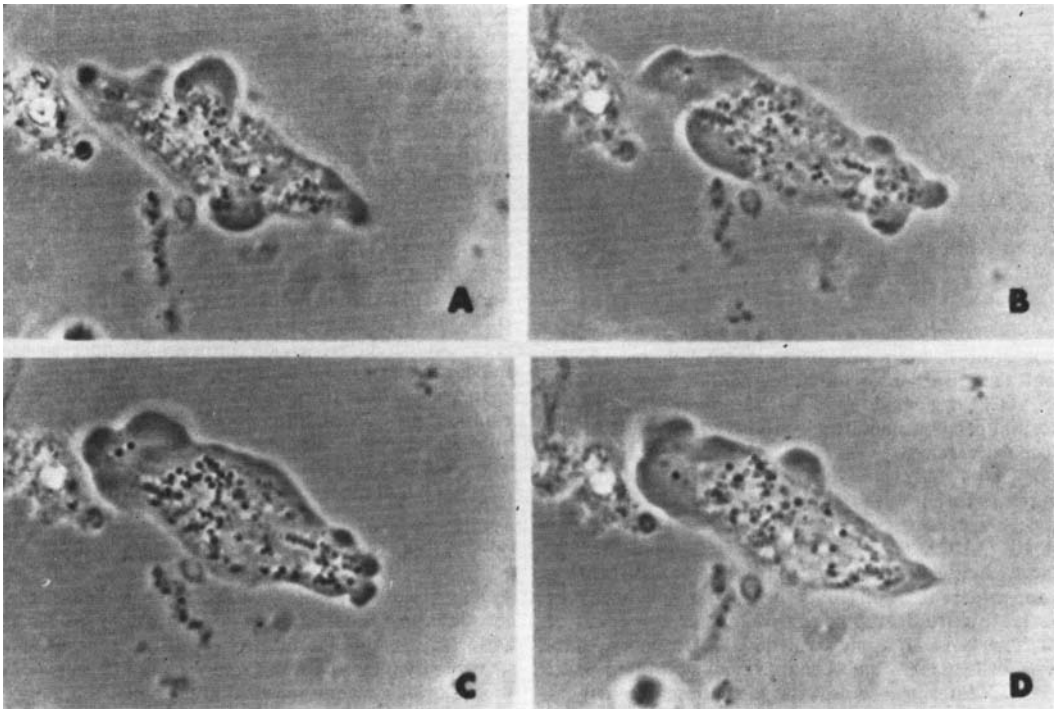

Fig. 1—Cardiac cell blebbing observed 6 hr. after perfusion with 10^{-6} M emetine. Photographs taken at 5-min. intervals (1,000×).

illustration of the typical immediate effects of 10^{-6} *M* emetine upon the beating rate of atrial and ventricular cells. It can be seen that the rate of beat decreased rapidly and progressively in both cell types. The strength of beat and synchrony of beat of the atrial cells was significantly more affected than the corresponding ventricular cells. The atrial cells appeared to maintain a regular rate, while superimposed on this rate were single or short bursts of weak, rapid contractions.

Observations up to 4 days after emetine dosing revealed progressive increases in the degree of vacuolization and signs of cell damage in doses ranging from 10^{-6} to 10^{-8} *M*. Cell growth and proliferation were observed to decrease at these dosage ranges also.

Ventricular cells dosed with 10^{-7} *M* and 10^{-8} *M* emetine exhibited short-term responses similar, yet not as pronounced, as those observed at the 10^{-6} *M* level. The atrial cells appeared to resist the effects of 10^{-7} *M* and 10^{-8} *M* emetine for periods of about 12 hr., after which were observed progressive decreases in rate, strength, and rhythmicity of beat similar to that found at the 10^{-6} *M* emetine level.

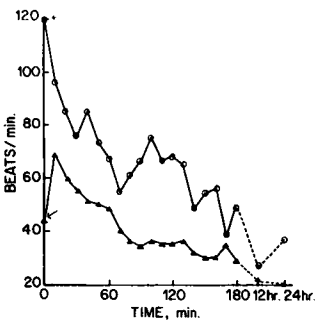

Fig. 2—Effect upon beating rate of 10^{-6} M emetine. Key: ○, atrial cell; ▲, ventricular cell; and ↑, perfusion.

Reversal Studies with Emetine-Dosed Cells—Having observed and characterized the usual effects of various concentrations of emetine upon both the atrial- and ventricular-type cells, it was decided to use 10^{-6} *M* emetine for subsequent studies. It was this concentration that elicited an observable toxic response from both atrial and ventricular beating cardiac cells. Subsequent experimentation consisted of perfusing the emetine-depressed cells with various compounds to determine their effects as possible toxicity-reversing agents. All initial perfusions with agents to counteract emetine toxicity were performed after the beating rate of the observed emetine-dosed cells had decreased to approximately one-half the preprocedural control rate.

Effect of Emetine Removal—The initial perfusion experiments consisted of removing the emetine from the culture chamber by washing with conditioned media containing no additional solutes. When compared to control cells dosed with emetine, multiple perfusion with conditioned media gave no observable protection against the toxicity, in terms of lengthened cell life, strengthening or retention of beating function, or reinitiation of beating after drug-induced cessation.

In order to evaluate and correlate the procedures reported by a variety of authors, using heart homogenates, isolated organs, or whole animals as test subjects, it was decided to appraise the activities of several biochemical compounds upon the eme-

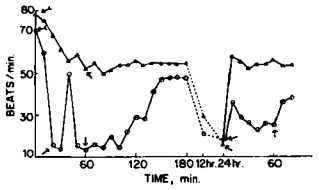

Fig. 3—Effect upon beating rate of 10^{-6} M emetine followed by 10^{-5} M NADH. Key: ▲, atrial cell; ○, ventricular cell; ⌇, emetine perfusion; ↑, NADH perfusion.

tine-poisoned cells used in this investigation. Control studies with adenosine triphosphate (ATP) and reduced nicotinamide-adenine dinucleotide (NADH) revealed that no significant effects on normal cells was elicited by these compounds in the dose ranges used.

Effect of NAD and NADH—Emetine-dosed cells were perfused with either NAD or NADH. No effects were observed with NAD at concentrations of 10^{-6}, 10^{-5}, or 10^{-4} *M*. NADH, on the other hand, induced significant stimulation of beating rate, apparent strength of beat, and synchrony of beat. Figure 3 graphically illustrates the effect of 10^{-5} *M* NADH upon both atrial- and ventricular-type cells which had been previously dosed with 10^{-6} *M* emetine. Following perfusion, the effects of the NADH were observable within 5–15 min. NADH appeared to possess more pronounced effects upon the ventricular cells than the atrial cells, although it should be noted that the atrial cells were not depressed as much as the ventricular cells. The total effects of NADH upon the observed emetine-dosed cells appeared to be a reversal of the symptoms chosen in this investigation to be considered toxicities. This reversal included three of the four measured parameters—rate of beating, synchrony of beat, and apparent strength of beat. No morphological differences were observable at the phase contrast level.

Following addition of the NADH to the cell environment, the cells would typically exhibit a brief time lag of usually from 5–10 min. before showing signs of response. After this brief period, the emetine reversal could be observed for periods lasting usually about 30–45 min. In a few instances, however, NADH reversed the observed toxic effects of emetine for periods up to several hours. Following the periods of emetine reversal the beating cells reverted back to a depressed status similar to that observed prior to the perfusion of NADH. Also noted was that the rate of beating following the effects of NADH was practically identical to the rate just prior to the NADH administration. After varying lengths of time, the rate of beating would diminish progressively until either cell death or additional NADH perfusion. After 24 hr., and in some experiments, 48 hr., further addition of NADH (also 10^{-5} *M*) to the depressed cells restored the rate, apparent strength, and rhythmicity of beat to a level at least equal to that obtained after the first administration of NADH. Often the observed reversal at this same time was in excess of reversals seen shortly after initiating the emetine toxicity. NADH (10^{-5} *M*) provided apparent protection against the effects of emetine (10^{-6} *M*) for periods up to 5 days after dosing.

Effects of Embryonic Chick-Heart Extract—The results of perfusion with an embryonic chick-heart extract indicated a slight, yet detectable, emetine-reversal effect.

Effects of ATP—Compared with the data obtained using the NADH as the perfusate compound, ATP at a dose of 10^{-6} *M* evoked a relatively brief, intense reversal of the emetine toxicity. The concentration of ATP eliciting maximum reversal was approximately 10-fold less than the most effective concentration of NADH.

It should be noted that the work of Harary and Farley (16) has demonstrated that cells such as those used in this study do respond to exogenous ATP and NADH, giving indirect support to the belief that these compounds do diffuse into the cells.

Effects of Other Compounds—None of the following compounds had significant effects upon atrial or ventricular cells: Adenine (10^{-6} *M*), flavin-adenine dinucleotide (FAD) (10^{-6} *M*), reduced FAD (10^{-6} *M*), and nicotinamide (10^{-5} and 10^{-6} *M*).

DISCUSSION AND CONCLUSIONS

The results of this investigation indicate that emetine possesses at least two possible modes of action upon the intact heart cell. The first, observable by phase-contrast microscopy in a relatively short period of time, occurs only at higher emetine concentrations, and is probably a nonspecific physical activity upon the plasma membrane. The second mode appears to be quite specific, and is believed to inhibit the production of high-energy phosphate compounds by the electron transport system.

Nonspecific Membrane Activity—Maintenance of an ionic membrane potential is known to be a requisite of the beating character of the cell types used in this investigation (12). Levinson and Green (13), also using cultured chick-heart cells, suggest that cell membrane injury from enzymatic trauma may result in swelling due to a loss of differential permeability to small electrolytes such as sodium and potassium. It seems reasonable, then, that the observed immediate cessation of beat is caused by an electrochemical imbalance resulting from the functional disruption of the cell membrane. It is also reasonable to assume that the irritation of the plasma membrane involves some mode of activity other than strictly a pH effect, or a phenolic-type protein denaturation since the media was buffered at pH 7.2–7.4, and since emetine is a nonphenolic alkaloid. One possible mode of activity upon the cell membrane in view of the abrupt change in activity observed between 10^{-5} and 10^{-6} *M* suggests a possible inhibition of enzymes of the plasma membrane. These enzymes, which catalyze and control the permeability of the membrane to most compounds, may be subject to inhibition by emetine only when the critical concentration of 10^{-5} *M* is attained.

Inhibition of Cellular High-Energy Phosphate—Whereas the physical irritation observed in the previous discussion appears to be totally based on membrane phenomenon, the second type of response to emetine is probably a result of the intracellular activities of the compound.

The importance of the TCA cycle and electron-transport system in providing sufficient energy to

support the periodic cellular contraction of cultured heart cells has been demonstrated by Harary and Farley (14). Succinate is oxidized directly by FAD, while the remaining TCA intermediates are oxidized first by NAD, which is in turn oxidized by FAD. The primary advantage of the NAD-mediated step resides in a considerable increase in ATP production. Succinate oxidation with concurrent inhibition of the oxidation of other normally occurring metabolites, results in an inhibition of heart muscle respiration (15). It is, therefore, apparent that a compound which inhibits the oxidation of NAD-mediated substrate, yet has no effect upon the succinoxidase system, would depress the energy-requiring processes of the myocardial cells.

Several facets of the experimental results from this investigation clearly indicate that cellular ATP production is seriously limited under the influence of emetine. The observed parameters of toxicity were reversed with NADH and NADPH. Perfusion of ATP had a similar, yet more rapid, intense, and short-lived reversal effect upon the beating activity of the emetine-dosed cells. It should be recalled that NADH must act throughout the electron transport system of the mitochondrion to produce the large amounts of ATP essential for myocardial activity. On the other hand, exogenously added ATP apparently by-passes the mitochondrial system and passes directly to the contractile myofibrils of the cell. This is then manifested as strengthening of beat and a rapid increase in the beating rate.

Applying this same concept, it is also logical that a longer time lag is observed before NADH reversal than ATP reversal. NADH represents potential ATP, while ATP, of course, is capable of utilization the moment it enters the cell. At this point, it should be noted that the investigations of Harary and Farley (16) offer indirect evidence that ATP does indeed permeate the ATP-deficient cell.

Of equal significance are the results in which NAD and NADP (oxidized forms) had no observable effect upon the emetine-dosed cells. This suggests that the activity of emetine is upon an enzyme, because uncatalyzed reduction of NAD requires relatively strong chemical measures which would not be available within the intact cell. In the absence of a functional enzyme system, exogenous NAD or NADP would, therefore, not be reduced and could provide no additional hydride to the electron transport system. In support of this view is the work of Marino *et al.* (17), who reported that the addition of NAD did not prevent electrocardiographic changes in the guinea pig heart after emetine administration. On the other hand, the results of this investigation and the work of Marino (18) indicate that embryonic heart extract has a reversal effect upon the observed emetine toxicity. Such an extract contains relatively high amounts of NADH and ATP, and would, therefore, be expected to elicit results similar to those observed when NADH is administered alone.

Additional indirect evidence that the emetine-dosed cell apparently lacks the ability to produce ATP was observed after performing two of the control experiments. Perfusing either ATP (10^{-6} M) or NADH (10^{-5} M) into previously untreated cultures of beating cells resulted in no observable response. The cells simply continued to maintain the beating function as though no extracellular changes had occurred. The cells apparently were producing sufficient amounts of high-energy phosphate compounds to efficiently maintain the functional status. Following emetine dosage (10^{-6} M), however, the cells appeared to rapidly utilize the exogenously provided NADH or ATP. This difference in response to exogenously provided NADH or ATP in normal cells and emetine-dosed cells, was interpreted as a facilitative mechanism of the cell to maintain chemical and functional homeostasis. This was indicated by the observavations that the normal cell, which produced sufficient endogenous ATP and NADH, had no apparent need for these compounds. However, when cellular energy production was impaired by emetine, the cell apparently turned to the ATP and NADH provided in the extracellular environment.

Two concepts have been proposed concerning the cause of cell blebbing, which was seen to occur at emetine doses of 10^{-6} M and less. The concept of Levinson and Green concerning cellular osmotic changes due to plasma membrane damage, has been discussed previously. Lettre (19), on the other hand, suggests that the plasma membrane, which requires ATP to maintain its integrity, receives an inadequate supply of the high-energy phosphates. The resultant weakening of the membrane is then manifest as the localized areas of spontaneous cell blebbing.

Lettre's concept appears to better explain the results observed at the lower emetine doses. More specifically, emetine enters the cell and effects an inhibition of the production of ATP by the electron transport system. The resultant decrease in ATP is then manifest first as relatively slow retraction of the hyaloplasmic veil. Soon following is progressive decrease in apparent strength, rhythmicity, and rate of beat, followed by cell blebbing, cessation of cell proliferation, and ultimately cell death. If the activity of emetine at this dosage level were directly upon the plasma membrane, as apparently was the case in the higher dosage levels, one would expect to see blebbing soon after emetine contacted the cells. However, such is not the case. Usually the blebbing phenomenon occurs at delayed periods after perfusion.

Based upon the reports of other authors (20), little biochemical significance can be placed upon the incidence of vacuolization in these cultured cells. However, the dose-related onset and degree of vacuolization of these emetine-treated cells lends itself to the concept of Yang *et al.* (21), who reported that the vacuole is an attempt by the cell to dilute the drug which is acting upon the cell.

Conclusions of Biochemical Effects of Emetine—The results of this study support, at the cellular level, the above-described findings of other investigators who used subcellular homogenates as the experimental system. The data suggest that emetine cardiotoxicity observed *in vitro* is primarily due to an affinity of the drug for the energy-producing portions of the cardiac tissue.

This study suggests that these isolated beating cells possess two levels of energy priorities, and budget the available ATP accordingly. Most fundamental to the survival of the cell is structural maintenance. However, only a relatively small portion of total cell energy is actually necessary for this function. The second level is maintenance of

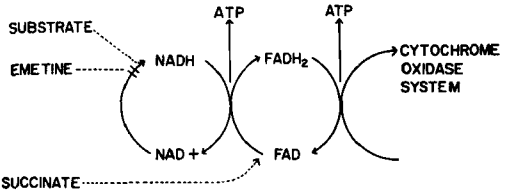

Fig. 4—Proposed site of cardiotoxic effects of emetine.

the contractile characteristics. These cells have differentiated to such an extent that the metabolism has been routed so as to provide continuously large quantities of energy for this latter function. From the observations of this study, it appears that rapid decreases in the available ATP supply, such as those induced by emetine, prevent the ability of the cell to "dedifferentiate." That is, enzymatic changes necessary to compensate for the drastic alteration in available energy cannot occur rapidly enough to maintain the cellular functions. Although much smaller quantities of ATP are produced, the same energy priorities exist. Inadequate energy is, therefore, provided to either of the abovementioned energy levels and the result is a loss of cellular function and integrity.

The proposed site of inhibition of cellular energy production is illustrated in Fig. 4. The results suggest that exogenous NADH by-passes the point of inhibition. It should be pointed out that despite the apparent functional ability of the electron transport system, the inhibition by emetine remains a major insult to the ability of the cell to efficiently produce high-energy phosphate compounds. This was evidence by the slow, yet progressive decreases in beating function observed when emetine and NADH were perfused simultaneously.

Pathological Implications of the Proposed Modes of Action—The apparent inhibition of mitochondrial activity by emetine points the way for a tentative explanation of pathological disturbances related to emetine toxicity. This may range from maintenance of electrolyte gradients, through fat and protein metabolism, to the maintenance of the cellular structure.

The reported gastrointestinal side-effects and prostration caused by emetine would be a direct result of the physical membrane effect associated with this drug. Goodman and Gilman (22) also believe the reported skeletal muscle effects are based solely upon the irritant effects upon the particular areas of drug administration.

The reported abnormalities in cardiac function indicate that emetine possesses a degree of preferential toxicity for the ventricular portions of the heart. The results of the present *in vitro* study have indicated also that isolated atrial cells can resist the toxic effects of emetine more effectively than ventricular cells.

Emetine has been reported to cause progressive cloudy swelling and fatty degeneration of the heart. It would be expected that emetine inhibition of NADH oxidation in the mitochondria would prevent the complete metabolism of the fatty acids being actively transported into the cell. The pathological result would be the observed fatty degeneration.

The dose-related inhibition of emetine upon cell growth, maintenance, and proliferation observed in this investigation indicates a likely inhibition of protein syntheses. Grollman (4) indicates that emetine may possess some structural similarity to the glutarimide antibiotics and acts similarly by physically binding at the ribosome and thereby inhibiting the aminoacyl-sRNA transfer reaction in protein biosynthesis. The results of this investigation neither confirm nor negate the possibility that emetine may act at the ribosomal level. However, it is suggested that the proposed concept of NADH inhibition by emetine is of more fundamental consequence to the cell. While protein synthesis may concomitantly be inhibited, it appears that the apparent inhibition of energy production more convincingly explains the cardiotoxic properties of this compound.

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Keyphrases

Emetine toxicity—embryonic chick-heart cells
Heart cells, embryonic chick—test cultures
Cell perfusion—emetine solution
Contractions, heart cells—emetine effect
NAD, NADH effect—emetine-dosed cells

Morphine Determination in Kaolin Pectin Formulations

By RONALD L. HOUTMAN

A method has been developed for the quantification of morphine in kaolin pectin formulations containing opium. The method consists of lyophilizing the suspension, washing of the solids with ammonia methanol, complexing with bromothymol blue, extraction, silanization, and gas chromatography.

THE ALKALOIDS of opium are frequently formulated with kaolin and pectin for the symptomatic relief of acute nonspecific diarrhea. The suspensions combine the adsorbent and detoxifying demulcent action of kaolin and pectin with the sedative and analgesic effects of opium alkaloids.

A number of assay methods are available for the quantification of opium alkaloids (1–3). Brochmann-Hanssen and Svendsen (4) describe the gas chromatography of opium alkaloids and Brochmann-Hanssen and Furuya (5) identified the alkaloids using a combination of gas, thin-layer, and paper chromatography. No procedure, however, has been reported involving the quantification of opium alkaloids in kaolin pectin suspensions. Bracey and Selzer (6) described a method combining liquid–liquid extraction and column chromatography for the determination of microgram quantities of belladonna alkaloids in kaolin pectin suspensions. Similar methods, when applied to the extraction of morphine from these suspensions, met with little success in this laboratory.

This paper presents a method for simple elution of morphine from the lyophilized kaolin pectin formulation. The morphine is then complexed with bromothymol blue (7, 8) and the complex extracted with chloroform to remove interfering excipients. The morphine is measured by gas chromatographing the trimethylsilyl derivative. Since the concentration of morphine in opium extracts is known (10%) the assay of the extracts in the product is based on the quantification of morphine.

EXPERIMENTAL

Instrument—An F & M model 402 gas chromatograph equipped with a flame-ionization detector and glass columns, 1.2 m. long × 3 mm. i.d. was used for this work. The columns were packed with 3.8% SE-30 on 80–100 mesh Diatoport S. The carrier gas was helium at a flow rate of approximately 50 ml./min. Hydrogen and air flows were adjusted for maximum response. The column was operated isothermally at 200°, the flash heater at 220°, and the detector at 260°. The recorder was equipped with a model 227 disk chart integrator for peak area measurements.

Solvents and Reagents—The following reagent grade materials were used: chloroform, dimethylformamide, sodium hydroxide, citric acid monohydrate, sodium phosphate dibasic anhydrous, anhydrous methanol, and ammonium hydroxide. Other reagents used were tetraphenyl tin,[1] bromothymol blue,[2] hexamethyldisilazane,[3] and tetramethylammonium iodide.[4]

Solutions—*Dye-Buffer Solution*—Prepare 500 ml. of a water solution containing 200 mg. bromothymol blue, 3.2 ml. of 0.1 *N* sodium hydroxide, 577.5 mg. of citric acid monohydrate, and 6.32 g. of sodium phosphate dibasic anhydrous. The pH of the resulting solution should be between 7.2 and 7.4.

Internal Standard Solution—Prepare a dimethylformamide solution containing 1 mg./ml. of tetraphenyl tin.

Reference Solution—Transfer an accurately weighed portion of morphine sulfate equivalent to about 100 mg. of morphine and dissolve it in exactly 100 ml. of water. Transfer 2.0 ml. of this solution to a separator, add 20 ml. of dye-buffer solution, and extract with four 30-ml. portions of chloroform. Take the combined chloroform extracts to dryness. Dissolve the residue in exactly 2.0 ml. of internal standard solution, add 2 ml. of hexamethyldisilazane, 10 mg. of tetramethylammonium iodide, and allow it to stand for 2 hr.

Sample Preparation—Accurately measure and lyophilize a volume of well mixed sample equivalent to about 2 mg. of morphine. To lyophilize or freeze-dry the sample, shell freeze it in a dry ice–acetone bath and place the sample in sufficient vacuum to maintain the frozen condition until the water is removed.[5] Quantitatively transfer the residues to a 2.5 × 40 cm. glass column with a

Received May 22, 1968, from Control Laboratories, The Upjohn Company, Kalamazoo, MI 49001
Accepted for publication August 27, 1968.
The author is grateful to P. B. Bowman for the interpretation of mass spectral data.

[1] Aldrich Chemical Co., Milwaukee, Wisc.
[2] Eastman Organic Chemicals, Rochester, N. Y.
[3] Applied Science Laboratories, Inc., State College, Pa.
[4] Matheson Coleman and Bell, Cincinnati, Ohio.
[5] Freeze dryer, model FDC-6-V15 MRS, available from Thermovac Industries, Inc., was used in this laboratory.

TABLE I—DETERMINATION OF OPIUM IN KAOLIN PECTIN FORMULATIONS

Formulation	Opium Content (Label)	Found
U	20.0 mg./30 ml.	19.4 mg./30 ml.
P	15.0 mg./30 ml.	15.2 mg./30 ml.
D	24.0 mg./30 ml.	23.5 mg./30 ml.

fritted disk. Wash the solids with 250 ml. of 5% ammonia methanol and evaporate the eluate to dryness. Dissolve the residue in 10 ml. of dye-buffer solution and 20 ml. of chloroform. Transfer this mixture to a separator and rinse with similar portions of dye-buffer solution and chloroform. Extract the dye with the chloroform and repeat with three additional 30-ml. portions of chloroform and take the combined extracts to dryness. Dissolve the residue in 2.0 ml. of the internal standard solution, add 2 ml. of hexamethyldisilazane, 10 mg. of tetramethylammonium iodide, and allow it to stand for at least 2 hr.

Chromatography—Chromatograph 5 μl. each of the sample and reference preparations. Using the conditions described under *Instrument*, retention times of 10 and 18 min. were obtained for morphine and the internal standard, respectively.

Calculations—Calculate the mg. of morphine in each 30.0 ml. of the formulation from the formula:

$$\frac{R_1 \times A \times 30}{R_2 \times B}$$

where

R_1 = sample morphine peak area/internal standard peak area
R_2 = reference morphine peak area/internal standard peak area
A = weight in milligrams of morphine in the reference preparation
B = volume in milliliters of the formulation used in the sample preparation

RESULTS AND DISCUSSION

The desirable absorptive properties of kaolin pectin formulations makes difficult the quantitative removal of morphine for analysis. Several approaches to wet extraction including salting out and complexing met with little success. Removal of the water by lyophilization from the clays apparently reduces their adsorptive properties permitting elution of morphine with an ammonia–methanol mixture. The complexing with bromothymol blue and subsequent extraction are necessary to separate the morphine from water-soluble excipients. The tetramethylammonium iodide is added to break the morphine bromothymol blue complex prior to silanization. There is no apparent chromatographic response from the bromothymol blue.

A sample was prepared in this laboratory containing 1.95 mg. of morphine/30 ml. of formulation. Ten replicate assays of this sample showed 99.5% recovery with a coefficient of variation of 1.96%.

Three commmercial preparations were assayed by this procedure and the results are shown in Table I.

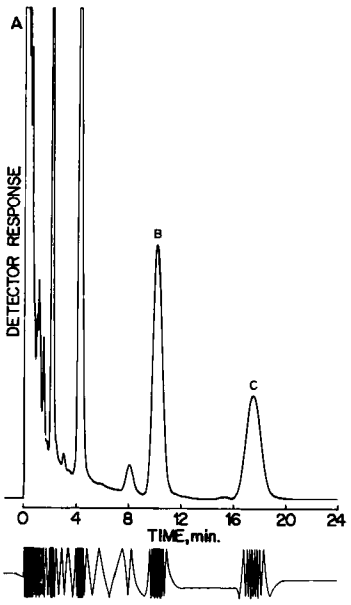

Fig. 1—Chromatogram of an assay preparation from a kaolin pectin formulation. Key: A, solvent; B, morphine; C, internal standard.

Figure 1 shows a chromatogram resulting from the assay of Formulation D. Combination gas chromatography-mass spectrometry (LKB-900GC-MS) was used to verify the morphine trimethylsilyl derivative peak. No effort was made to determine the nature of the excipient peaks.

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Keyphrases

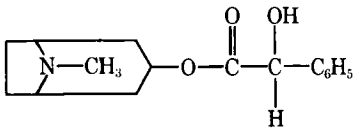
Morphine analysis—kaolin pectin formulations
Bromothymol blue-morphine complex-extraction
GLC—analysis
Dimethylformamide-tetraphenyl tin solution—internal standard

Stability Determination of Homatropine Hydrobromide by Direct Cerimetric Titration

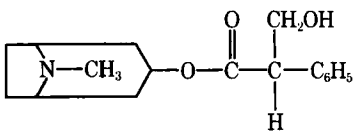
By LESTER CHAFETZ and ROBERT E. DALY

Using ceric nitrate as the titrant, the difference in titer between saponified and unhydrolyzed aliquots of homatropine solutions provides a measure of intact drug in the presence of its mandelic acid hydrolysis product. The titration is conducted on chilled solutions to circumvent interference by tropine, the other hydrolysis product, which is slowly oxidized by the titrant at room temperature. The proposed procedure is highly selective for the drug in the presence of its hydrolysis products, other tropine esters, and most other organic nitrogenous bases. The cerimetric titration method is rapid, absolute, and stability indicating.

HOMATROPINE IS A synthetic homolog of the solanaceous alkaloid atropine in which the tropic acid moiety of the ester alkaloid is replaced by *d,l*-mandelic acid. It is used in medicine as an ophthalmic anticholinergic agent, having the



Homatropine



Atropine

advantage over atropine of a shorter duration of activity (1). Since the hydrolysis products of homatropine, *i.e.*, tropine and mandelic acid, are inactive, the findings of Patel and Lemberger (2) that homatropine hydrolyzes at the pH of tears at several times the rate of atropine provide a rational basis for its shorter activity.

Patel and Lemberger (2) followed the rates of hydrolysis of homatropine under various experimental conditions by means of UV spectrophotometry after separation of intact mandelate ester from free mandelic acid by solvent extraction. Their scheme provides a valid stability method for homatropine, for the mandelic acid moiety comprises the UV chromophore. However, the procedure is somewhat tedious, and current practice requires the preparation or procurement of a reference standard for spectrophotometric comparison. Because the UV spectrum is benzenoid, the method is relatively nonselective.

Received June 26, 1968, from the Pharmaceutical Research and Development Laboratories, Warner-Lambert Research Institute, Morris Plains, NJ 07950

Accepted for publication August 5, 1968.

The assay for homatropine hydrobromide ophthalmic solution in USP XVII (3) does not discriminate between intact and hydrolyzed homatropine. The prescribed method is acetous perchloric acid titration of base extracted with chloroform from ammoniacal solution. (Measurements in the authors' laboratories—which will be described more fully in a future communication—indicate that tropine is well extracted from alkaline solution by chloroform. The partition coefficient, c_{CHCl_3}/c_{H_2O}, is 0.55.)

Chafetz and Gaglia (4) reported the direct titration of mandelic acid with ceric nitrate in dilute nitric acid and suggested application of the method to the assay of homatropine and other arylglycolate esters. The reaction was found to be highly selective for the free arylglycolic acids; their esters are not oxidized by ceric nitrate. Taken with quantitative data showing that homatropine is stable to hydrolysis in dilute acid and very labile at high pH (2), these observations afford a rational basis for an absolute assay of homatropine hydrobromide by direct cerimetric titration. A procedure for the determination of homatropine in the presence of its hydrolysis products and common preservatives and stabilizing agents is presented here and proposed for compendial use.

EXPERIMENTAL

Reagents and Supplies—Homatropine hydrobromide USP (Merck and Co., Inc.), chlorobutanol, and benzalkonium chloride (both USP) were used as received. The titrant was 0.05 *N* ceric nitrate, a 2.45% solution of ceric ammonium nitrate (G. Frederick Smith Co.) in 1 *N* nitric acid. It was standardized against primary standard ferrous ethylenediammonium sulfate (G. Frederick Smith Co.) as previously described (4). Nitroferroin T.S. was used as the end point indicator; it was prepared by dissolving 150 mg. of 5-nitro-1,10-phenanthroline in 15 ml. of freshly made aqueous 1.4% ferrous sulfate.

Assay—Dissolve an accurately weighed sample of homatropine hydrobromide in distilled water or

suitably dilute an accurately measured volume of homatropine hydrobromide ophthalmic solution to obtain a concentration of about 8 mg./ml. Transfer a 10.0-ml. aliquot of the solution or dilution to a 100-ml. beaker, add 5 ml. of 1 *N* sodium hydroxide, and heat the solution just to boiling on a hot plate. Add 10 ml. of 1 *N* nitric acid, dilute the solution to 50 ml. with water, and cool it to 0–5° in an ice bath. Concomitantly add 5 ml. of 1 *N* nitric acid to a second 10.0-ml. aliquot of the solution or dilution, dilute it to 50 ml. with water, and chill it in an ice bath. Add 1 drop of nitroferroin indicator to each of the beakers, and titrate the solutions to the disappearance of the pink color while stirring magnetically, using 0.05 *N* ceric nitrate as titrant from a 10-ml. buret. Similarly, titrate a blank consisting of 50 ml. of 0.1 *N* nitric acid and 1 drop of indicator, and make any indicated corrections. (The indicator requires acidity for a sharp color change.) Each milliliter of 0.05 *N* ceric nitrate is equivalent to 8.907 mg. of $C_{16}H_{21}NO_3 \cdot HBr$.

The titer of the saponified aliquot is a measure of the total amount of intact and hydrolyzed drug; the titer of the unsaponified aliquot affords a value for hydrolyzed ester. Thus, the difference in titers is equivalent to unhydrolyzed homatropine.

Stability Evaluations—No samples of homatropine hydrobromide ophthalmic solution were available to the authors for this study. In order to demonstrate the applicability of the method to partially degraded samples, solutions of the drug buffered at pH 9.95 were heated at 30° for periods of time monitored by means of a stopwatch, the saponification reaction was quenched by addition of nitric acid, and the samples were assayed for intact homatropine as described above. The apparent first-order rate constant obtained was compared with that calculated by the equation developed by Patel and Lemberger (2).

RESULTS AND DISCUSSION

Accuracy and Precision—A comparison of the results obtained by the proposed method with the USP XVII acetous perchloric acid titration method for homatropine hydrobromide is presented in Table I. An average of 99.3 ± 0.3% (*RSD*) was obtained by the cerimetric method. The precision was comparable to that previously reported (4) for mandelic acid in methenamine mandelate, and it is comparable to the result of 99.2 ± 0.33% (*RSD*) obtained in the USP assay. It may be noted that the cerimetric and USP methods measure different parts of the homatropine molecule. The titer for the unsaponified aliquot in the cerimetric determinations of homatropine hydrobromide bulk drug was the same as for the indicator blank, indicating no hydrolysis.

Selectivity—It has been noted previously (4) that, although ceric nitrate is a highly selective oxidant under the specified conditions, it is by no means specific. Tropine was found to be slowly oxidized at room temperature. Good values were obtained for the mandelic acid content of homatropine at room temperature, but the indicator end point was transient. This interference was circumvented by performing the titration on a chilled solution.

Chlorobutanol and benzalkonium chloride, two of the commonly used preservatives for ophthalmic solutions, were found to be unaffected by the ceric titrant. Many of the easily oxidized preservatives such as parabens could be removed by solvent extraction from the acidified solutions if they were present.

Table I—Comparison of USP and Cerimetric Methods for Homatropine

	% $C_{16}H_{21}NO_3 \cdot HBr$
USP XVII method	99.6, 99.1, 99.5, 98.7, 99.3, 99.0
Proposed cerimetric titration	98.8, 98.8, 99.6, 99.0, 99.3, 99.6
	99.6, 99.3, 99.3, 99.6, 99.6, 99.6

Previous work (4, 5) has shown that tropic acid, the acid moiety of the natural solanaceous alkaloids, is not oxidized by ceric salts, therefore, the test for *Atropine and Other Solanaceous Alkaloids* in the USP XVII monograph would be unnecessary if cerimetric titration were used for the assay of homatropine. The selectivity inherent in the assay method would also eliminate the need for the test for *Most Other Alkaloids* in the homatropine hydrobromide monograph.

Use in Stability Evaluations—The kinetic study reported by Patel and Lemberger (2) on homatropine hydrolysis demonstrated that solutions of the drug have limited stability at the pH range recommended in USP XVII. Thus, the availability of a convenient method for stability evaluation of homatropine solutions is of more than theoretical interest in assessing product potency.

The general rate equation (2) affords a calculated first-order rate constant of 6.6×10^{-3} min.$^{-1}$ for the hydrolysis of homatropine at pH 9.95 and 30°. The average of four duplicate samples taken over a period of about 100 min. yielded an estimate of 6.0×10^{-3} min.$^{-1}$ for this constant.

SUMMARY AND CONCLUSIONS

Cerimetric titration of mandelic acid liberated by the saponification of homatropine is proposed as an assay for homatropine hydrobromide and homatropine hydrobromide ophthalmic solution. The titration is conducted on a chilled solution to circumvent interference of the tropine component of the saponification products, for tropine is slowly oxidized at room temperature. The selectivity of the oxidation reaction for arylglycolic acids eliminates the need for the tests for *Atropine and Other Solanaceous Alkaloids* and *Most Other Alkaloids* in the USP XVII monograph for homatropine hydrobromide. The proposed assay has the important advantage in comparison with the USP XVII procedure of being stability indicating. Like the official assay, the proposed method is absolute, *i.e.*, it does not require use of a reference standard.

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Keyphrases

Homatropine HBr—stability determination
Cerimetric titration, direct—analysis
Hydrolysis, homatropine HBr—detection

Technical Articles

Study on Dosage Variations of Individual Capsules and Tablets of Desipramine and Imipramine Hydrochloride

By S. AHUJA, C. SPITZER, and F. R. BROFAZI

Automated methods of analyses based on UV absorption of desipramine and imipramine hydrochloride were developed in order to obtain information on intercapsule and intertablet variations of the following six dosage forms: desipramine hydrochloride capsules 10, 25, and 50 mg. and imipramine hydrochloride tablets 10, 25, and 50 mg. The automated methods require the use of an automatic analyzer and the same manifold is used for all six dosage forms. A careful review of the results obtained on individual capsules and tablets reveals that almost all of the capsules and tablets analyzed by these methods were within ±15 percent limits of the indicated dosage.

THE PHARMACEUTICAL INDUSTRY has long been interested in obtaining more information on drug content of individual tablets or capsules to assure high standard of drugs, from the standpoint of production, quality control, and therapeutic activity. This information is necessary for establishing suitable control measures in production and quality control in order to obtain reasonable dosage uniformity of drugs.

Analytical methods which are based on the analysis of sample composites cannot provide reliable indications of content uniformity because they express product dosage on individual dosage forms, whereas the analyses are actually run on sample composites. Therefore, such analyses not only average out small variations in composition between individual tablets or capsules, but may also mask large deviations.

Quantitative analysis of the active ingredient itself, in each of the individual capsules or tablets, should provide a good approach for studying dosage variations. However, this approach was rather difficult to carry out until the recent introduction of automated methods of analyses.

The automated methods of analyses based on UV absorption of desipramine hydrochloride[1] [10,11 - dihydro - 5 - (3-methylaminopropyl) - 5*H*-dibenz[*b*,*f*] azepine hydrochloride] and imipramine hydrochloride[2] [10,11 - dihydro - 5 - (3-dimethylaminopropyl) - 5*H*-dibenz[*b*,*f*]azepine hydrochloride] were developed with the object of gaining information on intercapsule and intertablet variations on the following six dosage forms: (*a*) desipramine hydrochloride capsules (hard gelatin) 10, 25, and 50 mg., and (*b*) imipramine hydrochloride tablets (sugar coated) 10, 25, and 50 mg.

A variety of methods (1–8) have been used for the analysis of desipramine and imipramine hydrochloride. For several years manual UV methods have been used for the analysis of these compounds in these laboratories. These pro-

Received May 29, 1968, from Geigy Chemical Corporation, Ardsley, NY 10502
Accepted for publication August 8, 1968.
Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

[1] Pertofrane, Geigy Chemical Corp., Ardsley, N. Y.
[2] Tofranil, Geigy Chemical Corp., Ardsley, N. Y.

Keyphrases

Homatropine HBr—stability determination
Cerimetric titration, direct—analysis
Hydrolysis, homatropine HBr—detection

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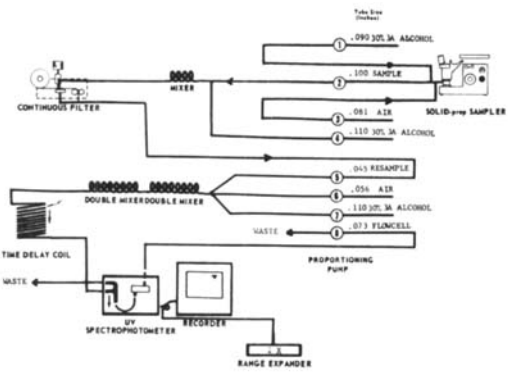

Fig. 1—Flow diagram.

cedures were automated by the use of the automatic analyzer.[3]

EXPERIMENTAL

Materials and Methods—Figure 1 shows a flow diagram of the analytical system including tubing sizes. The Solidprep sampler is programmed to operate at a rate of 13 samples/hr. The sample (tablet or capsule) is deposited in a cup placed on the turntable of the sampler. In turn, each sample is dumped into the homogenizer and homogenized with 125 ml. of 30% 3A alcohol[4] and a 0.9 ml./min. sample (segmented with air) is pumped from the sampler. The sample is diluted with 30% 3A alcohol, mixed, and filtered with the continuous filter. About 17% of it is resampled and diluted with additional 30% 3A alcohol, segmented with air, mixed with two double mixers, and then passed through a time delay coil.

The suitably diluted sample thus obtained is passed through a 15-mm. tubular continuous flow cell and its absorbance is recorded at an appropriate wavelength (imipramine = 250 mμ and desipramine = 252 mμ). Standards are run at various intervals to provide a means of sample concentration calculation and also to keep a check on any instrumental variations. The same manifold is used for all of the formulations and can handle concentrations of imipramine and desipramine ranging from 10 to 50 mg. per sample (tablet or capsule). Range Expander at 2× setting is used only for 10-mg. dosage forms.

The manifold was designed so that it can handle all six dosage forms without any loss of time in changeover procedures. Thirteen determinations can be made per hour with the automated methods as compared to 10 per day with the manual methods.

[3] AutoAnalyzer, Technicon, Chauncey, N. Y.
[4] US Industrial Chemical, Newark, N. J.

RESULTS AND DISCUSSION

A linear relationship was observed between the concentration of imipramine and desipramine hydrochloride and absorbance in the range of concentrations studied for this investigation. The precision of the method was checked by running standards and synthetic formulations (prepared in a manner similar to commercial formulations). Table I shows the precision of the results obtained on standards in various concentrations.

The percentage recovery (an average of 10 individual determinations) of two synthetic formulations, each containing 50 mg. of imipramine and desipramine hydrochloride, was 100.2% (*SD* = ±0.94) and 100.1% (*SD* = ±0.91), respectively. By running placebos of these formulations, it could be shown that there was no significant interference from the excipients. Examples of the recordings obtained for these formulations are shown in Figs. 2 and 3.

In order to study dosage variation a very large number of individual capsules (590) and tablets (730) were analyzed by these methods. The distribution of dosage in each of these dosage forms is presented in the form of a histogram in Figs. 4–9, where the ordinate indicates the number of capsules or tablets, and the abcissa, the number of milligrams of active ingredient present in an individual capsule or tablet. The capsules and tablets used for this study were randomly selected from several production batches, where possible, in order to obtain a good overall picture of dosage variation. The results on interbatch and intrabatch variations will be the subject matter of another publication. The results of the present investigations are summarized in Table II.

Of the 590 individual desipramine capsules analyzed, over 94% were within ±10% limits of the indicated dosage while 100% were within ±15% limits of indicated dosage. Of the 730 individual imipramine tablets analyzed, over 90% were within

Table I—Precision of the Automated Methods

No.	Sample	Results (Av. Abs.)	No. Determinations	Range Expander	*SD*
1	Imipramine HCl, 50 mg.[a]	0.372	10	—	±0.004
2	Imipramine HCl, 25 mg.[a]	0.176	10	—	±0.003
3	Imipramine HCl, 10 mg.[b]	0.152	10	2×	±0.003
4	Desipramine HCl, 50 mg.[a]	0.403	10	—	±0.004
5	Desipramine HCl, 25 mg.[a]	0.191	10	—	±0.001
6	Desipramine HCl, 10 mg.[b]	0.170	10	2×	±0.002

[a] In solid form. [b] In solution form.

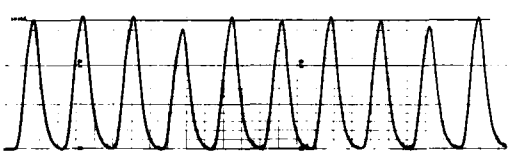

Fig. 2—Typical recording of desipramine capsules, 10 mg.

TABLE II—DOSAGE VARIATION OF DESIPRAMINE CAPSULES AND IMIPRAMINE TABLETS

Dosage, mg.	% Capsules or Tablets Within Indicated Dosage							
	± 2%		± 5%		± 10%		± 15%	
	Tabs.	Caps.	Tabs	Caps.	Tabs.	Caps.	Tabs.	Caps.
10	27.7	32.5	57.4	65.0	90.3	96.7	99.4	100.0
25	38.1	49.7	79.6	67.8	99.2	94.1	100.0	100.0
50	45.2	42.0	82.9	88.7	99.0	100.0	100.0	100.0

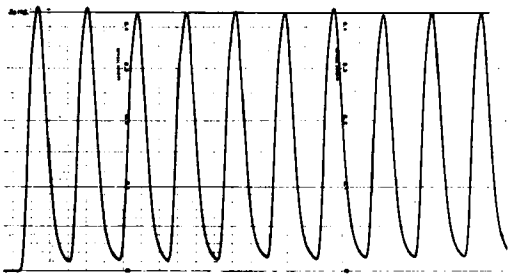

Fig. 3—Typical recording of imipramine tablets, 50 mg.

±10% limits of indicated dosage, and all except one tablet (10 mg.) were within ±15% limits of indicated dosage. The results, therefore, very strongly suggest that in the case of these capsules, the content uniformity is at least as good, if not better than that of tablets, in the dosage range studied for these formulations.

The current specifications for tablets requiring a content uniformity test are: the first 10 tablets analyzed out of a representative sample of 30 tablets (or 29 out of 30 tablets) should be within the limits of 85 and 115% of the average of the specified tolerances in the monograph. A content uniformity test for capsules is presently being considered for the new editions of USP and NF. Needless to say, these capsules and tablets would have passed the content uniformity test of tablets of the compendia. Furthermore, these results provide some indications as to the type of specifications that could be set for capsules.

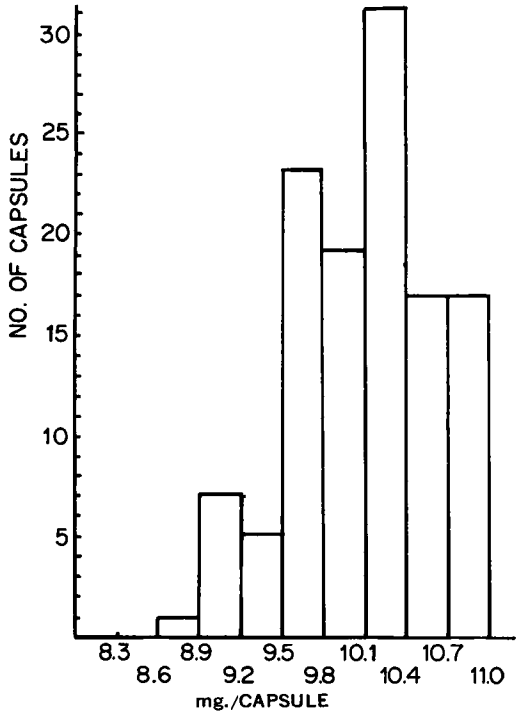

Fig. 4—Histogram of desipramine capsules, 10 mg.

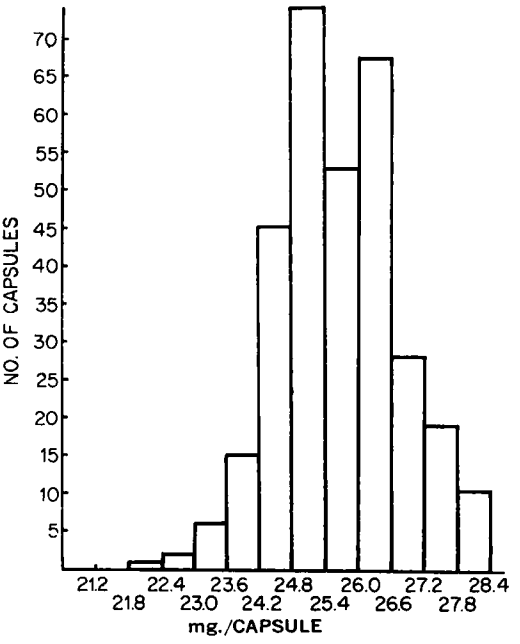

Fig. 5—Histogram of desipramine capsules, 25 mg.

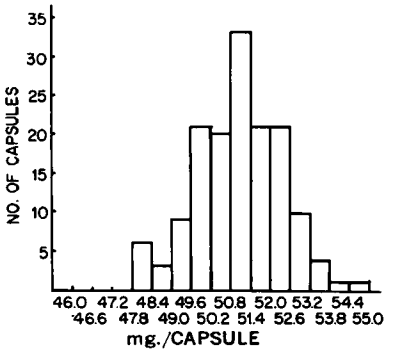

Fig. 6—Histogram of desipramine capsules, 50 mg.

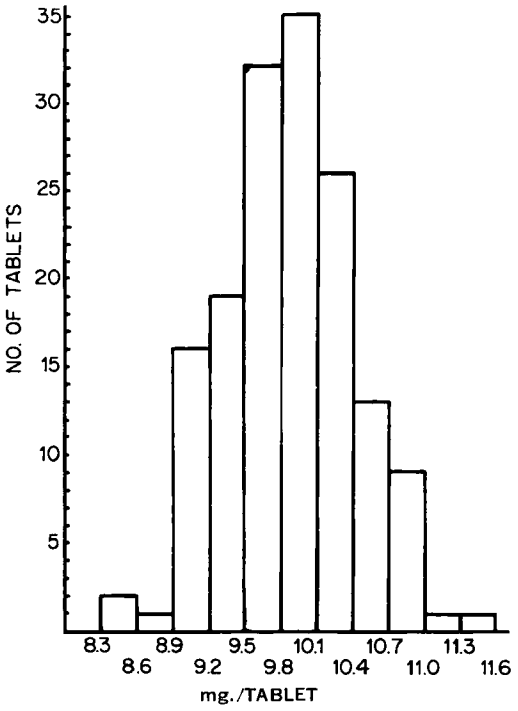

Fig. 7—Histogram of imipramine tablets, 10 mg.

CONCLUS ONS

A careful review of the results obtained from a large number of individual capsules and tablets reveals that of the 1,320 individual capsules and

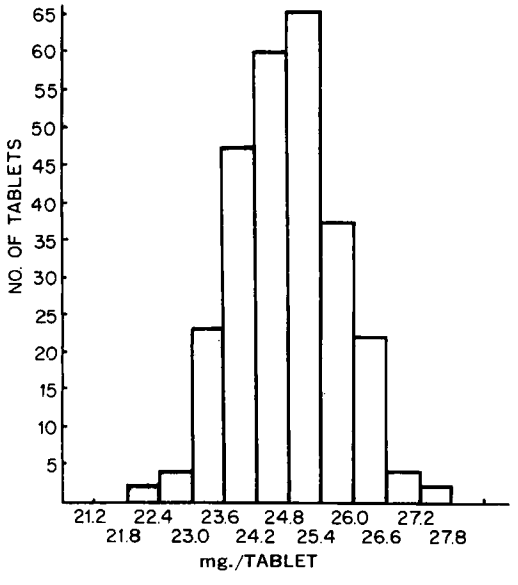

Fig. 8—Histogram of imipramine tablets, 25 mg.

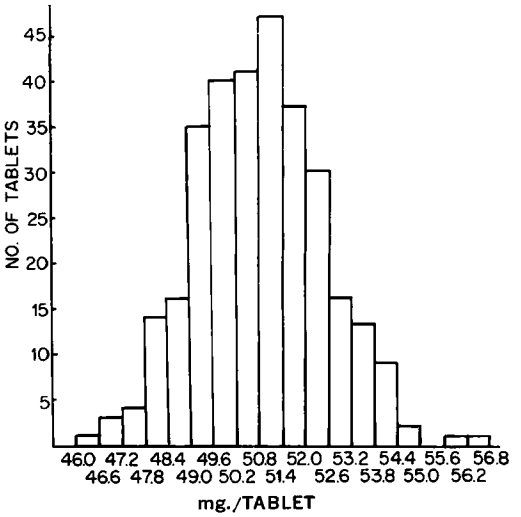

Fig. 9—Histogram of imipramine tablets, 50 mg.

tablets analyzed by the automated methods, only one tablet (10 mg.) was found to be outside the ±15% limits of the indicated dosage. Therefore, it may be logically concluded that with these formulations and with the equipment used to manufacture these tablets and capsules,[5] it is possible to produce capsules with dosage uniformity comparable to that of tablets.

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[5] Hard-shelled gelatin capsules (No. 4), used for these formulations, are filled with the Zanasi machines. The percentages (w/w) of the active ingredient in the capsule fills of the 10, 25, and 50-mg. capsules are 5.50, 13.75, and 27.50, respectively.

Keyphrases

Dosage variation—individual capsules, tablets
Desipramine HCl capsules, individual—analysis
Imipramine HCl tablets, individual—analysis
UV spectrophotometer—analysis
Diagram—automated apparatus

An Apparatus and Method for the Coating of Solid Particles

By MANFORD J. ROBINSON, GEORGE M. GRASS, and RUSSELL J. LANTZ

A method for the coating of discrete solid particles and a diagram of the apparatus used is presented. In this process, solid particles are educted from a fluidized bed by a turbulent gas jet, separated into discrete units, and transported upward through a converging cone into a coating application zone. In this zone, the solid particles are contacted by discrete liquid droplets comprising the coating material. These liquid droplets are entrained in a downward moving airstream which has a greater velocity than the upward moving solid particles. The velocity differential removes the wetted solid particles from the coating zone and returns them to the fluid bed. The coating liquid is spread over the surface of the solid particles in this bed prior to their reeduction into the turbulent gas jet. The cycle is repeated until coatings of the desired kind and thickness are obtained.

COATING PHARMACEUTICAL DOSAGE forms or their component particles is an old and extensive practice. The purpose of coating has been to modify chemical or physical properties, such as taste, appearance, solution rate, reactivity, sensitizing or irritation potential, flowability, compressibility, dusting propensity, *etc.* Methods or apparatus used in the pharmaceutical industry to coat solid particles include rotating pans (1), fluid and spouted beds (2, 3), and tablet presses (4). Coacervation (5), spray-drying (6–8), and emulsification (9) have been used to entrap or encapsulate solid particles.

The vast majority of coating is done in rotating pans. This predilection for one apparatus is due, in part, to tradition and to this industry's large investment in pan coating equipment and technology. It is also because the more esoteric processes are often complex, costly, and in some instances have minimal advantage over pan coating. This combination of factors has resulted in a tendency for the pharmaceutical industry to coat only those particles which can be handled in a rotating pan. This is unfortunate because it limits the obvious utility of coating to large monosized particles, and limits the coating materials to a few nonadhesive substances. It appeared desirable to develop a simple, inexpensive apparatus and method capable of coating most of the solid particles used in the pharmaceutical industry with a wide variety of coating materials. This report describes and outlines the method of operation for a coating device developed in this laboratory (10). This device has been used to coat solid particles that have mean diameters ranging from about 10 to 10^3 μ with materials having widely different properties. It is simple, inexpensive, and appears to have desirable characteristics not possessed by other coating equipment.

Subsequent reports will present an evaluation of coated particles prepared using this device and illustrate the utility of using coated particles in the formulation of pharmaceutical dosage forms.

Received June 14, 1968, from the Research and Development Division of Smith Kline & French Laboratories, Philadelphia, PA 19101

Accepted for publication August 13, 1968.

Presented in part at the 63rd National meeting of the A.I.Ch.E., St. Louis, Mo., February 20, 1968.

DISCUSSION

Description of the Coating Apparatus—A schematic drawing of a laboratory size particle coating unit is shown in Fig. 1. It consists of a conical base (A), vertical casing (B), expanded upper section (C), and a gas-vapor exhaust system (D). The bottom of the unit contains a receptacle for air inlet nozzles (E) of varying cross-section and design. A converging tube (F) is centered in the vertical column and this tube can be moved vertically up or down. An air ring (G) with a series of holes on its inner surface is located between the conical base and vertical casing; the air inlet holes are slightly above the base of the converging tube. A vertically adjustable tube (H) is centered in the expanded upper section and this tube contains gas and liquid feed lines. The lower end of tube H is designed to accept atomizing nozzles of varying design. The center of the atomizing nozzle is in precise alignment with the centers of E and F. The gas vapor exhaust system

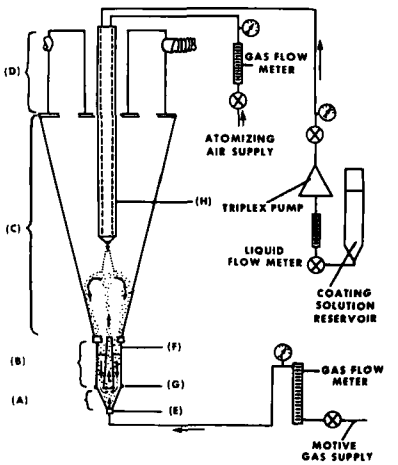

Fig. 1—A schematic representation of the particle coating device.

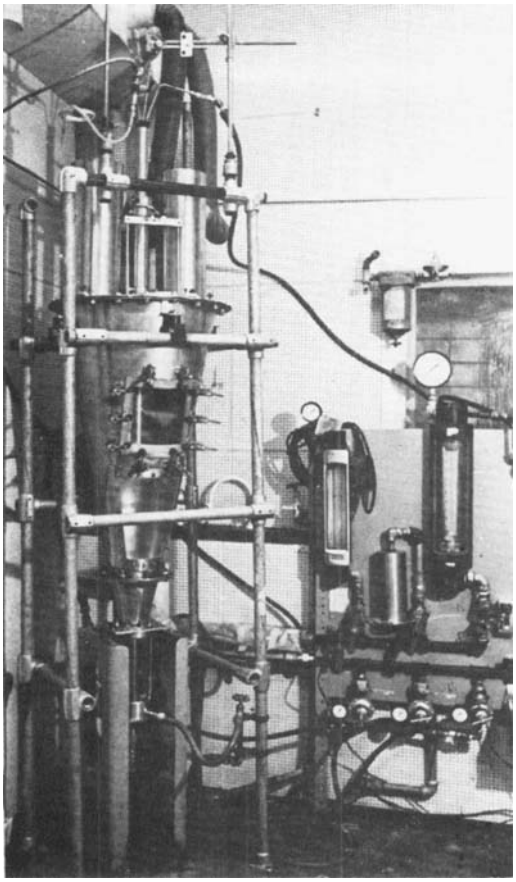

Fig. 2—The laboratory model particle coating device.

contains two frame-supported cloth bags enclosed in cylindrical caps. These caps contain outlets which are connected with flexible hoses to an exhaust blower. Combination sight and fill ports are located in the top and side of the expanded upper section. Air actuated vibrators[1] are attached to B and C. All metal parts are fabricated from stainless steel.

The gas and liquid feed systems are also shown in Fig. 1. Liquid wetted parts are either glass or stainless steel. Compressed air is obtained from a compressor[2] capable of supplying 100 s.c.f.m. at 100 psia. Air entering the device is filtered[3] and dried to a dew point of −40°C. by passing it through a silica gel column.[4] Gases other than air, such as nitrogen or carbon dioxide, are obtained from pressurized cylinders. A thermostatically controlled heater[5] having a range of 150–550°F. is used to heat the gas when indicated. Solutions of the coating material are pumped to the atomizing nozzle by a triplex metering pump.[6] Flowmeters[7] are used to measure both gas and liquid flow and this flow is controlled by conventional valves and pressure gauges. Photographs of a laboratory and pilot unit are shown in Figs. 2 and 3.

[1] Vibrolator, Martin Engineering Co., Neponset, Ill., and Piston Vibrator, Branford Vibrator Co., New Britain, Conn.
[2] Gardner Denver, Quincy, Ill.
[3] Dollinger, Rochester, N. Y.
[4] Oriad, C. M. Kemp Mfg. Co., Glen Burnie, Md.
[5] Chromalox, Edwin L. Weigand Co., Pittsburgh, Pa.
[6] Wallace and Tiernan, Belleville, N. J.
[7] Fischer-Porter, Warminster, Pa.

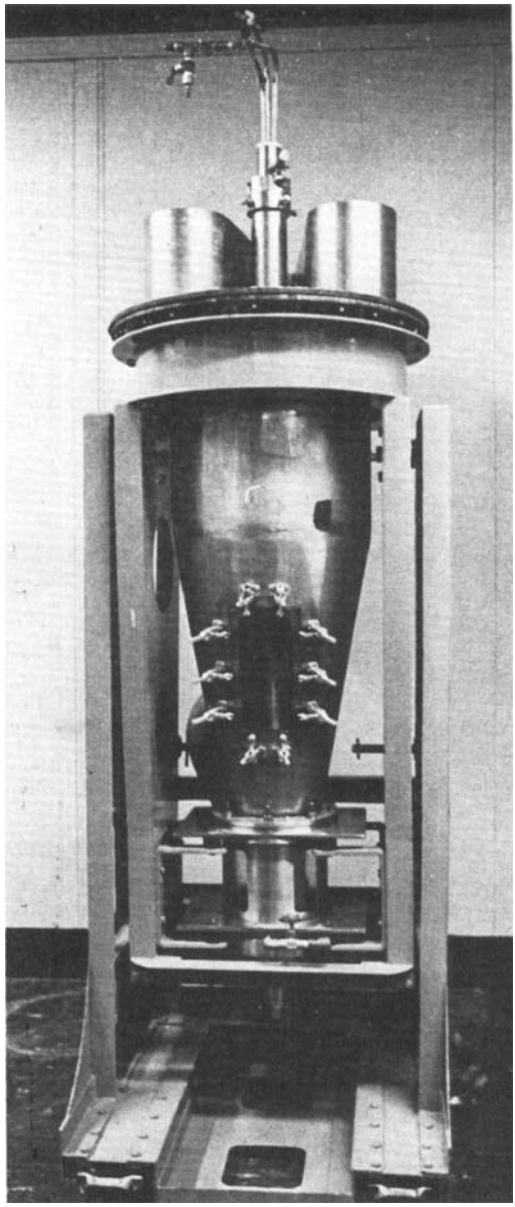

Fig. 3—The pilot model particle coating device.

Operating Procedure—The coating process can be subdivided into distinct phases. These phases include: (*a*) separating of the particle assemblage into discretely separate units of the desired size, (*b*) applying of liquified coating material onto the surface of these units, (*c*) spreading of the coating material uniformly over the surface of the units, (*d*) continuing of phases *a* through *c* until the required coating thickness is obtained, and (*e*) solidifying of the coating and collecting of the coated particles. The flow of particles in the coating device is shown schematically in Fig. 1. Particles introduced through an inlet port form a dense phase bed in the annular space between the walls of the vertical column B and the converging tube as shown in Fig. 4. The particles are fluidized by the action of the vibrators and this fluid state can be augmented by

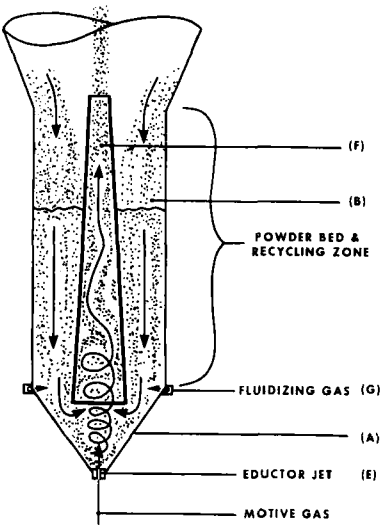

Fig. 4—A schematic representation of the particle collection and separation section.

passing gas through the holes in the air ring G. A jet of gas moving through nozzle E educts particles from the base of this bed as distinctly separate units and transports these units upward through the converging tube F. The frequency and amplitude of vibration, gas velocity, and particle flow rate are adjusted to provide particles of the desired size. The volume of gas moving through nozzle E is also adjusted so that the particle velocity in F exceeds the saltation or choking velocity but does not reduce the solid volume concentration below about 1.5%.

A solution of coating material and entraining gas moves under pressure through the tubing in H. The coating solution is atomized by a nozzle at the tip of H, as shown in Fig. 5. The gas velocity is adjusted so that it exceeds the velocity of the droplets and the spray of particles issuing from the outlet of tube F. The spray angles and distance between F and the liquid atomizing nozzle are adjusted to give maximum particle to droplet contact. The velocity of

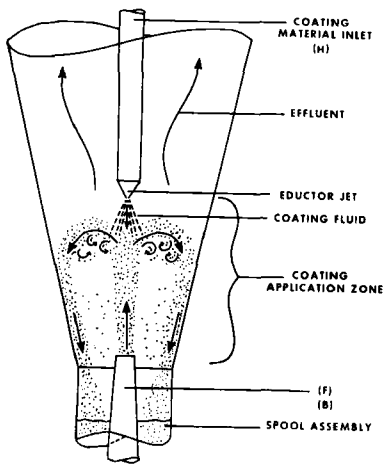

Fig. 5—A schematic representation of the coating application section.

the opposed gas streams creates a zone of intense mixing in which there is essentially no solidification of liquid particles. The velocities of the two opposed gas streams are controlled so that the downward motive force is greater. This results in the particles being scrubbed out of the upwardly moving gas stream and returned to the dense phase fluid bed. As the wetted particles move downward through this bed, they are again deagglomerated and the liquified coating is spread over the surface of the separated particles.

The particles are continuously cycled in this manner until the desired amount of coating has been applied. The liquid feed is then stopped and additional drying gas admitted through the air ring G. When the solvent has evaporated the particles can be removed through a port in the base section A. At this point, it is also possible to continue coating with a second material if desired.

A powder may be added during the coating cycle. This is accomplished by adding the solids to the top of the fluid bed during coating, or by mixing them with the liquified coating material, and then reducing the particle-separating forces to a level at which the added particles are not separated in the coating cycle.

Comparison of Coating Processes—A major difference between this coating method and others (rotating pan, fluid, and spouted bed) is the range and control of the particle-separating forces. The need for range and control is most clearly seen when the factors associated with particles separation are considered. To separate a particle from an assemblage requires that the separating force exceed the forces holding the assemblage together. In a bed of particles, the force resisting separation is the sum of the particle(s) weight plus the interparticle adhesive forces. The latter are related to properties of the particles and of the coating material. In the absence of coating material, particles agglomerate primarily because of van der Waals' attractive forces and/or adsorbed fluid. Electrostatic bonding forces are usually negligible (11, 12). These bonding forces act at points of particle contact and the area of contact depends on bed density, particle size, and particle shape. When an agglomerate has been separated into units of the desired size, and sufficient coating material applied to entirely cover the surface of the particle, the adhering force is proportional to the tensile strength of the liquified coating film. Depending on the properties of the coating material, this film tensile strength may increase or decrease as it solidifies. From this generalized description, it can be seen that the separating force needed to coat particles must vary considerably and will depend on the properties of substance to be coated and the coating materials.

The limitation of space does not permit a complete presentation of the theory and supporting experimental data associated with particle separation in this or other coating processes. Factors associated with particle separation in fluid beds have been reviewed in detailed by Zenz and Othmer (13). These factors will also be discussed in subsequent publications, in which theory and experimental data associated with solid particle coating in rotating pans and in this particle coating device will be presented. Differences in particle separation in various coating devices can be illustrated by comparing the relation-

ship between particle weight (G_p) and the interparticle adhesive forces (ψ). In a fluid or spouted bed a major force for particle separation is the drag resulting from the interaction among the particle and upward flowing gas. This drag force is given (12) by

$$F_d = \alpha\,^{1}/_{2}\, C_d \rho_f A_p V_f^2 \qquad \text{(Eq. 1)}$$

For particle separation to occur

$$F_d = G_p + \psi \qquad \text{(Eq. 2)}$$

The superficial gas velocity (V_f) to provide this drag then is

$$V_f = \left(\frac{G_p + \psi}{\alpha\,^{1}/_{2}\, C_d \rho_f A_p}\right)^{1/2} \qquad \text{(Eq. 3)}$$

However, at the required superficial gas velocity the ability to fluidize or spout a bed of particles and the quality thereof is determined by the relative magnitudes of G_p and ψ (12). When ψ is large relative to G_p the bed may not fluidize or spout because there is insufficient particle flow to break up the channels created by the upwardly moving gas stream. Alternatively, particle flow may be of a stick-slip type which results in an erratic, surging, slugging bed; this type of bed is poorly adapted for coating. Under this limiting condition ($\psi \gg G_p$), there will also be considerable elutriation of particles from the bed, because the superficial gas velocity required for particle separation must be greater than that required for conveying. Since G_p is proportional to particle size (D_p) there is a limit below which particles cannot be coated in either a fluid or spouted bed coater. This lower limit varies with the material used (both particle and coating), but in the authors' experience, the limiting value for D_p is near $10^2\,\mu$.

When particles are coated in a rotating pan, there is a similar relationship between G_p and ψ. When a bed of particles is rotated in a pan, that part of the bed adjacent to the wall is carried to a given height by wall friction or baffles. At this height the particles will flow or fall back into and through the bed. The particles closest to the wall will travel the greatest distance and will return to the bed under essentially free fall conditions. It is this condition of particles falling on and through the bed that provides, to a considerable extent, the mixing and separation required for coating. The size of the units which separate at a given height depends on the relationship between G_p and ψ. If the weight of a single particle is greater than ψ, the particles separate and flow through the bed as individual units. If it is not, the particles will separate as agglomerates, which will either move to the front of the pan or be compacted to form aggregates. These aggregates will increase in size until G_p is larger than ψ. There are also separating forces in a coating pan which are associated with (*a*) the weight of the bed above a given assemblage of particles and (*b*) the centrifugal force (torque) which induces the particles in a bed to rotate. Although related to particle weight (size) and ψ, these separating forces are probably small when ψ is large relative to G_p. In the writers' experience,the limiting value for D_p in a coating pan is about two to three times that of a fluid or spouted bed coater.

The relationship between G_p and ψ is considerably less critical for the particle coating device (PCD). When an assemblage of particles is introduced into this unit, it will break up into units with dimensions that are dependent on the magnitude of G_p and ψ. If ψ is large relative to G_p, these units will be agglomerates. As shown in Fig. 4, these units fall into and form a dense phase bed, whose mass (essentially plug) flow is controlled by the frequency and amplitude of vibration, use of fluidizing gas, and the cross-sectional area of the outlet. Vibration or fluidization may augment particle separation but the major separating force is the turbulent jet into which these particles are educted. Gas flow from this jet can be adjusted to provide the velocity needed for particle transport and separation (Eq. 3). The conveying gas velocity is relatively uniform in the converging nozzle, because the flow is turbulent. The volume flow of conveying gas is controlled by the cross-sectional area (or design) of the inlet nozzle (E). By controlling gas velocity and bed flow it is possible to reduce agglomerates to particles having a D_p as small as 5 μ, and to transport the deagglomerated particles upward through the converging tube without saltation, choking, or slugging. The motion of a particle as it leaves the converging nozzle is described by the relationship

$$\text{inertial force} + \text{drag forces} = -\text{gravitational force} \qquad \text{(Eq. 4)}$$

It is obvious from the foregoing that the magnitude of these forces is associated with the relationship between G_p and ψ. In addition to the force of gravity, the upward motion of the particles is opposed by the downward motion of the coating liquid droplets and their entraining gas stream. The inertial force (F_i) for both particles and droplets is given by

$$F_i = M\frac{dv}{dt} \qquad \text{(Eq. 5)}$$

A droplet inertial force greater than that of the solid particle can be obtained by control of droplet size and velocity. This is done in the PCD by proper selection and operation of the atomizing nozzle. For example if a two-fluid educting-type nozzle is used, droplet size and velocity are related to gas velocity and liquid feed rate (14). It is also possible to vary the gas velocity flowing from tube H, and thus create a net downward drag on the wetted particles in the area of opposing gas flow. Through control of these variables, upward particle motion is arrested and redirected towards the wall of the expanded chamber, as illustrated in Fig. 5. However, there is always a net upward flow of gas plus vapor to the exhaust. The drag created by this upward flow is sufficient to elute particles having low values of G_p from the particle assemblage as it flows towards the wall. These particles are either suspended in the expanded section or carried upward into the bag collectors; they are not coated. In practice, it has been found that this loss is significant only when the majority of particles have D_p less than about 5 μ, probably because there is considerable agglomeration of particles in the coating zone, which reduces elutriation and increases G_p.

Examples of Coated Particles—Through control of particle separation and transport, it is possible to coat particles having a large range of G_p and ψ, and also to use coating materials having large differences in film tensile strength. The utility of the PCD is illustrated by the following examples of coated solid

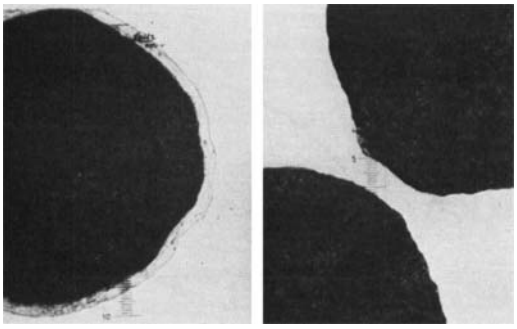

Fig. 6—Uncoated and ethycellulose coated sugar pellets.

particles. Photomicrographs of sugar pellets ranging in D_p from 710–840 μ which have been coated with ethylcellulose[8] are shown in Fig. 6. The solution rate of sugar from these pellets is shown in Table I. Solution rates were determined using the method of Souder and Ellenbogen (15).

TABLE I—DISSOLUTION OF SUGAR FROM ETHYLCELLULOSE-COATED PELLETS

Run No.	% Coating	% Dissolved, hr. 0.5	2	4.5
1	10.4	24	65	81
2	10.3	20	65	81
3	10.2	19	66	80
4	10.7	—	—	81
5	10.7	—	—	80
6	10.6	—	—	81

Photomicrographs of acetylsalicylic acid crystals coated with cellulose acetate phthalate[9] are shown in Fig. 7. The solution times for these enteric-coated crystals are given in Table II. Figure 8 shows the rate of CO_2 evolution when $NaHCO_3$ particles coated with hydrogenated castor oil[10] are intensively agitated in 0.1 N HCl solution. The acetylsalicylic acid and $NaHCO_3$ crystals were obtained from commercially available powders meeting USP standards. Photomicrographs of acetaminophen particles coated with 12-hydroxystearyl alcohol[11] are shown in Fig. 9. These closely sized particles were obtained by spray drying and had $D_p \approx 15$ μ prior to coating. The solution rate of acetaminophen in USP intestinal fluid at 37°C. is shown in Fig. 10. The size distributions for micronized saccharin and for saccharin coated with 12-hydroxystearyl alcohol are shown in Fig. 11. The dissolution of the coated saccharin particles in distilled water at 25°C. is shown in Fig. 12.

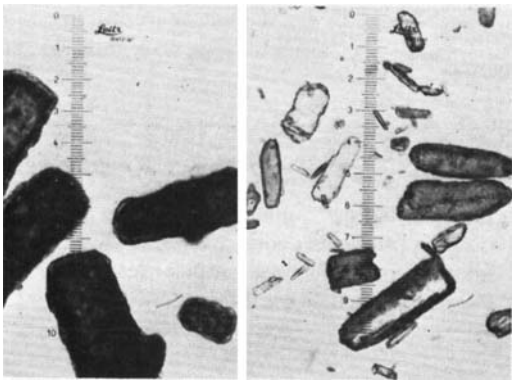

Fig. 7—Uncoated and C-A-P coated acetylsalicylic acid crystals.

TABLE II—SOLUTION TIMES FOR ENTERIC-COATED ACETYLSALICYLIC ACID CRYSTALS

Run No.	% Coating	% Dissolved—USP Gastric Fluid after 4 hr.	USP Intestinal Fluid after 15 min.
1	36.6	9	99
2	37.2	4	99
3	38.4	7	98

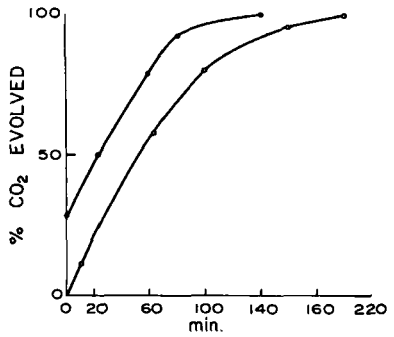

Fig. 8—Rate of CO_2 evolution from $NaHCO_3$ crystals coated with hydrogenated castor oil. Key: $NaHCO_3$: HCO, ●—●, 2:1; ○—○, 1:1.

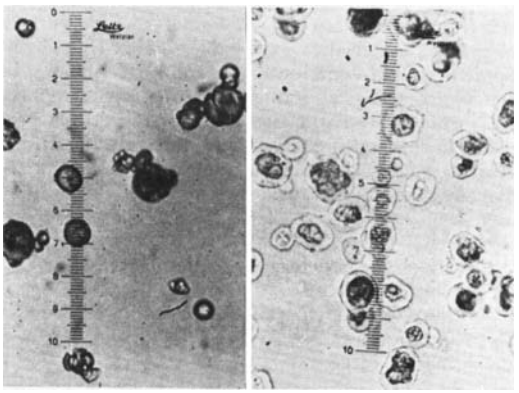

Fig. 9—Uncoated and coated particles of acetaminophen.

These few examples illustrate typical particles which have been coated using the particle coating device. They are representative of the majority of the solid particles found in the pharmaceutical industry. It is interesting that this flexibility was obtained with a single, simple device without the need to employ a variety of complex, expensive coating procedures.

[8] Ethocel, Std. Ethoxy 10 cps. Dow Chemical Co., Midland, Mich.
[9] C-A-P, Distillation Products, Rochester, N. Y.
[10] Castor Wax, Baker Castor Oil Co., Bayonne, N. J.
[11] Adol 45, Ashland Chemical Co., Minneapolis, Minn.

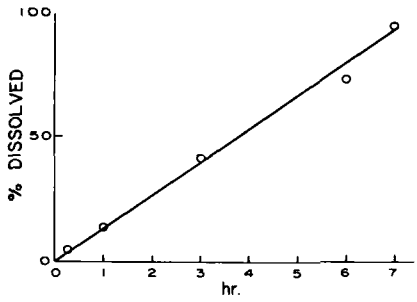

Fig. 10—Solution rate of coated acetaminophen particles in USP artificial intestinal fluid.

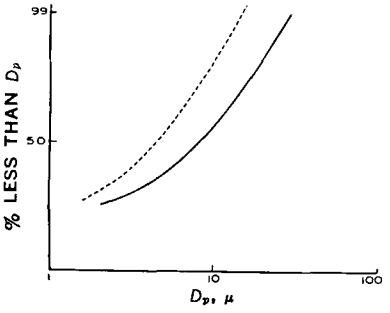

Fig. 11—Size distribution of micronized and coated saccharin particles. Key: - - -, uncoated; ——, coated.

SUMMARY

The description and method of operation for a solid particle coating device has been presented. It appears that, by control of particle separation and transport, it is possible to coat particles ranging in size from about 10 to 10^3 μ in diameter with a variety of coating materials. It is believed that this development should contribute to coating technology in the pharmaceutical industry. Subsequent reports will present an evaluation of coated particles prepared in this coating device and illustrate the utility of using coated particles in the formulation of pharmaceutical dosage forms.

NOMENCLATURE

A_p = effective particle cross-sectional area
C_d = drag coefficient
D_p = representative particle diameter
F_d = drag force
F_i = inertial force

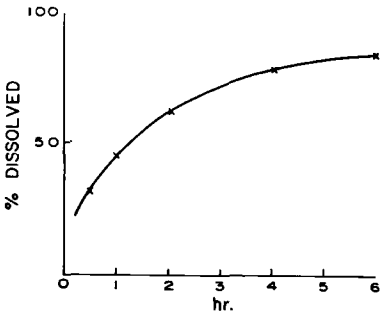

Fig. 12—Solution rate of coated saccharin particles in distilled water.

G_p = particle weight
M = mass
t = time
v = velocity
V_f = superficial velocity
α = constant
ρ_f = density of the gas
ψ = effective interparticle force

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Keyphrases

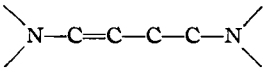
Particle coating—apparatus, method
Coating processes—comparison
Photomicrographs—coated particles
Diagram—particle coating apparatus

Mannich Bases from 2-Phenylindolizines III. 1,3-Bis(dialkylaminomethyl)-2-phenylindolizines

By WILLIAM B. HARRELL* and ROBERT F. DOERGE

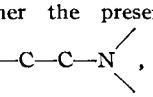
In seeking new therapeutic agents derived from indolizines, four new Mannich bases derived from 2-phenylindolizine have been synthesized. Preliminary pharmacological screening of 3-diethylaminomethyl-1,2-diphenylindolizine, a compound previously reported in these studies, has been carried out and the results are given.

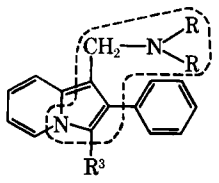
THE STRUCTURAL ANALOGY between indolizines with dialkylaminomethyl side chains and certain biologically interesting indoles has been pointed out in previous publications by the authors (1, 2). It is suggested that compounds such as reserpine, lysergic acid diethylamide, and psilocin might owe their activity in part to the presence of a certain spatial arrangement between the indole nitrogen and the extraindole nitrogen:



In Part I of this study the preparation of a series of Mannich bases derived from 2,3-disubstituted indolizines was reported. One compound in the series, 1-diethylaminomethyl-3-methyl-2-phenylindolizine, was tested and found to exhibit central nervous system depressant activity.

In these studies the Mannich reaction (3) has been employed because it is a convenient way to introduce dialkylaminomethyl side chains at the C-1 or C-3 positions of the indolizine nucleus. The primary purpose of this study was to synthesize a number of Mannich bases derived from selected 2-phenylindolizines and to evaluate them for possible activity on the central nervous system. If significant activity was observed, an attempt to correlate chemical structure with activity would be made.

To date three types of Mannich bases derived from 2-phenylindolizines have been synthesized. These compounds have the general structures represented below. The structural analogy between Types I and III with lysergic acid diethylamide (IV) is also shown; thus it will be of interest to determine whether the presence of the grouping,  , has any influence on the activity of these compounds.



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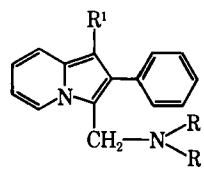

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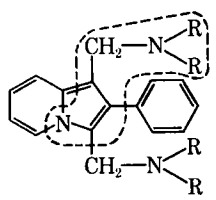

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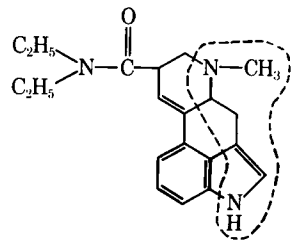
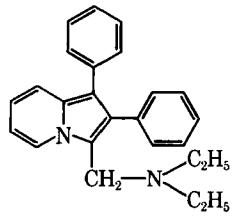


IV

The syntheses of Types I and II have been reported in previous publications by the authors (1, 2). In addition to the activity previously reported for 1-diethylaminomethyl-3-methyl-2-phenylindolizine, a compound of Type I, the following preliminary pharmacological data have been obtained for 3-diethylaminomethyl-1,2-diphenylindolizine (V), a compound of Type II:



V

When injected intraperitoneally in mice as a solution in propylene glycol, the compound, in doses of 10 mg./kg. body weight, exerted a pronounced central nervous system depressant effect. The animals did not appear to sleep and were easily aroused. Spontaneous motor activity was measured through the use of a photoelectric cell activity cage and was found to be decreased in mice. Rats, when given the compound intraperitoneally in propylene glycol exhibited a loss of aggressiveness. The compound

Received May 22, 1968, from the School of Pharmacy, Oregon State University, Corvallis, OR 97331
Accepted for publication June 25, 1968.
Presented to the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
Abstracted in part from a thesis submitted by William B. Harrell to the Graduate School, Oregon State University, in partial fulfillment of Doctor of Philosophy degree requirements.

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showed no significant anticonvulsant activity by the supramaximal electroshock method using mice. The LD_{50} was found to be 215 mg./kg. body weight using mice.

A more extensive biological evaluation of these compounds and others prepared in this study is currently being carried out and the results will be reported elsewhere.

Reports in the literature on the utilization of the Mannich reaction with indolizines have been very scarce and the scope of such investigations has been limited. Rossiter and Saxton (4) prepared 1-dimethylaminomethyl - 2,3 - dimethylindolizine by treating 2,3-dimethylindolizine with formaldehyde and dimethylamine. Carbon and Brehm (5), following the method of Rossiter and Saxton, reported the synthesis of 3-acetyl-1-dimethylaminomethylindolizine. To date no other Mannich bases derived from indolizines have been reported in the literature, except those previously described by the present authors. In these studies the syntheses of Mannich bases which involved the C-1 position have been described, the C-3 position, and both the C-1 and C-3 positions of the indolizine nucleus. A mechanism by which indolizines participate in the Mannich reaction in forming Mannich bases at these two positions (1, 2) has been proposed. In continuing medicinal chemical investigation of indolizines, further studies involving the Mannich reaction are planned.

EXPERIMENTAL

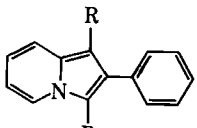
All melting points were taken on a Thomas-Hoover capillary melting-point apparatus and are uncorrected. Elemental analyses were provided by Weiler and Strauss Microanalytical Laboratory, Oxford, England. Preliminary pharmacological screening of V was carried out with the assistance of Dr. Edward J. Eugere at Texas Southern University.

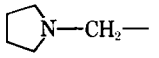
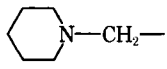
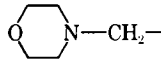
Materials—The parent indolizine employed in the syntheses of the Mannich bases in this series was 2-phenylindolizine (Table I). This compound was prepared by the Chichibabin synthesis (6) and has been previously reported in the literature (7).

1,3-Bis(dimethylaminomethyl)-2-phenylindolizine (VI)—A mixture containing 20 ml. dimethylamine (0.11 mole), 6 ml. of 37% aqueous formaldehyde (0.075 mole), and 0.1 ml. of 50% sodium hydroxide was allowed to stand in an ice bath for 20 min. The mixture was added slowly with shaking to a flask containing 2 g. 2-phenylindolizine (0.01 mole) dissolved in 50 ml. *N,N*-dimethylformamide. The flask was stoppered and allowed to stand at room temperature for 72 hr. Water was added in excess very slowly to the reaction mixture and the product precipitated as a heavy oil. Excessive shaking was avoided during the addition of water to prevent the formation of an emulsion. After allowing to stand overnight, the aqueous layer was carefully decanted. The semisolid residue was dissolved with heating in a minimum amount of hot ethanol. Water, equivalent to about 10% of the volume of the alcoholic solution, was added while the solution was still hot. After allowing to cool to room temperature, the clear solution was placed in the freezing compartment of the refrigerator. The product crystallized after 2 days in the refrigerator. The product was removed by filtration and washed with 50% ethanol. The yield was 2.7 g. (88%). Upon recrystallization from hot ethanol the compound gave m.p. 68.5–69.5°.

1,3 - Bis(pyrrolidinomethyl) - 2 - phenylindolizine (VII)—A mixture containing 10 ml. dioxane, 1.8 g. pyrrolidine (0.025 mole), and 1.8 ml. 37% aqueous formaldehyde (0.023 mole) was allowed to stand at room temperature for 15 min. To this mixture was added 0.97 g. 2-phenylindolizine (0.005 mole) and, after swirling occasionally for about 1 hr. to dissolve the indolizine, the resulting solution was allowed to stand for 48 hr. at room temperature. The mixture was poured into an evaporating dish and cold air was blown over the surface to remove the solvent. When evaporation was nearly complete, the oily residue was washed with water and dried over anhydrous sodium sulfate. The sodium sulfate was removed by filtration, washed with 5–7 ml. ether, and the filtrate placed in the freezer compartment of the refrigerator. After 3 days, the inside of the flask was scratched with a glass stirring rod and returned to the freezer. Crystals appeared within 1 hr. The mixture was allowed to remain in the freezer for 4 hr. longer to allow crystallization to be completed. The product was removed by suction, washed with cold ethanol, and recrystallized from

TABLE I—MANNICH BASES PREPARED



Compd.	Empirical Formula	R	M.p., °C.	Yield, %	Recrystn. Solvent		Anal., % Calcd.	Found
VI	$C_{20}H_{25}N_3$	$(CH_3)_2NCH_2$—	68.5–69.5	88	Ethanol	C	78.14	78.05
						H	8.20	8.09
						N	13.67	13.86
VII	$C_{24}H_{29}N_3$		106–107	70	Acetone–water	C	80.18	80.24
						H	8.13	8.24
						N	11.69	11.68
VIII	$C_{26}H_{33}N_3$		98.5–99.5	85	Ethanol	C	80.58	80.53
						H	8.58	8.57
						N	10.84	10.74
IX	$C_{24}H_{29}N_3O_2$		156–157	81	Ethanol	C	73.63	73.75
						H	7.47	7.56
						N	10.73	10.84

cold acetone–water. The yield was 1.2 g. (70%), m.p. 106–107°.

1,3 - Bis(piperidinomethyl) - 2 - phenylindolizine (VIII)—Six milliliters of 37% aqueous formaldehyde (0.075 mole) and 8.4 g. piperidine (0.10 mole) were combined with 75 ml. dioxane. One-tenth milliliter of 50% sodium hydroxide was added and the mixture was allowed to stand for 15 min. at room temperature. Two grams 2-phenylindolizine (0.01 mole) was dissolved in the mixture which was then allowed to stand at room temperature for 48 hr. The reaction mixture was transferred to an evaporating dish and cold air was blown across the surface. Evaporation was accompanied by vigorous scratching with a glass rod. The crystalline product which was obtained during the evaporation process was removed by filtration and washed with 50% ethanol. The yield was 3.3 g. (85%). On recrystallization from hot ethanol the compound gave m.p. 98.5–99.5°.

1,3 - Bis(morpholinomethyl) - 2 - phenylindolizine (IX)—Six milliliters of 37% aqueous formaldehyde (0.075 mole) and 8.7 g. morpholine (0.10 mole) were combined with 75 ml. dioxane. One-tenth milliliter of 50% sodium hydroxide solution was added and the mixture was allowed to stand for 15 min. at room temperature. Two grams 2-phenylindolizine (0.01 mole) was added and the flask swirled to achieve solution. The reaction mixture was allowed to stand at room temperature for 48 hr. and then transferred to an evaporating dish. Evaporation was carried out by blowing cold air across the surface accompanied by vigorous scratching with a glass rod. The crystalline product obtained was removed by filtration and washed with 50% ethanol. The yield was 3.2 g. (81%). The product was recrystallized from ethanol and gave m.p. 156–157°.

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Keyphrases

Mannich bases—synthesis
2-Phenylindolizine derivatives—Mannich bases
3-Diethylaminomethyl-1,2-diphenylindolizine—pharmacological screening

Effect of Perfusion Rate and Distribution Factors on Drug Elimination Kinetics in a Perfused Organ System

By RENPEI NAGASHIMA and GERHARD LEVY*

The effects of flow rate and volume of perfusate on the elimination of a drug from a perfusion fluid by an organ or tissue such as the liver, kidney, or intestine are examined. It is shown that the volume of perfusate (or the apparent volume of distribution of a drug) has a pronounced effect on the rate constant for the decline of drug concentration in the perfusate, but that perfusion rate has an effect only if the concentration of drug in the perfusate leaving the organ is appreciably lower than the concentration in the fluid entering the organ.

MANY KINETIC studies of drug absorption or metabolism are carried out by perfusing a segment of intestine, or an organ such as the liver, and determining the change in the concentration of the drug in the perfusate as a function of time. Two important variables in such experiments are the volume of perfusate and its rate of flow through the organ. These variables are encountered also *in vivo* since blood flow rate can be affected by shock, in certain disease conditions, and by some drugs, and since the apparent volume of distribution of drugs (which is analogous in certain respects to the perfusate volume in *in vitro* studies) differs as a function of plasma protein concentration, type and concentration of drug, and other factors. The pharmacokinetic analysis to be presented here will deal specifically with the isolated perfused liver system, but the principles which will be outlined apply equally to the other systems mentioned above.

The liver is viewed as a tissue with numerous parallel channels through which perfusate flows. The concentration of drug in the perfusate leaving the liver is lower than the drug concentration in the fluid entering the liver, due to biotransformation of the drug in that tissue. This decrease in the drug concentration is a function of the activity of the biotransformation process (assuming that drug transfer from perfusate to liver is not rate limiting), and the contact time of a given increment of perfusate with liver tissue (1). If the elimination is a zero-order process, the drug concentration in the

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outflow is given by:

$$C_o = C_i - k^\circ\tau \qquad \text{(Eq. 1)}$$

where C_i and C_o refer to the drug concentrations in the inflowing and outflowing perfusate, respectively, k° is the zero-order rate constant for drug elimination (concentration change per unit time), and τ is the contact (or transit) time. If drug elimination is a first-order process, the appropriate equation is given by:

$$C_o = C_i \exp(-k\tau) \qquad \text{(Eq. 2)}$$

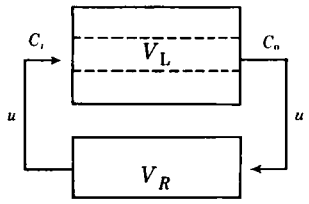
where k is the first-order rate constant for drug elimination, in reciprocal time units.

The mean contact time for perfusate in all the channels in the liver is given by:

$$\tau = V_L/u \qquad \text{(Eq. 3)}$$

where V_L is the total volume of the channels, and u is the flow rate of perfusate (2).

Consideration must now be given to the extrahepatic space, *i.e.*, the real or apparent (in the case of an *in vivo* system) volume of perfusate in the reservoir and tubings. This volume is designated as V_R. The described model is illustrated in Scheme I.



Scheme I

Immediately upon leaving the liver, the perfusate is incorporated in and mixed with V_R. The drug concentration in V_R is identical to C_i. The net rate of decrease in the amount of drug in V_R is given by:

$$-V_R \cdot \frac{dC_i}{dt} = u(C_i - C_o)$$

where dC_i/dt is the rate of drug concentration change in V_R.[1] Upon rearrangement,

$$-\frac{dC_i}{dt} = \frac{u}{V_R} \cdot (C_i - C_o) \qquad \text{(Eq. 4)}$$

Substituting for C_o in Eq. 4 from Eqs. 1 or 2, and for τ from Eq. 3 yields:

$$-\frac{dC_i}{dt} = \frac{V_L}{V_R} \cdot k^\circ \qquad \text{(Eq. 5)}$$

and

$$-\frac{dC_i}{dt} = \frac{u}{V_R} \cdot [1 - \exp(-kV_L/u)]C_i \qquad \text{(Eq. 6)}$$

respectively. The apparent rate constants for the decline of drug concentration are then:

$$k^\circ_{app.} = \frac{V_L}{V_R} \cdot k^\circ \qquad \text{(Eq. 7)}$$

and

$$k_{app.} = \frac{u}{V_R} \cdot [1 - \exp(-kV_L/u)] \qquad \text{(Eq. 8)}$$

If kV_L/u is smaller than 0.1, Eq. 8 may be reduced to:

$$k_{app.} = \frac{V_L}{V_R} \cdot k \qquad \text{(Eq. 9)}$$

The error introduced by this simplification is less than 5 %.[2] Note that Eqs. 7 and 9 do not contain a flow rate term.

Regardless of whether drug elimination in the perfused organ proceeds by zero- or first-order kinetics, the apparent rate constants are inversely proportional to the volume of perfusate (Eqs. 7 to 9). Therefore, when serial sampling of perfusate reduces the volume of that fluid as a function of time, appropriate corrections must be made to account for the volume change. This is shown in another report from this laboratory (4).

A change in the flow rate of perfusate has absolutely no effect on drug elimination rate constants when the kinetics are zero-order,[3] and essentially no effect when the kinetics are first-order and kV_L/u is small. Taking for example the results of an isolated rat liver perfusion study of bishydroxycoumarin elimination described in another report (4), u = 1,140 ml./hr., V_R = 100 ml., and the largest $k_{app.}$ = 0.565 hr.$^{-1}$. Thus, from Eq. 8, kV_L/u = 0.05, which shows that flow rate should have no effect on the elimination kinetics of bishydroxycoumarin.

A similar analysis can be carried out with respect to intact animals, assuming rapid distribution of drug between plasma and tissues. In this case,

$$V_R = V_d - W(C_L/C_P) \qquad \text{(Eq. 10)}$$

where V_d is the apparent volume of distribution of the drug, W is the weight of the liver, and C_L/C_P is the liver:plasma concentration ratio of the drug. Using data for bishydroxycoumarin elimination by intact rats presented in other reports (8, 9), where V_d = 100 ml./kg. of body weight, C_L/C_P = 0.44 ml./g., and W = 33.5 g./kg. of body weight (5), it was calculated that V_R = 85 ml./kg. of body weight. Considering a hepatic blood flow rate of about 50 ml./min./kg. of body weight (6) and a hematocrit value of 45% (4), the hepatic plasma flow rate in rats is 1,350 ml./hr./kg. of body weight. The value of kV_L/u may then be calculated from Eq. 8 and from the *in vivo* $k_{app.}$ value of 0.14 hr.$^{-1}$ (4). By these calculations, kV_L/u = 0.009, which shows that the elimination kinetics of bishydroxycoumarin in the intact rat are apparently not affected by hepatic blood flow. The same probably applies to man, considering the similar magnitude of hepatic blood flow (6), the lower $k_{app.}$ value (7), and the similar relative apparent volume

[1] See *Reference 1* for a similar derivation for chemical reactions in flow systems.

[2] Using the Mclaurin series (3):

$$\exp(-x) = 1 - x + \frac{x^2}{2!} - \frac{x^3}{3!} + \ldots$$

When $x \ll 1$: $\exp(-x) \simeq 1 - x$. Specifically when x = 0.1, the difference in $k_{app.}$ calculated by Eqs. 8 and 9, respectively, is 5%.

[3] Provided that the rate of supply of drug to the perfused organ is not elimination rate limiting.

of distribution of bishydroxycoumarin in rats and humans (8). On the other hand, it is possible that hepatic blood flow rate will affect the elimination kinetics of certain drugs with very short biologic half-lives which are metabolized in the liver.

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Keyphrases

Perfused organ system—drug elimination
Elimination kinetics—perfusion rate effect
Distribution factors, effect—elimination kinetics
Kinetic equations—perfused organ system

Effect of Deuterium Oxide on the Culturing of *Penicillium janczewskii* III. Antifungal Activity of Fully Deuterated Griseofulvin

By D. A. NONA, M. I. BLAKE, H. L. CRESPI*, and J. J. KATZ*

The *in vitro* antifungal activity of fully deuterated, partially deuterated, and protiogriseofulvin was compared. The antibiotics were evaluated by plate assay with *Microsporum gypseum* (ATCC 14683) as the test organism. A statistically significant enhancement in antifungal activity was observed in zones of inhibition when fully deuterated griseofulvin was compared with partially deuterated and protiogriseofulvin. No significant difference was apparent in a comparison of biological activity of partially deuterated and ordinary griseofulvin. The enhanced antifungal activity demonstrated by fully deuterated griseofulvin may be due to an increased efficiency of action. The antifungal target site may involve the metabolism of a C—D bond directly or indirectly. Since C—D bonds are generally more stable than C—H bonds, the increased stability of the molecule may alter the rate of griseofulvin metabolism by the fungus.

In the first paper (1) of this series the nutritional requirements for optimal growth of *Penicillium janczewskii* in heavy water were reported. The effects on antibiotic production were noted. The second study (2) described in detail the isolation, purification, and characterization of the deuterated griseofulvin biosynthesized by the organism in a replacement culture. In the present report comparison is made of the *in vitro* antifungal activity of ordinary griseofulvin and the antibiotics obtained from the organism grown in a deuterated environment.

EXPERIMENTAL

Antifungal activity of the antibiotic was determined by plate assay using *Microsporum gypseum* as the test organism. A modification of the USP (3) procedure for microbiological assay of griseofulvin was employed.

Medium I—The medium was composed of dextrose, 40.0 g.; peptone, 10.0 g.; agar, 15.0 g.; chloramphenicol, USP, 0.05 g.; and sufficient distilled water to make 1,000 ml. The pH was adjusted, if necessary, to 5.65 ± 0.05. The medium was placed into 100-ml. vials, fitted with rubber closures, sealed, and capped. The vials were autoclaved at 15 p.s.i. for 15 min. The medium was used immediately after cooling to 50°, or was stored for future use. After storage at room temperature, the solidified medium was melted by gentle warming on a steam bath.

Medium II—The composition of this medium differed from Medium I in that 2 ml. of a 1% aqueous solution of cycloheximide (Actidione, Upjohn) was added for each 100 ml. of the agar medium. The cycloheximide solution was injected through the rubber closure of the vial using a needle and syringe fitted with a Swinney adapter. The membrane filter had a porosity of 0.22 μ.

Preparation of Inoculum—The test organism, *Microsporum gypseum* (ATCC 14683), was grown

Received May 22, 1968, from the Department of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612, and the Chemistry Division,* Argonne National Laboratory, Argonne, IL 60439
Accepted for publication June 27, 1968.
Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
This investigation was supported by grant AI 06825-01 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and was performed in part under the auspices of the U. S. Atomic Energy Commission, Washington, D. C.

of distribution of bishydroxycoumarin in rats and humans (8). On the other hand, it is possible that hepatic blood flow rate will affect the elimination kinetics of certain drugs with very short biologic half-lives which are metabolized in the liver.

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Keyphrases

Perfused organ system—drug elimination
Elimination kinetics—perfusion rate effect
Distribution factors, effect—elimination kinetics
Kinetic equations—perfused organ system

Effect of Deuterium Oxide on the Culturing of *Penicillium janczewskii* III. Antifungal Activity of Fully Deuterated Griseofulvin

By D. A. NONA, M. I. BLAKE, H. L. CRESPI*, and J. J. KATZ*

The *in vitro* antifungal activity of fully deuterated, partially deuterated, and protiogriseofulvin was compared. The antibiotics were evaluated by plate assay with *Microsporum gypseum* (ATCC 14683) as the test organism. A statistically significant enhancement in antifungal activity was observed in zones of inhibition when fully deuterated griseofulvin was compared with partially deuterated and protiogriseofulvin. No significant difference was apparent in a comparison of biological activity of partially deuterated and ordinary griseofulvin. The enhanced antifungal activity demonstrated by fully deuterated griseofulvin may be due to an increased efficiency of action. The antifungal target site may involve the metabolism of a C—D bond directly or indirectly. Since C—D bonds are generally more stable than C—H bonds, the increased stability of the molecule may alter the rate of griseofulvin metabolism by the fungus.

In the first paper (1) of this series the nutritional requirements for optimal growth of *Penicillium janczewskii* in heavy water were reported. The effects on antibiotic production were noted. The second study (2) described in detail the isolation, purification, and characterization of the deuterated griseofulvin biosynthesized by the organism in a replacement culture. In the present report comparison is made of the *in vitro* antifungal activity of ordinary griseofulvin and the antibiotics obtained from the organism grown in a deuterated environment.

EXPERIMENTAL

Antifungal activity of the antibiotic was determined by plate assay using *Microsporum gypseum* as the test organism. A modification of the USP (3) procedure for microbiological assay of griseofulvin was employed.

Medium I—The medium was composed of dextrose, 40.0 g.; peptone, 10.0 g.; agar, 15.0 g.; chloramphenicol, USP, 0.05 g.; and sufficient distilled water to make 1,000 ml. The pH was adjusted, if necessary, to 5.65 ± 0.05. The medium was placed into 100-ml. vials, fitted with rubber closures, sealed, and capped. The vials were autoclaved at 15 p.s.i. for 15 min. The medium was used immediately after cooling to 50°, or was stored for future use. After storage at room temperature, the solidified medium was melted by gentle warming on a steam bath.

Medium II—The composition of this medium differed from Medium I in that 2 ml. of a 1% aqueous solution of cycloheximide (Actidione, Upjohn) was added for each 100 ml. of the agar medium. The cycloheximide solution was injected through the rubber closure of the vial using a needle and syringe fitted with a Swinney adapter. The membrane filter had a porosity of 0.22 μ.

Preparation of Inoculum—The test organism, *Microsporum gypseum* (ATCC 14683), was grown

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on Sabouraud dextrose agar slants at 25° for 10 days at which time the growth assumed a chamois-like appearance. The slants were stored in a refrigerator at 5° for as long as 2 months.

Three-hundred milliliters of Medium I was placed into a Roux bottle and allowed to solidify. The medium was inoculated by spreading an aqueous suspension of the test organism evenly over the surface of the agar with the aid of sterile glass beads. The culture was incubated at 25° for 2 weeks. At the end of this period, a stock spore suspension was prepared by collecting the surface growth in 50 ml. of sterile water. The spore suspension was stable for at least 2 months at 5°.

Test plates were prepared using a 6-ml. base layer of Medium I covered with a 4-ml. seed layer of Medium II inoculated with varying volumes of spore suspension to give a 1, 2, or 3% suspension. The most appropriate percent of inoculum was determined by observing the largest and clearest zones of inhibition produced by 5 mcg./ml. of standard griseofulvin on the test plate.

Procedure—Six milliliters of Medium I was added to a 20 × 100 mm. Petri dish and allowed to harden for use as a base layer. The Petri dishes were covered and chilled at 5°. Medium II was allowed to cool to approximately 50° and 2 ml. of spore suspension was added for each 100 ml. of medium. The culture and agar were mixed thoroughly and 4 ml. was added to each plate containing the uninoculated base agar. Each plate was tilted back and forth to spread the inoculated agar evenly over the surface. When the agar hardened, six cylinders were placed on the surface so that they were at 60° intervals on a 2.8-cm. radius. Stainless steel cylinders were used having an outside diameter of 8 ± 0.1 mm., and inside diameter of 6 ± 0.1 mm., and a height of 10 ± 0.1 mm.

A standard stock solution of protio-griseofulvin was prepared by dissolving 50 mg. in sufficient *N,N*-dimethylformamide (DMF, Eastman Organic) to give a concentration of 1,000 mcg./ml. A standard curve was prepared by diluting the 1,000 mcg./ml. griseofulvin solution to 64, 80, 100, 125, and 156 mcg./ml. in DMF. One part of each of these solutions was further diluted with 19 parts of 0.1 *M* phosphate buffer pH 8.0, to give final solutions of 3.2, 4.0, 5.0, 6.25, and 7.8 mcg./ml.

Three plates were used for the determination of each point of the curve, with the exception of the 5.0 mcg./ml. solution. On each of the three plates, three cylinders were filled with the 5.0 mcg./ml. solution and the other three cylinders were filled with the particular concentration of the standard curve under test. There were thirty-six 5.0 mcg./ml. determinations and nine determinations for each of the other points on the curve. The plates were incubated for 48 hr. at 30°, after which the diameters of the circle of inhibition were measured. The data obtained were corrected (4) and a potency curve was constructed by plotting the concentration of reference standard protio-griseofulvin *versus* the zone of inhibition on semilogarithmic graph paper. The griseofulvin concentrations were plotted on the logarithmic scale and the diameter of the zones on the linear scale, and the line of best fit was constructed. The procedure was repeated using deuteriogriseofulvin and the curve was plotted on the same semilogarithmic paper (see Fig. 1).

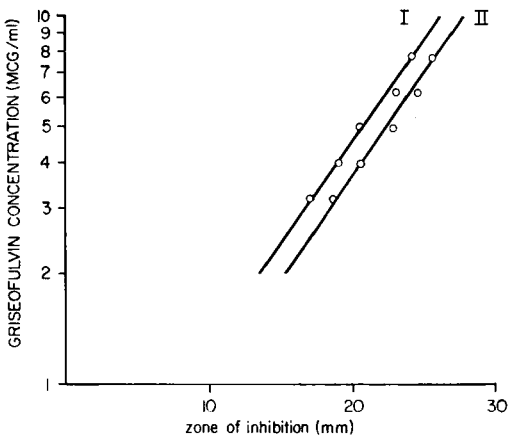

Fig. 1—The relationship of griseofulvin concentration and the diameter of zone of inhibition; (I) ordinary griseofulvin, (II) deuteriogriseofulvin.

Reference standard protio-griseofulvin, protio-griseofulvin isolated in this laboratory, partially deuterated griseofulvin (2), and fully deuterated griseofulvin (2) were assayed according to the method of Knoll *et al.* (4). The samples were diluted with DMF to give a concentration of 100 mcg./ml. These solutions were further diluted with 19 parts of 0.1 *M* phosphate buffer pH 8.0 to give a final concentration of 5.0 mcg./ml. of griseofulvin. A series of 18 assays were compared for each form of griseofulvin. The data are shown in Table I.

RESULTS AND DISCUSSION

The potency curves, Fig. 1, for the ordinary griseofulvin and the fully deuterated griseofulvin were found to be straight lines. The linearity of the curves was tested by calculating the regression equations, and their significance was established by analysis of variance using the *F* test as the test of significance. The value of *F* obtained for ordinary griseofulvin and fully deuterated griseofulvin was found to be significant at the 0.1% level and 1% level, respectively. This denotes that on the basis of probability there is a significant linear relationship over and above any curvilinear relationship.

The zones of inhibition were determined for a predetermined concentration (5 mcg./ml.) of standard griseofulvin, protio-griseofulvin obtained in this laboratory, partially deuterated griseofulvin, and fully deuterated griseofulvin (Table I). They were compared statistically by means of analysis of variance. The valid comparison of fully deuterated *versus* partially deuterated and ordinary griseofulvin, as well as partially deuterated griseofulvin *versus* ordinary griseofulvin are permitted since there are two degrees of freedom in this experiment. The difference between fully deuterated *versus* partially deuterated and ordinary griseofulvin was found to be highly significant ($p = 0.001$), indicating that there is a true difference in the zones of inhibition due to fully deuterated griseofulvin.

The computed value of *F* for comparison of partially deuterated griseofulvin with reference standard griseofulvin was found not to be significant. It was concluded therefore, that there is virtually no

TABLE I—COMPARISON OF THE DIAMETERS OF THE ZONES OF INHIBITION FOR THE GRISEOFULVINS AT A CONCENTRATION OF 5 MCG./ML.

Type of Griseofulvin			
Reference Standard,[a] cm.	Protio-,[b] cm.	Partially Deuterated,[b] cm.	Fully Deuterated,[b] cm.
2.20	2.17	2.13	2.37
2.21	2.21	2.08	2.40
2.21	2.20	2.15	2.34
2.23	2.12	2.14	2.38
2.16	2.16	2.09	2.40
2.23	2.09	2.18	2.41
2.20	2.22	2.29	2.42
2.13	2.18	2.21	2.50
2.20	2.23	2.13	2.36
2.20	2.10	2.21	2.41
2.22	2.11	2.20	2.50
2.20	2.21	2.09	2.42
2.15	2.14	2.12	2.36
2.16	2.16	2.20	2.33
2.09	2.18	2.21	2.40
2.16	2.12	2.16	2.36
2.17	2.17	2.11	2.41
2.15	2.16	2.21	2.36
$\bar{x} = 2.18 \pm 0.04$[c]	$\bar{x} = 2.16 \pm 0.03$[c]	$\bar{x} = 2.16 \pm 0.06$[c]	$\bar{x} = 2.40 \pm 0.05$[c]

[a] Obtained from McNeil Laboratories (Lot No. 2461). [b] Isolated in this laboratory (2). [c] Standard deviation, $n = 18$.

difference between the antifungal properties of the two samples.

It is apparent that there was no difference in zones of inhibition obtained with reference standard griseofulvin and protio-griseofulvin obtained from H_2O cultures in this laboratory. The mean for standard griseofulvin was found to be 2.18 ± 0.04 cm. and the mean for the laboratory-produced ordinary griseofulvin was 2.16 ± 0.03 cm.

The mode of action of protio-griseofulvin is not completely understood. Consideration has been given to the theory that the agent exerts its fungistatic properties by interfering with the synthesis of cell wall chitin, or by interference with nucleic acid metabolism. Recent evidence suggests that griseofulvin stimulates DNA synthesis and causes the formation of abnormal cells, leading to the proposal (5) that the site of action is in the replicatory system of the fungal cell. It is not unlikely that the basic mechanism of antifungal action for fully deuterated griseofulvin is the same as that for ordinary griseofulvin. However, the mean zone of inhibition for deuterio-griseofulvin at a concentration of 5 mcg./ml. was found to be 2.40 ± 0.05 cm. whereas the mean for standard protio-griseofulvin at the same concentration was 2.18 ± 0.04 cm. The data observed over the range of concentrations tested indicate that deuterio-griseofulvin is about 10% more active than protio-griseofulvin on a weight basis. Since the molecular weight of deuterio-griseofulvin is about 5% greater than protio-griseofulvin, comparison on a mole basis would provide an even greater effect for the deuterio-analog.

The enhanced antifungal activity demonstrated by the fully deuterated antibiotic may be due to an increased efficiency of action. This increase in efficiency may be the result of a primary deuterium isotope effect, where the target site may involve the cleavage of a C-D bond directly. A kinetic effect involving a rate-limiting step may be affected. Since C-D bonds are generally more stable than C-H bonds, the increased stability of the molecule may play a role in that the fungus may not be able to eliminate the fungistatic agent as readily. The biological effect could also be due to a secondary isotope effect where deuterium in molecular positions other than a reaction locus are involved.

No increase in antifungal activity was observed with partially deuterated griseofulvin. More extensive deuterium replacement is apparently required in the molecule, or at least at critical loci, before enhanced antifungal activity can be observed.

Deuterium isotope effects in biological systems are extremely complex, and a combination of several effects may well be involved. A better understanding of the mechanism of antifungal activity of ordinary hydrogen griseofulvin will probably be necessary before the effect of deuterium replacement in the molecule can be fully understood. More studies concerning stability, enzyme systems of the fungus, and the effect of the antibiotic upon them, are clearly necessary for a better understanding of the fungistatic properties of fully deuterated griseofulvin.

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Keyphrases

Deuterated griseofulvin—antifungal activity
Antifungal assay—*Microsporum gypseum*
Griseofulvin, protio, fully and partially deuterated—comparison

Phytochemical Investigation of the Flowers of *Cassia reticulata* Willd. (*Leguminosae*)

By W. M. MESSMER, N. R. FARNSWORTH, G. J. PERSINOS*, and J. D. WILKES*

A study of the flowers of *Cassia reticulata* has been made. By means of polyamide column chromatography, two anthraquinones, rhein and aloe-emodin, as well as a sterol, β-sitosterol, were isolated and identified.

ALTHOUGH VARIOUS SPECIES of *Cassia* have been investigated extensively as to their phytochemical composition, relatively little is known of the constituents of *Cassia reticulata* Willd. (*Leguminosae*). Cassic acid, a substance possessing antibiotic properties (1), and later identified as the anthraquinone rhein (2), has previously been isolated from the leaves of this plant.

Flavonoids (3, 4), anthraquinones (5), a sterol (5), and alkaloids (6) have been isolated from several species of this genus. A preliminary investigation of a small sample of *C. reticulata* flowers by means of thin-layer chromatography, indicated the presence of flavonoids and anthraquinones. Extraction and fractionation (Fig. 1) of a large sample of the coarsely milled flowers yielded an ethyl acetate fraction which contained the majority of compounds of interest, as evidenced by thin-layer chromatography on Silica Gel G plates. Column chromatography of this fraction on polyamide yielded rhein, aloe-emodin, and β-sitosterol.

EXPERIMENTAL

Materials and Methods—The plant material used in this investigation was collected in the area of Iquitos, Peru during February of 1968, and represented the flowers of *Cassia reticulata* Willd. (*Leguminosae*). Voucher specimens have been deposited at the Economic Herbarium of Oakes Ames, Botanical Museum, Harvard University, Cambridge, Mass.

All melting points were taken on a Thomas-Hoover melting-point apparatus and are uncorrected. IR spectra were taken using a Beckman spectrophotometer, model IR-8, and the UV absorption spectra were measured in 95% ethanol using a Beckman spectrophotometer, model DK-2. Thin-layer chromatography (TLC) was performed in most cases on Silica Gel G plates. Preparative TLC was performed on plates (20 × 40 cm.), coated with a 1-mm. thick layer of Silica Gel G, which were subsequently activated at 100° for 1.5 hr.

Preparation of Fractions—The plant material (1.0 kg.) was milled to a coarse powder and extracted in a continuous extraction apparatus with skellysolve B for 108 hr. Solvent was changed every 24 hr. to prevent excessive heating of the extract. All of the skellysolve B extracts were combined and evaporated *in vacuo* to yield 17.1 g. of Fraction A. The marc was removed from the continuous extraction apparatus, spread out to air dry for 24 hr., and repacked into the continuous extraction apparatus for extraction with 95% ethanol. Extraction was continued for 96 hr., with a fresh change of solvent every 24 hr. The combined ethanol extracts, when dried *in vacuo*, yielded 166.1 g. of a dark brown residue. Redissolving this viscous mass in 1.0 l. of ethanol, and refrigerating for 3 days, produced 16.4 g. of a dark greenish-brown residue, Fraction B. The filtrate was evaporated to dryness to give 133.8 g. of residue, to which was added 800 ml. of distilled water. This solution was then heated on a steam bath with frequent stirring for 30 min., cooled, filtered, and the residue on the filter paper dried (Fraction C, 13.0 g.). The filtrate then was extracted with ethyl acetate six times. After this extract was dried *in vacuo*, it produced 9.4 g. of Fraction D. The aqueous phase remaining after the ethyl acetate extractions were completed was labeled Fraction E (see Fig. 1).

Column Chromatography of Fraction D—Two-hundred grams of MN-polyamide powder[1] was washed with water and then methanol until the methanol filtrate, when taken to dryness using a flash evaporator, yielded no visible residue. The powder was then packed to a constant height (42 cm.) in a 5 × 64-cm. glass column. Fraction D (9.4 g.) was dissolved in a minimum volume of methanol and was added to the top of the polyamide column. One-hundred-milliliter fractions were collected. The fractions were eluted with methanol and grouped according to their TLC patterns using ethyl acetate–methanol–water (100:16:12.5) as the developer and 25% aqueous lead subacetate solution as a spray reagent, followed by drying the chromatograms and visualizing under long-wavelength UV light (366 mμ).

Isolation of β-Sitosterol—Fraction grouping 5–14 from the column on concentration yielded 301.8 mg. of a white amorphous powder which did not react with the spray reagent. A Liebermann-Burchard test (7) subsequently indicated this material to be steroidal. TLC, followed by spraying with 70% aqueous H_2SO_4 solution, followed by heating at 110° for 15 min., showed the isolate to be a mixture of three sterols. Preparative TLC, using the ethyl acetate–methanol–water eluent, followed by elution of the bands with methanol, and crystallization of the material from the highest band from 95% ethanol, yielded 5.8 mg. of β-sitosterol, m.p. 134.5–135.5°, lit. m.p. 139–140° (5). The IR spectrum of the isolate was identical with that of reference β-sitosterol.

Isolation of Rhein—Fraction grouping 30–60 from the column yielded 42.6 mg. of a yellow amorphous powder during slow evaporation, which gave a positive Bornträger's test (8, 9). TLC of the isolate, using the ethyl acetate–methanol–water eluent, and followed by spraying with 2 *N* aqueous potassium hydroxide solution, showed the isolate to be a single entity, m.p. 321–324° (dec.). An IR spectrum of the

Received June 25, 1968, from the Department of Pharmacognosy, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15213, and Research Pathology Associates,* Rockville, MD 20851

Accepted for publication July 29, 1968.

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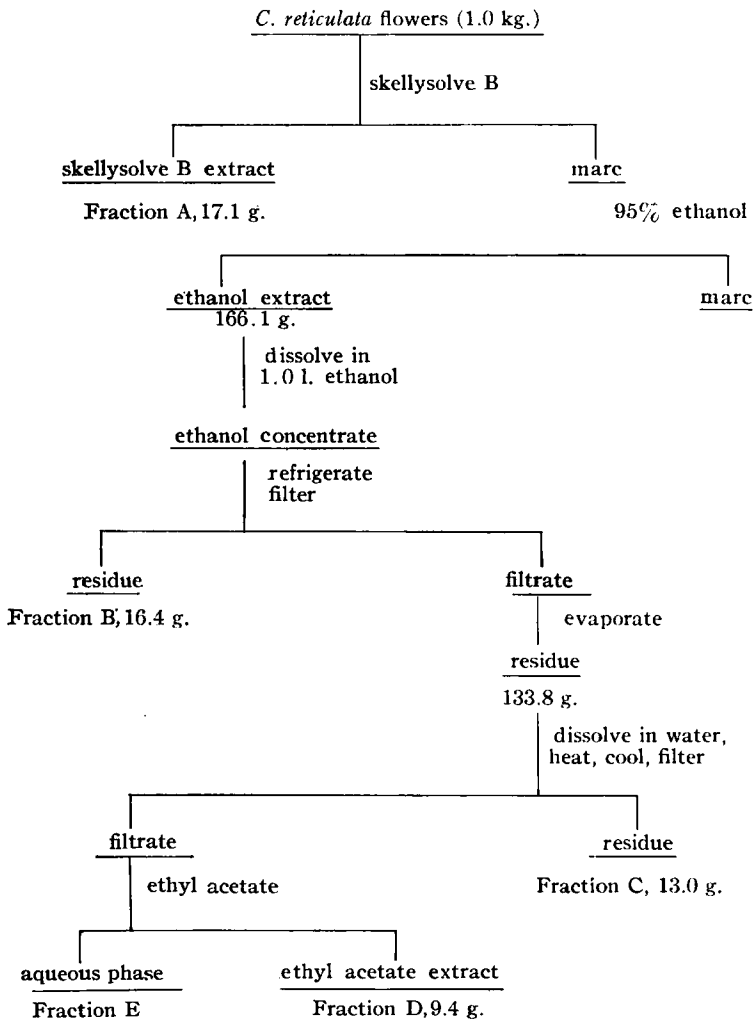

Fig. 1—Extraction and fractionation scheme for C. reticulata *flowers.*

isolate was superimposable with that of reference rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid).[2] UV absorption spectrum, $\lambda_{max.}^{EtOH}$ 229, 258, and 430 mμ (log ε 3.8, 3.5, and 3.4), compared favorably with that found in the literature, $\lambda_{max.}^{EtOH}$ 230, 260, and 430 mμ (2). TLC with reference rhein in several different solvent systems showed identical R_f values and chromogenic reactions to the KOH spray reagent. A molecular weight of 284 was confirmed by mass spectrometry.

Fractions 61–110 from the column were combined and slowly evaporated to yield 93.1 mg. of material which was shown by TLC to be a mixture containing mainly rhein, in addition to lesser amounts of a second anthraquinone having a slightly higher R_f value. Column chromatography of this material on a polyamide matrix resulted in an additional crop of pure rhein, but the second anthraquinone could not be separated.

Isolation of Aloe-emodin—The mother liquor from combined Fractions 5–14 from the column, in addition to Fractions 15–29, were combined and taken to dryness to yield 7.4 g. of residue. This was dissolved in distilled water and chromatographed over a column containing 200 g. of MN-polyamide which had been washed several times with distilled water and packed as an aqueous slurry. Elution was initiated with water and 100-ml. fractions were collected. Fractions 1–31 were eluted with water, 32–51 with 10% methanol, 52–67 with 25% methanol, 68–85 with 50% methanol, 86–137 with 75% methanol, 138–212 with methanol, and 213–278 with acetone. The aqueous fractions were frozen and lyophilized, the aqueous methanol fractions were concentrated *in vacuo* to remove methanol, and then were frozen and lyophilized, and the methanol and acetone fractions were taken to dryness *in vacuo*. Each fraction was then dissolved in *ca.* 10 ml. of methanol, followed by TLC to determine those fractions to be combined.

Fractions 113–122 were similar in their chromatographic pattern and were combined. Slow evaporation yielded an orange-yellow product having m.p.

[2] Rhein samples were generously provided by Professor J. W. Fairbairn, University of London, and Professor H. Wagner, Munich, Germany.

220–221°, and weighing 17.9 mg., lit. m.p. for aloe-emodin 223–225° (5). TLC in several different solvent systems showed the isolate to be homogeneous and identical in R_f value with reference aloe-emodin.[3] IR spectra of the isolate and reference aloe-emodin were superimposable, and the UV absorption spectrum for the isolate was λ_{max}^{EtOH} 226, 253, and 287 mμ (log ε 4.2, 3.8, and 3.5).

SUMMARY

A phytochemical investigation of *Cassia reticulata* flowers has resulted in the isolation of rhein, aloe-emodin, and β-sitosterol, which have not been previously reported from the flowers of this plant. At least one additional anthraquinone was observed to be present, in addition to three flavonoids, but these were not isolable using the methods employed in this study.

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[3] A reference sample of aloe-emodin was provided by Professor J. W. Fairbairn.

Keyphrases

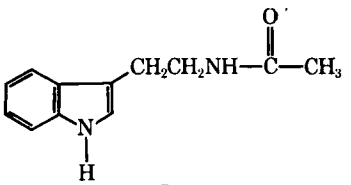
Cassia reticulata flowers—phytochemistry
Anthraquinones—isolated, identified
β-Sitosterol—isolated, identified
Column chromatography—separation
TLC—identity
IR spectrophotometry—identity
UV spectrophotometry—identity

Hydroxyindole-*O*-methyltransferase II. Inhibitory Activities of Some *N*-Acyltryptamines

By BENG T. HO, WILLIAM M. McISAAC, L. WAYNE TANSEY, and PATRICIA M. KRALIK

Several *N*-acyltryptamines were found to inhibit hydroxyindole-*O*-methyltransferase. Of these inhibitors the most active was *N*-phenylacetyltryptamine, having an affinity to the enzyme six times greater than the substrate *N*-acetylserotonin. These studies showed that the alkyl and aralkyl groups of the amides were most likely complexed with the enzyme by hydrophobic bonding.

In the previous paper (1), the binding of the substrate *N*-acetylserotonin to the enzyme hydroxyindole-*O*-methyltransferase (HIOMT) was studied. There was a good indication that the CH_3 of the amide I was complexed with the enzyme by



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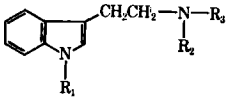
hydrophobic bonding. To explore this hydrophobic region on HIOMT, several substituted *N*-acyltryptamines (II–VIII) were synthesized and their inhibitory activities evaluated (Table I).

The finding that both *N*-cyclobutylcarbonyl-

TABLE I—INHIBITION OF HIOMT



Compound	R_1	R_2	R_3	I_{50},[a] (m*M*)
I	H	H	$COCH_3$	1.40[b]
II	H	H	COC_4H_7	0.88
III	H	H	COC_6H_{11}	0.85
IV	H	H	COC_6H_5	0.37
V	H	H	$COCH_2C_6H_5$	0.18
VI	H	H	$COCH_2CH_2C_6H_5$	0.22
VII	H	CH_3	$COCH_2C_6H_5$	0.16
VIII	CH_3	H	$COCH_2C_6H_5$	0.35
IX	H	H	$SO_2C_6H_5$	0.37

[a] Concentration of an inhibitor giving 50% inhibition of the enzyme. [b] Data from the previous paper (1).

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Accepted for publication July 12, 1968.

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The authors would like to thank Mr. Edward Fritchie for his technical assistance in providing the enzyme assay data.

220–221°, and weighing 17.9 mg., lit. m.p. for aloe-emodin 223–225° (5). TLC in several different solvent systems showed the isolate to be homogeneous and identical in R_f value with reference aloe-emodin.[3] IR spectra of the isolate and reference aloe-emodin were superimposable, and the UV absorption spectrum for the isolate was λ_{max}^{EtOH} 226, 253, and 287 mμ (log ε 4.2, 3.8, and 3.5).

SUMMARY

A phytochemical investigation of *Cassia reticulata* flowers has resulted in the isolation of rhein, aloe-emodin, and β-sitosterol, which have not been previously reported from the flowers of this plant. At least one additional anthraquinone was observed to be present, in addition to three flavonoids, but these were not isolable using the methods employed in this study.

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[3] A reference sample of aloe-emodin was provided by Professor J. W. Fairbairn.

Keyphrases

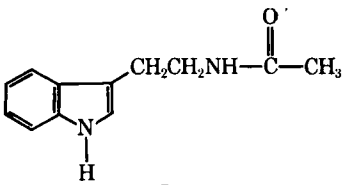
Cassia reticulata flowers—phytochemistry
Anthraquinones—isolated, identified
β-Sitosterol—isolated, identified
Column chromatography—separation
TLC—identity
IR spectrophotometry—identity
UV spectrophotometry—identity

Hydroxyindole-*O*-methyltransferase II. Inhibitory Activities of Some *N*-Acyltryptamines

By BENG T. HO, WILLIAM M. McISAAC, L. WAYNE TANSEY, and PATRICIA M. KRALIK

Several *N*-acyltryptamines were found to inhibit hydroxyindole-*O*-methyltransferase. Of these inhibitors the most active was *N*-phenylacetyltryptamine, having an affinity to the enzyme six times greater than the substrate *N*-acetylserotonin. These studies showed that the alkyl and aralkyl groups of the amides were most likely complexed with the enzyme by hydrophobic bonding.

In the previous paper (1), the binding of the substrate *N*-acetylserotonin to the enzyme hydroxyindole-*O*-methyltransferase (HIOMT) was studied. There was a good indication that the CH_3 of the amide I was complexed with the enzyme by



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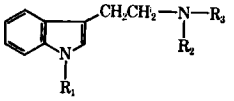
hydrophobic bonding. To explore this hydrophobic region on HIOMT, several substituted *N*-acyltryptamines (II–VIII) were synthesized and their inhibitory activities evaluated (Table I).

The finding that both *N*-cyclobutylcarbonyl-

TABLE I—INHIBITION OF HIOMT



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VIII	CH_3	H	$COCH_2C_6H_5$	0.35
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[a] Concentration of an inhibitor giving 50% inhibition of the enzyme. [b] Data from the previous paper (1).

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TABLE II—PHYSICAL CONSTANTS OF *N*-ACYLTRYPTAMINES

Compound	Method[a]	Yield, %	M.p.,[b] °C.	Anal. Calcd.	Anal. Found
II	A	67	110–111[c]	C, 74.4	74.3
				H, 7.49	7.48
				N, 11.6	11.5
III	A	63	105–106[c]	C, 75.5	75.4
				H, 8.20	8.19
				N, 10.4	10.3
IV[d]	B	39	130–132[e]		
V[f]	A	54	144–145[g]		
VI	B	88	74–75[c]	C, 78.0	77.9
				H, 6.90	6.99
				N, 9.58	9.63
VII	A	66	104–105	C, 78.0	78.3
				H, 6.90	7.10
				N, 9.58	9.47
VIII	B	79	76–76.5[c]	C, 78.0	77.9
				H, 6.90	6.81
				N, 9.58	9.47
IX	B	90	88.5–89.5[h]	C, 64.0	64.0
				H, 5.37	5.58
				N, 9.33	9.21

[a] See *Experimental*. [b] Melting points were taken on a Fisher-Johns apparatus and are corrected. [c] Recrystallized from aqueous ethanol. [d] Lit. (3) m.p. 137–138° (benzene). [e] Recrystallized from benzene. [f] A melting point of 142–143.5° (benzene) has been recorded for this compound prepared by the reaction of tryptamine with phenylacetic acid (3). [g] Recrystallized from ethanol. [h] Recrystallized from benzene–hexane.

tryptamine (II) and *N*-cyclohexylcarbonyltryptamine (III) were better inhibitors of HIOMT than I seemed to support the existence of a hydrophobic interaction of the cyclobutyl and cyclohexyl groups with the enzyme. A further increase in inhibitory activity was observed when the methyl group of I was replaced by a phenyl group. The resulting compound, *N*-benzoyltryptamine (IV) was nearly four times more active than I. This increase in activity was attributed to the greater affinity of the phenyl ring to the enzyme as compared to the methyl group. A phenyl ring is capable of complexing with the enzyme by both hydrophobic bonding and donor–acceptor interaction, whereas an alkyl group could only bind to the enzyme by hydrophobic inaction. This could account for the greater than twofold difference in activity between IV and II or III. Insertion of a methylene group between the phenyl and C=O of IV resulted in an inhibitory activity eight times greater than I (see V). It was observed that a maximum binding could be obtained with the $C_6H_5CH_2$ of V, since extension of aralkyl chain from $C_6H_5CH_2$ to $C_6H_5CH_2CH_2$ (VI) did not give further increase in inhibitory activity.

Substitution of the amide hydrogen of V by a methyl group (see VII) did not cause any change in the activity of V. This finding agreed well with the previous finding (1). Methylation of the indole nitrogen of V, however, resulted in a twofold decrease in activity (see VIII). Whether this decrease was due to the loss of the indolic hydrogen, which otherwise would form a hydrogen bond with the enzyme, or whether the 1-methyl group caused steric hindrance to the binding of the indole nucleus to the enzyme remains to be investigated.

Compound IX which has a structural resemblance to IV was found to be equally as active as IV.

Kinetics studies showed that V was bound to the enzyme six times and IV four times stronger than the substrate *N*-acetylserotonin, with $Km = 5.7 \times 10^{-5}$ *M*, Ki (V) $= 0.92 \times 10^{-5}$ *M*, and Ki (IV) $= 2.8 \times 10^{-5}$ *M* obtained by the reciprocal plot method. They were competitive inhibitors of the enzyme.

EXPERIMENTAL

Synthesis—*Method A*—To a solution of 10 mmoles of tryptamine or methyltryptamine in 25 ml. of chloroform was added 10 mmoles of triethylamine and an equivalent amount of acid chloride in chloroform. The mixture was stirred at room temperature for 30 min. The chloroform layer was separated, washed successively with 50 ml. of 10% HCl, 50 ml. of 2*N* NaOH, and 50 ml. of water, dried with anhydrous sodium sulfate, and evaporated *in vacuo*. In the cases of III and VII, the residue was an oil which solidified upon standing or drying in a desiccator *in vacuo*.

In the preparation of V, instead of chloroform 100 ml. of benzene was used. The product, which precipitated during a 30-min. period, was collected on a filter and washed successively with acid, base, and water.

See Table II for compounds prepared by this method and their physical constants.

Method B—A solution of 10 mmoles of tryptamine hydrochloride or 1-methyltryptamine hydrochloride (2) in 25 ml. of water was neutralized with 15 ml. of 10% aqueous sodium hydroxide. Dichloromethane (50 ml.) was added to dissolve the precipitate, followed by an addition of 10 mmoles of acid chloride. The mixture was stirred at room temperature for 1 hr. The organic layer was washed successively with 10% NaOH, 10% HCl, and water, and dried with anhydrous sodium sulfate. Evaporation of solvent under reduced pressure gave an oily residue which solidified upon standing.

In the preparations of IV and IX the organic solvent phase was omitted.

See Table II for compounds prepared by this method and their physical constants.

Assay—Hydroxyindole-*O*-methyltransferase was

isolated from beef pineal gland and purified according to the method of Axelrod and Weissbach (4).

The stock solutions of inhibitors II, III, IV, and VII were prepared in propylene glycol, and those of V, VI, VIII, and IX were prepared in dimethylsulfoxide. The previous findings showed that the same magnitude of inhibitory activity was obtained regardless of the use of these two solvents (1).

Incubation was carried out with *N*-acetylserotonin and *S*-adenosyl-L-methionine-methyl-^{14}C according to the previously described procedure (1).

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Keyphrases

Enzyme inhibitors
N-Acetyltryptamines—synthesis
Hydroxyindole-*O*-methyltransferase inhibition—*N*-acyltryptamines

Effect of Flow Rate on the Distribution Kinetics of a Drug From Perfusate to a Perfused Organ

By RENPEI NAGASHIMA and GERHARD LEVY*

A pharmacokinetic analysis of the distribution of a drug into a perfused organ or hypothetical compartment of the body yields mathematical expressions which may be useful for ascertaining if the distribution of a drug is rate limited by the flow of the perfusate, by the diffusion of the drug from the perfusate into the organ, or if it is a function of both of these processes. These expressions have been used to analyze the distribution of a drug from a perfusing fluid into the isolated perfused rat liver, and of several drugs from a hypothetical central compartment to a hypothetical peripheral compartment in man.

THE DISTRIBUTION of a drug from a perfusion fluid such as the blood to a perfused organ such as the liver is likely to be rate limited by the flow of the perfusate, by the diffusion of drug from perfusate to the organ, or it may be a function of both of these processes. It is useful therefore to undertake a kinetic analysis of such a system so that the rate-limiting step in the distribution of a given drug under defined conditions may be determined.

For the purpose of this analysis, the liver is viewed as a tissue with numerous parallel channels through which perfusate flows. The concentration of the drug in the perfusate leaving the liver is lower than the drug concentration in the fluid entering the liver, due to the diffusion of drug from perfusate into the liver. Elimination of the drug by the liver and back-diffusion of the drug from the liver to the perfusate are considered to be negligible during the early distribution phase. These assumptions lead to the same model as has been presented in an earlier report from this laboratory dealing with drug elimination kinetics in a perfused organ (1). Equation 8 of that report then takes the form:

$$k_{\text{dist.}} = \frac{u}{V_R}[1 - \exp(-k_{\text{trans.}}V_L/u)] \quad \text{(Eq. 1)}$$

where $k_{\text{dist.}}$ is the apparent first-order rate constant for the distribution of drug from perfusate to the perfused organ or tissue, u is the flow rate of perfusate, V_R is the real or apparent volume of the extrahepatic perfusate (which in practice usually is equivalent to the real or apparent volume of total perfusate), V_L is the volume of the channels in the organ or tissue through which the perfusate flows, and $k_{\text{trans.}}$ is a rate constant for the transfer of the drug from perfusate to the organ or tissue.[1] When drug distribution is rate limited by the flow of the perfusate, *i.e.*, $u \ll k_{\text{trans.}}V_L$, the bracketed term in Eq. 1 approaches unity, and

$$k_{\text{dist.}} = \frac{u}{V_R} \quad \text{(Eq. 2)}$$

If, on the other hand, the distribution of the drug is rate limited by the transfer of the drug from perfusate to the tissue, $k_{\text{trans.}}V_L/u < 0.1$ and it can be shown (1) that

$$k_{\text{dist.}} = \frac{V_L}{V_R} \cdot k_{\text{trans.}} \quad \text{(Eq. 3)}$$

It should be noted that Eq. 3 does not contain the flow-rate term.

One may now turn to a consideration of the more general case, namely, the distribution and elimination of the drug during the entire experimental

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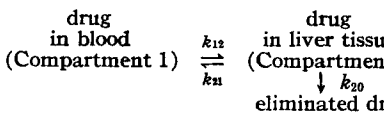
period. When sampling intervals are sufficiently short, it is possible to observe a bi-exponential decline in the drug concentration in the perfusate of an isolated liver perfusion system as a function of time (2). Then,

$$C = A \exp(-\alpha t) + B \exp(-\beta t) \quad \text{(Eq. 4)}$$

where C is the drug concentration in the perfusate at any time t, B is the extrapolated zero-time concentration and $-\beta/2.3$ is the slope of the terminal exponential phase in a plot of log C *versus* t, A is the extrapolated zero-time intercept and $-\alpha/2.3$ the slope obtained when the differences between the early experimentally determined drug concentrations and the values obtained by back-extrapolation of the terminal exponential phase of the concentration *versus* time curve are plotted semilogarithmically. The transfer of a drug between liver and blood and the elimination of drug from the system *via* the liver can be represented by the following model,



where the k's are apparent first-order rate constants defined in terms of the rate of the changes in the amount of drug in the respective compartments. It is evident that $k_{dist.}$ in Eqs. 1–3 is essentially equivalent to k_{12}. Considering the usual case where $V_R \gg V_L$, V_R approximates the total blood volume ($V_R + V_L$). The value for k_{12} can be determined from the equation:

$$k_{12} = (\alpha A + \beta B)/(A + B) \quad \text{(Eq. 5)}$$

which is derived from the relationship described by Rescigno and Segre for a more general model (3).

In a recent study of the pharmacokinetics of bishydroxycoumarin elimination in isolated perfused rat liver systems (4), the following average values were obtained: V_R = 100 ml., k_{12} = 0.063 min.$^{-1}$ (see *Footnote 2*), and u = 19 ml./min. These data yield a $k_{trans.}V_L$ value of 7.6 ml./min. Since this value is not much smaller than the flow rate, u, it can be concluded that the distribution of bishydroxycoumarin from the perfusate to the liver tissue is not rate limited solely by the diffusion of drug from the perfusate to the liver. Also, since $k_{12}V_R$ = 6.3 ml./hr. and $< u$, the distribution is not rate limited solely by the flow rate of perfusate. Rather, on the basis of the kinetic model used, the kinetics of the distribution of bishydroxycoumarin is a function of both the flow rate of the perfusate and the process described by the transfer rate constant, $k_{trans.}$, for the diffusion of drug from perfusate to liver tissue.

A similar kinetic analysis can be used to get some indication of the rate-limiting process (or the relative importance of blood flow and diffusion) in the distribution of a given drug from blood to other tissues, both *in vitro* and *in vivo*. Considering, for example, the distribution of a drug in man, $k_{dist.}V_R$ would equal the plasma flow rate if the latter is the rate-limiting step in the distribution of the drug (Eq. 2). A test of the mathematical model presented here is that $k_{dist.}V_R$ should never exceed a value equivalent to the known plasma flow rate through the compartment into which the drug is diffusing. Riegelman and his associates have pointed out recently that the distribution and elimination of certain drugs can be described adequately by a two-compartmental open-system model consisting of a central and a peripheral compartment (5). Using their reported values for α, β, A, and B, k_{12} values were calculated (6). Their V_p and k_{12} are equivalent to V_R and $k_{dist.}$ as defined here, and thus $k_{dist.}V_R$ can be determined. The results of these calculations for the five drugs reported on by Riegelman *et al.* (5) are listed in Table I. The highest $k_{dist.}V_R$ value, 0.80 l./min., was obtained with thiopental. If the peripheral compartment is considered to be equivalent to the poorly perfused tissues as classified by Price (7), the plasma flow rate through this compartment is 0.9 l./min. (see *Footnote 3*). This is in good agreement with the flow rate calculated from the thiopental data and it appears therefore that the distribution of thiopental into the peripheral compartment is essentially blood flow rate limited. On the other hand, the $k_{dist.}V_R$ values for the other drugs are such that blood flow rate does not appear to be the rate-limiting factor in their distribution from the hypothetical central compartment to the hypothetical peripheral compartment. Neither is their distribution rate limited by the transfer process represented by the rate constant, $k_{trans.}$, since their $k_{trans.}V_L$ values are not much smaller than u (Table I). It appears rather that their distribution is a function of both blood flow rate and transfer rate.

The significant result of this analysis is that the pharmacokinetic data for one drug yielded a calculated plasma flow rate essentially equal to the actual flow rate reported in the literature and, just as important, that none of the data yielded theoretical plasma flow rates ($k_{dist.}V_R$) that were higher than the actual value. One complicating factor in the pharmacokinetic analysis of drug distribution in intact animals or man (as opposed to isolated perfused organ systems) is the possible contribution to the $k_{dist.}$ values due to direct diffusion of drug between adjacent tissues (9, 10). This can lead to $k_{dist.}V_R$ values which are higher than the flow rate, or it can

TABLE I—DISTRIBUTION KINETICS OF SEVERAL DRUGS IN MAN[a]

Drug	k_{12}, min.$^{-1}$	V_p, l.	$k_{12}V_p$ or $k_{dist.}V_R$, l./min.	$(k_{trans.}V_L)/u$
Acetylsalicylic acid	0.085	6.3	0.54	0.92[c]
Salicylic acid	0.070	5.6	0.39	0.57
Griseofulvin	0.0038	59.3	0.23	0.29
Thiopental[b]	0.0132	60.5	0.80	2.21
Pentobarbital[b]	0.0118	62.0	0.73	1.67

[a] k_{12} and V_p values from *Reference 5* except where indicated otherwise. [b] Calculated using data from *Reference 5* and Eqs. 16 and 17 from *Reference 6*. [c] The value of u used in the calculations in this column is 0.9 l./min. (see *Footnote 3* in text).

[2] The perfusate was sampled initially every 10 min. In most cases, there was very little difference between the drug concentration in the perfusate at 10 min. and the theoretical 10-min. value obtained by back extrapolating the linear segment of the log concentration *versus* time plot. Therefore, the estimated value of α is only a minimum value, and thus, the calculated k_{12} is only a minimum value.

[3] Price lists a blood flow rate of 1.65 l./min. Considering an average hematocrit value of 45% (8), this equals a plasma flow rate of 0.9 l./min.

yield values equivalent to the flow rate although the latter is not distribution rate limiting.

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Keyphrases

Drug distribution—perfusate to rat liver
Flow rate, perfusate—drug-distribution effect
Pharmacokinetics—flow rate-drug distribution

Rapid Assay Method for the Determination of Methotrexate

By M. K. BALAZS, C. A. ANDERSON, and P. LIM

A rapid procedure for the assay of methotrexate samples (bulk or formulated) has been developed in these laboratories. This procedure, which employs quantitative paper chromatography, makes use of relatively inexpensive equipment and is simple to execute.

METHOTREXATE (MTX),[1] a clinically useful anticancer agent, is without exception an impure preparation consisting of MTX, contaminants related to MTX, and water. Efforts to assay MTX preparations have led to the development of column chromatographic procedures[2] that utilize adsorbents such as DEAE cellulose (1, 2) and diatomaceous earth.[3] These elegant procedures are adequate, but they require elaborate equipment and considerable time. The paper chromatographic procedure, which constitutes the basis of this note, requires relatively inexpensive equipment, is rapid and simple to execute, and affords a better separation of MTX from its contaminants.

The sensitivity of the method is limited only to the amount of MTX needed to give a useful UV spectrum. In this laboratory, a sample size of 1 mg. has been found to be convenient. The results of this procedure are comparable to those obtained by column methods.

Procedure—One milligram of methotrexate is dissolved in 0.2–0.3 ml. of 0.1 *N* sodium hydroxide and applied as a narrow band on Whatman No. 1 chromatographic paper 22.8 × 48.2 cm. (9 × 19 in.). The chromatogram is developed by descending chromatography with 0.5% aqueous sodium carbonate solution for 2.5 hr. The solvent front travels approximately 43 cm. (17 in.) during this time. The chromatogram is dried (in a fume hood) and the major band (MTX) is located ($R_f \simeq 0.6$–0.8) by illumination of the paper with shortwave UV light. This band is cut from the chromatogram and divided into 6 to 8 pieces, which are placed in a glass-stoppered, 125-ml. conical flask. Fifty milliliters of 0.1 *N* sodium hydroxide solution is added to the flask, which is then shaken vigorously until the paper is reduced to a pulp. A portion of the pulpy mixture is centrifuged at high speed for 3 min. or until a clear supernatant is obtained. The UV absorption of the supernatant solution is measured at 303 mμ, and the amount of methotrexate in the sample is calculated, using as a standard the molar absorptivity, 2.24×10^4.[4] A blank is prepared by the procedure cited above, and the slight increase in the base line is subtracted from the absorption of each sample. Due to the photo-instability of MTX, the entire analysis is carried out under minimal illumination.

Results and Discussion—The examples presented in Table I illustrate the precision of this method. The authors have observed that this procedure generally yields values as much as 2% lower than those obtained when the column chromatographic assay is used. The higher values obtained by column method are due to the inclusion of materials which contribute to the absorbance of the MTX but are in reality impurities. These impurities, which are absent in purified[5] samples of MTX, travel similarly to it but fluoresce brightly under UV light in contrast to the MTX, which absorbs. This visible difference permits excellent mechanical separation

Received May 21, 1968, from Stanford Research Institute, Menlo Park, CA 94025
Accepted for publication July 17, 1968.
This work was carried out under the auspices of the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, U.S. Public Health Service, contract No. PH-43-64-500. The opinions expressed in this paper are those of the authors and not necessarily those of the Cancer Chemotherapy National Service Center.

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yield values equivalent to the flow rate although the latter is not distribution rate limiting.

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TABLE I—ASSAY PROCEDURE FOR MTX

Lot[a]	Paper Chromatography	Column Chromatography
	In Bulk Material	
	% MTX	% MTX
A0301	88.8, 87.9, 88.5, 87.5, 88.3	88.8, 91.7, 89.7
	Av. %, 88.2	Av. %, 90.1
8105	91.5, 90.2	91.5
	In Formulated Samples	
250-mg. lyo vials[b]		
Vial No.	mg. MTX/vial, av.	mg. MTX/vial, av.
1	227, 225, 230 (227)	240, 226 (233)
2	230, 225 (228)	228, 236 (232)
3	225, 229 (227)	228
50-mg. lyo vials[c]		
		UV Assay, mg.
1	47.2, 48.0, 47.8 (47.7)	47.1
2	48.3, 46.7, 47.2 (47.4)	47.0
3	47.2, 49.2, 46.6 (47.7)	47.4
4	47.2, 48.5, 48.4 (48.0)	47.8

[a] Received from Cancer Chemotherapy National Service Center. [b] The lyo vials contained MTX as the sodium salt, 86 mg. of sodium chloride, and a small amount of sodium hydroxide. [c] The lyo vials contained MTX as the sodium salt, 34.4 mg. sodium chloride, 3.2 mg. methylparaben, 0.8 mg. propylparaben, and a small amount of sodium hydroxide.

of MTX from these contaminants and as a result the accuracy of the assay is enhanced. Agreement between the paper and column method is obtained if the first and last tube of the MTX band eluted from the column are eliminated. These tubes contain the fluorescent contaminants seen on the papers.

The total recovery of MTX from the chromatogram and evidence that no deterioration occurs during a run is illustrated by the analysis of a chromatographically homogeneous sample.[5] The comparison of the UV of the bulk with the UV of the band removed from the paper showed a 99.7% recovery (average of four runs).

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Keyphrases

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UV spectrophotometry—analysis

Heterocyclic Amines V. Electrophilic Substitution in Some Carbamate Derivatives of 3-Aminothiophene

By EMERY W. BRUNETT* and WALTER C. McCARTHY

t-Butyl N-(3-thienyl)carbamate has been brominated, and neopentyl N-(3-thienyl) carbamate has been chlorinated, brominated, iodinated, acetylated, nitrated, and coupled with a diazonium salt. In all cases the incoming substituent was shown by NMR spectrum to be in the 2-position.

VARIOUS ELECTROPHILIC substitution reactions were investigated for some carbamate derivatives of 3-aminothiophene in order to determine suitable conditions for such reactions with these sensitive compounds, and to confirm the orientation of the incoming group.

t-Butyl *N*-(3-thienyl)carbamate (1) was smoothly brominated in the 2-position with *N*-bromosuccinimide. Similarly, the neopentyl ester (1) could be chlorinated, brominated, or iodinated with the corresponding *N*-halosuccinimides. The neopentyl ester was acetylated under mild conditions using only acetic anhydride and acetic acid. Attempted nitration of the neopentyl ester with nitric acid in acetic anhydride, as used by Campaigne (2) to nitrate the similar 3-acetamidothiophene, gave only tar, but the nitration could be effected in good yield by Anderson's (3) reagent, cupric nitrate in acetic anhydride. The neopentyl ester also coupled readily with sodium *p*-nitrobenzenediazotate. In all cases the incoming substituent was shown by NMR spectrum to be in the 2-position.

Received May 3, 1968, from the College of Pharmacy, University of Washington, Seattle, WA 98105
Accepted for publication July 16, 1968.
* Pharmaceutical Manufacturers Association Fellow of the American Foundation for Pharmaceutical Education, 1965–1966.

EXPERIMENTAL[1]

t-Butyl N-(2-bromo-3-thienyl)carbamate—From 4.0 g. (0.02 mole) of *t*-butyl *N*-(3-thienyl)carbamate (1) and 3.6 g. (0.02 mole) of *N*-bromosuccinimide refluxed in carbon tetrachloride for 2 hr., after the usual workup of filtration, extraction with water to remove succinimide, evaporation of solvent, decolorization with carbon, and recrystallization from dilute alcohol, there was obtained a yield of 3.54 g. (64%), m.p. 69.5–71°, NMR spectrum in CCl_4: τ = 8.46 (s, CH_3, 9H), 3.46 (broad peak, NH, 1H), 2.81 (d, 4-H of ring, 1H), 2.37 (d, 5-H of ring, 1H) J_{45} = 5.8 c.p.s.

Anal.—Calcd. for $C_9H_{12}BrNO_2S$: C, 38.86; H, 4.35; Br, 28.73; N, 5.04; O, 11.50; S, 11.54.

[1] All of the analyses reported in this paper were performed in the laboratories of Dr. Alfred Bernhardt, Mülheim, Germany.

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[1] All of the analyses reported in this paper were performed in the laboratories of Dr. Alfred Bernhardt, Mülheim, Germany.

Found: C, 38.99; H, 4.43; Br, 28.60; N, 4.97; O, 11.48; S, 11.65.

Neopentyl N-(2-bromo-3-thienyl)carbamate—From 2.13 g. (0.01 mole) of neopentyl *N*-(3-thienyl)-carbamate (1) and 1.78 g. (0.01 mole) of *N*-bromosuccinimide refluxed in carbon tetrachloride for 2 hr., after the usual workup as indicated for the *t*-butyl ester above, and recrystallization from dilute alcohol, there was obtained a yield of 1.8 g. (62%), m.p. 81–81.5°, NMR spectrum in CCl_4: τ = 9.03 (s, CH_3, 9H), 6.17 (s, CH_2, 2H), 3.37 (broad peak, NH, 1H), 2.87 (d, 4-H of ring, 1H), 2.46 (d, 5-H of ring, 1H); J_{45} = 6 c.p.s.

Anal.—Calcd. for $C_{10}H_{14}BrNO_2S$: C, 41.10; H, 4.83; Br, 27.35; N, 4.79; O, 10.95; S, 10.97. Found: C, 41.06; H, 4.67; Br, 27.19; N, 4.81; O, 11.04; S, 11.13.

Neopentyl N-(2-chloro-3-thienyl)carbamate—A solution of 2.13 g. (0.01 mole) of neopentyl *N*-(3-thienyl)carbamate (1) in 50 ml. of chloroform was heated to boiling and 1.135 g. (0.01 mole) of *N*-chlorosuccinimide was added in small portions. The mixture was heated under reflux for 2 hr. The succinimide was removed by extraction with water, and the dried chloroform layer was evaporated. The residue was decolorized with charcoal and recrystallized from dilute alcohol to give a 1.8 g. (73%) yield, m.p. 86–87°, NMR spectrum in CCl_4: τ = 9.03 (s, CH_3, 9H), 6.16 (s, CH_2, 2H), 3.25 (broad peak, NH, 1H), 3.01 (d, 4-H of ring, 1H), 2.43 (d, 5-H of ring, 1H); J_{45} = 6 c.p.s.

Anal.—Calcd. for $C_{10}H_{14}ClNO_2S$: C, 48.48; H, 5.70; Cl, 14.31; N, 5.65; O, 12.92; S, 12.94. Found: C, 48.38; H, 5.73; Cl, 14.11; N, 5.67; O, 13.13; S, 13.10.

Neopentyl N-(2-iodo-3-thienyl)carbamate—Following a method similar to that for the corresponding bromo compound, there was obtained from 1.292 g. (0.006 mole) of neopentyl *N*-(3-thienyl)carbamate (1) and 1.364 g. (0.006 mole) of *N*-iodosuccinimide, after decolorization of the product with charcoal and recrystallization from dilute alcohol, 1.4 g. (69%) of white crystals, m.p. 81–82°. Attempted drying of these crystals in a drying pistol under vacuum over phosphorus pentoxide overnight resulted in decomposition with blackening, but the crystals could be suitably dried in the dark over activated alumina. Because this compound decomposed en route on sending a sample abroad for the customary elemental analysis, it was identified solely by means of its NMR spectrum in CCl_4: τ = 9.01 (s, CH_3, 9H), 6.12 (s, CH_2, 2H), 3.38 (broad peak, NH, 1H), 2.62 (d, 4-H of ring, 1H), 2.49 (d, 5-H of ring, 1H); J_{45} = 5.8 c.p.s.

Neopentyl N-(2-nitro-3-thienyl)carbamate—A solution of 0.533 g. (2.5 mmoles) of neopentyl N-(3-thienyl)carbamate (1) in 15 ml. of acetic anhydride was cooled to dry ice–acetone temperature and to this was added over a 10-min. period, a solution of 0.64 g. (2.65 mmoles) of cupric nitrate trihydrate (3) in 33 ml. of acetic anhydride. The cooling bath was removed and the reaction was allowed to proceed for 75 min. longer with gradual warming to room temperature. The color of the reaction mixture changed from blue to green. Water (50 ml.) was added and stirring was continued for an additional 10 min. The reaction mixture was shaken with 300 ml. of distilled water and this solution was extracted with three 50-ml. portions of dichloromethane. The combined organic extracts were washed with dilute ammonium hydroxide and then with water. The organic layer was dried over anhydrous magnesium sulfate and then evaporated to dryness. The residue was recrystallized from dilute alcohol to give 0.51 g. (79%) of pale yellow crystals, m.p. 98.5–100°, NMR spectrum in CCl_4: τ = 9.00 (s, CH_3, 9H), 6.12 (s, CH_2, 2H), 2.63 (d, 4-H of ring, 1H), 2.14 (d, 5-H of ring, 1H), 0.52 (broad peak, NH, 1H); J_{45} = 6.0 c.p.s.

Anal.—Calcd. for $C_{10}H_{14}N_2O_4S$: C, 46.50; H, 5.46; N, 10.85; O, 24.78; S, 12.41. Found: C, 46.43; H, 5.95; N, 10.73; O, 24.88; S, 12.54.

Neopentyl N-(2-acetyl-3-thienyl)carbamate—A mixture of 2.13 g. (0.01 mole) of neopentyl *N*-(3-thienyl)carbamate (1), 4 g. of acetic anhydride, and 4 g. of glacial acetic acid was warmed to effect solution of the carbamate and stirred for 18 hr. The mixture was poured into water to destroy the excess acetic anhydride and the product was extracted with ether. The ether extract was washed with sodium bicarbonate solution, dried over anhydrous sodium carbonate, and evaporated to dryness. The residue was decolorized with charcoal and crystallized from dilute alcohol. The NMR spectrum of this material indicated that it was a mixture of starting material and acetylated product, but attempts at fractional crystallization to purify the product were unsuccessful. This material, dissolved in petroleum ether, was chromatographed on 90 g. of aluminum oxide. Elution with 250 ml. of carbon tetrachloride and evaporation of the eluate gave a white crystalline product, which after recrystallization from dilute ethanol, showed a yield of 0.59 g. (23%), m.p. 99–100°, NMR spectrum in CCl_4: τ = 9.01 (s, CH_3, 9H), 7.62 (s, CH_3CO, 3H), 6.17 (s, CH_2, 2H), 2.67 (d, 4-H of ring, 1H), 2.11 (d, 5-H of ring, 1H), −0.3 (broad peak, NH, 1H); J_{45} = 5.5 c.p.s.

Anal.—Calcd. for $C_{12}H_{17}NO_3S$: C, 56.45; H, 6.71; N, 5.49; O, 18.80; S, 12.56. Found: C, 56.34; H, 6.69; N, 5.41; O, 18.91; S, 12.44.

Neopentyl N-(2-p-nitrobenzeneazo-3-thienyl) carbamate—A suspension of 0.565 g. (3 mmoles) of sodium *p*-nitrobenzene-antidiazotate (4) in 3 ml. of distilled water plus 4.5 g. of glacial acetic acid was added with stirring to a solution of 0.533 g. (2.5 mmoles) of neopentyl *N*-(3-thienyl)-carbamate (1) in glacial acetic acid. A red-orange precipitate began to form at once. The reaction mixture was stirred for 0.5 hr., 15 ml. of water was added, the precipitate was collected by filtration under suction, washed with 10 ml. of 50% alcohol and then with 3 ml. of 95% alcohol. Drying in a vacuum desiccator gave a yield of 0.61 g. (67%). After recrystallization from dilute alcohol, the orange crystals melted at 167–168°, NMR spectrum in CCl_4: τ = 8.97 (s, CH_3, 9H), 6.06 (s, CH_2, 2H), unresolved spectrum in lower field with apparent multiplets centered at 2.19 and 1.73 (benzene protons) and doublets at 2.50 and 2.15 (thiophene protons); J_{45} = 6 c.p.s.

Anal.—Calcd. for $C_{16}H_{18}N_4O_4S$: C, 53.03; H, 5.01; N, 15.46; O, 17.66; S, 8.85. Found: C, 53.15; H, 5.13; N, 15.61; O, 17.52; S, 9.04.

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Keyphrases

Heterocyclic amines
NMR spectroscopy—structure
3-Aminothiophene carbamates—electrophilic substitution

Cumulative Lethal Dose of Alcohol in Mice Given Amitriptyline

By GERALD MILNER

Because of the continued social use of alcohol and the wide prescription of ethical psychotropic drugs, it is essential to test for possible drug–alcohol interactions. It was found that pretreatment with amitriptyline (50 mg./kg.) significantly potentiated the toxic effects of multiple doses of alcohol when these were given at two hourly intervals. Thirty mice given amitriptyline required significantly fewer doses of alcohol to produce death than was the case in groups of mice which had been given placebo in place of amitriptyline.

THE PSYCHOTROPIC DRUGS are being more widely prescribed and, as most adults drink alcohol, adverse effects may occur due to drug–alcohol interaction. It is possible to test for and predict such interaction by animal studies. Adequately designed, reproducible tests should form part of the screening and preliminary evaluation of all new drugs.

A method of testing the effects of chronic treatment with psychotropic drugs on the acute toxicity of other agents was described by Meyers, Kanyuck, and Anderson (1). They used adult rats which had been maintained on a diet containing 0.04% nortriptyline HCl or 0.04% amitriptyline HCl. Five animals from each group were used for interaction tests. Doses of the challenging agent were given intraperitoneally every 30 min. to the control and thymoleptic pretreated rats, until death occurred. A cumulative lethal dose (CLD) for the challenging agent was then computed for the individual animals by multiplying the amount of drug (dose in mg./kg.) by the number of doses required to produce death. An index of interaction was later established by dividing the geometric mean of CLD values for the control animals by the geometric mean of CLD's in each of the thymoleptic pretreated groups. An interaction index significantly larger than 1.00 indicated a synergistic interaction. Meyers *et al.* did not find evidence of potentiation of toxicity of alcohol by either amitriptyline or nortriptyline in rats. Other studies involving measures of "length of loss of righting reflexes" and lethal dose levels have indicated that amitriptyline may add to the sedative and toxic effects of alcohol in mice and humans (2–4). This paper describes a study of the CLD of alcohol in mice given a single dose of amitriptyline.

METHOD

Adult albino mice (from the strain bred by the Institute of Medical and Veterinary Science, Adelaide, South Australia) were arranged in six groups of 10. Three of the groups were given 50 mg./kg. amitriptyline and three a placebo solution (water equal in volume to the amitriptyline solution). The mice were then dosed at two hourly intervals with a solution of alcohol. All treatments were administered orally. Four of the groups receiving the multiple doses of alcohol were given 12.5 ml./kg. 25%; the others were given 10 ml./kg. 25% alcohol. The mice were observed in individual rodent observation chambers (5) for loss of righting reflexes and time of death. The results were tested by Fisher's analysis of variance technique.

RESULTS

In a group of 10 mice given amitriptyline and multiple doses of 10 ml./kg. 25% alcohol, the average number of doses of alcohol required to cause death was 4.0; in the corresponding group given a placebo in place of amitriptyline, the average number of doses required to cause death was 6.1 (see Table I). This interaction index of 1.5 is significant at the 1% level (see Table II).

In the 20 mice given amitriptyline plus multiple doses of 12.5 ml./kg. 25% alcohol the average number of doses required to cause death was two;

Received May 23, 1968, from Claremont Hospital, Mental Health Services, Claremont, Western Australia.
Accepted for publication July 22, 1968.
The author acknowledges Dr. A. S. Ellis, Mental Health Services, Western Australia; Dr. I. Oswald, University of Western Australia, Department of Psychiatry; Dr. W. Laurie, Public Health Laboratory of Western Australia, who provided facilities for this research. Dr. M. F. Lockett (Department of Pharmacology, University of Western Australia) gave advice on the experimental methodology. Geigy, Roche, and Sandoz pharmaceutical companies assisted in a number of ways.
This work forms part of a thesis submitted for the Doctor of Medicine degree, University of Western Australia.

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Keyphrases

Heterocyclic amines
NMR spectroscopy—structure
3-Aminothiophene carbamates—electrophilic substitution

Cumulative Lethal Dose of Alcohol in Mice Given Amitriptyline

By GERALD MILNER

Because of the continued social use of alcohol and the wide prescription of ethical psychotropic drugs, it is essential to test for possible drug–alcohol interactions. It was found that pretreatment with amitriptyline (50 mg./kg.) significantly potentiated the toxic effects of multiple doses of alcohol when these were given at two hourly intervals. Thirty mice given amitriptyline required significantly fewer doses of alcohol to produce death than was the case in groups of mice which had been given placebo in place of amitriptyline.

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This work forms part of a thesis submitted for the Doctor of Medicine degree, University of Western Australia.

TABLE I—NUMBER OF DOSES OF ALCOHOL REQUIRED TO CAUSE DEATH IN MICE GIVEN AMITRIPTYLINE OR PLACEBO

Pretreatment Condition	Group	Number of Doses of Alcohol Required to Cause Death (Animal Number) 1	2	3	4	5	6	7	8	9	10
Amitriptyline, 50 mg./kg.	A[a]	2	3	3	3	4	5	5	5	5	5
Placebo	B[a]	3	4	5	6	6	7	7	7	8	8
Amitriptyline, 50 mg./kg.	C[b]	1	1	1	1	1	2	2	2	2	2
Amitriptyline, 50 mg./kg.	D[b]	1	1	1	2	3	3	3	3	3	4
Placebo	E[b]	1	2	2	2	3	3	3	3	4	4
Placebo	F[b]	2	2	2	3	3	3	3	3	3	3

[a] Dosed with alcohol 10 ml./kg. 25% (orally). [b] Dosed with alcohol 12.5 ml./kg. 25% (orally).

TABLE II—ANALYSIS OF VARIANCE OF THE CUMULATIVE LETHAL DOSE SCORES SHOWN IN TABLE I

Source of Variance	*df*	*SS*	*MS*	*F* Ratio Significance
Groups A & B				
Between conditions	1	22.05	22.05	10.75[a]
Between subjects within condition	18	36.90	2.05	
Total	19	58.94		
Groups C, D, E, & F				
Between conditions	1	5.625	5.625	8.76[a]
Between groups within condition	2	4.050	2.025	3.15
Within groups	36	23.100	0.642	
Total	39	32.775		

[a] Significant at the 1% level of probability.

the corresponding 20 mice which had been given placebo in place of amitriptyline took an average of 2.7 doses of alcohol before they died. This interaction index of 1.35 is significant at the 1% level.

DISCUSSION

This study of the cumulative dose of alcohol in mice given amitriptyline or placebo confirms the findings of Halliwell *et al.* (2), Lockett and Milner (3), and Milner (4), that amitriptyline increases the toxicity of alcohol in mice. Meyers *et al.* (1), in tests with rats, found no significant interaction of alcohol with amitriptyline, but 10-day chronically amitriptyline-fed rats did show an interaction with some other psychotropic agents. Meyers *et al.* used an interval between doses of 0.5 hr., whereas in the experiments described in this paper an interval of 2 hr. was used. When the cumulative loss of righting reflexes in mice given amitriptyline and alcohol or alcohol alone is plotted, little difference is found between the two groups in the first hour after dosing but, as the experiment progresses, the difference between the groups becomes progressively marked (6).

Meyers *et al.* do not state the dose of alcohol they used. If the concentration of alcohol used in their experiments was low, then it is likely that the rats were overloaded with the volume of alcohol needed to cause death and the effect of this would mask an interaction between the amitriptyline and the alcohol. If the alcohol were given in a concentrated solution, then protein denaturation and hemorrhagic erosion of the peritoneal membranes may have interfered with the absorption of the alcohol (7).

In addition to the above, the caution advised by Brodie, Cosmides, and Rall (8) may be quoted—"Since organic compounds can disappear from mice as much as a thousand times as rapidly as from man, it is disconcerting to hear proposals that certain drugs should be administered in small daily doses over the lifetime of a mouse without it being known whether or not the agent is inactivated so rapidly that it is almost equivalent to a placebo."

It seems reasonable to warn patients on amitriptyline that they should avoid drinking alcohol. Drug effects represent a complex interaction between the individual patient, the chemical compound, and the environment. Modern psychotropic agents are potent and have widespread effects—environmental factors which may contribute to the present high incidence of adverse drug reactions should be minimized.

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Keyphrases

Amitriptyline–alcohol interaction
Alcohol cumulative lethal dose—amitriptyline effect
Interaction index—amitriptyline–alcohol

Pharmaceutical Applications of the Near Infrared Determination of Water

By JOSEPH E. SINSHEIMER and NANCY M. POSWALK

The combination band for water at 1.9 μ has been observed in a series of solvent systems and for selected solid-state samples. Pharmaceutical applications of the measurement of this band are described.

ALTHOUGH SEVERAL STUDIES and analytical applications involving near IR water bands have been reported (1, 2), little has been developed concerning pharmaceutical systems. It is the purpose of this paper to expand pharmaceutical applications of measurements of the 1.9-μ combination band of water in the areas of (*a*) studies of water as a solute, (*b*) determinations comparable to Karl Fischer titrations of water, and (*c*) the direct determination of water in solids.

EXPERIMENTAL

Reagents—Spectrograde acetonitrile was employed. All other solvents were reagent grade and except for chloroform were used without further purification. The chloroform employed was passed through a column of alumina to remove ethanol and used within 5 days.

Instrumental Parameters—The Beckman DK-2A settings employed for absorbance spectra and 0 to 100 scale percent transmittance spectra were: sensitivity, 50; time constant, 0.2; recording speed, 18 mμ/min.; and wavelength expansion, 25 mμ/cm. These parameters were also employed for 100 to 90 expanded scale percent transmittance spectra with a change in time constant to 2.0 and recording speed to 3.6 mμ/min. Solid-state samples were recorded at a maximum sensitivity of 312, time constant of 2.0, and recording speed of 3.6 mμ/min. Wavelength calibration was on the basis of a spectrum of 1,2,4-trichlorobenzene (3).

Spectra—(*a*) Except where noted, water spectra were obtained in solution in 1 cm. near IR matching cells against the anhydrous solvents from 2,100 to 1,700 mμ.

(*b*) Water in hydrophilic petrolatum was observed as a thin film between silica plates against a silica plate as the reference and under nitrogen purge from 2,150 to 1,550 mμ. The 90 to 100% expanded transmittance range was employed with manual attenuation of the reference shutter required for hydrated ointment samples to establish a base line in this range.

(*c*) Solid-state absorption spectra were obtained against air and with manual attenuation of the reference shutter when required. From 25 to 40 mg. of compound was mixed with 60 to 90 mg. of powdered and dried analytical grade KCl in a plastic capsule of the Wig-L-Bug[1] for 30 sec. Thirteen-millimeter disks were formed at 20 tons pressure for 5 min. in a specially constructed die.[2] Hydrated samples were pressed without vacuum while anhydrous samples were held in an evacuated die for 5 min. before being pressed for an additional 5 min. at 20 tons. Anhydrous samples and dry powdered KCl were obtained by drying these materials for 48 hr. under vacuum at 110°.

Extraction Procedures—(*a*) From 0.1 to 0.3 g. of insoluble hydrate samples accurately weighed were allowed to equilibrate with 5 ml. of 5% pyridine in methanol for 1 hr. at 60° in stoppered 10-ml. flasks. The samples were centrifuged at 1,000 r.p.m. for 10 min. and a portion of the supernatant liquid was transferred by pipet to a cell. This solution was read against a blank pyridine–methanol solution processed in the same manner and compared to standard series of water in pyridine-methanol solutions.

(*b*) From 10 to 15 g. of crude drug accurately weighed was extracted in an electric blender (Waring) for 7 min. with 100 ml. of methanol. The methanol solution was decanted, centrifuged, and read against an aliquot of a 100-ml. methanol blank processed in the same manner.

RESULTS AND DISCUSSION

Greinacher, Lüttke, and Mecke (4) in establishing water bands in both the IR and near IR laid the basis for the assignment of the 1.9 μ water band as a combination band of fundamental stretching and bending vibrations of water. These authors also established the effect on bond shape, intensity, and wavelength of maximum absorbance by various solvents in regard to the 2.7-μ fundamental stretching vibrations. These effects are considered hydrogen bonding phenomena (5).

Similar hydrogen bonding effects are exhibited by the 1.9-μ band of water in solvents. Figure 1 shows these variations for 0.2% v/v solutions of water in miscible solvents of interest in this investigation. Table I lists the wavelength of maximum absorbance for water in such solvents as well as for saturated water solutions in solvents in which water has only limited solubility.

These results are consistent with the recent work of O'Laughlin *et al.* (6) in their near IR study of phosphine oxides as bases in association with water in carbon tetrachloride. Their investigation in turn takes note of the report of Mohr, Wilk, and Barrow (7) which assigns OH stretching fundamental vibrations for free water, water bonded with one molecule of base (solvent), B··HOH, and with water bonded to two molecules of base, B··HOH··B. Thus, based upon these references,

Received May 6, 1968, from the College of Pharmacy, The University of Michigan, Ann Arbor, MI 48104

Accepted for publication August 5, 1968.

Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

This investigation was supported in part by a Mead Johnson grant for undergraduate research.

The assistance of Mr. Henry Lee is acknowledged and appreciated.

[1] Crescent Dental Co., Chicago, Ill.

[2] Die constructed by Limit Div., Beckman Corp., Fullerton, Calif., according to the specification of Sister M. M. Stimson.

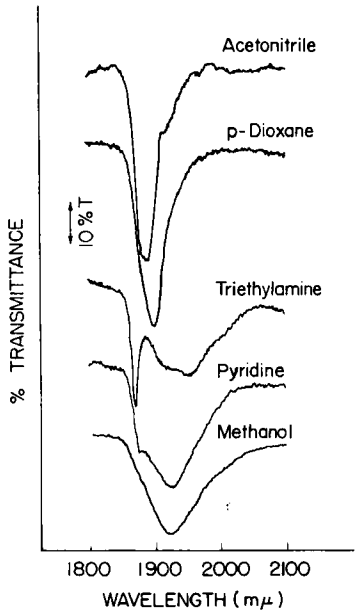

Fig. 1—Spectra of 0.5% v/v water in acetonitrile, p-dioxane, triethylamine, pyridine, and methanol.

the following assignments were made in the present investigation: (*a*) free water, 1.89 to 1.90 μ (values for dilute solutions in the inert solvents, carbon tetrachloride, and tetrachloroethylene); (*b*) free OH for water bonded at one H only, 1.90–1.92 (values for higher concentrations of water in intermediate basic strength solvents); and (*c*) bonded OH stretching, associated water and the bonded OH of both the B··HOH and B··HOH··B species, broad bands from 1.90 to 2.00 (values for higher concentrations of water in stronger basic solvents).

Figure 2 represents the Beer's law relationships

TABLE I—WAVELENGTH OF MAXIMUM ABSORBANCE FOR WATER IN VARIOUS SOLVENTS

Solvent	0.01% Water as Solute, mμ	0.2% Water and 12% Alcohol, mμ
n-Hexane	—	1,890; 1,932
Cyclohexane	—	1,885; 1,937
Triethylamine	1,882; 1,966[a]; 1,990[a]	—
Carbon tetrachloride	1,886	1,887; 1,936
Tetrachloroethylene	1,887	1,885; 1,935
Benzene	1,891	1,897; 1,937[a]
Toluene	1,891	1,897; 1,937[a]
Carbon disulfide	1,892	—
Chloroform	1,893	1,891; 1,944[a]
1,2-Dichloroethane	1,895	1,896; 1,945[a]
Bromoform	1,896	1,898; 1,947[a]
Ethyl acetate	1,897	—
Dibromomethane	1,899	1,900; 1,945
Acetonitrile	1,901	1,904
Acetone	1,903	1,903
Dioxane	1,911	1,915
Methanol	1,932	—
Ethanol[b]	1,887[a]; 1,935	—
Pyridine	1,943	1,894[a]; 1,939

[a] Wavelength reading at the center of a shoulder. [b] With 0.2% water.

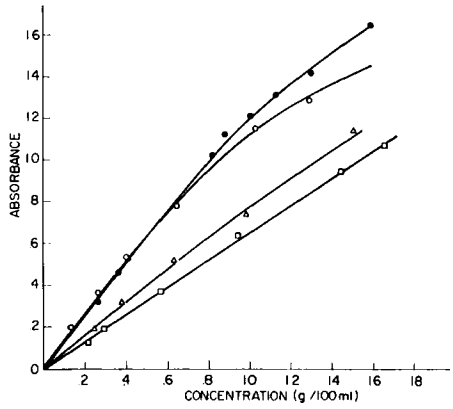

Fig. 2—Absorbance versus *concentration relationship for water in* p-*dioxane at 1.91 μ, acetonitrile at 1.90 μ, pyridine at 1.94 μ, and methanol at 1.93 μ. Key:* ●, p-*dioxane;* ○, *acetonitrile;* △, *pyridine;* □, *methanol.*

for some representative water-miscible solvents. The values plotted for the less basic solvents, acetonitrile and *p*-dioxane, are for the more intense peaks for these solvents at 1.9 μ while the broad bonded peaks are plotted for pyridine and methanol. Note the deviation from conformity to Beer's law at the higher concentrations for acetonitrile and *p*-dioxane which is consistent with the opportunity for more water to water association at higher water concentrations. This is also indicated by an increased ratio of absorbance in the 1.90 to 2.00-μ region as compared to 1.90 μ absorbance at higher water concentrations. Thus, while water can be quantitatively determined in a variety of solvents on the basis of its near infrared combination band, it is important to realize that range of conformity to Beer's law is limited in weakly basic solvents to the lower concentrations. It also follows that initial traces of water in the reagent employed has more effect in the weakly basic solvents. That is, while the absorbance of such water can be compensated in the reference cell, its contribution to increasing water associations is not compensated.

The sensitivity of the procedure is comparable to that of Karl Fischer titrations. Most measurements were made with normal 1 cm. near IR cells of 4-ml. capacity. Under these conditions, as shown in Fig. 2, less than 1% solution of water is required to obtain significant absorbance. Microcells, particularly a 50-μl. cell,[3] were also found to be of value. For example, 50 μl. of a methanol solution, which is a relatively opaque solvent at 1.9 μ, containing 0.5% water was readily measured in this cell against a methanol blank in a similar cell.

In an effort both to increase the solubility of water in the nonpolar solvents and to increase the range of conformity to Beer's law, a limited number of ternary systems were evaluated. These involved 0.2% water and 12% alcohol in various solvents. Typical results are illustrated in Fig. 3 and are summarized in Table I. It is particularly interesting that while there is an increase in the bonded OH bands (broad absorption in the 1.9 to 2.0-μ

[3] Cell No. 97250, Beckman Corp., Fullerton, Calif.

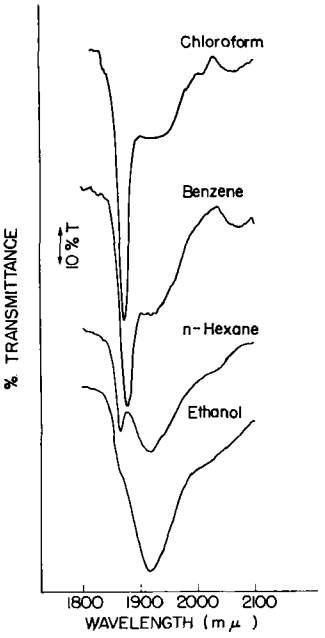

Fig. 3—Spectra of 0.2% v/v water and 12% v/v ethanol in chloroform, benzene, n-hexane as compared to 0.2% v/v water in ethanol.

region), there is also retention of essentially the same absorption of the sharp 1.9-μ bands which appear in the aprotic and the less basic solvents. It should be noted that a similar situation exists for the assignment of the lowest wavelength band in the fundamental stretching region. Errera, Gaspart, and Sack (8) pointed out in the fundamental region that low wavelength OH stretching absorption is retained in ternary systems.

Based upon conformity to Beer's law over a greater range, systems involving pyridine and methanol were evaluated for quantitative water determinations comparable to Karl Fischer determinations. Where a compound is soluble in these solvents and shows no interfering absorption in the 1.9-μ region, a direct measurement of the solution and comparison to standard water in solvent values becomes a possible moisture determination. Thus, as outlined in Table II, the water of crystallization for methylene blue and atropine sulfate was determined by solution in methanol. However, because methylene blue absorbs in the 1.9-μ region, as indicated by an atypical water in methanol spectrum, a base-line calculation method was used to obtain these results. The dehydration of a sample of cholesterol was also followed by dissolving it in pyridine and noting the decrease in absorbance at 1.92 μ. Ascorbic acid systems were of potential interest in that they cannot be determined directly in the Karl Fischer procedure. While water in presence of ascorbic acid can be determined in methanol as indicated in Table II, there again is the limitation of having to compensate for absorption by ascorbic acid at 1.92 μ.

The determination of water of hydration of stable hydrates by extraction can be illustrated by the successful results reported in Table II for ammonium oxalate and sodium tartrate. While

Table II—Near Infrared Water Determinations

System	Found, %	Comparison Values, %
Solutions		
Atropine sulfate	3.96, 3.98, 3.96	3.35[a]
Methylene blue	13.5, 13.4, 13.1	13.2[a]
Water in the presence of ascorbic acid	1.25	1.26[b]
	2.55	2.52[b]
	3.81	3.79[b]
Extracted water		
Stable hydrates		
Ammonium oxalate	12.3, 12.6, 12.3	12.68[c]
Sodium tartrate	15.3, 15.7, 15.7	15.66[c]
Crude drugs		
Belladonna leaves	6.04	6.62[a]
Cinchona bark	6.87	6.51[a]
Colchicum root	5.31	6.42[a]
Podophyllum	7.54	7.27[a]

[a] Water content based upon loss on drying. [b] Percentage of water actually added. [c] Amount present in pure crystal.

such water could not be efficiently determined with pyridine as the solvent, quantitative extraction was accomplished after 2 hr. at 60° with methanol. The addition of 5% pyridine permitted a reduction of the extraction time to 1 hr. Centrifugation was found to be a satisfactory method to remove traces of suspended solids without extended exposure to atmospheric moisture. In general, the stronger hydrogen bonding solvents were the most useful for quantitative measurements not only based upon their greater miscibility with water and conformity to Beer's law over greater range, but also because of their greater capacity to extract bonded water.

The Waring blender extraction procedure used by Mary (9) in the gas chromatography determination of water in crude drugs was applied for the assay of their moisture content by near IR measurements. It was found necessary to run a blank methanol determination in the electric blender in order to obtain consistent results in agreement with the NF (10) oven drying procedure. The results of these determinations are listed in Table II. The oven dehydrated drugs were also extracted and evaluated by the same procedure. Only the extract of the dried cinchona bark showed absorbance equivalent to water of about 0.5% of the hydrated drug.

The near IR moisture procedure was applied in connection with studies in progress[4] on the water content of the micellar system: Dioctyl sodium sulfosuccinate (Aerosol OT) (0.1413 g.), *n*-octane (6.925 g.), water (0.04 and 0.08 g.). Water could be determined directly in such a system against a reference anhydrous system and was not significantly different than water as a neat film. The quantitative comparison of water at the two levels (1:2) by direct measure of these systems was somewhat inhibited (1:1.93) by a nonlinear base line caused by light diffusion of the

[4] Samples provided by Drs. G. Zografi and S. Frank, University of Michigan.

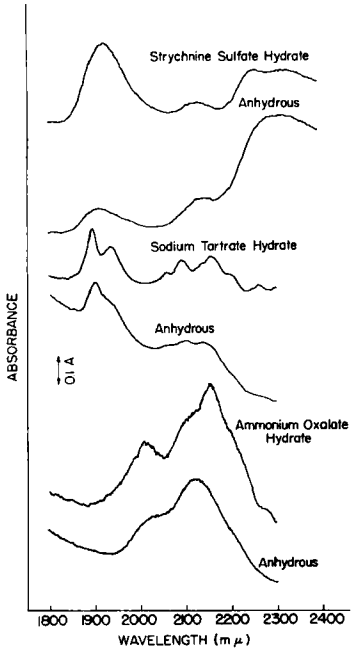

Fig. 4—Solid-state absorbance spectra for: (a) *strychnine sulfate hydrate (0.0323 to 0.0793 g. KCl to form 0.1154-g. disk) compared to strychnine sulfate anhydrous (0.0356 to 0.0891 g. KCl to form 0.1154-g. disk);* (b) *sodium tartrate hydrate (0.0355 to 0.0681 g. KCl to form 0.0654-g. disk) compared to sodium tartrate anhydrous (0.0412 to 0.0925 g. KCl to form 0.1162-g. disk); and* (c) *ammonium oxalate hydrate (0.0272 to 0.0619 g. KCl to form 0.0776-g. disk) compared to ammonium oxalate anhydrous (0.0266 to 0.0797 g. KCl to form 0.0546-g. disk).*

system. A quantitative ratio of absorbance was obtained, however, by quantitatively diluting 1 ml. of the micellar system with 4 ml. of methanol and determining the water content as compared to a standard water in methanol solution.

Water could also be readily observed in a hydrophilic petrolatum emulsion. Samples of ointment and water film were determined against a reference silica plate. While quantitative measurements of the water content of such an ointment were attempted using the intensity of CH overtone at 1.7 μ as an internal standard, these were only partially successful. Both diffusion and lack of stability at higher water content interfered with quantitative results.

The opportunity to observe water directly in solid-state samples is an attractive feature of near infrared measurements. In Fig. 4 is shown a comparison of three pairs of hydrated *versus* anhydrous samples. While changes in absorbance in the 1.9- and 2.0-μ region were expected, the changes above 2.1 μ as found in sodium tartrate and ammonium oxalate were not anticipated. As 100-mg. KCl disks were prepared containing at least 25 mg. of compound, it was difficult to control diffusion and to obtain quantitative results.

In common with other spectral procedures, the major limitation of the near IR water measurements is the necessity to account for background and reference absorbance. However, the ease of obtaining data, the potential for microdetermination, and the ability to examine redox compounds make this technique a valuable addition to the Karl Fischer method.

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Keyphrases

Water determination
Solvents, solids—water determination
IR spectrophotometry, near—analysis

Inhibition of Liver-Derived Monoamine Oxidase by Several Isopropyl Hydrazides

By M. R. WILEY, A. M. BURKMAN, and M. H. WEINSWIG*

Six aromatic hydrazide derivatives were examined for their ability to inhibit enzymatic oxidation of tyramine *in vitro* utilizing the Warburg technique. Two compounds, 1-*p*-chloro- and 1-*p*-hydroxybenzoyl-2-isopropyl hydrazide, were included in this study in order to compare their *in vitro* activities with their reported MAO inhibiting actions *in vivo*. Four of the acyl isopropyl hydrazines examined were recently synthesized substances whose biologically-relevant activities had not been previously investigated. Iproniazid served as a reference standard against which the other hydrazides were compared. Dose-dependent inhibitory effects were demonstrated for all compounds and activities were expressed in terms of interpolated ID_{50}'s, micromolar concentrations of drugs required to reduce the rate of oxygen uptake to 50 percent of the control rate. None of the substances examined exceeded iproniazid in potency although the 1-*p*-chlorobenzoyl analog proved to be equivalent to the standard in activity.

SINCE 1952 when Zeller *et al.* (1, 2) described the monoamine oxidase (MAO) inhibiting action of iproniazid, a large variety of acyl hydrazine derivatives have been fabricated and examined for their ability to suppress amine oxidase activity. On theoretical grounds, at least, there seemed to be great medicinal potential for substances having the ability to influence the metabolism of biogenic amines. The enormous interest in such substances is reflected in the comprehensively documented monograph on MAO inhibitors by Pletscher *et al.* (3) which includes 1389 references.

The isopropyl hydrazides whose *in vitro* activities are described in this report are all known in the chemical literature. Four of these compounds were originally synthesized by one of the authors (M.H.W.) but had never been screened for their ability to inhibit MAO (4). 1-*p*-Chlorobenzoyl-2-isopropyl hydrazide, a comparatively potent *in vivo* MAO inhibitor (5) and 1-*p*-hydroxybenzoyl-2-isopropylhydrazide, a comparatively weak *in vivo* MAO inhibitor (5) were included in this study in order to compare their *in vitro* activities with their reported actions *in vivo*. Iproniazid served as a reference standard inhibitor.

METHOD

Preparation of the Hydrazides—The six isopropyl hydrazides were completely dissolved in small volumes of 0.1 *N* hydrochloric acid and these stock solutions were subsequently diluted with Sorensen's 0.067 *M* phosphate buffer, pH 7.2.[1] Iproniazid, available as the phosphate salt, was immediately soluble in buffer.

Substrate—A stock solution of tyramine HCl was prepared using phosphate buffer as a vehicle.

Enzyme Preparation—Adult male albino rats were killed by cervical dislocation and their livers were quickly removed, washed with cold buffer, minced with a scissors, and homogenized with a Potter-Elvehjem tissue grinder. The homogenate, containing about 20% w/v of fresh liver, was centrifuged for 2 min. at 4000 $\times g$, the supernatant decanted and recentrifuged for 10 min. at 8500 $\times g$ in a refrigerated centrifuge. The mitochondrial pellet was resuspended in buffer so that each ml. of preparation contained the mitochondria in 300 mg. (wet weight) of liver. Monoamine oxidase activity was determined by the Warburg respirometer technique as described by Davison (6). Each reaction flask contained 1 ml. of mitochondrial preparation and 2.2 ml. of buffer. Tyramine HCl and inhibitor dissolved in 0.3 and 0.5 ml. of buffer, respectively, were each placed in side arms of the flasks. Inhibitors of varying concentrations were incubated with mitochondrial suspensions for 30 min. prior to substrate addition. Final tyramine concentration in all flasks was 0.01 *M*.

Duplicate determinations were made at each inhibitor concentration. The mean 1-hr. oxygen uptake data were expressed as percent of control (no inhibitor) and plotted against final inhibitor concentration. Lines of best fit were constructed by the method of least squares and median inhibitory doses (ID_{50}'s) were interpolated from the regression equations. Relative potencies were computed as ratios of ID_{50}'s (or alternatively, ratios of line slopes) using iproniazid as the standard.

RESULTS AND DISCUSSION

Four or five concentrations of each inhibitor provided sufficient data to reliably establish dose-effect relationships. Duplicate determinations made at each inhibitor concentration did not vary by more than $\pm$ 5% of the mean of duplicates.

The relative *in vitro* activities of the compounds are presented in Table I. With the exception of 1-*p*-chlorobenzoyl-2-isopropyl hydrazide, all of the acyl hydrazines were inferior to iproniazid in potency. Differences in the ID_{50} values for the chlorobenzoyl derivative and iproniazid were not significant and thus the compounds were judged to be approximately equivalent in terms of their influence on rat-derived MAO. Zeller *et al.* (5) reported this compound to have about 1.34 times the activity of iproniazid as determined by its *in vivo* effect on rat brain 5-hydroxytryptamine. 1-*p*-Hydroxybenzoyl-2-isopropylhydrazide had a relative *in vitro* activity about 0.6 that of iproniazid; considerably greater than its *in vivo* effect on rat brain-derived

Received May 22, 1968, from the Division of Pharmacology, College of Pharmacy, The Ohio State University, Columbus, OH 43210

Accepted for publication July 16, 1968.

Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

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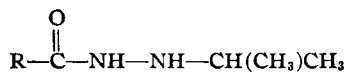
We are indebted to Dr. W. E. Scott, Hoffmann-La Roche, Inc. for providing us with iproniazid phosphate.

[1] In subsequent portions of this report "buffer" always refers to Sorensen's 0.067 *M* phosphate buffer, pH 7.2.

TABLE I—INHIBITION OF OXIDATIVE DEAMINATION OF TYRAMINE BY HYDRAZINE DERIVATIVES



Inhibitor	R	Slope of Regression Line[a]	$ID_{50} \pm SE$,[b] μmoles	Relative Activity
Iproniazid		−0.060	5.03 ± 0.28	1.00
1-*p*-Cl-benzoyl-2-IPH[c]		−0.054	5.59 ± 0.48	0.89[d]
1-*p*-OH-benzoyl-2-IPH		−0.038	7.95 ± 0.41	0.63
1-Benzoyl-2-IPH		−0.019	15.9 ± 1.9	0.31
1-α-Naphthylacetyl-2-IPH		−0.005	60.4 ± 2.0	0.08
1-Phenylacetyl-2-IPH		−0.003	100.7 ± 13.6	0.05
1-α-Naphthoyl-2-IPH		−0.002	151.0 ± 12.5	0.03

[a] In units of log percent (of control)/μmole of inhibitor base. The regression lines can be reconstructed by using a *y* intercept of 2.00 for all compounds. [b] Dose of inhibitor required to diminish oxygen uptake to 50% of control ± standard error. [c] Isopropylhydrazide. [d] Not significantly different from iproniazid standard.

enzyme which Zeller *et al.* (5) estimated to be about 0.11 that of iproniazid. 1-Benzoyl-2-isopropylhydrazide was approximately one-third as potent as the standard while the remaining compounds exhibited unimpressive degrees of activity.

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Keyphrases

Monoamine oxidase—liver derived

Isopropyl hydrazides—monoamine oxidase inhibition

Iproniazid—MOA inhibition standard

Tyramine HCl oxidation—isopropyl hydrazide inhibition

Membrane Filter Technique for Testing the Bactericidal Activity of Iodophors

By JAW-HUEY HORNG KO and RAYMOND W. VANDERWYK

A method has been devised for determining the contact killing time of a water-soluble antiseptic or disinfectant agent using a membrane filter as the test organism carrier. The method is useful in comparing the bactericidal activity of different concentrations of an agent since the number of organisms killed following a specific contact time can readily be determined on a percentage basis compared to that of a control. The problems of bacterial clumping, "wild plusses," and carry-over bacteriostasis are eliminated. A suspension of the test organism was filtered through a membrane filter, a solution of the agent was added, was allowed to remain in contact with the test organism for a specific time, and was filtered. The treated membrane was washed with a neutralizer, was rinsed, and was incubated on nutrient agar. The number of surviving organisms, if any, was determined by colony count. Solutions of povidone-iodine as well as commercial products containing it were tested against *Staphylococcus aureus*, FDA 209, ATCC 6538. Solutions containing a 1:15,000 concentration of available iodine gave a kill of 99.91 percent following a 1-min. contact.

A REVIEW of the history and descriptions of the methods currently used to test the bactericidal activity of antiseptics, disinfectants, and sanitizers has been published by Lawrence and Block (1). The Association of Official Agricultural Chemists recognizes two methods for testing disinfectants: the AOAC phenol coefficient method (2), originally devised in 1929, and the AOAC use-dilution method (3), introduced in 1953. Both of these methods measure the ability of an agent to elicit a 100% kill of the test organism. In 1961 Ortenzio and Stuart (4) introduced a modification of the use-dilution method where the percentage kill of various dilutions of test organism could be determined and where a result within 95% confidence limits is claimed. Two other methods which depend upon percentage kill and which are employed mostly for determining the effective dilutions of sanitizing agents are the Chambers modification of the Weber and Black method (5) and the modified Cade and Halvorsen method (6). All of the methods for evaluating bactericidal agents in use today show certain disadvantages and defects which are well known to workers in this field. This is evident from the many modifications in technique which have been made from time to time. In the seed tube to broth culture transfer techniques, the phenomenon of "wild plusses" is of frequent occurrence. In such instances, particularly with the quaternary ammonium compounds it often happens that one subculture will show growth while another one made from a less concentrated disinfectant solution or one made from the same solution after a shorter contact time will show no growth. Bacterial clumping and adherence to the sides of the seed tube have been suggested as reasons for these inconsistencies.

The problem of carry-over due to the bacteriostatic action of the test agent when transferred to subculture tubes must also be contended with. High activity substances such as quaternary ammonium compounds, mercurials, and iodophors must be neutralized by adding inactivators to the subculture medium. One can never be sure about the effect of neutralizers unless additional time-consuming control measures are taken. These steps involve making sure that the inactivator completely neutralizes the agent and at the same time does not itself inhibit bacterial growth. Kayser and van der Ploeg (7) have shown that sodium thiosulfate (0.25%) in an agar medium will completely inhibit the growth of some strains of *S. aureus*. They suggest that its use be avoided in growth media designed to neutralize iodine in disinfection efficiency tests. When stainless steel cylinders of the specifications described in the official use-dilution method (1) are used as carriers, the problem usually can be solved only by making additional transfers to secondary subculture tubes.

In an attempt to overcome some of these difficulties, a membrane filter technique was devised. It can be used to test any water-soluble agent which can be removed from a cellulose ester-type of membrane filter by washing with water or which can be inactivated rapidly by an appropriate neutralizer or both. It simplifies the determination of percentage kill, if this determination is sought after, eliminates the phenomenon of "wild plusses," and avoids the carry-over effect. The test agent selected was polyvinylpyrrolidone-iodine[1] (PVP-I) (povidone-iodine), and commercially available products containing it.

EXPERIMENTAL MATERIALS

Test Organism—*Staphylococcus aureus* FDA 209, ATCC No. 6538. Maintain stock cultures on nutrient agar slants by monthly subcultures. Incubate new stock transfer 2 days at 37°; then store at 2–5°. From the stock culture inoculate a tube of brain heart infusion broth (BBL) and make at least 4 consecutive daily transfers incubating at 37°, before using culture for testing. Use 22–26 hr. culture of organism growth in BHI at 37° in test. Mix well by high speed agitation on a Vortex Genie (Scientific Industries, Inc., Springfield, Mass.) for 1 min. before using.

[1] Betadine, Purdue Frederick Co., Yonkers, N. Y.

Received May 22, 1968, from the Department of Biological Sciences, Massachusetts College of Pharmacy, Boston, MA 02115

Accepted for publication August 6, 1968.

Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

Submitted to the Department of Biological Sciences, Massachusetts College of Pharmacy, Boston, Mass., by J. H. Ko in partial fulfillment of the Master of Science degree requirements, June 1967.

This investigation was supported by a financial grant from the Purdue Frederick Co., Yonkers, N. Y., and was aided by the Millipore Filter Corp., Bedford, Mass., who generously supplied the filtering equipment.

Culture Media—Brain heart infusion broth (BBL) for daily transfers of test organism and Trypticase Soy agar (BBL) for colony counts.

Inactivator (Neutralizer)—Dissolve 0.5 g. of sodium thiosulfate in 100 ml. of distilled water and sterilize by autoclaving.

Filtering Apparatus—Millipore membrane filter holder (XX1004700) used with a filter membrane (DAWG 04700). Assemble with a liter flask and sterilize with steam under pressure. Plastic Petri dishes (60 × 20 mm.).

Transfer Loop—Make a 4-mm. i.d. single loop at end of 5–7.5 cm. (2–3 in.) Pt or Pt alloy wire No. 23 B and S gauge. Fit other end in suitable holder.

PROCEDURE

Dilutions of PVP-Iodine—Prepare an aqueous stock solution of the PVP-iodine material (powder or solution) in sterile distilled H_2O such as to represent a 1:1,000 concentration of available iodine. Set up a series of 10 sterile culture tubes in a rack and prepare 5-ml. quantities of each of the 10 dilutions shown in Table I.

Preparation of Test Organism—Transfer a loopful of a 22–26 hr. old broth culture of *S. aureus* to 100 ml. of BHI broth. Mix well. This suspension (contact suspension), containing between 2×10^4 and 4×10^4 organisms/ml., is used for testing the activity of the PVP-iodine dilutions. Dilute the contact suspension in BHI (1:100) and mix well. This is used as the organism numbers control suspension and also to determine the effectiveness of the inactivator.

Organism Numbers and Inactivator Controls—Assemble a sterile filtering apparatus with membrane. Place 5 ml. of sterile H_2O in the funnel, add 2 ml. of organism numbers control suspension, swirl to mix, and filter by suction so as to form a layer of bacteria evenly spread over the membrane surface. Prepare five such membranes and place on Trypticase Soy agar. Incubate at 37° for 24 hr. Count the colonies on each plate, obtain the average, multiply by 100, and use this figure in calculating the percentage kill for the PVP-iodine dilutions. Filter 5 ml. of the most concentrated solution of PVP-iodine through a sterile membrane. Neutralize the iodine remaining on the membrane by overlaying with 10 ml. of inactivator. Filter after a few seconds of contact. Rinse immediately with 20–25 ml. of sterile H_2O. Impregnate the membrane by filtering 2 ml. of organism numbers control suspension. This is the "wash" control. Incubate at 37° for 24 hr. and compare the colony count with the average of the five controls. There should be no significant difference in colony counts showing that the iodine had been inactivated and the $Na_2S_2O_3$ had been removed.

Table I—Percentage Kill of PVP-I Solutions of Varying Concentrations of Available Iodine against *S. aureus* after a 1-min. Contact

Avail. I	Colony Count	% Kill
1:1,000	0	100
1:2,000	0	100
1:4,000	1	99.99
1:6,000	12	99.95
1:8,000	20	99.93
1:10,000	24	99.92
1:15,000	32	99.91
1:20,000	262	99.23
1:25,000	335	99.01
1:30,000	4,180	87.07
Control	3.39×10^4	

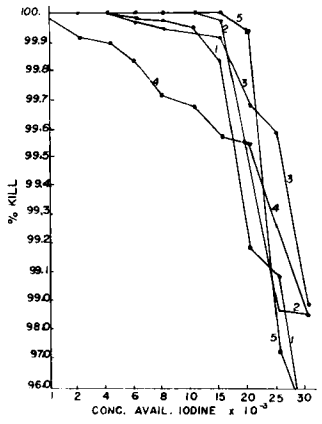

Fig. 1—Percentage kill of PVP-I products against S. aureus after 1-min. contact. Key: 1, mouthwash; 2, solution; 3, surg. scrub; 4, shampoo; 5, douche.

OPERATING TECHNIQUE

Prepare an organism impregnated membrane for each PVP-iodine solution to be tested using 2 ml. of contact suspension for each membrane. The membranes can be prepared, dried, and stored aseptically at 2–5° for several days prior to the test, if desired. When prepared extemporaneously, pack the contact suspension in an ice bath when not in use to inhibit bacterial growth. Remove any vacuum in the filtering flask and overlay an impregnated membrane with 5 ml. of each dilution of test agent. After exactly 60 sec. of contact, pour 10 ml. of inactivator into the funnel and filter rapidly. Immediately flush with 20–25 ml. of sterile H_2O. A contact period other than 60 sec. may be used if desired. Place the treated membranes on Trypticase Soy agar contained in 60-mm. Petri dishes and incubate at 37° for 24 hr. Count the number of colonies, if any, and determine the percentage reduction over the numbers control. The same filtering apparatus can be used for each membrane providing the platform and funnel are well rinsed between each change of membranes.

RESULTS AND DISCUSSION

Using this method, aqueous solutions of PVP-iodine containing the amounts of available iodine shown in Table I were prepared and tested. The percentage kill for each dilution is shown and the killing power can easily be assessed. Similarly five commercial products were tested and their activities were compared. The results are shown in Fig. 1.

The membrane filter technique seems to present certain features which are an improvement over the AOAC methods of testing for bactericidal activity, particularly for soluble antiseptics and disinfectants.

1. It eliminates the questionable pour plate technique for determining viable cell counts. Every organism contacted by the agent is cultured for viability not just an aliquot portion. Therefore,

[2] Betadine products, Purdue Frederick Co., Yonkers, N. Y.

there can be no "skipping" or "wild plusses," a phenomenon often seen when aliquot amounts are subcultured.

2. When a diluted bacterial suspension is filtered through a membrane, the organisms form an even, thin layer over its surface and they cannot be removed by washing as from stainless steel carriers. This distribution more closely resembles the spread of bacteria on human skin, mucosa, and contaminated surfaces. The problem of cell clumping which often occurs with bacteria suspended in solutions of quaternary ammonium compounds is eliminated completely.

3. There is no possibility of any carry-over of PVP-iodine activity, since available iodine is neutralized and thoroughly rinsed from the organism prior to culturing. The effectiveness of the neutralization can easily be determined without delay. Since the neutralizer is also removed by washing, the possibility of any interference with the growth of the test organism is eliminated.

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Keyphrases

Iodophors—bactericidal activity testing
Membrane filter technique—bactericidal activity test
Bactericidal activity—povidone-iodine solutions

Communications

Mechanism of Flow Improvement by the Addition of Fine Particles to Bulk Solids

Sir:

The flowability of a bulk particulate solid may be improved by the addition of a small proportion of fine particles. These fine particles, or glidants, may be chemically similar or dissimilar to the material with which they are admixed (1). In the former case the term "fines" may be used to describe the glidant particles.

Gold *et al.* (2) suggest that fine particles may act as glidants by filling the void spaces between particles, in addition to reducing interparticulate cohesive forces and reducing surface rugosity (3).

Now it can be shown that the voidage of a random array of spheres is 0.38 and 0.26 for a close packed array (4). Provided the size ratio of fine:coarse particles is less than 0.4 → 0.6, fine particles percolate into the interstitial void spaces. Thus, increasing the percentage of fines in a granulation should increase the bulk density until the void spaces are full.

Figure 1 is representative of a number of binary mixtures of magnesia and shows that the bulk density [determined by the British Standard Method (5)] increases as the intergranular voids are filled and then decreases due to bed expansion by the fine component.

If the flowing bulk solid is considered as a continuum, then it should follow that the higher the bulk density the greater the mass flow rate since more material can flow through the orifice due to a closer packing.

The flowability of the binaries outlined in Fig. 1 have been determined (3) and it can be seen

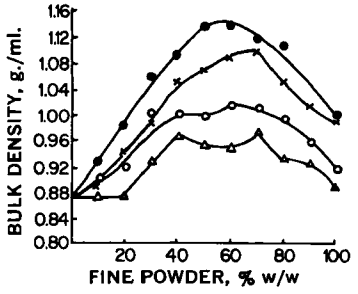

Fig. 1—The changes in bulk density in binary mixtures consisting of coarse granules (0.0561 cm.) with increasing concentrations of fine powders. Arithmetic mean diameter of fine powders. Key: △, 0.0158 cm.; ○, 0.0090 cm.; ×, 0.0059 cm.; ●, 0.0048 cm.

there can be no "skipping" or "wild plusses," a phenomenon often seen when aliquot amounts are subcultured.

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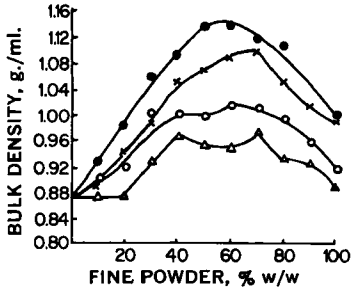

Fig. 1—The changes in bulk density in binary mixtures consisting of coarse granules (0.0561 cm.) with increasing concentrations of fine powders. Arithmetic mean diameter of fine powders. Key: △, 0.0158 cm.; ○, 0.0090 cm.; ×, 0.0059 cm.; ●, 0.0048 cm.

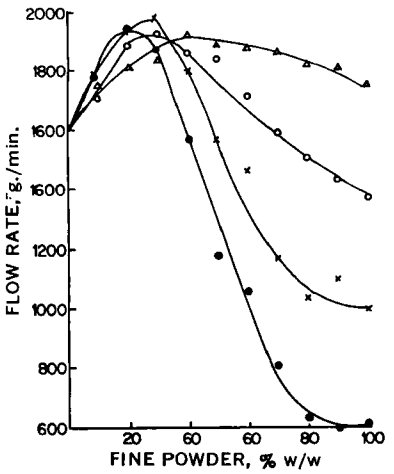

Fig. 2—The effect of fine powders on the flow rate of a coarse granulation (0.0561 cm.) from a horizontal-based hopper through an orifice of diameter 1.353 cm. Arithemetic mean diameter of fine powders. Key: △, 0.0158; ○, 0.0090 cm.; ×, 0.0059 cm.; ●, 0.0048 cm.

(Fig. 2) that the maxima in the two sets of graphs do not correspond, *i.e.*, improvement in flow rate cannot simply be due to void space filling.

Adopting a particulate approach to the kinematics of flow, it may be shown that particles in the bulk flow downwards and inwards towards an arch situated above the orifice from which they are discharged under the influence of gravity (6). This arch continuously forms and collapses producing a fluctuating voidage at the orifice. The stability of the arch and hence the rate of flow depends upon the number of particle components in its structure, *i.e.*, particle size.

Now the interposition of fine particles between the coarse components of an arch will increase the points of interparticulate slippage due to a displacement of their centers of mass. In addition, the frictional characteristics of the fine component will affect the coefficient of rolling friction of the constituent particles of the arch. Clearly, a limiting concentration of fine particles must be reached above which cohesive bridging will occur and thus a maximum is obtained in the relationship of flow rate to percentage fine component (2, 3).

Gold *et al.* (2) show that the flowability of fine lactose particles is increased by the addition of silica, talc, and magnesium stearate. On the basis of the theory that flow rate depends upon the stability of the natural free fall arch, these additives may act in two ways. First, it is likely that the frictional characteristics of the glidant lead to a reduction in particle-particle interaction within the arch and this may explain the behavior of the silica and talc glidants. However, it is unusual for magnesium stearate to produce glidant action (1) due to its long hydrocarbon tail increasing particle-particle interaction; it is, of course, a useful lubricant within the tablet die. Thus it is possible that a second mechanism of action applies in that the glidant may be of sufficiently large particle size to physically separate the fine particles of the granulation and thereby reduce the influence of cohesive bridging.

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Received June 24, 1968.
Accepted for publication August 27, 1968.

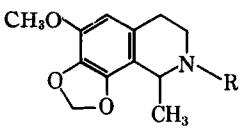
Keyphrases

Glidant action—bulk solid flow
Flow rate, solids—glidant effect
Particle size—flow-rate effect

Peyote Alkaloids VI. Peyophorine, a Tetrahydroisoquinoline Cactus Alkaloid Containing an *N*-Ethyl Group

Sir:

During a recent investigation (1–3) of the peyote cactus (*Lophophora williamsii*) alkaloids, anhalonine (I*a*) and lophophorine (I*b*) were isolated in addition to eight other compounds. However, gas chromatography[1] of the "purified" I*b* hydrochloride {m.p. 233–235.5°; $[\alpha]_{589}^{25} = -13.6°$ (c = 0.59, H_2O); lit. (4) $[\alpha]_{589}^{25} = -9.47°$ (c = 1, H_2O)} and the base {$[\alpha]_{589}^{25} = -45°$; $[\alpha]_{436}^{25} = -83.7°$ (c = 0.82, $CHCl_3$); lit. (5) $[\alpha]_{589}^{25} = -47°$ (c = 1, $CHCl_3$)} disclosed the presence (*ca.* 10%) of a new alkaloid. The structure of this alkaloid, designated peyophorine, was clearly established as *N*-ethylanhalonine (I*c*), the *N*-ethyl homolog of I*b*, by its characteristic mass spectrum [base peak *m/e* at 234 (P—CH_3)] completely analogous with I*b* [base peak *m/e* at 220 (P—CH_3), *cf.* pilocereine (6)]. The same compound I*c* was also found in authentic samples obtained from the collection of Spaeth and coworkers as well as a sample acquired from a commercial source.[2] To eliminate the possibility of an artifact (*e.g.*, ethanol plus acid, *etc.*), de-



I*a* (R = H)
I*b* (R = CH_3)
I*c* (R = C_2H_5)
I*d* (R = $COCH_3$)

fatted plant material was extracted with ethanol-free chloroform and the chloroform-soluble hydrochlorides were found to contain *ca.* 20% I*c* (compared to I*b*) as determined by the combined GLC-mass spectrum.

I*c* was separated from I*b* by TLC on Silica Gel G using benzene–*n*-propanol–triethylamine (65:20:1) and the isolated purified oily base afforded a crystalline picrate m.p. 155–156°.[3]

N-Ethylanhalonine was prepared by ethylation of I*a* with diethyl sulfate (removing unreacted I*a* by acetylation) and was found to be identical (IR, GLC, mass spectra) with natural I*c*.[4] The prepared I*c* picrate showed the same m.p., mixed m.p., and IR spectrum as the picrate of natural I*c* suggesting that they were of the same optical configuration.

Anal.—Calcd. for $C_{20}H_{22}N_4O_{10}$: C, 50.21; H, 4.64; N, 11.71. Found: C, 50.23; H, 4.63; N, 11.76.

The ethiodide of I*c* (m.p. 203–204°) derived from I*a* showed a negative Cotton effect with the first maximum at 289 mμ $[\alpha]_{289}^{25} = -232°$ (c = 0.52, H_2O); *cf.* the methiodide of natural lophophorine (I*b*, containing *ca.* 10% I*c*) first maximum at 289 mμ $[\alpha]_{289}^{25} = -257°$ (c = 0.016, H_2O).

Alkaloids containing *N*-ethyl group are extremely rare in nature (7), occurring mainly in terpenoid alkaloids (8), *e.g.*, delpheline, aconitine, and lycoctonine, where their presence is clearly related to acetate biosynthesis.

Battersby *et al.* (9) have recently shown that the *1*-methyl group of tetrahydroisoquinoline cactus alkaloids is not derived from methionine. Although acetate is a logical alternative [*cf.* the presence of *N*-acetylmescaline in the plant (10)] randomization of the label during 1-^{14}C acetate feedings was observed. However, the *N*-ethyl group of I*c* is more obviously derived from acetate than C_1 and its attached methyl group and tracer experiments are in progress to determine this point.

An examination of the mother liquor from which isolation of additional I*a* was reported by us (11), has indeed revealed the occurrence of the *N*-acetyl derivative (I*d*). The latter compound was identified by comparison with synthetic I*d* (m.p. 151.5–153°; $[\alpha]_{589}^{25} = 206°$) using the combined GLC-mass spectra technique.

Anal.—Calcd. for $C_{14}H_{17}NO_4$: C, 63.87; H, 6.51; N, 5.32. Found: C, 63.93; H, 6.64; N, 5.32.

By employing the same method the presence of I*d* has also been demonstrated in a neutral fraction of the peyote cactus. The occurrence of I*d* in the plant suggests that it may be a precursor of I*c*. Biosynthesis of I*c* and other peyote alkaloids is currently being studied.

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[1] GLC-mass spectra were determined on the LKB-Producter mass spectrometer using a 183-cm. (6-ft.) column containing 3% OV-7 on 100 mesh Gas Chrom Q at 200° at a helium flow rate of 15 ml./min. Mass spectra were obtained at 70 ev., 100 μamp. trap current at 3.5 kv., accelerating voltage.

[2] Authentic sample of lophophorine hydrochloride from the collection of the late Prof. E. Spaeth was obtained from Smith, Kline and French Laboratories, and a sample of the base hydrochloride was furnished by S. B. Penick & Co.

[3] The identification of I*c* in the "purified" sample of I*b* suggests that the recently reported (1) quaternary alkaloid lophotine, the iodide of which was found to be identical with the methiodide of the isolated I*b*, is a mixture of the metho-derivatives of I*b* and I*c*.

[4] The same compound was obtained by lithium aluminum hydride reduction of *N*-acetylanhalonine (see text). During the ethylation of I*a* with diethyl sulfate a minor reaction product isolated was identified by NMR and mass spectrometry as *N*-carboethoxyanhalonine. The formation of the latter compound is presumed to be due to the reaction of a halonine with phosgene (from chloroform, used as a solvent) and ethanol (from diethyl sulfate).

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Received June 12, 1968.
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Peyophorine—identity confirmation
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GLC—identity
IR spectrophotometry—identity
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REVIEW

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Staff review

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Each chapter contains previously unpublished facts, hypotheses, and conclusions which should stimulate discussion by those readers previously unacquainted with the present knowledge of this interesting antibiotic. Although the monograph does not contain all the available information on this antibiotic, those who carefully read the clearly written chapters and consult the bibliography will quickly grasp the essential features and properties of this important antibiotic.

The monograph contains few typographical errors and is reasonably free of other errors. The adequate subject index and bibliography (more than 700 references) are of use to both the newcomer and the antibiotic expert. This monograph should be included in any library on antibiotics.

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The Structural Basis of Antibody Specificity. By DAVID PRESSMAN and ALLAN L. GROSSBERG. W. A. Benjamin, Inc., One Park Avenue, New York, NY 10016, 1968. xvii + 279 pp. 16 × 23.5 cm. Price $16.75.

This monograph is based on a series of lectures given for a special biophysics course at the University of Rochester. The text is a quantitative appraisal of research on the chemical nature of antibody specificity since the earlier studies described by Landsteiner. It may be used as a text for senior-graduate level courses on chemical immunology or immunochemistry, and also as a reference for those in the field. The book is written for the reader who has some knowledge of immunologic nomenclature. The text treats those structural features of haptens or antigens and of antibodies which provide the basis for their specific interaction. The first two chapters introduce the chemical concepts which are involved in antibody-antigen interaction. Chapter 3 discusses a large number of antihapten systems in terms of the structural details important for antibody-hapten combination. The remaining chapters discuss the basis of the heterogeneity of antibody molecules and the use of antibodies to map out structures of small and large molecules in aqueous solution. Liberal use is made of drawings of chemical structures with their names, and the bibliography and appendix give access to many studies of antihapten systems.

Staff review

Cell Physiology and Pharmacology. By J. F. DANIELLI. Hafner Publishing Co., Inc., 31 East Tenth St., New York, NY 10003, 1968. (originally published 1950). vi + 156 pp. 15.5 × 23 cm. Price $8.00.

On the first examination, the volume under review gives the impression of questionable worth. It appears unlikely that a slender book of less than 200 pages could give a comprehensive coverage of the vast field of cell physiology and pharmacology. Secondly, the book is based on lectures given at University College, London, almost two decades ago and originally published in 1950. The domain of cell physiology as enlarged by the fantastic advances in biochemistry and molecular biology in the last twenty years is now almost a qualitatively new one. My initial reaction, therefore, was, "What could we possibly learn about a vast field like cell physiology and pharmacology in a volume of less than 200 pages which was published twenty years ago?"

However, as I read the book, I found there was much that could be learned. The author established the premise underlying his discussion in the preface. He stated that in the development of drugs, the biological side of the process does not proceed in a rational manner. The chemist will synthesize an array of compounds which are then subjected to biological testing. If our biological knowledge of drug action were adequate, it would be possible to predict which compounds or modifications of a compound would be biologically active and perhaps 90% of the work assigned to the chemist would be unnecessary and eliminated.

The book is organized into six chapters bearing the following titles: The Cell as a Physico-Chemical Unit; Possible Actions of Drugs on Surfaces; Membrane Permeability and Drug Actions; Enzymes and Drug Actions; The Actions of Narcotics; Responses of Cells on the Biological Level.

In general, the chapters are well written and well organized. The emphasis is on principle and mechanism rather than the presentation of masses of data. Literature references at the end of each chapter are few and of course relatively old, that is, prior to 1950.

In the opinion of this reviewer, this volume is a valuable contribution and should be of interest to all involved in pharmacological research and teaching. Although the author does point out some examples of drugs for which the mechanism of action is somewhat known, he makes quite clear the point that we have a great deal of work ahead of us if we are ever to establish cell pharmacology as a science well enough developed to permit prediction of the action of chemicals on biological systems.

Reviewed by Joseph Judis
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Studies of a Core Curriculum. Edited by M. R. GIBSON. American Association of Colleges of Pharmacy, 850 Sligo Ave., Suite 301, Silver Spring, MD 20910, 1968. 91 pp. 15 × 22.5 cm. Price $3.50. Paperbound.

This volume is a report of the Committee on Curriculum of the American Association of Colleges of Pharmacy and outlines what this Committee believes to be the basic core course content for pharmacy education. This report covers only those professional areas of pharmaceutical education—not prerequisites and liberal arts courses—which represent the basic core of information needed before specialization. No "how to" information is included, but merely what constitutes a complete professional curriculum. The five chapters of core course contents and the authors are: Pharmacognosy—Melvin R. Gibson; Pharmacology—Marvin H. Malone; Pharmaceutical Chemistry—Frank E. DiGangi; Pharmacy Administration—Raymond J. Dauphinias; and Pharmacy—August P. Lemberger.

Staff review

Topics in Pharmaceutical Sciences. Vol. 1. Edited by D. Perlman. Interscience Publishers, Inc., 605 Third Avenue, New York, NY 10016, 1968. 15.5 × 23.5 cm. ix + 136 pp. Price $7.95

This volume is a collection of papers presented at two symposia held during the 1967 annual meeting of the Academy of Pharmaceutical Sciences. The topics of the two symposia are "Penicillins and Cephalosporins" and "Effects of Hormonal Steroids on Cellular Processes."

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A publication of the American Pharmaceutical Association

Published monthly under the supervision of the Committee on Publications with the approval of the Council

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JANUARY TO DECEMBER, 1968

JOURNAL OF Pharmaceutical Sciences

December 1968 volume 57, number 12

Review Article

Drug Metabolism by Intestinal Microorganisms

By RONALD R. SCHELINE

STUDIES DEALING with the metabolic fate of drugs are of considerable importance as they may often elucidate significant aspects of the biological action of these compounds. The literature in this field is extensive and many excellent reviews are available including those by Williams (1), Gillette (2, 3), Ariëns and Simonis (4), Maynert (5), Boyland and Booth (6), Shideman and Mannering (7), Williams and Parke (8), Hayes (9), Remmer (10), and Bush and Sanders (11). Interest has hitherto been nearly exclusively devoted to the metabolic processes which take place in the tissues. However, drugs will often come into contact with the microorganisms which comprise the normal gastrointestinal flora. This situation is encountered when drugs are administered orally and especially when their absorption is delayed because of low solubility or other factors. Also, diffusion or secretion of drugs into the intestinal lumen or, more commonly, excretion of them or their metabolites in the bile may occur. These situations may lead to alteration of the compounds as a result of metabolic activity of the microorganisms in the gastrointestinal tract.

Several examples of the intestinal metabolism of drugs have been discussed by Smith (12), primarily in connection with the fate of biliary conjugates in the intestine. However, most of the information presently available is scattered throughout the literature and often stems from incidental observations made in the course of other studies. The incidence of such reports has increased recently and it is becoming more widely recognized that the gastrointestinal flora may be of great significance in determining the metabolic fate of drugs. This review therefore aims to summarize and assess the present knowledge of the metabolism of drugs by the gastrointestinal microorganisms. For this purpose the definition of a drug will be interpreted liberally. However, emphasis will be placed on compounds which can be considered as foreign to the animal organism.

GASTROINTESTINAL FLORA

Nature of Flora—The normal gastrointestinal flora in man and animals has not been extensively studied in the past although interest in this field of microbiology has increased considerably in recent years. The reasons for neglect are partly historical in that emphasis has been placed on pathogenic microorganisms and partly technical in that many members of the normal flora are difficult to cultivate. The complex nature of the gastrointestinal flora is emphasized by the fact that more than 60 bacterial species have been isolated from the in-

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testinal tracts or feces of healthy animals, including man (13). Many studies have dealt with the coliforms and enterococci and this reflects the ease with which these organisms can be cultivated. However, recent evidence suggests that more typical representatives of the flora are to be found among the lactobacilli, the anaerobic nonsporulating bacilli (*Bacteroides*), and the fusiform organisms (14). Spears and Freter (15) have recently shown that the predominant, strictly anaerobic bacteria of the flora are sensitive to even transient contact with atmospheric oxygen. Thus it must be emphasized that our conception of the flora is greatly dependent on the techniques used and also that our present knowledge may be both limited and somewhat misleading.

In spite of these reservations it is worthwhile to briefly summarize the findings now available. Table I lists the main groups of gastrointestinal microorganisms in mammals. Further information on the subject is available in the works of Rosebury (16), Wilson and Miles (17), and Dubos (18). Donaldson (13, 19, 20) and Raibaud *et al.* (21–24) have also published valuable reviews and other pertinent information is available (14, 25–34).

Species Differences—Smith (27) investigated the gastrointestinal flora in 15 homothermic species including the common laboratory animals when fed on conventional diets. Although great quantitative differences were noted, the florae were found to consist of the same types of organisms in all species except the rabbit. The major groups or species were bacteroides, lactobacilli, *E. coli*, streptococci, *Clostridium welchii*, veillonellae, and yeasts. Except for bacteroides which were confined to the large intestine, these organisms were distributed throughout the gastrointestinal tract. The rabbit differed greatly in that the flora of the large intestine consisted nearly entirely of bacteroides whereas the stomach and small intestine were usually sterile. This was probably due to the low pH of the stomach contents in rabbits. However, Bornside and Cohn (31) have reported a high incidence of bacteroides and bacilli in the small intestine of rabbits.

The human jejunum has been found to be sparsely populated and the microorganisms have generally been considered to be transients (13, 30, 31). The ileum has also often been reported to be sterile (13, 17), however this belief will no doubt have to be altered in view of recent findings (30, 31, 33). In general, it appears that the predominant bacterial groups in the intestinal tract of man are those found in many other species including dogs (31) and mice (14).

Effect of Diet—It is well known that the diet may influence the nature of the gastrointestinal flora. The earlier literature has been reviewed by Donaldson (13). The effects of high and low protein diets and starvation (27) and of high fat diets (29) on the flora in rats have been reported. Some of the findings are conflicting but it appears that a diet high in meat protein or casein will increase the numbers of clostridia and coliforms. Yeasts appear to be very sensitive to the nature of the diet and are numerous in animals fed mainly cereals but largely absent when meat or protein is fed (27). Lactobacilli also appear to be present in greater

TABLE I—MICROORGANISMS FOUND IN THE GASTROINTESTINAL TRACT

Group, Genus or Species	Description	Remarks
Bacteroides	Gram-negative, strictly anaerobic, nonsporulating rods	Found mainly in lower intestine
Lactobacilli	Gram-positive, microaerophilic or anaerobic rods	Numerous in all parts
Enterobacteria	Gram-negative, aerobic or facultatively anaerobic rods	Found in all parts but more numerous in lower intestine
E. coli		
Aerobacter		
Klebsiella		
Proteus		
Pseudomonas	Gram-negative, aerobic rods	
Ps. aeruginosa		
Streptococci		
Enterococci	Gram-positive, aerobic or facultatively anaerobic cocci	Found in all parts but more numerous in lower intestine
S. faecalis		
Anaerobic	Gram-positive, anaerobic cocci	Numerous in all parts
Clostridia	Gram-positive, anaerobic sporulating rods	Sometimes found in all parts but mainly in lower intestine
Bacilli	Gram-positive, aerobic or facultatively anaerobic sporulating rods	
Staphylococci	Gram-positive, aerobic or facultatively anaerobic cocci	Found in all parts
Fusobacteria	Gram-negative, anaerobic rods	
Veillonellae	Gram-negative, anaerobic cocci	Found in all parts but more numerous in lower intestine
Yeasts		Numerous in all parts

numbers in animals fed on cereals. High fat diets seem to have little effect on the flora in rats (29).

While radical changes in the nature of the diet may therefore alter the composition of the intestinal flora, it does not seem likely that appreciable variations will be encountered with ordinary diets. However, it is not uncommon to use fasted animals in metabolic studies and the effects of starvation on the flora may be extensive. Thus, Smith (27) found large reductions in the numbers of yeasts, moderate reductions in the numbers of *E. coli*, *Cl. welchii*, and lactobacilli and no change in the numbers of streptococci and bacteroides when rats maintained on a normal diet were starved for 24 hr.

Effect of Age—Appreciable variations in the types and numbers of microorganisms are observed in animals of different ages (13, 17, 22, 25, 28) with the greatest changes occurring during early life.

Effect of Drugs—The nature of the flora may be profoundly altered when drugs having an antibacterial effect are fed. Such substances are often added to animal feedstuffs. This subject will not be discussed here as it has been reviewed by Luckey (35). Further information is found in the articles by Donaldson (13), Evenson *et al.* (36), and Dubos *et al.* (37).

REACTIONS

Hydrolysis of Glucuronides—It is well known that glucuronides may undergo hydrolysis in the intestine and it seems likely that this is one of the most important of the intestinal reactions. Its importance arises from the fact that many drugs and foreign compounds are excreted in the bile as glucuronide conjugates (12, 38). These then pass along the intestine where they may undergo hydrolysis. The liberated aglycone may be absorbed from the intestine to varying degrees and thereby establish an enterohepatic circulation of the compound. It is also possible for the aglycone to undergo further intestinal metabolism and for these metabolites to be absorbed into the body or excreted in the feces. This interplay between tissue and intestinal metabolism can thus lead to a fairly complex metabolic picture for some compounds. On the other hand, some glucuronides may be stable to β-glucuronidase and thus be excreted unchanged in the feces.

The most common situation appears to be the one in which glucuronide hydrolysis is followed by absorption of the aglycone to a varying extent. Several such examples are listed in Table II. The first three deal with glucuronides administered orally as these compounds are not important biliary metabolites following the administration of phenol, *p*-hydroxybenzoic acid, or salicylic acid. The urinary recoveries are often considerably reduced following administration of the glucuronides and accounted for only 35 and 50% of the dose with salicylic acid glucuronide and phenyl glucuronide, respectively. A more pronounced example of this occurs with iopanoic acid [3-(3-amino-2,4,6-triiodophenyl)-2-ethylpropionic acid] ester glucuronide in cats (48, 49). Oral administration of this glucuronide resulted in about 10% of the dose being excreted in the urine and the remainder in the feces. As the nature of the material in the feces was not determined, it is not known if the high fecal excretion is due to the stability of the glucuronide to intestinal hydrolysis or the limited ability of the liberated iopanoic acid to be absorbed.

The other examples listed in Table II are of glucuronides which are biliary metabolites. Further examples have been reported although the fate of the aglycones is not so well understood. Thus the biliary metabolites of ethynylestradiol-3-cyclopentyl ether, which are predominately glucuronides, are absorbed to a

TABLE II—GLUCURONIDES WHICH ARE HYDROLYZED IN THE INTESTINE

Conjugate	Species	Fate of Aglycone	Reference
Phenyl glucuronide	Rabbit	Liberated phenol absorbed, metabolized to sulfate and glucuronide conjugates and polyphenols which are excreted in urine	(39)
p-Hydroxybenzoic acid diglucuronide	Man	Liberated *p*-hydroxybenzoic acid absorbed and excreted in urine free and combined with glycine	(40)
Salicylic acid ether glucuronide	Rat	Liberated salicylic acid absorbed and excreted in urine free and combined with glycine and glucuronic acid	(41)
Indomethacin [1-*p*-(chlorobenzyl)-5-methoxy-2-methylindole-3-acetic acid] glucuronide	Dog	Liberated indomethacin undergoes enterohepatic circulation and excretion in feces	(42)
Desacetylbisacodyl (4,4'-dihydroxydiphenyl-2-pyridylmethane) glucuronide	Rat	Liberated desacetylbisacodyl partly absorbed but mainly excreted in feces	(43)
Morphine glucuronide	Dog	Liberated morphine both absorbed and excreted in feces	(44, 45)
Thyroxine glucuronide	Rat, dog	Liberated thyroxine nearly completely absorbed	(46, 47)

small extent following their infusion into the small intestine (50). Extensive enterohepatic circulation of diethylstilbestrol has been shown to take place in the rat (51). The main biliary metabolite of this compound is the monoglucuronide (52) and it is likely that it is hydrolyzed in the intestine prior to reabsorption. Glutethimide (2-ethyl-2-phenyl-glutarimide) is extensively excreted in the bile as glucuronide conjugates when administered to rats (53). The slow reabsorption of the glutethimide metabolites from the intestine suggests that they are first hydrolyzed. A similar picture is shown by griseofulvin which is extensively excreted in the bile as a conjugate of 4-demethylgriseofulvin (54). A major biliary metabolite of butylated hydroxytoluene (3,5-di-*tert*-butyl-4-hydroxytoluene) in the rat is the ester glucuronide of 3,5-di-*tert*-butyl-4-hydroxybenzoic acid which is believed to be partly responsible for the enterohepatic circulation seen in this species (55). However, the free acid is the main fecal metabolite and it seems likely that intestinal hydrolysis occurs prior to its absorption. Naphthaleneacetic acid is excreted in the bile as a glucuronide which has been postulated to undergo intestinal hydrolysis and absorption (56).

Table III lists glucuronides which have been shown to be hydrolyzed and further metabolized in the intestine. These intestinal metabolites may be absorbed and appear in the urine as major metabolites. Ferulic acid glucuronide provides an interesting example in that it undergoes a sequence of intestinal reactions including glucuronide hydrolysis, reduction of a double bond, demethylation, and dehydroxylation before being absorbed and excreted in the urine as *m*-hydroxyphenylpropionic acid.

The above findings showing that glucuronides are very susceptible to hydrolysis in the intestine are not unexpected in view of the high β-glucuronidase activity found in the intestinal contents from many species including fowl, ruminants, and common laboratory species (59). Intestinal contents or feces have been found to hydrolyze chloramphenicol glucuronide (57), estriol glucuronide (60), and 4-methylumbelliferone glucuronide (61). High activity is always noted in the large bowel, low or no activity is seen in the stomach while activity in the small intestine is variable. These findings suggest that the β-glucuronidase activity is derived from one or more microorganisms of widespread occurrence which are able to populate a fairly large portion of the gastrointestinal tract. These ideas are in agreement with the fact that *E. coli* is an intestinal inhabitant in many species and is known to possess β-glucuronidase activity (57, 62). The numbers of this organism are nearly always greatest in the cecum or large intestine and this could very well explain the greater β-glucuronidase activity found there.

Hydrolysis of Glycosides—A common example of the intestinal hydrolysis of glycosides is that seen with the anthraquinone cathartics. The active constituents of these agents, which include cascara sagrada and senna, are related to emodin (1,3,8-trihydroxy-6-methylanthraquinone). These active substances are released from the glycosides by bacterial action in the large intestine (63).

Glycoside hydrolysis is important in the metabolism of the cardiac glycosides. These substances exist in plants as precursors which are called native, natural, or genuine glycosides. Examples of these are the lanatosides (digilanids) found in *Digitalis lanata* leaf and the desacetyldigilanids found in *D. purpurea* leaf. All of these contain one glucose and three digitoxose moieties attached to the aglycone. Incubation of lanatoside A with rat feces suspensions was shown to result in the rapid hydrolysis of the terminal glucose unit to give acetyldigitoxin which was then deacetylated to digitoxin (64). Hydrolysis of the native glycoside *k*-strophanthoside occurred under these conditions and resulted in the loss of two glucose units to give the glycoside cymarin. The latter finding has also been reported by Engler *et al.* (65).

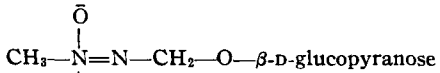
Studies on the intestinal 6-bromo-2-naphthyl glycosidases in rats showed that the cecal enzymes have higher pH optima than the corresponding enzymes from the small intestine (66). The cecal 6-bromo-2-naphthyl-α-glucosidase activity was found to be dependent on bacterial enzymes. However, the origin of the cecal 6-bromo-2-naphthyl-β-glucosidase and -β-galactosidase activities was not determined.

TABLE III—GLUCURONIDES WHICH ARE HYDROLYZED AND FURTHER METABOLIZED IN THE INTESTINE

Conjugate	Species	Fate of Aglycone	Reference
Chloramphenicol-3-glucuronide	Rat	Liberated chloramphenicol reduced to arylamines which are partly absorbed and excreted in urine	(57)
Ferulic acid glucuronide	Rat	Liberated ferulic acid metabolized to *m*-hydroxyphenylpropionic acid which is absorbed and excreted in urine	(58)

Much of the information presently available on the hydrolysis of glycosides in the intestine deals with the flavonoid compounds. It has been shown in several studies that the ingestion of flavonoid glycosides results in the urinary excretion of the aglycones and other metabolites which are primarily ring fission products. These results have been obtained with hesperidin (hesperetin-7-rhamnoglucoside) (67), diosmin (diosmetin-7-rhamnoglucoside) (67), and naringin (naringenin-7-rhamnoglucoside) (68). Similar studies with rutin (quercetin-3-rutinoside) show that it is converted to several metabolites lacking the sugar residue (69). It was subsequently shown that this degradation occurred when rutin was incubated with intestinal contents (70). This finding has been confirmed using rat cecal microorganisms and both the aglycone quercetin, as well as the subsequent ring fission metabolites, were detected (61). Also, hesperidin was metabolized to hesperetin and phenyl-β-D-glucoside to phenol under these conditions.

An interesting example of the role of the intestinal microorganisms in glycoside hydrolysis is found with cycasin, the β-glucoside of methylazoxymethanol (71). This compound is of in-



Cycasin

terest because it is present in cycad plants from which various products are used as foods in some parts of the world. Cycasin is not toxic when given by injection but is both hepatotoxic and carcinogenic when fed to small laboratory animals. This toxicity is also shown by the aglycone. Cycasin is neither absorbed nor toxic when given to germ-free rats. When these animals were contaminated with several strains of bacteria including *S. faecalis*, both the hydrolysis and toxicity of cycasin were observed. Intestinal microorganisms therefore convert cycasin to the toxic aglycone and variations in the intestinal flora probably have a role in determining the variations in toxicity found in animals given comparable doses of cycasin.

Hydrolysis of Ethereal Sulfates—Relatively little is known of the ability of the intestinal microflora to hydrolyze ethereal sulfates. Closon *et al.* (72) reported that 3,5,3′-triiodo-L-thyronine sulfuric acid ester was hydrolyzed to triiodothyronine when incubated with rat intestinal bacteria. However, *p*-nitrocatechol sulfate (2-hydroxy-5-nitrophenylsulfate) has been found not to be hydrolyzed by rat cecal microorganisms (61). This result is in agreement with earlier reports on the fate of ethereal sulfates in animals. Garton and Williams (39) found that phenylsulfate was excreted essentially unchanged after oral dosage in rabbits. The fate of phenylsulfate and 1- and 2-naphthylsulfate has been studied in rats (73). These compounds were well absorbed following oral dosage and only 4–12% of the dose underwent hydrolysis. However, the extent of hydrolysis was similar after injection and it was concluded that little hydrolysis of these arylsulfates occurred in the gastrointestinal tract. Sodium estrone sulfate has been shown to be hydrolyzed to the extent of about 75% of the dose when administered to female rats (74). This value was similar after gastric administration or injection which suggested that the hydrolysis occurred in the tissues. It was also found that rat liver homogenates hydrolyzed the ester. However, Stimmel (60) has reported that sodium estrone sulfate is hydrolyzed when incubated with human feces. A recent study has shown that sulfuric acid esters of phenols having laxative properties are not hydrolyzed in the intestine (75).

Based upon the above findings, it appears that the intestinal hydrolysis of ethereal sulfates is a reaction of limited occurrence and importance. Also of significance in this regard is the fact that biliary metabolites of phenols are generally glucuronides rather than sulfates (52, 76). The latter are mainly excreted in the urine and will therefore not come into contact with the intestinal contents to any significant extent. This has been shown in recent studies where it was found that only very small amounts of 3-aminophenyl sulfate (76) or dodecyl sulfate (77) were excreted in the bile following their injection in rats.

Hydrolysis of Glycine Conjugates—The metabolism of glycine conjugates in the intestine has been studied mainly with regard to the bile acids. Norman and Grubb (78) found that cultures of rat fecal suspensions could hydrolyze the peptide bond in glycocholic acid. Several strains of clostridia and enterococci were shown to carry out this reaction. They also reported that enterococci are able to hydrolyze hippurate to glycine and benzoic acid. A recent study by Hill and Drasar (79) dealt with the degradation of bile salts by pure cultures of intestinal bacteria. They found that taurocholate was readily deconjugated by many strains of the strictly anaerobic genera *Bacteroides*, *Veillonella*, *Bifidobacterium*, and *Clostridium* and also by about half of the tested strains of *S. faecalis* and a few strains of *Staphylococcus aureus*.

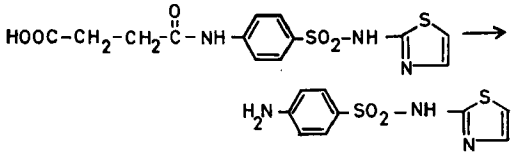
p-Aminohippuric acid and *p*-acetylaminohip-

puric acid undergo extensive hydrolysis in the alimentary tract following their oral administration to man (80). *p*-Aminohippuric acid is readily converted to *p*-aminobenzoic acid when it is incubated with rat cecal microorganisms (61).

N-Deacetylation—This reaction has been studied to only a limited extent and then largely in regard to the fate of *N*-acetyl derivatives of sulfonamides. Patki and Shirsat (81) found that N^4-acetylsulfadiazine was deacetylated to a negligible extent in the gastrointestinal tract in mice. No deacetylation was detected with N^4-acetylsulfanilamide when it was incubated with rat cecal microorganisms (61). However, *N*-deacetylation can occur under these conditions as *p*-aminobenzoic acid was formed from *p*-acetamidobenzoic acid. Also, N^1-acetylsulfisoxazole is known to undergo deacetylation when incubated with several intestinal bacteria including *E. coli*, *Lactobacillus acidophilus*, and *S. faecalis* (82).

N-Acetylhistamine was reported to be deacetylated when incubated with human fecal suspensions (83).

Hydrolysis of Amides—In addition to the examples in the two groups mentioned above, other compounds containing the amide bond have been found to undergo hydrolysis in the intestinal tract. This may often be of practical importance as several useful drugs are metabolized in this manner. Phthalylsulfathiazole and succinylsulfathiazole are intestinal antiseptics which owe their activity to the liberation of sulfathiazole in the large intestine as a result of bacterial hydrolysis. This reaction has been shown to occur with the latter compound when incubated with the rat cecal microflora (61) (Scheme I).



Scheme I—Metabolism of succinylsulfathiazole by rat intestinal microorganisms (61).

Hydrolysis of the amide group in other N^4-acyl derivatives of sulfonamides has been observed (81). The stability of the N^4-acetyl derivatives has been noted above but extensive gastrointestinal deacylation was found with N^4-propionyl- and N^4-butyrylsulfadiazine. Deacylation decreased with increasing chain length and was negligible with the N^4-lauryl and N^4-palmityl derivatives.

Another antibacterial substance known to undergo bacterial hydrolysis of the amide bond is chloramphenicol (84, 85). Some of the bacteria shown to carry out this reaction, including *E. coli* and *Proteus vulgaris*, are common intestinal inhabitants.

The bacterial amidases have been extensively studied in the case of penicillin where two types of hydrolysis are possible. One type, carried out by enzymes referred to as penicillinases or β-lactamases, opens the β-lactam ring and results in inactivation of the antibiotic. These enzymes are produced mainly by Gram-negative bacilli including coliforms, *Aerobacter aerogenes*, and *Proteus* present in the human intestine (86). In addition, β-lactamase is formed by many strains of certain Gram-positive organisms (*S. aureus*, *S. albus*, and *Bacillus cereus*) (86). The second type of hydrolysis removes the side chain and is carried out by an enzyme referred to as penicillin acylase or amidase. Acylase activity is found in many microorganisms belonging to the genera *Aerobacter*, *Micrococcus*, *Proteus*, and *Pseudomonas* and also in *E. coli* (87).

Hydrolysis of Esters—Studies on the absorption, metabolism, and excretion of the succinic acid esters of chloramphenicol have shown that they undergo intestinal hydrolysis prior to absorption (88). Similar findings have been made with erythromycin propionate (89).

Booth (90) found that the ester of citraurin, which occurs in orange peel, was hydrolyzed during passage through the alimentary tract of the rat. Similarly, the diesters of taraxanthan and zeaxanthol were found to be extensively hydrolyzed to free carotenol (91). The laxative bisacodyl [bis(*p*-acetoxyphenyl)-2-pyridylmethane] undergoes nearly complete hydrolysis in the intestine (92). Carbenoxolone, the hemisuccinate of β-glycyrrhetic acid is used in treating gastric ulcers. Studies using the ^{14}C-labeled compound have shown that it is principally hydrolyzed to β-glycyrrhetic acid in the gastrointestinal tract (93).

The origin of the esterase activity in the above examples was not determined but the splitting of carotenoid esters was assumed to be brought about by an intestinal lipase (91). Also, the hydrolysis of the antibiotic esters is sometimes described as being carried out by these enzymes. It is well known that the lipases of the pancreatic juice are capable of splitting esters and that this action takes place in the small intestine. Intestinal hydrolysis of esters at this site would be expected to result in appreciable absorption of the liberated compound. Chloramphenicol, which is itself extensively absorbed, has been shown to

give incomplete absorption when administered as the mono- and disuccinic acid esters (88). This may indicate that hydrolysis is carried out by bacterial esterases in the large intestine where absorption would be expected to be lower.

There is some evidence available which suggests that the bacterial esterases may play an important role in the intestinal hydrolysis of esters. Lanatoside A (digitoxigenin-bis-digitoxosido-acetyldigitoxosido-glucoside) and acetyldigitoxin were shown to be converted to digitoxin (digitoxigenin-tridigitoxoside) when incubated with intestinal microorganisms (64). Methyl gallate is readily hydrolyzed to gallic acid by rat cecal microorganisms (61). Chlorogenic acid, the quinic acid ester of caffeic acid, is hydrolyzed and further metabolized to *m*-hydroxyphenylpropionic acid when fed to rats (94). This reaction sequence is also seen when chlorogenic acid is incubated with rat intestinal microorganisms (95).

Dehydroxylation—The reaction of removal of hydroxyl groups was first shown to occur with the bile acids. Cholic acid was found to be transformed in rats and by rat fecal suspensions into a number of metabolites including some which lack a hydroxyl group at the 7-position (96, 97). These metabolites are not formed in germ-free rats (98). Several strictly anaerobic strains of microorganisms from rat and human feces have been isolated which are capable of dehydroxylating chenodeoxycholate at the 7-position (99). These were subsequently found to be strains of anaerobic Gram-positive, nonsporeforming rods belonging to the tribe *Lactobacilleae* (100). Recently, Hill and Drasar (79) reported that many strains of the strictly anaerobic genera *Bacteroides*, *Clostridium*, and *Veillonella* and also *S. faecalis* are able to remove the 7-hydroxyl group from cholate to yield deoxycholate. They also isolated strains which dehydroxylated cholate and deoxycholate at the 12-position.

The dehydroxylation reaction has also been observed in feeding experiments with other classes of compounds. Thus, 3,4-dihydroxyphenylacetic (homoprotocatechuic) acid was found to be metabolized partly to *m*-hydroxyphenylacetic acid when administered to rats and rabbits (101). This finding was subsequently confirmed using ^{14}C-homoprotocatechuic acid (102, 103). Nearly 7% of the dose (100 mg./kg.) was converted to *m*-hydroxyphenylacetic acid in rats while the amount was twice this in rabbits. In addition, a small amount of *meta*-dehydroxylation which yielded *p*-hydroxyphenylacetic acid was observed.

The metabolism in animals of 3,4-dihydroxybenzoic (protocatechuic) acid, 3,4-dihydroxycinnamic (caffeic) acid, and 3,4-dihydroxyphenylalanine (DOPA) has been studied (94, 104). Dehydroxylation occurred with the latter compounds yielding *m*-hydroxyphenylpropionic acid and *m*-hydroxyphenylacetic acid, respectively. No evidence was obtained for dehydroxylation of the benzoic acid derivative. However, Dacre and Williams (105, 106) were later able to demonstrate with the use of ^{14}C-protocatechuic acid that this compound was dehydroxylated to a minor extent in rats.

The site of formation of the *m*-hydroxyphenyl compounds was indicated by the findings of Shaw *et al.* (107) who reported that inhibition of the intestinal microorganisms with neomycin virtually abolished the excretion of these substances. Similar results have been obtained with neomycin-treated animals given ^{14}C-labeled protocatechuic and homoprotocatechuic acids (103, 106).

Further confirmation that the intestine is the site of dehydroxylation of phenolic acids has been made in studies of the metabolism of these compounds by intestinal contents. Microorganisms from intestinal contents or feces from many animal species were found to dehydroxylate caffeic acid, homoprotocatechuic acid and dihydroxyphenylalanine (70, 108). This reaction was inhibited by aerobic conditions and by several antibiotic substances. Recent studies using rat cecal microorganisms have confirmed the dehydroxylation of caffeic and homoprotocatechuic acids and shown that 3,4-dihydroxyphenylpropionic (hydrocaffeic) acid also undergoes this reaction (58, 109). The extent of dehydroxylation appears to be dependent upon side-chain length. Thus, caffeic and hydrocaffeic acids are extensively converted whereas only small amounts of *m*- and *p*-hydroxybenzoic acids are formed from protocatechuic acid (106). However, the carboxyl group is not essential for activity as pyrogallol was readily dehydroxylated to resorcinol by rat intestinal microorganisms (61, 110). Similar experiments using rabbit fecal extracts resulted in only traces of resorcinol being produced. Little has been reported on the characterization of the microorganisms responsible for the dehydroxylation of these phenolic compounds although Perez-Silva *et al.* (111) isolated a strain of *Pseudomonas* from rat feces which could dehydroxylate caffeic acid.

Dehydroxylation has also been shown to occur with quinaldic acid derivatives. Kynurenic (4-hydroxyquinaldic) acid and xanthurenic (4,8-dihydroxyquinaldic) acid are converted to quinaldic acid and 8-hydroxyquinaldic acid, respec-

tively, when given to animals (112, 113). It was subsequently shown that treatment of rabbits with neomycin in doses sufficient to depress the intestinal microflora prevented the dehydroxylation of xanthurenic acid (114). However, this ability was regained when neomycin administration was stopped. The role of the intestinal microorganisms in the conversion of xanthurenic acid has been confirmed in experiments using rabbit fecal and rat cecal extracts (61, 115).

Several other examples of dehydroxylation have been reported. Tyrosine and phloretic acid are converted to 3-phenylpropionic acid when incubated with sheep rumen liquor (116). An unusual reaction sequence has been reported to occur in the guinea pig treated with tolbutamide which resulted in the conversion of dopamine, norepinephrine, normetanephrine, and 4-hydroxy-3-methoxyphenylglycol to *m*-hydroxyphenylacetic acid (117). Thus, both aromatic and aliphatic dehydroxylations were observed.

Decarboxylation—Roche *et al.* (118) found 3,5,3′-triiodothyronamine in the intestinal contents and feces of rats after administration of 3,5,3′-triiodo-L-thyronine. As the decarboxylated metabolite was absent from the bile, it was concluded that it was formed in the intestine from the free hormone or its conjugates by bacterial action.

Orotic acid (1,2,3,6-tetrahydro-2,6-dioxo-4-pyrimidine carboxylic acid) is extensively decarboxylated in rats by the intestinal microflora (119). Germ-free rats fed a diet containing orotic acid excreted about one third of the compound unchanged in the feces. However, it was absent from the feces of conventional rats after the first few days of feeding.

Much of our present knowledge concerning intestinal decarboxylation has been obtained from experiments with phenolic acids. Tompsett (120) reported the presence of pyrogallol in human urine and suggested that it is probably derived from 3,4,5-trihydroxybenzoic (gallic) acid by decarboxylation in the alimentary tract. Gallic acid was subsequently shown to be metabolized partly to pyrogallol in rats and rabbits (121, 122) although the findings did not point to the gastrointestinal tract as the site of the reaction.

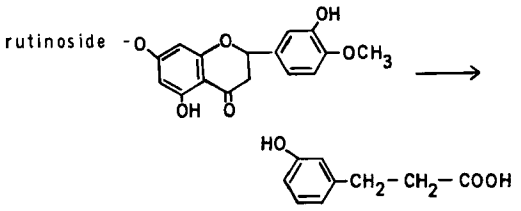
The metabolism of a related compound, 3,4-dihydroxybenzoic acid, by rat fecal and cecal extracts was reported by Booth and Williams (70) who found that it was decarboxylated to catechol. Subsequently, the metabolism of a large number of phenolic acids by the rat intestinal microflora was investigated (58, 109, 110, 123) and it was found that decarboxylation by the intestinal microorganisms is an important reaction with these compounds.

The phenolic benzoic acid derivatives have been most fully studied in regard to their metabolism by rat cecal microorganisms (110, 123). It was found that a free *p*-hydroxyl group is essential for the decarboxylation reaction. Thus *p*-hydroxybenzoic acid was extensively metabolized to phenol while *o*- and *m*-hydroxybenzoic acids were unaffected. Protocatechuic acid and gallic acid also underwent decarboxylation by the cecal microorganisms. The decarboxylation of protocatechuic acid by the rat microflora has been confirmed using ^{14}C-labeled compound (106). The reaction was reduced to varying degrees with substituents in the *meta* position and was abolished with *ortho* substitution. Decarboxylation was also observed with the phenolic phenylacetic and cinnamic acids but did not occur with the corresponding phenylpropionic acids (58, 109). In all cases, a *p*-hydroxyl group is essential for the reaction.

More than one intestinal microorganism and decarboxylase is involved in these reactions. This was indicated by the finding that pretreatment of the cecal microorganisms to destroy vegetative forms but not spores abolished the decarboxylation reaction with all but the cinnamic acid derivatives (110). Subsequently, strains of *Bacillus* were isolated from rat intestine which specifically decarboxylated *p*-hydroxycinnamic acids (124).

The above investigations have also made it clear that these reactions take place following the administration of the phenolic acids to animals. The decarboxylated metabolites of protocatechuic acid and gallic acid were excreted in the urine when rats were given the acids by oral but not by intraperitoneal administration (110). This reaction was reduced or abolished when the animals were pretreated with antibiotics to reduce the numbers of intestinal microorganisms. 4-Methylcatechol was excreted in the urine when homoprotocatechuic acid was given to rats orally but not by injection (109). Similar results were obtained with caffeic acid which was decarboxylated to 4-vinylcatechol (58). Since normal diets contain a large number of phenolic acids or their precursors, it seems reasonable to assume that the bacterial decarboxylation of these compounds in the intestine is an important source of urinary phenols.

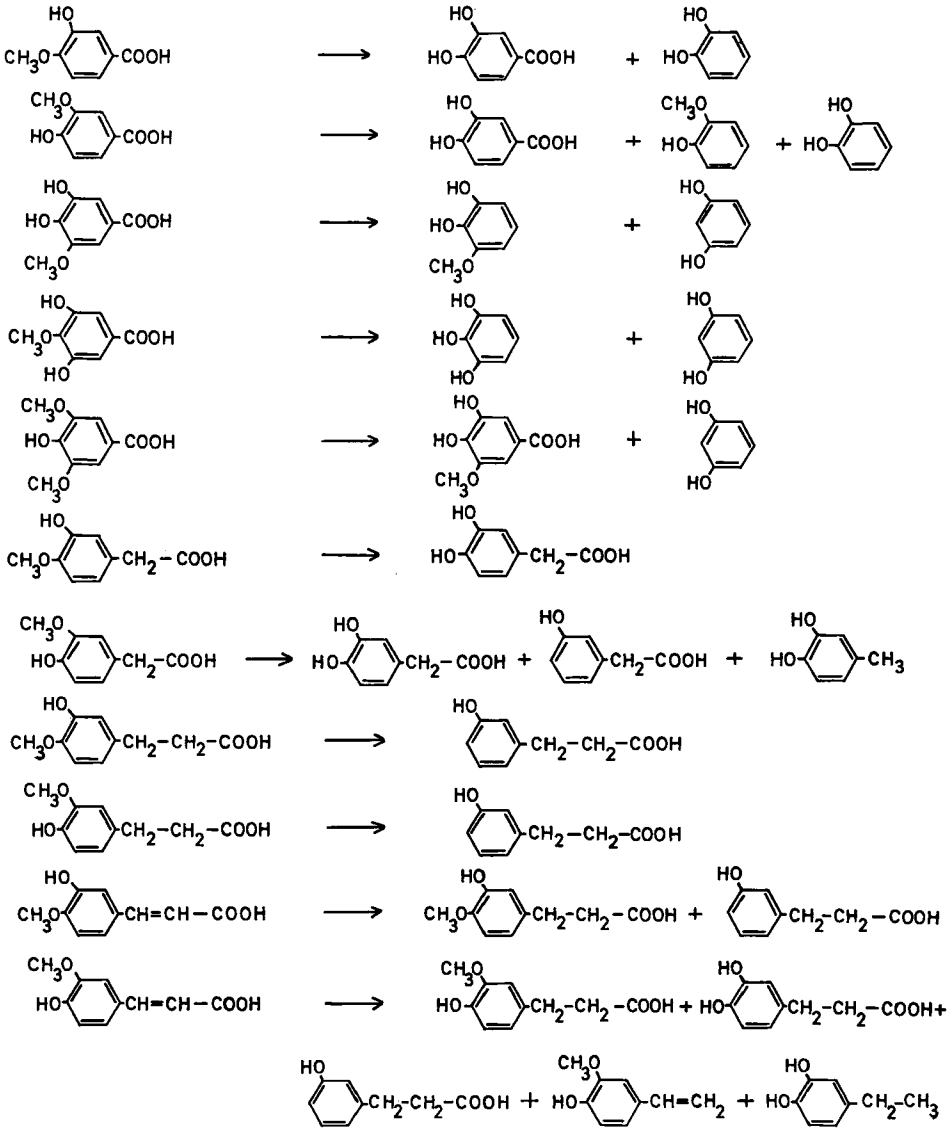
O-Demethylation—Until quite recently, the presence of an intestinal *O*-demethylation reaction appears to have gone unrecognized even



Scheme II—Metabolism of hesperidin by rat cecal microorganisms (61).

though some examples of demethylation could be more easily explained on this basis. An example is found with the flavonoid hesperidin which is partly metabolized in the rabbit to 3,4-dihydroxyphenylpropionic acid and *m*-hydroxyphenylpropionic acid (67). Demethylation of hesperidin is required for the formation of these metabolites and it has been suggested that this reaction occurs in the intestine (58). It was subsequently shown that rat cecal microorganisms are able to convert this flavonoid to *m*-hydroxyphenylpropionic acid (61) (Scheme II).

The metabolism of a large number of phenolic acids by the rat microflora has been investigated (58, 123) and it was found that many compounds containing methoxyl groups underwent demethylation. This reaction has been found to occur with methoxy derivatives of benzoic, phenylacetic, phenylpropionic, and cinnamic acids (see Scheme III).



Scheme III—Metabolism of methoxy compounds by rat cecal microorganisms (58, 123).

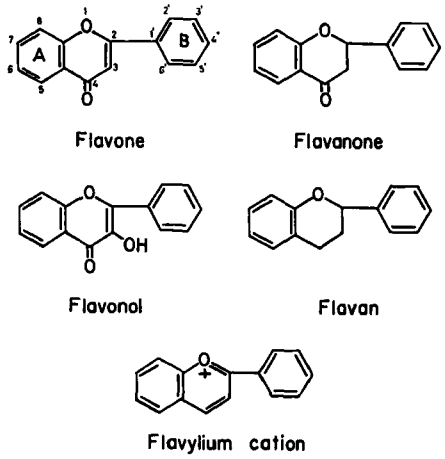


Fig. 1—Structural formulas of some of the major types of flavonoid compounds.

It is not unreasonable to assume that this reaction may be of significance in the metabolism of other classes of compounds including alkaloids, which often contain methoxyl groups. In this regard it is of interest to note that brucine is demethylated to strychnine derivatives in rabbits (125). However, the finding that the metabolites excreted in the urine were also excreted in the feces in nearly the same relative ratios, although in smaller amounts, suggests that the demethylation of brucine may have an intestinal origin.

Heterocyclic Ring Fission—Most studies of the fission of heterocyclic compounds by the intestinal microflora have been carried out with the flavonoids. These compounds make up a large and important group of phenolic substances which occur in all parts of higher plants. The flavone ring system and some of the major types of flavonoids are shown in Fig. 1.

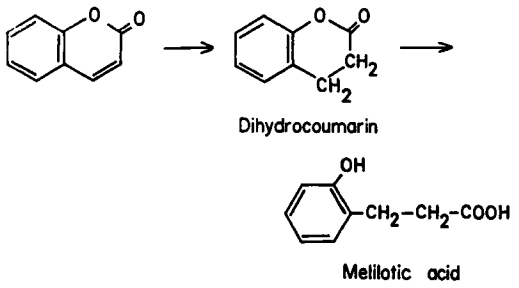
Flavonoids of the anthocyanin type occur widespread in nature as the pigments of fruits, flowers, and leaves. A cationic flavylium structure is common to these compounds and their aglycones, the anthocyanidins. Horwitt (126) found that the anthocyanin pigment from Concord grapes did not undergo loss of color when incubated with extracts of human feces. It appears that the intestinal microorganisms do not alter these flavylium compounds as cyanidin chloride (3,5,7,-3′,4′-pentahydroxyflavylium chloride) was not converted to phenolic metabolites when incubated with rat cecal microorganisms (61). The structural feature responsible for this stability appears to be the cation as (+)-catechin (3,5,7,-3′,4′-pentahydroxyflavan) is metabolized to *m*-hydroxyphenylpropionic acid by rat intestinal microorganisms (61, 70). Griffiths (127, 128) reported that the latter compound is a major urinary metabolite of (+)-catechin in rats and showed that its formation is dependent upon intestinal microorganisms.

Rutin, the glycoside of the flavonol quercetin (3,5,7,3′4′-pentahydroxyflavone), is perhaps the most frequently occurring flavonoid. It has been the subject of considerable investigation with regard to the ability of the flavonoids to modify capillary permeability (129). The metabolism of rutin by rat intestinal microorganisms was studied by Booth and Williams (70) who found that it was converted to *m*-hydroxyphenylpropionic acid. However, it was subsequently shown that *m*-hydroxyphenylacetic acid is also a product of rutin metabolism by rat intestinal microorganisms (61). Quercetin was metabolized similarly in these experiments. These findings are of interest because it is known that rutin and quercetin are excreted partly as phenylacetic acid and phenylpropionic acid derivatives when fed to rats (69, 130). The intestinal origin of these derivatives has also been demonstrated by indirect means as rats treated with neomycin to suppress the intestinal flora do not excrete them when fed quercetin (130).

Flavonoids of the flavanone type are illustrated by hesperidin and its aglycone hesperetin (5,7,3′-trihydroxy-4′-methoxyflavanone). Metabolism studies have shown that these compounds undergo ring fission when fed to rats and are excreted partly as phenylpropionic acid derivatives (67). It was recently reported that these two flavonoids are converted to *m*-hydroxyphenylpropionic acid by the rat intestinal flora (61).

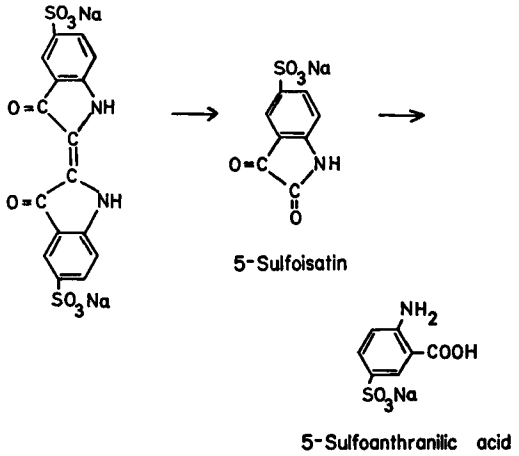
It therefore appears that ring fission by the intestinal microflora is a common reaction with heterocyclic oxygen compounds. With the flavonoids, it seems that a certain degree of hydroxylation of the heterocyclic ring system (A ring) is essential for fission as this reaction occurs to a very limited extent, if at all, with flavone itself (131). However, a hydroxylated aromatic ring may not be required in other classes and coumarin is converted to melilotic acid when incubated with rat cecal microorganisms (132) (Scheme IV).

Indigo carmine (sodium indigotin disulfonate) is metabolized to a small extent in rats to 5-sulfoisatin and further by heterocyclic ring fission to 5-sulfoanthranilic acid (133) (Scheme V). Incubation of this blue dye with rat intestinal contents resulted in fading of the color and in the appearance of the degradation products. It was suggested that these urinary metabolites may



Scheme IV—Metabolism of coumarin by rat cecal microorganisms (132).

arise partly from their absorption following the decomposition of the dye by intestinal bacteria.



Scheme V—Metabolism of indigo carmine by rat intestinal contents (133).

Heterocyclic ring fission by the intestinal bacteria has recently been shown to occur with tartrazine (134). When tartrazine labeled with ^{35}S in the *p*-sulfophenyl group of the pyrazolone moiety of the dye was given orally to rats, it was found that about 25% of the urinary sulfanilic acid was radioactive. Thus, this metabolite is produced partly by reduction of the 4-*p*-sulfophenylazo group and partly by degradation of the pyrazolone ring. The latter reaction was also found to occur when ^{35}S-tartrazine was incubated with rat intestinal contents. Small amounts of *p*-sulfophenylhydrazine were found in both experiments and it was suggested that the pyrazolone ring was cleaved to the hydrazine derivative which then underwent reductive fission to sulfanilic acid (Scheme VI).

Splitting of the β-lactam ring in penicillin has been mentioned in connection with the hydrolysis of amides.

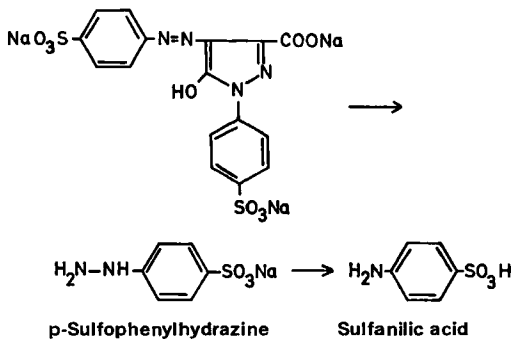
Reduction of Double Bonds—The reduction of double bonds by intestinal microorganisms has been most widely studied in connection with the metabolism of unsaturated fatty acids in the rumen and lower intestine. A recent report by Wilde and Dawson (135) described the hydrogenation of linolenic acid and oleic acid by sheep rumen microorganisms. Hydrogenation was carried out by a mixed population of microorganisms but not by several pure cultures of fecal bacteria including *Clostridium perfringens, S. faecalis, E. coli,* and a coliform organism.

Ricinoleic acid, a component of castor oil, is hydrogenated to hydroxystearic acid in both the rat and human intestine (136). This reaction is modified when neomycin is given with the castor oil so that much less hydroxystearic acid is found in the feces. This indicates that the conversion is a result of intestinal bacterial activity.

Booth and Williams (108) found that caffeic acid was partially reduced to 3,4-dihydroxyphenylpropionic acid when incubated with rat or rabbit cecal contents. A recent study of the metabolism of a number of phenolic acids by the rat cecal microorganisms showed that reduction of cinnamic acid derivatives was a major reaction of these compounds (58). This study included *o*-, *m*-, and *p*-coumaric acids, caffeic acid, ferulic acid, and isoferulic acid and reduction to the corresponding hydro derivatives was observed with all of the compounds.

Fecal microorganisms from monkeys have been shown to hydrogenate indolylacrylic acid and cinnamic acid to the corresponding propionic acid derivatives (137).

Nitro Reduction—The significance of the intestinal bacteria in the reduction of nitro groups has been recognized for some time. Studies on the metabolism of chloramphenicol in rats showed that parenteral administration resulted in the excretion of large amounts of inactive nitro compounds in the bile (138). These were partly reduced in the intestine to aryl amines which were excreted in the feces. Reduction of the nitro



Scheme VI—Metabolism of tartrazine by rat intestinal contents (134).

group in chloramphenicol has been shown to be carried out by several microorganisms including the common intestinal inhabitants *E. coli* and *P. vulgaris* (84, 139). It was subsequently found that rat cecum contents and *E. coli* hydrolyzed and reduced chloramphenicol glucuronide to aryl amines (57). The properties of an enzyme system from *E. coli* which will reduce the nitro groups in chloramphenicol and a number of simple organic nitro compounds have been reported (140, 141). This system differs in several respects from that occurring in rabbit liver and kidney which reduces the nitro groups of organic nitro compounds including chloramphenicol (142).

Bray *et al.* (143) studied the metabolism of tetrachloronitrobenzenes in the rabbit and found that some of the major metabolites were reduced in the intestine. They found that several nitro compounds including 2,3,4,5-tetrachloronitrobenzene, nitrobenzene, *m*-nitrophenol, *o*-, *m*-, and *p*-nitrobenzoic acid and their amides were readily reduced when incubated with rabbit intestine contents. *p*-Nitrophenol and *o*- and *p*-nitrotoluene were reduced very slowly. They concluded that nitro reduction by the intestinal bacteria is a factor which should be considered when the fate of aromatic nitro compounds is studied. This is especially important when the compounds are slowly absorbed as it is possible that virtually all the reduction observed may occur in the intestine prior to absorption.

The herbicide trifluralin (2,6-dinitro-*N*,*N*-di-*n*-propyl-α,α,α-trifluoro-*p*-toluidine) is poorly absorbed in rats following oral administration and nearly 80% of the dose is excreted in the feces (144). Both trifluralin and its monoamino derivative are prominent fecal metabolites. The latter compound was not detected in the urine although the corresponding compounds lacking one or both of the *N*-propyl groups were found. This suggests that trifluralin may be reduced in the intestine and the resulting amino metabolite absorbed and then dealkylated in the liver.

Azo Reduction—The reduction of azo compounds by the intestinal microflora has received considerable attention in recent years. Most investigations have dealt with water-soluble azo dyes containing sulfonic acid groups which are widely used as food coloring agents but the oil-soluble dyes have also been studied.

An early indication of the significance of the intestinal microflora in the metabolism of azo dyes was the finding that sodium 4-*p*-sulfophenylazo-1-naphthol was rapidly destroyed when incubated with dog intestinal contents (145). However, the nature of the breakdown products was not reported. Radomski and Mellinger (146) studied the fate of the disulfonated dyes amaranth [1-(4-sulfo-1-naphthylazo)-2-naphthol-3,6-disulfonic acid trisodium salt], Ponceau SX [2-(5-sulfo-2,4-xylylazo)-1-naphthol-4-sulfonic acid disodium salt], and sunset yellow (1-*p*-sulfophenylazo-2-naphthol-6-sulfonic acid disodium salt) in rats (see Fig. 2). They demonstrated that the liver azo reductase system is relatively unimportant in the metabolism of these compounds. However, the dyes were found to be nearly completely reduced to sulfonated amines by the intestinal bacteria. The reduction of amaranth is shown in Scheme VII. These metabolites were then absorbed from the intestine and excreted in the urine. Similar findings were obtained by Jones *et al.* (147) who studied the metabolism of tartrazine (3-carboxy-5-hydroxy-1-*p*-sulfophenylazopyrazole trisodium salt) in the rat, rabbit, and man (Scheme VII).

Acid yellow (4-aminoazobenzene-3,4′disulfonic acid disodium salt) has also been found to undergo fission of the azo linkage in rats following oral dosage (148) (Scheme VII). Azo reduction was not observed when the dye was given by injection. Acid yellow was shown to be readily reduced by extracts of rat feces and a strain of *S. faecalis* isolated from feces (61, 148).

Brown FK, which consists of a mixture of *p*-sulfophenylazo derivatives of 2,4-diaminotoluene and 1,3-diaminobenzene, underwent reductive fission to give sulfanilic acid, a phenazine-

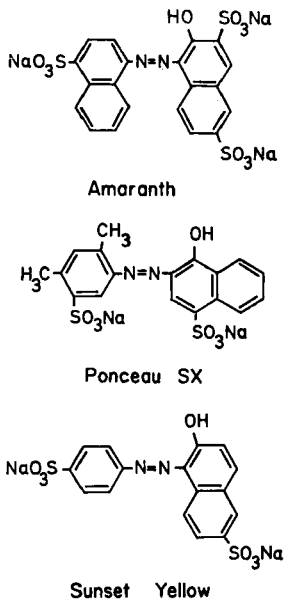
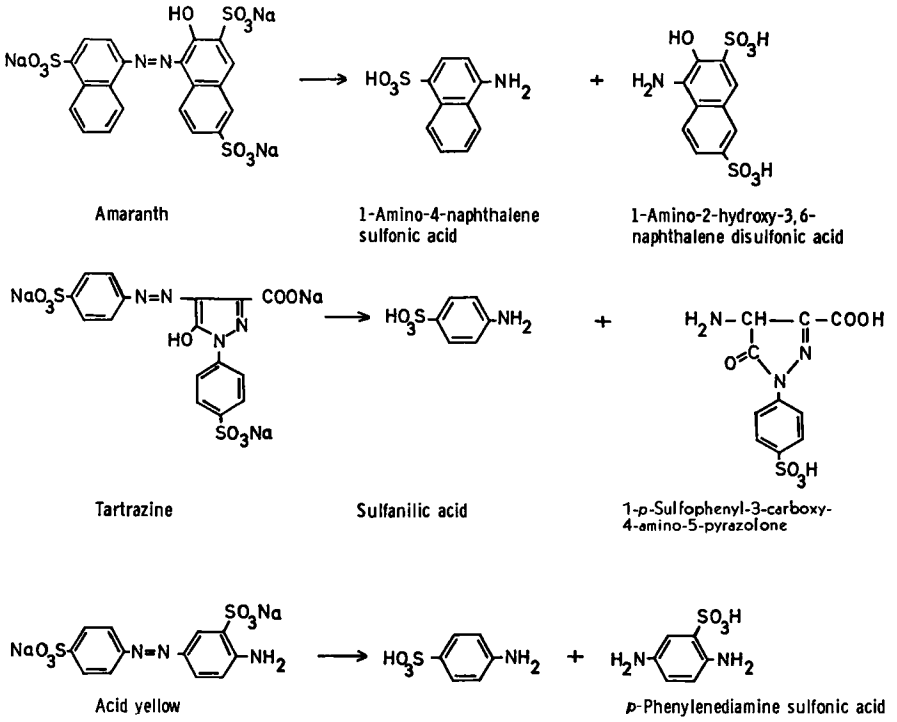


Fig. 2—Structural formulas of some water-soluble azo dyes.



Scheme VII—Reduction of azo dyes by rat intestinal microorganisms (146–148, 150).

like material and ill-defined products when incubated with rat intestinal contents (149). Several cecal microorganisms including *S. faecalis* were reported to be capable of decolorizing this dye.

Roxon *et al.* (150) reported that tartrazine was reduced when incubated with a *Proteus sp.* isolated from rat intestine. This organism was also able to reduce a number of other water-soluble azo dyes (151). Further studies on the mechanism of this reaction have shown that a soluble NADPH-dependent, FMN-flavoprotein is involved (152). It is probable that tartrazine and other azo dyes act as alternate electron acceptors for the respiratory chain under anaerobic conditions. It is of interest to note that the reduction of tartrazine occurs rapidly with cell extracts of *P. vulgaris* but not with freshly harvested microorganisms (150, 152). Reducing activity is present in aged cells and this is apparently due to permeability changes in the cell wall. However, it is known that intestinal microorganisms rapidly reduce azo dyes including tartrazine (148, 151) and it seems likely that much of the reduction that occurs when azo dyes are given orally to animals may be due to other intestinal microorganisms including *S. faecalis*.

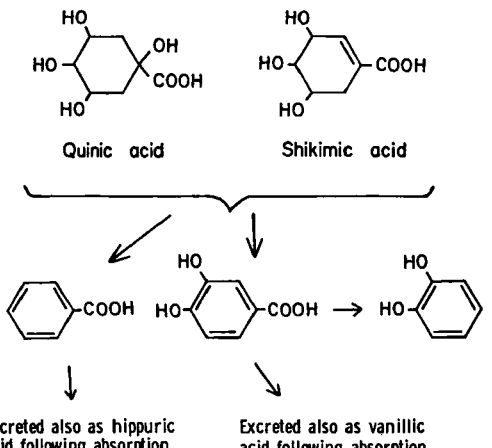
Oil-soluble azo dyes are also reduced by intestinal microorganisms. Childs *et al.* (153) found that 1-phenylazo-2-naphthol was reduced when incubated with rat intestinal contents or with *E. coli*, *A. aerogenes*, and *B. proteus* isolated from rat intestine. Similar results were obtained with methyl red (4-dimethylaminoazobenzene-2′-carboxylic acid) which was reduced to its component amines, *o*-aminobenzoic acid and *N,N*-dimethyl-*p*-phenylenediamine, when incubated with rat cecal microorganisms (61).

Aromatization—The reaction whereby nonaromatic cyclic compounds are converted to aromatic substances has been known for more than a century (154). Quinic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid), a common constituent of many fruits and vegetables, has been found by several investigators (154–159) to be metabolized to benzoic acid or hippuric acid. This is a major metabolic reaction of quinic acid in man and about 70% of the ingested dose is excreted as hippuric acid (158). Likewise, 30 to 60% of an oral dose is converted to hippuric acid in guinea pigs (157, 158). However, no conversion was found to occur when quinic acid was given to guinea pigs by subcutaneous injection (156, 158).

The differences in quinic acid aromatization following different routes of administration together with the knowledge that some strains of *A. aerogenes* could utilize quinic acid in aromatic biosynthesis (160) suggested that the intestinal bacteria might be involved in this reaction (158)

The role of the microflora was confirmed as inhibition of bacterial multiplication in the intestine with neomycin was found to prevent the conversion of quinic acid to hippuric acid in man (158). This finding was subsequently confirmed by Asatoor (159) using rats. An even greater rise in urinary hippuric acid excretion occurred when shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid) was fed and this conversion was also prevented by neomycin (159).

Quinic acid and shikimic acid have also been found to increase the urinary catechol excretion when given orally to rats maintained on a purified diet (161). In addition, vanillic acid was detected in the urines. The conversion of quinic acid to catechol was subsequently shown to be carried out by rat intestinal microorganisms (70). It has been proposed that these polyhydroxy acids can be converted by the intestinal microflora to 3,4-dihydroxybenzoic acid which can be partly absorbed and methylated to give vanillic acid and partly decarboxylated in the intestine to give catechol which can then be absorbed (110). This suggestion is supported by the finding that shikimic acid is metabolized to protocatechuic acid when incubated with rat cecal microorganisms (61). These pathways are summarized in Scheme VIII.



Scheme VIII—Proposed pathways in the metabolism of quinic and shikimic acids.

Dehalogenation—The metabolism of DDT [1,1,1-trichloro-2,2-bis (*p*-chlorophenyl)-ethane] in higher animals is of considerable interest due to its widespread use as an insecticide. DDT is partly metabolized by reductive dechlorination to DDD [1,1-dichloro-2,2-bis (*p*-chlorophenyl)-ethane] when given orally to animals and it has been believed that this reaction occurs in the liver (162–164). However, Barker and Morrison (165) showed that DDD is produced from injected DDT in decomposing animal tissue but not in living tissues. It was subsequently found that a strain of *P. vulgaris* isolated from mouse intestinal contents could carry out this reaction (166). As this organism is one of the primary invaders of animal tissues after death, it was concluded that it, in part at least, was responsible for the conversion of DDT to DDD in aminals which have been killed by DDT.

The conversion of DDT to DDD was also studied by Mendel and Walton (167) who found that DDD was formed after oral but not intraperitoneal administration of DDT to rats. This indicated that DDT must pass through the gastrointestinal tract for the formation of DDD to occur. Furthermore, they showed that *A. aerogenes* and *E. coli* isolated from rat intestine readily carried out the dechlorination of DDT. These findings indicated that the gastrointestinal flora must be the main agent for the formation of DDD and that the conversion takes place during the life of the animal. The intestinal microflora has been shown to play a major role in DDT detoxication in the rainbow trout, *Salmo gairdneri* (168).

Recently, Wedemeyer (169–171) carried out an extensive investigation of the anaerobic dechlorination of DDT by *A. aerogenes*. He found that whole cells or cell-free extracts could catalyze the degradation of DDT to at least seven metabolites. The terminal steps of this sequence led to the formation of DDA [2,2-bis (*p*-chlorophenyl) acetic acid] and finally to 4,4′-dichlorobenzophenone.

Other Reactions—The reduction of ketones by the intestinal flora has been shown to occur. Stimmel (60) found that estrone was converted to a large extent to β-estradiol when incubated with human feces. Reduction of the 3-keto group in bile acids was reported to be carried out by mixed cultures of anaerobic fecal microorganisms (99).

Studies on the metabolic fate of diethyltin dichloride in rats have shown that it is dealkylated and that this reaction occurs both in the tissues and in the intestine (172). Also, it was shown that the conversion of diethyltin to ethyltin could take place when the former was incubated with rat cecal contents.

Diquat and paraquat are bipyridilium compounds which are used as herbicides. Their metabolic fate in rats has been investigated (173) and it was found that appreciable amounts were excreted in the feces in a degraded form. Small amounts of these products were absorbed from the intestine and excreted in the urine. Incu-

bation of diquat and paraquat with a rat fecal homogenate resulted in extensive degradation of the compounds. However, the nature of these products was not determined.

The metabolism of chloramphenicol by intestinal bacteria has been cited previously in connection with amide hydrolysis and nitro reduction. However, this substance has also been found to undergo oxidation of the secondary hydroxyl group and cleavage of the propanediol portion of the molecule when incubated with strains of several intestinal bacteria (84).

Asatoor and Simenhoff (174) have demonstrated that urinary dimethylamine is derived partly from ingested choline and lecithin and that this conversion is dependent on the breakdown of choline to trimethylamine by intestinal bacteria.

Deamination by the intestinal flora is known to occur with amino acids (175, 176). Rat intestinal bacteria can deaminate and decarboxylate triiodothyronine to triiodothyroacetic acid (72). Tyrosine, phenylalanine, and tryptophan undergo deamination by sheep rumen microorganisms (116) and dopa is deaminated by rat fecal and cecal extracts (70). The deamination of tryptophan, histidine, and phenylalanine to give the corresponding acrylic acid derivatives has been reported to be carried out by bacteria from monkey feces (137). Also, 5-fluorocytosine was deaminated to 5-fluorouracil by the intestinal flora in rats (177).

The number of reports dealing with synthetic intestinal reactions is limited. However, it appears that both acetylation and esterification reactions can be carried out by the intestinal bacteria. Histamine was found to be partly metabolized to *N*-acetylhistamine when incubated with human feces suspensions (83). There is also indirect evidence which suggests that sulfanilic acid and *p*-phenylenediamine sulfonic acid, the fission products of acid yellow, are partly acetylated in the intestine (148). Fecal bacteria have been reported to esterify coprostanol and cholesterol (178).

CONCLUSIONS

The findings presented above amply demonstrate that the gastrointestinal microorganisms can play a significant role in determining the metabolic fate of drugs and other organic compounds. The diversity of the intestinal reactions is impressive, although hardly surprising in view of the tremendous range of metabolic capabilities found in microorganisms. The intestinal reactions generally have a common characteristic in that they involve degradative processes. Thus, the various compounds usually undergo reduction in molecular size. These processes very often alter the physicochemical properties of the compound to a considerable extent. Examples of this are seen in the deconjugation of glucuronides and glycosides, reduction of nitro or azo compounds, and decarboxylation of phenolic acids. It is of great significance that these alterations may be accompanied by increases in biological activity or toxicity. The above examples may lead to the liberation of an active aglycone, production of a toxic arylamine, or formation of a more toxic phenol. These reactions often contrast markedly with those occurring in the tissues where the metabolic sequence leads ultimately to products of lower biological activity.

Many aspects of the study of the metabolism of drugs by the intestinal microflora have received only very limited attention. For example, relatively few reports have been published which correlate particular intestinal reactions with particular bacterial species. However, much of this work may depend on more satisfactory methods for the culture of some of the more fastidious intestinal inhabitants. Differences in the metabolic fate of some compounds may be a reflection of differences in the microflora. This may occur among different animal species and, in this regard, it is interesting to note the previously mentioned differences in the flora of the rabbit compared with other common species. Also, the differences may occur among a single species as a result of nutritional or environmental factors.

It is sincerely hoped that the present review may serve to stimulate interest in the study of drug metabolism by the gastrointestinal microorganisms as this has truly been a neglected aspect in the field of drug metabolism. However, its significance in the metabolism of many compounds demands that this factor must be given adequate attention in the future.

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Keyphrases

Drug metabolism—intestinal microorganisms
Gastrointestinal flora—normal, variables affecting
Metabolic activity—gastrointestinal flora
Microorganism metabolism, intestinal—drugs

Isolation and Characterization of Some UV-Induced Mutants of *Chlorella vulgaris* Prings.

By MARK J. SOLOMON and FRANK A. CRANE

Clones of new UV-induced mutants of *Chlorella vulgaris* are reported, including two strains darker green than the wild type. The isolated strains were characterized as to relative UV survival and general mutability. Significant stimulation of growth after low doses of UV irradiation was obtained. A taxonomic criterion distinguishing *C. vulgaris* and *C. pyrenoidosa* was recognized. Diameter measurements of cells grown under uniform environmental conditions differentiated *C. vulgaris* from *C. pyrenoidosa,* and various mutant clones within an initial isolation. Various histochemical and aberrant cytological features of mutant strains were observed. Thin-layer chromatography of extracted pigments demonstrated several that differed from those of the normal cells.

MUTATIONS, SPONTANEOUSLY occurring in natural populations or artificially induced, are abrupt changes in the genetic makeup of the organism, manifesting themselves phenotypically as altered biosynthetic capabilities through succeeding generations. These changes may result in detectably altered physiology and morphology. Metabolites having no counterpart in normal cells may be found.

Chlorella is especially suitable for mutation studies because it has a haploid nuclear complement, short life cycle, and is autotrophic and unicellular.

A naturally occurring mutant, *Chlorella rubescens*, was reported by Chodat in 1929 (1). Beijerinck (2) obtained yellow and colorless colonies of *C. variegata* in 1940. The work of Beadle and Tatum (3) influenced Davis (4) to investigate some mutant strains of *Chlorella* to elucidate the photosynthetic process, while Granick (5) studied the protoporphyrin compounds of *Chlorella* mutants involved in the biosynthesis of chlorophyll. Butler (6) isolated several UV-induced mutants of *C. pyrenoidosa* and characterized them as to starch synthesis, plastid formation, cell size, and changes in pigmentation. In 1963 Kvitko (7) used X-ray irradiation to induce a large number of different types of mutants and reported a synthetic classification for them. This classification included photosynthetic, nonphotosynthetic, biochemical, and gross morphological mutants. Zakharov (8) reported the isolation of several auxotrophic mutants of *Chlorella* by replicating and characterized two of them as arginine and thiamine deficient strains.

Previous reports on the study of selected *Chlorella* mutants have not included a procedure for the isolation of pure clones. Initial isolations were assumed to be uniform strains. This report characterizes some new UV-induced mutant clones and compares them with their corresponding wild type. In addition to changes in pigmentation other phenotypic observations were made to test the possibility of multiple mutations occurring simultaneously in the same strain. Adaptive effects of UV irradiation and increased mutability were considered.

A survey of the literature (9–14) and consideration of the slow growth rate previously mentioned prompted examination of cytological and histochemical methods which require only small amounts of material for intensive study. Flaumenhaft *et al.* (15) applied histochemical stains to normal and deuterated *Chlorella* and *Scenedesmus* cultures to study differences in carbohydrates, lipids, proteins, nuclear material, and population cell size.

Davis (4) reported absorption spectra of total extracted pigments of *Chlorella* mutants, but did not separate the pigments. Strain (16) isolated the chlorophylls of normal and deuterated *Chlorella* and *Scenedesmus* while Allen (17)

Received May 22, 1968, from the Department of Pharmacognosy and Pharmacology, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60680

Accepted for publication August 15, 1968.

Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

Abstracted from a thesis submitted by Mark J. Solomon to the Graduate College of the University of Illinois at the Medical Center in partial fulfillment of the Master of Science degree requirements.

investigated various carotenoid fractions of a normal strain of *C. pyrenoidosa* and some selected mutants. To understand the biochemical changes occurring in abnormally pigmented mutants, the individual pigments should be considered. Since populations of pure clones were obtained, variations in pigment production could be attributed to the genetic constitution of the strains considered. Elucidation of the pigment changes which occurred further characterized the various mutants studied.

EXPERIMENTAL

Materials and Methods—Wild type cultures of *C. vulgaris* (13402-R. W. Krauss strain Pringsheim 211/11a.) and *C. pyrenoidosa* (ATCC 11469-R. W. Krauss strain Pringsheim 211-8b) were used. Mutants were produced entirely by UV irradiation of this *C. vulgaris* strain. Both strains were subcultured on glucose medium every 10 days. Mutants were subcultured on glucose medium periodically to maintain them in the logarithmic growth phase.

Cultures were grown on Myers' (13) salt medium modified by deletion of his "possible micronutrients" and use of sequestered iron. Glucose medium contained in addition to salts, 1.5% glucose at pH 5.5. In early growth experiments a complete medium was used consisting of Myers' salt solution, 1.5% glucose, 1.5% casein hydrolysate, and 0.5% yeast extract. Since growth was better on glucose medium, complete medium was discarded.

All cultures were grown under aseptic conditions. To ensure sterility, plates, slants, and liquid media were incubated for 3–4 days at 32° before inoculation.

Growth Conditions—All cells were grown in a specially designed growth room with the temperature and humidity continuously controlled and recorded. The mean temperature was 23.6 ± 0.5° with a range of 22.2–25°. The relative humidity was maintained at 50% ±5%.

Light was supplied to the growing cells by 21 fluorescent lamps adjusted to 121.9 cm. (48 in.) above the table surface.[1] Photometer readings at table top level with new lamps were recorded as 500 ftc. Cultures were given continuous light.

Petri dishes and slants held in wire racks were placed on the table top with the colonies facing the light source. Liquid medium was placed on a horizontal shaker regulated to give twenty-four 500-ml. flasks 150 r.p.m., thus providing aeration. No additional means of introducing sterile air or carbon dioxide was used.

Growth Rate—Growth rate was determined by daily measurements of packed cell volume, culture turbidity, or cell counts in a hemocytometer. The cells were harvested by scraping or washing solid media or centrifuging liquid cultures.

Cell Size—Diameter measurements of cell populations in the logarithmic growth phase mounted in water were taken with a calibrated ocular micrometer. The diameters of approximately 1,000 cells were measured for each strain to provide an adequate sampling to ensure statistical validity.

Irradiation—All irradiations were accomplished by a single 15-w. germicidal lamp[2] giving off most of its energy at the wavelength of 2537 Å. An individual lamp was used for 100 hr. and then discarded. The lamp was permanently situated in a fluorescent fixture suspended 30.5 cm. (12 in.) above the table top with a white cardboard shield around the lamp to protect the investigator. The UV irradiation/sec./sq. cm. was standardized.[3]

The cells to be irradiated, in the logarithmic growth phase, were washed from slants and serially diluted. Aseptic aliquots of the dilutions were counted in a hemocytometer and the dilution representing 1×10^4 cells/ml. was used. One-milliliter aliquots were spread to a monocellular layer on the surface of sterile glucose medium Petri dishes.

The inoculated plates were then placed under the irradiation lamp and the covers removed to allow UV penetration. Any contaminated plates were discarded. Initial experiments indicated that a 30-sec. dose of UV irradiation produced no mutations, even with replica plating screening procedures, while 5-min. irradiation produced apparently discolored mutants.

Of the surviving colonies, obvious mutants were aseptically transferred to glucose medium slants and allowed to multiply. The isolations surviving this transfer were labeled with letters of the alphabet (16 of the 55 isolations survived). To obtain pure clones, each isolation was serially diluted. Aliquots containing 1 cell/ml. were transferred to glucose plates and allowed to grow. Colonies of each of the 16 types were labeled with numerical subscripts and continued through at least three subinoculations before consideration as established mutants (Table I). At least 75 days of growth were needed to obtain enough cells of these pure strains to begin characterization.

Survival, Lethality, and Mutation—The mutation index for a strain was defined as the percentage of colonies demonstrating a color difference from the cells on the control plate (0 UV) per 100 colonies counted in the population of surviving irradiated cells. Such indexes were determined for each of the six major mutant strains investigated as well as the two wild types.

Survival (survival capacity) is defined as the mean number of surviving colonies receiving a particular dose of UV compared with the mean number of colonies on the respective control plate for each strain (× 100%).

Lethality is defined as 100% minus the survival capacity.

Histochemical Methods—Cells of the various strains in the late logarithmic phase on slant cultures were mounted in water. The various histochemical stains were applied directly to the slide. These stains included the periodic acid-Schiff test for complex carbohydrates (18), acridine orange for mucin (19), ruthenium red for pectin (18), sudans III and IV for lipids (20), mercuric bromophenol blue for proteins (21), and iron-hematoxylin for nuclear material (22). After a suitable time for these various colorimetric reactions had elapsed observations were made microscopically. Care was taken to treat

[1] General Electric F 6 T 6 Cool White lamps.

[2] General Electric lamp No. G15T8.

[3] Weston Electronic Instrument Corp., model 703 type 3.

TABLE I—OBSERVATIONS OF COLONIES AND CELLS OF MUTANT STRAINS OF *Chlorella* GROWING ON GLUCOSE AGAR MEDIUM

Strain	Color of Colony	Spreading Characteristics on Agar Surface	Gelatinous Sheath	Cellulose Wall	Chloroplast Structure	Pigment Differentiation	Cell Divisions	Spore Formation
C. vulgaris	Green	Thickly covers entire surface	Normal	Normal	Normal	Normal	Normal	Normal
C. pyrenoidosa	Green	Thickly covers entire surface	Normal	Normal	Normal	Normal	Normal	Normal
A-1	Golden-green	Thickly covers entire surface	—	—	—	—	—	—
A-2	Light-green	Thickly covers entire surface	Thinner	Less prominent	—	—	—	—
A-3	Light-green	Thickly covers entire surface	Thicker	—	—	—	Unequal	—
C-1	Yellow	Film over surface	—	—	Irreg. granular	Red spots on chloroplast	—	—
C-4	Golden-brown	Covers entire surface	Thinner	Not visible	Irreg. granular	No chlorophyll visible	—	—
C-5	Yellow-orange	Covers entire surface	—	—	—	—	—	—
C-7	Golden-brown	Covers entire surface	—	—	—	Discrete spots on chloropl.	—	—
C-8	Golden-brown	Covers entire surface	—	—	—	—	—	Increased over normal
C-9	Golden-brown	Covers entire surface	Thicker	Crenulated	—	Discrete spots on chloropl.	—	—
C-10	Golden-brown	Covers entire surface	None evident	More prominent	Cytopl. ridges	No chlorophyll visible	—	—
C-11	Yellow	Film over surface	Thinner	Crenulated	—	—	—	—
E-4	Dark-green	Covers entire surface	Thinner	Less prominent	—	Darker green	—	—
E-5	Dark-green	Covers entire surface	Thicker	Crenulated	—	Darker green	Unequal	—
F-4	Yellow-green	Covers entire surface	Thinner	Crenulated Less prominent	—	—	—	—
H-3	Light-green	Covers entire surface	—	—	—	—	—	—

the mutants and wild types concurrently with each stain as well as to compare the unstained counterpart for each strain.

Chromatography and Quantitation of Pigments—Initial paper and column chromatographic separation of plastid pigments proved unsatisfactory for this work. Thin-layer chromatography (23) provided finer separation, less tailing, and the rapidity necessary to avoid photocatalysis of the various constitutents.

Commercial sources of carotenes were satisfactory, but extraction of chlorophylls and xanthophylls of leaf tissue were necessary to obtain standards for chlorophylls A and B and the xanthophyll group. Action spectra of the leaf pigments provided proof of identity and purity.

Cells representing 1-month growths in the logarithmic phase (several successive cultures) for each strain were harvested by washing and centrifugation. They were combined and then frozen. When needed for use the frozen cells were thawed, treated with liquid nitrogen for 15 min., then extracted exhaustively with acetone. The pigment-solvent mixtures were combined for each strain and extracted exhaustively with petroleum ether (b.p. 30–60°). The petroleum ether fraction was evaporated in a vacuum desiccator to a minimal volume and quantitatively transferred to 10-ml. volumetric flasks and brought to final volume. Aliquots of these solutions were spotted on activated Silica Gel G plates, and developed in the following solvent mixture: petroleum ether–benzene–absolute ethanol (40:15:4) according to the method of Schaltegger (23). After development the solvent front was marked; the spots were outlined; notations were made as to color and R_f values; and comparisons of various compounds were made.

Elution and spectrophotometric determination of the separated pigments were attempted. The slow growth rate of the various strains and low concentrations of pigments limited further comparative quantitative work.

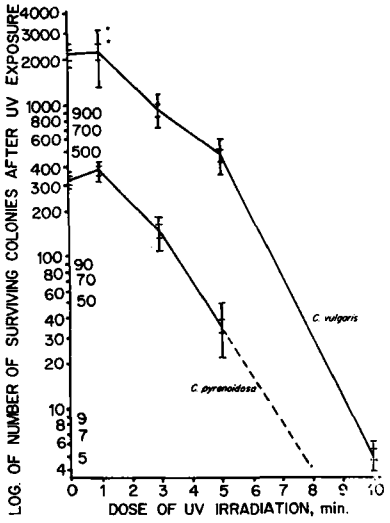

Fig. 1—Survival of C. vulgaris *and* C. pyrenoidosa *after exposure to UV irradiation under standardized conditions. Key: *, wider deviations are standard deviation; narrower deviations are standard error of the mean.*

RESULTS

Macroscopic Observations—The major discernible macroscopic feature differentiating the mutants from the wild types was color (Table I). Mutants C-1 and C-11 distributed themselves across the agar surface of glucose medium slants as a thin film while the other mutants and normal cells formed a thick layer. All mutants and the wild types were shiny while growing on glucose medium.

Test for Glucose Dependency—When the cells from each strain were inoculated on both glucose medium and salt medium, it was found that all of the strains were capable of growth on salt medium. All of the mutants were therefore classified as photosynthetic mutants according to Kvitko's classification system (7).

Comparative Survival After UV Exposure—Strains *C. vulgaris*, *C. pyrenoidosa* A-1, A-2, C-1, C-11, E-4, and H-3 were each exposed to 0, 1, 3, 5, and 10 min. of UV light and their relative survival compared (Fig. 1 and Table II). In all of the strains except E-4 there was a slight increase in survival over that of the control at low dosages of UV, though high doses produced the expected decrease. Mutant E-4 decreased in survival even at low doses of UV while its survival after high doses was greatly reduced. Strains H-3, A-2, *C. pyrenoidosa* were significantly more resistant to the lethal effects of UV than the wild type ($p < 0.01$ in Table II).

Mutation Indexes—With increasing doses of UV the mutation rate of the respective strains became

TABLE II—RELATIVE SURVIVAL OF SELECTED STRAINS OF *Chlorella* AFTER 1-MIN. AND 5-MIN. EXPOSURES TO UV IRRADIATION

Strain	Percent Increase, 1-min. UV[a]	Probability That This Culture Has Same Survival Capacity as its Control	Index of Survival, 5-min. UV	Probability That This Culture Has Same Survival Capacity as *C. vulgaris*
C. vulgaris	15.59	$p < 0.01$	11.59	$p = 1.00$
C. pyren.	6.33	$p > 0.40$	22.22	$p < 0.01$
A-1	0.21	$p > 0.20$	5.98	$p < 0.10$
A-2	15.59	$p < 0.02$	52.15	$p < 0.01$
C-1	10.84	$p > 0.20$	9.77	$p > 0.20$
C-11	12.93	$p > 0.2$	4.94	$p < 0.05$
E-4	−11.49	$p > 0.2$	7.43	$p > 0.2$
H-3	20.62	$p < 0.005$	23.47	$p < 0.005$

[a] Surviving colonies at 0-min. UV—surviving colonies at 5-min. UV/surviving colonies at 0-min. UV × 100%.

TABLE III—RELATIVE MUTATION INDEXES[a] OF SELECTED STRAINS OF *Chlorella* AFTER 3-MIN. AND 5-MIN. EXPOSURE TO UV IRRADIATION

Strain	Mutation Rate[b] After Exposure to 3-min. UV, %	Probability That This Culture Has Same Mutation Rate as *C. vulg.*	Mutation Rate After Exposure to 5-min. UV, %	Probability That This Culture Has Same Mutation Rate as *C. vulg.*
C. vulg.	0.04	$p = 1.00$	3.93	$p = 1.00$
C. pyren.	0.24	$p < 0.01$	2.83	$p > 0.4$
A-1	6.40	$p < 0.005$	22.22	$p < 0.005$
A-2	2.63	$p < 0.005$	6.11	$p < 0.20$
C-1	14.00	$p < 0.005$	19.23	$p < 0.005$
C-11	0.20	$p < 0.005$	37.17	$p < 0.005$
E-4	—	—	63.31	$p < 0.005$
H-3	15.43	$p < 0.005$	32.47	$p < 0.005$

[a] Mutation index = probability of pigmented mutant/probability of any mutation $\simeq$ constant. [b] Number of colonies (per 100 colonies counted) having a color other than that of the control plate, respectively, for each strain.

greater (Table III). All of the mutants had mutation rates significantly higher than the wild type. At 5-min. UV the differences in mutability were not statistically significant. The mutation rates of *C. pyrenoidosa* and *C. vulgaris* were not statistically different at the 1% level.

Size Measurements—The mean diameters of all of the mutants were found to be larger or smaller than the normal cells ($p < 0.05$–0.005), but with no uniform pattern. The normal cells had a smaller range of size than the mutants. The strains were arranged from smallest to largest (Table IV). Mutant E-5 had the greatest standard deviation and demonstrated unequal divisions of the cells. Contrary to the report of Retovsky (11), note should be taken that *C. vulgaris* and *C. pyrenoidosa*, under uniform environmental conditions, can be differentiated by the criterion of cell size ($p < 0.005$, Table IV). Comparison of cell size variation about the means of particular clones within an initial isolation (C and E but not A) demonstrated significant differences. If initial isolations were pure clones such variations would not be expected under uniform environmental conditions.

Histological Observations—The periodic Schiff reaction demonstrated the presence of complex carbohydrates in the walls of all cells, but this test as well as the acridine-orange test did not demonstrate any significant difference between the mutants and the normal strains. Ruthenium red did not differentiate *C. vulgaris* from *C. pyrenoidosa* as reported by Kessler (18), but did differentiate mutants A-1, C-5, and C-11 as having no pectin present. In all strains the mucilaginous envelope appeared to contain the greatest concentration of lipids. Mutants A-1 and A-2 appeared to contain less lipids than any of the others.

No significant differences could be found in the protein and nucleic acid contents of the respective strains.

Cytology of Irradiated Cells—Cytological observations indicated differences in the gelatinous envelope, the cell wall, chloroplast structure, cell divisions, formation of spores, appearance of the protoplast, and pigment differentiation. The aberrations from the normal are summarized in Table I.

Chromatography and Comparison of Pigments—*Chlorella vulgaris* had a yellow carotene at R_f 0.93 but *C. pyrenoidosa* had a yellow compound at R_f 0.82. Mutants A-2, A-1, E-4, and H-3 had orange, red, yellow-orange, and red-orange spots, respectively, at R_f values of 0.82, 0.82, 0.87, and 0.90. No carotene compounds were observed in C-1 and C-11 (Fig. 2).

Mutant E-4 was visibly darker green than *C. vulgaris*. On the TLC plate it contained 5 chlorophyll spots instead of the 4 found in the other strains tested. Mutant C-1 demonstrated no chlorophylls, and C-11 had only minute amounts of 3 chlorophylls, though the poor growth rate of these strains and the quantity of compounds available for extraction were probably responsible. Mutants A-1, A-2, E-4, and H-3 produced a reddish spot at R_f 0.25 while both wild type strains had none (Fig. 2).

TABLE IV—CELL SIZE (DIAMETER) OF NORMAL AND MUTANT *Chlorella* STRAINS

Cell Type	Number of cells	Mean Diameter, μ	$\pm$ *SD*	*SEM*	Probability
C-1	912	6.45	±1.92	0.04	0.005
C-8	1313	7.80	±1.91	0.05	0.005
C-10	1068	8.16	±2.14	0.01	0.005
C-9	688	8.22	±1.69	0.11	0.05
A-2	1298	8.35	±1.46	0.05	0.005
A-1	1202	8.45	±1.53	0.05	0.005
C. vulg.	1127	9.02	±1.89	0.06	—
C-5	1243	9.07	± .82	0.02	0.01
E-4	1153	9.10	±1.65	0.05	0.005
C-7	903	9.18	±2.08	0.09	0.05
C-11	1327	9.39	±2.12	0.06	0.005
C. pyr.	1333	9.42	±2.07	0.09	0.005
H-3	944	9.51	±2.12	0.07	0.005
E-5	1382	10.10	±2.20	0.06	0.005

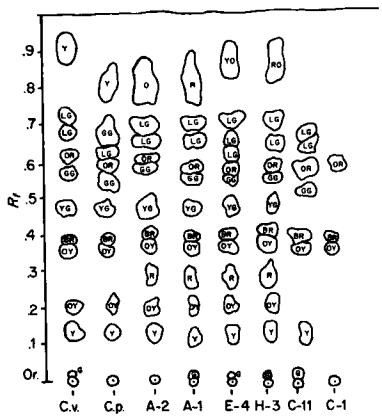

Fig. 2—Scale representation of thin-layer chromatogram of pigments extracted from Chlorella *mutants. Key: G, green; R, red; O, orange; OP, orange-pink; GG, gray-green; BR, brown-red; OR, orange-red; LG, light green; OY, orange-yellow; Y, yellow.*

DISCUSSION

With the minor modifications noted, Myers' (13) medium appears to be highly satisfactory for mutation studies with *Chlorella* species. Addition of yeast extract and casein hydrolysate did not enhance growth as might be expected, but actually reduced the volume of packed cells obtained.

All of the strains were able to grow on the salt medium but growth on glucose medium was faster and resulted in larger volumes of packed cells. Because of the decreased growth rate of the mutant strains relative to *C. vulgaris*, glucose medium was used to stimulate growth. No error in carbohydrate metabolism could be found in any of the mutant strains.

Redford and Myers (24) reported a slight increase in survival capacity of normal *C. vulgaris* irradiated with low doses of UV but they did not statistically document their finding. When the increase of the mean survival at 1-min. UV exposure was compared with the controls for each strain, respectively, it was found that *C. vulgaris* ($p < 0.01$) had a statistically significant increase while *C. pyrenoidosa* did not ($p > 0.4$). *C. pyrenoidosa* had a significantly larger survival from its control at 5-min. UV than did *C. vulgaris* over its control ($p < 0.01$).

Taken together, these two phenomena may be useful to differentiate the two species taxonomically (Table II).

This growth increase of *C. vulgaris* at low doses of UV irradiation may have practical applications for mass production of *Chlorella* as a source of food or other industrial uses. To enhance the growth and vitality of "seed cultures" for each batch, low doses of UV light may be significant. Low doses of UV to *Chlorella* represent rather high doses of UV light for bacterial contaminants and such doses may help ensure a more aseptic culture than would otherwise be expected.

Mutants A-2, H-3, and *C. pyrenoidosa* were more resistant to the lethal effects of 5-min. UV exposure than the normal cells ($p < 0.01$) based on survival percentage at 5-min. UV (Table II). It is evident that in the process of selecting mutants for color differences from the normal, some strains were selected which, upon further irradiation, had the ability to survive additional exposure better than their controls. All of the mutant strains had a mutation rate greater than the normal strains. As the lethality of the UV irradiation increased for the strains, the respective mutation rates (after 5-min. UV) also increased.

Retovsky (11) reported that the mean diameter of *Chlorella* cells in nature, *i.e.*, not grown under uniform conditions, did not provide an adequate taxonomic criterion for differentiating the two wild type species. With the qualification of growth under uniform conditions significantly different mean cell diameter distributions could be obtained. On several bases Soeder (25) concluded that *C. pyrenoidosa* should be reclassified. In view of the large spread of cell diameter measurements about the mean reported in this work for *C. pyrenoidosa* compared with *C. vulgaris*, Soeder's view is further strengthened (see Table IV).

Kessler (18) reported a distinction between *C. vulgaris* and *C. pyrenoidosa* using ruthenium red. Such a distinction could not be made in this work, but mutants A-1, C-5, and C-11 demonstrated a lack of pectins by this method. Mutants A-1 and A-2 appeared to contain less lipid than the other strains. Possible biochemical errors were indicated.

The glucose-dependency test described previously (1) indicated no carbohydrate metabolic error present. The PAS reaction verified this result.

Most of the morphological differences described by previous workers were macroscopic colony changes. The unequal division of mutant cells in certain strains is noteworthy. The characteristic thinner or not-evident gelatinous sheath (A-2, C-11, E-4, C-10, C-4, and F-4) has a rough correlation with the characteristically less prominent or not-evident wall (A-2, E-4, C-4, C-9, and F-4). Strains A-2, E-4, C-4, and F-4 had both characteristics in common and differ in that C-9 had a prominent gelatinous sheath with a nonprominent wall. It may be surmised that the genetic mechanism governing wall thickness may have some control over the gelatinous sheath. Both processes include glucose residues, polyuronides, and polysaccharides. It should be noted also that C-10 had a more prominent wall than *C. vulgaris*, but also had no thin gelatinous sheath evident.

The location of discrete chlorophyll spots appears to be distinctive for the C strains (C-1, C-4, C-7, and C-9) although not universal for all of the C strains. All of the other characteristics described appear to be distinctive for individual strains (Table I).

Excellent separation of pigments was obtained by application of thin-layer chromatography. The presence of compounds with R_f values similar to the carotenes of *C. vulgaris* provides substantial theoretical interest. The fact that spots did occur implies that some kind of carotene was formed. The determination of whether these spots represent the build-up of a precursor from a genetic block or an entirely new substance produced by the cell could not be ascertained because the relatively slow growth rate of the strains restricted this work.

Mutant E-4 appeared darker green to the naked eye. On the thin-layer plate E-4 had an extra chlorophyll spot. In almost every test E-4 demon-

strated difficulty adjusting to a new environment. This behavior might be explained in part if E-4 were synthesizing an inactive form of chlorophyll.

Mutants C-1 and C-11 had the slowest growth rate. Upon examination by TLC, fewer chlorophyll spots were found than would be expected. It is postulated that chlorophylls were present, however, because both C-1 and C-11 were photosynthetic on salt agar.

It is noted that less than 50 mg. of cells (fresh weight) of each strain was available for cell extraction.

SUMMARY

Thirteen UV-induced strains of *C. vulgaris* are reported. Especially interesting were mutants E-4 and E-5 which were darker green than normal to the eye.

This research represents an attempt to characterize pure mutant clones by use of a variety of methods.

Selected mutants were tested for susceptibility to UV damage and mutability. All of the mutants were found to be more mutable than the normal but several mutants were more resistant to the damage of UV irradiation than the normal.

A new taxonomic criterion for distinguishing the two species of *C. vulgaris* and *C. pyrenoidosa* became apparent. The increase in survival capacity over controls (0-min. UV for each strain) and the percent decrease after 5-min UV were statistically documented.

It was suggested that irradiated seed cultures of *Chlorella* cells may enhance the initial growth rate and help sterilize cultures for industrial algal growth.

Chlorella vulgaris and *Chlorella pyrenoidosa* could be differentiated by cell size when grown under uniform environmental conditions.

Clones within particular initial isolations demonstrated significant differences in cell size from the normal.

C. vulgaris and *C. pyrenoidosa* were not distinguished with ruthenium red but certain mutants could be distinguished from the normals with this stain.

Cytologic aberrations were observed including those of the sheath, cellulose wall, chloroplast, pigmentation, patterns of cell division, and spore formation.

TLC demonstrated that the mutants produced certain pigments with similar R_f values to the normal pigments but of different color, and also produced several pigments apparently not synthesized by the normal.

Mutant E-4, visibly darker green to the naked eye, chromatographed an extra chlorophyll spot when compared with the normal.

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Keyphrases

Chlorella vulgaris mutants–UV—induced
UV irradiation—*Chlorella* cultures
Mutation indexes—*Chlorella*
Cells, *Chlorella*—size, growth rate
TLC–separation, plastid pigments

In Vivo Evaluation of Local Anesthetics Applied Topically

By ARTHUR H. CAMPBELL, JEANETTE A. STASSE, GEOFFREY H. LORD, and JOHN E. WILLSON

A method has been offered for the screening of topically applied anesthetic agents of varying molecular structure. It is based on the observation that injuring, but not necessarily rupturing the skin, reduces its resistance to penetration.

THE ADMINISTRATION of chemical agents through the skin has long been the object of considerable investigation which, in isolated instances, has proved fruitful; but generally has been frustrated by as yet undefined or unproved explanations of normal skin function.

Among the types of compounds with which there has been limited success (1) has been the agent which reduces discomfort resulting from relatively minor accessible injuries—the local anesthetic.

A variety of topical anesthetic testing methods are contained in the literature. These have included studies in the lingual receptors of the frog (2), the nasal and bucco-pharyngeal mucosae of the rabbit (3, 4), the tail of the earthworm (5), the goldfish (6), the eyes of cats, dogs, guinea pigs, and mice (7–10), as well as a host of *in vitro* techniques.

No method could be found by which the potency of an anesthetic agent could be determined, following its application to the surface of the skin of the more commonly used laboratory animals. In an effort to fill this need, a series of testing procedures was developed. They have been successfully employed in these laboratories for several years. They are based on the observation that superficial injuries which need not necessarily rupture the skin, may nonetheless alter the natural dermal barrier and thereby permit penetration of local anesthetic molecules.

METHODS

Each of the studies presented herein conforms to a basic six-step pattern: (*a*) determination of preinjury skin sensitivity; (*b*) the infliction of the injury; (*c*) the determination of postinjury, or pretherapy, skin sensitivity; (*d*) application of the agent; (*e*) determination of posttherapy sensitivity; and (*f*) evaluation of the results.

Twenty-four hours before the study, the backs of the guinea pigs were shaved with mechanical clippers and depilated with a commercial depilatory.[1]

During the test, no more than two animals were used at one time by one investigator. This rule tended to minimize the time gap between infliction of the injury and application of the agent. In addition, the simultaneous handling of but two animals permitted adequate opportunity for observation of sometimes subtle, but nonetheless discernible, reactions.

Because there is no other way of determining whether pain has been abolished, in the treated tissue of the laboratory animal, a stimulus must be applied to the test site and an absence of response is interpreted as being indicative of a state of anesthesia. Thus, even though almost any type of pain-inducing stimulation may be used, it is important that the nature, duration, intensity, and frequency be sufficiently controllable as to make the stimulus reproducible.

The manner in which each guinea pig reacted to pain varied and because of this, its individual type of response had to be established before the experimental agents were applied. Only the more demonstrable animals, which reacted in unmistakable fashion to the stimuli, were used.

The type of stimulus selected was relatively superficial, from the point of view of penetration; but it was of constant duration and was also controllable. It consisted of pricking the test area on the back of the immobilized guinea pig with the point of a 22-gauge hypodermic needle which was attached to the plunger of a 10-ml. plastic disposable syringe. The rubber tip of the plunger had been first removed to facilitate its free movement within the barrel. The barrel of the syringe was attached to a ring stand in a vertical position at a constant height. The distance from which the plunger (with the attached needle) was dropped before striking the test site on the guinea pig's back, as well as the addition or subtraction of weights taped to the top of the plunger, determined the intensity of the stimulus applied. Furthermore, by dropping the plunger from the same height (as determined by the calibrations on the barrel of the syringe) a stimulus could be duplicated.

The sensitivity levels were determined by applying stimuli, the intensities of which were only of sufficient magnitude to have elicited consistent and reliable responses, and a state of anesthesia was considered to have been induced when the previously established postinjury sensitivity had been abolished following administration of the experimental agent.

In order to determine whether any specific area of the denuded back may have been more suitable, preliminary studies were run on varying sections.

Received April 21, 1967, from the Johnson & Johnson Research Foundation, New Brunswick, NJ 08903
Accepted for publication August 11, 1968.

[1] Nair, Carter Products Div. of Carter-Wallace, Inc., New York, N. Y.

It soon became apparent that the skin directly over the scapulae was more sensitive and yielded higher levels of consistency. Thereafter, those areas were used exclusively.

Two test sites were therefore used on the back of each animal (one over each scapula) for all but the Type II burn study.

The Type I burn was made by momentarily touching the skin with the broad side of a 2.21-cm. (7/8 in.) steel spatula. Heating was effected by holding the spatula above the 3- to 5-mm. high blue portion of a flame emitted from a Fisher Wide Flame gas burner for 15 sec.

The Type II burn (the scald) was inflicted by immersing the test area in a 80° bath for 5 sec. Here, as has been mentioned, only a single burn was made on the back of each animal.

Type III burns were caused by immobilizing the animals beneath a 250-w. Lo-Glare ceramic IR lamp for periods of 5 min. at a distance of 21.59 cm. (8 1/2 in.). Windowed shields of aluminum foil attached to the backs, restricted the areas of exposure to approximately 1 sq. in. at each of the two sites.

The experimental preparations were also tested on an abrasion which was induced by scraping the skin (5 strokes in one direction; then 5 more strokes at a right angle) with 150 (2/0) garnet carborundum paper.

As was mentioned above, the sensitivity (threshold) was again established after each injury and it was consistently noted that a stimulus of much less intensity was required to cause reactions after inflictions of the injuries.

Of significant importance was the observation that all portions of the skin, damaged by Type I burns, were equally sensitive to stimuli of constant intensity. This was not the case, however, following the infliction of Type II burns. The periphery of the scalded areas seemed very sensitive and lightly applied pricks elicited markedly exaggerated protests from the guinea pigs. As points nearer the center of the burn were stimulated, reactions tended to diminish and, in the center of the burn, levels of response which were inferior to those detected prior to burning, were noted. Whether this apparent inhibition of impulse conduction was due to damage of the superficial nerve fibers, or was due to a dampening effect created by the presence of edema fluid, was not established. Because of this variation, the application of all stimuli was restricted to a zone approximately 6 mm. in width, around the circumference of the scald...even though the experimental compounds were applied to the entire burned area. This zone was the most sensitive and yielded the most constant results. As was true following the Type I burns, those areas which had been injured by Type III burns, as well as abrasions, each exhibited increased sensitivity and all portions of the injured areas reacted equally to constant stimuli.

To illustrate the method, three known anesthetic agents diluted in a 80:20 propylene glycol–water mixture were applied at two concentrations, 0.125 and 1.000% (w/v). These agents were lidocaine-(diethylaminoacet-2, 6-xylidide); benzocaine(ethyl aminobenzoate); and diperodon[3-(1-piperidyl)-1,2-propanediol dicarbanilate].

Following the administration of each test preparation, stimuli equivalent to those required to have caused acceptable postinjury responses were applied at 1-min. intervals until a total of 10 had been made. Following this, they were again stimulated 15 to 20 min. after the application.

Every preparation was tested on a minimum of six sites to which the same type of injury had been inflicted; but on no occasion was the same agent applied to more than one site on the same animal.

Finally, in addition to being subjected to the procedures described, it was felt that efficacy, following application to optic tissue, should be considered when screening for topical anesthetic potency. Toward this end, the preparations were tested in the rabbit and guinea pig *via* the method of Chance and Lobstein (9). The validity of their procedure, as well as that of the newly proposed method, depends on acceptance of the above-stated premise that absence of response in a treated site, following application of a normally effective stimulus, is indicative of a state of anesthesia. In the eye, the relative potencies of topically administered agents were determined on the basis of responses to corneal stimuli. These, in the form of gentle strokes on the cornea with a single camel hair, were applied once each min. for the next 18 consecutive min. following treatment.

RESULTS

The number of "no responses" to stimuli applied to each test area have been presented individually in Table I. As recommended by Chance and Lobstein, optic sites were each stimulated 18 times; whereas only 12 stimuli were applied to injured skin sites.

The number of negative responses for each test group of six were totalled and then divided by the number of stimuli—thereby yielding single values (see Table II) which represented the anesthetic potencies of the preparations in the respective sites. A score of 1.000 was indicative of an onset of action of less than a min. and a duration of at least 18 min. in the eye or 20 min. in the skin. Values of lesser magnitude were proportional to the degree of anesthesia elicited. Also presented in Table II are the fiduciary limits for each value, calculated with a probability limit of 95%. Comparisons of these supported the following conclusions.

Rabbit Eye—(*a*) The 0.125% concentrations of none of the three test agents was significantly more potent than the water control. (*b*) Diperodon (1.0%) was more potent than 1.0% lidocaine. (*c*) Lidocaine (1.0%) was more potent than 1.0% benzocaine.

Guinea Pig Eye—(*a*) As compared with the water control, all six experimental solutions were significantly more active. (*b*) None of the three agents, when applied at concentrations of 0.125%, caused significantly different levels of response. (*c*) Lidocaine (1.0%) was more potent than 1.0% benzocaine. (*d*) Diperodon (1.0%), with a potency value which was between those of lidocaine and benzocaine, proved to have been no less potent than 1.0% lidocaine but simultaneously, no more potent than 1.0% benzocaine. The confidence limits of the diperodon value overlapped the confidence limits of both of the other solution values.

Abraded Skin—(*a*) All solutions, except the

TABLE I—TOTAL NUMBERS OF NEGATIVE RESPONSES OBSERVED AT EACH OF THE SIX SITES WHICH WERE EMPLOYED PER COMPOUND PER ANALYSIS

Compd.	Rabbit Eye	Guinea Pig				
		Eye	Abraded Skin	Burns Type I	Burns Type II	Burns Type III
0.125% benzocaine	0,0,1,0, 0,0	0,2,3,1, 4,2	0,0,0,0, 0,0	2,2,0,1, 0,0	0,0,0,0, 0,0	2,3,2,0, 1,2
0.125% diperodon	0,1,1,0, 0,0	2,0,1,3, 1,2	3,4,4,3, 5,3	3,0,2,2, 1,2	0,0,0,0, 0,0	0,0,0,0, 0,0
0.125% lidocaine	0,0,0,0, 0,0	0,2,4,1, 1,2	2,2,0,3, 1,1	0,0,2,1, 0,0	0,0,0,0, 0,0	0,0,0,0, 0,0
1.000% benzocaine	3,2,2,0, 2,3	7,4,6,6, 5,6	4,2,5,3, 2,4	2,1,2,1, 3,2	5,4,5,6, 4,6	5,4,4,4, 3,3
1.000% diperodon	9,12,16, 15,12,14	7,10,8 8,11,9	6,7,6,4, 5,5	4,1,2,2, 4,3	1,2,2,0, 1,0	0,0,0,0, 0,0
1.000% lidocaine	8,5,7,7, 8,9	10,11,12, 15,14,9	2,1,3,3, 3,2	1,2,0,0, 3,0	2,4,2,3, 1,2	0,0,0,0, 0,0
Water control	0,0,0,0, 0,0	0,0,0,0, 0,0	0,0,0,0, 0,0	0,0,0,0, 0,0	0,0,0,0, 0,0	0,0,0,0, 0,0

0.125% benzocaine, were significantly more active than the water control. (*b*) Diperodon (0.125%) was more potent than 0.125% lidocaine. (*c*) Diperodon (1.0%) was more potent than 1.0% lidocaine. (*d*) Benzocaine (1.0%), with an intermediate value, was no less active than 1.0% diperodon, but no more active than 1.0% lidocaine.

Type I Burn—(*a*) Of the six solutions tested, only 0.125% lidocaine exhibited no more potency than the water control. (*b*) There was no significant difference between the potency of 0.125% benzocaine and that of 0.125% diperodon. (*c*) There were no significant differences between the potencies of 1.0% diperodon, 1.0% lidocaine, and 1.0% benzocaine.

Type II Burn—(*a*) None of the three agents examined, when applied at concentrations of 0.125%, were more potent than the water control. (*b*) Benzocaine (1.0%) was significantly more potent than both 1.0% diperodon and 1.0% lidocaine. (*c*) The difference between the potency values of 1.0% diperodon and 1.0% lidocaine was not significant.

Type III Burn—(*a*) Of the three agents tested, only benzocaine, at both concentrations employed, proved to have been measurably effective in this type of injury. These findings have been subsequently confirmed in different vehicles as well as in blindly run studies.

From these data it seemed apparent that no single agent was consistently the local anesthetic of preference for all situations. Of those examined, diperodon may be the most effective in the eye and in the abraded skin—while any of the three may be correctly chosen to abolish pain in the Type I burn. Benzocaine, on the other hand, seemed most effective in the Type II and Type III burns. It was interesting to have noted that Adriani, following studies in the human, also found (11) that anesthetic agents which exhibited considerable potency after topical application to one type of site are not necessarily as active in another site.

DISCUSSION

Attention is directed to the quality of the data presented in Table I. An examination of these had led to the conclusion that (*a*) these are valid observa-

TABLE II—ANESTHETIC POTENCY SCORES (TOTAL "NO RESPONSES" DIVIDED BY THE TOTAL NUMBER OF APPLIED STIMULI PER COMPOUND PER ANALYSIS) AND THEIR RESPECTIVE 95% FIDUCIARY LIMITS

Compd.	Rabbit Eye	Guinea Pig				
		Eye	Abraded Skin	Burns Type I	Burns Type II	Burns Type III
0.125% benzocaine	0.009 (−0.009 to 0.027)	0.111 (0.052 to 0.170)	0.000	0.069 (0.011 to 0.128)	0.000	0.139 (0.059 to 0.219)
0.125% diperodon	0.018 (−0.007 to 0.044)	0.083 (0.031 to 0.135)	0.306 (0.200 to 0.412)	0.139 (0.059 to 0.219)	0.0000	0.0000
0.125% lidocaine	0.000	0.093 (0.038 to 0.147)	0.125 (0.049 to 0.201)	0.042 (−0.004 to 0.088)	0.000	0.000
1.000% benzocaine	0.111 (0.052 to 0.170)	0.315 (0.227 to 0.402)	0.278 (0.174 to 0.318)	0.153 (0.070 to 0.236)	0.417 (0.303 to 0.531)	0.319 (0.212 to 0.427)
1.000% diperodon	0.722 (0.638 to 0.807)	0.491 (0.396 to 0.585)	0.458 (0.343 to 0.573)	0.222 (0.126 to 0.318)	0.083 (0.019 to 0.147)	0.000
1.000% lidocaine	0.407 (0.315 to 0.500)	0.657 (0.568 to 0.747)	0.194 (0.103 to 0.286)	0.083 (0.019 to 0.147)	0.194 (0.103 to 0.286)	0.000
Water control	0.000	0.000	0.000	0.000	0.000	0.000

TABLE III—DATA ILLUSTRATING LEVELS OF CONSISTENCY FOLLOWING REPEATED TESTING OF 1.0% LIDOCAINE IN DERMAL INJURIES

Experiment No.	Skin Abrasion		Type I Burn		Type II Burn		Type III Burn	
	Individual Scores	Potency Value	Individual Scores	Potency Value	Individual Scores	Potency Value	Individual Scores	Potency Value
1	2,1,3,3,2,0	0.153	0,2,1,3,1,1	0.111	1,3,2,2,0,1	0.125	0,0,0,0,0,0	0.000
2	3,3,0,3,1,2	0.166	0,0,1,0,2,0	0.042	4,0,2,2,4,2	0.194	0,0,0,0,0,0	0.000
3	1,5,4,2,1,3	0.222	1,1,0,2,0,0	0.056	3,1,0,2,4,1	0.153	0,0,0,0,0,0	0.000

tions and not the result of chance, and (*b*) the values within groups have consistent variance and therefore are considered compatible.

Of course the potency scores in Table II, which are derived from the data in Table I, differ significantly only when the confidence limits do not overlap.

The importance of reproducibility cannot be too strongly emphasized. An example of the level of consistency which may be expected by use of this procedure has been presented in Table III. Contained therein are the results of repetitive studies of the above described 1.0% lidocaine solution. These results, it will be noted, neither differ significantly from each other nor from those in Tables I and II. Consistency of approximately the same order has been yielded by repeated topical studies of other experimental compounds.

The breadths of the confidence limits, which embraced the respective potency scores, were of concern. It was found, however, that these ranges could be narrowed considerably by merely supplementing the numbers of sites employed. After consideration, it was decided that the initial use of but six sites was sufficient to provide results which satisfied requirements of an initial screen; and that the strengthening process of adding data should probably best be limited to accurate appraisal of the more promising compounds.

With regard to the mechanics of the method, it may be felt that receptors and nerve fibers may have been sufficiently damaged by the burns to possibly inhibit the transmission of impulses. If this were to occur, it may be argued that applied stimuli may fail to elicit responses and that erroneous results as to the potency of the experimental compounds may be yielded.

The fact that measurable decreases in the threshold responses can be demonstrated after injuring the skin certainly refutes this argument. Tissues within and beneath the dermal layers are highly sensitive to the painful influences of a histamine-like H-substance (12) which is liberated following infliction of skin injuries. With increased tissue damage, the source of the pain lies further from the surface and the challenge to the penetrating ability of the anesthetic agent is correspondingly increased. It should furthermore be remembered that the same injury which causes the pain also alters the permeability, thereby providing the means by which the anesthetic may be permitted to penetrate.

It should again be emphasized that it is not the intent here to become involved in such matters as structure-activity relationships, penetration of the dermal barrier, biochemical or pathologic changes in skin following thermal injury, or any of a countless number of other related problems of a fundamental nature.

Instead, a method has been offered which, despite acknowledged minor fluctuations in response, due to variation in the animals as well as slight differences in the severities of the injuries, has repeatedly proven to have been accurate and reproducible; and has permitted the simultaneous evaluation of the spectra of activity of structurally different anesthetic agents.

CONCLUSION

A series of procedures for the *in vivo* testing of topical anesthetic agents on the superficially injured skin of the rodent has been presented. The relative potencies of three known agents were evaluated and the results indicated that (*a*) diperodon was the anesthetic of choice in nullifying pain in the abrasion; (*b*) lidocaine, benzocaine, and diperodon were equally effective in one type of burn; and (*c*) benzocaine exhibited superior activity in two additional types of burns.

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Keyphrases

Anesthetics, topical—*in vivo* evaluation
Burns, controlled infliction—guinea pig back
Abrasions, inflicted—guinea pig back
Eyes, rabbit, guinea pig—anesthesia testing
Stimuli application—anesthesia determination

Effect of Deuteration on the Growth and Antibiotic Production of a Number of Strains of *Penicillium*

By KASHEED MOHAMMED, DANIEL A. NONA, and MARTIN I. BLAKE

Thirteen strains of two species of *Penicillium* were surveyed for their ability to grow and produce antibiotic in a medium of high concentrations of heavy water. Mycelial dry weights, pH variation, and penicillin potency were noted over a 90-day study period. In the deuterio cultures autolysis and penicillin degradation did not occur. Phenylacetic acid was found to stimulate penicillin production in both the protio and deuterio cultures.

THE EFFECTS of heavy water on the growth rate and morphology of *Penicillium notatum* and *Aspergillus fonsecaeus* have been reported by Shaffer *et al.* (1). The production and isolation of antibiotic were not considered in that investigation. More recently Nona *et al.* (2, 3) studied the effects of D_2O on the culturing of *Penicillium janczewskii*. Deuterated griseofulvin was isolated and its properties studied. A brief review of the literature dealing with the effects of deuteration on algae, bacteria, fungi, and higher plants was included. These represent the only studies concerning the effects of D_2O on antibiotic-producing organisms.

In the present study 13 strains of two species of *Penicillium* were surveyed for their ability to grow and produce antibiotic in high concentrations of heavy water.

EXPERIMENTAL

Penicillium Strains—The penicillin-producing strains used in this investigation and the commercial sources of supply are indicated in Table I. The organisms were stored on agar slants at 5° and were reslanted every 3 months.

The agar slants used for maintenance of the molds were prepared in the following manner: Flask 1 contained peptone, 0.6 g.; tryptone, 0.4 g.; yeast extract, 0.3 g.; dextrose, 0.1 g.; beef extract, 0.2 g.; distilled water, 60 ml. Flask 2 contained potassium dihydrogen phosphate, 2.0 g.; malt extract, 2.0 g.; agar, 3.0 g. The pH of Flask 1 was adjusted to 6.7. The contents of Flask 2 were added to Flask 1 and the volume was diluted to 100 ml. with distilled water. The medium was heated to dissolve the agar, and was then dispensed into test tubes in 5-ml. aliquots. The tubes were sterilized and slanted.

Sterilization—A portable electric steroclave (Wisconsin-Aluminum) was used for sterilizing the slants at 15 p.s.i. for 20 min. Sterilization of all protio media was done under similar conditions.

Sterilization of deuterio media was effected in a pressure cooker Presto (model KE03A). Pure deuterium oxide (99.6%) was used as the sterilizing agent in place of water. Sterilization time was 15 min. in all instances.

Protio Nutrient Medium—The culture medium had the following composition in each 100 ml. of solution: sodium nitrate, 0.3 g.; magnesium sulfate, 0.025 g.; potassium dihydrogen phosphate, 0.05 g.; zinc sulfate, 0.005 g.; copper sulfate, 0.0003 g.; ferrous sulfate, 0.005 g.; dextrose, 3.0 g. The pH was adjusted to 6.5 with a 25% potassium hydroxide solution. Finally 0.5 g. of calcium carbonate was added.

For each *Penicillium* strain a series of 10 cultures was prepared in 250-ml. conical flasks. Forty milliliters of the nutrient medium was added to each flask. The flasks were plugged with nonabsorbent cotton, capped with aluminum foil, and sterilized. Protio cultures served as controls.

Deuterio Nutrient Medium—In the deuterio culture medium, 99.6% deuterium oxide was used in place of ordinary water. Otherwise, the composition of the nutrient was the same as that used in the water cultures. All exchangeable hydrogen was previously replaced with deuterium by dissolving weighed quantities of the nutrient mixture in deuterium oxide, and then lyophilizing the solution. This procedure was repeated to ensure complete exchange. The final nutrient solution contained better than 98% deuterium oxide. Ten 40-ml. cultures for each strain were prepared and sterilized as described under *Protio Nutrient Medium* and *Sterilization*, respectively.

Preparation of Seed Inoculum—A concentrated suspension of healthy spores in protio nutrient medium was prepared for each mold according to the formula shown under *Protio Nutrient Medium*, except for the dextrose content which was increased to 9.0 g. Fifty milliliters of the medium was placed in a 250-ml. conical flask and sterilized. One loopful of spores from each stock test tube slant was used for the seed inoculum. Submerged culture shake technique, as described below, was used in preparing the seed inoculum.

Culture Methods—*Shake Cultures*—The inoculum for the submerged cultures consisted of a suspension of tiny pellets, about 1 mm. in diameter obtained from the seed culture. Inoculation for the protio nutrient medium was performed using 5-ml. portions of a heavy suspension of the spores. In the case of the deuterio nutrient medium, the 5-ml.

Received May 22, 1968, from the Department of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612

Accepted for publication August 5, 1968.

Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

This investigation was supported by grant AI 06825-02 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

TABLE I—MYCELIAL DRY WEIGHT AND ANTIBIOTIC PRODUCTION OF *Penicillium* STRAINS GROWN IN AN H_2O AND D_2O ENVIRONMENT

Strain	Culture Method	Mycelial Dry Weight, g./l. H_2O	Mycelial Dry Weight, g./l. D_2O	Antibiotic Production, Units/ml. H_2O	Antibiotic Production, Units/ml. D_2O
PN[a]-35548[c]	Surface	4.80	3.80	8.6	1.1
	Shake	4.00	3.85	7.5	1.4
PC[b]-37767[d]	Surface	4.88	4.11	1.1	1.1
	Shake	5.00	4.80	3.2	0.0
PN-15378[d]	Surface	6.50	6.11	10.0	2.8
	Shake	15.51	10.80	11.1	0.0
PC-167[e]	Surface	20.10	15.89	3.0	2.1
	Shake	30.36	22.88	1.1	3.1
PC-1428[e]	Surface	6.38	6.60	8.1	3.2
	Shake	8.13	6.30	6.1	2.1
PC-1531[e]	Surface	9.88	7.30	8.7	3.8
	Shake	18.91	12.01	8.0	0.0
PN-230[f]	Surface	35.50	18.98	6.6	0.0
	Shake	38.00	23.91	8.0	0.0
PN-284[f]	Surface	8.00	3.82	2.9	0.5
	Shake	8.75	5.72	1.8	0.1
PC-451[f]	Surface	10.01	10.34	10.1	0.0
	Shake	16.00	8.72	8.3	0.0
PN-612[f]	Surface	8.80	9.02	3.9	4.0
	Shake	23.60	16.71	3.0	2.1
PC-1177[f]	Surface	12.00	10.00	4.0	4.0
	Shake	21.66	13.81	1.9	1.2
PN-821[g]	Surface	10.25	3.00	10.0	3.1
	Shake	6.21	4.00	3.2	0.5
PN-1249B21[g]	Surface	5.00	3.75	28.1	0.0
	Shake	8.50	4.89	12.1	0.0

[a] *Penicillium notatum.* [b] *Penicillium chrysogenum.* [c] Centraalbureau Voor Schimmelcultures, Baarn, Nederland. [d] Commonwealth Mycological Institute, Kew, Surrey, England. [e] Imperial Chemical Industries Ltd., Macclesfield, Cheshire, England. [f] Laboratorie de Cryptogamie, Paris, France. [g] Northern Utilization Research & Development Div., Peoria, Ill.

portions of spores were lyophilized before inoculation. The cultures were incubated at 24–25° on an Eberbach rotary shaker at 215 r.p.m., for as long as 3 months in certain instances.

Surface Cultures—Protio and deuterio cultures were inoculated with 5-ml. aliquots of the seed inoculum. However, the water was removed by lyophilizing prior to inoculating the deuterio nutrient medium. Incubation was at 24–25°. Care was taken to ensure that the surface pellicle was not disturbed. It was noted that when the pellicle sank below the surface of the nutrient solution, growth ceased and autolysis followed immediately.

Phenylacetic Acid Additive—In several experiments 0.3% phenylacetic acid was added to the nutrient medium. The pH of the solution was adjusted to 6.5 with potassium hydroxide before sterilization.

Analysis for Penicillin Potency—All cultures were periodically assayed for antibiotic activity. The cylinder-plate method described in USP XVII (4) was employed.

Mycelial Dry Weight—At varying intervals during the experiment, the mycelium from a culture was separated from the fermented broth by vacuum filtration. The mycelium was dried to constant weight in a convection air oven at 70°.

Determination of pH and pD—At intervals throughout the study period the pH of the protio cultures and the pD of the deuterio cultures were determined. A Beckman pH meter (model 76A) was used for both series. In the case of the deuterio cultures the apparent pH was obtained and an appropriate correction (5) to the reading was made.

Deuterium Analysis—At the conclusion of each experimental period, an aliquot of the deuterio culture broth was analyzed spectrophotometrically for deuterium oxide content according to the method of Crespi and Katz (6).

RESULTS AND DISCUSSION

Growth Observations—Growth was evident after 2 days of incubation in all surface and shake protio nutrient cultures. In the surface cultures rapid growth was evidenced by the enlargement of the surface pellicle. In the shake cultures this was demonstrated by the production of numerous globular pellets ranging in size from 2 to about 10 mm. in diameter. This was followed by a progressive thickening of the nutrient medium. In water all organisms sporulated in 5 to 7 days. The cultures appeared tan, light brown, or faint yellow in color.

A somewhat different pattern was noted in the deuterio nutrient cultures. There was no sign of growth after 2 days of incubation for any of the cultures, surface or shake. However, within 10 days after incubation the surface cultures did show definite signs of growth. Clusters of colonies, varying in size from 10 to 25 mm. in diameter, were apparent over the surface of the culture. One strain, PC-37767, produced very tiny brown colonies. The others ranged in color from white to gray to light tan and generally were lighter than the corresponding protio cultures. In general the shake cultures showed better growth than the surface cultures. In 6 days the shake cultures developed a sizable number of pellets though smaller in diameter (1.5 to 6.0 mm.) when compared with those formed in the protio nutrient

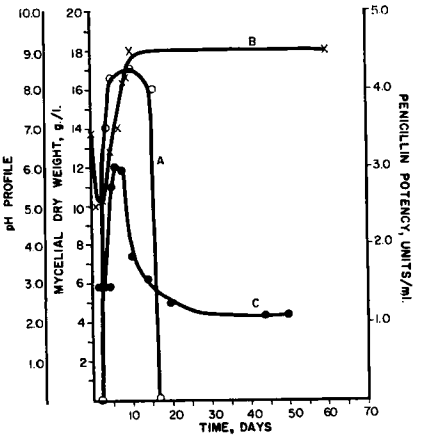

Fig. 1—The penicillin potency, pH profile, and mycelial dry weight of PC-1177 grown in protio medium by surface culture method. Key: A, penicillin potency; B, pH profile; C, mycelial dry weight.

medium. Growth was considerably slower for the 13 strains of *Penicillium* grown in the deuterio nutrient, requiring roughly twice the time to produce the same amount of growth in the deuterio cultures as in the corresponding protio cultures.

Mycelial Dry Weights—Dry weights of the mycelial tissue from the strains of *Penicillium* studied are listed in Table I. The values represent the maximum weights obtained by plotting dry weight *versus* time as typified in Figs. 1 and 2 for one of the strains in the study group. Considerable variation is apparent among the strains in both H_2O and D_2O and there appears to be little correlation between good growth and antibiotic production. Preliminary studies indicated that optimal growth was obtained when corn steep liquor was included in the nutrient medium. However, due to its indefinite composition and since the introduction of hydrogen atoms into a deuterio nutrient medium would preclude the biosynthesis of a fully deuterated antibiotic, this additive was not employed in subsequent studies.

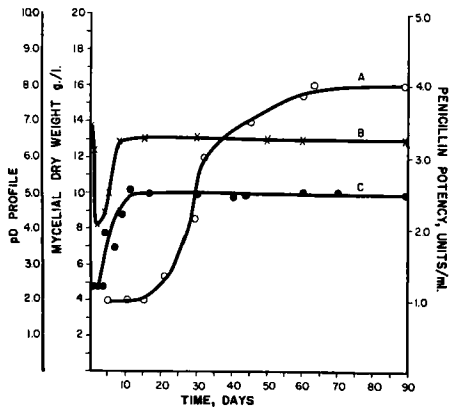

Fig. 2—The penicillin potency, pD profile, and mycelial dry weight of PC-1177 grown in D_2O medium by surface culture method. Key: A, penicillin potency; B, pD profile; C, mycelial dry weight.

Figure 1 shows the change in mycelial dry weight with time for the PC-1177 strain grown in a protio nutrient. There is a rapid increase in tissue formation for about 7 to 10 days followed by a rapid breakdown over the next 6 weeks. A different situation is apparent for the deuterio culture as shown in Fig. 2. There is a rather rapid formation of tissue over a 10-day period with little indication of autolysis in the following 80 days of the growth study period. This may be attributed to the fact that the enzymes responsible for autolysis are inhibited or inactivated in the presence of high concentrations of D_2O. Another contributing factor may be that the more stable carbon-to-deuterium bonds comprising the deuterated tissue may tend to resist autolysis

Penicillin Production—The *Penicillium* strains in this study varied markedly in their ability to produce antibiotic in a protio nutrient medium ranging from a low of 1.10 units/ml. to a high of 28.11 units/ml. of fermented broth. Preliminary studies indicated that much higher production was possible when corn steep liquor was incorporated in the nutrient medium. This was not used in the present study for reasons already noted. In the deuterio nutrient medium penicillin production was severely depressed and in most cases was completely inhibited. The repressive effects of D_2O on the production of alkaloids in belladonna have been reported by Uphaus *et al.* (7). Repression in griseofulvin production by the fungus *P. janczewskii* in the presence of high concentrations of D_2O was observed by Nona *et al.* (2). Nevertheless, in the present investigation two of the strains surveyed, PN-612 and PC-1177, appeared to be less adversely affected by D_2O than were the remaining strains. The penicillin production curves for the PC-1177 strain by the surface culture method in H_2O and D_2O nutrient media are shown in Figs. 1 and 2.

Maximum production in protio medium is found to occur in about 10 days. Peak production is followed by a rapid loss in activity and within a

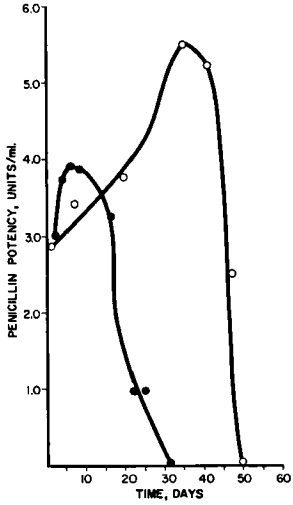

Fig. 3—Effect of adding phenylacetic acid on penicillin production by PN-612 in protio medium. Key: ●, protio medium; ○, 0.3% phenylacetic acid added to protio medium.

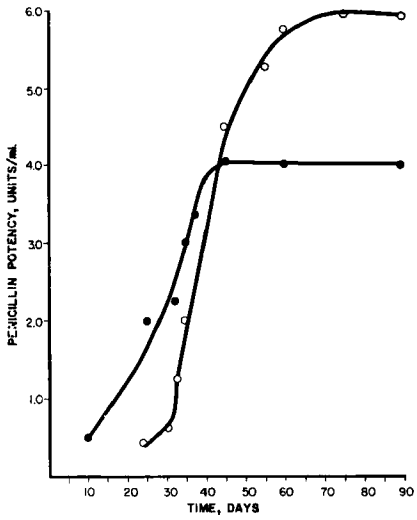

Fig. 4—Effect of adding phenylacetic acid on penicillin production by PN-612 in deuterio medium. Key: ●, deuterio medium; ○, 0.3% phenylacetic acid added to deuterio medium.

few days the penicillin titer reaches zero. In the deuterio cultures, however, the situation is quite different. There is noted a gradual increase in penicillin titer which reaches a maximum in about 60 days. There is no indication of antibiotic degradation over the 90-day study period. In the protio culture penicillin destruction took place after the pH of the medium reached its maximum value of 9.0 (see Fig. 1). Since penicillin is most stable in solutions having a pH range of 6.4 to 7.0 the observed breakdown and disappearance of penicillin is expected. In alkaline conditions the β-lactam ring of the penicillin molecule is broken producing a biologically inactive product.

In the deuterio culture, Fig. 2, the pD values remained below 7 throughout the study period. This fact apparently explains why the penicillin was not destroyed.

Effect of Phenylacetic Acid Additive—The effect of adding phenylacetic acid to the extent of 0.3% in the protio and deuterio nutrient media is illustrated in Figs. 3 and 4. Phenylacetic acid has been shown (8) to be a precursor in the biosynthesis of benzyl penicillin and to be useful in stimulating antibiotic production by fungi. This effect is clearly demonstrated in the protio culture (Fig. 3) and in the deuterio culture (Fig. 4) where the response to the phenylacetic acid additive is compared with the corresponding controls. It should also be noted that in the deuterio cultures degradation of the antibiotic did not occur after a peak in production as in the case of the protio culture.

At the conclusion of each fermentation period an aliquot of the culture medium was analyzed for D_2O content. In the series reported here all of the cultures showed a D_2O concentration above 87% and most were over 90%. Dilution of the deuterium content is accounted for by the 3% protio-dextrose in the nutrient and by exchange over the 3-month study period with atmospheric moisture. The opening of the culture flasks was plugged with cotton which may have permitted moisture exchange to a small extent.

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Keyphrases

Penicillium species—deuterium effect
Deuterium effect—*Penicillium* growth, antibiotic production
Protio nutrient media—*Penicillium* cultures
Phenylacetic acid effect—*Penicillium* cultures

Ultraviolet Spectrophotometric Determination of Phenylbutazone in Biologic Specimens

By JACK E. WALLACE

A rapid spectrophotometric method for determining phenylbutazone in biologic specimens in the presence of its metabolites and other acid-extractable drugs is described. Extensive separation and extraction of the drug is not required. Alkaline permanganate oxidation of phenylbutazone to azobenzene is the basis for the analytical technique. The strong absorption of azobenzene at 314 mμ makes for a high order of specificity. The method is sufficiently sensitive for the determination of phenylbutazone in blood of patients taking therapeutic amounts of the drug. Data showing the distribution of the drug in the rat are presented.

PHENYLBUTAZONE[1] IS A DRUG which has achieved certain measures of success in the treatment of rheumatoid disorders and other inflammatory conditions. The patients should be under medical care during treatment with the drug for a number of side reactions may occur. The most frequent side effects are various dyspeptic disorders, nausea, and drug rash. In addition, agranulocytosis is a rare but most feared complication. Phenylbutazone is rapidly absorbed from the gastrointestinal tract for it has been observed that 2 hr. after a single dose 80–90% of the drug can be recovered in the blood (1), and only negligible amounts are excreted in the urine. Therefore, it is desirable during treatment with phenylbutazone to obtain blood levels as an aid to the control of drug toxicity.

Several authors have reported blood concentrations of phenylbutazone in humans after dosage with the drug. Pulver (2) first estimated phenylbutazone in serum by hydrolyzing the substance to hydrazobenzene which subsequently rearranged to benzidine. This latter substance was diazotized and measured colorimetrically. Moss (3) and Tophøj (4) independently modified Pulver's original method and obtained serum concentrations after therapeutic dosing. The colorimetric procedures all require 4–6 hr. of supervised hydrolysis and exhibit a low order of specificity, sensitivity, and reproducibility. Burns *et al.* (5) described a direct UV spectrophotometric procedure which determines phenylbutazone at 265 mμ, the absorption maximum for the compound in aqueous alkali. This procedure is sensitive but it affords no specificity since many acidic drugs particularly barbiturates absorb strongly in this region of the spectrum. Certain normal tissue constituents also absorb in the 250–270 mμ region and if present in the final extract contribute to significant absorption at 265 mμ. Further, published spectrophotometric methods for analysis of phenylbutazone are not adequate for use in medical-legal investigations. Phenylbutazone is widely used and the need for a specific, rapid quantitative method for its determination in blood and tissues is evident.

The method described in this report is rapid, sensitive, and highly specific for phenylbutazone and its thio derivative, sulfinpyrazone. No other acidic extractable drug including the hydroxy metabolite of phenylbutazone interferes. In the determination, alkaline permanganate oxidation at controlled pH converts phenylbutazone to azobenzene which is extracted into refluxing *n*-heptane for final spectrophotometric assay.

EXPERIMENTAL

Instrumentation—Ultraviolet absorption measurements were performed by using a ratio recording spectrophotometer (Beckman DK-2A) equipped with linear wavelength presentation. The sample path was 1 cm. throughout. A double-beam IR spectrophotometer (Beckman IR-4) was used for IR spectral characterization of the reaction product. Elemental carbon, hydrogen, and nitrogen analyses were performed (F & M model 185 CHN analyzer).

Method—Five to ten milliliter or gram amounts of whole blood, urine, gastric contents, or homogenized tissue are adjusted to pH 1 to 2 with 1 *N* hydrochloric acid. The mixture is extracted by vigorous shaking with 200 ml. of *n*-heptane. If an emulsion occurs the hydrocarbon can be recovered by centrifugation. After separation the *n*-heptane is removed from the acidified solution and is subsequently extracted with 5 ml. of 0.5 *N* sodium hydroxide. Four milliliters of the aqueous layer is transferred to a 250-ml. round-bottom flask to which is added 20 ml. of 1% potassium permanganate in a 0.4 *M* sodium triphosphate–0.4 *M* sodium hydroxide solution (pH 12.4), 10 ml. of *n*-heptane (spectrophotometric grade), and a Teflon-coated magnetic stirring bar. The contents of the flask are refluxed

Received March 28, 1968, from the Forensic Toxicology Branch, USAF Epidemiological Laboratory, Lackland Air Force Base, TX 78236

Accepted for publication August 21, 1968.

The author gratefully acknowledges the technical assistance of John D. Biggs.

[1] Marketed as Butazolidin by Geigy Pharmaceuticals, Ardsley, N. Y.

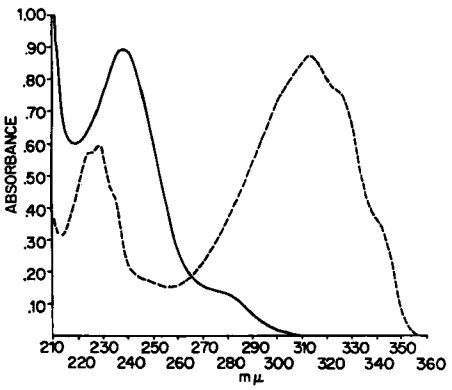

Fig. 1—UV absorption spectra of phenylbutazone and of the phenylbutazone product both corresponding to a concentration of 15 mcg./ml. in n-*heptane. Key: ——, phenylbutazone; ---, phenylbutazone reaction product.*

for 30 min. with constant stirring. After the flask has cooled, the *n*-heptane is separated from the aqueous permanganate, washed with 5 ml. of 0.5 *N* HCl, and scanned on a spectrophotometer from 260–380 mμ against an *n*-heptane blank. If absorption at a single wavelength is desired, it may be determined at 314 mμ. If large or trace amounts of phenylbutazone are suspected in the original specimen, the amount of *n*-heptane in the oxidation step may be varied from 5 to 50 ml. with no adverse effect to the efficiency of the method. A standard curve is prepared from aqueous solutions of the drug which have been carried through the procedure as described above.

RESULTS AND DISCUSSION

The UV absorption curve of the product of phenylbutazone oxidation is well defined. It exhibits an absorption maximum at 314 mμ and a minimum at 257 mμ. The absorbance at 314 mμ, illustrated in Fig. 1, provides the increase in specificity of the present method over that of techniques which rely on colorimetry or direct spectrophotometric analysis of unconverted phenylbutazone. Sulfinpyrazone yields a product, which has UV absorption identical to that of the product from phenylbutazone, except that the curve for the sulfoxide product provides a lower absorbance per unit concentration of original drug. Inability of the method to distinguish between phenylbutazone and its sulfoxide derivative is considered to be no serious problem since the medical use and pharmacologic effect of the two drugs are very similar. The reaction product has a linear absorbance relationship, at the levels investigated, to the concentration of drug in the original sample (Table I). The recovery of phenylbutazone which had been added in known amounts to whole blood and urine is summarized in Table II. Average recovery from blood was approximately 88% and from urine 91%.

Other drugs, which might interfere with the determination of phenylbutazone and do so with other analytical techniques, were investigated for interference. Each substance in an aqueous solution was added to permanganate and *n*-heptane. With the exception of phenylbutazone and sulfinpyrazone,

TABLE I—STANDARD CURVE DATA OF PHENYLBUTAZONE REACTION PRODUCT

Phenylbutazone in Sample, mcg./ml.	Absorbance in *n*-heptane	Absorbance/ Concn.
20	1.157	0.058
16	0.923	0.058
12	0.691	0.058
8	0.459	0.057
4	0.229	0.057

TABLE II—RECOVERY STUDIES OF PHENYLBUTAZONE

Phenylbutazone Added, mcg./ml.	No. of Detn.	Recovery, Mean ± *SD*, mcg./ml. Whole Blood	Urine
100.0	6	98.7 ± 0.6	91.2 ± 0.7
50.0	7	44.8 ± 0.3	45.9 ± 0.4
25.0	8	23.5 ± 0.3	23.7 ± 0.2
10.0	10	8.4 ± 0.2	8.9 ± 0.2
5.0	7	4.2 ± 0.1	4.3 ± 0.1
Av. recovery		88.3%	90.6%

TABLE III—COMPOUNDS INVESTIGATED FOR INTERFERENCE WITH THE DETERMINATION OF PHENYLBUTAZONE[a]

Compd.	Absorbance of Product[b]
Phenylbutazone	1.157
Sulfinpyrazone	0.868
Blank	0.000
Acetophenetidin	0.000
Bishydroxycoumarin	0.002
Caffeine	0.000
Diphenylhydantoin	0.000
Methyprylon	0.000
Oxyphenbutazone	0.000
Phenaglycodol	0.000
Phenobarbital	0.000
Phenol	0.000
Phensuximide	0.000
Picrotoxin	0.000
Salicylamide	0.002
Salicylate	0.000
Secobarbital	0.000
Sulfadiazine	0.000
Theobromine	0.000
Theophylline	0.000
Trimethobenzamide	0.000

[a] Each compound was added directly to the reaction flask. The level corresponds to a concentration of 20 mcg./ml. of the compound per ml. of *n*-heptane. Each value is the average of three determinations. [b] Read at 314 mμ.

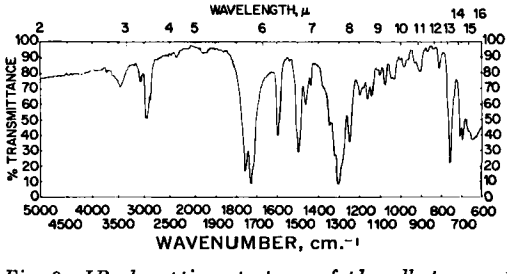

Fig. 2—IR absorption spectrum of phenylbutazone, 1 mg./400 mg. of potassium bromide.

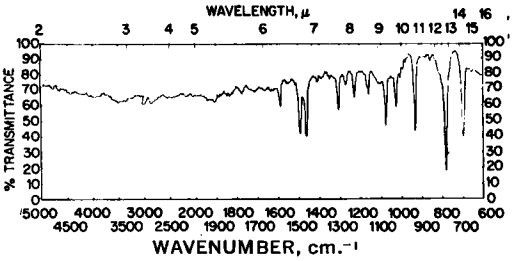

Fig. 3—IR absorption spectrum of phenylbutazone reaction product, 1 mg./400 mg. of potassium bromide.

no compound produced significant absorbance at 314 mμ (Table III).

Oxyphenbutazone, 1-(*p*-hydroxyphenyl)-2-phenyl-4-butyl-3,5-pyrazolidinedione, a primary metabolite of phenylbutazone (6) and itself a therapeutic agent does not interfere. Barbiturates, which make direct UV determination of phenylbutazone impracticable, if present, afford no interference in the method of this report.

The IR absorption spectra of phenylbutazone (Fig. 2) and its reaction product (Fig. 3) show several distinct differences. Strong carbonyl absorption bands which exist at 1598 cm.$^{-1}$ and 1720–1760 cm.$^{-1}$ in the spectrum of the drug are absent in the spectrum of the product. These bands are probably due to amide stretching vibrations. Further, methylene carbon absorption at 2950 cm.$^{-1}$, though present in the spectrum of phenylbutazone, is absent in that of the product. These observations suggest that the pyrazolidinedione

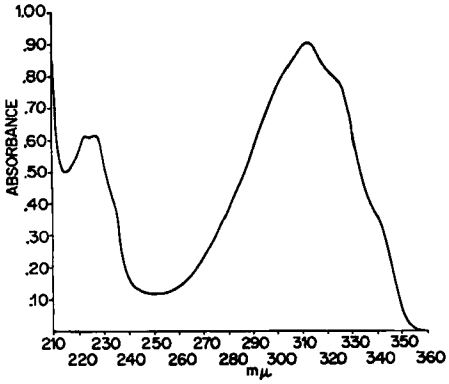

Fig. 4—UV absorption spectrum of azobenzene, 7.5 mcg./ml. in n-heptane.

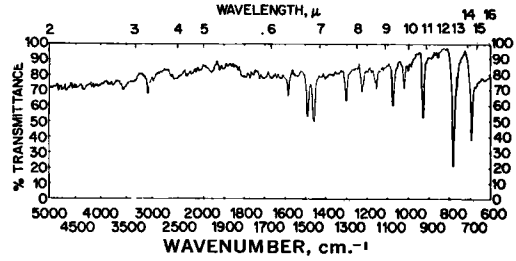

Fig. 5—IR absorption spectrum of azobenzene, 1 mg./400 mg. potassium bromide.

TABLE IV—PHENYLBUTAZONE LEVELS IN RAT

Tissue or Biologic Fluid	Concn. in Specimen, mcg./g. (ml.)[a] Male[b]	Female[b]
Blood	32.2	63.2
Brain	5.3	8.8
Fat	18.4	34.6
Kidney	37.9	52.3
Liver	54.5	77.4
Muscle	8.0	14.3

[a] Specimens were obtained 6 hr. after rats were fed by mouth 50 mg./kg. of body weight. Rats were fasted for 16 hr. prior to ingestion. [b] Average of results from ten rats.

ring is destroyed during permanganate oxidation. The absorption bands at 692 cm.$^{-1}$ and 775 cm.$^{-1}$ in the spectra of the product indicate no loss of the aromatic rings during the oxidation process. Randall *et al.* (7) have reported the occurrence of absorption at 700 cm.$^{-1}$ ± 10 cm.$^{-1}$ in numerous monosubstituted aromatic compounds and this band range is accepted as being specific for the monosubstituted phenyl group.

Ultraviolet (Fig. 4) and infrared (Fig. 5) spectra of azobenzene were identical to the spectra of the phenylbutazone product. The weak intensity of the —*N*=*N*— band, and the knowledge that it arises in the 1600 cm.$^{-1}$ region along with aromatic absorptions precludes in this study any useful structural correlations from this functional group. Carbon, hydrogen, and nitrogen data demonstrate the product compound to be azobenzene. By comparison of the UV spectra of the two compounds, it is apparent that approximately 82% of the phenylbutazone is converted to azobenzene in the analysis.

The distribution of the drug among various tissues of the rat 6 hr. after the animals had received 50 mg./kg. of body weight is shown in Tables IV and V. The levels of drug in various biologic fluids and organs in decreasing order are liver, kidney, whole blood, fat, muscle, and brain.

It is of interest that tissues from female rats consistently exhibit significantly higher levels of the drug than corresponding specimens from male rats. A similar observation was made for serum levels in rats by Kampmann and Frey (8). A faster and more efficient absorption rate from the gut in females is indicated yet hormonal influences may contribute to this difference. If this effect were observed in humans, it could account for the alleged greater toxicity of phenylbutazone in the female of this species (9).

TABLE V—PHENYLBUTAZONE DISTRIBUTION IN THE RAT

Tissue	Distribution Ratio[a], Mean ± *SD* Concn. in Tissue/Concn. in Blood Male[b]	Female[b]
Blood	1.0	1.0
Brain	0.15 ± 0.02	0.16 ± 0.03
Fat	0.57 ± 0.09	0.58 ± 0.15
Kidney	0.85 ± 0.12	1.33 ± 0.15
Liver	1.25 ± 0.15	1.74 ± 0.14
Muscle	0.24 ± 0.02	0.28 ± 0.04

[a] Specimens were obtained 6 hr. after albino rats were fed by mouth 50 mg./kg. of body weight. Rats were fasted for 16 hr. prior to ingestion of drug. [b] Average of results from ten rats.

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Keyphrases

Phenylbutazone—analysis
Biologic specimens—phenylbutazone analysis
Sulfinpyrazone—analysis interference
IR spectrophotometry—identity
UV spectrophotometry—analysis

Synthesis of Some Iodopurine Derivatives

By R. T. KODA, J. A. BILES, and W. WOLF

Several new 6- and 8-iodopurine derivatives have been prepared. It was found that a nucleophilic displacement of a chloro- or mercapto- function by iodide ion was most successful for the introduction of iodine into the purine ring. The resultant iodopurine derivatives were also screened against the lymphoid leukemia L-1210 test system for biological activity.

RECENT STUDIES (1) indicate that the antitumor activity of purine and pyrimidine derivatives is associated with (*a*) their incorporation into DNA or RNA, thereby altering or terminating normal replication; (*b*) the blocking of the incorporation of normal nucleic acid bases into the structure of DNA or RNA; or (*c*) inhibition of the *de novo* synthesis of naturally occurring purine or pyrimidine bases.

Irrespective of the mechanism by which they act, many halogen and mercapto derivatives of purine and pyrimidine have been shown to possess antitumor activity (2–5). This activity has been generally correlated to both steric and electronic effects contributed by the substituent group.

An iodine substitution on various purine derivatives may exert both a steric effect, because of its large molecular diameter (van der Waals radius = 2.15 Å.), or an inductive effect, due to its electronegativity.

The synthesis of a number of iodopurine derivatives was attempted with an iodine atom residing in the three available positions—C-2, C-6, and C-8. The latter position is not normally involved in intermolecular hydrogen bonding in the polydeoxynucleotide strands but would contribute mainly by its inductive effect on the electron distribution in the purine ring. Iodo substitutions at both C-2 and C-6 are in close proximity to those regions on the purine ring at which intermolecular hydrogen bonding normally occurs and may exert both steric and inductive effects by a purine base which has been incorporated into a polydeoxynucleotide strand.

DISCUSSION

Previous synthetic routes for the preparation of iodopurine derivatives involved either the direct iodination of the purine ring (6–9) with various electrophilic agents or nucleophilic displacement reactions (10–16) of functional groups such as mercapto, alkylmercapto, or halogen by iodide ion. Since direct iodination procedures have proved to be only partially successful, the displacement of a mercapto function on various purine derivatives by iodide ion was attempted. The synthesis of mercaptopurines was usually carried out by the displacement of a hydroxy function at C-6 or C-8 by phosphorus pentasulfide in pyridine (10, 17–20) or

Received May 22, 1968, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Southern California, Los Angeles, CA 90007
Accepted for publication August 22, 1968.
Abstracted from a portion of the dissertation submitted by R. T. Koda to the Graduate School, University of Southern California, in partial fulfillment of Doctor of Philosophy degree requirements.
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The synthesis of a number of iodopurine derivatives was attempted with an iodine atom residing in the three available positions—C-2, C-6, and C-8. The latter position is not normally involved in intermolecular hydrogen bonding in the polydeoxynucleotide strands but would contribute mainly by its inductive effect on the electron distribution in the purine ring. Iodo substitutions at both C-2 and C-6 are in close proximity to those regions on the purine ring at which intermolecular hydrogen bonding normally occurs and may exert both steric and inductive effects by a purine base which has been incorporated into a polydeoxynucleotide strand.

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TABLE I—MERCAPTOPURINE DERIVATIVES

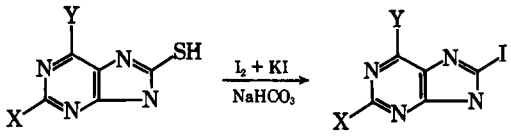
R_1	R_2	R_3	Method of Preparation[a]	Reference
NH_2	OH	SH	Fusion	—
H	NH_2	SH	Fusion	(21)
NH_2	H	SH	Fusion	(31)
H	OH	SH	Fusion	(22)
OH	H	SH	Fusion	(32, 33)
OH	NH_2	SH	Fusion	(30)
NH_2	SH	H	Displacement	(10)
H	SH	SH	Fusion	—
SH	NH_2	SH	Fusion	—
SH	NH_2	H	[b]	—
SH	OH	H	[b]	—

[a] Fusion indicates a condensation between thiourea and 4,5-diaminopyrimidine derivatives to yield the corresponding 8 mercaptopurine. Displacement indicates the substitution of a hydroxy function at C-6 and C-8 on purine derivatives using phosphorus pentasulfide in pyridine to yield the mercaptopurine. [b] Cyclo Chemical Corp.

the fusion of 4,5-diaminopyrimidine derivatives with thiourea (21, 22, 30–33) to yield the corresponding 8-mercaptopurine derivatives (Table I).

The displacement of an 8-mercapto function on purine nucleosides by iodide ion has previously been reported as successful (12). However, a corresponding study of the displacement of an 8-mercapto function on purine bases has not been previously reported.

As an example, when 6-amino-8-mercaptopurine (21) (8-mercaptoadenine) was treated with a mixture of iodine, potassium iodide, and sodium bicarbonate in aqueous solution, a good yield of 8-iodoadenine (I) was isolated. This reaction was subsequently found to be general for all 8-mercaptopurine derivatives having a single additional functional group (amino or hydroxy) at C-2 or C-6 (Scheme I) but not for those which were disubsti-



X = H when Y = NH_2 or OH
Y = H when X = NH_2 or OH

Scheme I

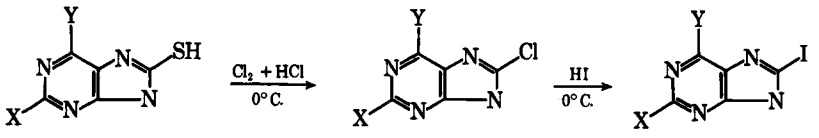
tuted in the pyrimidine ring as discussed below. Thus, the synthesis of 2-amino-8-iodopurine (II), 6-hydroxy-8-iodopurine (III), 2-hydroxy-8-iodopurine (IV), in addition to 8-iodoadenine (I), was attained in good yield using this procedure.

An alternate method for the synthesis of 8-iodopurine derivatives involved the initial displacement of the 8-mercapto function by chlorine, using a mixture of chlorine gas and hydrochloric acid at 0°, followed by the displacement of the 8-chloro function by treatment with hydriodic acid at 0° (Scheme II). The UV and IR spectra of the iodopurine product obtained by either synthetic route were identical.

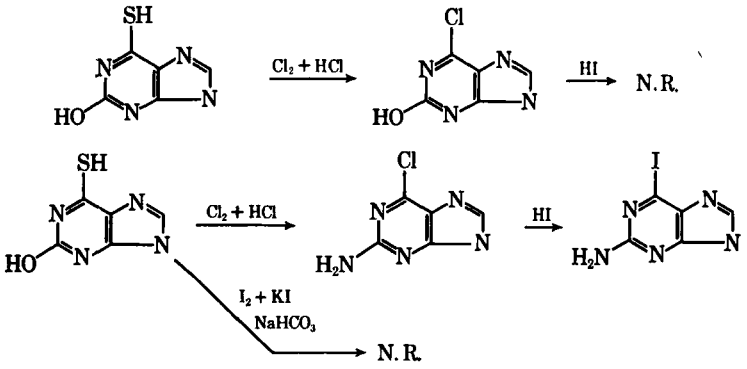
It is surprising that either displacement reaction described above did not operate on more heavily substituted purine derivatives. Attempts to selectively displace one of the mercapto functions on 6,8-dimercaptopurine (22) by iodide ion were not successful, and attempts to displace an 8-mercapto function by iodide ion on purine derivatives having simultaneous substitutions at C-2 and C-6 presented some unusual problems. While treatment of 6-amino-2-hydroxy-8-mercaptopurine (8-mercaptoisoguanine) with a mixture of iodine, potassium iodide, and sodium bicarbonate in water at room temperature resulted in the displacement of the 8-mercapto function to yield 8-iodoisoguanine (V), under similar reaction conditions, 8-mercaptoguanine or 2,8-dimercaptoadenine did not yield the corresponding 8-iodo derivatives.

It is possible that the presence of a hydroxy or mercapto function at C-2 and/or C-6, in addition to an amino substitution, increased the electron density at C-8 so that a stabilized carbonium ion intermediate cannot exist, making the usual displacement of the 8-mercapto function by iodide ion unsuccessful. It also has been reported that the thione tautomeric form at C-8 predominates in neutral or weakly acidic conditions (23).

The synthesis of several 6-iodopurine derivatives has previously been reported (24–28). The method for their synthesis generally involved a displacement of a 6-chloro function with hydriodic acid. However, attempts to extend this procedure to the displacement of a 6-mercapto function did not succeed. Thus, the authors were unable to displace the mercapto group on 2-amino-6-mercaptopurine (thioguanine) by iodide ion. However, 2-amino-6-iodopurine (VI) was successfully obtained by initially converting thioguanine to its 6-chloro derivative (using chlorine gas and hydrochloric acid), then treating the chloropurine derivative with hydriodic acid as shown in Scheme III.



Scheme II



Scheme III

A similar attempt to synthesize 2-hydroxy-6-iodopurine from 2-hydroxy-6-mercaptopurine did not succeed. Treatment of 2-hydroxy-6-mercaptopurine with a mixture of hydrochloric acid and chlorine gas gave a chlorinated product which when treated directly with hydriodic acid failed to yield the expected iodinated purine derivative. One may rationalize again that a hydroxy group at C-2 has increased the electron density at C-6 to such an extent that displacement cannot occur due to the lack of stabilization of the required carbonium ion intermediate.

The most serious problem encountered was the attempt to introduce an iodine at C-2. The authors have not been successful in this attempt. The 2-mercapto function of 6-amino-2-mercaptopurine and 6-hydroxy-2-mercaptopurine could not be displaced under any conditions used. This failure to displace a mercapto function at C-2 is in agreement with similar results obtained by Robins (15) who reported that treatment of 2-methylthiopurines with chlorine yielded only the 2-methylsulfonyl derivatives, while a similar reaction on 6- and 8-methylthiopurines resulted in a facile displacement reaction. This inability to displace a substituent at C-2 as opposed to the uncomplicated displacement at C-8, where both carbons appear to be equivalent (—N=CH—N) may be explained by the substantial difference that exists in the chemistry of the imidazole and pyrimidine ring systems.

It has been reported (6, 7) that several purine nucleosides—guanosine, xanthosine, and 2′-deoxyguanosine—could be directly iodinated with *N*-iodosuccinimide in dimethylsulfoxide using catalytic amounts of *n*-butyl disulfide. However, attempts to directly iodinate adenine and guanine using similar procedures to the above failed to yield any product other than the starting material. Further studies of the direct iodination of adenine or guanine with iodine monochloride in either glacial acetic acid or dimethylformamide, or with mixtures of iodine and nitric acid or iodine-iodic acid in glacial acetic acid also failed to lead to the iodinated derivatives.

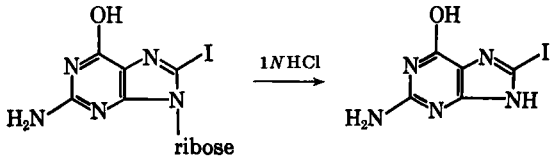
However, treatment of adenine with iodine monochloride in a mixture of *N*-ethylacetamide and dry carbon tetrachloride gave a product containing iodine whose UV and IR spectra varied considerably from that of the original material. Elemental analyses for carbon, hydrogen, and iodine did not agree with calculated values for iodoadenine. Favorable results were not achieved even after recrystallization from water. As it was observed that one of the characteristics of the substance was its ability to react with starch-iodide paper, it is likely that the product obtained was the *N*-iodo derivative of adenine at N-9. This would account for its oxidizing properties and may also explain the difficulty encountered in the purification of the product due to the relative instability of the *N*-iodo function.

As neither nucleophilic displacement or electrophilic substitution methods has allowed the synthesis of 8-iodoguanine (VII), this was finally accomplished by the hydrolysis of the iodinated nucleoside 8-iodoguanosine (6), under acidic conditions to yield the iodinated purine base (Scheme IV).

A summary of the results of the synthesis of iodopurines is shown in Table II.

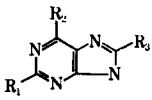
BIOLOGICAL EVALUATION OF IODOPURINES

The biological evaluation of the iodopurine derivatives was conducted under the auspices of the Cancer Chemotherapy National Service Center, National Cancer Institute, according to previously described protocols (29). The results of this study are shown in Table III. The results indicate that none of the compounds tested possessed any significant biological activity against the L-1210 lymphoid leukemia test system. All of the compounds, with the exception of 8-iodohypoxanthine (III) and 8-iodoisoguanine (V), possessed toxicity at elevated dosages. When the compounds were submitted for



Scheme IV

TABLE II—IODOPURINE DERIVATIVES



R_1	R_2	R_3	Calcd.	Found	pH	$\lambda_{max.}$	$\epsilon \times 10^{-3}$
NH_2	OH	I	C, 19.2 H, 1.59	C, 19.0 H, 1.83	1.3 11.9	275 279	8.60 7.37
OH	NH_2	I	C, 17.44 H, 2.03	C, 17.43 H, 2.39	1.0 14.0	299 296	15.2 13.3
H	NH_2	I	C, 19.6 H, 1.96 N, 22.5	C, 19.7 H, 2.3 N, 22.4	1.0 11.9	272 274	17.3 15.4
NH_2	H	I	C, 19.4 H, 1.61	C, 19.5 H, 2.14	1.0 12.85	222 309	30.0 9.44
H	OH	I	C, 22.9 H, 1.15	C, 22.85 H, 1.6	1.0 12.85	259 267	14.4 14.1
OH	H	I	C, 20.8 H, 2.08	C, 20.87 H, 2.21	1.0 12.85	306 223 299	7.18 17.95 6.77
NH_2	I	H	C, 23.0 H, 1.53	C, 23.2 H, 1.84	1.0 12.85	319 213 313 224	7.55 23.0 6.80 22.90
H	NH_2	H 9-iodo	C, 20.2 H, 2.69 I, 42.7 N, 23.6	C, 20.0 H, 2.68 I, 33.25 C, 18.69 H, 2.69 I, 38.56 N, 19.75			

evaluation, it was found that 2-amino-6-iodopurine had previously been submitted for testing under NSC No. 45254. The results showed that it also did not possess any significant antitumor activity against the L-1210 tumor test system.

Although the iodopurine derivatives did not show any significant biological activity against the L-1210 tumor test system, it is suggested that further evaluation against other tumor test systems be made in order to determine the complete antitumor spectrum for this group of compounds.

EXPERIMENTAL

Melting points were determined on a Fisher-Johns melting point apparatus and are given uncorrected. Microanalyses were conducted by Elek Microanalytical Laboratory, Torrance, Calif. All compounds were dried over phosphorus pentoxide at 2 mm. Hg and 100° for at least 12 hr. IR spectra were run as mineral oil mulls between sodium chloride windows on a spectrophotometer (Perkin Elmer 137 Infracord). UV spectra were run on a spectrophotometer (Beckman DB) using water as a solvent.

Preparation of 8-Iodoadenine Hydrochloride (I)—To a suspension of 0.160 g. (0.96 mmole) of 6-amino-8-mercaptopurine (23) and 0.90 g. of sodium bicarbonate in 5.0 ml. of water was added, over a 30-min. period, a solution of 0.90 g. of iodine and 0.60 g. (3.75 mmoles) of potassium iodide in 2.0 ml. of water. After the addition was completed, the mixture was allowed to stir for an additional 18 hr. at room temperature and filtered. The precipitate was washed with 5.0 ml. of 10% potassium iodide solution and finally with water. The yellow product was recrystallized from 5% hydrochloric acid to yield 0.120 g. (75%) of 6-amino-8-iodopurine hydrochloride (I), which darkened above 250°.

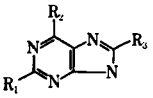
Anal.—Calcd. for $C_5H_5ClIN_5 \cdot 0.5H_2O$: C, 19.6; H, 1.96; N, 22.4. Found: C, 19.7; H, 2.3; N, 22.4. UV data: pH 1, $\lambda_{max.}$ 272 mμ (ϵ = 17,300); pH 7, $\lambda_{max.}$ 271 mμ (ϵ = 14,900); 215 mμ (ϵ = 20,300); pH 11.9, $\lambda_{max.}$ 274 mμ (ϵ = 15,400).

2-Amino-8-iodopurine Sulfate (II)—*Method A*—To a suspension of 0.165 g. (0.99 mmoles) of 2-amino-8-mercaptopurine (31) and 0.90 g. (3.75 mmoles) of sodium bicarbonate was added a solution of 0.90 g. of iodine and 0.60 g. potassium iodide in 2.0 ml. of water. The mixture was stirred for 18 hr. at room temperature and filtered. The precipitate was washed with water and recrystallized from 1 *N* sulfuric acid to yield 0.110 g. (36%) of 2-amino-8-iodopurine sulfate (II) which gradually decomposed above 160° when heated on a melting point block.

Anal.—Calcd. for $C_{10}H_{10}I_2N_{10}O_4S$: C, 19.4; H, 1.61. Found: C, 19.5: H, 2.14. UV data: pH 1, $\lambda_{max.}$ 222 mμ (ϵ = 30,000); pH 6.65 $\lambda_{max.}$ 312 mμ (ϵ = 9,000); $\lambda_{max.}$ 220 mμ (ϵ = 22,300); pH 12.85, $\lambda_{max.}$ 309 mμ (ϵ = 9,440).

Method B—To a mixture of 0.5 ml. of absolute methanol and 1.5 ml. of concentrated hydrochloric acid, previously cooled to −10°, was added 0.25 g. (1.5 mmoles) of 2-amino-8-mercaptopurine (31). Chlorine gas was passed into the mixture for 30 min., never allowing the temperature to rise above 0°. At the end of the reaction period, a white solid separated. The reaction mixture was neutralized with ammonium hydroxide, filtered, and washed with water. The white precipitate, without further purification, was added to 3.0 ml. of hydriodic acid (57%) which had been previously cooled to −10°. The suspension was stirred for 1.5 hr. at 0° then filtered. The precipitate was washed with water

TABLE III—ANTITUMOR ACTIVITY OF SOME IODOPURINE DERIVATIVES AGAINST LYMPHOID LEUKEMIA L-1210



R_1	R_2	R_3	Dose, mg./kg.	Survivors	Animal Weight Diff., T/C	Survival, Days—Test	Survival, Days—Control	% T/C
H	NH_2	I	400	0/4				
			200	4/4	−2.1	9.3	9.4	98
			100	4/4	−2.4	8.8	9.4	93
NH_2	H	I	400	0/4				
			200	0/4				
			100	0/4				
			80	0/6				
			40	0/6				
			20	3/6	−1.2	8.0	8.3	
			10	3/6	0.0	8.7	8.3	
			5	6/6	0.6	8.2	8.3	98
			5	6/6	−0.7	8.7	9.1	95
H	OH	I	400	4/4	−0.7	9.3	9.4	98
			200	4/4	1.0	9.3	9.4	98
			100	4/4	1.4	9.8	9.4	104
NH_2	OH	I	400	1/4	−4.3	10.0	9.4	
			200	1/4	−4.9	9.0	9.4	
			100	0/4				
			80	0/6				
			40	5/6	−0.9	8.6	8.3	103
			20	6/6	0.4	8.2	8.3	98
			20	6/6	−0.7	9.0	9.1	98
			10	6/6	0.1	8.5	8.3	102
			5	6/6	0.1	8.2	8.3	98
OH	H	I	400	2/6	−0.6	9.0	8.6	
			200	0/6				
			100	4/6	−0.1	8.3	8.6	96
			50	6/6	−0.2	9.8	10.1	97
OH	NH_2	I	400	5/6	−1.9	9.8	8.6	113
			200	5/6	−4.3	8.6	8.6	100
			100	5/6	−0.3	8.4	8.6	97
NH_2	I	H	112[a]	7/7	0.0	8.7	9.0	96

[a] Previously evaluated under NSC No. 45254, Oct. 14, 1962.

and recrystallized from 1 *N* sulfuric acid to yield 0.24 g. (51.5%) of 2-amino-8-iodopurine sulfate (II). UV and IR spectral evidence indicated that the compound was identical to that prepared by Method A.

6-Hydroxy-8-iodopurine (III)—To a suspension of 0.168 g. (1 mmole) of 6-hydroxy-8-mercaptopurine (24) and 0.90 g. of sodium bicarbonate in 5.0 ml. of water was added, over a 30-min. period, a solution of 0.60 g. of iodine and 0.60 g. (3.75 mmoles) of potassium iodide in 2.0 ml. of water. Stirring was continued for an additional 18 hr. at room temperature. The mixture was acidified to pH 5 with glacial acetic acid then filtered to yield a light yellow precipitate which was washed with 5.0 ml. of 10% potassium iodide solution, then with water. Recrystallization from water gave 0.150 g. (57%) of 6-hydroxy-8-iodopurine (III) which gradually darkened above 245°.

Anal.—Calcd. for $C_5H_3IN_4O \cdot 0.5H_2O$: C, 22.9; H, 1.15. Found: C, 22.85; H, 1.6. UV data: pH 1, $\lambda_{max.}$ 259 mμ (ϵ = 14,400); pH 6.65 $\lambda_{max.}$ 263 mμ, (ϵ = 13,800); pH 12.85, $\lambda_{max.}$ 267 mμ (ϵ = 14,100).

2-Hydroxy-8-iodopurine (IV)—To a suspension of 0.165 g. (1 mmole) of 2-hydroxy-8-mercaptopurine (32, 33) and 0.90 g. of sodium bicarbonate in 5.0 ml. of water was added a solution containing 0.60 g. of iodine and 0.90 g. of potassium iodide in 2.0 ml. of water. The dropwise addition of the iodine–potassium iodide solution was conducted over a 30-min. period. The reaction mixture was allowed to stir an additional 48 hr. at room temperature, then acidified with glacial acetic acid and filtered. The dark brown precipitate was washed with water and recrystallized from water to yield light yellow crystals of 2-hydroxy-8-iodopurine (IV) which decomposed above 250°. Yield: 0.130 g. (49.5%).

Anal.—Calcd. for $C_5H_3IN_4O \cdot 1.5H_2O$: C, 20.8; H, 2.08. Found: C, 20.87; H, 2.21. UV data: pH 1, $\lambda_{max.}$ 306 mμ (ϵ = 7180); $\lambda_{max.}$ 223 mμ (ϵ = 17,950); pH 6.65, $\lambda_{max.}$ 290 mμ (ϵ = 4260); pH 12.85, $\lambda_{max.}$ 299 mμ (ϵ = 6770).

6-Amino-2-hydroxy-8-iodopurine (V)—A solution of 0.60 g. iodine and 0.90 g. of potassium iodide in 2.0 ml. water was added dropwise, over a 30-min. period, to a suspension of 0.187 g. (1 mmole) of 6-amino-2-hydroxy-8-mercaptopurine and 0.90 g. of sodium bicarbonate in 5.0 ml. of water. Stirring was continued for an additional 24 hr. at room temperature. The mixture was filtered and the precipitate washed with 2 ml. of 10% potassium iodide solution, then with water. Recrystallization from 1 *N* sulfuric acid gave 0.135 g. (40.5%) of a

white crystalline product which gradually darkened above 235°.

Anal.—Calcd. for $C_5H_4IN_5O \cdot 0.5\ H_2O \cdot 0.5\ H_2SO_4$: C, 17.44; H, 2.03. Found: C, 17.43; H, 2.39. UV data: pH 1, $\lambda_{max.}$ 299 mμ (ϵ = 15,200); pH 6.65, $\lambda_{max.}$ 297 mμ (ϵ = 15,650); pH 12.85, $\lambda_{max.}$ 296 mμ (ϵ = 13,300).

2-Amino-6-iodopurine (VI)—To a mixture of 1.0 ml. of absolute methanol and 3.0 ml. of concentrated hydrochloric acid, previously cooled to −10° was added 0.50 g. (3 mmoles) of 2-amino-6-mercaptopurine (12). Chlorine gas was passed into the mixture for 30 min., never allowing the reaction temperature to rise above −5°. The mixture was neutralized with ammonium hydroxide and the white precipitate of 2-amino-6-chloropurine was filtered, washed with water, and dried.

To 5.0 ml. of hydriodic acid (57%), previously cooled to −10°, was added 0.50 g. (2.95 mmoles) of 2-amino-6-chloropurine. The mixture was stirred for 1.5 hr. at −10°, then filtered. The precipitate was suspended in water and the pH adjusted to neutrality with ammonium hydroxide. The mixture was filtered and the precipitate recrystallized from ethanol–water (1:1) to give 0.32 g. (25%, based upon 2-amino-6-chloropurine) of 2-amino-6-iodopurine (VI), which gradually darkened above 245°.

Anal.—Calcd. for $C_5H_4IN_5$: C, 23.0; H, 1.53. Found: C, 23.2; H, 1.84. UV data: pH 1, $\lambda_{max.}$ 319 mμ (ϵ = 7550), $\lambda_{max.}$ 213 mμ (ϵ = 23,000); pH 6.65, $\lambda_{max.}$ 314 mμ (ϵ = 8000), $\lambda_{max.}$ 240 mμ (ϵ = 9120); $\lambda_{max.}$ 217 mμ (ϵ = 27,900); pH 12.85, $\lambda_{max.}$ 313 mμ (ϵ = 6800), $\lambda_{max.}$ 224 mμ (ϵ = 22,900).

8-Iodoguanine Hydrochloride (VII)—A suspension of 0.50 g. (1.22 mmoles) of 8-iodoguanosine (8, 9) in 15.0 ml. of 1 *N* hydrochloric acid was heated on a steam bath for 2 hr. During the heating period, light yellow needles separated from solution. The reaction mixture was cooled in an ice bath and additional precipitation occurred. The crystals were filtered and washed with water. Recrystallization from 1 *N* hydrochloric acid gave 0.15 g. (40%) of 2-amino-6-hydroxy-8-iodopurine (VII).

Anal.—Calcd. for $C_5H_5ClIN_5O$: C, 19.2; H, 1.59. Found: C, 19.0; H, 1.83. UV spectral data: pH 1.3, $\lambda_{max.}$ 275 mμ (ϵ = 8600); pH 6.65, $\lambda_{max.}$ 252 mμ (ϵ = 9450); pH 11.9, $\lambda_{max.}$ 279 mμ (ϵ = 7370).

6,8-Dimercaptopurine (VIII)—One gram (7.05 mmoles) of 4,5-diamino-6-mercaptopyrimidine and 3.0 g. (39.5 mmoles) of thiourea were thoroughly mixed, then fused at 200–210° in an oil bath for 30 min. After cooling, the mass was dissolved in 30 ml. of 1 *N* potassium hydroxide, decolorized twice with charcoal, and filtered. The filtrate was acidified with acetic acid whereupon precipitation occurred. The yellow precipitate of 6,8-dimercaptopurine was filtered, washed with water, and dried. Purification was accomplished by reprecipitation from 1 *N* potassium hydroxide using glacial acetic acid. The UV spectral data at pH 1 and 11 were identical to that reported by Robins (14).

6-Amino-2,8-dimercaptopurine (IX)—A mixture of 2.0 g. (14.1 mmoles) of 2-mercapto-4,5,6-triaminopyrimidine sulfate and 8.0 g. (0.105 mole) of thiourea was heated at 200° for 60 min. in an oil bath. The fusion mixture was cooled and dissolved in 30 ml. of 1 *N* potassium hydroxide, decolorized with charcoal, and filtered. The filtrate was acidified with glacial acetic acid and a bright yellow precipitate separated. The precipitate was filtered and purified by reprecipitation from 1 *N* potassium hydroxide. Fine yellow microcrystals of 6-amino-2,8-dimercaptopurine (IX), which were slightly hygroscopic, were isolated and found to gradually darken above 240°.

Anal.—Calcd. for $C_5H_5N_5S_2$: C, 30.1; H, 2.51. Found: C, 29.9; H, 2.75. UV spectral data: pH 1, $\lambda_{max.}$ 302 mμ (ϵ = 15,900), $\lambda_{max.}$ 234 mμ (ϵ = 8730); pH 6.65, $\lambda_{max.}$ 238 mμ (ϵ = 12,100); pH 12.85, $\lambda_{max.}$ 313 mμ (ϵ = 16,200), $\lambda_{max.}$ 230 mμ (ϵ = 19,100).

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Keyphrases

Iodopurine derivatives—synthesis
Antitumor activity—iodopurine derivatives
IR spectrophotometry—structure
UV spectrophotometry—structure

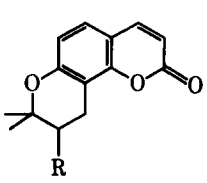
Coumarins VIII

Mass Spectra of Lomatin and Some of Its Derivatives

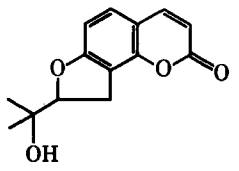
By M. SHIPCHANDLER and T. O. SOINE

The fragmentation behavior of lomatin and certain of its derivatives has been studied. Two distinct fragmentation pathways have been observed, one leading to aromatization of the chroman ring and the other leading to its fission. These findings are in complete agreement with the observations of other workers. Probable fragmentation mechanisms have been proposed.

THE AUTHORS' PAST INTEREST in naturally occurring coumarins had led them to the isolation and structural characterization of several new coumarins (1–5). Among these, lomatin (I) was subsequently obtained as the angelate ester (II) by Shanbhag *et al.* (6), Seshadri *et al.* (7), as well as by this laboratory (3, 5). Additionally, lomatin senecioate (III) was shown to be of natural occurrence (5) and given the



	R
I	—OH
II	—Angeloxy
III	—Senecioyloxy
V	—Acetoxy
VI	=O



IV

provisional name of *nuttallin*. The present authors have previously studied the mass spectral characteristics of columbianetin (IV) and certain of its esters (8) as well as of certain glycosidic derivatives (9). Because of the close relationship of I to IV it seemed desirable to examine the mass spectral characteristics of I and its naturally occurring congeners (II and III) as well as certain other closely related derivatives. The mass spectrum of II has been reported by Seshadri *et al.* (7) but, nevertheless, it has been reexamined in the present work along with the other esters and studied in greater detail. The observations reported herein are concerned with the fragmentation patterns of lomatin (I), lomatin acetate (V), lomatin angelate (II), lomatin senecioate (III), and the ketone (VI) derived from I.

There is a difference of opinion among different groups of workers regarding the structure of the ion arising from 2-pyrones by loss of CO from the lactone carbonyl upon electron impact. It has commonly been assumed to have a furan-type structure although Pirkle (10) and Pirkle and Dines (11) have adduced evidence to show that this is not true. Dean *et al.* (12) support Pirkle's view as do Pike and McLafferty (13) and Bursey and Dusold (14). However, Green and Brown (15) consider the matter to be an open question and Nakata and Tatematsu (16) suggest that the fragmentation occurs, at least in part, through an intermediate with a C—O bond (*i.e.*, a furan-like system). For the sake of simplicity, a practice which is not unprecedented in this field, the present authors prefer to employ a benzofuran structure for the ion arising by loss of CO from the coumarin lactone. This representation, it may be pointed out, is consistent with that of many other workers (17–25).

Metastable ion peaks have been helpful in supporting many of the fragmentation pathways presented in this study and will be represented by a heavily lined arrow. Fragmentation pathways not substantiated by metastable ions but, nevertheless, feasible on mechanistic grounds or because of past precedent, are suggested and represented with ordinary arrows.

The fragmentation characteristics of a number of oxygen-containing heterocyclic compounds have been studied by Willhalm *et al.* (26) and their findings indicate that there is a strong tendency for stable aromatic ions (*e.g.*, benzopyrilium ion in the case of chromenes) to be formed without heterocyclic ring fission. Such fission is observed in the corresponding reduced compounds (*e.g.*, chromans) where such aromatization is not feasible. The appearance of the benzopyrilium ion is a very characteristic feature in the mass spectra of chromenes, arising by expulsion of a methyl radical from the molecu-

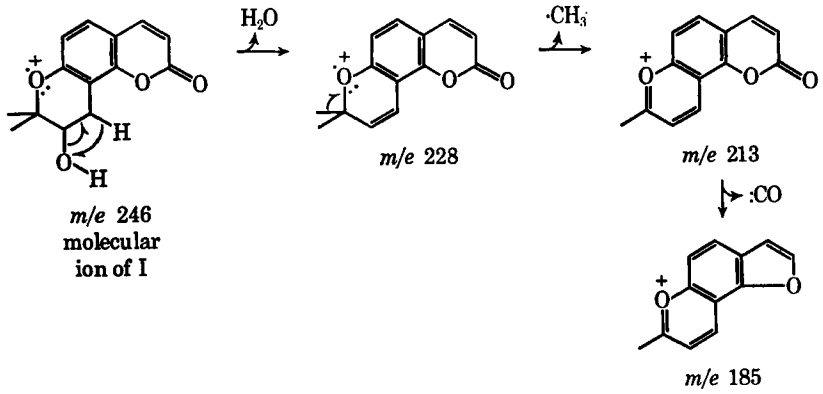
Received July 24, 1968, from the Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455

Accepted for publication August 27, 1968.

This work was supported by grant HE 07101 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

Send communications concerning this paper to Dr. T. O. Soine.

Assistance in the interpretation of the spectra by Dr. Thomas Watson, Pharmacy Department, University of Sydney, Sydney, Australia, is gratefully acknowledged.



Scheme I

lar ion (27). Its highly stable aromatic character minimizes further fragmentation and, therefore, this ion appears as a prominent peak (17). In compounds containing chroman systems with an oxygenated substituent, the benzopyrilium ion is generated in two steps as noted by Willhalm *et al.* (26) and confirmed in the present study. The first step involves a loss of ROH to generate a chromene system and this is followed by expulsion of a methyl radical. Thus, in the mass spectrum of I (see Fig. 1) the molecular ion loses HOH to give an ion of *m/e* 228 which then expels the methyl radical to provide the benzopyrilium ion at *m/e* 213 (see Scheme I). A loss of CO as in the case of seselin (17) is observed and gives a small peak at *m/e* 185. In the mass spectrum of deuterated lomatin (see Fig. 2) the peaks at *m/e* 228 and 213 remain undisplaced. The pathway M → 228 → 213 → 185 is also operative in the case of columbianetin and its derivatives (8, 9). The benzopyrilium ion appears in their mass spectra even though this class of compounds lacks a chroman ring and a mechanism leading to ring expansion has been proposed (8). The pathway M → 228 → 213 → 185 is of minor consequence in the case of I, however, although it is the principal route in the case of the *esters* of I (se below).

The principal route of fragmentation in the case of I is the fission of the chroman ring with a loss of two ring carbons and is in agreement with the observations of Willhalm *et al.* (26). The ring fission, in the present study, occurs with one as well as two hydrogen transfers and generates the ions of *m/e* 175 and 176 (base peak), respectively. As mentioned earlier, such ring fission is characteristic of chroman and 2,2-dimethylchroman and occurs with or without hydrogen transfer in the former and with hydrogen transfer in the latter (26). The mechanism of the ring fission can be rationalized as shown in Scheme II and is partially supported by the fact that the base peak at *m/e* 176 is displaced to *m/e* 177 in the mass spectrum of the deuterated sample (see Fig. 2) indicating that one of the transferred hydrogens arises principally from the hydroxy group. Loss of a hydrogen from the ion of *m/e* 176 would provide the ion of *m/e* 175 which could also arise from the molecular ion directly by a fission with a single hydrogen transfer. The ion of *m/e* 175 very likely rearranges to the corresponding tropylium ion which can lose three molecules of CO in a stepwise manner, a process characteristic of coumarins (27). As a result, the ions of *m/e* 147,

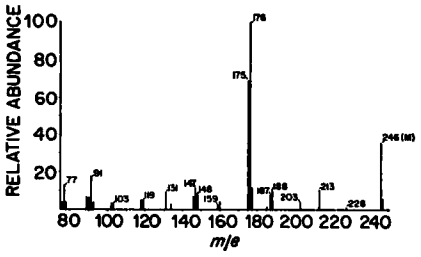


Fig. 1—Mass spectrum of I. Peaks of abundance ratio less than 3% have been omitted in all spectra unless a reference has been made to them in the text.

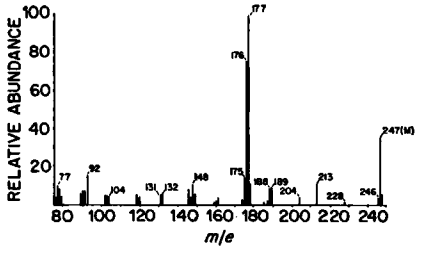
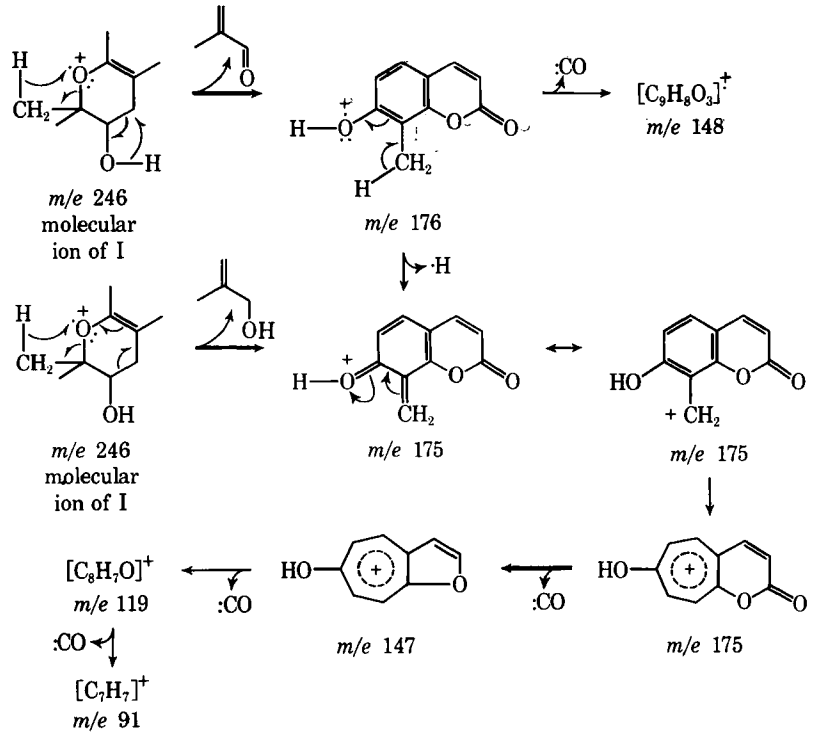


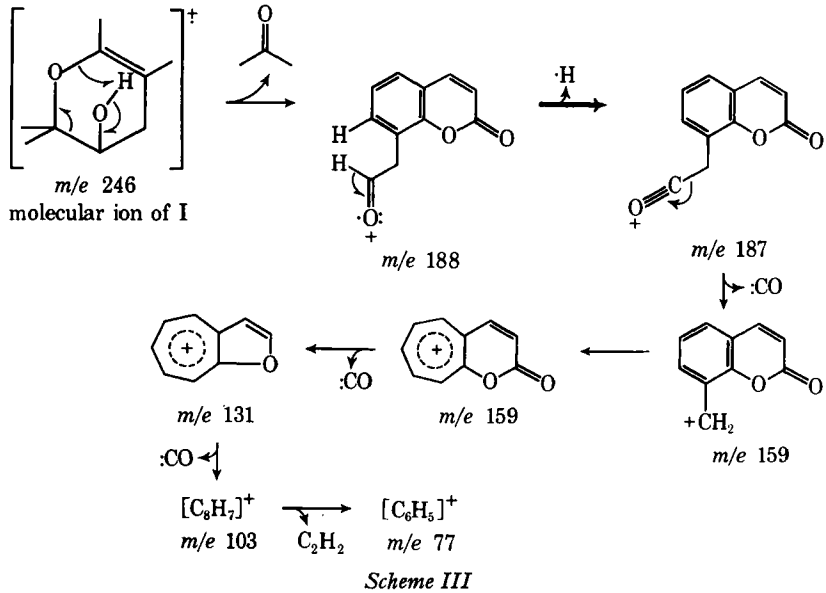
Fig. 2—Mass spectrum of deuterated I.



Scheme II

119, and 91 are produced. The ion of *m/e* 148 can be envisioned as arising from the ion of *m/e* 176 by loss of CO. A very broad metastable ion peak may be due to either the transformation M → 175 (124.5 theory) or to 175 → 147 (123.5 theory) or both. However, the peak at *m/e* 125.9 is distinguishable and corresponds to the transformation M → 176 (125.9 theory).

Another minor pathway observed is a loss of acetone from the molecular ion giving an ion of *m/e* 188 which loses a hydrogen giving rise to an ion of *m/e* 187 (see Scheme III), the latter being



Scheme III

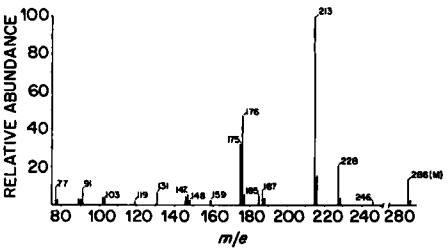


Fig. 3—Mass spectrum of V.

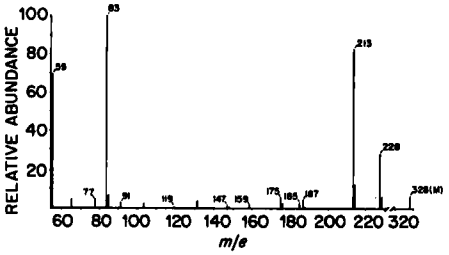
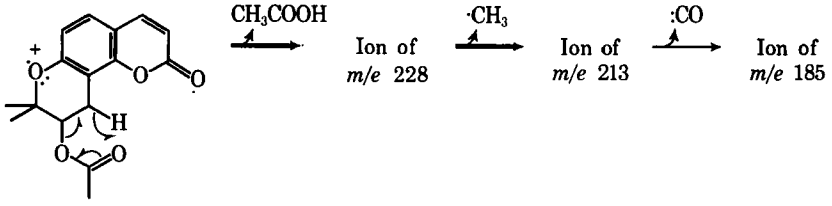


Fig. 4—Mass spectrum of II.

supported by the appearance of the corresponding metastable ion peak. In the deuterated sample, the peaks at *m/e* 188 as well as 187 are displaced to one higher unit, indicating that the transferred hydrogen originates from the hydroxy group. The isomeric IV also shows a loss of acetone followed by a loss of hydrogen (8). However, in this case the pathway is the main fragmentation route giving the base peak at *m/e* 187. Once again, in the present study, a stepwise loss of three molecules of CO is observed giving rise to ions of *m/e* 159, 131, and 103. Finally, the ion of *m/e* 103 can lose a molecule of acetylene to account for the ion of *m/e* 77. The pathway 131 → 103 → 77 is very similar, and probably analogous, to that of IV where it is supported by visualized as resulting from the loss of ketone by the molecular ion (see Scheme V) and it would be expected that this ion would then fragment further just as in the case of lomatin provided that the fragmentation generated ion is similar to that arising by direct electron impact. Therefore, it is expected that almost all of the peaks found in the spectrum of the parent compound would also be found in the spectrum in the present case. Moreover, the ions of *m/e* 176 and 175 could also arise directly from the molecular ion of the acetate as shown in Scheme V.

The mass spectrum of lomatin angelate[1] (II) (see Fig. 4) was reported by Seshadri *et al.* (6) and comparison with the present spectrum shows



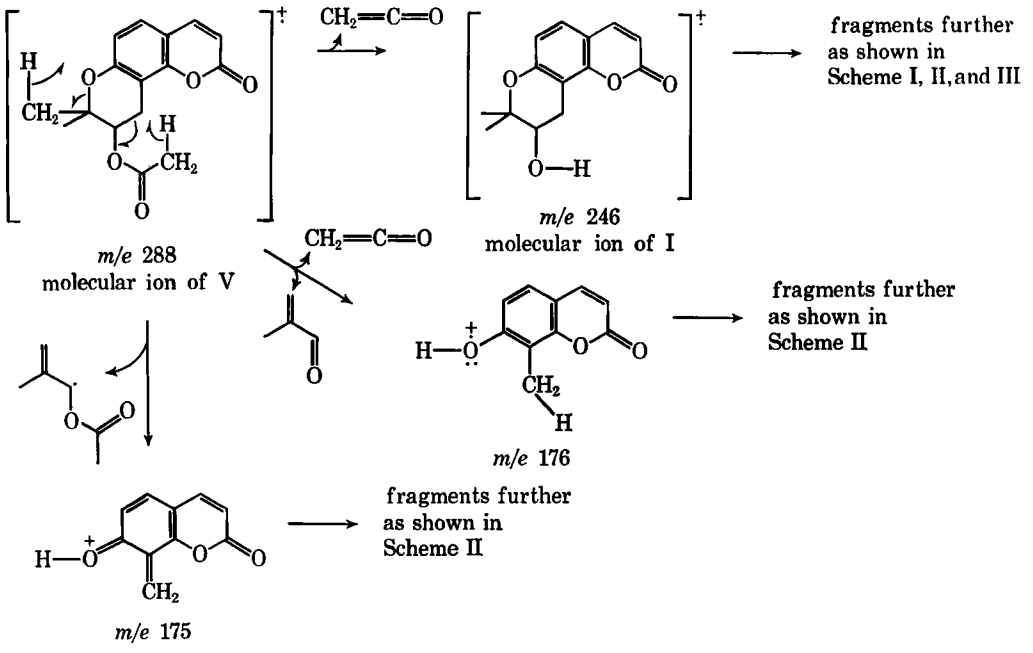
Scheme IV

the corresponding metastable ions. Finally, the proposed structures for the ions of *m/e* 175, 159, 147, and 131 are in accordance with the corresponding ions found in the mass spectra of osthol and dihydroosthol (17, 27).

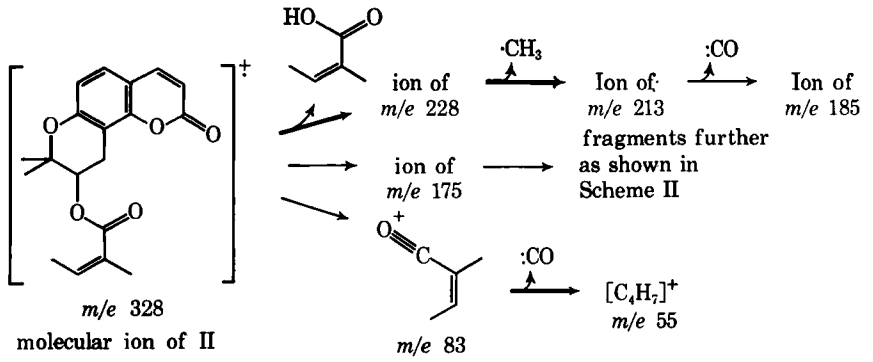
The mass spectrum of lomatin acetate (V) (see Fig. 3) shows a predictable mode of fragmentation as described in Scheme IV. Loss of a molecule of acetic acid by the molecular ion provides the ion of *m/e* 228 which expels a methyl radical to give the stable benzopyrilium ion as the base peak at *m/e* 213 (doubly charged ion at *m/e* 106.5). This ion then loses a molecule of CO to produce the ion of *m/e* 185 (doubly charged ion at *m/e* 92.5). This pathway is analogous to those observed in the case of esters of IV (8) and seselin (17). The M-42 ion can be marked similarity with the principal differences being in peak heights, a behavior not uncommon for identical compounds examined under differing instrumental conditions. The fragmentation pathways have been outlined in Scheme VI and are in agreement with those of the previous workers. In addition, corresponding metastable peaks have been found for the transformations M → 228, 228 → 213, and 83 → 55. Fission of the heterocyclic ring with one hydrogen transfer is observed, giving the ion of *m/e* 175.

The mass spectrum of lomatin senecioate (III) (see Fig. 5) resembles that of the isomeric angelate ester and, presumably, all of the fragmentation pathways for II also hold true for III.

[1] Lomatin angelate was named selinidin by Leshadri *et al.* (7) and jatamansin by Shanbhag *et al.* (6).

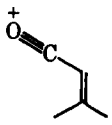


Scheme V



Scheme VI

The ion of *m/e* 228 arises by loss of senecioic acid from the molecular ion and the ion appearing at *m/e* 83 (base peak) probably has the isomeric structure VII.



m/e 83

VII

The proposed fragmentation pathways for the ketone (VI) have been outlined in Scheme VII. The molecular ion appears as an intense peak in this spectrum (see Fig. 6) and most of the peaks arise from either a loss of CO or an expulsion of methyl radical from the molecular ion and then from the subsequent fragments. For example, the molecular ion shows a loss of CO, giving an ion of *m/e* 216 which expels a methyl radical to give the ion of *m/e* 201 (base peak). The alternate route, *i.e.*, initial expulsion of a methyl radical followed by a loss of CO, is also operative. The ion of *m/e* 201 shows a loss of CO to give the

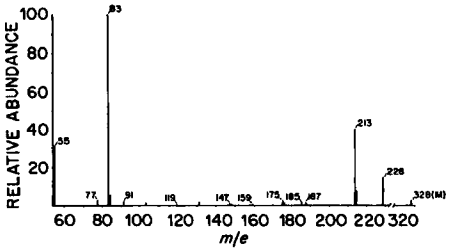
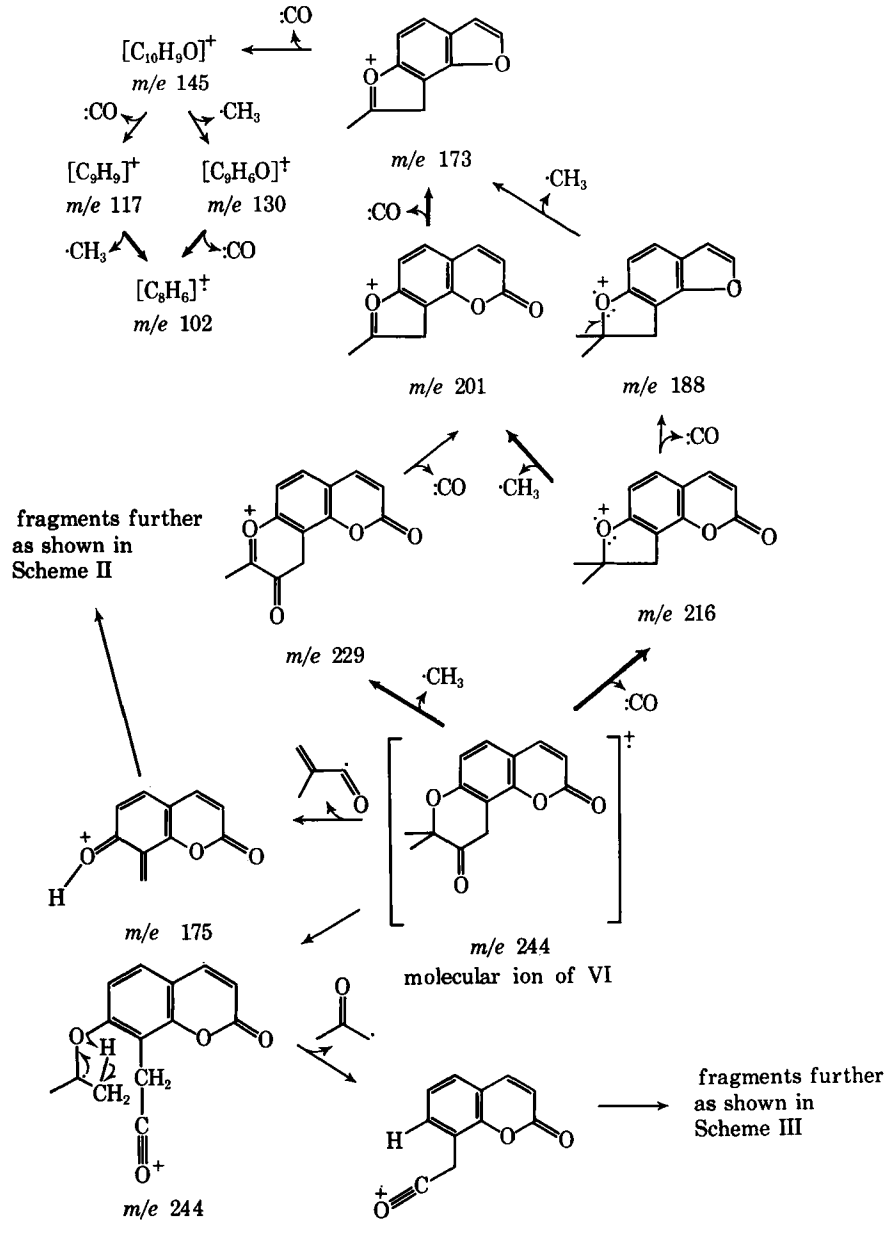


Fig. 5—Mass spectrum of III.



Scheme VII

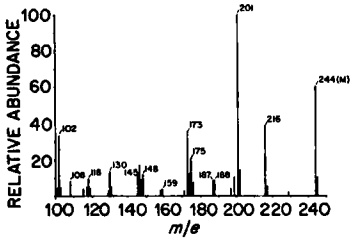


Fig. 6—Mass spectrum of VI.

ion of *m/e* 173 which then fragments further as shown in Scheme VII. Fission of the chroman ring with one hydrogen transfer would result in the ion of *m/e* 175 as in the case of I. A peak at *m/e* 187 could be proposed as resulting from ion formation by the mechanism shown in Scheme VII.

EXPERIMENTAL

Materials—Lomatin (I), its angelate (II), and its senecioate (III) were available in these laboratories

from the studies of Lee and Soine (5). The acetate (V) as well as the ketone (VI) were available in these laboratories from the studies of Gupta and Soine (28).

Deuteration of lomatin was achieved by heating a small amount of it overnight in a sealed tube at 130° in the presence of enough D_2O to form a clear solution during the heating period. After cooling, the crystallized product was removed by filtration and dried. The ratio of the molecular ion peaks (see Fig. 2) indicated approximately 90% incorporation.

Mass Spectra—These were carried out by Mr. A. R. Swanson and Mr. D. L. Hobbs, School of Chemistry, University of Minnesota, employing a mass spectrometer (Hitachi-Perkin-Elmer RMU-6D). The instrument was operated with a source temperature of 250° and an ionizing voltage of 50 ev. The direct sample inlet temperature was 90° in the case of I, deuterated I, II, V, and VI and 135° in the case of III.

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Keyphrases

Coumarins
Lomatin, derivatives—mass spectra
Mass spectra—lomatin, derivatives, fragmentation patterns
Deuterated lomatin—mass spectrum

Radiosynthesis of 3′-Chloro-3-nitrosalicylanilide and Determination of Its Uptake by Lamprey Larvae

By LARRY L. FRENCH and HOWARD A. SWARTZ

Tritium radiosynthesis of 3′-chloro-3-nitrosalicylanilide was accomplished by an exchange reaction with tritiated phosphoric acid-boron trifluoride reagent. Radiochemical purity was established by means of paper chromatography and autoradiography. Residual activity in lamprey larvae was determined at intervals of 1, 2, and 3 hr. The values observed were 3.046, 3.16, and 3.512 percent, respectively, representing a total residue of 0.0182 mg./g., 0.0186 mg./g., and 0.021 mg./g. at the stated time intervals.

THE LAMPREY EEL of the Great Lakes has inflicted considerable damage to the fishing industry of this region, and for many years attempts have been made to kill the eel and inhibit its reproduction.

The lamprey eel is a vertebrate of the Cyclostomi, the "round mouthed" class, constituting the *Petromyzon* genus. Although there are five species indigenous to the Great Lakes region, both

Received April 29, 1968, from Department of Bionucleonics, College of Pharmacy, Butler University, Indianapolis, IN 46208
Accepted for publication September 12, 1968.

from the studies of Lee and Soine (5). The acetate (V) as well as the ketone (VI) were available in these laboratories from the studies of Gupta and Soine (28).

Deuteration of lomatin was achieved by heating a small amount of it overnight in a sealed tube at 130° in the presence of enough D_2O to form a clear solution during the heating period. After cooling, the crystallized product was removed by filtration and dried. The ratio of the molecular ion peaks (see Fig. 2) indicated approximately 90% incorporation.

Mass Spectra—These were carried out by Mr. A. R. Swanson and Mr. D. L. Hobbs, School of Chemistry, University of Minnesota, employing a mass spectrometer (Hitachi-Perkin-Elmer RMU-6D). The instrument was operated with a source temperature of 250° and an ionizing voltage of 50 ev. The direct sample inlet temperature was 90° in the case of I, deuterated I, II, V, and VI and 135° in the case of III.

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Coumarins
Lomatin, derivatives—mass spectra
Mass spectra—lomatin, derivatives, fragmentation patterns
Deuterated lomatin—mass spectrum

Radiosynthesis of 3′-Chloro-3-nitrosalicylanilide and Determination of Its Uptake by Lamprey Larvae

By LARRY L. FRENCH and HOWARD A. SWARTZ

Tritium radiosynthesis of 3′-chloro-3-nitrosalicylanilide was accomplished by an exchange reaction with tritiated phosphoric acid-boron trifluoride reagent. Radiochemical purity was established by means of paper chromatography and autoradiography. Residual activity in lamprey larvae was determined at intervals of 1, 2, and 3 hr. The values observed were 3.046, 3.16, and 3.512 percent, respectively, representing a total residue of 0.0182 mg./g., 0.0186 mg./g., and 0.021 mg./g. at the stated time intervals.

THE LAMPREY EEL of the Great Lakes has inflicted considerable damage to the fishing industry of this region, and for many years attempts have been made to kill the eel and inhibit its reproduction.

The lamprey eel is a vertebrate of the Cyclostomi, the "round mouthed" class, constituting the *Petromyzon* genus. Although there are five species indigenous to the Great Lakes region, both

Received April 29, 1968, from Department of Bionucleonics, College of Pharmacy, Butler University, Indianapolis, IN 46208
Accepted for publication September 12, 1968.

parasitic and nonparasitic, the species *marinus* was chosen for this work because they are the most abundant and harmful to the industry. Young eels, called ammocoetes, remain in the larval stage some 3 years. Although delectible food for other fish, they spend most of their time buried in the soft mud and sand at the bottom of the lake for protection. It is the larva that is affected by chemical methods of eradication. Partial eradication has been attributed to the widespread treatment of the larval habitat with 3-trifluoromethyl-4-nitrophenol, known commercially as TFM (1). The larvacidal LD_{100} for this compound varies from 3–10 p.p.m., depending on the physical and chemical characteristics of the water in which it is used. The LD_{25} for 11 species of fish indigenous to this area has been reported to be between 5–42 p.p.m. (2). During a continuing screening program to detect more potent and selectively toxic lamprey larvacides, substituted 3-nitro- and 5-nitrosalicylanilides were found to be highly active with relation to TFM. One compound, 2′-5-dichloro-4′-nitrosalicylanilide,[1] has been found and used successfully as a synergist to TFM in the control of the lamprey. Many derivatives of the salicylanilide series have been found to exhibit a diverse bioactivity spectrum, including antimicrobial activity (3), molluscidal activity (4), antitumor activity (5), and as a taenecide (6). Preliminary mammalian oral toxicity studies with substituted 3-nitro- and 5-nitrohalo compounds of this series indicate lack of side effects in dosages less than 2.5 g./kg. of body weight (7). Prompted by the preliminary larvicidal screening studies and relatively low mammalian toxicity, it was decided to evaluate the structural-activity relationships of a series of 3-nitro and 5-nitrosalicylanilides (8). From these studies it was found that the obligatory molecular requirement for activity was the orthophenolic substituent on the carboxylic acid moiety of the salicylanilide. In addition, maximum toxicity and selectivity required the optimum combination of a halogen and a nitro group in the molecule. Substitution of more than two of the halogens in the aniline moiety, however, decreased selectivity and toxicity due to a saturation effect (9).

Studies by Starkey and Howell (10) showed that the combination 3′-chloro-3-nitrosalicylanilide, known experimentally as 33NCS, produced maximum toxicity and selectivity. The LD_{100} for this compound for the larvae was determined to be 0.3 p.p.m. and an LD_{25} for fingerling rainbow trout to be 0.9 p.p.m. The compound was selected for the study reported here.

A primary problem facing the investigator desiring to employ radiotracer methods is securing the suitable labeled compounds. The two major elements of radiolabeling are ^{14}C and ^{3}H. Chemical syntheses employing these two isotopes are used frequently to prepare labeled compounds in many tracer studies. Tritium-labeled compounds have been increasingly used in recent years, even though the mean energy of the β-particles emitted is only 5.5 kev. However, with the advent of highly sensitive scintillation systems, the usefulness of this isotope has been greatly exploited. Tritium-labeled compounds are usually "hotter" in terms of specific activity than other isotopic compounds available for biological investigation. Tritium radiosynthesis may be accomplished by several methods, none offering a simple procedure but having the distinct advantage of the high specific activity at relatively low cost.

The method of choice for most biological tracer studies with tritium is the reduction of a suitable unsaturated precursor with the carrier-free tritium gas, carried out in a nonoxidizing solvent under highly controlled criteria of study and evaluation. The advantages of this system are a very high specific activity, purity of the product, and the known position of labeling of the compound. The major limitation to this method, however, is the availability of the suitable precursor of the desired compound and the criteria for the reaction procedure.

Random tritium labeling may also be secured by simple exchange methods, with or without a catalytic system. Usually this method employs either tritiated water or glacial acetic acid as the exchange medium and platinum black as the catalyst (11). Although high specific activities are available through this method, much of the tritium exchanged product will be labile, and will exchange, or reverse its initial exchange, upon contact with nonlabeled material. The removal of the labile tritium and subsequent purification may be very difficult to achieve.

A third method, the Wilzbach reaction (12), or the Wilzbach gas exposure method, is largely responsible for the increased popularity of tritium as a tracer. In this method, the compound to be labeled is exposed to curie amounts of carrier-free tritium gas in a sealed reaction vessel for a period of from a few days to several weeks. External energy such as electric discharge, microwaves, UV irradiation, and γ irradiation have been used to hasten the process, yielding, however, many

[1] Bayer 73.

more degradation products. The energy released in the disintegration of the tritium and absorbed by the system provides the activation necessary to effect labeling. The major disadvantage of such a system is the formation of a great number of by-products, due largely to radiolysis. Rigid and extensive purification steps are necessary to insure radiochemical purity.

An excellent method for tritium exchange was reported in the literature by Yavorsky and Gorin (13), and has been successfully used to label several compounds (14). The method involves the preparation of an exchange reagent, tritiated phosphoric acid-boron trifluoride ($3H_3PO_4 \cdot BF_3$). Many organic compounds can be labeled with tritium by simply mixing the compound to be labeled with the reagent and agitating this mixture in a sealed vessel for several hours. The ultimate specific activity theoretically can be calculated from the formula (14):

$$Sx = \frac{S_R{}^0}{(3/166)\,(M_X/N_H) + (W_X/W_R)}$$

where $S_R{}^0$, initial specific activity of the reagent; M_X, molecular weight of the product; W_X/W_R, weight ratio of product to reagent; N_H, number of exchangeable hydrogen positions of the compound to be labeled.

Studies with this procedure have shown that compounds having substituent groups that tend to withdraw electrons from the aromatic ring inhibit substitution of the tritium. This includes groups such as the nitro- and carboxyl- substituents. Substituent groups that contribute electrons to the ring, such as alkyl and aryl moieties, tend to activate exchange. This method appeared to offer a suitable approach to labeling the selected compound, 33NCS, with the distinct advantages of low radiation hazard and reduced purification requirements.

EXPERIMENTAL

Preparation of the Labeled Reagent—Tritiated phosphoric acid saturated with boron trifluoride (BF_3) was prepared by the method of Yavorsky and Gorin (13). Two-tenths milliliter of tritiated water, with an initial specific activity of 500 mc./ml., was diluted to 5 ml., using triple-distilled deionized water. This combination was thoroughly mixed and added to 13.1 g. of anhydrous phosphorus pentoxide, in a sealed reaction chamber that had previously been purged with nitrogen. The apparatus employed is illustrated in Fig. 1. When all of the tritiated water had been added to the reaction flask and the dense fumes had dissipated, the mixture was saturated with the boron trifluoride (see Fig. 1).

The stoichiometric equation for the reaction is as follows:

$$6BF_3 + 9\ {}^3H_2O + 3P_2O_5 \rightarrow 6\ {}^3H_3PO_4 \cdot BF_3$$

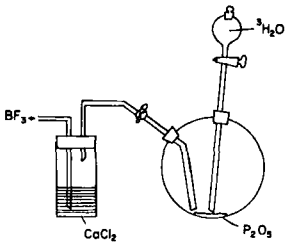


Fig. 1—Apparatus for radiosynthesis.

The final product is a very dense, viscous, yellow liquid.

Preparation of the Tritium-Labeled Compound—To the labeled reagent, 2.000 g. of the compound 33NCS[2] was added. This mixture was then heated to 40° and mixed by means of magnetic stirrers for a period of 24 hr. At the conclusion of this period, the mixture was allowed to reach equilibrium at room temperature and the flask containing the labeled mixture removed from the apparatus and the labeled 33NCS precipitated from solution by the addition of a 50-ml. divided quantity of purified deionized distilled water. The precipitate was collected on filter paper and dried in an oven at 37°. The total weight of the dried, but unpurified, product was 1.818 g.

Radiochemical Purity of the Compound—Several solvent systems were evaluated for recrystallization of the labeled product for purification purposes. The most efficient and convenient of these was acetone. This solvent proved to be superior for two reasons: first, upon solution and recrystallization, excellent yield was obtained, and second, acetone allowed sufficient removal of labile tritium on the labeled compound.

The labeled compound was recrystallized four times from acetone in each of three separate quantities. Samples were taken from each division on each occasion of recrystallization, and it was observed that constant specific activity was obtained after the third recrystallization. After the total recrystallization effort, the combined weight of labeled 33NCS was 1.050 g.

A 20-mg. sample of the purified product was added to 50 ml. of toluene, agitated to obtain complete solution, and allowed to reach equilibrium. Two 0.01-ml. samples of this solution were spotted on separate Whatman No. 1 chromatographic paper. The spots were air dried, the papers placed in a chromatographic chamber, allowed to attain equilibrium. One chromatogram was prepared using a solvent system of butanol 4 parts, water 4 parts, and glacial acetic acid 1 part (No. 1). The other solutions consisted of butanol 4 parts, and water 1 part (No. 2). The individual chromatograms were placed on sheets of 8 × 11 film (No-Screen Kodak X-Ray), and exposed for 10 days. At the end of this period, the films were removed and developed. In both instances, a single, well-defined exposed area was observed, indicating complete radiochemical purity.

Chemical Yields and Final Specific Activity—The final specific activity of the labeled 33NCS was determined by taking 1 ml. of solution, containing 200 mg. of labeled compound in 20 ml. of toluene, and diluting again to 10 ml. in toluene. A 0.1-ml. aliquot was taken for counting in a liquid scintillation detec-

[2] 3′-Chloro-3-nitrosalicylanilide, supplied by Ben Venue Laboratories, Inc., Bedford, Ohio.

tor.[3] These samples were counted at the determined tritium balance point for this instrument.

Each sample was counted under these conditions and the necessary corrections made. The quenching effect was corrected by means of an internal standard of tritiated hexadecane. The true net counts per minute (c.p.m.) for each sample were then calculated and the specific activity in net c.p.m./mg. determined. This value was calculated and found to be 1.32 μc./mg. of labeled compound 33NCS.

Determination of Residual Activity of Lamprey Larvae—A stock solution of the labeled 33NCS was prepared by dissolving 200 mg. in 2 l. of deionized water. Aliquots of 6 ml. of this solution, containing 0.6 mg. of labeled compound, were diluted to 3 l. to give the maximum toxic dose of 0.2 mg./l., or 0.2 p.p.m. The water employed for the 3-l. volumes was aged to afford removal of chlorine. The volumes were aerated and six lamprey larvae[4] added to each of three volumes. Aeration was continued at a constant rate throughout the experiment. One group of the lamprey larvae was removed at the end of the first hour after exposure to the labeled 33NCS. The second group was removed at the end of 2 hr., showing extremely toxic effects of the compound. The final group was removed at the end of the third hour. These larvae were completely immobilized, though not yet dead at the time that they were removed. Each individual larva for each group was accurately weighed (see Table I for individual weights) and placed into individual glass vials containing 25 ml. of a solution of 1 *M* diisobutylcresoxy ethoxyethyl dimethyl benzyl ammonium chloride[5] in methanol. The vials were then sealed by means of aluminum lined caps and incubated at 37° to facilitate digestion and solubilize the larvae. When these processes were complete, 1-ml. samples were taken for counting.

All samples were then counted by means of a liquid scintillation counter.[6] The 1-ml. samples for counting were added to 15 ml. of a liquid scintillation counting solution contained in Wheaton No-Sol-Vite glass vials. This solvent system employed consisted of PPO (2,5-diphenyloxazole), 0.3%; POPOP [1,4-bis-2-(5-diphenyloxazoyl) -benzene], 0.1%; naphthalene, 5%; cellusolve, 300 ml., dioxane, 300 ml.; and toluene, a quantity sufficient to yield 1 l.

The activity of the initial 0.2 p.p.m. solution was determined by taking 1 ml. of the original solution and adding to 15 ml. of the solvent system. The activity of the standards were determined at the determined balance point for tritium. A counting time of 10 min. was employed for all samples. The counting error was calculated to be less than 1% in all instances. Quenching again was corrected by means of an internal standard of tritiated hexadecane.

The percent residue for each larvae at each interval was determined by comparing the residual activity/g. to the total activity of the 3-l. solution. These values are tabulated in Table I and illustrated in Fig. 2.

From the known activity/ml. of initial solution and the total activity per body weight of each larva, the total milliliters of the solution of the compound 33NCS cleared by each eel were calculated, and the values tabulated in Table II.

TABLE I—PERCENT RESIDUE OF LABELED SOLUTION PER GRAM OF LAMPREY LARVAE

No.	Wt., g.	% Residual Activity/g.
	1-hr. Interval	
1	0.6914	2.72
2	1.310	3.32
3	0.3914	3.37
4	0.5202	2.95
5	0.4720	2.88
6	0.7242	3.04
$\bar{X}$	—	3.046
SX	—	0.232
	2-hr. Interval	
1	1.330	2.59
2	1.610	2.69
3	0.4916	4.24
4	0.6712	3.16
5	0.4932	3.19
6	1.061	3.09
$\bar{X}$	—	3.16
SX	—	0.533
	3-hr. Interval	
1	1.4912	2.90
2	0.8394	3.88
3	1.1204	3.40
4	0.7236	3.73
5	0.4974	3.47
6	0.5286	3.69
$\bar{X}$	—	3.512
SX	—	0.319

DISCUSSION

Radiosynthesis—Tritium labeling of compound 33NCS (3′-chloro-3-nitrosalicylanide) was achieved by use of tritiated phosphoric acid saturated with boron trifluoride. This method offers a distinct advantage over the recoil methods in the ease of recovery and purification. Three to four recrystallizations gave a radiochemical pure product, with very little loss of compound. The final specific

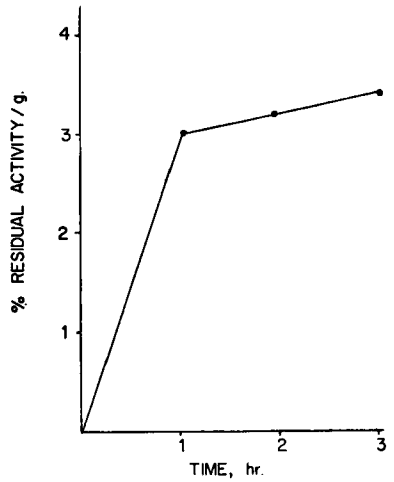


Fig. 2—Percent residual activity versus *time.*

[3] Ecko detector model N610A, Beta spectrometer, model N664A.
[4] The larvae were supplied by Mr. John Howell, Hammond Bay *Biological Station*, United States Department of the Interior, Millersburg, Mich.
[5] Hyamine, Rohm and Haas, Philadelphia, Pa.
[6] Packard Tri-Carb Counter and Detection System, model 314-S.

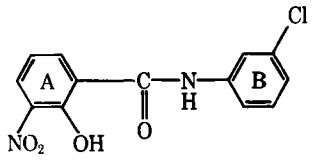
TABLE II—TOTAL MILLILITERS OF COMPOUND 33NCS SOLUTION CLEARED BY EACH LARVA

No.	ml./g.[a]	ml./Body Weight[a]
	1-hr. Interval	
1	81.55	56.38
2	99.60	129.48
3	101.13	39.58
4	88.52	46.05
5	86.29	46.73
6	91.10	65.97
$\bar{X}$	91.36	63.03
SX	6.992	31.101
	2-hr. Interval	
1	75.88	113.17
2	80.81	67.83
3	127.42	142.76
4	94.52	68.39
5	95.81	47.65
6	92.75	49.03
$\bar{X}$	94.53	81.47
SX	16.444	34.915
	3-hr. Interval	
1	87.09	115.83
2	116.54	187.62
3	102.04	50.06
4	112.11	75.24
5	104.04	51.31
6	110.91	117.68
$\bar{X}$	105.76	99.628
SX	9.663	47.813

[a] Calculated from percent residue per gram and body weight and total initial activity.

activity was 1.32 μc./mg. or 1/32 mc./g. which proved satisfactory for the uptake study even at the low concentration of 0.2 p.p.m. (0.2 mg./l.).

The position of the label, as with all tritium exchange methods, was random. The structure of the product, (I) has a bearing on the degree of exchange



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(14). The nitro and carboxyl groups in Ring A would tend to suppress tritium exchange in this ring as they are ring-deactivating groups. This would perhaps be overcome to some extent by the ring-activating hydroxyl group. The chlorine in Ring B would have little influence on exchange. The hydrogen of the A ring hydroxyl and the amino on Ring B would readily exchange, but being labile, would be removed in purification. On the basis of the electrophilic action of the carboxyl and nitro groups, with their inhibition of exchange in Ring A, it is estimated that Ring B contained the predominate number of tritium atoms and the label is principally found in this ring.

Larvae Residual Activity—The mean value in percent for the residual activity/g. were 3.046 ± 0.232 at 1 hr., 3.16 ± 0.533 at 2 hr., and 3.512 ± 0.319 at 3 hr. As illustrated in Fig. 3, these values are indicative of a very rapid uptake from the surrounding medium with a slight but gradual increase from 1 hr. to the 3-hr. period. At this time, all of the larvae were immobilized and showing severe toxic effects. None were dead. The mean values for the milliliters of solution cleared of the labeled compound for the time intervals involved were 91.36 ± 6.992/g. or 63.03 ± 31.101/total body weight, 94.53 ± 16.44/g. or 81.47 ± 34.915/total body weight, and 105.46 ± 96.63/g. or 99.628 ± 47.813/total body weight, respectively. These values do not take into consideration that the actual concentration was being reduced with time, and are therefore somewhat low. Based on these values the total uptake of the labeled compound, calculated from 0.0002 mg./ml. × ml. cleared/g., are 0.0182 mg./g. at 1 hr., 0.0186 mg./g. at 2 hr., and 0.021 mg./g. at 3 hr. Similar, but slightly higher, values are obtained by calculating the total uptake from the percent residues/g. and the initial concentration of the compound (% residue/g./100 × 0.6 mg.). Thus on the values observed, it appears that an uptake of some 0.02 mg./g. of 33NCS produces severe toxicity in the larvae. This could be low also, as the figure is based on the observed residual activity/g. and it must be recognized that metabolic products could have been formed and excreted, which would reduce the residual activity. The milliliters of water cleared/g. at each time interval would not apply for the natural habitat as in this study a uniform or homogeneous distribution was employed which could not be expected in a lake or river.

SUMMARY

Radiosynthesis of the compound 33NCS proved quite satisfactory. The distinct advantages of the method for tritium labeling gave a radiochemically pure product in good radio yields.

The calculated dose of 0.2 p.p.m. appears to be quite toxic to the larvae. Toxic reactions and indications were observed at the end of 1 hr. and by the end of 2 hr., the larvae were having extreme difficulty in maintaining the righting reflex. By the end of 3 hr., all the larvae were completely immobilized and very near death.

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Keyphrases

3′-Chloro-3-nitrosalicylanilide, tritium labeled—synthesis
Paper chromatography—analysis
Radiochromatography—analysis
Liquid scintillation counting—radioactivity
Lamprey larvae—3′-chloro-3′-nitrosalicylanilide effect

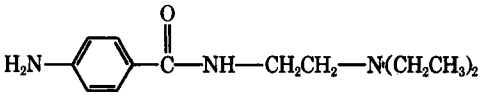
Potential Antiarrhythmic Agents I

Synthesis and Pharmacological Evaluation of Some Piperazine and Ethylenediamine Analogs of Procaine Amide

By D. K. YUNG*, L. G. CHATTEN, and D. P. MACLEOD*

Fourteen 1-alkyl-4-benzoyl-, 1-alkyl-4-*p*-substituted benzoylpiperazine hydrochlorides and eight *N,N′*-dibenzoyl-, *N,N′*-di(*p*-substituted benzoyl)-*N*-alkylethylenediamines were synthesized as procaine amide analogs. These compounds were tested for their effect on the effective refractory period of isolated guinea pig atria. 1-*n*-Propyl-4-benzoylpiperazine hydrochloride, 1-*n*-butyl-4-benzoylpiperazine hydrochloride, 1-*n*-propyl-4-*p*-methoxybenzoylpiperazine hydrochloride, 1-*n*-propyl-4-*p*-chlorobenzoylpiperazine hydrochloride, 1-isobutyl-4-*p*-chlorobenzoylpiperazine hydrochloride, 1-*n*-pentyl-4-*p*-chlorobenzoylpiperazine hydrochloride, and the eight *N,N′*-dibenzoyl-, *N,N′*-di(*p*-substituted benzoyl)-*N*-alkylethylenediamines were further screened for antiarrhythmic activity in cats. Only 1-*n*-propyl-4-*p*-chlorobenzoylpiperazine hydrochloride showed some degree of protection against aconitine-induced cardiac arrhythmias.

THE RELATIONSHIP between the structure and the antiarrhythmic activity of procaine amide [*N*-(2-diethylaminoethyl)-*p*-aminobenzamide, I] has not been thoroughly studied. A few



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workers, however, have studied the alteration of antiarrhythmic activity caused by various substituents on the benzene ring. Clinton *et al.* (1) reported that several of the higher members of the *N*-(2-diethylaminoethyl)-4-amino-2-alkoxybenzamides, in particular the 2-hexoxy derivative, showed outstanding properties as antiarrhythmic agents. Reisner and Cordasco (2) synthesized 2-chloroprocaine amide and found that this compound was approximately four times as potent as procaine amide in blocking atrial fibrillation in dogs. Libonati and Segre (3) studied the antiarrhythmic activity of *N*-(2-diethylaminoethyl)-*p*-4-hydroxybutylbenzamide and *N*-(2-diethylaminoethyl)-*p*-isopropylbenzamide and reported that these compounds showed an activity of 75 and 10 times, respectively, that of procaine amide. Similar compounds, such as *N*-(2-diethylaminoethyl)benzamide and *N*-(2-diethylaminoethyl)-*p*-methoxybenzamide, have also been evaluated for antiarrhythmic activity (4). The former compound was found to have a lower toxicity but the same potency as procaine amide and the latter compound to have a higher potency and toxicity than procaine amide.

The first systematic synthesis of procaine amide analogs was reported by Thyrum and Day (5). In the series of compounds they prepared, the following showed significant antiarrhythmic activity when tested on isolated

Received July 17, 1968, from the Faculty of Pharmacy, University of Alberta, Edmonton, Alberta, Canada.
Accepted for publication September 3, 1968.
Grateful acknowledgment is made to the National Research Council of Canada for financial assistance and to Dr. L. P. Chénier, Miss Joan Maxwell, and Mr. Leo Fleming of Frank W. Horner Ltd., Montreal, Quebec, Canada for performing the toxicity studies and testing some of the compounds for antiarrhythmic activity.
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Keyphrases

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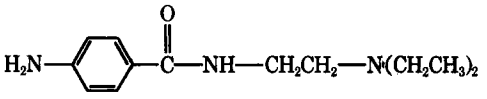

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workers, however, have studied the alteration of antiarrhythmic activity caused by various substituents on the benzene ring. Clinton *et al.* (1) reported that several of the higher members of the *N*-(2-diethylaminoethyl)-4-amino-2-alkoxybenzamides, in particular the 2-hexoxy derivative, showed outstanding properties as antiarrhythmic agents. Reisner and Cordasco (2) synthesized 2-chloroprocaine amide and found that this compound was approximately four times as potent as procaine amide in blocking atrial fibrillation in dogs. Libonati and Segre (3) studied the antiarrhythmic activity of *N*-(2-diethylaminoethyl)-*p*-4-hydroxybutylbenzamide and *N*-(2-diethylaminoethyl)-*p*-isopropylbenzamide and reported that these compounds showed an activity of 75 and 10 times, respectively, that of procaine amide. Similar compounds, such as *N*-(2-diethylaminoethyl)benzamide and *N*-(2-diethylaminoethyl)-*p*-methoxybenzamide, have also been evaluated for antiarrhythmic activity (4). The former compound was found to have a lower toxicity but the same potency as procaine amide and the latter compound to have a higher potency and toxicity than procaine amide.

The first systematic synthesis of procaine amide analogs was reported by Thyrum and Day (5). In the series of compounds they prepared, the following showed significant antiarrhythmic activity when tested on isolated

Received July 17, 1968, from the Faculty of Pharmacy, University of Alberta, Edmonton, Alberta, Canada.
Accepted for publication September 3, 1968.
Grateful acknowledgment is made to the National Research Council of Canada for financial assistance and to Dr. L. P. Chénier, Miss Joan Maxwell, and Mr. Leo Fleming of Frank W. Horner Ltd., Montreal, Quebec, Canada for performing the toxicity studies and testing some of the compounds for antiarrhythmic activity.
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TABLE I—1-ALKYLPIPERAZINES

Compd.	Alkyl	Formula	Yield, %	B.p., °C./mm. Found	B.p., °C./mm. Reported
II	$-CH_2CH_3$[a]	$C_6H_{14}N_2$	42	57–60/40	64/50 (6)
III	$-CH_2CH_2CH_3$	$C_7H_{16}N_2$	48	75–77/37	65/20 (6)
IV	$-CH_2CH_2CH_2CH_3$	$C_8H_{18}N_2$	42	97–100/40	84/10 (6)
V	$-CH_2CH(CH_3)_2$	$C_8H_{18}N_2$	57	88–90/47	78–80/13 (7)
VI	$-CH_2CH_2CH_2CH_2CH_3$[b]	$C_9H_{20}N_2$	58	116/48	105–110/9 (8)

[a] M.p. of the dipicrate 245.8–246.0° dec., reported m.p. 249° dec. (9). [b] M.p. of the di-HCl 265.0–266.8° dec., reported m.p. 267–269° dec. (8).

rabbit heart: *N*-[2-(2-indolinyl)ethyl]-*p*-aminobenzamide, *N,N*-[bis-2(*p*-aminobenzamido)ethyl]-*n*-butylamine, *N*-(2-piperidinoethyl)-*p*-aminobenzamide, and *N*-[2-(*N*-methylanilino)ethyl]-*p*-aminobenzamide. The latter compound was reported to have a potency 15 times that of procaine amide.

It would thus appear that studies of the structure-activity relationship of procaine amide so far reported have been concerned with replacement of the aromatic amino group in the molecule by other groupings, substitution on the benzene ring, and modification of the diethyl substituent on the terminal tertiary nitrogen atom. The effects on activity caused by joining the amide nitrogen and the terminal tertiary amine nitrogen together by cyclization or conversion of the terminal tertiary amine nitrogen to a tertiary amide nitrogen in the procaine amide molecule have not been examined. The present work set out to study these molecular varients in an attempt to provide additional information regarding the structure-activity relationship of procaine amide and to prepare some potential antiarrhythmic agents. It was also the intention of the authors to attempt to map a receptor for the antiarrhythmic agents.

EXPERIMENTAL

Chemical Synthesis

The synthesis of the compounds was initiated by treating excess piperazine or ethylenediamine with the various alkyl halides to produce the 1-alkylpiperazines or *N*-alkylethylenediamines. These monoalkylated piperazines and ethylenediamines were then allowed to react with benzoyl, anisoyl, *p*-chlorobenzoyl, or *p*-nitrobenzoyl chloride to yield the desired compounds. The 1-alkyl-4-*p*-nitrobenzoylpiperazines and *N,N'*-di(*p*-nitrobenzoyl)-*N*-alkylethylenediamines were reduced to the corresponding amino compounds with iron and hydrochloric acid.

Melting points were taken with a Thomas-Hoover capillary melting-point apparatus and are corrected. Elemental analyses were performed by Weiler and Strauss, Oxford, England.

1-Alkylpiperazines (Table I)—Anhydrous piperazine (50.0 g., 0.56 mole) was dissolved in absolute ethanol (150 ml.). To this solution was added the alkyl bromide (0.20 mole). The reaction mixture was refluxed for 10–12 hr. and then neutralized by sodium carbonate (0.25 mole). The ethanol was then removed by distillation, and after the addition of ice cold water (*ca.* 300 ml.), the reaction mixture was extracted with chloroform. The combined chloroform extracts were dried over anhydrous sodium sulfate and then filtered. After the removal of the solvent by distillation *in vacuo*, the products were distilled under reduced pressure.

N-Alkylethylenediamines (Table II)—The above procedure for preparing the 1-alkylpiperazines was followed, with the exception that 4 parts of anhydrous ethylenediamine were used to react with 1 part of an alkyl bromide.

1-Alkyl-4-benzoyl- and 1-Alkyl-4-p-substituted Benzoylpiperazine Hydrochlorides (Table III)—The 1-alkylpiperazine (0.2 mole) was dissolved in a mixture of dry benzene and anhydrous ether (1:1, 150 ml.). The acyl chloride (0.2 mole) in dry benzene (30 ml.) was added dropwise to the solution kept at a temperature of 0–5°. Stirring was continued for 90 min at 10° after the addition was complete. The precipitate was then collected, washed with a small amount of anhydrous ether, and recrystallized from 2-propanol.

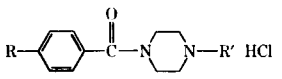
N,N'-Dibenzoyl- and N,N-Di(p-substituted benzoyl)-N-alkylethylenediamines (Table IV)—To a mixture of 10% sodium hydroxide (40 ml.) and *N*-alkylethylenediamine (0.05 mole) in chloroform (100 ml.) was slowly added a chloroform solution of the acyl chloride (0.10 mole). The resulting mixture was kept at a temperature of 0–5°. After the addition was complete, stirring was allowed to continue for 4–5 hr., while maintaining the temperature below 10°. The chloroform layer was separated and the

TABLE II—*N*-ALKYLETHYLENEDIAMINES

Compd.	Alkyl	Formula	Yield, %	B.p., °C./mm. Found	B.p., °C./mm. Reported
VII	$-CH_2CH_2CH_3$[a]	$C_5H_{14}N_2$	40	52–56/38	153–154/749 (10)
VIII	$-CH(CH_3)_2$	$C_5H_{14}N_2$	57	134/724	137–138/752 (10)
IX	$-CH_2CH_2CH_2CH_3$[b]	$C_6H_{16}N_2$	50	74–76/37	78–80/25 (10)
X	$-CH_2CH(CH_3)_2$	$C_6H_{16}N_2$	47	72–73/51	—
XI	$-CH_2CH_2CH_2CH_2CH_3$[c]	$C_7H_{18}N_2$	56	98–100/51	135–140/90 (11)

[a] M.p. of the di-HCl 206.0° dec., reported m.p. 204–205° dec. (10). [b] M.p. of the di-HCl 230.5–231.8° dec., reported m.p. 230° dec. (10). [c] M.p. of the di-HCl 235.4–236.8° dec., reported m.p. 236–237° dec. (12).

TABLE III—1-ALKYL-4-BENZOYL- AND 1-ALKYL-4-*p*-SUBSTITUTED BENZOYLPIPERAZINE HYDROCHLORIDES

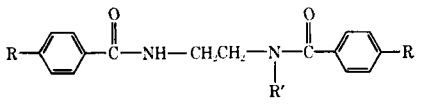


Compd.	R	R′	Formula	M.p., °C.	Yield, %		Anal., % Calcd.	Found
XII	—H	—CH_2CH_3	$C_{13}H_{19}ClN_2O$	243.0–244.5 dec.	48	C,	61.29	61.34
						H,	7.52	7.42
						N,	11.00	10.81
XIII	—H	—$CH_2CH_2CH_3$	$C_{14}H_{21}ClN_2O$	214.0–214.8 dec.	95	C,	62.55	62.60
						H,	7.88	7.74
						N,	10.43	10.10
XIV	—H	—$CH_2CH_2CH_2CH_3$	$C_{15}H_{23}ClN_2O$	224.0–224.9 dec.	88	C,	63.70	63.53
						H,	8.20	8.11
						N,	9.91	10.02
XV	—OCH_3	—CH_2CH_3	$C_{14}H_{21}ClN_2O_2$	190–192.5 dec.	93	C,	59.04	59.00
						H,	7.43	7.40
						N,	9.84	10.00
XVI	—OCH_3	—$CH_2CH_2CH_3$	$C_{15}H_{23}ClN_2O_2$	218.0–220.0 dec.	84	C,	60.29	60.19
						H,	7.76	7.71
						N,	9.38	9.62
XVII	—OCH_3	—$CH_2CH_2CH_2CH_3$	$C_{16}H_{25}ClN_2O_2$	192.0–193.0	93	C,	61.43	61.53
						H,	8.06	7.98
						N,	8.96	9.00
XVIII	—Cl	—CH_2CH_3	$C_{13}H_{18}ClN_2O$	240–243.0 dec.	76	C,	53.99	54.06
						H,	6.27	6.27
						N,	9.69	9.58
XIX	—Cl	—$CH_2CH_2CH_3$	$C_{14}H_{20}Cl_2N_2O$	249.0–250 dec.	92	C,	55.45	55.35
						H,	6.65	6.70
						N,	9.24	9.40
XX	—Cl	—$CH_2CH_2CH_2CH_3$	$C_{15}H_{22}Cl_2N_2O$	228.5–229.2 dec.	78	C,	56.78	56.76
						H,	6.99	7.06
						N,	8.83	8.58
XXI	—Cl	—$CH_2CH(CH_3)_2$	$C_{15}H_{22}Cl_2N_2O$	240.0–241.6 dec.	94	C,	56.78	56.80
						H,	6.99	7.09
						N,	8.83	8.92
XXII	—Cl	—$CH_2CH_2CH_2CH_2CH_3$	$C_{16}H_{24}Cl_2N_2O$	223.6–225.0 dec.	93	C,	58.01	58.07
						H,	7.30	7.41
						N,	8.46	8.29
XXIII	—NO_2	—CH_2CH_3	$C_{13}H_{18}ClN_3O_3$	about 289 dec.	49	C,	52.09	52.41
						H,	6.05	6.10
						N,	14.02	14.49
XXIV	—NH_2	—CH_2CH_3[a]	$C_{13}H_{19}N_3O$	137.4–138.1	56	C,	66.92	66.91
						H,	8.21	8.19
						N,	18.01	17.86
XXV	—NH_2	—$CH_2CH_3 \cdot 2H_2O$	$C_{13}H_{24}ClN_3O_3$	foams 180	45	C,	51.06	51.01
						H,	7.91	7.73
						N,	13.74	13.95
XXVI	—NO_2	—$CH_2CH_2CH_3$	$C_{14}H_{20}ClN_3O_3$	277.2–280.5 dec.	94	C,	53.59	53.54
						H,	6.43	6.41
						N,	13.39	13.43
XXVII	—NH_2	—$CH_2CH_2CH_3 \cdot H_2O$[a]	$C_{14}H_{23}N_3O_2$	96.2–102.0	81	C,	63.36	63.22
						H,	8.74	8.74
						N,	15.84	15.83
XXVIII	—NH_2	—$CH_2CH_2CH_3 \cdot 2H_2O$	$C_{14}H_{26}ClN_3O_3$	above 300	76	C,	52.57	52.46
						H,	8.20	8.24
						N,	13.14	13.10
XXIX	—NO_2	—$CH_2CH_2CH_2CH_3$	$C_{15}N_{21}ClN_3O_3$	256.0–256.6 dec.	87	C,	54.96	55.00
						H,	8.87	8.92
						N,	12.82	12.90
XXX	—NH_2	—$CH_2CH_2CH_2CH_3$[a]	$C_{15}H_{23}N_3O$	103.0–103.5	53	C,	68.93	69.13
						H,	8.87	8.92
						N,	16.08	15.72
XXXI	—NH_2	—$CH_2CH_2CH_2CH_3 \cdot H_2O$[b]	$C_{15}H_{27}ClN_3O_2$	above 280	45	C,	51.14	51.11
						H,	7.73	7.71
						N,	11.9	12.35

[a] Free base. [b] Dihydrochloride salt.

aqueous layer was washed with chloroform. The combined chloroform solutions were washed with water, dried over anhydrous sodium sulfate, and the solvent removed under vacuum. Ether or *n*-hexane was stirred into the residue until a white solid was obtained. The product was removed by filtration, washed with ether or *n*-hexane, and recrystallized from acetone and *n*-hexane.

TABLE IV—*N,N'*-DIBENZOYL- AND *N,N'*-DI(*p*-SUBSTITUTED BENZOYL)-*N*-ALKYLETHYLENEDIAMINES



Compd.	R	R'	Formula	M.p., °C.	Yield, %		Anal., % Calcd.	Anal., % Found
XXXII	$-NO_2$	$-CH_2CH_2CH_3$	$C_{19}H_{20}N_4O_6$	190.0–190.5	64	C,	56.99	57.25
						H,	5.04	4.86
						N,	13.99	14.12
XXXIII	$-NH_2$	$-CH_2CH_2CH_3$	$C_{19}H_{24}N_4O_2$	89.0–92.0	77	C,	67.03	67.50
						H,	7.11	7.11
						N,	16.46	16.10
XXXIV	$-NO_2$	$-CH_2CH(CH_3)_2$	$C_{20}H_{22}N_4O_6$	178.0–178.9	81	C,	57.96	57.95
						H,	5.35	5.69
						N,	13.52	13.58
XXXV	$-NH_2$	$-CH_2CH(CH_3)_2$	$C_{20}H_{26}N_4O_2$	156.0–156.6	93	C,	67.77	67.95
						H,	7.40	7.41
						N,	15.81	15.71
XXXVI	—Cl	$-CH(CH_3)_2$	$C_{19}H_{20}Cl_2N_2O_2$	184.2–184.9	66	C,	60.16	60.18
						H,	5.32	5.48
						N,	7.39	7.35
XXXVII	—Cl	$-CH_2CH_2CH_2CH_3$	$C_{20}H_{22}Cl_2N_2O_2$	111.8–112.5	62	C,	61.07	60.97
						H,	5.64	5.68
						N,	7.12	7.09
XXXVIII	$-OCH_3$	$-CH_2CH_2CH_3$	$C_{21}H_{26}N_2O_4$	125.9–127.0	87	C,	68.08	68.13
						H,	7.08	7.18
						N,	7.56	7.41
XXXIX	$-OCH_3$	$-CH_2CH_2CH_2CH_3$	$C_{22}H_{28}N_2O_4$	109.4–110.0	91	C,	68.73	68.74
						H,	7.34	7.37
						N,	7.29	7.47
XL	—H	$-CH_2CH(CH_3)_2$	$C_{20}H_{24}N_2O_2$	144.6–145.6	42	C,	74.04	73.68
						H,	7.46	7.50
						N,	8.64	8.65
XLI	—H	$-CH_2CH_2CH_2CH_3$	$C_{21}H_{26}N_2O_2$	105.0–106.0	47	C,	74.52	74.26
						H,	7.74	7.49
						N,	8.27	8.58

1-Alkyl-4-p-aminobenzoylpiperazines and Their Hydrochlorides (Table III)—A suspension of powdered iron (0.30 mole) in water (*ca.* 80 ml.) and concentrated hydrochloric acid (2 ml.) was heated to 85° and a warm aqueous solution of the 1-alkyl-4-*p*-nitrobenzoylpiperazine hydrochloride (0.05 mole) was added in small portions with stirring. The mixture was stirred and heated for 2 hr. at 75–80° and filtered while hot. The black residue was washed with hot water and the filtrate chilled, filtered and made alkaline with 10% sodium carbonate solution. The solution was then extracted with chloroform. The combined chloroform extracts were washed with water and dried over anhydrous sodium sulfate overnight. After removal of the solvent, the residue was dissolved in a small amount of acetone and *n*-hexane was added until the solution became cloudy. The solid 1-alkyl-4-*p*-aminobenzoylpiperazines were later collected and recrystallized from acetone and *n*-hexane, or from ethanol.

The hydrochlorides were prepared by passing dry hydrogen chloride gas into a solution of the base in anhydrous methanol and recrystallized from methanol and ether.

N,N' - Di(p - aminobenzoyl) - N - alkylethylenediamines (Table IV)—These compounds were prepared according to the method described by Clinton *et al.* (13) for the synthesis of ethyl 2-benzoyloxy-4-aminobenzoate.

Pharmacological Testing

The pharmacological testing was carried out in two stages. The first stage involved a determination of the relative activities of all the compounds synthesized on the effective refractory period of isolated electrically driven guinea pig atria. This was done by measuring the changes induced by a compound in the maximum rate of stimulation (MSR) that the suspended atria would follow with a 1:1 stimulus–contraction ratio (Table V). The rationale for this determination is based on the fact that prolongation of the refractory period of cardiac muscle is an important factor in terminating and preventing the occurrence of arrhythmias. The method of determination was that reported by MacLeod and Reynolds (14) and was essentially as follows: after the atria were properly mounted for recording of contractile activity in the tissue bath, they were driven by a Grass model S4C stimulator at progressively increasing rates with frequency increments of 12 shocks per minute for 20-sec. intervals until they failed to follow the stimulus; this was manifested in the recording as a dropped beat followed by a contraction of greater than normal amplitude. Recording of contractile activity was done by means of a force displacement transducer on a physiograph. The rate at which the atria failed to follow the stimulus was taken as the MSR. At least four control determinations were carried out, depending upon their reproducibility. The

TABLE V—EFFECT OF COMPOUNDS ON THE MAXIMUM STIMULATION RATES (MSR) OF ISOLATED GUINEA PIG ATRIA[a]

Compd.	Concn.	MSR, Shocks/min. ± SD		Average % Depression, MSR
		Control	Compd.	
XII	1:5,000	416±6.9	396±12.2	No effect
XIII	1:5,000	525±12.2	356±6.9	32.2±1.3
	1:10,000	417±24.9	360±9.8	13.7±2.3
XIV	1:5,000	438±14.7	324±12.0[b]	26.0±2.7
	1:10,000	491±7.5	357±14.8	27.3±3.1
XV	1:5,000	354±12.6	363±15.1	No effect
XVI	1:5,000	354±12.6	[c]	—
	1:10,000	453±12.6	430±8.7	5.1±1.9
XVII	1:10,000	343±4.6	[c]	—
	1:50,000	324±12.6	[c]	—
XVIII	1:5,000	431±14.4	416±6.9	No effect
XIX	1:5,000	375±6.0	[c]	—
	1:10,000	412±27.3	304±25.0	26.2±6.1
XX	1:5,000	325±12.0	320±6.9	No effect
XXI	1:10,000	354±12.6	[c]	—
	1:100,000	416±6.9	[c]	—
XXII	1:50,000	427±25.8	[c]	—
	1:100,000	375±7.5	[c]	—
XV	1:5,000	312±3.2	310±10.2	No effect
XXVIII	1:5,000	371±19.6	336±13.9	9.7±3.2
XXXI	1:5,000	410±6.9	268±6.9	34.6±1.7
	1:10,000	381±6.0	304±6.9	20.2±1.8
XXXIII	1:100,000	424±12.6	[c]	—
XXXV	1:100,000	312±6.9	[c]	—
XXXVI	1:100,000	377±6.6	[c]	—
	1:200,000	389±12.0	[d]	—
XXXVII	1:100,000	438±11.5	[c]	—
	1:200,000	354±6.9	[d]	—
XXXVIII	1:100,000	354±12.6	[d]	—
XXXIX	1:100,000	381±12.0	[c]	—
XL	1:100,000	461±7.5	460±8.7	No effect
XLI	1:200,000	336±7.5	[c]	—
Procaine amide hydrochloride	1:5,000	443±15	318±25.5	28.2±5.2
	1:10,000	440±21	376±13.9	14.5±3.1
	1:50,000	536±7.5	495±15.0	7.7±2.8
	1:100,000	499±7.5	490±6.9	No effect

[a] Unless otherwise indicated, data in this table were obtained at the 15-min. interval. [b] Calculation based on the 10-min interval, because atria became too depressed to be measured at the 15-min. interval. [c] Atria stopped beating. [d] Atria too depressed to be measured.

control value was taken as the average of these determinations. The test compound was then added to the tissue bath and the MSR was determined at 5-, 10-, and 15-min. intervals. Determinations were repeated three times after the test compound was washed out of the tissue bath and the MSR had returned to approximately control levels. The piperazine hydrochlorides were dissolved in demineralized water, and the ethylenediamine compounds in 95% ethanol so that 1 ml. of the resulting solution added to the tissue bath gave the desired concentration.

The second stage of the pharmacological testing was the evaluation of some of the compounds for their ability to prevent or to terminate arrhythmias in cats (Table VI). The method as provided by Frank W. Horner Ltd. for inducing the arrhythmias was local application of aconitine nitrate solution (25%) on the right atrium of the cat. A somewhat different procedure was employed in testing Compound XIV for antiarrhythmic activity in this laboratory. The arrhythmias were induced by petroleum ether and adrenaline (14). After a control arrhythmia produced by adrenaline following petroleum ether inhalation, the cat was then treated with the compound at doses varying between 2 and 24 mg./kg. The petroleum ether–adrenaline challenge was repeated within 15 min. of the injection of the compound. Electrocardiogram and blood pressure recordings were taken before petroleum ether inhalation had begun and continuously from just before adrenaline injection to the end of the arrhythmias. Procaine amide hydrochloride was employed as the standard.

RESULTS AND DISCUSSION

In order to determine whether any effect on the antiarrhythmic activity of procaine amide was produced by joining the amide nitrogen and the terminal tertiary amine nitrogen in the molecule by cyclization, 1-ethyl-4-*p*-aminobenzylpiperazine hydrochloride dihydrate (XXV) was synthesized by inducing a piperazine ring. It has been reported by Ellis and Sivertsen (16) that some *o*-alkylated benzhydrylpiperazines showed considerable antiarrhythmic activity. Another piperazine derivative, hydroxyzine has been introduced in clinical use for treatment of arrhythmias (17). This appears to indicate that the piperazine ring might be making a significant

TABLE VI—ANTIARRHYTHMIC ACTIVITY OF SOME COMPOUNDS[a]

Compd.	LD_{50}[b] mg./kg.	Heart Frequency Before Compd. after Aconitine per min.	Dose, mg./kg. I.V.	Heart Frequency After Compd. per min.
XIII	240	Atrial = 900 Vent. = 300	20	Atrial = 240 Vent. = 240
XVI	218	Atrial >1,000 Vent. = 600	20	Atrial = 500 Vent. = 150
XIX	60	Atrial = 400 Vent. = 200	20	Atrial = 200 Vent. = 200
XIX	60	Atrial = 600 Vent. = 200	20	[c]
XIX	60	Atrial >1,000 Vent. = 200	20/10/10[d]	Atrial = 200 Vent. = 200
XIX	60	Atrial >1,000 Vent. = 200	20	Atrial = 200 Vent. = 200
XXI	360	Atrial >1,000 Vent. = 200	20	Atrial = 280 Vent. = 140
XXVIII	142	Atrial = 600 Vent. = 300	20	Atrial = 500 Vent. = 250
XXXI	128	Atrial = 600 Vent. = 300	20	Atrial = 600 Vent. = 300
Quinidine sulfate	235	Atrial >1,000 Vent. = 200	10	Atrial = 200 Vent. = 200
Quinidine sulfate	235	Atrial >1,000 Vent. = 180	10	Atrial = 150 Vent. = 150

[a] Data supplied by Frank W. Horner Ltd. [b] Calculated by the method of Litchfield and Wilcoxon (15) and based on 3-day observations. [c] Cardiac arrest. [d] Consecutive dose.

contribution to the antiarrhythmic activity of these compounds. However, according to the data listed in Table V, Compound XXV showed no activity in reducing the MSR of isolated guinea pig atria at a concentration of 1:5,000, whereas procaine amide hydrochloride at this concentration reduced the MSR by 28.2 ± 5.2%. Since this compound did not prolong the refractory period of isolated guinea pig atria, it was considered to be devoid of antiarrhythmic activity. Therefore, joining the amide nitrogen and the aliphatic amine nitrogen together in the procaine amide molecule by cyclization appears to reduce or eliminate its antiarrhythmic activity.

Compound XXV was modified by replacing the *N*-ethyl side chain by a propyl group (XXVIII) or a butyl group (XXXI). Although the resulting compounds were capable of reducing the MSR (Table V), they failed to show any antiarrhythmic property when tested in intact animals (Table VI). Therefore, no further attempt was made to modify the *N*-alkyl chain length of Compound XXV.

Other modifications were carried out in which the aromatic amino substituent was replaced by a hydrogen, methoxy, or chloro group. Accordingly, Compounds XII–XX were synthesized. From the data in Table V it is noted that Compounds XIII, XIV, XVI, and XIX exhibited an effect on the refractory period of atrial muscle and for this reason they were tested for antiarrhythmic activity in intact animals. The results summarized in Table VI show that only Compound XIX was found to possess antiarrhythmic activity in these experiments. In three out of four trials with this material, a sinus rhythm of normal frequency was established within 2 min. and lasted from 40 min. to 3 hr. In the fourth experiment cardiac arrest brought about the death of the animal. Control cats treated with quinidine also showed a sinus rhythm maintained from 2 to 4 hr. It appears from these experiments that the antiarrhythmic activity of Compound XIX is approximately half that of quinidine. Since procaine amide hydrochloride is less potent than quinidine on a weight basis (18), Compound XIX may be considered to have an activity similar to that of procaine amide hydrochloride.

Compound XIV was investigated to determine whether it provided protection against arrhythmias induced by the combined use of adrenaline and petroleum ether in the cat. These tests showed that this compound exhibited no antiarrhythmic activity at a dose of 24 mg./kg. The dosage for procaine amide hydrochloride for preventing the occurrence of arrhythmias induced by the same technique was 8 mg./kg.

Compounds XXI and XXII were prepared following the detection of antiarrhythmic activity in Compound XIX. These two substances showed strong depressant action on the force and rate of contraction of isolated guinea pig atria even at a concentration of 1:100,000. The atria were usually completely arrested at this concentration and therefore the effect of Compounds XXI and XXII on the MSR could not be determined. Nevertheless, Compound XXI was tested for antiarrhythmic activity in intact animals and was found to be inactive.

A survey of the antiarrhythmic agents showed that the great majority of them possess a tertiary amine nitrogen in the molecule. Conn and Luchi (19) pointed out that the tertiary amine nitrogen in quinidine played an important role in the effectiveness of this drug. However, in the antiarrhythmic agent diphenylhydantoin (20) only two amide nitrogens are present in the molecule. Thyrum *et al.* (21) found that substitution of an aromatic group for one of the ethyl groups at the tertiary amine nitrogen of procaine amide gave highly active compounds. According to these workers, the basis for the increased activity of the aromatic substituents was probably due to the additional hydrophobic binding provided by the aromatic rings. Such additional binding would improve the facility for interaction between the drug and the receptor. Therefore, the

introduction of a benzoyl group on the tertiary amine nitrogen in procaine amide, as typified by the ethylenediamine compounds in this investigation, should be expected to produce the same effect. However, this alteration also changes the character of that nitrogen to an amide from an amine. Consequently, it is no longer possible for the molecule to be protonated at physiological pH. Any information with regard to the relationship between the activity and the ability of the molecule to be protonated at physiological pH would be valuable in determining whether an anionic site exists at the receptor site. The presence of a charged group on an active compound suggests a counter charged group in the receptor.

In light of this, it was considered of interest to synthesize some ethylenediamine analogs of procaine amide in which the tertiary amine nitrogen in the parent compound was replaced by a tertiary amide nitrogen and to study the effect of this modification on the antiarrhythmic activity. Consequently, Compounds XXXIII, XXXV–XLI were prepared. As shown in Table V these compounds did not reduce the MSR to any measurable extent at the concentrations used. On the other hand, these compounds exhibited a remarkable depressant action on the force and rate of contraction of the atria, even at a concentration as low as 1:100,000. At this concentration, the atrial beat was usually completely inhibited, but the activity returned shortly after several washings. In one instance, however, a concentration of 1:20,000 of Compound XXXVII was found to completely abolish the contraction of the atria despite repeated washings.

To be certain that the various effects on the isolated atria were due to the compounds themselves and not to the 95% ethanol which was employed as the solvent, control experiments with 95% ethanol were conducted. Results showed that the small quantity (0.3 ml.) of ethanol used had no effect on either MSR or force of contraction.

Despite the fact that the ethylenediamine analogs (Compounds XXXIII, XXXV–XLI) did not exhibit any activity in depressing the MSR of isolated guinea pig atria, they were tested for antiarrhythmic activity in intact animals. The results of these experiments showed that compounds XXXIII, XXXV–XLI were absolutely without antiarrhythmic activity. Therefore, it would appear that the terminal amine nitrogen in procaine amide is essential for activity.

When the effective refractory period studies demonstrated that some compounds had marked depressant action on the contraction of isolated guinea pig atria, it was considered desirable to determine whether the nature of the depressant action was due to parasympathomimetic activity. If these compounds did act by this mechanism, then a parasympatholytic substance, such as atropine, would block the depressant action. For this reason, a solution of atropine sulfate was added to the tissue bath to give a concentration of 3 mcg. of the substance and after 5 min., the test compounds were administered. The experiments were repeated three times and on two different atria. The compounds chosen for the test were Compounds XXI and XXXIX, because both showed strong depressant activity (see Table V). The concentration used was 1:100,000 for both compounds. Results of the experiments indicated that these two compounds did not appear to possess parasympathomimetic activity, since the atropine sulfate did not prevent their depressant effect in the slightest manner. The same concentration (3 mcg.) of atropine sulfate, however, was found to block the depressant action of acetylcholine in a concentration of 1:100,000. These observations confirmed that those compounds which depressed atria did so by a direct action on the muscle.

The results of this investigation have shown that the tertiary amine nitrogen in procaine amide must not be changed to an amide nitrogen, thus indicating the possibility of an anionic site on the receptor surface. This view is supported by the fact that such a tertiary amine nitrogen is found in agents of proven antiarrhythmic activity, such as quinidine, lidocaine (22), antazoline (23), hydroxyzine (17), amotriphene (24), pronethalol (25), and Compound XIX of this work. These agents can be protonated at physiological pH and therefore can bind to the anionic site on the receptor by ionic bonding. Another surface feature that can be present on the receptor is a flat area which allows binding with the area of aromaticity provided by the aromatic ring of the antiarrhythmic agents through hydrophobic or van der Waals' forces. Such an area of aromaticity is found in all antiarrhythmic agents mentioned above.

In order to establish the relative position of the anionic site to the flat area on the receptor surface, a Fisher-Hirschfelder-Taylor molecular model was prepared for all the antiarrhythmic agents cited above. It was found that despite the differences in the chemical structures, all these compounds can adopt a common orientation in which the tertiary amine nitrogen is approximately 4.5–5.0 Å. away from the center and 3.0–3.5 Å. above the plane of the area of aromaticity. The configuration considered here may not be the most stable, but one which could occur on the receptor.

This proposed receptor cannot explain why diphenylhydantoin has antiarrhythmic activity, since this molecule apparently can only bind to the flat area and not to the anionic site of the proposed receptor due to its lack of a basic nitrogen. It is probable that diphenylhydantoin exerts its antiarrhythmic action through a different mechanism.

SUMMARY

1. In the procaine amide molecule conversion of the tertiary amine nitrogen to an amide nitrogen causes complete loss of activity, as indicated by the ethylenediamine analogs (Compounds XXXIII, XXXV–XLI).

2. Converting the amide nitrogen and the terminal tertiary amine nitrogen in procaine amide to a piperazine ring produced a compound, namely, 1-ethyl-4-*p*-aminobenzoylpiperazine hydrochloride dihydrate, which has no antiarrhythmic activity.

3. The ethylenediamine analogs (Compounds XXXIII, XXXV–XLI) showed marked depressant activity on the force and rate of contraction of isolated guinea pig atria. Some 1-alkyl-4-*p*-chloro- and 1-alkyl-4-*p*-methoxybenzoylpiperazine hydrochlorides show similar effect. This depressant activity does not appear to involve the autonomic nervous system and is likely the result of a direct depressant action on the atrial muscle.

4. 1-*p*-Propyl-4-*p*-chlorobenzoylpiperazine hy-

drochloride was found to have antiarrhythmic activity in cats. It appears to have a potency similar to that of procaine amide hydrochloride. Replacement of the chloro substituent in 1-*n*-propyl-4-*p*-chlorobenzoylpiperazine hydrochloride by a hydrogen, methoxy, or amino group, or the *n*-propyl group by other alkyl groups abolished the activity.

5. A receptor for antiarrhythmic agents has been proposed to consist of an anionic site and a flat area. The anionic site is believed to be approximately 4.5–5.0 Å. away from the center and 3.0–3.5 Å. above the plane of the flat area.

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Atria, guinea pig—antiarrhythmic activity determination
Structure-activity relationship–procaine amide analogs

Linear Nonisothermal Stability Studies

By M. A. ZOGLIO, J. J. WINDHEUSER, R. VATTI, H. V. MAULDING, S. S. KORNBLUM, A. JACOBS, and H. HAMOT

A method for evaluation and utilization of data from linear nonisothermal kinetics has been developed. The studies have yielded energy of activation, reaction rate, and stability predictions from a single experiment. The relatively short length of time needed to complete a study and the comparatively few analytical experiments required present a significant advantage over classical kinetic methods. Large volumes of temperature-controlled space and the need for preliminary screening studies have been eliminated in the method. The advantage of this approach over other nonisothermal kinetic methods lies in the simplicity of equipment required and the ease of analysis of concentration-time (temperature) curves. The hydrolysis of *N*-acetyl-*p*-aminophenol and procainamide hydrochloride has been followed to demonstrate the validity of the theory and the advantages of the method.

THE USE OF stability predictive methods, such as that described in this report and other approaches (1), cannot be expected to replace the formal stability program. The predictive methods should rather be employed in cutting down the number of formulations which are subjected to formal testing by eliminating poor preparations through preformulation as well as formulation screening. The procedures which can be used to predict stability have reached a reasonable degree of sophistication. The recent upsurge of pre-

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Accepted for publication September 19, 1968.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
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formulation testing programs will most likely increase the use of predictive methods and result in further technicological growth in the field.

The classical isothermal approach to drug stability prediction has not disappeared from the industrial pharmacy scene for two reasons. First, some of the newer methods require estimates of reaction rate through slope determinations at points on a nonlinear curve and second, time-temperature relationships in the nonisothermal studies reported in the literature to date are complex and somewhat difficult to accomplish without specially designed equipment. The slope estimate method is employed in differential thermal analysis (2). A spectrophotometric method (3), which utilizes the slope of a continuous spectrophotometric curve plotted against time while the temperature of the sample cell is raised also requires a slope estimate. The first mathematically precise nonisothermal stability study was reported by Rogers (4) and uses the time-temperature relationship.

$$\frac{1}{T} = \frac{1}{T_0} - 2.303 \log (1 + t)$$

The use of Rogers' method by Cole and Leadbeater in two recent reports (5, 6) is indicative of the value of the nonisothermal technique. Limitations to this procedure which are applicable to other programmed kinetic methods including the linear method are put forth in an excellent article by Carstensen (7). The second treatment was reported by Eriksen (8) using the time-temperature relationship.

$$\frac{1}{T} = \frac{1}{T_0} - at$$

The method described in this report involves a linear relationship of time and temperature ($t = bT + C$) Energy of activation is determined by comparing analytical data to model degradation curves. The curves are obtained through digital computer solution of equations derived in the linear nonisothermal approach. The curves, once obtained for a given temperature range, can be used in any number of experiments which consider this range so that an understanding of the mathematics involved in obtaining model curves is not necessary in employing the method. After obtaining the energy of activation a simple calculation is made from the analytical and computer data to arrive at a reaction rate or stability prediction at any desired temperature. The temperature range of the experiment may be controlled by analysis of the drug degradation during the early stages of the experiment. An important feature of the method is the concentration-time (temperature) curve. It allows a visual interpretation of the effect of temperature on the degradative rate of the formulation. In the curves it can be seen quite clearly that there are fairly prominent shoulders, especially at the higher activation energies where a drug degradation rate will begin to accelerate rapidly with increased temperature.

THEORETICAL

In order to integrate the general differential equations for zero-, first-, or second-order reactions where temperature is varying with time, one must be able to express and integrate the observed rate constant as a function of time. The expression

$$k_{obs.} = ae^{-E/RT} \qquad \text{(Eq. 1)}$$

cannot be integrated for the case where temperature is changing linearly with time. It is not possible, therefore, to obtain a concentration-time equation which would yield energy of activation and reaction rate directly for the linear case.

The approach used to handle the linear time-temperature relationship is based on the following:

Consider the theoretical drug degradation in Fig. 1. In this degradation the reaction order remains unchanged throughout the experiment. The temperature is changing linearly as a function of time.

$$t = bT + C \qquad \text{(Eq. 2)}$$

The curve for the degradation can be described by a series of slopes or rate constants each representing the instantaneous rate of change of drug concentration with time at some time (temperature). The linear method is based on the assumption that the arithmetic average of these rates of change is equal to the total degradation of the drug during the experiment divided by the time span required for the

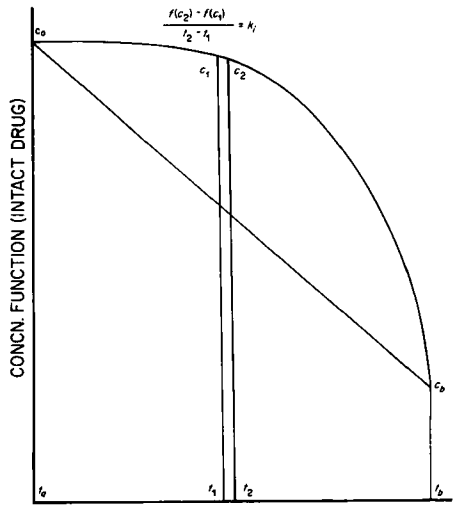

Fig. 1—Hypothetical degradation of a drug during a linear nonisothermal stability study. $t_b - t_a$ *is the total time span of the experiment. In the studies considered in this report* $b' = 1$. *Time* = b' *temperature* + *constant.*

experiment.

$$\frac{f(b) - f(a)}{b - a} = \frac{k_1 + k_2 + k_3 + \ldots k_i \ldots k_n}{n} \quad \text{(Eq. 3)}$$

If $k_i = f'(c_i)$, then the average of k over the interval (a,b) is (9):

$$\bar{k} = \frac{1}{b - a}\int_a^b f'(c)dc = \frac{f(b) - f(a)}{b - a} \quad \text{(Eq. 4)}$$

For very large n, a discrete curve should approach the smooth curve and the average of k should not differ significantly from the quantity $f(b) - f(a)/(b - a)$. The question still remains as to whether the average of k over a finite set of intervals as defined in this treatment indeed approaches $\bar{k}$ and if so how large must n be to minimize error. Both of these questions will be dealt with after formulation of the basic expressions used in applying the linear nonisothermal method.

To solve Eq. 3 for an individual rate constant, the rate constant must be expressed in terms of all other rate constants in Eq. 3. This can be done through Eq. 1. For the rate constant k_i

$$k_i = ae^{-E/RT_i} \quad \text{(Eq. 5)}$$

For the rate constant $k_{(i+1)}$

$$k_{(i+1)} = ae^{-E/RT_{(i+1)}} \quad \text{(Eq. 6)}$$

Dividing Eq. 6 by Eq. 5 yields

$$k_{(i+1)} = k_i e^{E/R\{[T_{(i+1)} - T_i]/[T_iT_{(i+1)}]\}} \quad \text{(Eq. 7)}$$

If the increment $T_{(i+1)} - T_i$ is some constant increment c then the solution for $k_{(i+2)}$ in terms of k_i becomes

$$k_{(i+2)} = k_i e^{E/R\{c/[T_{(i+1)}T_i]\}} \times e^{E/R\{c/[T_{(i+2)}T_{(i+1)}]\}} \quad \text{(Eq. 8)}$$

In this manner each rate may be expressed in terms of k_i. These expressions can be substituted for the values k_1 through k_n in Eq. 3. If $k_1 = k_i$ the following expression is obtained:

$$\frac{f(b) - f(a)}{b - a} = \frac{k_1}{n}\{1 + e^{E/R[c/(T_1T_2)]} + e^{E/R[c/(T_1T_2) + c/(T_2T_3)]} + \ldots e^{E/R[c/(T_1T_2) + c/(T_2T_3) + c/(T_3T_4) + \ldots c/(T_{(n-1)}T_n)]}\} \quad \text{(Eq. 9)}$$

The quantity in brackets can be determined using the digital computer. Since the quantity $[f(b) - f(a)]/(b - a)$ is known through experiment, rate k_1 can be calculated for a particular activation energy. In the same manner equations similar to Eq. 9 may be derived and solved for k_2 through k_n. The question as to whether the finite set of rates obtained in this manner approaches $\bar{k}$ is somewhat clarified by the following:

In dealing with a first-order reaction

$$\frac{f(c_{i+1}) - f(c_i)}{t_{(i+1)} - t_i} \quad \text{(Eq. 10)}$$

implies that $f(c) = \ln C$.

The rate constant at time zero is

$$k_0 = a^*e^{-E/RT_0} \quad \text{(Eq. 11)}$$

If this temperature is maintained for a time increment t, then the concentration at time t will be

$$C_1 = C_0 \exp(-t \cdot a^* e^{-E/RT_0}) \quad \text{(Eq. 12)}$$

If the temperature is changed to T_1 and maintained for the same increment t, after which the temperature is changed to T_2, and so on, then after n increments the concentration will be

$$C_n = C_0 \prod_{i=0}^{n-1} \exp(-t \cdot a^* \cdot e^{-E/RT_i}) \quad \text{(Eq. 13)}$$

Therefore,

$$\frac{\ln C_n - \ln C_0}{nt} = \frac{1}{nt}\left[\ln C_0 + \sum_{i=0}^{n-1} t \cdot a^* e^{-E/RT_i} - \ln C_0\right] = \frac{a^*}{n}\sum_{i=0}^{n-1} e^{-E/RT_i} = \bar{k}_n \quad \text{(Eq. 14)}$$

$\bar{k}_n$ represents the average using n discrete values.

If we consider the function:

$$\frac{a^*}{T_n - T_0} e^{-E/RT} \quad \text{(Eq. 15)}$$

then an upper Rieman sum for the function in the interval (T_0, T_n) formed by dividing the interval into n equal parts would be

$$\sum_{i=1}^{n-1}\left(\frac{T_n - T_0}{n}\right)\frac{a^*}{T_n - T_0} e^{-E/RT_i} = \frac{a^*}{n}\sum_{i=1}^{n-1} e^{-E/RT_i} \quad \text{(Eq. 16)}$$

This sum, as n approaches infinity, will tend towards:

$$\frac{1}{T_n - T_0}\int_{T_0}^{T_n} a^* e^{-E/RT}dT = \bar{k} \quad \text{(Eq. 17)}$$

such as pointed out in Eq. 4, the average of the rate constant over the integral (T_0, T_n). Hence it is reasonable to assume that for a first-order reaction arithmetic average in this treatment is adequate. The question as to how large a value of n would be to approach a smooth curve is demonstrated by testing the convergence of the bracketed expression in Eq. 9.

The reciprocal of the expression:

$$\frac{1 + \sum_{k=1}^{n-1} \exp\left[\frac{E \cdot c}{R}\sum_{i=1}^{k} T_i^{-1} T_{(i+1)}^{-1}\right]}{n} \quad \text{(Eq. 18)}$$

was evaluated for values of E at various levels of n using the values for T_0 and T_n corresponding to those used experimentally. The results, are tabulated in Table I and show rapid convergence as n approaches 384. The line of reasoning as outlined here for the first-order case can be employed for zero- and second-order reactions. For this study k_1 through k_{384} are considered and a set of 384 rates obtained for a

TABLE I—CONVERGENCE OF EXPRESSION

$$n \cdot \left\{1 + \sum_{k=1}^{n-1} \exp\left[\frac{E \cdot c}{R} \sum_{i=1}^{k} T_i^{-1} T_{(i+1)}^{-1}\right]\right\}^{-1}$$

Value of n	Value of Expression for E = 10 kcal.	E = 20 kcal.	E = 30 kcal.
8	0.322	0.0710	0.0141
16	0.292	0.0610	0.0111
48	0.271	0.0542	0.00923
96	0.267	0.0527	0.00879
192	0.267	0.0527	0.00869
384	0.267	0.0527	0.00869

particular activation energy. The rates were then used to synthesize families of model curves on a first-order or logarithmic scale. For the first-order acid-catalyzed degradation of *N*-acetyl-*p*-aminophenol and procainamide hydrochloride analytical data are superimposed over the model curves to determine activation energies (Figs. 2 and 3). The activation energy, actual experiment degradation, and computer data are then used in calculating a specific reaction rate. The reaction order is then utilized in predicting stability. The Arrhenius equation may be used with these rates to calculate rates outside of the temperature range studied.

EXPERIMENTAL

Initial experiments were carried out utilizing a temperature programmed convection oven. The equipment consisted of a convection oven (Thelco model 17) equipped with thermocouple (West program controller JSBGB-2), and saturable core reactor (West). Temperature was continuously recorded (YSI model 425C Tele-thermometer and the model G14A Varian recorder). The temperature range was arbitrarily set at 35–83° with a linear rise of 1°/hr. The accuracy of the programmed oven was found to be ±0.3°. The oven represented very

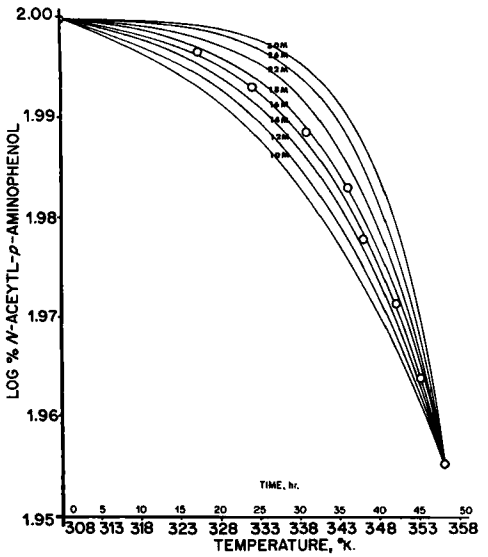

Fig. 2—Nonisothermal hydrolysis of N-*acetyl-p-aminophenol in aqueous solution (pH = 2). Temperature range, 308–356° K.;* E_a *= 17 kcal./mole;* E_a *literature = 16.69 kcal./mole.*

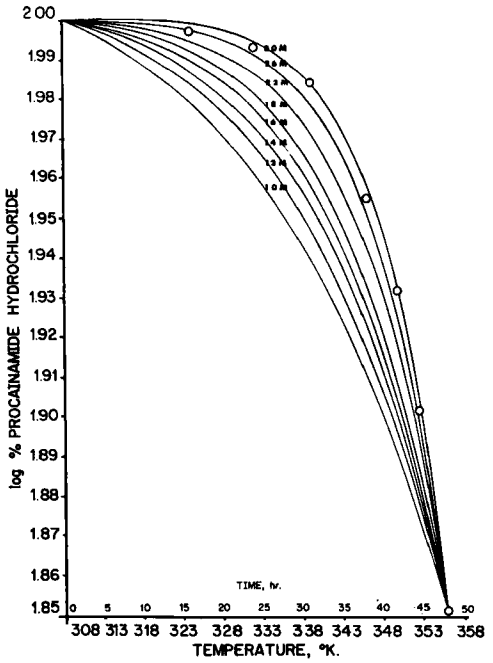

Fig. 3—Nonisothermal hydrolysis of procainamide hydrochloride in 0.934 M perchloric acid. Temperature range, 308–356°K.; E_a *= 29 kcal./mole;* E_a *literature = 13 kcal./mole.*

flexible instrumentation since the set point unit cam could be cut for a variety of temperature functions. The accuracy, however, for the linear program could be improved to ±0.1° by using a thermoregulator (Bronwill) driven by a synchronous clock motor as described by Scott (10). Temperature recording for this unit was accomplished in the same manner as for the oven.

Hydrolysis of N-Acetyl-p-aminophenol—A 0.15% solution of *N*-acetyl-*p*-aminophenol[1] in 0.2 *M* phosphate buffer (pH = 2.0) was prepared, filled into 10-ml. ampuls, and the ampuls sealed. Samples were then placed into the programmed oven and allowed to equilibrate at 35° for a period of 2 hr. A 0-hr. sample was removed and the temperature program begun. Samples were removed every 2 hr. during the first 8 hr. and analyzed for intact *N*-acetyl-*p*-aminophenol. The program of sampling was scheduled according to the results of these assays. Subsequent to the initial sampling, the ampuls were placed in a deep freeze immediately upon removal from the oven. At a convenient time the samples were quickly brought to room temperature by heating over a steam bath and analyzed for *N*-acetyl-*p*-aminophenol in the following manner.

A chromatography column (10 mm. diameter) containing 1 g. of ion-exchange resin[2] was prepared. Five milliliters of ampul solution was transferred to a 50-ml. volumetric flask and diluted to the mark with distilled water. Ten milliliters of this solution was pipeted into the chromatography column. The column was eluted with water to a volume of 100 ml. and the absorbance of the eluate determined at 243 mμ in a recording spectrophotometer (Cary model 11).

[1] Eastman Organic Chemicals, m.p. 167–168°.
[2] Rohm and Haas Co., Philadelphia, Pa.

The absorbance of the 0-hr. sample was used as the standard reading. Severely degraded samples, which distort the UV absorbance curve, were not encountered during the experiment.

Hydrolysis of Procainamide Hydrochloride (Nonisothermal)—A 0.4 % solution of procainamide hydrochloride[3] in 0.939 *N* perchloric acid solution was prepared, filled into 10-ml. ampuls, and ampuls sealed. The sampling procedure was the same as that described for *N*-acetyl-*p*-aminophenol. The programmed oven which had been used for three experiments with *N*-acetyl-*p*-aminophenol was used twice for procainamide. The third experiment with procainamide was performed using a motor-driven thermoregulator. The sampling schedule remained the same as with the oven experiments. Analysis for intact procainamide hydrochloride was accomplished in the following manner.

Four milliliters of the ampul solution was pipeted into a separator. Twenty-five milliliters of pH 9.5 buffer (sodium carbonate–sodium bicarbonate) was added. The aqueous layer was extracted with 30-ml. portions of chloroform and the extracts collected in a 100-ml. volumetric flask. The extracts were diluted to the mark with chloroform and 5.0 ml. of this solution transferred to a 50-ml. volumetric flask. This sample was diluted to the mark with chloroform and the absorbance determined at 272 mμ in a recording spectrophotometer (Cary model 11).

Hydrolysis of Procainamide Hydrochloride (Isothermal)—Hydrolysis of procainamide hydrochloride in 0.939 *N* perchloric acid was studied isothermally at constant temperatures of 35° (±0.01°), 59° (±0.01°), and 83° (±0.01°). Sampling was scheduled according to early assay results. Analytical determinations were performed in the same manner as the nonisothermal experiments.

RESULTS AND DISCUSSIONS

The analytical data for the first-order linear nonisothermal hydrolysis of *N*-acetyl-*p*-aminophenol and procainamide hydrochloride are superimposed on model degradation curves in Figs. 2 and 3. The analytical results are average results from three separate experiments and represent a total breakdown of 9.8% for *N*-acetyl-*p*-aminophenol and 28.9% for procainamide hydrochloride. The results indicate an activation energy of 17,000 cal. for *N*-acetyl-*p*-aminophenol and 29,000 cal. for procainamide hydrochloride hydrolysis.

The first activation energy is in fine agreement with the value of 16,700 cal. reported by Koshy (11). The activation energy of 29,000 cal. for procainamide hydrochloride is not in agreement with the literature value of 13,000 cal. (12). Classical isothermal techniques were employed as experimental conditions for obtaining this lower value. Reaction rates were studied at 85.3, 91.3, and 97.3°. To resolve this discrepancy, the isothermal experiment was repeated in these laboratories for the temperature range 35 to 83° and an activation energy of 27,000 cal. found (Fig. 4). The low value reported in the literature could be due to the high temperatures and narrow temperature range chosen for the study.

The information obtained through the linear non-

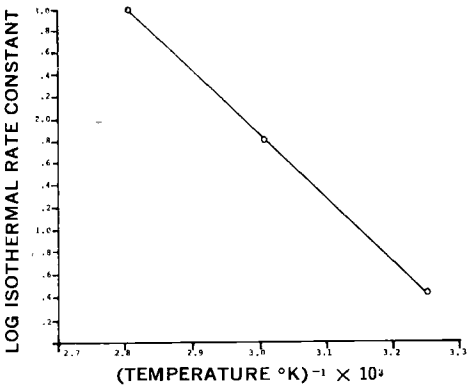

Fig. 4—Arrhenius plot showing the temperature dependency of the hydrolysis of procainamide. The energy of activation as found from this plot is 27.0 kcal./mole.

isothermal method can be used to calculate a rate constant or shelf-life prediction at some desired temperature. Using *N*-acetyl-*p*-aminophenol as an example, the following procedure would be employed in calculation of the apparent rate constant at 35°. From the left side of Eq. 9:

$$\frac{f(b) - f(a)}{b - a} = \frac{2.3 \log 100/90.2}{48}$$

$f(b) - f(a)$ represents the total first-order degradation and $b - a$ the total hours of the experiment. 90.2 is the percentage of intact drug left at the end of the experiment. From the right side of Eq. 9:

$$\frac{k_{35}\,[1 + e^{E/R[c/(T_1T_2)} + \ldots]}{n} = \frac{k_{35}\,(4278)}{384}$$

In this expression n represents the number of rate constants being considered in describing the total curve. The value of the bracketed quantity was obtained by digital computer solution of the expression with E equal to 17,000 cal. Values were generated through equations solved for k_1 through k_{384} for an activation energy range of 10–30,000 cal. in increments of 1000 cal.[4] The value for k_{35} calculated for the nonisothermal method is 1.95×10^{-4} hr.$^{-1}$. The value calculated from isothermal literature data is 2.52×10^{-4} hr.$^{-1}$. Knowing reaction order and rate, it is an easy task to integrate the appropriate rate equation to obtain a shelf-life estimation. For calculations of rates outside of the temperature range studied the Arrhenius equation may be employed.

Figures 2 and 3 are not only useful with regard to shelf-life estimation, but also present an excellent picture of the effect of temperature on reaction rate at a particular activation energy level. Analysis of these families of curves demonstrates the inadequacies of single isothermal high-temperature experiments often employed in pre formulation and formulation screening. The slopes or first derivatives at the higher temperatures can be very close yet the rates at lower temperatures widely different. If, for example, a procainamide hydrochloride solution were studied at 75° it would appear to be very unstable, whereas in fact if one examines the rates at lower

[3] The authors wish to express appreciation to the Squibb Institute of Medical Research for the generous sample of procainamide (Pronestyl).

[4] IBM 360 Fortran program will be provided to interested parties upon request.

temperature the solution is stable at these temperatures by virtue of the high activation energy of the hydrolytic reaction.

SUMMARY

Linear nonisothermal kinetic data from a single experiment can be used to obtain activation energy, reaction rate, and shelf-life prediction. The method described in this report involves a comparison of model degradation curves to experimental data for the determination of activation energy. Calculation of reaction rate and shelf-life predictions at a specific temperature are made utilizing the total experimental degradation and activation energy. The advantages of the method over the classical isothermal method lie in the use of a single experimental unit, the analysis of one set of samples, the shorter time required for completion of the experiment, and the ability to change the temperature range of the experiment without interruption of the study. The advantages over other nonisothermal methods lie in the ease of analysis of data, simplicity of time-temperature relationship, and the use of readily available laboratory equipment. The approach is limited only by assay precision, constancy of activation energy, and applicability of the Arrhenius equation. The method is proposed and is currently being evaluated for preformulation and formulation screening of ampul solutions.

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Keyphrases

Stability studies—linear, nonisothermal
Nonisothermal method—stability prediction
Kinetic equations—nonisothermal hydrolysis
Acetaminophen, procainamide HCl—test compounds
Column chromatography—separation
UV spectrophotometry—analysis

Separation and Determination of Chlorpheniramine and Its Dealkylated Metabolites from Urine

By K. ALBERT and J. J. WINDHEUSER

Application of ion-pair extraction to partition chromatographic techniques has been used effectively to separate chlorpheniramine and its dealkylated metabolites from one another in urine. Gas-liquid chromatography was employed to detect the amines. The ion-pair partition column employed selectively separated the amines prior to detection by gas chromatography. The chromatograms indicated symmetric peaks with little or no tailing typical of the secondary and primary amine metabolites. Recovery studies from urine demonstrated that at the parts-per-million level, complete recovery of chlorpheniramine and its metabolites was achieved after certain precautions against loss were taken. On the basis of these studies, an analytical procedure to separate chlorpheniramine and its metabolites from urine was developed. Recoveries of chlorpheniramine and its dealkylated metabolites were quantitative. The method offers the advantage of complete isolation of all components from one another and suggests the possibility of using ion-pair partition columns as a method to quantitatively separate other nitrogen-containing compounds from one another.

THE SEPARATION AND DETECTION of drugs from mixtures of closely related compounds is becoming more and more important. Of particular interest is the analysis of drugs and their metabolites in blood and urine in microgram quantities. This communication deals with a model system utilizing the concept of ion-pair separation of related amines from urine.

The suitability of ion-pair extraction as a process for separation and isolation of nitrogen-containing compounds in analytical samples was recently demonstrated by Higuchi *et al.* (1, 2).

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Accepted for publication September 4, 1968.
Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
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These researchers, using dextromethorphan and chlorpheniramine as examples of drugs containing one and two nitrogen centers, respectively, studied solvation effects and anion polarizability as factors responsible for the extractive equilibrium of ion pairs.

Kamm and Van Loon (3) recently reported that in dogs and rats, the major metabolite of chlorpheniramine was the *N*-desdimethylated analog while the minor metabolite was *N*-desmonomethylchlorpheniramine. Kabasakalian *et al.* (4, 5), in their study of the urinary excretion of chlorpheniramine, brompheniramine, and pheniramine in humans, reported that *N*-demethylation accounted for the metabolic degradation of the drugs.

On the basis of the above results, it was considered of interest to study the use of ion-pair formation as a method to separate and quantify nitrogen-containing compounds that undergo *N*-demethylation from their metabolites in urine. Chlorpheniramine was chosen as a model of such a drug. The general method would be applicable to other drugs of the chlorpheniramine type.

EXPERIMENTAL

All spectrophotometric measurements were made on a recording spectrophotometer (Cary model 11) using 5-cm. cells. A gas chromatograph (Barber-Coleman model 5000) equipped with a flame-ionization detector and a 1-mv. recorder was used with the following conditions: 182.9 cm. (6 ft.) × 4 mm. borosilicate column containing 2% polyethylene glycol[1] and 2.5% KOH on 100/120 mesh Gas-Chrom Q operated at 235°; detector temperature 248°; injector temperature 240°; nitrogen at 70 ml./min.

The liquid–liquid partition chromatographic columns containing buffered ion-pair formers were 19 mm. × 300 cm. and were equipped with Teflon stopcocks. The liquid level for the mobile phase was maintained constant by using a reservoir with a distended capillary tube placed approximately 5 cm. into the column.

Tapered centrifuge tubes with a capillary tip about 2 mm. internal diameter capable of containing a volume of 15 ml. were scored at 50 μl.

Chemicals and Reagents

All chemicals and reagents were analytical reagent grade unless otherwise indicated. Polyethylene glycol[1] and diatomaceous earth[2] were obtained from Applied Sciences Labs, and Johns-Manville, respectively; chlorpheniramine maleate was supplied by Schering Corp.; *N*-desdimethylchlorpheniramine and *N*-desmonomethylchlorpheniramine were furnished by Smith, Kline & French.

General Procedure for Preparation of Liquid–Liquid Chromatographic Columns—To 10 g. of diatomaceous earth 12 ml. of buffered anion solution was added and mixed well until the diatomaceous earth appeared uniformly wet and homogeneous. Enough water-saturated chloroform was added to thoroughly wet the diatomaceous earth, and portions were added to the chromatographic column containing a small pledget of silanized glass wool and about 10 ml. of chloroform. Using a suitable length of glass dowling, the diatomaceous earth was thoroughly homogenized and tamped gently, but firmly. Subsequent to packing, about 150 ml. of water-saturated chloroform was passed through the column to remove impurities. The flow rate was maintained constant at 2 drops/sec.

Table I—Comparison of Extraction Constants for Chlorpheniramine Ion Pairs at 1 *M* $CHCl_3$ Concentration

	K_e = P.C.app./$[X^-]$ $(1 + H^+/K_a)$
Perfluorohexanoic acid	10
Trifluoroacetic acid	2.1×10^{-3}
Bromide	1.4×10^{-5}

General Procedure for Recovery Studies of Chlorpheniramine and Its Metabolites from Urine—All glassware used in this study was treated with hydrophobic monomers[3] to eliminate possible drug adsorption. The hydrophobic monomers were freely applied and the glass allowed to air dry. Following a chloroform rinse, the glass surface was rinsed with ethyl ether and allowed to dry.

To 25 ml. of urine contained in a 50-ml. glass-stoppered centrifuge tube, 25 mcg. (1 p.p.m.) of drug was added. The solution was brought to a pH of 10 with 6 *M* NaOH and extracted with three 15-ml. portions of anhydrous ethyl ether. The solution was centrifuged and the ether layer withdrawn and collected in a beaker containing 500 mg. of diatomaceous earth. The hydrochloride salt of the drug was formed by bubbling in HCl gas prior to volatilization of the ether under nitrogen gas. Five-tenths milliliter of 6 *M* NaOH was added followed by quantitative transfer to the liquid partition column. The beaker was dry rinsed with an additional 200 mg. of diatomaceous earth, the rinsings added to the column, and the column tamped.

The drug was eluted with the appropriate chloroform phase and the eluate collected in a round-bottom flask. The chloroform was slowly evaporated under nitrogen at room temperature to about 10 ml. and quantitatively transferred to a 15-ml. tapered tube. After evaporating the chloroform nearly to dryness, the residue was dissolved in dimethylformamide and diluted to the 50-μl. mark with dimethylformamide. A 2-μl. aliquot was injected onto the polyethylene glycol–KOH column and the area corresponding to the drug compared against a suitable standard solution in dimethylformamide.

RESULTS AND DISCUSSION

It has been established that chlorpheniramine formed ion pairs whose extraction constants varied depending upon the anion employed (2). Table I lists a comparison of the extraction constants for chlorpheniramine obtained in this study and by Higuchi and Kato. Because the metabolites of chlorpheniramine are secondary and primary amines, their extraction constants into chloroform should be

[1] Carbowax 20M, Applied Science Labs, State College, Pa.
[2] Celite 545, Johns-Manville, New York, N. Y.
[3] Desicote, Beckman Instruments, Inc., Fullerton, Calif.

TABLE II—EFFECT OF VARIOUS ANIONS ON THE COLUMN CHROMATOGRAPHIC SEPARATION OF CHLORPHENIRAMINE AND ITS DEMETHYLATED METABOLITES

	% Eluted[a]	
	Chlorpheniramine	Desmonomethylchlorpheniramine
Perfluorohexanoic acid, 0.25 *M*		
Fraction		
1	100	100
2		
Trifluoroacetic acid, 0.25 *M*		
Fraction		
1	100	85
2	—[b]	15
3	—	—
Sodium bromide, 0.25 *M*		
Fraction		
1	80	—
2	20	—
3	—	22
4	—	60
5	—	15
6	—	3

[a] *N*-desdimethyl chlorpheniramine was not eluted under these conditions. [b] Dashes indicate no detectable drug.

TABLE III—EFFECT OF VARYING BROMIDE ANION CONCENTRATION ON THE COLUMN CHROMATOGRAPHIC SEPARATION OF CHLORPHENIRAMINE AND ITS DEMETHYLATED METABOLITES

	% Eluted[a]	
	Chlorpheniramine	Desmonomethylchlorpheniramine
Sodium bromide, 1 *M*		
Fraction		
1	100	55
2	—[b]	45
3	—	—
Sodium bromide, 0.50 *M*		
Fraction		
1		
1	99	30
2	1	65
3	—	5
4	—	—
Sodium bromide, 0.25 *M*		
Fraction		
1	80	—
2	20	—
3	—	22
4	—	60
5	—	15
6	—	3

[a] *N*-Desdimethylchlorpheniramine was not eluted under these conditions. [b] Dashes indicate no detectable drug.

different enough from chlorpheniramine to allow separation of the amines from one another.

Liquid–liquid partition columns containing 0.25 *M* perfluorohexanoic acid, trifluoroacetic acid, and sodium bromide in 0.01 *M* citrate buffer at pH 3.5 were prepared, and 0.5 mg. each of the amines was eluted from individual columns using water-saturated chloroform as eluate. Twenty-five-milliliter fractions were collected and determined spectrophotometrically from 300–250 mμ. The results of this experiment are presented in Table II. It can be seen that only the bromide column effectively separated each of the amines. An examination of Table I will reveal a difference of almost 10^6 in the extraction constants for chlorpheniramine using perfluorohexanoic anion and bromide anion as ion-pair formers. The same differences could be anticipated for ion pairs of the metabolites. However, if the partition coefficients of all three amine-ion pairs into chloroform were high, regardless of their individual differences in magnitude, then it would be difficult to separate them effectively on a partition column since all three would be eluted rapidly. To further illustrate this point, Table III exhibits the results of an experiment in which partition columns containing 1 *M*, 0.5 *M*, and 0.25 *M* bromide ion were prepared, buffered as before. It would be expected that the magnitudes of the partition coefficients for the amines from 1 *M* bromide to 0.25 *M* bromide would be considerably less than 10^6, yet only 0.25 *M* bromide effectively separated chlorpheniramine from its secondary metabolite. Consequently, anion polarizability as well as concentration are factors that contribute to separation. The primary metabolite was not eluted from any of the columns.

In an attempt to explain the lack of elution of the *N*-desdimethyl metabolite, it was suggested that the compound either did not form an ion pair or was so strongly adsorbed to the diatomaceous earth that even though an ion pair was formed, it was not eluted. Partition coefficients were determined for chlorpheniramine and its metabolites using equal volumes of mutually saturated pH 3.5, 0.25 *M* bromide in 0.01 *M* citrate buffer, and chloroform. The data, tabulated in Table IV, indicate that although the primary metabolite formed an ion pair with bromide with a partition coefficient close to that of the secondary amine, the more polar, basic primary metabolite was probably adsorbed by the diatomaceous earth during column separation.

Chloroform saturated with ammonia was used to elute *N*-desdimethylchlorpheniramine which was found between the fourth and tenth 25-ml. fractions.

Table V illustrates the complete separation as well as quantitative and reproducible results obtained for elution of 500 mcg. each of the amines from the 0.25 *M* bromide partition column. Eluting with water-saturated chloroform, the first 35 ml. containing only chlorpheniramine was determined spectrophotometrically and the percent recovery of chlorpheniramine calculated. In a similar manner, the next 150 ml. of eluate containing only the secondary metabolite was collected in 25-ml. fractions, and the recovery of *N*-desmonomethylchlorpheniramine calculated.

TABLE IV—PARTITION COEFFICIENTS USING 0.25 *M* BROMIDE ION IN 0.01 *M* CITRATE, pH 3.5 OF ION PAIRS OF CHLORPHENIRAMINE AND ITS METABOLITES

Tertiary Amine	Secondary Amine	Primary Amine
1.500[a]	0.280[a]	0.193[a]

[a] Corrected for free base extraction.

TABLE V—RECOVERIES OF AMINES FROM BROMIDE-PARTITION COLUMN

Trial	3° Amine, %	2° Amine, %	1° Amine, %
1	100.0	99.7	100.0
2	100.0	99.7	100.0
3	100.1	100.1	100.1
4	100.0	100.0	99.7
5	100.1	100.1	99.9

The eluent was changed to ammonia-saturated chloroform, the first 75 ml. discarded, and the following 175 ml. containing only *N*-desdimethylchlorpheniramine collected in 25-ml. fractions. The percent recovery of the primary metabolite was calculated from spectrophotometric data.

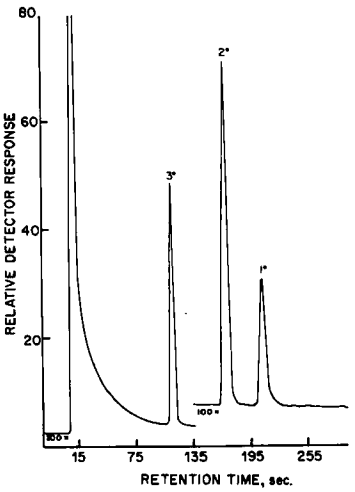

Fig. 1—Chlorpheniramine and its demethylated metabolites, 2 mcg. in dimethylformamide. Column: Gas-Chrom Q with 2.5% KOH, 2% polyethylene glycol. Nitrogen flow rate 70 ml./min. Injector 240°, column 235°, and detector 248°. Attenuation as noted on plot.

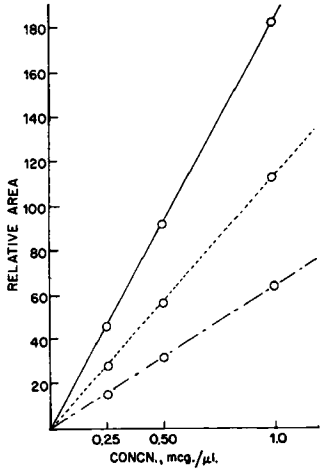

Fig. 2—Detector response for chlorpheniramine and its metabolites. Key: ——, chlorpheniramine maleate; - - -, N-desmonomethylchlorpheniramine; — - —, N-desdimethylchlorpheniramine.

TABLE VI—RECOVERIES OF CHLORPHENIRAMINE AND ITS METABOLITES AT 1 p.p.m. FROM URINE

Chlorpheniramine, %	Secondary Metabolite, %	Primary Metabolite, %
97.6	96.3	97.6
101.1	98.3	99.1
98.6	98.2	96.9
102.2	99.7	98.4
Av. 100.0	98.1	98.0

Recovery of microgram quantities of the drug and its metabolites from urine necessitated the need for a sensitive method of detection. Gas chromatography was chosen. However, since the analytical method itself is specific, any sensitive, nonselective means of determination would be suitable. Figure 1 illustrates the symmetrical peaks obtained for 2 mcg. of chlorpheniramine and its metabolites in dimethylformamide on the polyethylene glycol –KOH column. For chlorpheniramine, 2,500 theoretical plates were calculated, 3,010 for the secondary metabolite, and 2,580 for the primary metabolite. The linear flame-ionization response for the amines is illustrated in Fig. 2.

Recovery studies were next initiated at the 1-p.p.m. level from urine. Aqueous solutions of chlorpheniramine and its metabolites were prepared and 25 mcg. of each drug added to 25 ml. of urine. During evaporation of the ether extract under nitrogen gas, an all-glass system was used to eliminate contamination of the sample with plasticizer. Loss of the secondary and primary amines as free bases from ethereal solution was eliminated by forming hydrochloride salts. Further, since the primary metabolite was eluted from the bromide partition column as a free base, the hydrochloride salt of the drug was formed in the chloroform solution prior to volatilization. Table VI exhibits the quantitative and reproducible recoveries of chlorpheniramine and its metabolites from urine.

CONCLUSIONS

In the present study, an attempt has been made, using chlorpheniramine as a model drug undergoing *N*-demethylation, to evaluate the feasibility of using ion-pair formation as a method to selectively separate the drug from its metabolites. In this investigation the selectivity of various anions on the column chromatographic separation of chlorpheniramine and its demethylated metabolites was studied. On the basis of the studies described, a general method for selectively separating *N*-demethylated metabolized drugs and their metabolites is proposed. The method offers the advantage of complete isolation of all components prior to determination, permitting a nonselective means of analysis.

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Keyphrases

Chlorpheniramine, dealkylated metabolites—analysis
Urine—chlorpheniramine, metabolites, separation, determination
Ion-pair partition chromatography—separation
GLC—analysis
UV spectrophotometry—analysis

Comparative Effects of Chlorpromazine Hydrochloride and Quaternary Chlorpromazine Hydrochloride on the Central Nervous Systems of Rats and Mice

By N. WATZMAN*, A. A. MANIAN†, H. BARRY, III, and J. P. BUCKLEY

The effects of quaternary chlorpromazine HCl (QCPZ) and the tertiary parent compound chlorpromazine hydrochloride (CPZ) on the central nervous system were investigated in male rats and mice. CPZ was much more potent than QCPZ in inducing depressant effects on spontaneous activity of mice, forced motor activity of rats, continuous shock avoidance of rats, and potentiation of hexobarbital sleeping time in mice. Neither compound significantly altered the threshold of convulsive seizures induced by either pentylenetetrazol or strychnine. Although quaternization of the CPZ molecule at the terminal side chain nitrogen produced a decrease in central nervous system activity, the resulting compound was much more toxic than CPZ.

THE QUATERNIZATION of chlorpromazine (CPZ) by introducing a functional ^{14}C-methyl group into the terminal side chain nitrogen provides a highly sensitive and convenient method for the quantitative analysis of minute amounts of this compound and some of its metabolites in human blood plasma (1). Quaternary ammonium compound formation, in all likelihood, could also be used for determining plasma levels of other psychoactive amines employed in the treatment of the mentally ill.

Scientific literature discloses two references to biological information on this quaternary compound.[1] The first appears in a summary of an investigation by Seeman (2) on *in vitro* membrane stabilization by the phenothiazines, and the other by Fog *et al.* (3) describing the effects of quaternary phenothiazines administered *via* intracerebral injection in rats. Some quaternary phenothiazines such as 10-(α-dimethylamino-propionyl)-phenothiazine methobromide[2] (4, 5) have anticholinergic and ganglion-blocking actions while others such as 1-(10-phenothiazinylmethyl)-ethyl-2-hydroxyethyldimethyl ammonium chloride[3] (6–9) possess antihistaminic activity. Excellent review articles on the chemistry and pharmacologic actions of quaternary ammonium compounds, in general, have been presented by Cavallito and Gray (10) and D'Arcy and Taylor (11, 12).

This current study was undertaken to investigate the comparative pharmacologic activity of the quaternary chlorpromazine, 1-(2-chloro-10-phenothiazinyl)propyl - 3 - trimethylammonium chloride (QCPZ) (I) and the tertiary parent drug chlorpromazine HCl (CPZ),[4] in a battery of biological tests.

Received July 29, 1968, from the Department of Pharmacology, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15213
Accepted for publication September 11, 1968.
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[2] Secergan, Aktiebolaget Astra, Sodertalje, Sweden.
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Keyphrases

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Urine—chlorpheniramine, metabolites, separation, determination
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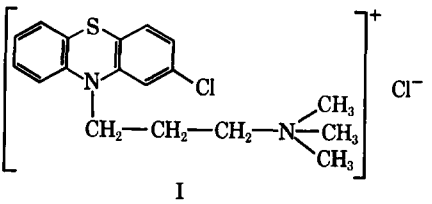


I

METHODS

Subjects—Male albino mice (Swiss-Webster) and rats (Wistar descendant) were supplied by Hilltop Laboratory Animals, Inc., Scottdale, Pa.

Drug Administration—Both QCPZ and CPZ were injected intraperitoneally in an isotonic saline solution of 0.1 ml./g. for mice or 1.0 ml./kg. for rats. The same volume of the isotonic saline served as the placebo.

Gross Behavior—QCPZ was evaluated by a gross-observation rating scale which reflects qualitative drug effects on gross behavior. The scale is divided into four major drug actions: (*a*) stimulation, (*b*) depression, (*c*) autonomic activity, (*d*) reflexes and tone. Each of these actions is, in turn, subdivided into component characteristic responses; for example, motor activity and body tremors are two of the components of stimulation. Each trial consisted of the observation of a drug-treated animal simultaneously with a nontreated one. Items on the scale were checked 15, 30, 60, 120, and 240 min. after drug administration, so that the time course of the drug effect might be ascertained. QCPZ was administered intraperitoneally to two to six rats and mice at each dose level, ranging from 6.25 to 100 mg./kg. The effects of CPZ, in comparable doses, were observed in the rats used in the spontaneous activity test and in the mice used in the hexobarbital potentiation test. In addition, the 72-hr. LD_{50} was determined for both compounds, with 12–14 mice at each dose level. Toxic doses of QCPZ were also tested in a small number of rats.

Spontaneous Motor Activity—The effect of both compounds on the spontaneous motor activity of rats was measured in four photocell activity cages (Actophotometer, Metro Industries, Inc., New York, N. Y.). A single animal was placed in each photocell cage 1 hr. after drug administration and counts were recorded every 15 min. for a total of 1 hr. The doses were selected from the data obtained in the gross-observation studies. Animals tested simultaneously were administered saline, 1, 2, and 4 mg./kg. in one experiment, and saline, 6.25, 12.5, and 25.0 mg./kg. in the other experiment. Each dose was tested in a factorial design in each of the four activity cages in order to negate the differences in sensitivity among the units. This design, requiring 16 animals, was replicated once so that a total of 32 animals was tested for each compound (eight animals per dose level, including saline). The ED_{50} of each compound, defined as the dose which decreased the level of performance to 50% of the control scores, was calculated according to the method of Miller and Tainter (13).

Forced Motor Activity—The effects of the two compounds on forced motor activity of mice were compared on a rotarod, 5.08 cm. (2 in.) in diameter. The procedure was similar to that described by Watzman and Barry (14): the wooden rod rotated at 4 r.p.m. during the first 30 sec. of each trial, at 6 r.p.m. during the next 30 sec., and at progressively increasing speeds thereafter at 30-sec. intervals until the animals fell off. Six animals, tested simultaneously, were given 10 trials, with two spaced several hours apart on each of 5 consecutive days. The tenth trial was preceded at an interval of 1 hr. by the administration of saline or one of three doses of either compound (1, 2, and 4 mg./kg.). The use of eight groups of six animals provided a total of 48 test subjects with 12 animals per dose level, including the saline group. The animals tested simultaneously included each of the four dosage conditions; each dose was tested an equal number of times in each of the six rod positions in a factorial design. The drug or placebo effects for each animal were computed as the ratio of performance time on the tenth trial divided by performance time on the ninth trial. A second experiment was performed with QCPZ only, using 6.25, 12.5, and 25 mg./kg.

Continuous Shock Avoidance—Standard operant-conditioning chambers (Lehigh Valley Electronics, Inc., Fogelsville, Pa.), each with two levers on one wall, were used for a two-lever continuous shock-avoidance task with a separate shock-escape response. Painful electric shocks (250 v. a.c. through a series resistance of 150,000 ohms) were programmed to be delivered on the grid floor for a duration of 2 sec. with a 5-sec. interval between shocks. A press on one lever (Escape) terminated the shock, initiating the 5-sec. S-S interval. Each press on the other lever (Avoidance), provided the shock was not currently on, initiated a longer, 20-sec. R-S interval until the next shock. Effects of the compounds were tested in 17 rats which had acquired a stable and proficient level of avoidance performance in more than 20 previous 3-hr. sessions. The test sessions were preceded at an interval of 1 hr. by injection of CPZ, QCPZ, or saline. Drug sessions alternated with saline sessions. The first two drug treatments were 2 mg./kg. CPZ and 2 mg./kg. QCPZ. Each of these treatments was given first to half the animals in order to counterbalance the sequence. Subsequent drug sessions consisted of progressively higher doses of QCPZ (4, 8, and 16 mg./kg.). Eight of the animals were selected to receive additional doses of QCPZ (24, 32, and 48 mg./kg.) without intervening saline sessions.

Hexobarbital Sleeping-Time Potentiation—A total of 40 mice was divided into four groups of 10 animals each; three groups received 4, 8, or 16 mg./kg. QCPZ 1 hr. prior to the intraperitoneal administration of 100 mg./kg. of hexobarbital; the last group received 100 mg./kg. hexobarbital only. The same drug regimen was used for CPZ, except that only the two lowest doses (4 and 8 mg./kg.) were administered. Sleeping times for the animals of all groups were recorded as the interval between the loss and return of righting reflex. The criteria for the loss and return of the reflex are defined as the inability and ability, respectively, of the animal to right itself within 5 sec. in three successive trials when placed on its back. The trials were arbitrarily terminated 3 hr. after the loss of righting reflex if the animals continued to sleep.

Pentylenetetrazol Antagonism—The protection of mice from tonic convulsions and death produced by pentylenetetrazol was studied in the following manner. A total of 77 male albino mice was divided into

four treatment groups: the first five of these (10 mice each) received 4 mg./kg. CPZ or QCPZ, 8 mg./kg. CPZ or QCPZ and saline, respectively, 1 hr. prior to the subcutaneous administration of 100 mg./kg. pentylenetetrazol; the last three groups (nine animals) received 16 mg./kg. CPZ or QCPZ and saline, respectively, 1 hr. prior to the administration of pentylenetetrazol. Protection from convulsions was indicated if tonic extension of the hind limbs for 5 sec. or longer did not occur. Time of convulsions and onset of death were recorded for each animal.

Strychnine Antagonism—The same procedure and same number of animals as for pentylenetetrazol antagonism were used except that strychnine (2 mg./kg., i.p.) was the convulsant employed. The highest dose level (16 mg./kg.) of both CPZ and QCPZ was tested simultaneously on a different day than the two lower dose levels, thus requiring a separate control group (saline).

RESULTS

Gross Behavior—The onset of action of QCPZ usually occurred within 15 min. in mice and 30 min. in rats. Minimal depression was produced in rats by doses of 6.25, 12.5, and 25.0 mg./kg. and consisted of decreased motor activity, decreased irritability, and decreased startle response with no signs of autonomic effects or effects on reflexes and tone. Mice, however, did not display central nervous system depression, but autonomic activity was indicated by exophthalmos, mydriasis, and increased urination. Both mice and rats treated with the same doses of CPZ were so profoundly depressed that they did not move even when prodded.

Toxic effects of QCPZ were noted in doses ranging from 30–100 mg./kg. These effects usually consisted of ataxia and labored respiration. Death usually occurred within 5 min. in mice, which was much quicker than in rats. The 72-hr. LD_{50} in mice was 170 ± 69 mg./kg. for CPZ and 35.5 ± 2.1 mg./kg. for QCPZ. A lethal effect of QCPZ occurred in one of two rats after 30 mg./kg. and in three of four rats after 100 mg./kg. The LD_{50} for CPZ injected intraperitoneally was 115 mg./kg. for mice and 85 mg./kg. for rats in one prior study (15) and 215 mg./kg. for mice and 150 mg./kg. for rats in another prior study (16). The LD_{50} values of the present study are similar for CPZ but much lower for QCPZ.

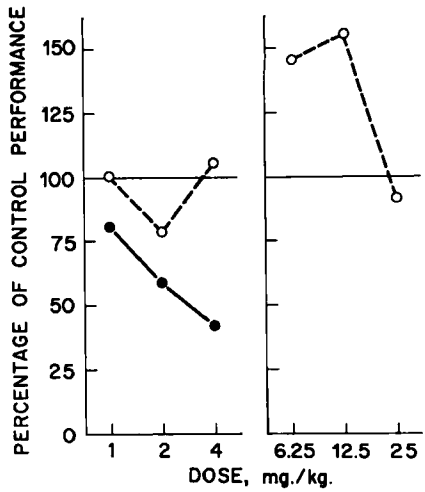
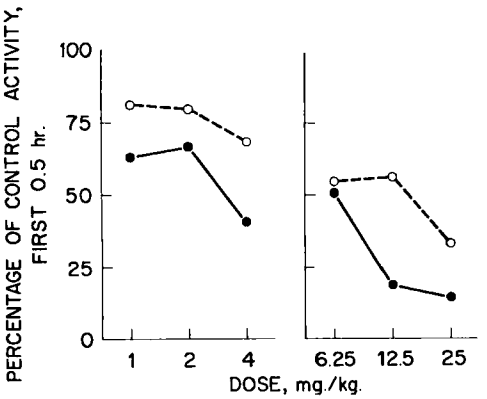


Fig. 2—Comparative effects of chlorpromazine hydrochloride and quaternary chlorpromazine hydrochloride on rotarod performance of mice. Key: O--O, QCPZ; ●—●, CPZ.

Spontaneous Motor Activity—Figure 1 shows the comparative effects of CPZ and QCPZ on spontaneous activity of rats in two separate experiments. In both the lower and higher dose ranges, CPZ decreased activity to a greater degree. The ED_{50} values were 12.0 mg./kg. for QCPZ and 4.3 mg./kg. for CPZ. Thus QCPZ appears to have much less potency than CPZ.

Forced Motor Activity—Figure 2 shows the comparative effects of CPZ and QCPZ on the rotarod performance times of mice. In doses of 1, 2, and 4 mg./kg., CPZ clearly decreased motor coordination, whereas QCPZ had no reliable inhibiting effect. In the second experiment, higher doses of 6.25, 12.5, and 25 mg./kg. QCPZ likewise had no reliable inhibitory effect. In this test of forced motor activity, the ED_{50} for CPZ was 2.8 ± 1.1 mg./kg., whereas the



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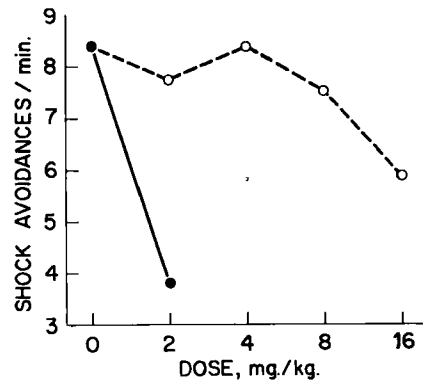

Fig. 3—Comparative effects of chlorpromazine hydrochloride and quaternary chlorpromazine hydrochloride on continuous shock avoidance by rats. Key: O-O, QCPZ; ●—●, CPZ.

TABLE I—COMPARATIVE EFFECTS OF QUATERNARY CHLORPROMAZINE HCl AND CHLORPROMAZINE HCl ON THE HEXOBARBITAL SLEEPING TIME OF MICE IN MINUTES

Mouse No.	QCPZ				CPZ		
	Control[a]	4[b]	8[b]	16[b]	Control[a]	4[b]	8[b]
1	50	100	32	77	17	101	172
2	41	94	135	82	21	95	180+
3	34	155	23	101	—	89	180+
4	46	151	65	41	33	117	180+
5	41	109	70	84	40	119	180+
6	56	90	76	82	54	161	127
7	48	155	60	33	25	116	167
8	56	126	67	66	45	97	126
9	56	93	66	77	23	111	140
10	78	143	40	90	35	157	180+
$\bar{X}$	50.6	121.6	63.4	73.3	32.6	116.3	163.2
± *SE*	3.8	8.7	9.7	6.7	4.1	7.8	7.3
% (incr.)	—	142.0	25.3	45.0	—	256.7	400.6

[a] = hexobarbital (100 mg./kg. i.p.) only. [b] = mg./kg.

ED_{50} for QCPZ could not be determined but was definitely above 25 mg./kg.

Continuous Shock Avoidance—Figure 3 shows the rate of shock avoidances for the entire 3-hr. session in four placebo sessions averaged together and after CPZ (2 mg./kg.) and QCPZ (2, 4, 8, and 16 mg./kg.). Performance was decreased to 46% of the control rate by 2 mg./kg. CPZ, whereas a reliable effect of QCPZ was found only at 16 mg./kg., with performance at 70% of the control rate. A precise ED_{50} could not be determined for CPZ because of the single dose used, but appears to be less than 2 mg./kg., in agreement with previous reports that shock avoidance is impaired by low doses of this compound (17). The ED_{50} for QCPZ could not be determined but appears to be greater than 16 mg./kg. Evidence for severe toxicity of QCPZ at 16 mg./kg. was found in that five of the 17 animals died within 24 hr. after this dose. Among the eight animals tested with higher doses of QCPZ, two died within 4 hr. after 32 mg./kg. and three within 4 hr. after 48 mg./kg. In spite of these lethal effects in the majority of animals, no consistent performance decrement was found in the survivors, even at 48 mg./kg.

Hexobarbital Sleeping-Time Potentiation—Table I shows the comparative effects of CPZ and QCPZ on the hexobarbital sleeping times of mice. CPZ, 8 mg./kg., prolonged average sleeping time almost to the maximum of 180 min., whereas QCPZ was much less effective and the responses were not dose related.

Pentylenetetrazol Antagonism—Pentylenetetrazol produced both clonic and tonic convulsions, the former occurring approximately 3–5 min. after injection, whereas tonic extension of the hind limbs and arching of the back began approximately 10 min. after administration. Death usually occurred immediately following the tonic seizures. Neither CPZ nor QCPZ had any statistically significant effects, but only seven of the ten animals convulsed after 8 mg./kg. of QCPZ, and there was a slight increase in the time before death after 4 and 8 mg./kg. of CPZ.

Strychnine Antagonism—Strychnine produced seizures in the form of tonic extension of the hind legs and arching of the back about 3–7 min. after administration, usually followed by death. CPZ and QCPZ had almost exactly the same effects as in the pentylenetetrazol test; only seven of the nine animals convulsed after 16 mg./kg. QCPZ, whereas all nine control animals convulsed, and CPZ showed slight evidence of increasing the time of onset before convulsions or before death. None of these effects of QCPZ or CPZ was statistically reliable.

DISCUSSION AND CONCLUSION

QCPZ and CPZ were studied in a battery of tests designed to compare their effects on the central nervous system. The tests were: gross behavior, spontaneous motor activity, forced motor activity, continuous shock avoidance, hexobarbital sleeping-time potentiation, and pentylenetetrazol and strychnine antagonism. In each of the first five tests, CPZ clearly appeared to be the more potent compound, although the LD_{50} was much higher for CPZ than for QCPZ. In the gross-observation experiments, both compounds produced a sedative profile in doses ranging from 1–25 mg./kg. CPZ produced generalized depression to a large degree, whereas QCPZ-induced depression was minimal. From the standpoint of the central nervous system activity of QCPZ, the ED_{50} and doses producing minimal depression are close to the lethal dose range, so that the theoretical therapeutic index is much smaller than that of CPZ. Both compounds potentiated hexobarbital sleeping times with CPZ producing the greater effect. However, neither compound protected the test animals from death due to strychnine or pentylenetetrazol. The data suggest that QCPZ, in common with 1-(10-phenothiazinylmethyl)-ethyl-2-hydroxyethyldimethyl ammonium chloride (9), fails to penetrate the blood-brain barrier to an appreciable degree. Therefore, the effect of low doses of CPZ on various measures of performance, especially shock avoidance (17), can be attributed to the central rather than peripheral actions of CPZ.

In conclusion, quaternization of the CPZ molecule at the terminal side chain nitrogen produces a marked increase in toxicity and a decrease in central nervous system activity.

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Keyphrases

Chlorpromazine HCl—quaternary, tertiary forms
Quaternary, tertiary chlorpromazine HCl—activity compared
Motor activity, spontaneous, forced—chlorpromazines
Shock avoidance—chlorpromazines
Hexobarbital sleep-time—chlorpromazines

Interaction of Methyl and Propyl Parabens with Selected Sucrose Esters

By CARLOS VALDEZ, EUGENE I. ISAACSON, and FRANK P. COSGROVE*

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NUMEROUS REPORTS in recent years have noted the inactivation of various preservatives in the presence of surface-active agents commonly employed in pharmaceutical preparations (1–11). Examples which have been frequently cited are the inhibition of preservative activity of phenolic compounds in the presence of polyether derivatives of fatty acid esters.

The introduction, in 1956, of a novel series of nonionic surfactants, the fatty acid esters of sucrose, and their proposed use in forming emulsion bases and as dispersing agents in pharmaceutical suspensions warrants a consideration of their possible interaction with preservatives commonly used in such systems. A previous investigation by Blaug and Ebersman (12) using the dialysis method revealed evidence of interaction between fatty acid mono and diesters of sucrose and fatty acid monoesters of propoxylated sucrose and derivatives of benzoic acid. The present study employs the solubility method to investigate the interaction between methyl and propyl *p*-hydroxybenzoates with sucrose monotallowate and sucrose monococoate. The effect of elevation of temperature and change of concentration of surfactant on the association is considered. Microbiologic studies have been designed so as to permit a direct correlation of preservative activity and the degree of binding of the preservative.

EXPERIMENTAL

Materials—Recrystallized methyl *p*-hydroxybenzoate, m.p. 124–126°; recrystallized propyl *p*-hydroxybenzoate, m.p. 95–96°, Eastman Organic Chemicals; sucrose monotallowate; sucrose monococoate, Sucro-Chemical Division, Colonial Sugar Co., Gramercy, La.

Solubility Method—The interaction of methyl and propyl parabens with sucrose monotallowate and sucrose monococoate was studied at different concentrations and temperatures according to the Higuchi and Zuck (13–15) solubility method.

Received April 11, 1968, from the College of Pharmacy, University of Texas, Austin, TX 78712
Accepted for publication September 12, 1968.
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Keyphrases

Chlorpromazine HCl—quaternary, tertiary forms
Quaternary, tertiary chlorpromazine HCl—activity compared
Motor activity, spontaneous, forced—chlorpromazines
Shock avoidance—chlorpromazines
Hexobarbital sleep-time—chlorpromazines

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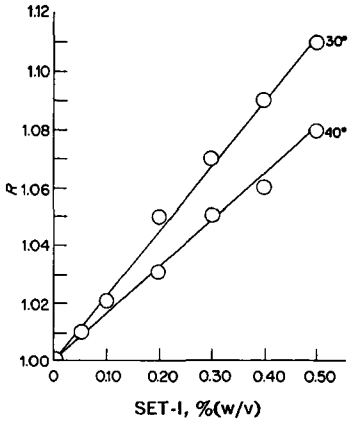

Fig. 1—Ratio R *(total methyl paraben to free methyl paraben) as a function of the concentration of sucrose monotallowate (SET-I) at 30° and 40°. Slope 30°, 0.232; slope 40°, 0.154.*

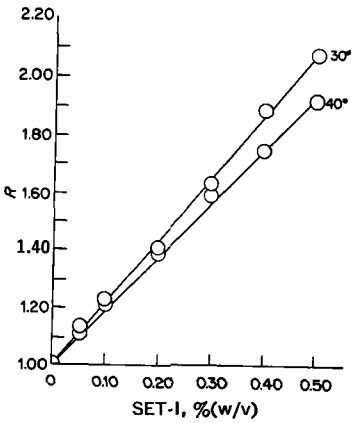

Fig. 3—Ratio R *(total propyl paraben to free propyl paraben) as a function of the concentration of sucrose monotallowate at 30° and 40°. Slope 30°, 2.148; slope 40°, 1.818.*

Quantities (0.5000 g.) of the parabens were weighed and placed in 100-ml. volumetric flasks together with 50 ml. of varying concentrations of the sucrose ester. The stoppered flasks were placed in a mechanical shaker in a constant-temperature bath. The shaking time for each reaction was determined beforehand from equilibration studies. After equilibration, 1-ml. volumetric pipets, the tips wrapped with Whatman No. 1 filter paper, were used to remove aliquot quantities free from excess solid paraben. After appropriate dilution, samples were assayed for paraben content using the spectrophotometer (Beckman DB) at a wavelength of 255 mμ.

Interaction of Methyl Paraben with Sucrose Esters—Data obtained in this study were plotted so as to show the value R, the ratio of total paraben to unbound paraben, as a function of the concentration of the sucrose ester. The degree of association between the reactants can be seen from the diagrams (Figs. 1–4).

A study of Figs. 1 and 2 indicates that the solubility of methyl paraben at 30° increases with an increase in macromolecular concentration in the range of 0.05–0.50% surfactant. An almost identical increase in paraben solubility is noted in the sucrose monotallowate and sucrose monococoate solutions, although the surfactants differ in the range of 4–5 carbon atoms in their fatty acid moieties. This increase in solubility is attributable to the formation of a soluble complex which continues to form without reaching a saturation point in the range studied.

Examination of the slope values for similar studies at 40° indicates that at the elevated temperature, the solubility of the parabens diminishes somewhat from that expected with a 10° temperature rise.

Interaction of Propyl Paraben with Sucrose Esters—It is evident that propyl paraben exhibits a greater solubilizing tendency with sucrose monotallowate and sucrose monococoate than was demonstrated by the methyl ester. A greater than twofold increase in the solubility of propyl paraben at 30° is noted in the presence of 0.5% sucrose monotallowate and 0.5% sucrose monococoate solutions. At 40°, the propyl paraben exhibited a similar diminished solubility compared to that shown by the methyl paraben. It may be observed that, in

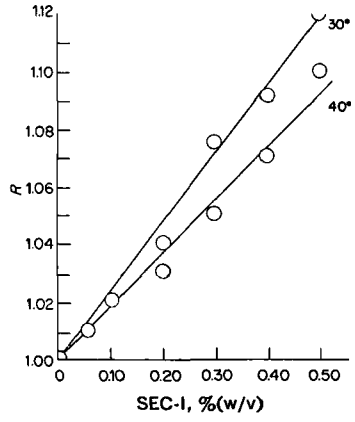

Fig. 2—Ratio R *(total methyl paraben to free methyl paraben) as a function of the concentration of sucrose monococoate (SEC-I) at 30° and 40°. Slope 30°, 0.238; slope 40°, 0.189.*

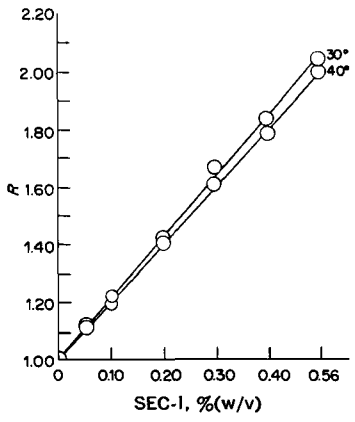

Fig. 4—Ratio R *(total propyl paraben to free propyl paraben) as a function of the concentration of sucrose monococoate at 30° and 40°. Slope 30°, 2.075; slope 40°, 1.969.*

general, the solubility which the parabens exhibit in the presence of the sucrose esters increases with the molecular weight of the parabens. This phenomenon has been reported for the interaction of methyl, ethyl, propyl, and butyl parabens with the polyoxyethylene ester type molecules (16).

DISCUSSION

The Nature of the Paraben-Surfactant Association—The exact nature of the paraben-surfactant association is not completely known. A number of forces have been implicated in other drug-macromolecule associations to account for the experimental data generally obtained (1, 17–21). In aqueous solutions the possibilities exist for hydrogen bonding both in the monomer and in the micellar states. It is possible to visualize that at the dilute concentrations of surfactant employed in this study, paraben molecules may become incorporated within the micelles and associate through a combination of hydrogen and hydrophobic bonding to form a somewhat stable complex.

Binding sites on the hydrophilic portion of the sucrose ester are equivalent to a minimum of 10 ethylene oxide groups based on water-solubility data for the oxygen molecules on the sucrose moiety (22). Results obtained by Blaug and Ebersman (12) and in this study indicate that the macromolecules in question are appreciably less reactive than the polyoxyethylene sorbitan ester series because of the lack of ethylene oxide groups in the sucrose portion of the molecule.

In accordance with the low order of association exhibited by methyl and propyl parabens with sucrose monotallowate and sucrose monococoate, the interaction is postulated to involve primarily hydrogen bonding forces and hydrophobic association. The negative center of the alcoholic hydroxyl of the surfactant is seen as competing for the proton of the phenolic hydroxyl of the paraben more favorably than the carbonyl oxygen of the paraben for the alcoholic hydroxyl hydrogen of the surfactant, although both interactions may occur. The interaction tendencies at 30 and 40° indicate polar forces are operative since these are weakened with an increase in thermal energy. Additionally, however, the small decrease in binding tendency with an increase in reaction temperature of 10° suggests that hydrophobic binding, which is not affected to a great extent by increasing thermal energy (10), is also operative. That hydrophobic binding is operative is supported by the fact that there is a pronounced increase in interaction in going from methyl paraben to the more nonpolar propyl paraben.

MICROBIOLOGIC STUDIES

Microorganisms—The organisms selected for these studies were *Aerobacter aerogenes* and *Escherichia coli*. The strain of *A. aerogenes* exhibited a more suitable growth rate in the simple medium used, however, and was solely used in the study.

Culture Medium—A simple 10% dextrose solution was selected and the strain of *A. aerogenes* was adapted to it. The possibility of an interaction between propyl paraben and the medium was investigated by determining the solubility of the paraben in the 10% dextrose solution at various concentrations of sucrose monotallowate. *R* values obtained did not differ significantly from the aqueous solutions.

Table I—Interaction of Propyl Paraben and Sucrose Monotallowate at 30°

Concn. Set-I (w/v)	Concn. Total Paraben (moles/l. × 10³)	R^a (Biologic)	R^a (Physical Chemical)
0	2.11	1.00	1.00
0.1	2.60	1.23	1.21
0.3	3.52	1.67	1.63
0.5	4.49	2.13	2.08

[a] R = Total paraben/free paraben.

Sterilization—The propyl paraben and sucrose monotallowate were found to be stable to autoclaving procedures used in this study.

Influence of Sucrose Monotallowate on Growth—The presence of sucrose monotallowate in the culture medium exhibited no detectible influence on the growth rate of *A. aerogenes*.

Procedure—Growth studies were carried out in sterile test tubes 25 × 150 mm. (Pyrex). Tubes containing 20 ml. of medium with the desired concentration of sucrose monotallowate and paraben were inoculated with 0.2 ml. of a 72-hr. suspension of *A. aerogenes* grown in plain 10% dextrose medium and standardized to a Klett reading of 100. Tubes were incubated at 30°. Growth was followed using a Klett-Summerson photoelectric colorimeter with a No. 42 blue filter. Turbidity measurements were made after 3 weeks and the minimum inhibitory concentration (M.I.C.) was determined by taking as the inhibitory concentration the range between the lowest paraben concentration not showing growth and the next lowest paraben concentration.

Discussion—Data on the degree of binding of the preservative by the surfactant may be used to predict preservative activity if the assumption is made that preservative activity is a function of the concentration of the free or unbound paraben. On this basis:

$$\text{M.I.C. (predicted)} = R\ \text{(M.I.C.)}$$

at any given concentration studied.

In order to permit a direct correlation of preservative activity in binding, propyl paraben and sucrose monotallowate were studied at 30° and the minimum inhibitory concentrations of propyl paraben were obtained for *A. aerogenes* in a control medium containing no sucrose monotallowate and in media containing several concentrations of sucrose monotallowate.

The method is similar to that used by Pisano and Kostenbauder (7) to study the preservative activity of *p*-hydroxybenzoic acid esters in the presence of polyoxyethylene sorbitan monooleate. Table I illustrates the close correlation of binding data and preservative activity obtained by employing physical chemical and biologic procedures. Microbiologic studies reveal a loss in preservative activity of the propyl paraben in proportion to the degree of binding exhibited by the paraben in the presence of sucrose monotallowate.

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Parabens, methyl, propyl-sucrose esters—interaction
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Microbiological analysis—parabens-sucrose esters
UV spectrophotometry—analysis

Identification of Medicinal Barbiturates by Means of Mass Spectrometry

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The mass spectra of the eight barbiturates most commonly prescribed in North America (amobarbital, barbital, butabarbital, mephobarbital, pentobarbital, phenobarbital, secobarbital, and thiopental) and, for comparison purposes, the spectra of butethal (butobarbitone B.P.) and itobarbital were recorded. The weakly acidic material present in capsules or tablets of four different barbiturate-containing products was extracted into ether and the mass spectrum of each extract was recorded. Evidence is presented that it is possible to identify positively individual barbiturates, and mixtures of barbiturates in pharmaceutical dosage forms, by means of mass spectrometry. The mass spectrum of phenacetin (present in one of the capsules studied) is discussed.

THE MASS SPECTRA of various barbiturates of medicinal importance (1) and other barbiturates (2) have been recorded and interpreted. The former study has revealed that under electron impact, barbiturate molecules undergo fragmentations in characteristic manner and the formation of the major ions in their mass spectra has been explained. The data as they are presented, however, do not permit easy differentiation between closely related structures. A positive identification of a particular barbiturate, such as would be desirable in toxicology studies, would not be possible using the reported (1) data.

The present study, therefore, was undertaken to determine whether it was possible to identify positively individual barbiturates, and to see whether mixtures of barbiturates in pharmaceutical dosage forms could be determined qualitatively, using mass spectrometry.

EXPERIMENTAL

Materials—Some of the barbiturates used in this study were purchased from the sources indicated: May and Baker (Canada) Ltd., Montreal (amobarbital, butethal, butabarbital, pentobarbital, and secobarbital); The British Drug Houses Ltd., London (barbital); Merck and Co., Ltd., Montreal (phenobarbital). The remainder were gifts from various drug houses.[1]

Isolation Procedure—*Capsules*—A portion (5 mg.) of the capsule contents was dissolved or suspended in water (2 ml.), concentrated hydrochloric acid (0.1 ml.) was added, and the whole extracted with ether (2 × 5 ml.). The combined ether extracts

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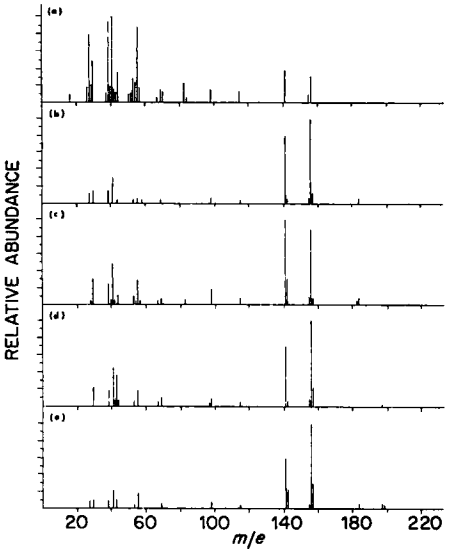


Fig. 1—Mass spectra of (a) barbital; (b) butabarbital; (c) butethal; (d) pentobarbital; and (e) amobarbital.

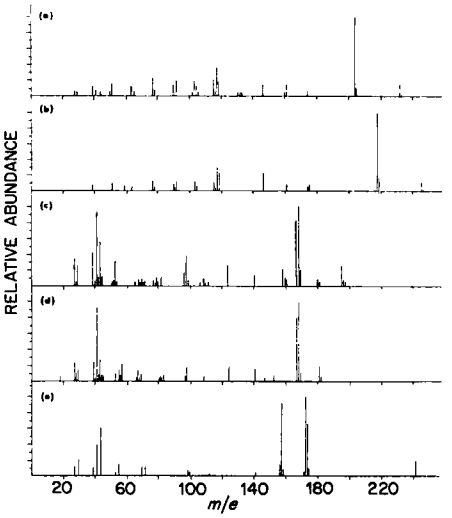


Fig. 2—Mass spectra of (a) phenobarbital; (b) mephobarbital; (c) secobarbital; (d) itobarbital; and (e) thiopental.

was extracted with sodium bicarbonate solution (2 × 2 ml., discarded), washed with water (2 ml.), and dried (Na_2SO_4). The ether solution so obtained was used for mass spectrum determination.

Tablets—A portion (10 mg.) of the powdered tablet was treated in the manner described for capsules.

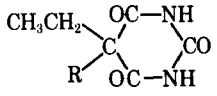
Mass Spectra—The mass spectra of the solids and ether extracts were determined using an A.E.I. M.S.9 instrument equipped with a heated inlet system operating at 150–170°. The electron beam energy was 70 ev. Samples were introduced by direct probe. Line spectra were drawn from the mass spectra and in each instance the most abundant ion was set at 100. Lines of relative abundance less than 2% were ignored in all figures except Fig. 3.

RESULTS AND DISCUSSION

Many deliberate and accidental deaths can be attributed to barbiturate poisoning. Summaries of cases of poisoning have been published (3, 4) and these have revealed that the vast majority of deaths are the result of ingestion of one or more of a relatively small number of barbiturates; amobarbital, bartital, butethal (butobarbitone), pentobarbital, phenobarbital, and secobarbital (quinalbarbitone) are the ones most commonly used. The mass spectra of these six barbiturates were recorded. In addition, the spectra of three other barbiturates, which are present in a significant number of preparations available in North America (5) were run. For comparison purposes, the spectrum of itobarbital was also recorded. Line diagrams of all 10 spectra were drawn (Figs. 1 and 2). These spectra are discussed conveniently under four groups.

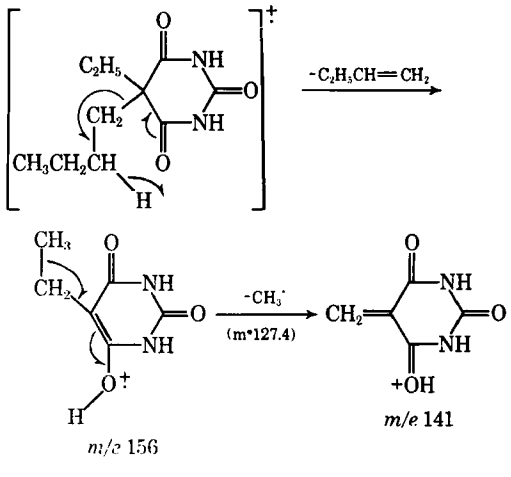
The first group comprises compounds of General Structure I. None of the compounds in this group (barbital, butabarbital, butethal, pentobarbital, and amobarbital) gave a parent ion, an observation which is contrary to what was reported previously (1). The mass spectrum of each of these five compounds, however, showed a very weak M+1 peak (rel. abund. 0.2–1.7%). Its presence together with the virtual absence of an M peak was at first confusing, but this observation can be explained in terms of the abstraction of a proton from the neutral barbiturate molecule by the molecular ion, to give a protonated molecular ion which is more stable than the molecular ion (6).

All of the compounds of General Structure I are



I, R = alkanyl

barbiturate	R
barbital	CH_3CH_2—
butabarbital	$CH_3CH_2CH(CH_3)$—
butethal	$CH_3CH_2CH_2CH_2$—
pentobarbital	$CH_3CH_2CH_2CH(CH_3)$—
amobarbital	$CH_3CH(CH_3)CH_2CH_2$—



Scheme I

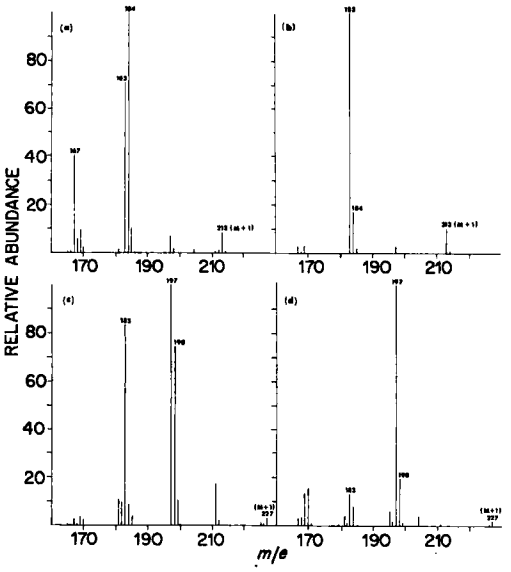
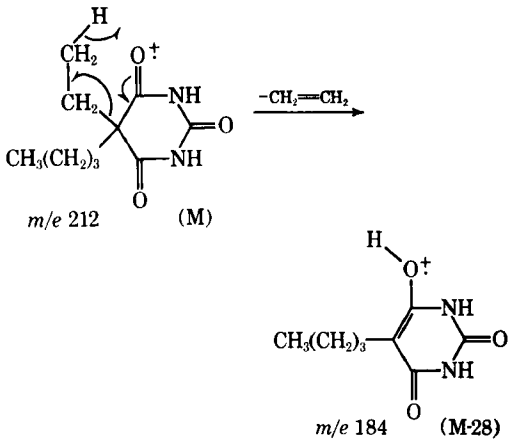


Fig. 3—Portions of mass spectra of (a) butethal; (b) butabarbital; (c) amobarbital; and (d) pentobarbital in the molecular ion region. The relative abundance of the strongest ion within the region is considered as 100.

known (1) to fragment in the manner illustrated in Scheme I for butethal, and give rise to strong ions of *m/e* 156 and *m/e* 141. The direct *m/e* 156→*m/e* 141 fragmentation is supported by the presence of a particularly strong metastable ion (m*) of *m/e* 127.4. Barbital is readily distinguished from the other four structures. It gave peaks of only medium intensity at *m/e* 156 and *m/e* 141 as well as the metastable ion at 127.4; abundant ions were located at *m/e* 55, 41, 39, 29, and 27 (Fig. 1*a*). In contrast, the mass spectra of butabarbital, butethal, pentobarbital, and amobarbital (Figs. 1*b*–1*e*) are extremely similar. Under electron impact, all readily lose the alkyl side chain. The resultant abundant ion, *m/e* 156, fragments in virtually identical fashion in all instances. Thus, to identify these compounds, reference must be made to that part of the spectrum in which masses greater than 156 are recorded. Appropriate line diagrams were drawn (Fig. 3).

The presence of the peak at *m/e* 197 in the spectra of amobarbital and pentobarbital readily distinguished these two barbiturates from butethal and butabarbital, in which this ion is absent. Butethal and butabarbital are isomeric, but nevertheless, it is possible to distinguish the one from the other by means of its mass spectrum. The former has an unbranched butyl substituent at C_5. This increases the amount to which the fragmentation depicted in Scheme II occurs and thus increases the relative abundance of the M-28 ion. A weak metastable ion, *m/e* 151.7, supports the subsequent loss of an OH radical from the M-28 ion, which results in the formation of an ion, *m/e* 167 (*i.e.*, M-45 ion), of significant abundance. In contrast, the 1′-methyl substituent of the C_5-isobutyl side-chain of butabarbital apparently minimizes an M→M-28 fragmentation in this compound. The result is that the M-28 ion of butabarbital is of very low abundance, the M-45 ion is virtually absent, and a metastable ion at *m/e* 151.7 is not observed.



Scheme II

The other two barbiturates, amobarbital and pentobarbital, are also isomeric compounds, and they can be differentiated in a similar way. The former does not have a 1′-methyl substituent in the C_5H_{11} side chain. The abundance of the M-28 (*m/e* 198) and the M-43 (*m/e* 183) ions from this compound are significant. The latter fragment is reputed (1) to be the M-HNCO ion. In addition, a weak metastable ion, *m/e* 165.5, in the spectrum of amobarbital supports a direct fragmentation of M-28→M-45 (loss of ·OH). The presence of the 1′-methyl substituent in the C_5H_{11} side chain of pentobarbital again minimizes an M→M-28 transition and thus the *m/e* 198 ion from this compound is of very low abundance. Also, no metastable ion is observed at *m/e* 165.5 in this spectrum.

Phenobarbital and mephobarbital (II*a* and II*b*) are readily identified by means of their mass spectra (Figs. 2*a* and 2*b*). Both spectra show a parent ion, of low relative abundance, of *m/e* 232 and 246, respectively, and the base peak in both spectra is the result of the loss of the ethyl side chain as an ethylene molecule (*cf.* Scheme II). The presence of a strong metastable ion, *m/e* 179.2, in the spectrum of phenobarbital and the equivalent ion, *m/e* 193.2, in the mephobarbital spectrum support a direct M→M-28 cleavage of both molecules. The presence of these metastable ions is of value in identifying these two barbiturates.

The third group of compounds investigated possess the General Structure III. Parent peaks (*m/e* 238 and 224, respectively) were absent from the mass spectra of secobarbital and itobarbital; M+1 ions (*m/e* 239 and 225, respectively) were present in less than 1% abundance. The most diagnostic features of the mass spectra of secobarbital (Fig. 2*c*) and itobarbital (Fig. 2*d*), as well as butalbital (1), are the presence of two very abundant ions of *m/e* 168 and 167, both of which arise by the loss of the saturated C_5-substituent. Thus, the ion of *m/e* 168 in the spectrum of secobarbital is formed by loss of $CH_3CH_2CH_2CH{=}CH_2$ from the molecular ion; loss of the radical $CH_3CH_2CH_2\dot{C}HCH_3$ accounts for the formation of the ion, *m/e* 167. Itobarbital and butalbital fragment in a similar way. The result is that the mass spectra of secobarbital, itobarbital, and butalbital are virtually identical. Minor, but significant differences are observed above *m/e* 168,

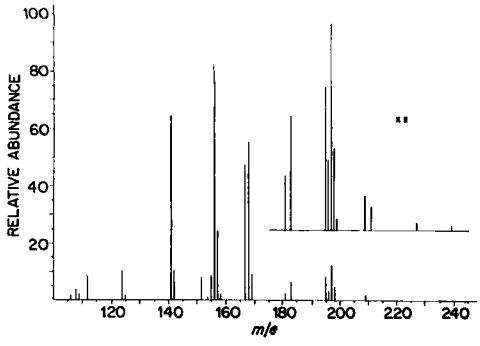


Fig. 4—Mass spectrum of an extract from a capsule which contained amobarbital and secobarbital.

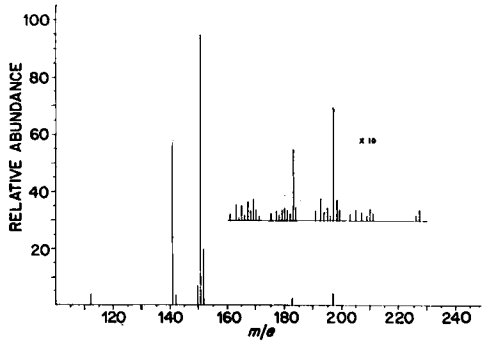
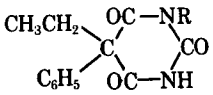


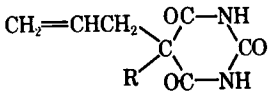
Fig. 5—Mass spectrum of an extract from a capsule which contained pentobarbital and butabarbital.

and in this way, it is possible to differentiate secobarbital from itobarbital, even though molecular ions are absent from both spectra. The former molecule loses a propyl radical from the alkanyl side chain of the molecular ion, and gives an ion of significant abundance (26%) of *m/e* 195 (*m/e* calcd.



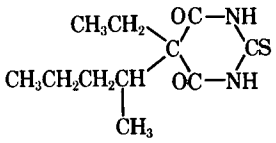
II

	R
(*a*) phenobarbital	H
(*b*) mephobarbital	CH_3



III

	R
(*a*) secobarbital	$CH_3CH_2CH_2CH(CH_3)$—
(*b*) itobarbital	$CH_3CH(CH_3)CH_2$—
(*c*) butalbital	$CH_3CH_2CH(CH_3)$—



IV

for $C_9H_{11}N_2O_3$: 195.0770; measured: 195.0764). Similarly, the loss of an ethyl radical gives an ion of low abundance (3%) of *m/e* 209. The former ion is absent from the mass spectrum of itobarbital.

The structure of thiopental (IV) is related to those compounds of General Structure I and fragments in a similar manner (Fig. 2*e*). The presence of the sulfur atom stabilizes the molecular ion, however, and an M peak (rel. abund. 20%), *m/e* 242, is observed. The other major diagnostic features in the mass spectrum of this molecule are abundant ions of *m/e* 172 and 157 and a strong metastable ion of *m/e* 143.3. These ions correspond to the ions of *m/e* 156, 141, and 127.4, respectively, in compounds of Structure I, and are formed in the same manner (*cf.* Scheme I).

To determine whether mass spectrometry could be used to identify barbiturates in pharmaceutical dosage forms, four commercially available products, each of which contained at least one of the barbiturates already discussed in this study in combination with other medicaments, were selected. Three of the products were capsules and one was a sugar-coated tablet. Ether extracts of the weakly acidic materials (and neutral materials, if present) contained in 5 mg. of each capsule or 10 mg. of the powdered tablet were made, and the mass spectra of all four ether extracts were recorded. As ions of small mass are of limited value in fragmentation studies, a line diagram of the ions of *m/e* 100 (arbitrarily chosen) and greater mass was drawn from each spectrum. The four diagrams so obtained (Figs. 4–7) differed appreciably from each other.

In the spectrum (Fig. 4) of the extract from a capsule which contained sodium amobarbital and sodium secobarbital,[2] the abundant ions of *m/e* 156 and 141 together with the strong metastable ion *m/e* of 127.4 confirmed the presence of a barbiturate of General Structure I; the two abundant ions of *m/e* 168 and 167 indicated that a barbiturate of Structure III was also present. Consideration of the *m/e* 180–240 region permitted more positive identification. The very weak peaks of *m/e* 239 and 209 and the weak peak of *m/e* 195 are all found in the spectrum of secobarbital and correspond to the M+1, M-29, and M-43 ions, whose formation has been discussed earlier. The presence of a very weak peak of *m/e* 227 and weak peaks of *m/e* 198, 197, and 183 suggest amobarbital, rather than its isomer (pentobarbital).

The simple mass spectrum (Fig. 5) of the extract from a capsule containing sodium pentobarbital and butabarbital,[3] with its abundant ions of *m/e* 156 and 141 (and a very strong metastable ion of *m/e* 127.4) clearly indicates that one or more compounds of General Structure I are present in this preparation. The absence of M peaks and the very low abundance of M+1 peaks makes a positive identification of pentobarbital and butabarbital impossible. However, when reference is made to the *m/e* 160–230 portion of the spectrum of the extract and this is compared with the information included in Fig. 3, then some compounds of Structure I may be eliminated as possible components of the preparation. The intensities of the ions of *m/e* 184 and 198 (Fig. 5) would tend to eliminate butethal and amobarbital from consideration, for example.

[2] Tuinal capsules (Eli Lilly and Co. Ltd.).
[3] Twinbarb capsules (C. E. Frosst and Co.).

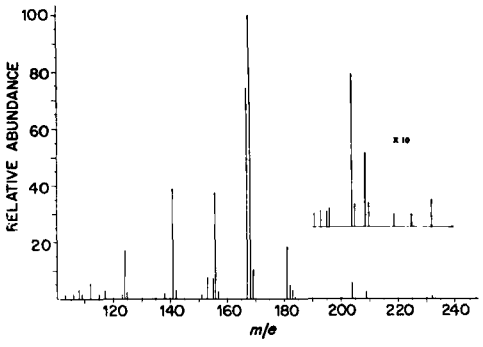


Fig. 6—Mass spectrum of an extract from a tablet which contained barbital, phenobarbital, butalbital, and other medicinal agents.

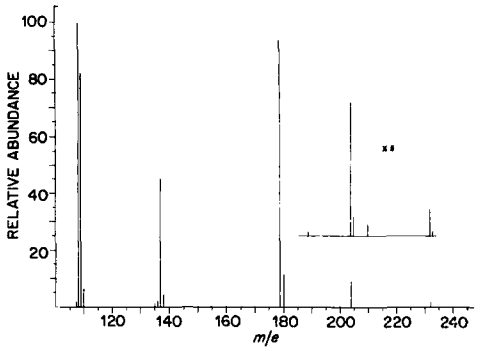
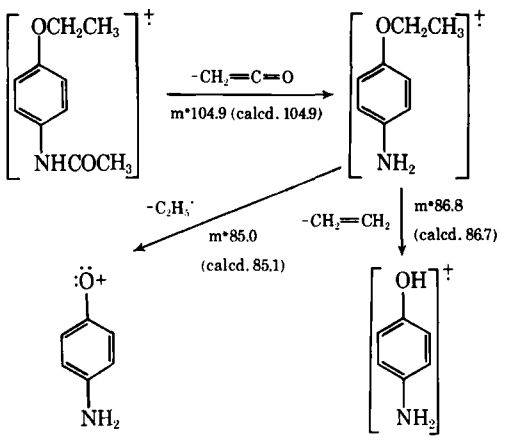


Fig. 7—Mass spectrum of an extract from a capsule which contained phenobarbital, phenacetin, and other medicinal agents.

The tablet studied contained sodium barbital, sodium phenobarbital, sodium butalbital, dihydroergotamine methanesulfonate, and scopolamine hydrochloride.[4] The mass spectrum (Fig. 6) of the ether extract of this tablet is quite diagnostic and indicates that a mixture of three barbiturates are present in the extract. A barbiturate of General Structure I is indicated by the presence of abundant ions of *m/e* 156 and 141 and the strong metastable ion of *m/e* 127.4. A positive identification of this barbiturate is not possible, although a comparison of



Scheme III

the region *m/e* 182–232 with the data in Fig. 3 eliminates butethal, amobarbital, and pentobarbital as possibilities. The presence of itobarbital in the mixture is indicated by the abundant ions of *m/e* 168 and 167 and by the absence of a significant ion of *m/e* 195 (which is present in the spectra of secobarbital and butalbital). The weak peaks of *m/e* 232 and 204 confirm the presence of phenobarbital in the mixture.

The dominant features in the spectrum (Fig. 7) of the ether-soluble neutral and acidic material present in the fourth commercial product[5] studied (which contained phenacetin, acetylsalicylic acid, phenobarbital, and hyoscyamine sulfate) are abundant ions of *m/e* 179, 137, 109, and 108, all of which arise from electron bombardment of the phenacetin molecule in the manner shown (Scheme III). The presence of strong metastable ions (m*) of appropriate masses confirm these fragmentation pathways.

In addition to these abundant ions, significant ions of *m/e* 232 and 204 were observed. Their presence together with a metastable ion of *m/e* 179.2, which was somewhat obscured by the strong parent peak of phenacetin, indicated the presence of phenobarbital in the extract. No attempt was made to remove the phenacetin before recording the mass spectrum. Had this been done, the spectrum would have been characteristic of many preparations containing phenobarbital as the sole barbiturate. The retention of phenacetin in the ether extract makes the mass spectrum much more diagnostic.

CONCLUSION

It has been demonstrated that it is possible to identify individual barbiturates by means of mass spectrometry. Barbiturates in pharmaceutical dosage forms can be identified also in this way. The results obtained in this study suggest that a library could be assembled of the mass spectra of extracts from pharmaceutical preparations containing barbiturates. The spectra so collected could be used as reference spectra in forensic studies, when only a very small portion of the pharmaceutical preparation is available.

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Keyphrases

Barbiturates—identification
Dosage forms, barbiturates—analysis
Mass spectroscopy—identity, structure
Electron impact—barbiturate fragmentation

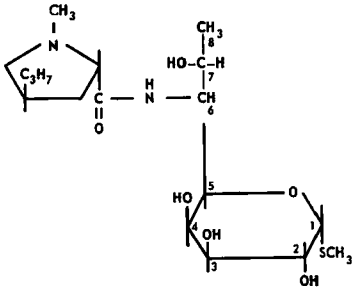
[4] Plexonal tablets (Sandoz Pharmaceuticals).
[5] Phenaphen capsules (A. H. Robins Co. Ltd.).

Absorption of Lincomycin and Lincomycin Esters from Rat Jejunum

By H. PATRICK FLETCHER, HEATHER M. MURRAY, and TODD E. WEDDON

The absorption of lincomycin equivalents from rat intestinal loops was improved when short-chain (3 and 4 carbons) fatty acid esters of the drug were used. The 7-butyrate and 7-propionate esters of lincomycin appear to be absorbed more efficiently than corresponding 2-esters. *In vivo* and *in vitro* experiments indicate that this difference in absorption results from intestinal hydrolysis of the 2-esters. The first-order rate constants for the disappearance of the esters from the intestinal loops were obtained from an equation which expresses the disappearance of lincomycin as the sum of two first-order expressions.

THE INCOMPLETE ABSORPTION of lincomycin (I) from the rat gastrointestinal tract was



I

indicated by results of Meyer and Lewis (1). It was estimated that 45% of the lincomycin disappeared from the small intestine after oral dosing and that most of the absorption occurred in the first quarter of the small intestine.

Fatty acid esters of lincomycin were synthesized in an attempt to find a tasteless derivative for a pediatric preparation. The monoesters used in this study were formed at the 2- and 7-positions and were hydrochloride salts. Since the addition of the fatty acid moiety increased the lipid solubility of the lincomycin molecule (2), the absorption from the small intestine was investigated. Results reported in the literature (3, 4) have indicated that increased lipid solubility may play an important role in the absorption of certain drugs.

The object of this study was to find an ester or analog which would be more efficiently absorbed from the intestine and result in higher serum antibiotic levels. Thus, the absorption of these compounds from ligated loops was studied in the rat jejunum.

EXPERIMENTAL

Animals—Male, albino rats of the Sprague-Dawley strain weighing between 180–210 g. were used throughout this study. Food and water were available to the animals up to the time of surgery. Five rats were used for each time interval in the intestinal absorption studies.

Drug Solutions—The drug solutions were prepared so that 0.5 ml. yielded 6.50 μmoles of lincomycin base. The compounds were placed into distilled water, and no suspending agents or solubilizers were used.

Intestinal Loops—The method for preparing intestinal loops in the rat jejunum was described by Levine and co-workers (5). Under ether anesthesia, a midline incision was made, and the first 10 cm. of the jejunum were exposed. After the lumen of the exposed jejunum had been cleared, a proximal ligature was loosely placed around the intestine and a distal ligature secured 10 cm. below it. Care was taken so that the ligatures did not interfere with the major blood vessels supplying the intestinal loop. The drug solutions were placed into the lumen of the loop *via* a 1-ml. syringe attached to a 26-gauge needle after the proximal ligature had been tightened around the needle. After delivery of the drug solution, the needle was withdrawn and the proximal ligature was completed. The intestine was replaced into the abdomen, the incision closed, and the rat was allowed to recover.

At the end of appropriate time intervals, the rats were again given ether anesthesia, sacrificed by decapitation, and blood samples were taken. The intestinal loops were removed and individually homogenized in saline with glass homogenizers. The homogenized loops from rats given lincomycin HCl were placed into volumetric flasks and brought to a 100-ml. volume with 0.1 *M* phosphate buffer (pH 7.9).

The antibiotic activity of the lincomycin esters are much less than lincomycin HCl. Since the amount of lincomycin ester remaining in the loop was determined by the microbiological assay for lincomycin base, it was necessary to hydrolyze the esters by subjecting such loops (as the homogenates) to alkaline hydrolysis. The homogenized loops from rats given the lincomycin derivatives were quantitatively placed into 20-ml. beakers and subjected to alkaline hydrolysis with 0.4 *N* NaOH over steam

Received June 13, 1968, from the Bioassay Section Product Control, The Upjohn Co., Kalamazoo, MI 49001

Accepted for publication August 5, 1968.

This study was presented at the AAAS (Np Section Symposium) New York, N. Y., December 1967.

The authors would like to express their appreciation to: Walter Morozowich, who synthesized the lincomycin esters; to John G. Wagner for his assistance in the kinetic interpretation of the data; to Robert J. Cole and Carl Metzler for the computer work involved in the statistical analysis, and to W. E. Hamlin and P. B. Bowman for gas chromatography and mass spectroscopy support.

for 5 min. After the hydrolysis, the homogenates were cooled, adjusted to pH 7.9–8.0 with HCl, and brought to a volume of 100 ml. with 0.1 *M* tris buffer (pH 7.9). Preliminary experiments demonstrated that the hydrolysis procedure did not degrade lincomycin as determined by the microbiological assay.

The amount of lincomycin or lincomycin derivatives originally placed into the loops was also determined by microbiological assay. The drug solution, 0.5 ml., was placed into 3 ml. of homogenates of jejunum from nontreated rats and subjected to the same procedures as given above for loops from experimental rats. Five of these spiked homogenates were prepared for each absorption study.

In Vitro Hydrolysis by Intestinal Homogenates—Segments of jejunum were taken from nontreated rats immediately after sacrifice and homogenized with chilled Kreb's Ringers bicarbonate solution containing glucose (5 g./l.). Three milliliters of homogenate, equivalent to 0.5 g. of intestine, was placed into 25-ml. conical flasks. Control flasks received the 3 ml. of homogenate which had been coagulated by heat. After 0.5 ml. of drug solution containing 2.65 mg. of lincomycin base equivalents of the lincomycin ester was deposited into each flask, it was placed into the Dubnoff metabolic shaker for appropriate time intervals. The flasks were incubated under a constant pressure of 95% O_2 and 5% CO_2 at 37°. At the end of the incubation period, all homogenates were coagulated with heat and the contents were brought to appropriate volume using a 0.1 *M* phosphate buffer pH 7.9. The amount of hydrolysis was estimated by an increase in microbiological activity from the intrinsic activity level observed in the control flasks.

Statistical Treatment of Data—The intestinal absorption of the esters were compared using semilog plots based on the disappearance of lincomycin from the intestinal loop. A regression analysis was used to determine the slopes of the absorption plots. These slopes were compared to lincomycin HCl and the significance of the differences was tested by analysis of covariance. The computations for these studies were obtained by use of the IBM 1620 computer. The curve resulting from nonlinear regression analysis was completed by the IBM 360.

Microbiological Assay—The microbiological assay for lincomycin in the serum described by Vavra *et al.* (6) was used for both serum and homogenates. The test organism was *Sarcina lutea* grown in Penassay seed agar. The samples from the absorption study of a particular ester were assayed on the same day. The results were corrected for day to day assay variation using the spiked homogenates and the theoretical content of lincomycin base.

RESULTS AND DISCUSSION

Absorption from Intestinal Loops—The absorption of lincomycin HCl from the loops was repeated four times during the study. The absorption plots were reproducible and the slopes were not significantly different when compared by analysis of covariance. A representative lincomycin absorption plot is shown in Fig. 1.

The semilog plots (Fig. 1) indicated that the

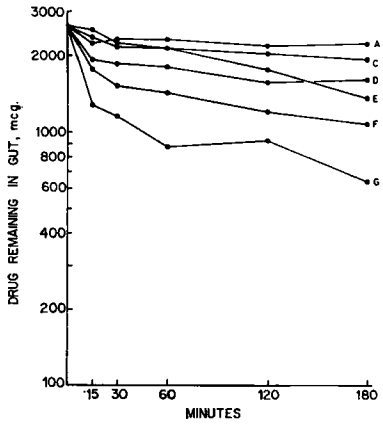


Fig. 1—Absorption of lincomycin and lincomycin esters from jejunal loops of rats. Each point is a mean of five rats. Key: A, 2-laurate; C, 2-octanoate; D, 2-hexanoate; E, linco HCl; F, 2-butyrate; G, 2-propionate.

absorption of the 2-monoesters of lincomycin increased as the chain length of the fatty acid group decreased. Also, unlike the absorption of lincomycin HCl, this figure shows that the absorption of the esters was rapid at the early time intervals and much slower in the later intervals. Thus, the semilog plots of these means resulted in a curved line, especially for the 2-butyrate and 2-propionate esters. A possible explanation for this curvature will be given later.

By taking the results (mcg. remaining in loop *versus* time) in the early time intervals prior to the "leveling off" of the absorption curve, the slopes of the esters were compared to lincomycin HCl in the same time intervals.

The increased slopes (Table I) from the regression analysis in the early time intervals appear to correspond with the percent dose absorbed after 2 hr. The analysis of covariance indicated that 2-butyrate, 7-butyrate, 2-propionate, and 7-propionate esters are significantly better absorbed (as reflected by the slopes) than lincomycin HCl (Table I).

The results in Table I also show the increase in lipid solubility resulting from the addition of the fatty acid groups to the lincomycin molecule. It appears that as far as the esters are concerned, the amount absorbed decreases as the lipid solubility increases. This may be a result of the decreased aqueous solubility of the longer chain esters. This decreased aqueous solubility may partially explain the poor absorption of the 2-laurate and 2-octanoate, since a precipitate was noted when they were placed in loops or intestinal homogenates. Thus, the better absorbed esters probably possess a balance between an increased lipid solubility and an adequate aqueous solubility.

The 7-butyrate and 7-propionate esters were better absorbed than their corresponding 2-esters (Table I). The comparison of the 2-butyrate and 7-butyrate esters (Fig. 2) revealed that the means in the later time intervals of the 2-butyrate absorption run parallel to lincomycin HCl. This was also apparent when the slopes in the later time intervals were not significantly different when compared by analysis of covariance. This parallelism in the

TABLE I—ABSORPTION OF LINCOMYCIN ESTERS

Lincomycin Compd.	pH[a]	Initial Slope[b]	Results of Analysis of Covariance[c]	Intrinsic Partition Coefficients[d]	Apparent % Absorbed at End of 2 hr.
Lincomycin HCl	5.7	−0.0015	—	0.06	33
2-Laurate HCl	3.8	−0.0009	0.05	7,800	17
2-Octanoate HCl	3.9	−0.0017	N.S.	2,750	27
2-Hexanoate HCl	5.0	−0.0051	N.S.	390	40
2-Butyrate HCl	4.6	−0.0084	0.05	40	54
2-Propionate HCl	4.4	−0.0120	0.01	14	64
7-Butyrate HCl	5.2	−0.0094	0.01	4	82
7-Propionate HCl	5.2	−0.0170	0.01	1	91

[a] pH of drug solutions placed in loops. [b] Based on mcg. remaining in the intestine at 0, 15, and 30 min. [c] Statistically compared to lincomycin HCl. [d] Ether–water: values were reported by W. Morozowich and F. Young (personal communication).

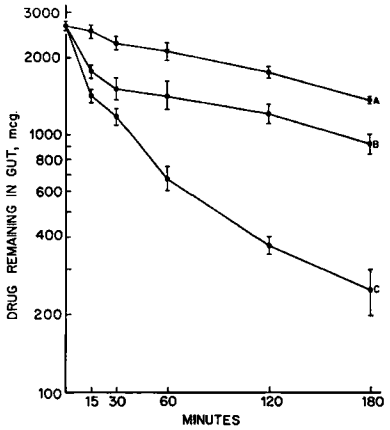


Fig. 2—Absorption of 2- and 7-butyrate from rat jejunal loops. (Each point represents a mean of five rats ± SE.) Key: A, linco HCl; B, linco-2-butyrate; C, linco-7-butyrate.

later part of the absorption plots was also found for the 2-propionate ester. These results indicate that lincomycin HCl was being absorbed in the later time intervals after the ester had been placed into the loop. This leads to the investigation of the hydrolysis of the ester by the intestinal enzymes.

In Vitro Hydrolysis—The increase in antibiotic activity from the control flask level (Table II) was assumed to be due to the presence of lincomycin base resulting from hydrolysis of the less active ester. Even though hydrolysis rates as such were not obtained due to the presence of antibiotic activity in the control flasks, it is obvious from Table II that the 7-esters, butyrate and propionate, are more resistant to the hydrolysis of intestinal enzymes than their corresponding 2-esters. The differing hydrolysis of the 2- and 7-butyrates in homogenates was also confirmed by gas chromatographic results. By measuring the resulting lincomycin peaks, approximately four times as much lincomycin was noted in the case of the 2-butyrate which confirmed the results obtained with microbiological assay. Also, the apparent resistance of the 7-butyrate to hydrolysis was demonstrated *in vivo* when intestinal loops were removed 30 min. after dosing and a microbiological assay was run before and after alkaline hydrolysis. The pre- and posthydrolysis antibiotic activities of the lincomycin-2-butyrate loops were not significantly different. However, the posthydrolysis antibiotic activity for the 7-butyrate ester was four times that of the prehydrolysis activity and this difference was statistically significant. Although the absorption of the 7-hexanoate was not studied, its hydrolysis in intestinal homogenates revealed again the apparent protection from hydrolysis afforded by the 7-position.

Serum Levels—The serum levels (Table III) reflected the amount of absorption from the intestinal loops as far as the 2-esters are concerned. The results also indicate that the serum levels for the better absorbed 2-esters appear to reach higher levels earlier than lincomycin HCl. These higher levels were especially apparent when lincomycin-2-butyrate and lincomycin-2-propionate were placed into ligated loops.

The antibiotic serum levels obtained after the more efficiently absorbed 7-esters were no higher than lincomycin HCl. An explanation for these results may be that a portion of the lincomycin in the serum was present as the 7-ester which is essentially inactive in the assay. Alkaline hydrolysis of serum from animals with lincomycin-7-butyrate and 7-propionate in ligated loops increased the antibiotic activity (Table IV). It is probable that this increase in antibiotic activity resulted from the hydrolysis of 7-esters to the active lincomycin.

Lincomycin-7-propionate was identified in the serum of rats which had the ester in intestinal loops. This identification was made by mass spectrometric results from chloroform extracts of the rat serum.

TABLE II—HYDROLYSIS OF LINCOMYCIN ESTERS BY HOMOGENATES OF RAT INTESTINE

Derivative	mcg.[a] Placed into Flask	Intrinsic Activity, mcg. in Control Flask[b]	Lincomycin, mcg. after Incubation
2-Butyrate	2,280 ± 28[c]	421 ± 2	2,220 ± 48
7-Butryate	2,050 ± 68	60 ± 6	610 ± 62
2-Propionate	2,640 ± 10	400 ± 18	2,630 ± 45
7-Propionate	2,700 ± 56	<15	<15

[a] Determined by microbiological assay. [b] Contained boiled homogenate. [c] Mean of six flasks ± *SE.*

TABLE III—SERUM LEVELS OF LINCOMYCIN ACTIVITY (mcg./ml.) AT VARIOUS TIME INTERVALS

Lincomycin Derivatives	Postdose, min. 15	30	60	120	180
Lincomycin HCl	0.4[a]	1.3	0.6	0.5	0.3
2-Laurate	0.8	0.5	<0.3	<0.3	<0.3
2-Octanoate	0.7	0.7	0.4	<0.3	<0.3
2-Hexanoate	1.6	0.9	0.6	0.5	<0.3
2-Butyrate	2.1	1.2	0.8	0.6	<0.3
2-Propionate	4.7	1.9	1.0	0.3	<0.3
7-Butyrate	1.3	1.0	0.4	<0.3	<0.3
7-Propionate	1.0	0.9	0.5	<0.3	<0.3

[a] Mean of 5 rats.

The hydrolysis of serum from rats receiving lincomycin HCl and lincomycin-2-butyrate in ligated loops did not increase the antibiotic activity in the serum. These results indicated that the increased antibiotic activity was not a result of hydrolysis of some inactive conjugate of lincomycin.

If the 7-ester were present in the serum, then the ester, as such, was absorbed from the intestine and the enzymes outside the intestine must also have a decreased ability to hydrolyze the 7-esters. Studies with liver homogenates indicated that the enzymes in the liver also have a decreased ability to hydrolyze the 7-butyrate ester when compared with the 2-butyrate ester.

Stephens and Conine (7) reported increased blood levels resulting from oral administration of fatty acid esters of erythromycin to humans. As in the lincomycin study, the highest levels were obtained with the propionate and butyrate esters.

Effect of pH of Drug Solution—The pH of the various solutions of esters used in this study varied from 3.8 to 5.2 (Table I). No effect on absorption was detected when the 2-propionate solution was adjusted from 4.4 to 6.0, when the 2-octanoate was adjusted from 3.8 to 4.9, and when lincomycin HCl was adjusted from 5.0 to 3.0.

Proposed Model for Intestinal Absorption—The proposed model for the absorption of the lincomycin esters assumes that the esters are better absorbed than lincomycin HCl. This increased absorption probably results from increased lipid solubility as reflected by the intrinsic partition coefficients of the ester, or perhaps other properties which were not examined in this study. Thus, the amount of lincomycin or its ester absorbed depends upon how well the ester resists hydrolysis and remains in the ester form while in the intestinal loop (Fig. 3). Evidence presented thus far *in vivo* and *in vitro* indicates that this could explain the difference in absorption between the 2- and the 7-esters.

TABLE IV—LINCOMYCIN SERUM LEVELS IN HYDROLYZED AND NONHYDROLYZED SERUM

Postdose, min.	mcg./ml.[a] 15	30	60	120	180
7-Butyrate					
Nonhydrolyzed	1.3	1.0	0.4	<0.3	<0.3
Hydrolyzed	2.2	1.8	1.0	0.3	<0.3
7-Propionate					
Nonhydrolyzed	1.0	0.9	0.5	<0.3	<0.3
Hydrolyzed	2.8	1.8	1.2	0.3	<0.3

[a] Results from the pooling of samples from five rats.

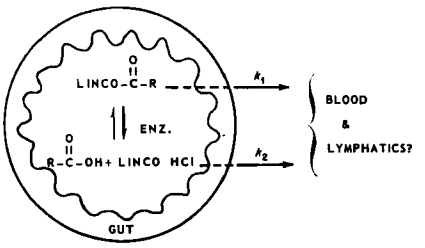


Fig. 3—Diagram for the proposed model for the absorption of lincomycin esters.

The equation for the proposed model is

$$y = x_1 e^{-k_1 t} + x_2 e^{-k_2 t}$$

where

k_1 = first-order rate constant for the disappearance of lincomycin ester
k_2 = first-order rate constant for the disappearance of lincomycin HCl
x_1 = amount of lincomycin ester
x_2 = amount of lincomycin HCl
t = time
y = amount of lincomycin in intestine

This equation states that the amount of drug remaining in the intestine (y) is the sum of two first-order expressions representing the disappearance of two different compounds from the intestine. The first-order rate constants, k_1 and k_2, were estimated from the absorption curves (Fig. 1) for the particular 2-esters. The k_2 constant was estimated from a line drawn through the means in the later time intervals. The k_1 was estimated from plotting the residuals in the first three time intervals of the absorption curve. It was assumed that this constant represented the estimate of the constant for the disappearance of the lincomycin ester from the intestine. Thus, k_2 was used as the rate constant for the disappearance of lincomycin HCl.

These rough estimates for k_1 and k_2 and the individual rat data were used in the computer program which calculated the k_1 and k_2 based on nonlinear regression analysis. This is a modification of a method described by Wagner and Metzler (8).

The 2-propionate curve calculated by the above method by IBM computer is shown in Fig. 4. The

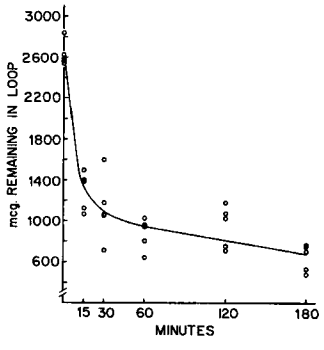


Fig. 4—Calculated curve (using nonlinear regression analysis) for the absorption of lincomycin-2-propionate. Key: O, observed data; ——, computer-calculated curve.

TABLE V—ESTIMATES OF FIRST-ORDER RATE CONSTANTS[a] FOR THE DISAPPEARANCE OF LINCOMYCIN AND LINCOMYCIN ESTER

	k_1[b], 95% C.L.	k_2,[c] 95% C.L.
Lincomycin-2-propionate	8.32 (2.29–14.35)	0.17 (0.05–0.30)
Lincomycin-2-butyrate	6.06 (0.00–12.4)	0.13 (0.01–0.25)
Lincomycin-2-hexanoate	9.67 (0.00–20.9)	0.06 (0.02–0.11)

[a] Obtained from nonlinear regression analysis. [b] k_1 rate constant for ester. [c] k_2 rate constant for lincomycin.

first-order rate constants for the better absorbed 2-ester are shown in Table V. Even these calculated rate constants must be considered estimates, since this equation does not take into consideration the rate of enzymatic hydrolysis in the intestine. The amounts of lincomycin base and the ester in the intestine are not only affected by the disappearance of each through absorption, but also by the amount of ester being hydrolyzed. However, even with these limitations, this method allows one to estimate k_1 and k_2 when the semilog absorption plots are not linear. Because of the linearity of its semilog absorption plots (Fig. 1), the rate constant for the absorption of lincomycin HCl was calculated by the usual method using the slope obtained by linear regression analysis. This rate constant was 0.21 hr.$^{-1}$ for lincomycin HCl. The estimates for lincomycin HCl are lower in Table V, 0.17 hr.$^{-1}$, 0.13 hr.$^{-1}$, and 0.06 hr.$^{-1}$.

This model and the data presented in this study indicate that the 2-ester and its hydrolysis product, lincomycin HCl, are both absorbed at the early time intervals, while only lincomycin HCl is absorbed in the later time intervals, the ester being completely hydrolyzed.

SUMMARY

The absorption of lincomycin was improved by using derivatives of lincomycin which consisted of esters of short-chain fatty acids (3 and 4 carbons). The absorption efficiencies of the various esters were modified by intestinal hydrolysis. The explanation for absorption of these esters is based on a model which states that the lincomycin ester and lincomycin base both contribute to this absorption.

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(1) Meyer, C. E., and Lewis, C., "Antimicrobial Agents and Chemotherapy," American Society for Microbiology, 1963, p. 169.
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Jejunum, rat—ligated loops
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Intestinal homogenates—lincomycin HCl, esters hydrolysis

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By C. F. HISKEY and F. F. CANTWELL

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ACETARSONE (3-acetamido-4-hydroxy phenylarsonic acid) and arsthinol (cyclic 3-hydroxypropylene ester of 3-acetamido-4-hydroxy dithiobenzene arsenous acid) have the following structures:

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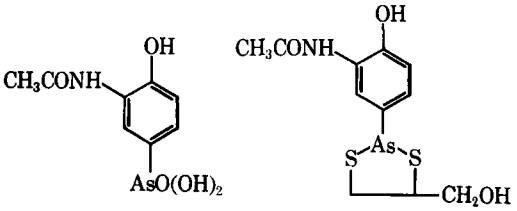


Acetarsone Arsthinol

The first of these has been known for many years being Ehrlich's 596. Its synthesis has been described by Raiziss and Fisher (1). The synthesis of arsthinol (by the reaction of 3-acetamido-4-hydroxy phenyl arsenoxide with 2,3-dimercapto-propanol) was reported by Friedheim (2) in 1953.

Some of the chemical properties of this substance and methods of assay for it in the various dosage forms have been briefly described (3). In order to understand more about its acid-base chemistry and its absorption spectra for analytical purposes an experimental study was made of it, of acetarsone, and of some of their surrogates.

An examination of the literature revealed that Gex and Kreimer (4) had done differential titrations of acetarsone and reported four (sic) ionization constants having the following values: $pK_1 = 3.3$; $pK_2 = 4.2$; $pK_3 = 7.6$, and $pK_4 = 9.4$. They appear to have been under the misapprehension that the amide group had amine-like characteristics. They were also troubled by extensive precipitation of the acetarsone as they made their solutions acidic and seem to have made no provision to take this into account. They also present some data on the UV absorption spectrum of acetarsone.

EXPERIMENTAL

The arsthinol used in this study was regular production grade material, corresponding in its characteristics to its description in Tests and Standards of the N.N.R. It was used without further purification.

The acetarsone used in the titration study was dissolved in aqueous alkali solutions, filtered to remove any insoluble matter, and then precipitated with hydrochloric acid. The precipitates were washed repeatedly with distilled water until all traces of chloride were removed, after which they were dried at 105° to constant weight. This produced a fine powder which dissolved rapidly during the titration experiments as described below.

The absolute methanol was analytical grade and the propylene glycol was USP grade material.

The acid-base titrations were performed in a wide-mouth conical flask with magnetic stirring, using a Teflon-coated stirring bar, and employing a model 7 Corning pH meter. About 1.5 mmoles of the compound was suspended in a known volume of carbon dioxide-free water and titrated with approximately 0.1 *N* sodium hydroxide. Throughout the course of the titration a vigorous stream of nitrogen was blown into the solution to prevent carbon dioxide uptake.

A Beckman model DK-2 ratio-recording spectrophotometer was used in obtaining the spectrograms in this study and a Beckman model DU spectrophotometer was used for measuring absorbances at isolated wavelengths. Measurements were made in matched 1.00-cm. quartz cells.

Solutions for spectrophotometric measurements were prepared in the following manner: An amount of pure substance, approximately equal to a quarter of a millimole, was accurately weighed and transferred to a 100-ml. volumetric flask. Solution was effected using either anhydrous methanol in one group of studies or more commonly by the addition of 0.01 *N* sodium hydroxide. The solutions were brought to volume after which an appropriate aliquot was transferred to a second 100-ml. volumetric flask, and there again diluted with appropriate reagents to give the desired solution.

When determining the various spectra associated with the conjugate acid-base species of acetarsone, solutions were buffered so as to have a pH in the region where each species was dominant.

Solutions in approximately 0.1 *N* sodium methoxide were prepared by dissolving about 0.1 mole of metallic sodium in anhydrous methanol previously distilled over sodium. A 5.00-ml. aliquot of a stock solution of arsthinol in anhydrous methanol was transferred to a 100-ml. volumetric flask and the sodium methoxide solution was added to mark. A reference solvent for spectral work was prepared in an identical manner, using 5.00 ml. of methanol in place of the arsthinol stock solution.

Iodometric titrations employed in this study were done as follows: An aliquot was acidified with sulfuric acid, an equal amount of propylene glycol, and then an excess of standard iodine solutions was added. The iodine is back titrated with thiosulfate to a water-white solution. The starch-iodine end point cannot be used in this instance because the high concentration of propylene glycol present precludes its formation.

RESULTS

Titration of Acetarsone—Acetarsone has a very limited solubility in water at room temperature. Consequently it was necessary in the initial stages of the titration to wait after each addition of alkali for the pH meter to drift to its final steady-state values. During this period, more undissociated acetarsone went into solution until both the solubility and the ionization equilibria were satisfied. This situation continued until about 80% of the first equivalent of alkali had been added, at which point all of the undissolved acid was in solution and from then on equilibrium was established quickly after each addition of alkali. Typical of the data obtained are those presented in Fig. 1 where they are plotted as the individual points. The titration curve shown in the figure is that calculated, using the ionization constants determined in this study.

As expected, it was found that acetarsone titrates as a triprotic acid, *i.e.*, two of the protons come from the arsonic moiety and one from the phenolic group *para* to it. The first stage of ionization is separated

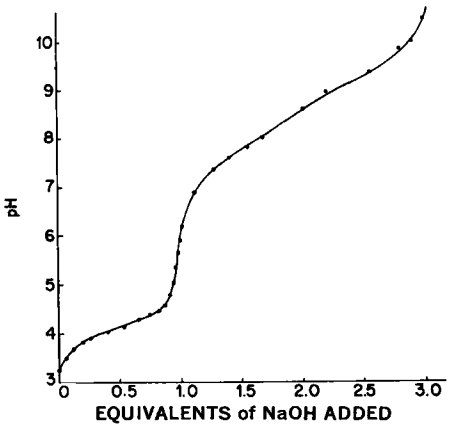


Fig. 1—Potentiometric titration curve of acetarsone with sodium hydroxide. Points shown are experimental results and the line is that calculated from the evaluated constants.

from the second stage by about four orders of magnitude, while the second and third stages of ionization are so close to each other that there is only a barely perceptible break observable in that region of the titration curve.

The evaluation of the successive ionization constants was performed as follows:

First it was assumed that the ionization of this triprotic acid could be represented by the equilibria:

$$H_3A \overset{K_1}{\rightleftharpoons} H_2A^- \overset{K_2}{\rightleftharpoons} HA^= \overset{K_3}{\rightleftharpoons} A^{\equiv} \qquad \text{(Eq. 1)}$$

Since the first stage of ionization is so widely separated from the second and third stages, its constant may be evaluated separately according to the equilibria:

$$H_3A_{solid} \rightleftharpoons (H_3A)_{aq.} \overset{K_1}{\rightleftharpoons} (H_2A^-)_{aq.} \qquad \text{(Eq. 2)}$$

The first ionization constant is then

$$K_1 = \frac{(H^+)\,(H_2A^-)}{(H_3A)} = \frac{(H^+)\,(H_2A^-)}{S} \qquad \text{(Eq. 3)}$$

In this expression S is the solubility of the undissociated acetarsone. The latter term in Eq. 3 applies when dilute slurries of acetarsone are being considered while the middle term applies to its solutions.

The equation for the titration function of this monoprotic acid is as follows:

$$H - OH = \left[H_2A^- \right] C_B \qquad \text{(Eq. 4)}$$

In the above expression, since these data are only approximate, only the H and OH terms represent the activities of the H_3O^+ and OH^- in the solution. The other terms refer to the concentration of strong base, C_B, and to the concentration of the conjugate base H_2A^-. Substitutions for the H_2A^- term may be made to treat the situations where acetarsone is (*a*) partly and (*b*) completely dissolved. These are, respectively:

$$H - OH = \frac{K_1 S}{H} - C_B \text{ and} \qquad \text{(Eq. 5a)}$$

$$H - OH = \frac{mK_1}{H + K_1} - C_B \qquad \text{(Eq. 5b)}$$

where m is the formal concentration of acetarsone.

To use Eq. 5*a* the solubility of the undissociated acetarsone must be known. It was determined spectrophotometrically in 0.1 *N* hydrochloric acid (0.478 g./l.; 1.74×10^{-3} *M*) and in water (0.616 g./l.; 2.24×10^{-3} *M*).

Since the solubility in water is substantially greater than it is in 0.1 *N* hydrochloric acid, it is evident that considerable ionization has occurred in the pure water solvent, *i.e.*, 23%. From these data and Eq. 3, K_1 is calculated to be approximately 1.5×10^{-4}; $pK_1 = 3.8$. However, a more accurate value of 3.73 was determined by fitting the potentiometric titration data to Eqs. 5*a* and 5*b*.

The evaluation of K_2 and K_3 was performed by a method originally developed by Linderstrom-Lang and subsequently modified by Bjerrum (5). With it the data of Fig. 1 were evaluated; the slope at the second equivalence point was found to be 0.66 which gives a value for the spreading factor of 5.0. From this it follows that pK_2 equals 8.6 − log 5.0 = 7.9 and pK_3 equals 8.6 + log 5.0 = 9.3.

Thus all three constants for this triprotic acid have been determined and it now becomes possible to calculate the relative abundance of all of the four conjugate species as a function of pH. The results of these calculations are plotted in Fig. 2. These results were used in assigning spectra to the different conjugate species.

Titration of Arsthinol—The only acidic functional group in the arsthinol molecule is the phenolic one. Consequently the initial part of its titration curve prior to the addition of a full equivalent of alkali was consistent with this view and consistent with a pH for that phenolic group of about 9.4 to 9.6. Inasmuch as the phenolic ionization constant is so small, there is little of the compound in solution when a slurry of arsthinol in water is made. The solubility as determined in water at 25° was 0.32 g./l. or 0.00116 *M*. Less than 1% of the dissolved arsthinol would be ionized. However, somewhat before the first equivalent of alkali was added, all of the arsthinol had dissolved, leaving a water-white solution.

Addition of more than one equivalent of alkali did not cause the pH to change from about 11.2 even though two, three, and four equivalents were added. This indicated that hydrolytic cleavage of the arsenic sulfur bonds was occurring, and that the sulfhydryl groups and the arsenous acid were consuming the alkali. This formulation is consistent with

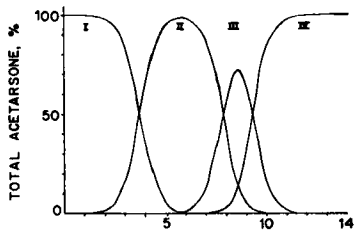
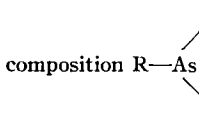


Fig. 2—Relative abundance of the various conjugate acid-base species of acetarsone as a function of pH. Key: I, H_3A; II, H_2A^-; III, $HA^=$; IV, $A^{\equiv}$.

the experimental observations of Cohen *et al.* (6) on the reversible hydrolysis of dithioarsenites of the

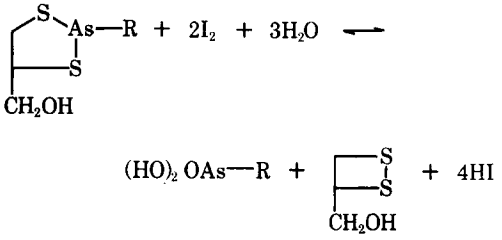


composition R—As(S—R′)(S—R′) in aqueous solution.

When air was substituted for the nitrogen, the pH began to fall immediately, suggesting that the trivalent arsenic was being oxidized to make acetarsone which, with its greater acid ionization constants, would cause a shift to lower values of the pH. This continued until a pH of about 10.2 was reached and then as the air bubbling continued, the solution became turbid while the pH abruptly shifted to higher values.

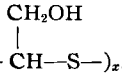
The explanation of this phenomenon is also easily understood in terms of the reactions given above. After all, or the bulk of the trivalent arsenical was oxidized to acetarsone, the fully ionized 2,3-dimercaptopropanol is next oxidized to give disulfido linkages producing the insoluble matter observed and simultaneously releasing two equivalents of alkali to the solution, this accounting for the sudden increase in pH. The precipitate that is formed resembles the precipitate formed when 2,3-dimercaptopropanol is oxidized.

Additional evidence for aqueous alkaline air oxidation of arsthinol was obtained from a measurement of the iodometric titer of previously alkaline arsthinol solutions which had been aerated at room temperature for various periods of time. In these studies a 0.029 *M* arsthinol solution in 0.1 *N* sodium hydroxide was aerated. At periodic intervals an aliquot was taken and titrated according to the procedure described above. The arsthinol is titrated to acetarsone according to the following reaction:



$$(HO)_2OAs—R + \text{(cyclic S, S, CH}_2\text{OH)} + 4HI$$

It is entirely possible that the disulfide compound is



polymeric, *i.e.*, $(—S—CH_2—CH(CH_2OH)—S—)_x$. The elastic, gummy nature of the precipitate suggests this. The data obtained are listed in Table I and show that with time, continued aeration will eventually oxidize the arsthinol to acetarsone and disulfide.

When a similar type of experiment was done, using anhydrous methanol as the solvent, with sodium methoxide as the alkali, little or no oxidative cleavage of the arsenic-sulfur bonds occurred. This fact made it possible to obtain the phenolate spectrum of arsthinol.

Further supporting evidence for the hydrolytic cleavage of the sulfur arsenic bonds and the oxidation of the sulfhydryl groups in alkaline solution was elicited by means of the nitroprusside test (3). When arsthinol solutions are made alkaline in the presence of nitroprusside, a red-violet color is observed indicating the presence of free sulfhydryl groups. If these solutions are acidified with a bicarbonate buffer to a pH of about 7.0 to 8.0, the nitroprusside test for the mercaptan group fails. However, if additional alkali is again added, cleavage of the linkages occurs and the nitroprusside test is again positive. This is indicative of a reversible hydrolysis of the —S—As bonds of arsthinol. In the presence of oxygen, however, this nitroprusside test weakens in intensity and in due course fails as all of the mercaptan groups are oxidized.

TABLE I—AIR OXIDATION OF ARSTHINOL IN ALKALINE SOLUTION

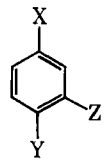
Aeration Time, min.	Molarity of Arsthinol	Initial Molarity, %
0	0.0288	100.0
10	0.0261	90.4
20	0.0234	81.2
30	0.0220	71.3
60	0.0021	7.0

Finally, as will be demonstrated below, the spectrum of $10^{-5}M$ arsthinol in aqueous alkali solution, prepared with air-saturated water, gave only the spectrum of acetarsone. This could be proved easily by acidulating these solutions, whereupon the spectrum which was obtained was that of unionized acetarsone and not that of arsthinol. In order to observe the spectrum of phenolate species of arsthinol it was necessary to use a sodium methoxide solution in anhydrous methanol. This result is exactly what would be anticipated in the light of the failure to oxidize this substance in the experiment cited previously.

Spectrophotometric Studies and Their Interpretations—Some of the spectra determined in the course of this study are presented in Figs. 3–8 and if examined before reading the following will facilitate the understanding of the interpretations.

Spectra of Acetarsone—Considering acetarsone first, it may be noted that the spectra of the H_3A, H_2A^-, and $A^=$ species (Fig. 3) were obtained by scanning solutions at pH's of about 1, 5.16, and about 13, respectively, where it will be seen (Fig. 2) that the particular species accounts for at least 98% of the formal acetarsone concentration. The spectrum of $HA^=$ was deduced from scans made on solutions with pH values in the vicinity of 8.6 by subtracting the fractional contribution of the other conjugate species present from the observed spectrum.

The spectrum of acetarsone, a trisubstituted benzene derivative, is essentially the spectrum of benzene considerably modified by the substituent groups (7).[1] It has been shown that the B and C bands (designated first primary and secondary by Doub and Vandenbelt) of compounds of the type:



[1] The absorption band nomenclature used in this discussion is that proposed by Moser, C., and Kohlenberg, A. I., *J. Chem. Soc.*, **1951**, 804.

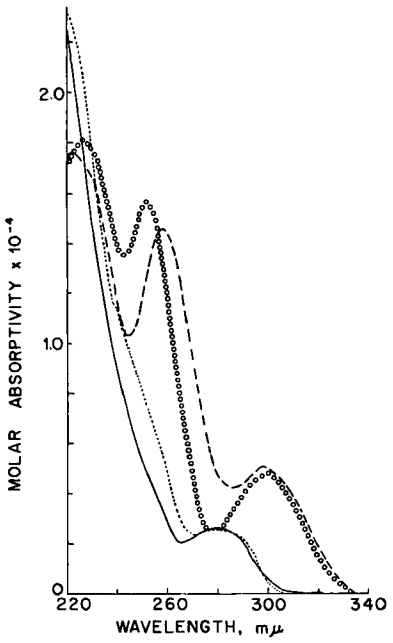


Fig. 3—Spectra of conjugate acid-base species of acetarsone in aqueous solvents. Key: ···, H_3A; ——, H_2A^-; - - -, $HA^=$; ooo, $A^{\equiv}$.

where Y and Z are electron donating and X is electron attracting often resemble that band which is the most displaced among corresponding bands of the three constituent disubstituted compounds.

The *o-p*-directing properties of the hydroxyl and acetamido groups are well established and their bathochromic effect on benzene bands is evident in the spectra of phenol and acetanilide. The spectrum of phenylarsonic acid (Fig. 4), on the other

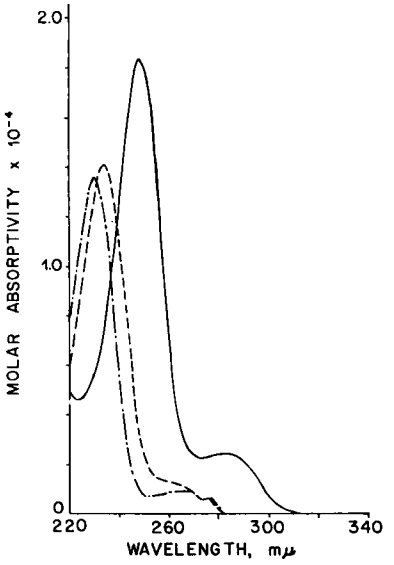


Fig. 5—Spectra of p-hydroxyphenylarsonic acid in 0.1 N HCl, 5% MeOH, - - -; pH = 6, 5% MeOH, - · -; and in 0.1 N NaOH, 5% MeOH, ——.

hand, would indicate little or no *m*- or *o-p*-directing properties for the arsonic group in all three states of protonation since the C band is very similar to that of benzene being shifted only a few mμ and increased in intensity two to five times.

However, the *m*-directing nature of the arsonic group when under the influence of a strongly *o-p*-directing group *para* to it is inferred from the shift in the B band of *p*-hydroxyphenylarsonic acid produced by the loss of the first proton (Fig. 5). The loss of the proton from an electron-attracting group would be expected to diminish its electron-attracting prop-

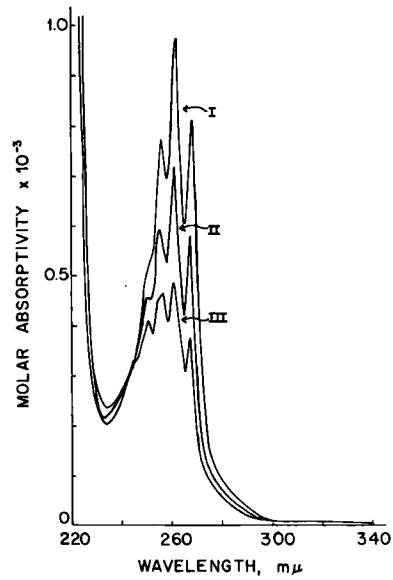


Fig. 4—Spectra of phenylarsonic acid in 0.1 N HCl, 5% MeOH (I); pH = 6, 5% MeOH (II); and in 0.1 N NaOH, 5% MeOH (III).

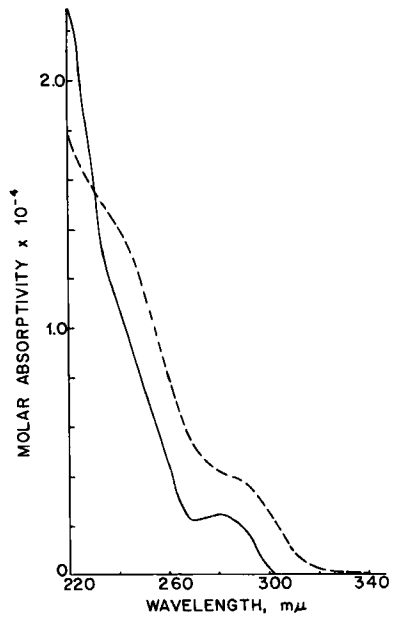


Fig. 6—Spectra of acetarsone, —— and arsthinol, - - - in 0.1 N HCl, 5% MeOH.

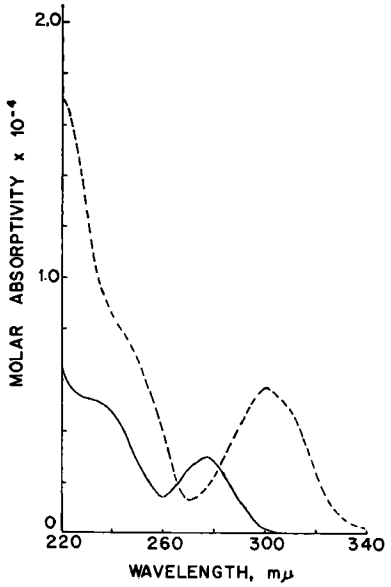


Fig. 7—Spectra of o-hydroxyacetanilide in 0.1 N HCl, 5% MeOH, ——; and in 0.1 N NaOH, 5% MeOH, - - -.

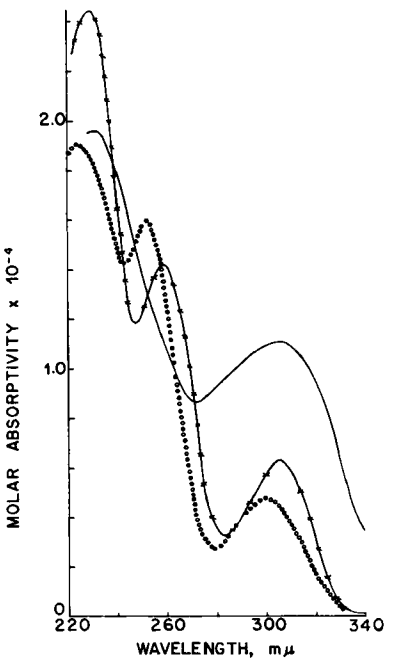


Fig. 8—Spectra of acetarsone in 0.1 N NaOH, 5% MeOH, I: ooo; in 0.1 N NaOH, 95% MeOH, II: ×; and of arsthinol in methanol, 0.1 N NaOMe, III: ——.

erties and move the band to shorter wavelengths (7).

An examination of the spectrum of the H_3A species of acetarsone (Fig. 6) reveals a C band at 280 mμ resembling the C band of *o*-hydroxy acetanilide (Fig. 7) at 278 mμ. Loss of the first proton, which undoubtedly comes from the arsonic group, produces little change in the C band but appears to have a hypsochromic effect on the B band below the spectral range investigated. Loss of the second proton produces a marked bathochromic effect on the B and C bands which are now located at 251 mμ and 300 mμ, respectively. The C band now resembles closely the band of the phenolate form of *o*-hydroxyacetanilide. This type of effect is frequently observed upon loss of a proton from an electron-donating substituent group (*i.e.*, phenol). Finally, loss of a third proton from acetarsone produces little effect on the C band but has a hypsochromic effect on the B band; analogous to that observed with the loss of the first (arsonic) proton. It would therefore seem that the second ionization step can only be associated with the phenolic hydroxyl group and the third ionization step with the remaining arsonic proton.

This finding is not completely unanticipated since Pressman and Brown (8) have postulated a similar ionization sequence for *p*-hydroxyphenylarsonic acid. They reasoned that since the analinium ion of aniline has its acidity increased by about 1.6 pK units as a consequence of having a singly ionized arsonic acid group *para* to it in *p*-aminophenylarsonic acid, the same should apply to the phenolic group in *p*-hydroxyphenylarsonic acid (pK_1 = 3.88, pK_2 = 8.37, pK_3 = 10.05) as compared to phenol.

Designation of the peak at 226 mμ in the fully ionized acetarsone spectrum as an A band or as an additional B band is uncertain. The effect of the less polar solvent, methanol, on both the 226-mμ and the 251-mμ bands is to shift them to the red about 6–7 mμ although the absorptivities of the two are affected differently (Fig. 8). This similar bathochromic solvent effect would seem to favor the designation of the higher energy band as an additional B band possibly associated with the *o*-hydroxyacetanilide portion of the acetarsone molecule. The 251-mμ band closely resembles in position and peak form, the B band of the fully ionized *p*-hydroxyphenylarsonic acid and is probably associated with that portion of the acetarsone molecule.

Spectra of Arsthinol—A consideration of the spectra of arsthinol in aqueous acid (Fig. 6) and methanolic alkali (Fig. 8) solvents reveals a greater similarity to *o*-hydroxyacetanilide than to acetarsone. The disappearance of the acetarsone B band at 257 mμ in methanolic alkali upon substitution of the cyclic 3-hydroxypropyldithioarsenous groups for the arsonic acid group indicates the different character of these groups and further supports the association of that band with the *p*-hydroxyphenylarsonic portion of the acetarsone molecule.

Analytical Applications—It is self-evident from these data that the analyst is afforded a wide range of choice in devising spectrophotometric methods of assay for these two pharmaceutical entities. If one uses anhydrous alkaline methanol as the solvent medium, the spectra of arsthinol will be that of the intact molecule. Any oxidative hydrolysis which may have occurred to it as a consequence of aging in its various pharmaceutical formulations can be readily detected by the appearance of a peak at 260 mμ due to acetarsone as well as a substantial reduction in its absorption in the 307-mμ region. Coupling this method with the iodometric titration affords corroborative data.

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Arsthinol—acid-base chemistry
Ionization constants—acetarsone, arsthinol
Acid-base titration—acetarsone, arsthinol
Iodometric titration—acetarsone, arsthinol
UV spectrophotometry—analysis

Hypoglycemic Activity and Chemical Structure of the Salicylates

By VICTOR FANG*, WILLIAM O. FOYE, SUMNER M. ROBINSON†, and HOWARD J. JENKINS

The results obtained in a study undertaken to clarify both the structural requirements of salicylate hypoglycemic activity in the hope of diminishing the unpredictability associated with it and the mechanism of such activity are presented in an effort to shed further light on the pathophysiology of diabetes mellitus. Results of the structure studies point to the carboxyl group as being essential to the salicylate hypoglycemic activity, the hydroxyl radical in the *ortho* position as being beneficial but not imperative with respect to this activity. Results of the action mechanism studies involve suppression of the release of fatty acids from adipose tissues in salicylate hypoglycemic activity.

THE SALICYLATES have been used for nearly a century in the treatment of diabetes mellitus but their usefulness as hypoglycemic agents has been limited by the fact that the doses required to bring about a significant blood glucose lowering response are relatively large and the response is somewhat unpredictable in that a wide variety of effects other than the hypoglycemic effect is elicited concomitantly. The advent of insulin and the oral hypoglycemics signaled a loss of interest in the antidiabetic action of these compounds.

The effectiveness of salicylates in decreasing glycosuria and hyperglycemia in various animal preparations (1–6) and in man (7–12) is, however, well established. The discovery of the cause of their unpredictability and the mechanism of their action has not been achieved.

Salicylates diminish the hyperglycemia of some, not all, human diabetics, both of maturity-onset and juvenile types. Salicylate-induced hypoglycemia can be demonstrated in alloxan or pancreatectomized diabetic animals of different species.

In general, a fairly large dose of salicylates is required to produce significant lowering of blood sugar. In man the maximal hypoglycemic activity is reached at plasma salicylate concentrations of between 20 and 30 mg./100 ml. Salicylate levels as low as 6 to 10 mg./100 ml. have been claimed to reduce hyperglycemia effectively (13–16). The effect of salicylates to reduce hyperglycemia in the rat is not abolished by hypophysectomy (6, 17) or by bilateral adrenalectomy (5, 18).

The blood sugar response to salicylates in humans and animals is related to blood salicylate level and to initial blood glucose concentration. The hypoglycemic activity of salicylates is more

Received May 20, 1968, from the Edwin Avery Brewer and Howard D. Brewer Pharmacology Laboratories, Massachusetts College of Pharmacy, Boston, MA 02115
Accepted for publication September 6, 1968.
Abstracted in part from a thesis submitted by Victor S. Fang to the Massachusetts College of Pharmacy in partial fulfillment of Doctor of Philosophy degree requirements.
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pronounced when the hyperglycemia of diabetes is marked and is more pronounced when fasting rather than postprandial blood levels are involved. It would appear that salicylates do not have a significant hypoglycemic effect in normal (euglycemic) man or animals. On the other hand, many salicylate poisoning cases have been reported to be complicated by hyperglycemia and glycosuria (19, 20).

The study reported was undertaken to determine the structural requirements of the hypoglycemic activity of the salicylates and the mechanism of this activity.

EXPERIMENTAL

The animals used were alloxan-diabetic male and female rats, weighing between 130 and 150 g., which were the offspring of Carworth Wistar males and Charles River females. The rats selected for the production of diabetic animals were starved for 24 hr. but were given water *ad libitum* during this period. Glucose determinations on blood samples withdrawn from the stubs of the cut tails of the rat just prior to their alloxan injections served as control values. Each rat was given a subcutaneous injection of the first alloxan dose of 150 mg./kg. body weight. Two days later, each rat received a subcutaneous injection of the second dose of the diabetogenic drug of 120 mg./kg. The incidence and severity of the resultant diabetes could be judged by the appearance of characteristic symptoms and the subsequent blood glucose determination. Sometimes it was necessary to give a third and even a fourth dose of the drug of 100 mg./kg. each at 2-week intervals until the diabetic symptoms became very apparent and the fasting blood glucose was more than 200 mg.%.

Most diabetic rats could survive for at least 1 year without any insulin treatment, provided good care was maintained. Adequate food and water, optimal room temperature, and clean cages were indispensable. Usually the diabetic rats developed the characteristic "poly triad" shortly after the first two injections. Retinopathy was recognizable within 1 to 2 months after alloxan treatment. Retardation of growth was easily observed in a comparison of the injected animals with their normal littermates. The most reliable criterion of diabetes was, of course, the lowered glucose tolerance with the pronounced hyperglycemia.

The diabetic rats were not used in the experiments of this study unless they had survived for at least 3 months after the alloxan treatment. A diabetic rat would be used repeatedly for many experiments, but it was required to have a 1-month period of rest after each experiment to ascertain the clearance of any residual drug effect.

Screening of salicylate derivatives and compounds similar in structure to salicylate for their effects on the blood sugar levels of the rats was carried out as follows.

The food but not the water intake of normal as well as diabetic rats was restricted for 18 to 20 hr., in order to establish a rather stable fasting blood glucose level. Blood samples from tail stumps were taken before the rats received any treatment. Then the animals were given the test compound of specified dosage by subcutaneous injection. If the compound could not be made soluble enough to prepare an aqueous solution for injection, it was administered orally to the animal in a freshly-prepared CMC medium in accord with a modified Dulin's formula (benzyl alcohol omitted); sodium carboxymethylcellulose, 0.5%; polysorbate 80,[1] 0.4%; sodium chloride, 0.9%. blood samples were collected 0.5, 1, 2, 3, 5, and in most cases 7 hr. after injection or 1, 2, 3, and 5 hr. after oral administration.

Nondrug control tests were accomplished with normal and diabetic rats receiving either saline injection or oral administration of CMC medium only. In most experiments one diabetic rat receiving a subcutaneous injection of 4 mmoles of sodium salicylate/kg. body weight served as the drug control. Furthermore, glucose tolerance tests for a few normal and a few diabetic rats were performed and served as a reference.

The maximal dose level was 4 mmoles/kg. body weight. If a toxic reaction were observed at such a dose, lower levels were employed until there was no evidence of toxicity. The minimal dose, 0.5 mmole/kg., was reached in some instances. The limit for the volume of the injection or oral dose was fixed at 5 ml./kg. body weight. The pH of the injected solution was adjusted to the range between 6.5 and 8.5.

It was found important to handle the animals as gently as possible and to avoid any unnecessary stress and injury. If the animal were too feeble, its response to drug was not considered reliable. The animals undergoing testing had to be able to tolerate the 18-hr. fast plus the 7-hr. test period. Care was taken to prevent coprophagy during the fast. Test rats were not used more frequently than once each month.

The blood glucose concentration of the samples was determined by the enzymatic method described by Saifer and Gerstenfeld (21) and Washko and Rice (22) slightly modified to conserve the blood of the animal.

In this method, when the animal was immobilized, 50 μl. of blood was taken from the stump of the severed tail tip by means of a lambda pipet and diluted to 1 ml. with distilled water. To this 1 ml. (20-fold dilution), 0.5 ml. each of barium hydroxide solution and zinc sulfate solution were added with thorough mixing to precipitate the proteins. The now 40-fold diluted specimen was centrifuged and 0.5 ml. of the clear supernatant liquid was pipeted to a test tube. To the tube containing this blood specimen 4.5 ml. of Glucostat reagent solution was added. The test tube was incubated in a water bath at 37° for at least 30 min. At the end of incubation 2 ml. of 17.1 N H_2SO_4 was added to develop a pink color. The color intensity was measured by the absorbance of the sample at 540 mμ in a spectrophotometer (Coleman). Water, instead of blood, processed in the same way, served as a blank.

A standard curve was prepared during each assay with standards of known amounts of glucose, as was the case in the different dilutions of the glucose stock solution. The concentration of glucose in a specific

[1] Tween 80, Altas Chemical Industries, Inc., Wilmington, Del.

blood sample in mg./100 ml. of blood could thus be read directly by converting its absorbance to the standard curve glucose equivalence.

The Novak colorimetric ultramicro method (23), slightly modified, was used for the determination of plasma nonesterified fatty acids (NEFA) in samples.

In this method, 50 μl. of blood was taken from the stump of the severed tail tip and mixed immediately with 75 μl. of heparinized saline. The blood suspension was centrifuged and an aliquot of 70 μl. of plasma solution was taken and kept frozen until the start of the assay. To the frozen plasma solution 0.4 ml. of Dole's extraction mixture (24) was added. The liquids were mixed by vibration and the test tube was cooled in an ice bath. After 0.3 ml. of heptane and 0.5 ml. of water were added, the contents of the tube were thoroughly mixed again. Three-tenths milliliter was drawn from the upper heptane phase and transferred to another test tube. To it 0.5 ml. of chloroform–heptane mixture and 0.6 ml. of the cobalt reagent were added. The solution was thoroughly mixed and centrifuged. Six-hundredths milliliter of the upper chloroform–heptane layer was transferred to a third test tube containing 0.75 ml. of the indicator solution and an orange color developed. The color intensity was measured by the absorbance of the sample at 500 mμ (Coleman spectrophotometer). A tube which contained heparinized saline instead of plasma solution was processed in the same way to serve as a blank.

A standard curve was prepared in each assay by measuring the absorbance of standard palmitic acid solutions containing known quantities of the fatty acid. The concentration of NEFA in the assay sample in meq./l. of plasma could be read directly from the corresponding absorbance on the standard curve.

RESULTS AND DISCUSSION

The effect of salicylic acid on the blood glucose of alloxan diabetic rats is illustrated in Fig. 1. The effect of each of the compounds studied on the blood sugar level of the diabetic rat is listed in Table I.

Chemical Structure–Biological Activity Relationships—Acetylsalicylic acid possessed almost the same hypoglycemic activity as salicylic acid. Salicylamide *elevated* blood sugar levels.

Block of carboxyl group, as in semicarbazone and hydrazine derivatives, caused abolition of the glucose lowering action of salicylic acid. Benzoic acid was less effective than salicylic acid as an hypoglycemic agent, yet it had significant hypoglycemic activity.

Insofar as the hydroxyl group is concerned, phenol had no significant activity on blood sugar concentration. Catechol also had no activity. Both were toxic. An additional hydroxyl radical on the benzene ring of salicylic acid eliminated hypoglycemic activity. If the hydroxyl group were replaced by an amino group, such as anthranilic acid, the hypoglycemic effect was reduced but was still significant. If it were substituted by a nitro or mercapto group or by fluorine, blood sugar tended to rise and toxicity was increased.

If the benzene ring were alkylated, the toxicity was increased and the hypoglycemic activity disappeared. The toxicity of alkylated salicylic acid was increased in proportion to the number of carbon

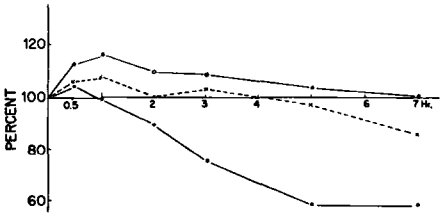

Fig. 1—Effect of salicylic acid (S.A.) on blood glucose of diabetic rats. Key: ○, 11 rats, 86.4–444.0 mg./100 ml., 5 ml. saline/kg., s.c.; ×, 5 rats, 130.0–193.6 mg./100 ml., 2 mmoles S.A./kg., s.c.; ●, 7 rats, 88.0–375.0 mg./100 ml., 4 mmoles S.A./kg., s.c.

atoms of the alkyl group or groups. Also, the position of alkylation made no difference. If the benzene ring were replaced by a naphthalene ring, the effect on blood glucose was like that of alkylation. Replacement of the benzene ring with a 5-membered ring seemed to increase hypoglycemic potency.

Nicotinic acid analogs had greater hypoglycemic activity than salicylic acid itself. The sugar-lowering activity of nicotinic acid and 2-hydroxynicotinic acid was even more pronounced. Isonicotinic acid derivatives did not follow this pattern. Pyrazine and pyrimidine analogs of salicylic acid had little effect to alter blood sugar level.

In summary, the carboxyl group of salicylic acid appeared to be responsible for the hypoglycemic activity. The hydroxyl group at the *ortho* position was not imperative but beneficial to the hypoglycemic action.

Mechanism of Action of Salicylates—A considerable difference of opinion exists on the matter of the mechanism of salicylic acid's hypoglycemic effect, some investigators being of the opinion that these agents suppress gluconeogenesis, some that they promote glycogen formation. In addition, a number of other mechanisms representing possibilities for hypoglycemic activity have been introduced. None is supported by available evidence. Salicylates do not diminish the *in vivo* rate of intestinal absorption of glucose in either animal preparations (25, 26) or in man (10, 27). Also, they do not reduce dietary intake of glucose because they are not responsible for a reduction in body weight of those animals to which they are administered. They have no effect on glomerular filtration or reabsorption of glucose because the glycosuria of diabetic patients treated with salicylates diminishes to an extent parallel to that of the reduction in blood sugar (10). Any observed salicylate effect of expanding the plasma volume is quantitatively inadequate to account for the fall in blood sugar (27). The formation of salicylate glucuronide is unlikely to be a mechanism of salicylate hypoglycemia (28). The conversion of glucose to liver and muscle glycogen might represent a possible mechanism of blood glucose lowering. But salicylates either have no glycogenetic effect or they deplete liver glycogen. Smith, Meade, and Bornstein (3) observed that large doses of salicylates reduced the glycosuria and blood glucose in the diabetic rat but caused no changes in the liver glycogen content and that in the normal rat the same treatment caused no alteration in blood glucose but a depression of liver glycogen. The glycogenolytic effect of salicylates in normal man and animals is responsible for the hyperglycemia of salicylate in-

TABLE I—EFFECT OF COMPOUNDS TESTED ON BLOOD GLUCOSE LEVELS OF DIABETIC RATS

Compd.	% Change in Plasma Glucose at 5 hr.[a]
Hypoglycemic Effect	
Salicylic acid, 4 mmoles/kg., s.c., oral	57.8
Benzoic acid, 4 mmoles/kg., s.c.	93.5
Anthranilic acid, 4 mmoles/kg., s.c.	90.8
Nicotinic acid, 2 mmoles/kg., s.c.	41.3
2-Hydroxynicotinic acid, 4 mmoles/kg., s.c.	47.5
2-Hydroxypyrazine-3-carboxylic acid, 2 mmoles/kg.	88.0
Indole-3-acetic acid, 4 mmoles/kg., s.c.	81.1
2-Thiophenecarboxylic acid, 2 mmoles/kg., s.c.	85.0
Hyperglycemic Effect	
Salicylamide, 4 mmoles/kg., s.c.	141.6
o-Mercaptobenzoic acid, 1 mmole/kg., s.c.	138.2
o-Nitrobenzoic acid, 4 mmoles/kg., s.c.	128.7
α-Resorcylic acid, 4 mmoles/kg., s.c.	150.4
m-Cresotic acid, 4 mmoles/kg., s.c.	143.2
o-Thymotic acid, 1 mmole/kg., s.c.	130.7[b]
3,5-Diisopropylsalicylic acid, 0.5 mmoles/kg., s.c.	138.3
o-Fluorobenzoic Acid, 1 mmole/kg., s.c.	163.3
1-Hydroxy-2-naphthoic acid, 1 mmole/kg., s.c.	227.9
3-Hydroxyisonicotinic acid, 1 mmole/kg., s.c.	129.4
No Significant Effect	
Nicotinamide, 4 mmoles/kg., s.c.	85.5
Salicylaldehyde semicarbazone, 4 mmoles/kg., oral	106.3
Salicylhydrazide, 2 mmoles/kg., oral	111.7
3,6-Dimethylsalicylic acid, 2 mmoles/kg., s.c.	105.9
3-*tert*-Butyl-6-methylsalicylic acid, 0.5 mmole/kg., s.c.	99.2
Phenol, 4 mmoles/kg., s.c.	93.5
Catechol, 1 mmole/kg., s.c.	105.9
Saligenin, 2 mmoles/kg., s.c.	83.9
2,4-Dihydroxybenzoic acid, 4 mmoles/kg., s.c.	90.7
Gentisic acid, 4 mmoles/kg., s.c.	80.1
2-Hydroxy-3-naphthoic acid 1 mmole/kg., s.c.	135.9
2-Aminopyrimidine-3-naphthoic acid 1 mmole/kg., s.c.	97.4
4-Aminopyrimidine-5-carboxylic acid 2 mmoles/kg., s.c.	121.8
4-Hydroxy-2-methylquinoline-3-carboxylic acid, 4 mmoles/kg., s.c.	117.1
Methyl-3-hydroxythiophene-2-carboxylate, 4 mmoles/kg., oral	116.1
Ethyl-4-hydroxy-2-methyl-thiazole-5-carboxylate, 4 mmoles/kg., oral	107.5

[a] *t* value significance never as much as 0.05. [b] % change in plasma glucose at 3 hr.

toxication. Niederlund (29) showed that both single and continuous doses of salicylates in the range of 230 to 810 mg./kg. body weight caused depletion of glycogen in the skeletal muscles and the myocardium of rabbits. Stowers (27) concluded that a long-term hypoglycemic action such as that induced by salicylates in man could not be explained by a change in distribution of or conversion to carbohydrates which could have only a transient action on blood glucose levels. Absence of relationship between salicylate uncoupling oxidative phosphorylation effect and hypoglycemia was reported by Gilgore (11) and Stowers (27). The hypoglycemic effect of salicylates does not appear to depend on either an insulin-like action or on liberation of insulin (10, 30–33). That the inhibition of lactate dehydrogenase by salicylates as a result of the reversible competition between them and pyridine nucleotide enzymes (NAD or NADP), is responsible for the hypoglycemia effect of the salicylate is unlikely because it is improbable that there is any difference in this inhibition in normal and diabetic animals. Attempts to correlate the metabolic effects of salicylates and their effects to diminish the protein binding of insulin and thyroxine (31, 34, 35) have not been convincing simply because the mode of action of these hormones does not fit the pattern of the hypoglycemic response to the salicylates.

Accumulating evidence suggests a reciprocal correlation between glucose utilization by peripheral tissues and release of nonesterified fatty acid (NEFA) from adipose tissue into the blood stream (36, 37). Carlson and Ostman (38) discovered that calcium acetylsalicylate in normal and diabetic subjects caused blood glucose and NEFA concentrations to fall.

Salicylate appears to depress NEFA release from adipose tissue. Inadequate glucose utilization by adipose tissue will induce a high level of NEFA in blood plasma (39). Observations by Shipp *et al.* (40) and Randle *et al.* (41) have shown that plasma fatty acids at high concentrations can inhibit the tissue utilization of glucose. These observations have been interpreted to suggest that the increase in NEFA occurring in diabetes can block the utilization of glucose from the blood stream. Thus, agents which will depress NEFA such as salicylate, should secondarily increase glucose utilization in diabetics and may lower blood sugar.

Table II illustrates the relationship between blood glucose and plasma NEFA. The effect of salicylic acid on plasma NEFA is seen within the first hour following administration, while the effect on blood glucose shows up toward the end of the third hour.

To determine whether or not the blood glucose

TABLE II—RELATIONSHIP BETWEEN BLOOD GLUCOSE AND PLASMA NEFA

D.R.[a]	Treatment	Blood Glucose Contents, mg./100 ml. 0 hr. Range	0 hr. Av.	Changes in % 1 hr.	3 hr.	5 hr.	Plasma NEFA Contents in meq./l. 0 hr. Range	0 hr. Av.	Changes in % 1 hr.	3 hr.	5 hr.
16	Saline control, 5 ml./kg., s.c.	86.9–350.1	188.2	117.0	115.6	115.8	0.55–1.65	0.92	93.4	104.3	129.6
10	Salicylic acid, 4 mmoles/kg., s.c.	94.2–350.7	182.4	103.7	91.6	72.7	0.51–1.27	0.81	17.9	53.1	93.0
8	Salicylamide, 4 mmoles/kg., s.c.	132.5–535.7	328.1	107.0	104.3	108.3	0.75–1.69	1.26	87.7	111.7	106.1
8	Benzoic acid, 4 mmoles/kg., s.c.	96.7–371.4	229.6	88.8	88.8	112.2	0.54–1.51	0.93	20.1	76.9	141.1
8	Thiosalicylic acid, 1 mmole/kg., s.c.	158.0–392.9	280.9	108.3	107.0	110.4	1.12–2.00	1.37	58.5	76.8	94.1

[a] Number of diabetic rats.

TABLE III—EFFECT OF EXOGENOUS NEFA TO BLOCK THE HYPOGLYCEMIC ACTIVITY OF SALICYLIC ACID IN DIABETIC RATS

D.R. Group	No.	Injected Serum[a]	Plasma NEFA Contents, meq./l. 0 hr. Range	0 hr. Av.	Changes in % 1 hr.	3 hr.	5 hr.	Blood of Injected Serum[a]	Blood Glucose Contents, mg./100 ml. 0 hr. Range	0 hr. Av.	Changes in % 1 hr.	3 hr.	5 hr.	t
I[b]	5	—	0.76–2.48	1.44	96.6	100.7	109.8	—	337.7–387.1	364.5	104.4	96.5	94.2	—
II[c]	5	—	0.70–1.69	1.30	24.1	39.8	53.0	—	198.0–408.6	349.5	96.3	69.2	45.4	0.005
III[d]	5	1.24–1.56	1.00–1.29	1.15	156.4	121.0	105.5	56.1–68.3	193.6–466.5	330.1	116.4	130.4	130.7	0.005
IV[e]	3	1.28–1.56	1.08–1.86	1.55	51.3	76.8	87.0	56.1–58.7	133.3–505.7	349.6	116.1	103.3	101.7	0.1

[a] The plasma NEFA and blood glucose contents of the blood samples, from which the serum was obtained and used for injection. [b] Normal saline control, 5 ml./kg., s.c. [c] Salicylic acid, 4 mmoles in 5 ml./kg., s.c. [d] Normal saline, 5 ml./kg. s.c and then serum from starved normal rats, 5 ml./kg., i.v. [e] Salicylic acid, 4 mmoles in 5 ml./kg., s.c. and then serum from starve normal rats, 5 ml./kg., i.v.

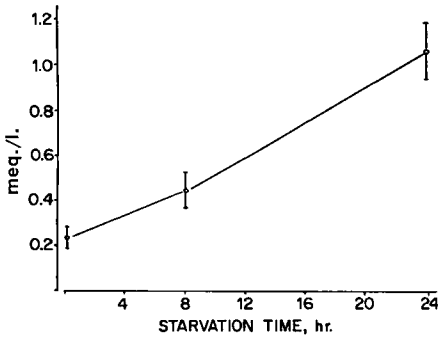

Fig. 2—Relationship between plasma NEFA level and starvation time (mean ± SD, 10 normal rats).

lowering capacity of salicylic acid is dependent upon the supression of plasma NEFA and therefore secondary to it, exogenous NEFA was supplied to salicylic acid pretreated animals.

The relationship between plasma NEFA level and starvation time is shown in Fig. 2. The effect of exogenous NEFA to block the hypoglycemic activity of salicylic acid in diabetic rats is seen in Table III. Thus, when the primary effect of salicylic acid to depress blood NEFA concentration is nullified by the addition of exogenous NEFA, the secondary hypoglycemic effect of salicylic acid is abolished.

SUMMARY

1. The carboxyl group of salicylic acid appears to be responsible for the hypoglycemic activity of this compound. The hydroxyl group at the *ortho* position is not imperative but beneficial to the hypoglycemic activity.

2. It would appear that the hypoglycemic action of salicylates is achieved through a primary effect to suppress the release of fatty acids from adipose tissues.

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Keyphrases

Salicylates—hypoglycemic activity
Structure-activity relationship—salicylates
Hypoglycemic acitivity mechanism—salicylates
Fatty acid, nonesterified—salicylate effect
Colorimetric analysis—spectrophotometer

Identification of a Prednisolone Derivative Interacting with Calf Thymus Histones

By KIYOSHI SUNAGA* and S. S. KOIDE

Prednisolone dissolved in 0.2 *M* phosphate buffer, pH 7.4, was analyzed by TLC and found to undergo degradative changes. One of the products on the chromatogram was identified as 21-dehydroprednisolone which interacted with calf thymus histones. Minimal or no binding occurred with prednisolone and the carboxylic derivative. The transformation of prednisolone to 21-dehydroprednisolone was blocked by EDTA and facilitated by cupric ions. However, the binding of histones with prednisolone in phosphate buffer was depressed when cupric ions were added to the media. The results of this study suggest that prednisolone in phosphate buffer is transformed to 21-dehydroprednisolone which interacts with calf thymus histones.

THE BINDING OF glucocorticoids with calf thymus histones was shown to be dependent on a preliminary incubation of the steroids in phosphate buffer (1). In this study, the transformation of prednisolone[1] in phosphate buffer was studied. A derivative was isolated and identified as 21-dehydroprednisolone which interacted with histones. Comparative studies of the binding capacity of histones with 21-dehydroprednisolone and prednisolone in phosphate buffer at various pH values were carried out.

MATERIALS AND METHODS

The material and methods for the determination of the binding of histones with steroids were the same as previously reported. (1–3). 1,2,4-prednisolone-^{3}H with specific activity of 721 mc./mmole was purchased from Schwarz Bio-Research Inc., New York, N. Y. The steroids were purified on silica gel chromatogram (6061, Eastman Kodak Co., Rochester, N. Y.) before use. The 21-dehydro and 21-carboxylic derivatives of prednisolone were prepared according to the method of Lewbart and Mattox (4). The amount of steroid was estimated by measuring absorbance at 240 mμ or by the radioactivities and by the blue tetrazolium (5) and Porter-Silber reactions (6). A liter of phosphate buffer was washed with 100 ml. of 0.001% dithizone in CCl_4, v/v. The aldehyde group was detected by the Sawicki reaction (7).

Chromatogram sheets were developed by the ascending technique. Two solvent systems were used; namely, chloroform–ethanol, 96:4, v/v, and ethyl acetate–*tert*-butanol–5*N* NH_4OH, 50:40:20 by vol. The sheets were developed and dried at room temperature. The unlabeled steroids were detected by UV lamp. The labeled steroids were determined by cutting the sheets in 1-cm. sections and placing each strip in a counting vial. Ten milliliters of phosphor in toluene were added to each vial and the samples were counted as previously reported (1–3). Each steroid component separated on the chromatogram was extracted with 2 ml. of ethanol for 24 hr. at room temperature. The extract was dried under vacuum and the residue was dissolved in distilled water. The eluted components were rechromatographed. About 4% of the material remained at the origin and other metabolites were not detected. The binding of the steroid fraction with arginine-rich histones was performed as previously reported (1–3) with slight

Received May 31, 1968, from Laboratories of The Population Council, Rockefeller University, New York, NY 10021
Accepted for publication August 21, 1968.
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[1] Trivial names and abbreviations used: Prednisolone, pregna-1,4-diene-11β,17α,21-triol-3,20-dione; 21-dehydroprednisolone, pregna-1,4-dien-21-al-11β,17α-diol-3,20-dione; adrenosterone, androst-4-ene-3,11,17-trione; EDTA, ethylenediamine tetraacetate; dithizone, diphenylthiocarbazone.

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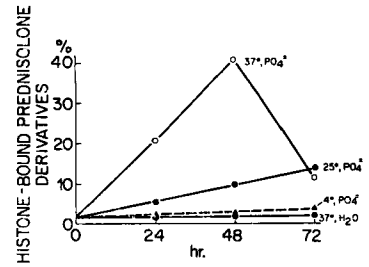

Fig. 1—Binding affinity of arginine-rich histones of calf thymus with prednisolone incubated at different temperatures for 72 hr. Prednisolone was dissolved in 0.2 M potassium phosphate buffer, pH 7.4.

modifications. A mixture was prepared containing 0.2 ml. (3 mg.) of aqueous histone solution, 0.2 ml. of 0.2 *M* potassium phosphate buffer, pH 7.4, and 0.05 ml. of ^{3}H-steroid extracted from the chromatogram. The final concentration of the steroids ranged from 36 to 170 mμM. For comparative study the final concentration was 50 mμM. The mixture was incubated at 37° for 30 min. and filtered on a column (Sephadex G-25) (0.8 × 32 cm.) in the cold (0–4°). The column was eluted with 0.2 *M* potassium phosphate buffer, pH 7.4.

RESULTS

In a preliminary experiment, the monobasic and dibasic potassium phosphates were recrystallized from 0.001 *M* EDTA and several times from deionized water. The transformation of prednisolone to 21-dehydroprednisolone in phosphate buffer prepared with recrystallized phosphates occurred at the same rate as buffer prepared from reagent grade phosphates. Similarly, the transformation occurred in phosphate buffer washed with dithizone.

Figure 1 shows the binding of prednisolone (derivatives) with arginine-rich histones of calf thymus on storage of the steroid dissolved in phosphate buffer or water and incubated at 37° for 72 hr. The maximum binding occurred with the sample stored at 37° for 48 hr. in phosphate buffer. This finding suggested that prednisolone undergoes transformation in phosphate buffer. When EDTA was added

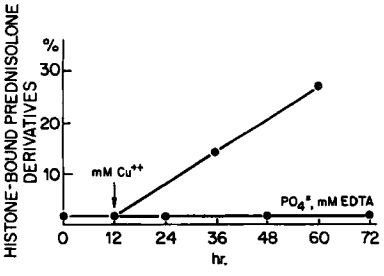

Fig. 2—Binding of arginine-rich histones of calf thymus with prednisolone treated with EDTA and various cations. The following salts were used: $CuSO_4 \cdot 5H_2O$, $MgCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, and $CaCl_2$ at a final concentration of 1 mM. The salts were added 12 hr. after the preparation of the prednisolone solution and incubated at 37°. Aliquots of the prednisolone solution were assayed for binding affinity at 12-hr. intervals for 72 hr.

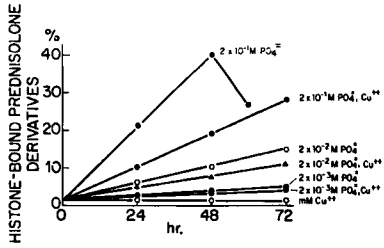

Fig. 3—Binding affinity of arginine-rich histones of calf thymus with prednisolone dissolved in phosphate buffer with and without added cupric ions. Prednisolone was dissolved in potassium phosphate buffer, pH 7.4, and incubated at 37° for 72 hr. The final concentration of cupric ions was 1 mM. The pH of the copper sulfate solution without phosphate buffer was 4.6.

to the solution, the binding capacity was markedly depressed (Fig. 2). The addition of cupric salts reversed the inhibition; whereas Ca^{2+}, Mn^{2+}, and Mg^{2+} ions did not. On the other hand, the addition of cupric salts to prednisolone in various concentrations of phosphate buffer decreased its binding with calf thymus histones (Fig. 3). Other trace metals may be present in analytical grade potassium phosphates which may influence this transformation. No binding was observed when prednisolone was incubated in aqueous cupric sulfate in the absence of phosphate ions (Fig. 3).

Figure 4 shows the TLC of prednisolone dissolved in 0.2 *M* phosphate buffer, pH 7.4, and incubated at 37° for 48 hr. The chromatogram was developed with 4% ethanol in chloroform. Three major and one minor components were detected. These components were identified as the 21-carboxylic derivative (Sp-1), prednisolone (Sp-2), and 21-dehydroprednisolone (Sp-3). The fourth component (Sp-4) which ran with the solvent front was tentatively identified as androsta-1,4-dien-11β-ol-3,17-dione. The Sp-4 spot appeared when 21-dehydroprednisolone was heated at 100° for 10 min. That this component may be androsta-1,4-dien-11β-ol-3,17-

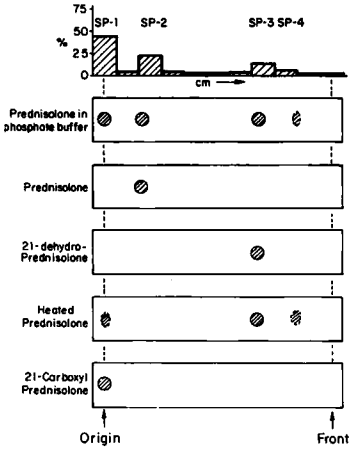

Fig. 4—Thin-layer chromatogram of prednisolone in phosphate buffer. The analysis was performed with prednisolone dissolved in 0.2 M potassium phosphate buffer, pH 7.4, at 37° for 48 hr.

TABLE I—PROPERTIES OF STEROID DERIVATIVES SEPARATED ON THIN-LAYER CHROMATOGRAM

Components	Proportion of 240 mμ: BT: PS	Aldehyde Reaction	R_f
Sp-1	1:0.4:0.4	0	0
Sp-2	1:1:1	0	0.2
Sp-3	1:0.1:0.3	+	0.7

dione is supported by the reports that adrenosterone was formed when cortisone was heated to 90° in ethyl ether peroxides (8) and that the main decomposition product of cortisol-4-^{14}C was androst-4-en-11β-ol-3,17-dione (9). Adrenosterone migrates with the solvent front when developed on silica gel chromatogram with this system. When the chromatogram was developed with a mixture containing ethyl acetate–*tert*-butanol–5 *N* NH_4OH, prednisolone and 21-dehydroprednisolone migrated with the solvent front. With this system, the R_f of Sp-1 was identical with the carboxylic derivative.

Table I shows some of the properties of these three components. When the amounts were determined by measuring the absorbance at 240 mμ and with the blue tetrazolium (BT) and Porter-Silber (PS) reactions, the proportions of the amounts determined by these three methods were markedly different. Only Sp-2 gave equivalent amounts by all three methods and was identified as prednisolone. Only Sp-3 gave a positive aldehyde reaction with the Sawicki reagent identifying it as the 21-dehydro derivative. These results suggest that the α,β-unsaturated 3-keto group remains intact and that the side chain had been altered.

Figure 5 shows the relationship of the binding capacity of histones with prednisolone in phosphate buffer which was incubated at varying lengths of time at 37° to the content of the various steroid derivatives. The transformation of prednisolone in 0.2 *M* potassium phosphate, pH 7.4, and 37° was followed and the contents were analyzed by TLC at 24, 48, and 72 hr. The carboxylic derivative (Sp-1) increased with time; whereas the unchanged prednisolone (Sp-2) decreased. The binding capacity correlated with the content of 21-dehydroprednisolone (Sp-3). These results indicate that the transformation of 21-dehydroprednisolone (Sp-3) to the carboxylic derivative (Sp-1) increased at 72 hr.

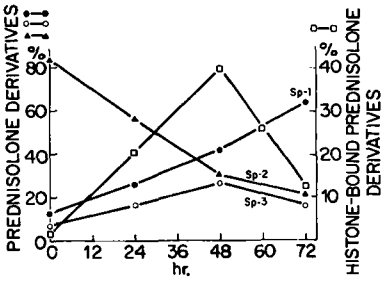

Fig. 5—Content of prednisolone derivatives during storage of prednisolone in phosphate buffer. The binding affinity was determined with the original prednisolone solution dissolved in 0.2 M potassium phosphate buffer, pH 7.4, and with the various derivatives. The final concentration of the steroids was 50 mμM. At time zero, the steroid solution was analyzed immediately after preparation.

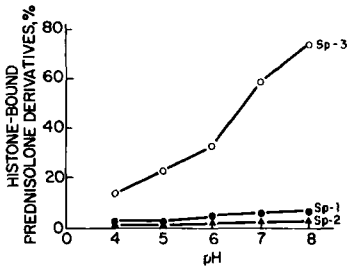

Fig. 6—Binding affinity of prednisolone derivatives with arginine-rich histones of calf thymus at various pH values. The steroids were isolated from the thin-layer chromatogram and dissolved in water. 0.2 M potassium phosphate buffers at the indicated pH values were used as the incubating media. Gel filtration was performed with 0.2 M potassium phosphate buffer, pH 7.4. The final concentration of the steroids was 50 mμM.

Figures 6 and 7 show the binding of these three components isolated from the thin-layer chromatogram with arginine-rich and lysine-rich histones of calf thymus at various pH values. Interaction occurred principally with 21-dehydroprednisolone (Sp-3). Little or no binding was detected with prednisolone (Sp-2) and the carboxylic derivative (Sp-1).

DISCUSSION

Although cupric ions catalyze the degradation of prednisolone to 21-dehydroprednisolone in phosphate buffer, the transformation which proceeds unabated in phosphate buffer washed with dithizone or prepared with recrystallized phosphates remains unexplained. The transformation is in accord with the reports that corticosteroids undergo degradation in aqueous solutions and that the decomposition is catalyzed by cupric ions and inhibited by EDTA (10, 12). It is conceivable that trace amounts of cupric ions are required for the transformation and that an excess may be inhibitory. Cupric ions may influence the binding of prednisolone derivatives with histones. Although the degradation occurs in alkaline media in the absence of cupric ions, the transformation catalyzed

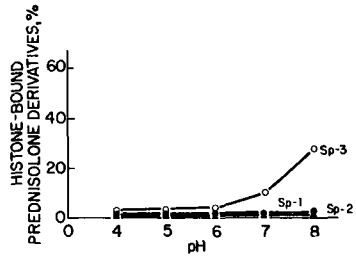

Fig. 7—Binding affinity of prednisolone derivatives with lysine-rich histones of calf thymus at various pH values. The steroids used were isolated from the thin-layer chromatogram and dissolved in water. 0.2 M potassium phosphate buffers at the indicated pH values were used as incubating media. Gel filtration was performed with 0.2 M phosphate buffer, pH 7.4. The final concentration of the steroids was 50 mμM.

by cupric ions in aqueous solutions at neutral pH can be demonstrated only in the presence of phosphate ions.

It has been demonstrated in this study that 21-dehydroprednisolone interacts with histones. This is in accord with the report of Kripalani and Sorby (10) that the degradative products of hydrocortisone interact strongly with human serum albumin. The different binding characteristics of histones with prednisolone and 21-dehydroprednisolone (Fig. 6) suggest that they interact with different loci on the histone molecule. The binding characteristics of these two steroids with transcortin and serum albumin were distinctly different (13). Preliminary studies on the interaction of 21-dehydrocorticosteroids with amino acids in this laboratory suggests that the reaction occurs between the 21-aldehyde and the α-amino group to form Schiff bases.

The possibility that prednisolone (21-dehydrocortisol, is formed under physiological conditions is suggested by the demonstration of 21-hydroxysteroid dehydrogenase in rat and sheep liver and bovine adrenal gland (14–16) although the reaction is strongly in the direction of the alcohol formation. It is noteworthy that the 21-dehydrosteroids are less active or inactive in biological systems in comparison to the parent corticosteroids (17, 18), suggesting that they need to be converted to the alcohol form to be biologically active. Since the biologically active prednisolone did not bind appreciably with histones in comparison with prednisolone, the mechanism of action of prednisolone probably does not depend on its interaction with histones.

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Keyphrases

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21-Dehydroprednisolone binding—calf thymus histones
TLC—separation, identity

Copper-Catalyzed Oxidation of Ascorbic Acid in Gels and Aqueous Solutions of Polysorbate 80

By ROLLAND I. POUST and JOHN L. COLAIZZI

The stability of ascorbic acid in aqueous solution has been studied as a function of polysorbate 80 concentration. The range of polysorbate concentrations studied was sufficiently wide to provide systems ranging from solutions of low viscosity to hydrogels. The study has been conducted at 30, 40, 50, and 60°. First-order rate constants for the oxidative reaction were calculated, and they appear to decrease as a function of polysorbate 80 concentration up to 10 percent, except at 30°, in which case the rate constants decreased up to 30 percent polysorbate 80. The rate generally leveled off at higher surfactant concentrations. It appears that this phenomenon is probably a result of micelle formation, or, more specifically, the increases in micellar aggregation number and micellar concentration which occur up to about 30 percent polysorbate 80. It does not appear that increased viscosity has a significant influence on the rate of ascorbic acid oxidation in these systems.

THE POSSIBILITY OF ENHANCING the stability of drugs by incorporating them into structured vehicles, such as thixotropic gels, has been suggested. Drugs such as vitamins, which are susceptible to decomposition, have been found to be stable for longer periods of time in thixotropic preparations (1). Macek (2) noted that a vehicle of peanut or sesame oil gelled with 2%

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aluminum monostearate reduces the rate of solution (and thus the hydrolytic tendency) of penicillin due to a physical entrapment of the penicillin within the cells of the hydrophobic structural network of the gel. However, relatively little experimental evidence exists describing specific studies performed to investigate this potential means of drug stabilization.

Several studies have indicated the stabilizing effects of nonionic surfactants on various drugs. Kern and Antoshkiw (3) found that aqueous dispersions containing 20% (w/v) polyoxyethylene (20) sorbitan monolaurate[1] stabilized vitamin A to a greater extent than did cottonseed oil containing 2% tocopherol and 4% lecithin, the optimum antioxidant combination for the oil. Swarbrick and Rhodes (4), in a study on the oxidation of linoleic acid in aqueous solutions of polyoxyethylene (23) lauryl ether,[2] found that increasing the surfactant concentration significantly decreased the rate of oxidation. In a series of studies, Carless *et al.* (5–9) found that the rate of oxidation of certain aldehydes and oils was decreased by increasing the concentration of nonionic surfactants used (cetomacrogol[3] and betaine[4]). In a similar study, Mitchell and Wan (10) also found that for a fixed concentration of aldehyde, an increase in surfactant concentration caused a decrease in the rate of oxidation. Swarbrick (11) has reviewed most of these studies in a review article dealing with solubilized pharmaceutical systems.

With such observations in mind, the authors have investigated the stability pattern of ascorbic acid at several temperatures in a range of concentrations of polysorbate 80[5] sufficiently wide to provide vehicles ranging from aqueous solutions to hydrogels. Thus it was possible to investigate relative effects of surfactant concentration, viscosity, and gelation on the stability of this particular drug.

Several attempts have been made to stabilize aqueous solutions of ascorbic acid against oxidation to dehydroascorbic acid by employing various pharmaceutical vehicles and additives, all of which tend to cause slight increases in the viscosity of the solution. Giral (12) hints that the viscosity of a pharmaceutical vehicle such as simple syrup might be a protective factor in stabilizing ascorbic acid. Bandelin and Tuschhoff (13) found that natural hydrophilic colloids (methylcellulose, carboxymethylcellulose, tragacanth, and pectin) which were added to produce solutions of greater viscosity, seemed to accelerate oxidation. This could have been due to trace metals or other impurities introduced by these gums. They also stated that aqueous solutions of sucrose, sorbitol, glycerin, and propylene glycol stabilized ascorbic acid. In a similar study, Bartilucci and Foss (14) found that ascorbic acid was most stable in high concentrations of propylene glycol with either (*a*) water and glycerin or sorbitol, or (*b*) a mixture of glycerin and sorbitol. In a study similar to this one, Nixon and Chawla (15) determined the effects of a wide range of polysorbate 20 concentrations on the oxidation rate of ascorbic acid in an aqueous solution buffered at pH 3.4. They found that the oxidation rates for the copper-catalyzed oxidation rise slightly in aqueous solutions of polysorbate 20 up to a concentration of about 40% (w/w). From that concentration, the oxidation rate then decreased quite rapidly until a concentration of 75% surfactant was reached. Here a leveling off of the rate of oxidation was observed up to about 93% where a slight decrease occurred. At the same time they noted that the aqueous systems showed a fairly rapid increase in viscosity from about 30 to 60% polysorbate 20. From the maximum, which occurred at 62.8%, a gradual decrease in viscosity was noted as the concentration of surfactant approached 100%. In all cases, the rheological properties of these systems were found to be Newtonian in nature. From these results the authors concluded that incorporation of ascorbic acid within the micelle appeared to be responsible for an increase in the oxidation rate at low surfactant concentrations, but that the high viscosity of the concentrated polysorbate 20 solutions caused a much larger reduction in oxidation rate due to a decrease in the diffusion of oxygen to the reaction site.

The viscosity-building and gelation characteristics of aqueous polysorbate 80 mixtures have been reported by Atlas Chemical Industries, Inc. (16). For example, it was reported that the viscosity of aqueous polysorbate 80 systems increased to 740 cps. at a surfactant concentration of 40% (w/w). Actual gel formation occurred from 45 to 65% surfactant concentrations. The viscosity then decreased from 4,700 to 500 cps. between 70 and 95% polysorbate 80. Polysorbate 80 was chosen for this study because of its nontoxic nature (17), gelling characteristics, viscosity-building effects, and the apparent physical, chemical, and microbial stability of the systems.

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[1] Marketed as Tween 20, Atlas Chemical Industries, Inc., Wilmington, Del.
[2] Marketed as Brij 35, Atlas Chemical Industries, Inc., Wilmington, Del.
[3] Polyethylene glycol 1000 monocetyl ether.
[4] *N*-Alkyl, *N*,*N*-dimethylglycines.
[5] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.

MATERIALS AND METHODS

Ascorbic acid was well suited for this study because its fairly rapid copper-catalyzed oxidation can be followed by a simple UV spectrophotometric assay (18). This method enables one to distinguish between ascorbic acid, which absorbs UV light in acidified solutions at 245 mμ, and its main product of oxidation, dehydroascorbic acid, which shows virtually no absorbance in this region of the spectrum.

Preparation and Storage of Test Systems—Exactly 3.00 g. of ascorbic acid[6] was weighed and dissolved in a small quantity of deionized water.[7] The remaining water, enough to give a total mass of 100 g., was then added. Polysorbate 80 USP XVII[5] in sufficient quantities to make concentrations of 0, 0.015, 0.05, 5, 10, 30, 40, 50, 60, and 70%[8] was then gradually stirred into the aqueous solution (using a Fisher Fultork Labmotor[9]) until a homogeneous mixture was obtained. For concentrations of polysorbate 80 above 40%, the mixture was heated on a steam bath to facilitate the escape of air which became entrapped as gels were formed in this concentration range. Initial readings indicated that this heating was not deleterious to the stability of the drug. Finally, 1.0 ml. of a 1×10^{-2} *M* cupric sulfate[10] solution was pipeted into the mixture to give a concentration of 1×10^{-4} *M* in cupric ions. Each test system was assayed immediately upon the completion of its preparation, and was then poured into a 4-oz. amber glass jar, sealed, and stored at either 30, 40, 50, or 60°. Temperature control was achieved by a water bath (30°) Sargent Thermonitor[11] or one of two ovens employed.[12] Temperatures were kept within ±0.5°.

Periodic Assays of Test Systems—All test systems were assayed immediately upon completion of their preparation at various predetermined time intervals. This was accomplished by accurately weighing a 1-g. sample of the test system, quantitatively transferring it to a 100-ml. volumetric flask, and diluting to volume with deionized water. Then 5.0 ml. of this solution was pipeted into another 100-ml. volumetric flask, 10.0 ml. of a 0.2 *N* hydrochloric acid solution was added, and the solution diluted to volume with deionized water. The absorbance of this solution was then determined at 245 mμ on a spectrophotometer (Beckman DB-G)[13] against a suitable reagent blank. The concentration of ascorbic acid remaining was determined from a Beer's law plot[14] of absorbance as a function of ascorbic acid concentration. From these values, the percentage of ascorbic acid remaining was calculated.

Table I—Viscosity Values of Various Concentrations of Polysorbate 80 in Water at 30°

Concn. of Polysorbate 80, % w/w	Viscosity, cps.
40	1304
50	— [a]
60	— [a]
70	4154
100	609

[a] Gel formation.

RESULTS

The physical appearance of the test systems in the concentration range studied was somewhat varied. Those systems containing no polysorbate 80 were clear, colorless solutions at all temperatures. The systems containing 0.015 and 0.05% polysorbate 80 were clear, faintly yellow solutions. The systems containing 5, 10, 30, and 40% surfactant were all clear solutions of increasing viscosity and increasing intensity of yellow color, due to the increasing concentrations of polysorbate 80. The 50% polysorbate 80 system was a translucent yellow gel at 30 and 40°, and a clear, yellow, highly viscous liquid at 50 and 60°. The 60% polysorbate 80 system was a clear yellow gel at 30° and a clear, yellow, highly viscous liquid at the other temperatures. The systems containing 70% surfactant were clear, yellow, viscous liquids at all temperatures. The liquid systems exhibited Newtonian rheological properties, and the gels behaved as non-Newtonian systems when analyzed in the rotational viscosimeter (Stormer). Viscosity values for some of the Newtonian liquids, as determined at 30°, are summarized in Table I.

The results of the stability studies at 30, 40, 50, and 60° are summarized in Tables II–V, showing the percentage concentrations of ascorbic acid remaining at various times in each concentration of polysorbate 80 used. At certain concentrations of surfactant, some protection from oxidation was afforded the ascorbic acid. In the cases of 60 and 70% polysorbate 80 at 30 and 40°, the reaction seemed to stop after a few days. The logarithms of the percentages of ascorbic acid remaining were plotted as a function of time for all concentrations of polysorbate 80 used at all temperatures. Four such plots representing the reaction in water, 10, 40, and 60% polysorbate 80 appear as Figs. 1–4. From these plots it appeared that the kinetics of these reactions is first-order. This is in agreement with the findings of Dekker and Dickenson (20) who stated that the copper-catalyzed oxidation of ascorbic acid in water is a first-order kinetic process. The straight lines shown were obtained by the method of least squares (21), but the points shown represent the experimental values. The slopes obtained from the least-squares calculations were used to calculate values (k) for the first-order rate constants (22). First order-rate constants were calculated for each experimental situation and were plotted as a function of polysorbate 80 concentration at all temperatures. These graphs appear as Fig. 5.

[6] Analytical reagent grade, Fisher Scientific Co., Pittsburgh, Pa.

[7] Deionized water was used throughout the study and was prepared by passing distilled water through two glass percolation columns containing a mixture of cationic- and anionic-exchange resins (Rexyn AG 501, Fisher Scientific Co.). Conductivity was below 0.1 p.p.m. (as sodium chloride) as determined with a Barnstead purity meter (Barnstead Still and Sterilizer Co., Boston, Mass.)

[8] Percentage values indicate percent by weight.

[9] Fisher Scientific Co., Pittsburgh, Pa.

[10] Analytical reagent grade, Merck and Co., Rahway, N. J.

[11] E. H. Sargent and Co., Chicago, Ill.

[12] Blue M Stabil-Therm Mechanical Convection Oven and Labline Radiant Heat Oven, Aloe Scientific, St. Louis, Mo.

[13] Beckman Instruments, Inc., Fullerton, Calif.

[14] These plots were prepared by determining absorbance values of solutions containing 2.00, 1.75, 1.50, 1.00, 0.50, and 0.25 mg.% ascorbic acid against a suitable blank. This was done for each concentration of polysorbate 80 used in the study.

TABLE II—PERCENTAGE CONCENTRATION OF ASCORBIC ACID REMAINING AS A FUNCTION OF TIME IN VARIOUS CONCENTRATIONS OF POLYSORBATE 80 AT 30° IN THE PRESENCE OF 1×10^{-4} *M* CUPRIC ION

Time, days	Water	Concn. of Polysorbate 80, % w/w							
		0.015	0.05	10	30	40	50	60	70
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1	95.00	95.90	95.04	92.86	—	—	—	—	—
2	89.20	91.02	90.87	94.00	98.71	98.35	92.46	96.15	94.91
3	92.93	91.02	90.08	91.26	—	—	—	—	—
4	90.86	89.40	90.87	90.43	—	—	—	—	—
7	86.70	86.15	85.52	85.87	—	—	—	—	—
8	—	—	—	—	96.62	95.75	—	83.65	93.62
11	83.33	81.27	81.82	82.47	96.08	94.93	92.46	87.56	93.62
18	78.73	75.22	78.51	79.54	94.53	93.22	83.73	84.95	93.62
25	73.73	69.11	72.70	72.91	92.03	88.09	79.13	81.52	91.52
32	71.66	64.17	62.78	71.70	90.34	88.09	79.13	81.52	91.52
39	70.96	60.92	55.38	69.34	88.65	86.79	79.13	74.72	91.52
46	67.06	55.26	49.17	68.13					

TABLE III—PERCENTAGE CONCENTRATION OF ASCORBIC ACID REMAINING AS A FUNCTION OF TIME IN VARIOUS CONCENTRATIONS OF POLYSORBATE 80 AT 40° IN THE PRESENCE OF 1×10^{-4} *M* CUPRIC ION

Time, days	Water	Concn. of Polysorbate 80, % w/w							
		0.015	0.05	10	30	40	50	60	70
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1	95.00	95.08	95.83	96.13	97.52	100.00	100.00	93.53	89.01
5	—	—	—	—	—	96.64	91.41	87.53	88.13
6	86.70	85.24	86.78	91.80	92.52	—	—	—	—
8	83.33	80.78	83.01	88.52	91.02	94.04	91.41	93.33	89.01
12	76.67	75.80	78.51	86.88	89.79	91.51	84.13	90.86	89.01
19	68.33	65.57	66.49	81.83	84.13	85.49	84.13	90.00	89.01
26	58.33	54.75	56.17	77.44	81.27	80.36	76.44	85.00	88.54
33	50.40	46.29	49.57	70.09	74.38	78.45	76.44	83.33	88.13

TABLE IV—PERCENTAGE CONCENTRATION OF ASCORBIC ACID REMAINING AS A FUNCTION OF TIME IN VARIOUS CONCENTRATIONS OF POLYSORBATE 80 AT 50° IN THE PRESENCE OF 1×10^{-4} *M* CUPRIC ION

Time, days	Water	Concn. of Polysorbate 80, % w/w							
		0.015	0.05	10	30	40	50	60	70
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1	92.93	95.00	96.56	84.85	98.71	99.17	98.71	100.00	100.00
4	80.00	84.52	86.78	91.80	96.08	94.04	97.42	98.33	92.40
7	73.73	75.16	75.21	85.24	92.03	88.50	90.23	92.06	91.52
11	68.73	60.14	61.92	77.44	84.40	81.66	83.86	83.73	84.74
14	63.33	54.48	52.87	72.13	81.02	76.94	75.38	81.66	81.35
18	52.06	46.74	42.96	67.60	72.18	70.51	71.59	75.00	77.55
21	45.00	40.63	38.00	61.44	65.83	66.68	67.79	71.73	71.18
25	36.66	32.89	31.59	56.52	59.55	61.55	63.11	66.67	69.49
28	32.06	27.63	27.23	54.09	55.70	58.13	57.22	61.66	66.91

TABLE V—PERCENTAGE CONCENTRATION OF ASCORBIC ACID REMAINING AS A FUNCTION OF TIME IN VARIOUS CONCENTRATIONS OF POLYSORBATE 80 AT 60° IN THE PRESENCE OF 1×10^{-4} *M* CUPRIC ION

Time, days	Water	Concn. of Polysorbate 80, % w/w							
		0.05	5	10	30	40	50	60	70
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1	91.67	95.00	96.67	99.67	100.00	96.67	91.33	90.87	99.67
5	71.67	78.33	80.80	90.00	92.53	86.67	85.00	88.33	92.53
8	58.80	65.00	69.10	83.40	88.45	83.40	80.80	85.00	85.00
12	45.00	50.75	58.80	75.80	80.80	75.00	76.70	80.00	76.70
15	38.33	45.87	55.00	70.00	75.00	70.86	75.00	76.70	75.00
19	30.87	35.87	50.87	64.13	68.33	65.00	68.33	70.00	71.67
22	25.87	33.33	48.33	61.67	66.67	60.87	64.13	66.67	66.67
26	20.00	26.67	44.13	57.53	60.00	54.20	60.67	62.47	62.47
29	17.53	25.87	41.67	54.20	56.67	51.67	55.00	56.67	60.87

As can be seen from the plots in Fig. 5, the rate of oxidation in 0.015% (slightly below the CMC of 0.03%)[15] and 0.05% (slightly above the CMC) is slightly higher than that of water at all temperatures except at 60° where a slightly lower value for 0.05% polysorbate 80 was observed. These increases are followed by a lowering of the rate at a 10% polysorbate 80 concentration at all temperatures. At the lower temperatures, this is followed by a further decrease at 30% polysorbate 80, somewhat of a leveling effect at 40%, an increase at 50%, and subsequent decreases at 60 and 70%. At the higher temperatures, the general trend seems to be a leveling off between 10 and 40% polysorbate 80 followed by a gradual decrease up to 70%, the limiting concentration to which the study was performed.

DISCUSSION

Weissberger *et al.* (23) have reported that the copper-catalyzed oxidation of ascorbic acid is a

[15] This value for the CMC was determined at 30° with a Fisher Surface Tensiomat, Fisher Scientific Co., Pittsburgh Pa.

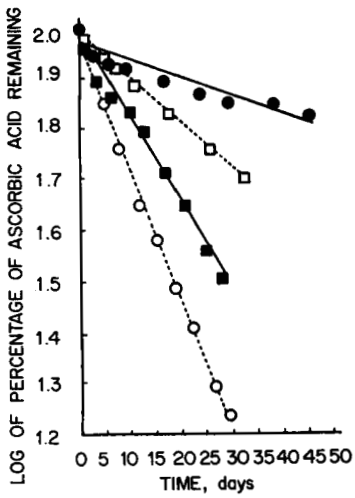

Fig. 1—Log of percentage of ascorbic acid remaining as a function of time in water. Key: ●, 30°; □, 40°; ■, 50°; ○, 60°.

first-order kinetic process and that the rate is proportional to the oxygen concentration in neutral and acid solutions. (While not carefully controlled through the use of buffers, pH values for the systems employed in this study fell within a range of 2.5 to 4.0.) They indicated that the rate-determining step is the formation of an intermediate semiquinone-like structure from the ascorbate ion, and that the formation of this intermediate is catalyzed by the presence of the cupric ion. Subsequent oxidation of this intermediate to dehydroascorbic acid is therefore dependent upon the concentration of oxygen present. Dekker and Dickenson (20) have reported much the same findings and are in agreement with Weissberger *et al.* as to the proposed mechanism of oxidation. As was mentioned previously, the decomposition kinetics of ascorbic acid for the systems investigated in the present study appears to be first-order, which is in agreement with the literature references indicated here.

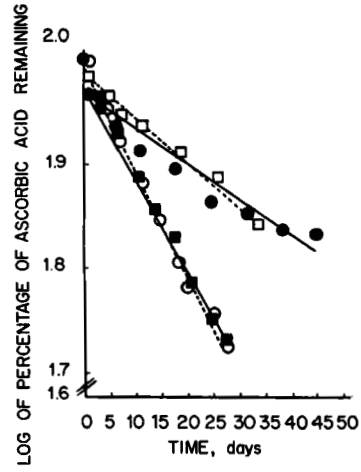

Fig. 2—Log of percentage of ascorbic acid remaining as a function of time in 10% polysorbate 80. Key: ●, 30°; □, 40°; ■, 50°; ○, 60°.

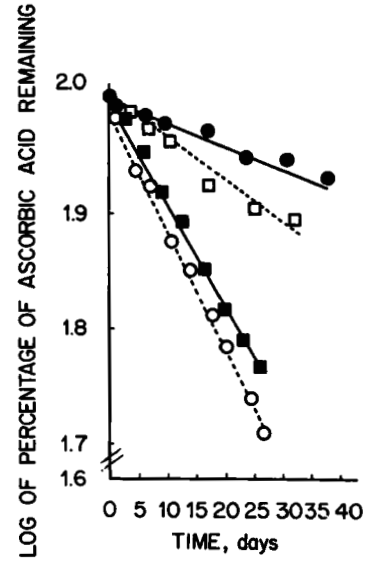

Fig. 3—Log of percentage of ascorbic acid remaining as a function of time in 40% polysorbate 80. Key: ●, 30°; □, 40°; ■, 50°; ○, 60°.

As illustrated in Fig. 5, the general effect observed upon the addition of polysorbate 80 to aqueous solutions of ascorbic acid is the occurrence of a reduction in rate constant. However, it should be noted that this reduction seems to be significantly influenced by surfactant concentration only up to a polysorbate 80 concentration of approximately 30% at 30° and 10% at the other temperatures. Then there was a general leveling off of the rate constants at higher surfactant concentrations. In all studies except that carried out at 60°, an initial increase in rate constants was observed in the systems containing 0.015 and 0.05% polysorbate 80. This may be due to an association complex formed

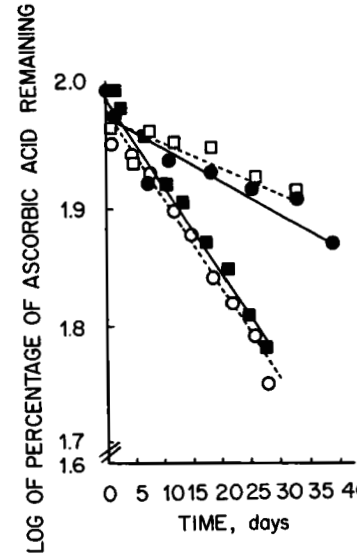

Fig. 4—Log of percentage of ascorbic acid remaining as a function of time in 60% polysorbate 80. Key: ●, 30°; □, 40°; ■, 50°; ○, 60°.

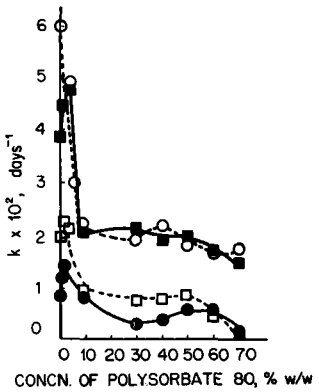

Fig. 5—First-order rate constants as a function of polysorbate 80 concentration. Key: ●, 30°; □, 40°; ■, 50°; ○, 60°.

between the ascorbic acid and polysorbate 80 molecules in which the ascorbic acid is made more readily susceptible to oxidative degradation. Another possibility might be that the ascorbic acid molecules are adsorbed onto the surface of the surfactant molecules, again making them more susceptible to oxidative attack due to a surface-type catalysis. These findings are in agreement with the observation of Nixon and Chawla (15) that low surfactant concentrations caused a rise in the rate of oxidation of ascorbic acid. Whatever the reasons for this phenomenon, these effects are apparently overcome at higher polysorbate 80 concentrations where a decrease in the rate of oxidation (as compared with the rate in systems containing no surfactant) occurred at all concentrations of polysorbate 80 studied. This decrease in the rate, or protection from oxidation, is most probably related in some way to micelle formation.

As was mentioned previously, Nixon and Chawla (15) carried out a stability study with ascorbic acid which in some respects resembles the present study. However, there are significant differences in the results of the two studies which could be accounted for in terms of differences in experimental conditions. The results in the present study showed that the greatest decrease in the copper-catalyzed oxidation rate occurred between 0.05 and 10% surfactant except at 60° where there was no initial increase in the rate with the very low surfactant concentration. At polysorbate 80 concentrations higher than 10%, a general leveling off of the rate constants occurred with an additional slight decrease occurring between 50 and 70%. However, in their study, Nixon and Chawla found an increase in the copper-catalyzed oxidation rate at the lower concentrations up to about 40%, where a sharp decrease in the rate occurred which continued up to a concentration of about 75% polysorbate 20, followed by a leveling off. It should be pointed out that different surfactants were utilized in each of the studies—polysorbate 80 in the present study and polysorbate 20 in the other. Also, with polysorbate 20, no gel formation, such as that which occurred in the present study, was observed. Perhaps the most significant difference was the method of assay employed by Nixon and Chawla in which a Warburg apparatus was used to measure the oxygen uptake for the total system, thus introducing the possibility that substances other than ascorbic acid may utilize oxygen and alter the rate of oxygen uptake. However, in the present study, the assay method was specifically designed to determine only the concentration of ascorbic acid remaining in the system thus eliminating any interference introduced by other components of the system. The initial concentration of ascorbic acid could also be responsible for differences in the results. Nixon and Chawla employed saturated solutions of ascorbic acid in polysorbate 20 whereas 3% (w/w) ascorbic systems were employed in the present study. The use of buffers, such as the dibasic sodium phosphate–citric acid buffer utilized by Nixon and Chawla, may have caused some difference in the results since no buffers were used in the present study. Finally, the stability studies carried out by Nixon and Chawla were at 25° exclusively, while four different temperatures—30, 40, 50, and 60°—were employed in the present study.

If the stabilizing effects of polysorbate 80 are, in fact, related to the presence of micelles, then those factors which are known to alter the micellar properties of a system should also influence stabilization profiles such as those observed in this study. Becher (24), in a discussion of micelle formation has stated that the aggregation number (number of monomer units per micelle) of a micelle appears to increase with increasing surfactant concentration up to those concentrations that are between 100 and 1,000 times the CMC. Thus, based on this idea, micelles in the surfactant systems of this study would increase in aggregation number and hence in micelle size between concentrations of 3 and 30% polysorbate 80.[16] At higher surfactant concentrations, the aggregation number levels off and thus the size of the micelles is somewhat the same at the various higher concentrations of polysorbate 80 employed in this study. Micellar shape can also vary with surfactant concentration as has been indicated by Nakagawa (25) who stated that micelles in dilute solutions are spherical or globular, but in more concentrated solutions the micelles tend to assume a lamellar structure. It has been found (24) that increased temperatures cause increases in the micellar weight suggesting that larger micelles would occur at higher temperatures. The addition of electrolytes has been found to cause a drop in the CMC, but causes very little, if any, change in the size of the micelle. Thus, the addition of ascorbic acid to these systems will have no appreciable effect on the size and shape of the micelle.

Based on the ideas in the preceding discussion and the plot of rate constants presented in Fig. 5, it appears possible that increases in the number of micelles and increases in the micellar aggregation number, *i.e.*, increases in the size of the micelles, which occur up to about 30% polysorbate 80, may cause the sharp decrease in the rate constants which is observed in this concentration range. The more extensive formation of lamellar-type micelles may also come about in this concentration range, and this might be another factor responsible for the decrease in rate constants. This decrease in oxidation rate may be due to a decrease in the collision

[16] These figures are based on the previously mentioned CMC of 0.03% for polysorbate 80 at 30°.

frequencies caused by the increasing size of the micelles. The larger micelles may slow down the rate of oxygen diffusion and thus slow down the rate of oxygen attack of the semiquinone-like ascorbic acid intermediate. As was indicated previously, the micellar size remains essentially constant at polysorbate 80 concentrations above 30% as does the rate of oxidation as can be seen by the general leveling off in Fig. 5 at higher surfactant concentrations. The increase in micellar size would be even larger at the higher temperatures possibly causing the sharper drop in the rate constants which was observed. In fact, different micellar properties at the higher temperatures might be responsible for the interesting grouping and similarity of slope observed with the results at 30 and 40° as compared to the results at 50 and 60° as illustrated in Figs. 2–4.

Based on these observations, it would seem that the increased number of micelles and changes in micellar shape that may occur with increasing polysorbate 80 concentrations (above 30%) have no appreciable effect on the rate of oxidation. It was noted that a slight decrease in the rate of oxidation occurred between 50 and 70% polysorbate 80, a region where gels and the liquid of maximum viscosity occurred. Nixon and Chawla (15) stated that at very high polysorbate 20 concentrations water was enclosed as a discontinuous pseudophase inside the micelle and that the ascorbic acid dissolved in it was protected from the oxygen by the surfactant. This might account for the slight additional decrease in oxidation rate at these higher surfactant concentrations. Nixon and Chawla have also proposed that the stabilizing effect of polysorbate 20 is closely related to the increasing viscosity of the systems which decreases the rate of oxygen diffusion. The work of Bartilucci and Foss (14) indicates that it would be possible that retardation of oxidation of ascorbic acid may be due primarily to the decrease in water content as surfactant concentrations increase. The results of this study, however, would seem to indicate that increased viscosity or simple reduction in water content are not the primary factors responsible for surfactant stabilization of ascorbic acid in the case of polysorbate 80. The viscosity of the systems employed in this study increased markedly between 40 and 50% polysorbate 80 (where gel formation occurred), whereas no significant change in the rate constants occurred in this region. The 60% polysorbate 80 gel and the 70% liquid also have viscosity values which are much higher than the 40% surfactant system, but no corresponding decrease in the rate constants was observed at these concentrations as would be expected if viscosity were the primary factor responsible for stabilization of ascorbic acid. Atlas Chemical Industries (16) has reported that only a gradual increase in viscosity of polysorbate 80–water systems occurs between 20 and 40% surfactant concentrations. Such a gradual increase in viscosity does not account for the large decrease in rate constants which was observed around 10% polysorbate 80. Similarly, a more gradual decrease in rate constants would probably have been observed if simple reduction in water content had been the stabilizing factor. Thus, it would seem that changes in micellar structure provide a more suitable explanation for the results obtained in this study.

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Keyphrases

Ascorbic acid stability—polysorbate 80 solutions, gels
Polysorbate 80 effect—ascorbic acid oxidation
Copper-catalyzed oxidation—ascorbic acid
UV spectrophotometry—analysis

Some Pharmacokinetic Aspects of 5-(Dimethyltriazeno)-imidazole-4-carboxamide in the Dog

By TI LI LOO, BRENDA B. TANNER, GLENN E. HOUSHOLDER, and BEVERLY J. SHEPARD

The renal clearance of 5-(dimethyltriazeno)-imidazole-4-carboxamide (DIC, NSC-45388) has been determined in the dog using radioactive inulin as the reference compound. The clearance ratio of DIC to inulin was greater than one, suggesting tubular secretion of DIC. The volume of distribution of DIC in the dog was determined from plasma clearance curves. In experiments lasting 1 hr., this volume was greater than total body water, indicating the localization of the drug in some tissues. However, when the experimental period was extended to 2 hr., the localized drug was eliminated from the body by both biotransformation and excretion, and the result was a reduction in the volume of distribution. A new mathematical treatment is described for analysis of the plasma clearance curves from 2-hr. experiments.

A POTENT CANCER chemotherapeutic agent of recent development, 5-(dimethyltriazeno)-imidazole-4-carboxamide (DIC, NSC-45388) (1), compares quite favorably with existing clinically useful agents such as cyclophosphamide and 6-mercaptopurine against the solid form of mouse Ehrlich carcinoma (2). In addition, it has shown activity against certain human malignant melanomas during clinical trials (3). Studies of its pharmacology have been reported (4). In this paper, certain pharmacokinetic aspects of DIC in the dog are described.

The renal clearance of DIC has been determined using radioactive inulin as the reference compound (5). The drugs were administered either by a single, rapid intravenous injection (6), or by constant infusion (7). Radioactive inulin was given either in trace dosage or diluted with unlabeled inulin.

The volume of distribution of the drug is estimated by a single, rapid intravenous injection technique (8–11). Frequent blood samples are taken at intervals. The plasma levels of DIC are plotted against time on a semilog scale. In experiments lasting 1 hr., the data are fitted by two exponential functions of time. This is interpreted to mean that the body behaves like a two-compartment open system in which the drug equilibrates between an immediately permeable compartment and a second compartment not immediately permeable to DIC. In addition, the drug is eliminated from the body by biotransformation and excretion following first-order kinetics (10). An alternative interpretation similar to that above is that the drug leaves the first compartment both by elimination and by passive diffusion into a second compartment in accordance with Fick's law (8). In either case a second-order linear homogeneous differential equation with constant coefficients is derived. The volumes of the two compartments and the two clearance constants are computed from the graphic data. By either model, exactly the same numerical values are obtained in each experiment.

However, in volume of distribution experiments lasting over 1 hr., the data can best be fitted by three instead of two exponential functions of time. In other words, the differential equation relating plasma levels of DIC to time is now a third-order linear homogeneous one with constant coefficients. Again, two models can be constructed to arrive at such an equation. Either the body can be thought of as a three-compartment open system (9) or the previous two-compartment concept may be retained but the intercompartmental clearance written as a linear function of time. The volumes of distribution of the reference compounds, inulin and mannitol, are invariant with time in the dog, and their values agree with published data (8).

EXPERIMENTAL

Materials—Both DIC and DIC-2^{14}C were supplied by the Drug Development Branch of the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md. For intravenous administration to the dog, DIC was dissolved in 0.1 *N* hydrochloric acid containing 0.88% of sodium chloride. No significant pH change in plasma after such an injection was ever observed in the experiments. Inulin carboxyl-^{14}C, inulin methoxy-^{3}H, and mannitol-1-^{14}C were supplied by New

Received June 17, 1968, from the Department of Developmental Therapeutics, The University of Texas M.D. Anderson Hospital and Tumor Institute, Houston, TX 77025

Accepted for publication September 4, 1968.

Presented in part before the spring meeting of the American Society for Pharmacology and Experimental Therapeutics, April 20, 1968, Atlantic City, N. J.

Supported by contract PH43-66-1156 with the National Cancer Institute, National Institutes of Health.

England Nuclear Corp., Boston, Mass., and dissolved in normal saline for administration.

Colorimetric Determination—DIC was determined by a colorimetric method developed by the authors (12). The principle involves the photodecomposition by ultraviolet light irradiation of a solution of DIC in dilute acid in the presence of *N*-(1-naphthyl)-ethylenediamine (Bratton-Marshall reagent), followed by measurement of the color intensity of the azo compound thus formed. The method is specific for dialkyltriazeno compounds, and metabolite interference has not been encountered. Plasma and urine standards were prepared from samples collected just before the experiment.

Radioactivity Determination—A liquid scintillation spectrometer (Packard Tri-Carb, model 3375) was used. All plasma and urine samples (0.2 ml.) were counted directly as a suspension in 11 ml. of counting solution described previously (13). Quenching was determined by channel ratios of an automatic external standard. Solutions containing precisely measured amounts of radioactive drugs were counted together with the samples.

Dogs—Mongrel dogs, 13–30 kg., of both sexes were lightly anesthetized with phenobarbital. Drug administration and blood sampling were performed in different veins. Heparin was used as an anticoagulant. Urine was collected by an indwelling Foley catheter.

Renal Clearance—The renal clearance of DIC in comparison with inulin was determined by both constant infusion and single i.v. techniques. Prior to all experiments the bladder was emptied and preinjection blood samples obtained.

Blood samples were taken at the midpoint of urine collection intervals over a 2-hr. period. A Sigmamotor 18 SH pump (Sigmamotor Inc., Middleport, N.Y.) was used in constant infusion experiments. The priming dose consisted of 20 mg./kg. DIC in all experiments while inulin ranged from 0.25 mg./kg. (radioactive inulin only) to 60 mg./kg. (a mixture of radioactive inulin and unlabeled inulin). The sustaining dose for DIC was 10–15 mg./kg./hr. and for inulin was 0.49 (radioactive only) to 131 mg./kg./hr.

In single i.v. experiments the dose of DIC was 20 mg./kg. and inulin ranged from 0.65 mg./kg. (radioactive only) to 100 mg./kg.

Volume of Distribution—Intravenous infusion of normal saline (about 2 ml./min.) was started 30 min. prior to each experiment. The bladder was emptied and a blood sample taken immediately before the injection. DIC was administered at a dose range of 10–20 mg./kg. The dose of the reference substance used in combination with DIC (mannitol and inulin) was 100 mg./kg. Blood samples were taken every 5 min. over a 2-hr. period.

RESULTS AND DISCUSSION

Renal clearance of DIC and that of inulin are compared in Table I. The sustaining dose of DIC used to maintain a fairly constant plasma level of the drug in the dog is based on previous experience (4). The sustaining dose of inulin follows a recommendation in the literature (14). There appears to be no significant difference in renal clearance whether the single intravenous injection technique or the constant-infusion technique is adopted. Also, the amount of inulin used does not seem to be critical.

The authors inulin clearance value is lower than that reported in the literature (14) because the urine flow in the anesthetized dog is much lower than the published average. However, since the clearances of both DIC and inulin are equally affected by the low urine flow, clearance ratios of the two substances must be substantially correct. The average clearance ratio of DIC to inulin is 1.8, indicating tubular secretion of DIC. Compounds with dipolar structure, such as *p*-aminohippuric acid (I) are known to be secreted by renal tubules; DIC (II) also has a dipolar structure as shown below. Renal tubular secretion of DIC is therefore not surprising.

A typical 1-hr. plasma clearance curve plotted on a semilog scale is illustrated in Fig. 1. It is evident that the data can be adequately described by two exponential functions of time, thus:

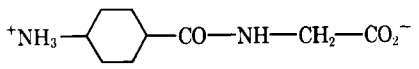
$$C_1 = Ae^{-r_1 t} + Be^{-r_2 t} \qquad \text{(Eq. 1)}$$

In other words, the plasma clearance curve is resolvable into two exponential (straight lines on a semilog

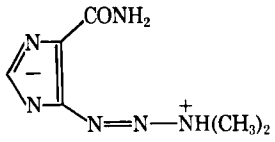
TABLE I—RENAL CLEARANCE OF DIC IN COMPARISON WITH INULIN

Dog No.	Wt., kg.	Priming Dose, mg./kg. DIC	Priming Dose, mg./kg. Inulin	Sustaining Dose, mg./kg./hr. DIC	Sustaining Dose, mg./kg./hr. Inulin	Clearance ml./min./kg. DIC	Clearance ml./min./kg. Inulin	Clearance Ratio, DIC Inulin
				Single i.v.				
53	14.6	20	100			2.6	1.4	1.9
85	21.6	20	100			5.1	2.6	2.0
87	19.3	20	100			4.3	2.6	1.7
277	20.3	20	0.65[a]			1.7	1.6	1.1
					Average	3.4	2.1	1.7
				Infusion				
16	14.8	20	50	10	131	3.0	2.0	1.5
18	19.9	20	50	15	98	3.9	3.9	1.0
37	19.6	20	0.25[a]	15	0.49[a]	4.4	2.2	2.0
41	16.0	20	50	15	121	9.4	3.0	3.1
279	26.0	20	60	10	75	3.6	2.2	1.6
					Average	4.9	2.7	1.8

[a] Radioactive material only.



I

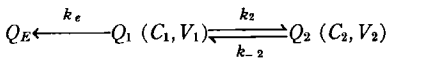


II

scale) curves. The differential equation of which Eq. 1 is the general solution is

$$D^2C_1 + (r + r_2)DC_1 + r_1r_2C_1 = 0 \quad \text{(Eq. 2)}$$

Consider now the distribution of the drug in a two-compartment open system according to Scheme I.



Scheme I

Eq. 2 may be rewritten in terms of the rate constants of Scheme I. Thus,

$$D^2C_1 + (k_2 + k_{-2} + k_e)DC_1 + k_{-2}k_eC_1 = 0 \quad \text{(Eq. 3)}$$

From the graphically obtained values of A, B, r_1, and r_2, the rate constants are readily computed (11):

$$k_2 = \frac{AB(r_2 - r_1)^2}{(A + B)(Ar_2 + Br_1)} \quad \text{(Eq. 4)}$$

$$k_{-2} = \frac{Ar_2 + Br_1}{A + B} \quad \text{(Eq. 5)}$$

$$k_e = \frac{(A + B)r_1r_2}{Ar_2 + Br_1} \quad \text{(Eq. 6)}$$

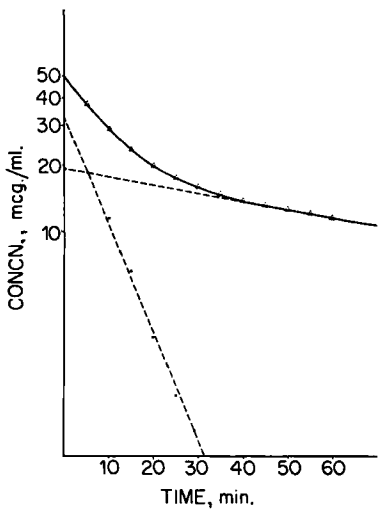


Fig. 1—Plasma level of DIC in dog No. 57 after a single i.v. injection (20 mg./kg.). A 1-hr. experiment. $C_p = 19.2\ e^{-0.008t} + 32.7\ e^{-0.111t}$.

V_1 is simply

$$V_1 = \frac{Q_I}{A + B} \quad \text{(Eq 7)}$$

The volume of the second compartment which is not immediately permeable to DIC, and the volume of distribution are also explicit in A, B, r_1, and r_2.

$$V_2 = \frac{AB(r_1 - r_2)^2Q_I}{(A + B)(Ar_2 + Br_1)^2} \quad \text{(Eq. 8)}$$

$$V_d = \frac{(Ar_2^2 + Br_1^2)Q_I}{(Ar_2 + Br_1)^2} \quad \text{(Eq. 9)}$$

The derivation of Eqs. 8 and 9 will be discussed later.

Results of eight experiments are shown in Table II. In all cases the volumes of distribution of DIC in the dog are in excess of total body water content of the animal, suggesting local concentration of DIC in some tissues of the dog.

An alternative model for the fitting of the experimental data by two exponential terms has been offered by Sapirstein *et al.* (8). These authors considered the mass transfer from the first compartment by both elimination and passive diffusion into the second compartment. If the elimination follows first-order kinetics and the diffusion obeys Fick's law, the following equation is readily derived;

$$D^2C_1 + \left(\frac{\alpha + G}{V_1} + \frac{\alpha}{V_2}\right)DC_1 + \frac{\alpha G}{V_1V_2}C_1 = 0 \quad \text{(Eq. 10)}$$

If the drug suffers biotransformation, and if the method of drug determination is specific for the drug alone, the rate constant for biotransformation can be absorbed into G. Equation 10 can be rewritten

$$D^2C_1 + \left(\frac{\alpha}{V_1} + \frac{\alpha}{V_2} + \frac{G}{V_1}\right)DC_1 + \frac{\alpha G}{V_1V_2}C_1 = 0 \quad \text{(Eq. 10}a\text{)}$$

A comparison of Eq. 10a with Eq. 3 reveals

$$\frac{\alpha}{V_1} = k_2,\ \frac{\alpha}{V_2} = k_{-2},\ \text{and}\ \frac{G}{V_1} = k_e$$

or

$$\alpha = k_2V_1 = k_{-2}V_2 \quad \text{(Eq. 11)}$$

and

$$G = k_eV_1 \quad \text{(Eq. 12)}$$

Since

$$\alpha = \frac{AB(r_1 - r_2)^2Q_I}{(A + B)^2(Ar_2 + Br_1)} \quad \text{(Eq. 13)}$$

Equations 8 and 9 are readily derived from Eqs. 11, 12, and 13.

When the volume of distribution experiments were extended to 2 hr., the plasma clearance curve could best be resolved into three instead of two exponential terms (Fig. 2), thus:

$$C_1 = Ae^{-r_1t} + Be^{-r_2t} + Fe^{-r_3t} \quad \text{(Eq. 14)}$$

Apparently under these circumstances the body behaves like a three-compartment open system. This situation is frequently encountered in tracer problems in which the body is treated not as a three-compartment system but as a multiple-compartment open system (15–17). At steady state, the number

TABLE II—VOLUME OF DISTRIBUTION OF DIC (1 hr.)

Dog No.	Wt., kg.	Dose, mg./kg.	G, ml./min.	α, ml./min.	V_1, l.	V_2, l.	Volume of Distribution, % Body wt.	$k_1 \times 10^2$, $min.^{-1}$	$k_{-1} \times 10^2$, $min.^{-1}$	$k_e \times 10^2$, $min.^{-1}$
57	30.3	10	162	578.1	9.3	25.7	115.5	6.3	2.2	1.8
57	30.3	20	225	628	11.7	13.6	83.5	5.4	4.6	1.9
63	13.5	10	104	255.2	4.1	7.0	82.2	6.3	3.6	2.6
63	13.5	20	174	256.9	7.3	5.9	97.8	3.5	4.3	2.4
72	22.3	20	512	444	10.2	9.8	89.7	4.4	4.5	5.0
76	23.6	20	146	481.2	9.5	7.3	71.2	5.1	6.9	1.5
87[a]	19.3	20	146.1	548	5.7	6.0	60.6	9.6	9.2	2.6
91[b]	18.5	20	170.6	203.5	8.0	5.4	72.4	2.6	3.7	2.1

[a] DIC-2-^{14}C used in combination with unlabeled DIC and inulin-methoxy-^{3}H. The volume of distribution of inulin was 32% of body weight. [b] Unlabeled DIC used in combination with mannitol-1-^{14}C. The volume of distribution of mannitol was 18% of body weight.

of postulated compartments and the rate constants of intercompartmental transport (namely, intercompartmental clearances) are estimated by nonlinear statistical regression analysis. Such a treatment requires a certain amount of mathematical sophistication and skill, and computer programs have been written to aid the analysis. Even in the present three-compartment problem, when the rate equations are written down, the solutions, though by no means difficult, are extremely cumbersome (see *Appendix*). Finally, on pharmacokinetic grounds alone, one finds it hard to explain why DIC becomes distributed in three instead of two functional compartments as the period of observation is extended.

However, the simpler two-compartment treatment can still be made applicable to the present problem if all functional body compartments except the one immediately permeable to the drug (plasma water in most cases) are conceptually combined and considered as one composite compartment. Intuitively one may write V_c, the volume of the composite compartment, as a weighted mean of all the compartments

$$V_c = \frac{\alpha_1 V_1 + \alpha_2 V_2 + \alpha_3 V_3 + \ldots + \alpha_n V_n}{\alpha_1 + \alpha_2 + \alpha_3 + \ldots + \alpha_n} \quad \text{(Eq. 15)}$$

The intercompartmental clearance between the central compartment and the composite peripheral compartment is the mean of all the intercompartmental clearances.

$$\alpha = \frac{\alpha_1 + \alpha_2 + \ldots + \alpha_n}{n} \quad \text{(Eq. 16)}$$

To derive a third-order linear homogeneous differential equation with constant coefficients for the description of the above two-compartment open system, one writes α' as a linear function of t, thus:

$$\alpha' = \alpha + \beta t, \beta \ll \alpha \quad \text{(Eq. 17)}$$

Consider the rate of decrease of a quantity of the drug from the central compartment both by elimination (and biotransformation) and by passive diffusion into the composite peripheral compartment (8):

$$DQ_1 = V_1 DC_1 = -GC_1 - \alpha'(C_1 - C_2) \quad \text{(Eq. 18)}$$

$$Q_I = C_1 V_1 + C_2 V_2 + \int_0^t GC_1 dt$$

or

$$C_2 = \frac{1}{V_1}\left(Q_I - C_1 V_1 - \int_0^t GC\,dt\right) \quad \text{(Eq. 19)}$$

Substitution of Eqs. 17 and 19 into Eq. 18 followed by rearrangement gives

$$DC_1 + \left(\frac{\alpha + \beta t + G}{V_1} + \frac{\alpha + \beta t}{V_2}\right) C_1 + \frac{(\alpha + \beta t)G}{V_1 V_2} \int_0^t C_1 dt - \frac{(\alpha + \beta t)Q_I}{V_1 V_2} = 0 \quad \text{(Eq. 20)}$$

The integral is removed by differentiating Eq. 20 twice with respect to t

$$D^3 C_1 + \left(\frac{\alpha + \beta t + G}{V_1} + \frac{\alpha + \beta t}{V_2}\right) D^2 C_1 + \left(\frac{2\beta V_1 + 2\beta V_2 + G(\alpha + \beta t)}{V_1 V_2}\right) DC_1 + \frac{2\beta G}{V_1 V_2} C_1 = 0 \quad \text{(Eq. 21)}$$

The general solution of Eq. 21 is complex. How-

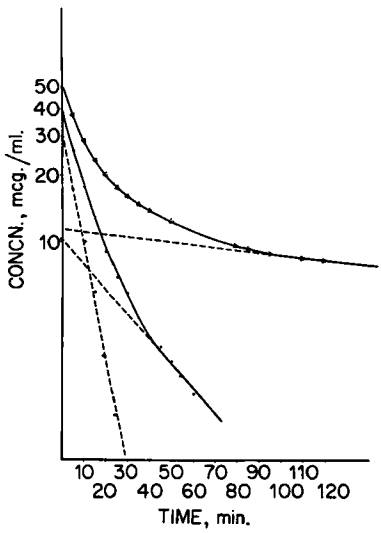


Fig. 2—Plasma level of DIC in dog No. 57 after a single i.v. injection (20 mg./kg.). A 2-hr. experiment. $C_p = 11.4\ e^{-0.003t} + 10.3\ e^{-0.028t} + 30.5\ e^{-0.117t}$.

TABLE III—VOLUME OF DISTRIBUTION OF DIC (2 hr.)

Dog No.	Wt., kg.	Volume of Distribution, % Body wt.	G, ml./min.	α', ml./min.	β, ml./min.[2]
57	30.3	50.8	585	279	0.32
72	22.3	49.3	862	100	0.39
76	23.6	52.5	354	136	0.28
85[a]	21.6	35.2	322	68	0.09
87[a]	19.3	47.6	309	259	0.12

[a] DIC-2-^{14}C used.

ever, remembering $\beta \ll \alpha$, provided t is not too large, $\alpha + \beta t \rightarrow \alpha'$. Under these conditions Eq. 21 is therefore reduced to

$$D^3C_1 + \left(\frac{\alpha' + G}{V_1} + \frac{\alpha'}{V_2}\right) D^2C_1 + \frac{\alpha' G}{V_1 V_2} DC_1 + \frac{2\beta G}{V_1 V_2} C_1 = 0 \quad \text{(Eq. 22)}$$

This is precisely the equation desired because the general solution of Eq. 22 is Eq. 14, (*ie.*,

$$C_1 = A_e^{-r_1 t} + Be^{-r_2 t} + Fe^{-r_3 t}).$$

The usual boundary conditions are: *at* $t = 0$,

$$C_1 = C_0 = A + B + F \quad \text{(Eq. 23)}$$

and also

$$DC_1 = -(Ar_1 + Br_2 + Fr_3) = -(\alpha + G)C_0/V_1 \quad \text{(Eq. 24)}$$

By simple algebraic manipulations, V_1, V_2, G, and α are now expressed in A, B, F, r_1, r_2, and r_3.

Therefore:

$$V_1 = Q_I/(A + B + F) \quad \text{(Eq. 25)}$$

$$V_2 = \alpha' G/(r_1 r_2 + r_2 r_3 + r_1 r_3)V_1 \quad \text{(Eq. 26)}$$

$$G = \frac{(r_1 r_2 + r_2 r_3 + r_1 r_3)Q_I}{A(r_2 + r_3) + B(r_1 + r_3) + F(r_1 + r_2)} \quad \text{(Eq. 27)}$$

$$\alpha' = \frac{(Ar_1 + Br_2 + Fr_3)V_1}{A + B + F} - G \quad \text{(Eq. 28)}$$

$$\beta = \frac{r_1 r_2 r_3}{2G} V_1 V_2 \quad \text{(Eq. 29)}$$

and, finally, the volume of distribution, V_d, is

$$V_d = \frac{V_1 + V_2}{W} \times 100 \quad \text{(Eq. 30)}$$

Table III shows the numerical values of the volume of distribution and G, α', and β. In the 2-hr. experiments, the volume of distribution of DIC averages 47% of body weight of the dog, or 80% of the total body water content. It may be recalled that, in all experiments of 1-hr. duration, the volumes of distribution exceed total body water content, suggesting the localization of the drug in some tissues. This contention receives support from recent preliminary studies. Using DIC-2-^{14}C, it has been shown that 15 min. after an i.p. injection in the mouse radioactivity is indeed localized in the liver and the small intestine.[1] When the volume of distribution experiment is carried on to 2 hr., the localized drug is eliminated from the body by both biotransformation and excretion, and consequently a reduction in the volume of distribution is observed.

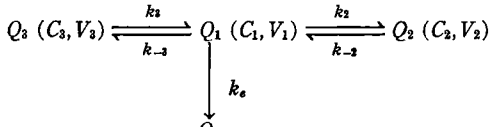
[1] Housholder, G. E., unpublished observation.

NOTATIONS

- C = Concentration of DIC. The subscripts denote individual compartments.
- A,B,F = Intercepts of plasma clearance curve (plotted on a semilog scale) on the y-axis (concentration).
- r_1,r_2,r_3 = Slopes of the individual exponential lines.
- t = Time.
- D = The differential operator d/dt.
- k_2 = First-order rate constant of drug transport from the central, immediately permeable compartment (plasma) to Compartment 2, with the dimension of t^{-1}.
- k_3 = Similar constant for Compartment 3.
- k_{-2} = First-order rate constant of reverse drug transport from Compartment 2 back to the central compartment.
- k_{-3} = Similar constant for Compartment 3.
- Q = Quantity of DIC. The subscripts refer to individual compartments.
- Q_E = Quantity of DIC eliminated from the body.
- k_e = First-order rate constant of drug elimination, with the dimension of t^{-1}.
- G = Glomerular filtration rate, with dimension L^3t^{-1}. (L stands for length.)
- V = Volume (dimension L^3) of individual functional compartment. The subscripts have the usual meaning.
- α,α' = Intercompartmental clearance with a dimension L^3t^{-1}.
- Q_I = Quantity of DIC injected.
- β = A constant of dimension L^3t^{-2}.
- V_d = Volume of distribution.
- V_c = Volume of the composite compartment.
- W = Body weight.

APPENDIX

Distribution of a Drug in a Three-Compartment Open System—Consider Scheme II.



Scheme II

$$DC_1 = -(k_2 + k_3 + k_e)C_1 + k_{-2}C_2 + k_{-3}C_3 \quad \text{(Eq. 31)}$$

$$DC_2 = k_2C_1 - k_{-2}C_2 \quad \text{(Eq. 32)}$$

$$DC_3 = k_3C_1 - k_{-3}C_3 \quad \text{(Eq. 33)}$$

Substitute Eqs. 32 and 33 into Eq. 31 and rearrange

$$D^3C_1 + (k_2 + k_3 + k_{-2} + k_{-3} + k_e)D^2C_1 + (k_2k_3 + k_3k_{-2} + k_ek_{-2} + k_ek_{-3} + k_{-2}k_{-3})DC_1 + k_{-2}k_{-3}k_e\,C_1 = 0 \quad \text{(Eq. 34)}$$

TABLE IV—FIRST-ORDER RATE CONSTANT ($\times 10^2$ IN MIN.$^{-1}$) OF TRANSPORT OF DIC[a] IN THE DOG IN 2-hr. EXPERIMENTS

Dog No.	k_2	k_3	k_e	k_{-2}	k_{-3}
57	3.8	2.5	1.2	6.0	1.4
72	2.7	1.9	4.3	5.3	2.2
76	3.1	0.2	1.3	5.3	1.5
85	2.3	2.5	1.1	6.0	1.7
87	4.5	2.6	0.1	14.0	1.0

[a] Dose of DIC injected: 20 mg./kg.

The general solution of Eq. 34 is Eq. 14. Therefore,

$$r_1 + r_2 + r_3 = k_2 + k_3 + k_{-2} + k_{-3} + k_e \quad \text{(Eq. 35)}$$

$$r_1r_2 + r_1r_3 + r_2r_3 = k_2k_{-3} + k_3k_{-2} + k_ek_{-2} + k_ek_{-3} + k_{-2}k_{-3} \quad \text{(Eq. 36)}$$

$$r_1r_2r_3 = k_{-2}k_{-3}k_e \quad \text{(Eq. 37)}$$

The boundary conditions are, at $t = 0$: $C_1 = C_0 = A + B + F$, which is Eq. 23, and

$$DC_1 = -(k_2 + k_3 + k_e)C_0 = -(Ar_1 + Br_2 + Fr_3) \quad \text{(Eq. 24}a\text{)}$$

It follows that

$$k_2 + k_3 + k_e = \frac{Ar_1 + Br_2 + Fr_3}{A + B + F} \quad \text{(Eq. 38)}$$

From Eqs. 35 and 38,

$$k_{-2} + k_{-3} = \frac{B + F)r_1 + (A + F)r_2 + (A + B)r_3}{A + B + F} \quad \text{(Eq. 39)}$$

Since

$$C_0 = k_e\int_0^\infty C_1dt = k_e\int_0^\infty (Ae^{-r_1t} + Be^{-r_2t} + Fe^{-r_3t})dt = k_e\left(\frac{A}{r_1} + \frac{B}{r_2} + \frac{F}{r_3}\right) \quad \text{(Eq. 40)}$$

therefore,

$$k_e = \frac{(A + B + F)r_1r_2r_3}{Ar_2r_3 + Br_1r_3 + Fr_1r_2} \quad \text{(Eq. 41)}$$

and from Eq. 36,

$$k_{-2}k_{-3} = \frac{Ar_2r_3 + Br_1r_3 + Fr_1r_2}{A + B + F} \quad \text{(Eq. 42)}$$

From Eqs. 39 and 42, k_{-2} and k_{-3} are computed; and from Eqs. 36 and 38, k_2 and k_3 are computed. Table IV presents the numerical values of these rate constants.

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Keyphrases

5-(Dimethyltriazeno)-imidazole-4-carboxamide(DIC)—pharmacokinetics
Renal clearance—DIC
Volume of distribution—DIC, DIC-2^{14}C
Pharmacokinetic equations—plasma clearance, DIC
Colorimetric analysis—spectrophotometer
Liquid scintillation counting—radioactivity determination

Thin-Layer Chromatography of Certified Coal Tar Color Additives

By MELVIN H. PENNER

A comprehensive thin-layer chromatographic procedure has been developed for the systematic separation and identification of 19 water- and/or alcohol-soluble coal tar dyes currently permitted for use in pharmaceutical dosage forms. TLC on cellulose-coated chromatoplates and development in one of the following solvent systems: (A) ethyl acetate–*n*-butanol–pyridine–water (5:5:6:5); (B) ethyl acetate–*n*-butanol–concentrated ammonia (20:55:25); (C) ethyl acetate–*n*-propanol–concentrated ammonia–water (35:35:20:20); (D) *n*-propanol–ethyl acetate–concentrated ammonia (65:75:60) offers rapid, sharp separations into compact zones of the most common colorant combinations used to achieve special color effects. Quantitation of the colorants may then be achieved by densitometric scanning of the developed plates. The method has also been applied for the detection of dye incompatibility in a liquid preparation.

DYES ARE USED in the pharmaceutical industry to achieve esthetic color characteristics required for consumer acceptance of drug products and for the identification and differentiation of dosage forms. Only those colorants which have been certified by the Food and Drug Administration may be used in drug products marketed in the United States. The array of coloring agents previously available has been considerably reduced because of the delisting of a number of certified dyes, necessitating the reformulation of many products. Since colors are generally produced by blending two or more dyes to achieve a desired effect, a rapid chromatographic system for the separation and identification of the component dyes in a color was highly desirable for formulation studies. Since the separation of all dyes is more of theoretical than practical interest, this investigation was limited to 19 certified colorants most frequently encountered in pharmaceutical formulations.

Prior separation of dyes in mixtures is necessary for identification and quantitation in most cases, for absorption spectrophotometry usually presents a confusing picture of overlapping bands. Color analysis based on partition of the dyes between two immiscible solvents was reported by Koch (1). However, at best the separations are only fractional and several of the permitted dyes are discolored by the treatment.

The chromatography of coal tar colorants has been the subject of numerous investigations. Separation of colors by paper chromatography (2–9) gives sharper separations than partition or adsorption column chromatography (10–13), electrophoresis (14–16), or ion-exchange methods (17, 18). However, no one solvent can effect satisfactory separation of mixtures of dyes, particularly the red dyes which give almost the same R_f value with most solvents.

Absorption spectrophotometric methods have been most widely used for the quantitative estimation of separated colors. Stanley and Kirk (3) have also applied reflectance measurements to the isolated dyes on paper chromatograms and achieved better results.

This report describes a scheme for the separation, identification, and quantitation of the most frequently used certified water- and/or alcohol-soluble colorants by TLC in a limited number of solvent systems. An example of the use of this technique in studying dye incompatibility is presented.

EXPERIMENTAL

Chromatoplates—Commercially prepared plates, 20 × 20 cm., coated with microcrystalline cellulose[1] to a layer thickness of 250 μ and purchased from Analtech, Inc., were used in this study without prior activation.

Solvent Systems—Four monophasic solvent systems were found to be the most useful: A, ethyl acetate–*n*-butanol–pyridine–water (5:5:6:5); B, ethyl acetate–*n*-butanol–concentrated ammonia (20:55:25); C, ethyl acetate–*n*-propanol–concentrated ammonia–water (35:35:20:20); D, *n*-propanol–ethyl acetate–concentrated ammonia (65:75:60).

Dye Solutions—Stock solutions of the following certified dyes were prepared at a concentration of 1 mg./ml. of water: FD&C Red No. 2 (R2), FD&C Red No. 3 (R3), FD&C Red No. 4 (R4), D&C Red No. 19 (R19), D&C Red No. 22 (R22), D&C Red No. 28 (R28), D&C Red No. 33 (R33), FD&C Blue No. 1 (B1), FD&C Blue No. 2

Received May 22, 1968, from the Pharmaceutical Research and Development Labs., Warner-Lambert Research Institute, Morris Plains, NJ 07950

Accepted for publication August 19, 1968.

Presented to the Drug Standards, Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

The author wishes to express his gratitude to Mr. D. M. Schoeffel and his associates for their technical assistance, and to Drs. T. I. Fand and L. Chafetz for their invaluable aid in preparing this manuscript.

[1] Trade marked as Avicel by American Viscose Div. of FMC Corp., Marcus Hook, Pa.

(B2), FD&C Yellow No. 5 (Y5), FD&C Yellow No. 6 (Y6), D&C Yellow No. 10 (Y10), D&C Orange No. 4 (O4), FD&C Violet No. 1 (V1), FD&C Green No. 3 (G3), and D&C Green No. 5 (G5). The stock solutions of D&C Yellow No. 11 (Y11), D&C Green No. 6 (G6), and D&C Orange No. 5 (O5) were prepared at the 0.1% concentration in alcohol since they are not water soluble.

Chromatographic Development—To determine the R_f values of the individual dyes under consideration and to determine the most effective solvent system to achieve the desired separation, 1 μl. of each stock solution, dispensed from a 1-μl. disposable pipet, was spotted to separate points on the origin on each of four chromatoplates, approximately 2.5 cm. from the bottom edge. The plates were then placed in the chromatographic chamber which has been equilibrated with the respective solvent system for 30–60 min. and the solvent allowed to travel a distance of 10–15 cm. from the point of origin. The plates were removed from the development chamber and air dried.

Detection—The developed chromatograms were viewed under long-wavelength UV light in addition to ordinary illumination. The colors and R_f values were noted.

Quantitation—A standard mixture containing four certified dyes, *i.e.*, FD&C Red No. 2 (0.5 mg./ml.), D&C Red No. 33 (0.5 mg./ml.), FD&C Yellow No. 6 (0.75 mg./ml.), and D&C Green No. 5 (0.1 mg./ml.), was prepared in aqueous alcohol. This stock solution was then serially diluted to contain 0.4, 0.3, 0.25, 0.2, 0.15, and 0.1 mg. of D&C Red No. 33/ml. solution. Five microliters of each solution, employing self-filling micropipets calibrated to deliver accurately known volumes, was spotted to alternate 1-cm. wide channels inscribed in a cellulose-coated chromatoplate (20 × 20 cm.) and allowed to air dry. The plate was developed in a chamber equilibrated with solvent system C to a distance of 12 cm. from the origin, removed from the chamber, and air dried. A Schoeffel spectrodensitometer model SD 3000, a double-beam ratio-type scanning instrument equipped with a high-intensity continuous xenon light source, quartz prism monochromator, and an integrating recorder was used for quantitation. Each dye was scanned and recorded with the monochromator set at the wavelength of maximum absorbance for the respective dye as determined on the chromatoplate.

RESULTS AND DISCUSSION

Qualitative Separations—The average R_f values of the 19 dyes considered in this investigation in each of the four solvent systems and the respective colors observed under long-wavelength UV light are recorded in Table I. The values reported in the parentheses represent minor impurities detected in the raw materials.

In conjunction with the R_f values, further verification of identities of the colorant mixture components may be ascertained by viewing the developed chromatogram under long-wavelength UV light and/or spraying the plate with various reagents noting the change in color as reported by numerous investigators (3, 4, 7, 19). It should be noted that FD&C Blue No. 2 has a tendency to fade in systems containing ammonia.

In 1944, Peacock (20) published colorant formulas

Table I—R_f Values of Certified Dyes by TLC

Dye	Solvent System A	B	C	D	Color Under UV Light
Blue					
FD & C No. 1	0.53	0.24	0.72	0.56	Violet
FD & C No. 2	0.33	0.09	0.47	—	Blue
Green					
FD & C No. 3	0.49	0.11	0.57	0.38	Pink
D & C No. 5	0.64	0.38	0.77	0.63	Blue
D & C No. 6	1.0	1.0	1.0	1.0	Blue
Orange					
D & C No. 4	0.76	0.55	0.89	0.79	Yellow-brown
D & C No. 5	0.74 (0.94)	0.28 (0.23)	0.63 (0.67)	0.55	Yellow
Red					
FD & C No. 2	0.28	0.00	0.28	0.10	Red
FD & C No. 3	0.94	0.46	0.79	0.68	Dull orange
FD & C No. 4	0.53	0.05	0.30	0.21	Reddish-orange
D & C No. 19	0.93	0.96	0.98	1.0	Brilliant orange
D & C No. 22	0.88	0.37	0.73	0.61	Yellow-green
D & C No. 28	0.79	0.51	0.83	0.74	Orange
D & C No. 33	0.43	0.12	0.54	0.39	Scarlet
Yellow					
FD & C No. 5	0.26	0.00	0.15	0.06	Deep yellow
FD & C No. 6	0.47 (0.26)	0.15	0.62	0.41	Red
D & C No. 10	0.62 (0.70)	0.29 (0.42)	0.72 (0.77)	0.58 (0.69)	Green
D & C No. 11	0.95	1.0	1.0	1.0	Green
Violet					
FD & C No. 1	0.61	0.38	0.80	0.66	Violet

TABLE II—TLC SEPARATION OF SOME COLORANT MIXTURES

Color	Dye Mixture	Solvent System
Black	R2, Y6, B1	C, D
Black	R2, Y6, B2, Y5	C
Blue	R3, G5	B
Deep blue/grape	R2, B1	A, C, D
Caramel/chocolate	R2, Y5, Y6, B1	C
Mint-green	Y11, G6	A
Lime	B1, Y5	A, C
Reddish-orange	R2, Y6	C, D
Medium orange	R4, Y5	A, C, D
Amber	R2, Y5	C
Orangeade	R4, Y5, Y6	C, D
Wine	R2, Y6, B1	C, D
Cola	R2, Y5, B1	C
Maroon	R2, V1	A, C, D
Cherry	R2, R4	A, D
Strawberry	R2, R4, Y6	D
Pink	R3, Y6	A, B, C, D
Raspberry	R2, Y6	C, D
Egg-yellow/lemon	R2, Y5, Y6	C

for use in proprietary mixtures to obtain various color effects, such as mint-green, caramel, strawberry, *etc.* Since each of these formulas contained some proportion of FD&C Orange No. 1 or FD&C Green No. 2, which have since been delisted, new formulas had to be devised. Several such replacements were reported by Peacock (21) and Zuckerman (22). Table II lists the colorant mixtures used to produce some popular color effects and the solvent system(s) best suited in each case to resolve the dye components.

As evident from Tables I and II, solvent systems C and D are the best choice for general screening of all the dyes and the latter especially where the presence of one or more red colorants is indicated. The remaining two systems were developed for special cases as shown in the tables.

Quantitation—Both absorbance and reflectance measurements for the quantitation of the chromatographically resolved dyes have been reported by Stanley and Kirk (3). However, the quantity of dye required in their method was considered excessive, since repeated evaporation of a dilute dye solution may be accompanied by sufficient build-up of excipient materials to affect the migration of the dye material.

Spectrodensitometry offered a significant increase in sensitivity. A plot of the integrated area obtained *versus* the concentration applied is shown in Fig. 1. Statistically, the best straight line was constructed for each colorant by means of linear regression analysis. A linear relationship was obtained over the concentration range considered which is also evidenced by the calculated value of the correlation coefficient, r, for each dye being extremely close to 1. From Fig. 1, the standard error of estimate, $s_{y \cdot x}$, for each colorant was calculated. Table III summarizes the r and $s_{y \cdot x}$ values obtained in each case.

The lower limit of detection of 0.2 mcg. of D&C Green No. 5 was achieved utilizing a more sensitive recorder range than for the remaining three dyes.

Standard calibration curves were necessarily prepared for each dye since the slopes of the four lines varied which may be attributed to several

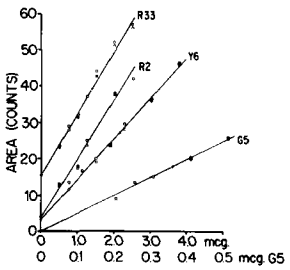

Fig. 1—Relationship of the spot area of various dyes to the concentrations applied. Slit width = 1 mm.; monochromator: R2 and R33 = 515 mμ, Y6 = 485 mμ, G5 = 628 mμ; recorder range: R2, R3, Y6 = 0–3 full scale, G5 = 0–0.5 full scale.

factors such as the intensity of stray light reaching the photocell detector, the purity of the standards, and variation in adsorbent layer thickness. Deviations from linearity resulting in curvature of the lines as the amount of dye spotted increases may be caused by saturation so that the peak density no longer increases proportionally with concentration. Differences in scans are then mainly caused by increases in spot size.

In order to ensure reproducibility and accuracy of the densitometric quantitation, several experimental parameters were found to be critical. The spectrodensitometer employed is designed to minimize errors due to moisture and to variations in thickness of the adsorbent material by scanning the sample channel *versus* the adjacent strip untouched to serve as the reference. Since the reference and sample beams are closely adjacent, positioning of the well-defined strips evenly under the sample and reference beam is important. The scribe line must be positioned evenly between the two light beams for reproducible results. Although the width of the channels is approximately 1 cm., care should be taken when depositing the sample to minimize diffusion of the sample laterally toward the edges of the strip. If this occurs, the subsequently chromatographed strip may exhibit the edge phenomenon which will seriously affect the results. The slit covers virtually the entire channel which has the advantage that the full width of the absorbing spot is scanned and all the material in the spot contributes to absorption of light. This eliminates errors due to small changes in shape and centering of the spot. Care should be taken, however, to deposit the initial spot as uniformly as possible. Excessive tailing or overlapping of zones decrease the precision of area measurements. The densitometry error, calculated from repetitive scanning of the same spot, was 1.3%. The micropipets used for spotting the dyes are capable of delivering a known volume with an accuracy of ±3%.

Dye Incompatibility—The present chromatographic procedure has also been applied to deter-

TABLE III—CALCULATED VALUES OF r AND $s_{y \cdot x}$

Dye	r	$s_{y \cdot x}$, %
R2	0.988	±6.1
R33	0.993	±4.1
Y6	0.997	±3.3
G5	0.992	±3.8

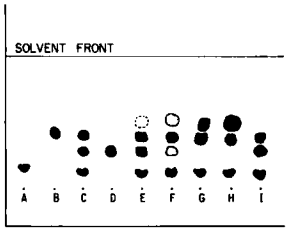

Fig. 2—Dye incompatibility study. Solvent system: ethyl acetate–n-butanol–pyridine–water (5:5:6:5). Plate: microcrystalline cellulose. Key: A, FD & C Red No. 2; B, FD & C Red No. 4; C and I, mixture of reds 2, 4, and 33; D, D & C Red No. 33; E, sample after 4 days at 60°; F, sample after 12 days at 60°; G and H, sample after 18 months at room temperature (3 and 5 μl., respectively).

mine the cause of color darkening observed visually in a liquid dosage upon aging at room temperature. The proposed formula contained a mixture of FD & C Red No. 2, No. 4, and D & C Red No. 33. A 100-ml. aliquot was extracted according to the procedure reported by Bandelin and Tuschhoff (7) and redissolved in 5 ml. alcohol. Standard solutions of the individual dyes and the sample extract were spotted on a microcrystalline cellulose plate and chromatographed in solvent system A. The resulting TLC pattern indicated the depletion of D & C Red No. 33 with the concomitant formation of a violet-colored species. Further experiments demonstrated that a reaction between D & C Red No. 33 with a flavoring component, anise oil, in the presence of formaldehyde was responsible for the formation of the violet moiety. This was clearly demonstrated when a synthetic mixture of the three red colorants with anise oil and formaldehyde were heated at 60° for 4 and 12 days and then chromatographed. The TLC pattern obtained is shown in Fig. 2.

SUMMARY

A thin-layer chromatographic procedure has been described which allows the rapid screening of 19 water- and/or alcohol-soluble certified colorants frequently used in the pharmaceutical industry. Since colorants are usually present in minute quantities in dosage forms, TLC is ideally suited due to the sharp separations and compact zones which result in its use.

A standard sample containing four certified dyes was scanned with a spectrodensitometer after thin-layer chromatographic separation. Plots of area *versus* concentration resulted in reasonably linear relationships with high sensitivity allowing for direct quantitation of the colorants on the chromatoplate.

The present chromatographic procedure has also been used to determine the cause of color darkening in a liquid preparation. Chemical reaction of one of the dyes with a flavor component resulted in the formation of a violet species responsible for the color change upon storage.

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Keyphrases

Color additives, coal tar—separation, identification
TLC—separation, identity
Chromatographic development systems—coal tar dyes
UV light—spot visualization
Densitometric scanning—color quantitation

Induction of Photohemolysis by Tetrachlorosalicylanilide

By W. S. OLENIACZ, E. J. SINGER, A. B. DOYLE, and L. J. VINSON

The antibacterial agent 3,3′,4′,5-tetrachlorosalicylanilide (TCSA) enhanced the sensitivity of erythrocytes to long-wave UV light *in vitro*. The exposure of TCSA-treated red cells to UV radiation resulted in photohemolysis; the extent of photohemolysis was related to the duration of irradiation. The photohemolytic reaction appeared to be a consequence of UV excitation of TCSA to the triplet state and subsequent reaction of the latter molecule with the erythrocyte membrane. The interaction of the excited TCSA molecule with the red cell membrane was manifested as increased permeability towards Mg^{2+}, K^+, or Na^+ ions. Photohemolysis was prevented by the UV absorbers urocanic acid and 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid. In addition to TCSA, 3,3′,4′,5- and 2′,3,4′,5-tetrabromosalicylanilides caused photohemolysis. Monobromo-, dibromo-, and tribromosalicylanilides failed to induce significant photohemolysis. The results suggest that some instances of photodermatitis due to TCSA may be related to changes in cell permeability and lysis.

PHOTODERMATITIS resulting from the topical application of the antibacterial agent 3,3′,4′,5-tetrachlorosalicylanilide (TCSA) has been reported by several investigators (1–5). The inflammatory reaction is elicited upon exposure of the skin to long-wave UV light within the range 300 to 425 mμ (6). The manner by which TCSA produces skin damage has not been definitely established. Primary irritant, phototoxic, contact allergic, and contact photoallergic processes have been suggested as possible mechanisms. Recent preliminary work has demonstrated the induction and passive transfer of TCSA photocontact hypersensitivity in guinea pigs (4). These results indicate that photodermatitis due to TCSA may be related to a delayed light-mediated immunological process, although further confirmation is required. However, it is well known that certain factors can contribute to photosensitization. The factors include the concentration of the agent used, the vehicle employed, the nature of the light source, and the length of exposure of the skin to light. Since the latter factors can influence the skin response, it is conceivable that TCSA may also cause photodermatitis by a mechanism other than that responsible for delayed hypersensitivity.

In addition to TCSA, various medicinal agents have been incriminated as photosensitizers (7). With the exception of the furocoumarins (8), it is generally believed that photosensitizing agents produce photodermatitis as a consequence of increased cell permeability and concomitant cell lysis. Increased permeability is the resultant manifestation of a photochemical reaction involving a photosensitizer, oxygen, and an unknown membrane component. Evidence has been presented which indicates that the disruption of lysosomal and mast cell membranes may be the primary event in photosensitization (9).

The possibility that photodermatitis due to TCSA may also be related to changes in cell permeability has not been considered nor substantiated in clinical investigations reported. This report presents the results of *in vitro* studies which demonstrate that the interaction of long-wave UV radiation with TCSA can cause altered membrane permeability, as evidenced by photohemolysis of erythrocytes. The value of erythrocytes as models for assessing photosensitization effects has been documented (9–11).

EXPERIMENTAL

Preparation of Erythrocytes—Adult rats, derived from the Sprague-Dawley strain, were used throughout this investigation. The animals were killed by decapitation and blood was collected from the severed vessels into heparinized tubes. Red cells were sedimented by centrifugation, washed three times with isotonic saline–phosphate buffer of pH 7.4 (0.14 *M* NaCl, 8.8×10^{-3} *M* Na_2HPO_4, 1.4×10^{-3} *M* NaH_2PO_4), and then resuspended in buffer to original volume.

Photohemolysis Assay, General Procedure—The erythrocyte suspension was diluted 1:200 with 10^{-4} *M* TCSA contained in saline–phosphate buffer. Appropriate controls consisted of erythrocytes diluted 1:200 with saline–phosphate buffer lacking TCSA. The samples were dark-incubated (low-actinic test tubes, 18 × 150 mm.) for 30 min. at 25° on a reciprocating shaker, operated at 68 strokes/min. Five milliliters of the suspension was then transferred to an open Stender dish (4.9 cm. diameter) and irradiated on the shaker. All assays were performed in triplicate. The radiation factors employed were: exposure time, 60 min.; target distance, 10 cm.; radiation source, multiray lamp

Received July 15, 1968, from Lever Brothers Co., Research and Development Division, Edgewater, NJ 07020
Accepted for publication August 29, 1968.

fitted with a long-wave UV light having maximum energy output at 366 mμ; intensity of incident radiation, 1.44×10^7 ergs/cm.^{2}, as measured with a YSI model 65 radiometer (Yellow Springs Instrument Co., Ohio). Following the irradiation period, the samples were returned to the dark and stationary incubated for 15 min. The suspensions were then diluted 1:1 with 0.44 *M* $NaNO_2$ to terminate hemolysis.[1] Cellular debris was removed by centrifugation and the hemoglobin content of the supernates determined by measuring the absorbance at 420 mμ with a colorimeter (Klett-Summerson). Samples, similarly prepared, which had been allowed to remain in the dark for 105 min. were used to correct for spontaneous hemolysis[2] (not due to UV light). Results were quantitated in terms of percent photohemolysis with red cells hemolyzed by 5.8×10^{-2} *M* NaCl serving as the standard for 100% hemolysis. The latter standard was also diluted with 0.44 *M* $NaNO_2$ to insure uniform conditions.

Chemicals—3,3′,4′,5 - Tetrachlorosalicylanilide (TCSA) was obtained from Geigy Chemicals, Ardsley, N. Y. 3,3′,4′,5-Tetrabromosalicylanilide was supplied by the Organics Section of Lever Brothers Co., Edgewater, N. J. Other salicylanilides employed were obtained from Fine Organics, Inc., Lodi, N. J. Urocanic acid was purchased from Mann Research Laboratories, Inc., New York, N. Y. (2 - Hydroxy - 4 - methoxybenzophenone - 5 - sulfonic acid) Uvinul MS-40 was obtained from Antara Chemicals, New York, N. Y.

RESULTS AND DISCUSSION

Figure 1 shows the relationship of the length of irradiation to the percent photohemolysis noted in the presence of TCSA. Photohemolysis greater than the control value was observed initially when the TCSA-treated cells were subjected to UV light for 30 min. The amount of photohemolysis increased as the irradiation time was extended, maximum lysis occurring with a 60-min. exposure. The latter irradiation time was therefore chosen for use in the subsequent phases of this investigation.

The exposure of TCSA-treated red cells to long-wave UV light produced marked increases in membrane permeability towards Mg^{2+}, K^+, and Na^+ ions, as compared to irradiated control erythrocytes. Equivalent degrees of photohemolysis (63%) were obtained when the TCSA-treated cells were irradiated in buffer containing 0.14 *M* NaCl, 0.12 *M* KCl, or 0.12 *M* $MgCl_2$. Irradiation of erythrocytes in buffer which contained TCSA and isotonic[3] $CaCl_2$, glucose, or sucrose (0.28 *M*, 0.3 *M*, and 0.25 *M*, respectively) yielded insignificant photohemolysis when compared to irradiated controls. Irradiated TCSA-deficient samples had photohemolytic values of 0% ($CaCl_2$), 12% (glucose), and 10% (sucrose). The results therefore indicate that UV light does not increase permeability of TCSA-treated cells to either

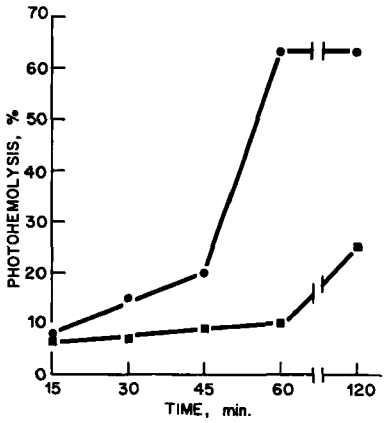

Fig. 1—Photohemolysis versus *time of long-wave UV irradiation for 3,3′,4′,5-tetrachlorosalicylanilide TCSA) at 25°. Intensity of incident radiation, 4 × 10^3 ergs/cm.^{2}/sec.; TCSA, 10^{-4} M in isotonic saline–phosphate buffer, pH 7.4. Key: ●, TCSA; ■, control.*

Ca^{2+}, glucose, or sucrose. The impermeability to Ca^{2+} ions is noteworthy; Ca^{2+} has a role in maintaining the normal low permeability of erythrocyte membranes (12).

Cations leaking across electrochemical gradients of normal erythrocytes are constantly being balanced by cations actively transported against these gradients (13). When the cell membrane is damaged by UV light, there is an isosmotic movement of salts and water into the cell. The accelerated diffusion rates cannot be overcome by active transport mechanisms. The erythrocytes therefore swell and lyse (14). In order to establish that the photohemolysis

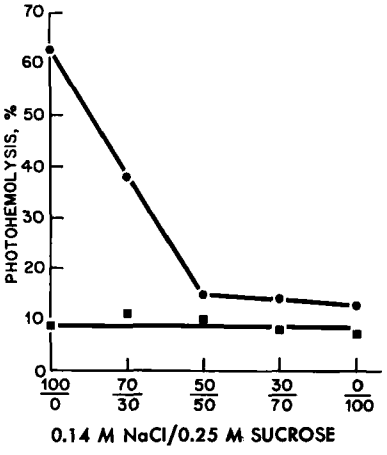

Fig. 2—Effect of NaCl content of the extracellular environment on the induction of photohemolysis by 3,3′,4′,5-tetrachlorosalicylanilide (TCSA). Intensity of incident radiation, 1.44 × 10^7 ergs/cm.^{2}; TCSA (10^{-4} M) and concentrations of NaCl and sucrose shown were contained in phosphate buffer, pH 7.4. Key: ●, TCSA; ■, control.

[1] The hypertonicity of the $NaNO_2$ solution stops hemolysis by causing cell shrinkage and its reducing action prevents further photochemical reactions (10).

[2] Hemolytic values of approximately 35 and 30% were usually noted for the nonirradiated samples with and without TCSA, respectively. These values were subtracted from the hemolytic values of the corresponding irradiated samples to give the degree of hemolysis due to UV light.

[3] Isotonic concentrations of $CaCl_2$, KCl, $MgCl_2$, glucose, and sucrose, respectively, were calculated by determining the amount required to exert approximately the same osmotic pressure as 0.85% NaCl (isotonic to blood).

of TCSA-treated cells, reported here, was due to the facilitated cellular uptake of cations, the red cells were irradiated in various isotonic NaCl + isotonic sucrose mixtures. The results obtained are illustrated in Fig. 2. It is apparent that photohemolysis of TCSA-treated cells was related to the Na^+ content of the external environment; photohemolysis decreased as the NaCl content of the medium was lowered.

Cation leakage associated with irradiated red cells is dependent upon the radiation dose and proceeds after irradiation is terminated (14). Thus, percent hemolysis usually becomes a function of incubation time after irradiation. Higher lytic values are obtained when the postirradiation period is prolonged. An evaluation of the relationship of the length of the postirradiation incubation period to the photohemolysis of TCSA-treated cells, observed here, was therefore of interest. The TCSA-treated cells were irradiated in saline–phosphate buffer and the percent photohemolysis determined 0, 5, 10, and 15 min. after irradiation. At each time interval, a photohemolytic value of 63% was obtained. These results thus indicate that the extent of photohemolysis was independent of the postirradiation sampling time under the conditions employed. Maximal lysis occurred during the exposure of erythrocytes to UV light. These findings suggest that, in this case, hemolysis is probably due to extensive alterations in membrane permeability as a result of the interaction of UV radiation, TCSA, and a membrane component responsible for maintaining cellular integrity.

It is well known that UV light causes the electronic excitation of photosensitive molecules to higher energy states (triplet or singlet). The excited molecules are extremely reactive and can transfer their energy to other molecular species by collision; the latter species then undergo a chemical reaction. Excited molecules can also dissipate their energy by dissociation into either radicals or more stable molecules. The interaction of long-wave UV radiation with TCSA is known to result in free radical production (15, 16). The photoconversion of TCSA to 3′,4′,5-trichlorosalicylanilide has also been reported (16). In the present study, it is conceivable that UV light may have excited TCSA to the long-lived metastable (triplet) state. The reaction of the highly energized TCSA molecule with the red cell membrane could therefore be responsible for the induction of structural alterations and associated permeability changes. Indirect evidence that this may be the case was obtained here through the use of KI and the paramagnetic ions, Cu^{2+} and Mn^{2+}, known quenchers of triplet state molecules (17). Photohemolysis failed to occur if the TCSA-treated cells were irradiated in isotonic KI (0.15 *M*), rather than in isotonic NaCl. When either KI (10^{-3} *M*) or $MnCl_2$ (2.5×10^{-3} *M*) was included in the saline–phosphate buffer, photohemolysis of TCSA-treated cells decreased by approximately 50% in comparison to the value obtained with saline–phosphate buffer alone (Table I). The inclusion of $CuCl_2$ (2.5×10^{-3} *M*) in the buffer prevented the TCSA-induction of photohemolysis, as evidenced by a photohemolytic value identical to that of the corresponding irradiated control. Average photohemolytic values of 10% were obtained when TCSA-deficient cells were irradiated in the presence of KI, $MnCl_2$, or $CuCl_2$. The results thus indicate that the triplet state of TCSA is the reactive species in the photohemolytic reaction. It should be noted that biological photosensitization due to psoralens likewise involves light excitation of the latter agents to the triplet state (18).

Table I—Effect of Various Chemicals on the Induction of Photohemolysis by Tetrachlorosalicylanilide (TCSA)

Chemical[a]	Concn., m*M*	Photohemolysis[b], %
None	—	60
$MnCl_2 \cdot 4H_2O$	2.5	31
KI	1.0	29
$CuCl_2 \cdot 2H_2O$	2.5	10

[a] TCSA, 10^{-4} *M*, and chemical indicated were contained in isotonic saline-phosphate buffer, pH 7.4. [b] Erythrocytes irradiated with long-wave uv light for 60 min. Intensity of incident radiation, 1.44×10^7 ergs/cm.^{2}.

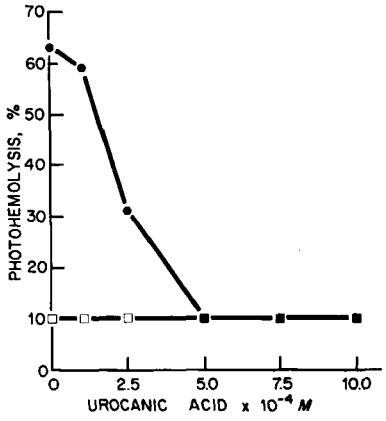

Fig. 3—Effect of urocanic acid on the induction of photohemolysis by 3,3′,4′,5-tetrachlorosalicylanilide (TCSA). Intensity of incident radiation, 1.44 × 10^7 ergs/cm.^{2}; TCSA (10^{-4} M) and urocanic acid concentrations shown were contained in isotonic saline–phosphate buffer, pH 7.4. Key: ●, TCSA; □, control.

Urocanic acid, the principal acid-soluble UV absorber present in the epidermis (19), was found to have an effect on the photohemolytic reaction. The addition of increasing concentrations of urocanic acid to the saline–phosphate buffer produced decreases in the percent photohemolysis of TCSA-treated cells (Fig. 3). Values identical to those of irradiated control cells were noted when urocanic acid was present in the irradiation mixture at a content of 5×10^{-4} *M*. 2-Hydroxy-4-methoxybenzophenone-5-sulfonic acid, a commercial sun screener used in cosmetic preparations, also protected TCSA-treated cells against UV radiation (Fig. 4). The effective concentration (5×10^{-3} *M*) required for inhibition of the photohemolytic reaction was greater than the quantity of urocanic acid needed.

To determine whether the enhanced light sensitivity of erythrocytes was unique to TCSA, red cells were irradiated for 60 min. in saline–phosphate buffer containing various salicylanilide isomers. In addition to TCSA, only 3,3′,4′,5- or 2′,3,4′,5-tetrabromosalicylanilide induced significant photohemolysis (Table II). Although the failure of the other isomers tested to cause photohemolysis significantly greater than the control value may be related to the

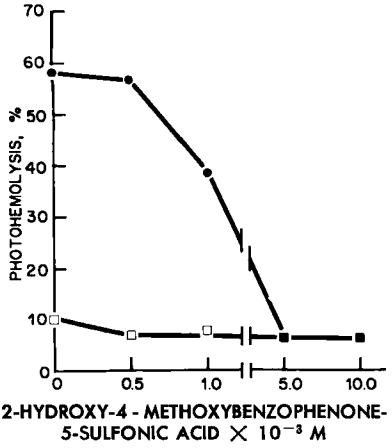

Fig. 4—Effect of 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid on the induction of photohemolysis by 3,3′,4′,5-tetrachlorosalicylanilide (TCSA). Intensity of incident radiation, 1.44 × 10⁷ ergs/cm.²; TCSA (10⁻⁴ M) and 2-hydroxy-4-methoxylbenzophenone-5-sulfonic acid concentrations shown were contained in isotonic saline–phosphate buffer, pH 7.4. Key: ●, TCSA; □, control.

concentration employed (10^{-4} *M*), a study of concentration effects was precluded by limited solubilities of the isomers in aqueous solvents. Similarly, the possibility exists that enhanced photohemolysis may have been observed if a longer irradiation exposure was used. Nevertheless, the monobromo-, dibromo-, and tribromosalicylanilides lack the photohemolytic capabilitity of TCSA since the irradiation time employed was optimal for the TCSA induction of photohemolysis (Fig. 1). Photohemolysis may therefore be of value for comparing the potential photosensitizing effects of select salicylanilides with those of TCSA.

The membrane component damaged by the interaction of long-wave UV radiation with TCSA was not determined in the present investigation. Lipoproteins may be involved, however, since it is well known that lipoproteins have an important role in the structure and permeability characteristics of cell membranes.

The data reported here have shown that TCSA can cause light-mediated permeability changes in cell membranes and that the extent of cell lysis is related to extraneous factors (*i.e.*, exposure time, external cellular environment, UV absorbers). Although the experiments performed were *in vitro* studies, it seems likely that some instances of photodermatitis due to TCSA may be related to increased cell permeability and lysis. The established role of lysosomes in inflammatory processes (20) suggests that lysosomes may be the target organelles *in vivo*. UV light causes increased lysosomal fragility (21); it is therefore conceivable that TCSA may enhance the light sensitivity of lysosomes with concomitant clinical developments (*i.e.*, edema, erythema, wealing, *etc.*). Additional studies are planned to determine if this is actually the case.

TABLE II—INDUCTION OF PHOTOHEMOLYSIS BY SALICYLANILIDE ISOMERS

Isomer[a]	Photohemolysis,[b] %
3,3′,4′,5-Tetrabromosalicylanilide	69
3,3′,4′,5-Tetrachlorosalicylanilide	63
2′,3,4′,5-Tetrabromosalicylanilide	47
3,4′,5-Tribromosalicylanilide	16
3,5-Dibromosalicylanilide	13
5-Monobromosalicylanilide	13
Salicylanilide	12
4′,5-Dibromosalicylanilide	11
None	10

[a] Isomer concentration 10^{-4} *M* contained in isotonic saline–phosphate buffer, pH 7.4. [b] Erythrocytes irradiated with long-wave UV light for 60 min. Intensity of incident radiation, 1.44 × 10^7 ergs/cm.².

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Keyphrases

Photohemolysis induction—tetrachlorosalicylanilide
Erythrocytes—photohemolysis determination
UV irradiation—photohemolysis
Cell permeability, cations—UV light effect

Determination of First pKa′ Value and Partition Coefficients of Bromothymol Blue

By V. DAS GUPTA and D. E. CADWALLADER*

The first pKa′ value and partition coefficients (between buffered aqueous phases and organic solvents; benzene, carbon tetrachloride, chloroform, ethylene dichloride, methylene chloride, and toluene) of bromothymol blue have been determined. The pKa′ value was determined using a method similar to that of Reilley and Swayer. Chloroform showed the highest affinity for bromothymol blue, followed by methylene chloride, ethylene dichloride, benzene, toluene, and carbon tetrachloride. Even at low concentrations (6×10^{-5} *M*) bromothymol blue appears to undergo polymerization, since partition coefficient values change with pH.

MANY AMINES FORM SALTS with acid dyes. These salts can be extracted by organic solvents and the dye component measured spectrophotometrically. This method has been used to assay many drugs (1). The amount of amine-dye salt extracted by an organic solvent is dependent upon the pH of the aqueous phase (2). Gupta *et al.* (3) derived an equation which can be used to predict the optimum assay conditions, such as pH, of the aqueous phase. To solve this equation, the first pKa value and the partition coefficient of the dye between the organic solvent to be used for extraction and the buffered aqueous phase are required. In the authors' previous paper (3) on the effect of pH and dye concentration on the extraction of a thiamine-dye salt by an organic solvent, they had reported the determination of the partition coefficient of bromothymol blue only between chloroform and water, using the approximated pKa value. The approximated pKa value did not agree with the reported value of Schill (4).

This paper describes the determination of the first pKa′ value and the partition coefficients of bromothymol blue between buffered aqueous phases and the organic solvents (benzene, carbon tetrachloride, chloroform, ethylene dichloride, methylene chloride, and toluene) which are most commonly used for the extraction of amine-dye salts (1).

EXPERIMENTAL

Reagents—All chemicals and reagents used were either USP, NF, or ACS grade. Bromothymol blue was purchased from W. H. Curtin & Co. and used without further purification.

Preparation of Solutions—*Bromothymol Blue Solutions for the Determination of* pKa′ *Value*—Bromothymol blue (100.0 mg.) was dissolved in 10 ml. of ethyl alcohol in a 50-ml. volumetric flask and enough distilled water added to make up to volume. Solutions of different pH values were prepared by diluting 0.5 ml. of this solution to 100 ml. with hydrochloric acid of various concentrations. All the solutions contained 0.001% of bromothymol blue. Hydrochloric acid (12.0 *N*) was standardized according to USP procedure (5). This hydrochloric acid solution was diluted with distilled water to obtain various other strengths as needed.

Bromothymol Blue Solutions for the Determination of Partition Coefficients—Aqueous buffer solutions of bromothymol blue (6×10^{-5} *M*) were prepared by dissolving 18.7 mg. of dye in approximately 0.5 ml. of 0.2 *M* sodium hydroxide solution and then adding enough 0.05 *M* phosphate buffer solutions of various pH values to make 500 ml. The pH of each solution was determined with a pH meter (Corning 12). Buffer solutions of various pH values were prepared according to the directions in the USP (6).

Determination of pKa′ Value—The pKa′ value was determined according to the procedure of Reilley and Swayer (7). Solutions of bromothymol blue (0.001%) in hydrochloric acid solutions of various concentrations were scanned between 360 and 600 mμ using a Beckman DK 2 spectrophotometer. Distilled water was used as the blank. Two wavelengths were chosen where the H_2I and HI^- forms of the dye showed a maximum difference in their absorbance. Plots of absorbance *versus* theoretical pH concentration ($-\log [H^+]$) were prepared for each of the two wavelengths selected. Two horizontal lines across each of the plots were drawn; one corresponding to the absorbance of the dye solution of theoretical pH -1.08, and the other corresponding to the absorbance of solution of 0.92 pH value (Figs. 1 and 2). The first line (A in Fig. 1 and B in Fig. 2) gave the absorbance of the dye when present entirely in the H_2I form. The second line (A in Fig. 2 and B in Fig. 1) gave the absorbance of the dye when present entirely in the HI^- form. The midpoint of each curve between the Lines A and B represented the equal concentration of H_2I and HI^- form of bromothymol blue. This was marked as the pKa′ value on each curve.

From each figure, the ratio of (HI^-/H_2I) at various pH values was obtained by measuring the relative distances of the point from HI^- and H_2I lines. Plots of log (HI^-/H_2I) (vertically) *versus* pH concentration were prepared (Figs. 3 and 4) for each of

Received May 24, 1968, from the College of Pharmacy, University of Houston, Houston, TX 77004
Accepted for publication September 19, 1968.
The authors express their appreciation to Dr. Harvey B. Herman, Department of Chemistry, University of Georgia, Athens, Ga., for his valuable comments.
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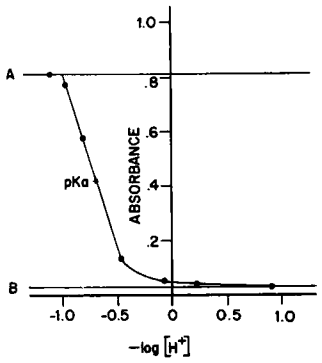

Fig. 1—Determination of pKa′ value using absorbance values at 558 mμ.

the two wavelengths selected. The point where the curve crossed the pH axis corresponded to pKa′ value, since $H_2I \rightleftarrows HI^- + H^+$ (dissociation of HI^- to I^{2-} and H^+ can be neglected in the pH range of −1.08 to 0.92 since K_2 is 1×10^{-7}). Therefore, $K_1 = [(HI^-)(H^+)]/H_2I]$, or $\log K_1 = \log HI^- + \log H^+ - \log H_2I$; hence, first pKa′ = pH − $\log HI^- + \log H_2I$, or $\log (HI^-/H_2I) =$ pH − pKa′. Therefore, when $HI^- = H_2I$, $\log (HI^-/H_2I) = 0$ and pH = pKa′. Results from Figs. 1–4 are presented in Table I.

Determination of Partition Coefficients—To determine the partition coefficients (D) of bromothymol blue, 25.0 ml. of dye solution (6×10^{-5} M) of appropriate pH value was mixed with 25.0 ml. of an organic solvent in a 250-ml. conical flask, and the flask shaken at high speed (temperature 24.5 ± 0.5°) for 30 min. using an Eberbach shaking machine. Preliminary investigations indicated that an equilibrium between the aqueous and organic phases was established within 30 min. After shaking, the mixture was placed in a 125-ml. separator and polar and nonpolar layers were allowed to separate and the organic phase collected. A portion of the organic phase was centrifuged at high speed for 10 min. using an International clinical centrifuge. The absorbance of clear solution so obtained and of the original dye solution used in each experiment was read at 503 mμ, the isosbestic point of bromothymol blue (7) using a spectrophotometer (Bausch & Lomb Spectronic 20). Distilled

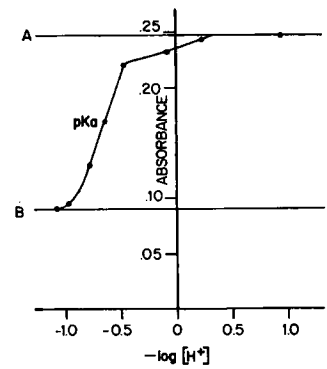

Fig. 2—Determination of pKa′ value using absorbance values at 470 mμ.

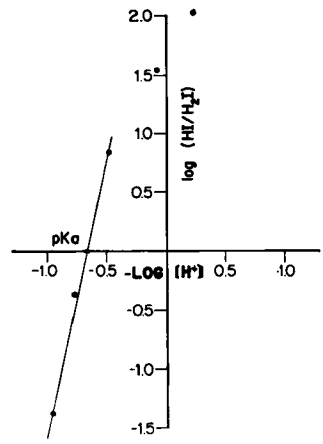

Fig. 3—Determination of pKa′ value using log (HI^-/H_2I) (from Fig. 1) versus pH concentration plot.

water was used as the blank. The amount of dye present in the organic phase was calculated by difference between the original and final absorbance values. To determine the partition coefficient $D = [(H_2I)_0]/[(H_2I)_a]$ the amount of dye present in the organic phase was assumed to be undissociated and therefore represented $(H_2I)_0$. The fraction of undissociated dye present in the aqueous phase, that is $(H_2I)_a$, at each pH value was calculated as follows (8):

$$(H_2I)_a = \frac{(H^+)^2}{(H^+)^2 + K_1(H^+) + K_1K_2}(C_{H_2I})_a$$

Results are presented in Table II.

DISCUSSION

pKa′ Value—The pKa′ value of −0.662 (Table I) did not compare with the value (1 to 1.48) reported by Schill (4). Three-point straight lines crossing the axis (Figs. 3 and 4) predict that the pKa′ value lies between −0.95 and −0.47. Two points did not

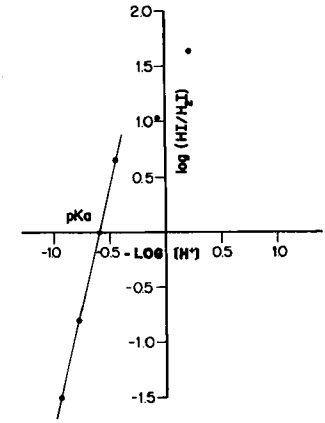

Fig. 4—Determination of pKa′ value using log (HI^-/H_2I) (from Fig. 2) versus pH concentration plot.

TABLE I—pKa′ VALUES AS DETERMINED FROM FIGURES ONE THROUGH FOUR

Figure No.	First pKa′ Value	K_1
1	−0.670	4.68
2	−0.648	4.45
3	−0.668	4.66
4	−0.600	3.98
	Av. −0.662[a]	Av. 4.60[a]

[a] Average of first three values.

TABLE II—EFFECT OF CHANGES IN pH ON THE PARTITION COEFFICIENTS OF BROMOTHYMOL BLUE[a]

Solvent	Partition Coefficient × 10^{-5} at pH 5.0	5.5	6.0	6.4
Benzene[b]	2.18	2.81	5.61	—
Carbon tetrachloride[b]	0.69	1.26	1.46	—
Chloroform[c]	—	12.15	18.70	27.42
Ethylene dichloride[c]	—	8.11	10.32	16.13
Methylene chloride[c]	—	10.03	15.00	23.95
Toluene[b]	1.29	1.98	2.50	—

[a] Each value is an average of two experiments. [b] Not done at pH 6.4 due to lack of sensitivity in the method to calculate the amount of dye in the organic phase. [c] Not done at pH 5.0 due to lack of sensitivity in the method to calculate the amount of dye in the aqueous phase.

fall on these straight lines, probably due to lack of sensitivity in the method to calculate either H_2I or HI^-. It was not possible to take into account the activity coefficients at these high concentrations of hydrochloric acid. This will, no doubt, cause some error in the determination of the pKa′ value.

Partition Coefficients—Partition coefficients varied with changes in pH (Table II). This may be due to the formation of polymers. The HI^- has been reported (4) to form dimers and tetramers. Error will probably be higher at higher pH value since the concentration of HI^- (present only in the aqueous phase) will increase with the pH. An error in the first pKa′ value does not appear to affect the relative differences between the partition coefficient values with changes in pH (Table III).

It is apparent that chloroform has the highest partition coefficient value (Table II), followed by methylene chloride, ethylene dichloride, benzene, toluene, and carbon tetrachloride (arranged in order

TABLE III—EFFECT OF CHANGES IN pKa′ VALUE ON THE RATIO OF PARTITION COEFFICIENTS AT pH 5.0 AND 5.5 USING BENZENE AS THE MODEL

pKa′	Partition Coefficient × 10^{-5} at pH 5.0	5.5	Ratio[a]
−0.845	3.37	4.33	1.29
−0.662	2.18	2.81	1.29
−0.544	1.68	2.16	1.29
−0.301	0.954	1.24	1.30
1.0	0.482	0.619	1.28

[a] Calculated taking pH 5.5 value and dividing by pH 5.0 value.

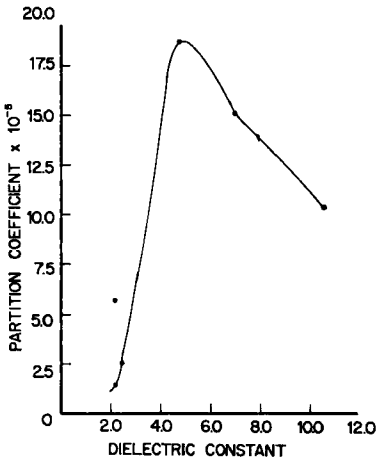

Fig. 5—Plot of partition coefficient versus *dielectric constant.*

of decreasing partition coefficient values). This is true when partition coefficient values are compared at any particular pH. Further analytical application of this information will be discussed in another report.

A plot of partition coefficient at pH 6.0 (Table II) *versus* dielectric constant (9) at 20° (benzene, 2.28; carbon tetrachloride, 2.24; chloroform, 4.81; ethylene dichloride, 10.65; methylene chloride, 9.08; and toluene, 2.39) passes through a maximum (Fig. 5). The maximum occurs at a dielectric constant value of 4.81 which corresponds to chloroform.

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Keyphrases

Bromothymol blue—acid dye
Partition coefficients—bromothymol blue
pKa′ value, first—bromothymol blue
Colorimetric analysis—spectrophotometer

Interdependence of Physiological Surfactant and Drug Particle Size on the Dissolution Behavior of Water-Insoluble Drugs

By SONG-LING LIN, JOHANNE MENIG, and LEON LACHMAN

A series of *in vitro* experiments was performed to demonstrate the interdependence of physiologic surfactant and drug particle size on the dissolution rate of glutethimide, griseofulvin, and a new diuretic Compound A. The presence of physiological concentrations of lysolecithin (a naturally occurring biosurfactant) is shown to exhibit micellar solubilizing properties on the drugs investigated. The data obtained from the dissolution rate studies showed that aqueous lysolecithin solution caused significant enhancement of the extent of solution of the drugs investigated. However, the reduction of particle size through micronization may not necessarily increase the *in vitro* dissolution rate. Data to support this statement are presented, and a plausible explanation for its occurrence is the electrostatic charge that develops on the solids after milling. This results in aggregates which can be larger in particle size than the unmilled drug.

SURFACE-ACTIVE AGENTS are commonly used as adjuvants in pharmaceutical preparations. Through the reduction of interfacial tension and micelle formation, these materials are capable of enhancing the aqueous solubility of poorly soluble drugs.

In biological systems, naturally occurring physiological surfactants are present in various tissues and organs at varying levels of concentration. There are a number of literature reports pertaining to the micellar solubilizing properties of physiologic surfactants for water-insoluble or slightly soluble medicinal agents. The physiologic surfactants most extensively employed in studying drug solubilization and dissolution are the bile salts. It has been shown that various bile salts are capable of solubilizing strychnine, quinine, quinoline, and camphor (1), dyes (2, 3), steroid hormones (4–6), carcinogenic polycyclic hydrocarbons (7–9), fatty acids, monoglycerides and cholesterol (10–14), glutethimide, griseofulvin and hexestrol (15), and inorganic salts (1).

In 1959 Robinson and Saunders (16) reported on the micellar solubilizing characteristics of the nonionic surfactant lysolecithin with triolein, monostearin, and cholesterol. More recently it was reported that lysolecithin exhibited marked solubilizing properties for and increased the dissolution rates of dienestrol, hexestrol, and griseofulvin (17).

It is generally believed that in order for a solid drug to be absorbed to any appreciable extent across the gastrointestinal membrane, it is preferable to have the drug go into solution in the gastrointestinal fluid prior to the absorption process. The rate-determining step in the diffusion-controlled absorption process for orally administered water-insoluble drugs is usually the dissolution rate of drugs in the biological fluids of the gastrointestinal tract. The significance of this on the transport and absorption processes is well recognized and has been the subject of several detailed discussions (18–21).

The factors influencing the dissolution rate are many and varied but one important factor is the surface characteristics of the solid particles (22–25). Following the disintegration of tablet and capsule dosage forms in the gastrointestinal tract, the particles break down into their initial particulate state or form small aggregates of drug. The surface characteristics of these aggregates would be different from the individual particles of drug and their subsequent dissolution behaviors would not be the same as for nonaggregated drug particles. Since most of the dissolution studies performed in the presence of physiologic surfactants have been reported to have been carried out without controlling the particle size of the medicinal agent, the present investigation was undertaken to study the influence of varying degrees of available surfaces on the dissolution rate of glutethimide, griseofulvin, and a new diuretic Compound A. The dissolution rate was determined in distilled water, 0.1 *N* hydrochloric acid, and 0.05% w/v aqueous lysolecithin solution at 37°.

EXPERIMENTAL

Materials—Glutethimide NF was dried at 45° over phosphorus pentoxide and was used without further purification. Analysis by NF assay (26)

Received June 19, 1968, from the Development and Control Dept., Ciba Pharmaceutical Co., Summit, NJ 07901
Accepted for publication August 20, 1968.
Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
The authors would like to express their gratitude to Miss Ann Nolan for her valuable assistance.

indicated a purity of better than 99.95%. Part of the samples were sieved through U. S. standard sieves and fractions of 20/40 and 100/120 mesh particles were collected for use in this investigation. The remaining material was micronized through a fluid energy mill[1] to obtain material less than 5 μ in size.

The new diuretic Compound A was dried at 100° for 8 hr. Analysis by a nonaqueous titrimetric method indicated a purity of better than 99%, which was substantiated by TLC analysis. Samples consisting of material being less than 5 μ and particles of 12/16 and 60/80 mesh were prepared as described for glutethimide.

Griseofulvin,[2] micronized and nonmicronized, was used as received. The nonmicronized powders were sieved and the particles of 12/16 and 20/40 mesh were employed in this study.

Lysolecithin[3] was reported to be better than 99% pure by paper chromatographic analysis. Elemental analysis of the lysolecithin sample was reported previously (17). The sample was dried in vacuum for at least 24 hr. prior to use.

Analytical Method—Beer's law curves were constructed individually for glutethimide (256 mμ), diuretic Compound A (282 mμ), and griseofulvin (292.5 mμ). The solvent systems employed were methanol–water in the ratio of 1:4, 1:1, and 3:2 for glutethimide, diuretic Compound A, and griseofulvin, respectively. In the dissolution rate determination the concentration of lysolecithin present in the diluted sample aliquots was found not to interfere with the spectrophotometic assay at the aforementioned wavelengths. A Beckman DB spectrophotometer with a Sargent recorder was used to obtain absorbance readings throughout the study.

Determination of Dissolution Rate—The procedures of Shefter and Higuchi (27) were employed with slight modification. A quantity of drug, in large excess of its predetermined solubility in 0.05% w/v aqueous lysolecithin solution was weighed and rapidly introduced to a 250-ml. water-jacketed beaker containing exactly 100 ml. of dissolution medium maintained at 37 ± 0.5°. The solution was agitated by means of an overhead stirrer. The three-blade stirrer was 2.5 cm. in diameter and located 2 cm. below the surface of dissolution medium. Constant agitation intensity was achieved by utilizing a constant torque unit (Servodyne). In order to insure complete contact of the hydrophobic drug with the fluid of the dissolution medium, a relatively high intensity of agitation, 600 r.p.m., was employed throughout the study. Water, 0.1 *N* hydrochloric acid, or 0.05% w/v aqueous lysolecithin solution was used as the dissolution medium. A 5-ml. aliquot was withdrawn at prescribed time intervals from the dissolution flask and replaced immediately with the same volume of dissolution medium maintained at 37°. The aliquot was rapidly filtered through 0.45-μ pore size Millipore filter paper to remove remaining undissolved drug. The clear filtrate was assayed spectrophotometrically after proper dilution with appropriate solvent. A cumulative correction was made in accordance with the method reported by Wurster and Taylor (28) to account for the previously removed samples in determining the total amount of drug dissolved at any specific time.

The photomicrographs of the drugs before and after the dissolution study were taken with a Polaroid camera attached to the photomicrographic apparatus.[4]

Electrostatic Charge of the Powders—The electrostatic properties of the various sized fractions of glutethimide, diuretic Compound A, and griseofulvin were determined at 37% R.H. and 25° using the apparatus and procedure described in an earlier publication (29).

RESULTS AND DISCUSSION

The solubilization of a water-insoluble drug, *D*, in the presence of surfactant can be treated as a process in which the drug is distributed between a micellar phase and an aqueous phase. Above the CMC of a surfactant, the solution is saturated with respect to surfactant monomers and an equilibrium exists between monomers and micellar aggregates. Therefore, the following equilibrium state can be written:

$$D_{\text{solid}} \rightleftarrows D_M + D_{NM} \qquad \text{(Eq. 1)}$$

where D_M is the concentration of drug in micelle and D_{NM} is the drug concentration in the nonmicellar phase. The equilibrium is influenced not only by the parameters of temperature, pressure, and surfactant concentration, but also by the characteristics of the solid particles employed. The reduction of particle size results in an increase of available surface area as well as a considerable increase in surface free energy. In general, the smaller the particle size of the solid, the greater is the shift of the equilibrium to the right-hand side of Eq. 1. This phenomenon of increasing solubility with decreasing particle size has been reported (30–32). The importance of identifying the characteristics of the physical state of the solid in solubility measurement as well as in the determination of dissolution rate is evident.

Figure 1 demonstrates the dissolution behavior of diuretic Compound A using 12/16 mesh, 60/80 mesh, and micronized powders, in water and 0.05% aqueous lysolecithin solution at 37°, respectively. Each curve is drawn through points which represent an average of at least two dissolution runs. In all instances the reproducibility is within the experimental error. It can be seen from these curves that the presence of lysolecithin in the dissolution medium significantly enhances the extent as well as the rate of dissolution of diuretic Compound A when compared to the extent and the rate of dissolution found in water. Since the dissolution rate is directly proportional to the solubility term in the Noyes-Whitney equation, the modification of the nature of the dissolution media which will effectively increase the solubility term should increase the dissolution rate. The ratio of the quantity of the solubilized drug in lysolecithin to the quantity of drug in water at the same time interval is expressed as relative extent of dissolution. As shown in Table I the lysolecithin micelle displays

[1] Trost Jet Mill, Helme Products Inc., Helmetta, N. J.
[2] Generously supplied by Dr. H. Wolkoff, Schering Corp., Bloomfield, N. J.
[3] Purchased from Nutritional Biochemicals Co., Cleveland, Ohio.
[4] Phaphot, Ernest Leitz Ltd., Germany.

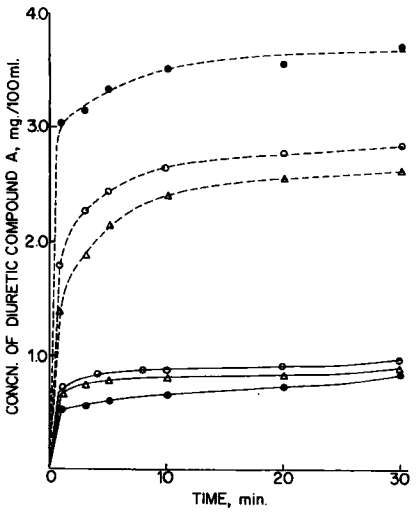

Fig. 1—Dissolution profiles of diuretic Compound A in water (——) and 0.05% aqueous lysolecithin solution (···) at 37°. Key: ●, micronized powder; ○, 60/80 mesh powder; △, 12/16 mesh powder.

approximately threefold increase in relative extent of dissolution for the 12/16 and 60/80 mesh powders of diuretic Compound A, while about fivefold increase was observed for micronized diuretic Compound A during the time period of 10–30 min. The ratio persists even after 2 hr. The enhancement in the extent as well as the rate of dissolution in the presence of lysolecithin is due to the lowering of the interfacial tension between the drug and the dissolution medium, as well as to micellar solubilization. The latter mechanism is most likely more significant since the concentration of lysolecithin employed in the dissolution rate determination is much higher than the CMC of lysolecithin.

As expected, the reduction of particle size from 12/16 mesh to 60/80 mesh slightly increases the extent of dissolution behavior of diuretic Compound A in water and 0.05% lysolecithin solution. However, when the particle size is further reduced to the micron range, the extent of dissolution of diuretic Compound A in water decreases somewhat when compared with the 12/16 or 60/80 powders. The probable explanations for these surprising results are (*a*) that the hydrophobic properties of the powder increases with the degree of subdivision of the powders because of absorption of air caused by micronization, and (*b*) the relative magnitude of the interparticulate binding force, such as electrostatic charge, is increased as the particle size is greatly reduced. Clumping of the diuretic Compound A micronized particles was observed during the dissolution experiments. Large particulate agglomerates, some being larger in particle size than the unmilled drug, were found to persist in the water even after 2 hr. of dissolution study. As shown in photomicrographs A and B of Fig. 2, the micronized sample observed after a 2-hr. dissolution run in water is present as aggregates having diameters larger than the 60/80 mesh powders. The measurement of the electrostatic charge of the various particle sizes of diuretic Compound A indicated that the electrostatic charge of the powders increased with increasing surface area through particle size reduction (33).

When 0.05% lysolecithin solution was employed as the dissolution medium, the dissolution rate of diuretic Compound A increased with the decreasing particle size as illustrated by the upper three curves of Fig. 1. This difference in effect of dissolution in water and lysolecithin solution can be explained by the surfactant wetting the hydrophobic surface of diuretic Compound A and breaking the large particle agglomerates into their original individual particulate state. This is illustrated with photomicrographs A and C or B and D of Fig. 2. The interparticulate interactions observed in photomicrograph B are reduced greatly in the presence of lysolecithin, as demonstrated in photomicrograph D of Fig. 2. This accounts for the fact that micronized diuretic Compound A exhibits a greater extent of dissolution than the unmilled powder in lysolecithin solution but not in water. The photomicrographs were taken of the samples before filtration through the Millipore assembly. If the photomicrographs are taken on the filtered powders upon dispersion in a suitable medium, such as mineral oil, it is not realistic of the state of the micronized drug during the dissolution run.

A similar phenomenon was observed for griseofulvin. The dissolution behaviors of micronized, 20/40, and 12/16 mesh samples of griseofulvin are depicted in Fig. 3. The extent of dissolution is increased at least twofold, when the dissolution medium is changed from water to 0.05% lysolecithin solution.

Inspection of the data in Table I shows that after 10 min., the ratios of the amount of drug dissolved in the 0.05% lysolecithin solution to that amount dissolved in water are 2.3, 2.1, and 2.4 for 12/16 mesh, 20/40 mesh, and micronized griseofulvin, respectively.

Table I—Relative Extent of Dissolution at 37°

Compound	Particle Size	Relative Extent of Dissolution[a] Time, min. 1	2	5	10	20	30
Diuretic Compound A	12/16 mesh	2.12	2.65	2.74	3.00	3.08	2.89
	60/80 mesh	2.50	2.37	2.52	2.98	3.04	2.96
	Micronized	5.81	5.55	5.39	4.86	4.97	4.86
Griseofulvin	12/16 mesh	5.11	3.47	2.64	2.28	2.29	2.32
	20/40 mesh	2.01	2.05	2.16	2.13	2.17	2.25
	Micronized	2.68	2.62	2.40	2.38	2.41	2.46
Glutethimide	20/40 mesh	1.00	1.02	1.01	1.01	0.99	1.01
	100/120 mesh	1.12	1.14	1.10	1.07	1.09	1.09
	Micronized	1.03	1.03	1.01	1.00	1.01	1.02

[a] Ratio of the quantity of solubilized drug in lysolecithin to the quantity of drug in water at the same time intervals.

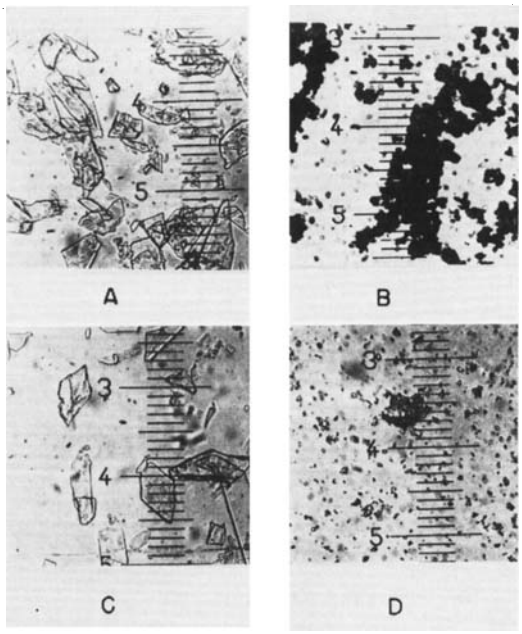

Fig. 2—Photomicrographs illustrating the physical state of diuretic Compound A after 2 hr. of dissolution study. Key: A, 60/80 mesh powders in water; B, micronized powders in water; C, 60/80 mesh powders in 0.05% aqueous lysolecithin solution; D, micronized powders in 0.05% aqueous lysolecithin solution.

In lysolecithin solution, the extent as well as the rate of the dissolution increases as the particle size is decreased from 12/16 mesh to micronized powder. However, in water the dissolution profile of micronized griseofulvin lies between that of 20/40 and 12/16 mesh samples.

It has been reported that when micronized griseofulvin is used, the blood levels can be achieved with approximately half the dose of the larger particle size material (34). Thus it is clear that the increase of *in vivo* drug availability in the biological system through particle size reduction may not readily be predicted by *in vitro* dissolution studies in aqueous systems. This is especially true when the dissolution rate of the water-insoluble hydrophobic drug is studied in water. Although the addition of synthetic or naturally occurring surfactant in the dissolution medium gives a better indication of the influence of particle size reduction on increasing dissolution rate, the ultimate means of providing the evidence to demonstrate that particle size reduction decreases the dose needed to produce an equal pharmacological response is by *in vivo* rather than *in vitro* study.

An interesting dissolution behavior was observed for glutethimide. The results of the dissolution rate study in water and 0.05% lysolecithin solution are depicted in Fig. 4. The extent of dissolution at the first 10-min. period was found to decrease in the following order:

100/120 mesh > micronized > 20/40 mesh glutethimide;

whereas after 20 min. the order becomes

100/120 mesh > 20/40 mesh > micronized glutethimide.

The same trend is found in both water and 0.05% lysolecithin solution. In contrast to the common belief that the dissolution rate is increased or reaches a plateau with respect to the decreasing particle size, it seems that an optimal particle size is desirable for glutethimide since the dissolution rate increases up to a particular particle size then decreases. As discussed previously and indicated in Table I the relative extent of dissolution of diuretic Compound A and griseofulvin was considerably enhanced by the presence of lysolecithin solution. However, the dissolution rate of the 20/40 mesh and micronized glutethimide was not affected and 100/120 mesh glutethimide was increased only 10% by the incorporation of the biosurfactant as seen in Fig. 4. This case seems to be an excellent example where particle size rather than the presence of biosurfactant has the predominant effect on dissolution rate.

Photomicrographs of the glutethimide taken after the 2-hr. dissolution rate study in water and

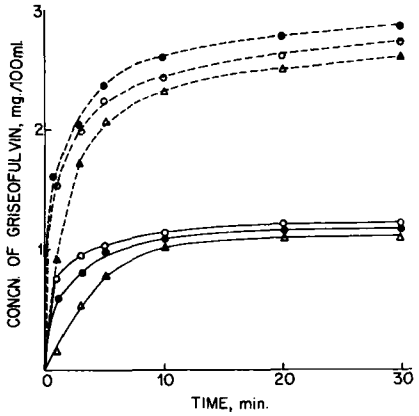

Fig. 3—Dissolution profiles of griseofulvin in water (——) and 0.05% aqueous lysolecithin solution (···) at 37°. Key: ●, micronized powder; ○, 20/40 mesh powder; △, 12/16 mesh powder.

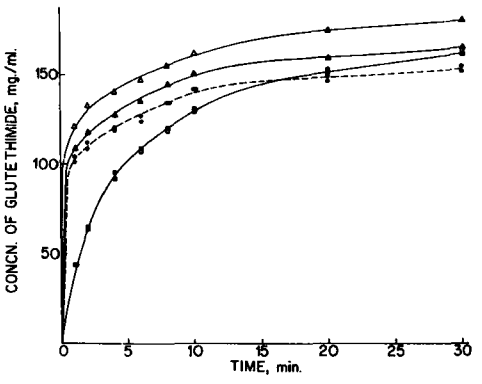

Fig. 4—Dissolution profiles of glutethimide in water (closed symbols) and 0.05% aqueous lysolecithin solution (open symbols) at 37°. Key: ○, ●, micronized sample; △, ▲, 100/120 mesh sample; □, ■, 20/40 mesh sample.

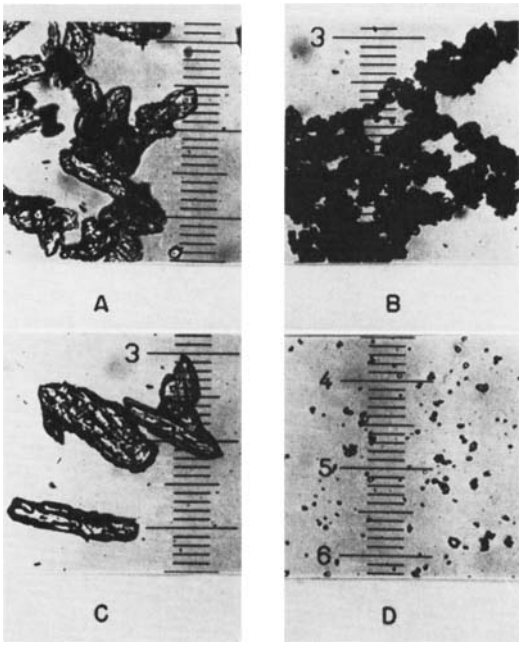

Fig. 5—Photomicrographs illustrating the physical state of glutethimide after 2 hr. of dissolution study Key: A, 20/40 mesh powders in water; B, micronized powders in water; C, 20/40 mesh powders in 0.05% aqueous lysolecithin solution; D, micronized powders in 0.05% aqueous lysolecithin solution.

0.05% lysolecithin solution are shown in Fig. 5. The decrease in the dissolution rate by changing from 20/40 mesh powder to micronized glutethimide as shown in Fig. 4 can be attributed to the fact that the particulate agglomerates of the micronized sample (photomicrograph B, Fig. 5) are larger in size than the 20/40 mesh particles (photomicrograph A, Fig. 5). When 0.05% lysolecithin solution is employed, the hydrophobic surface of glutethimide is wetted by the surfactant and the particulate agglomerates were dispersed to their individual particles as shown in photomicrographs C and D of Fig. 5. However, a corresponding increase of the dissolution rate was not observed. A possible explanation for this phenomenon may be the fact that micellar aggregates of lysolecithin have little affinity for glutethimide, as demonstrated by the equilibrium solubility curve of Fig. 6. The equilibrium solubility of glutethimide in 0.05% aqueous lysolecithin is increased only 15% as compared with the equilibrium solubility of glutethimide in water. It has been demonstrated, *in vivo*, that the blood level of glutethimide is not influenced by the reduction of particle size. On the contrary, griseofulvin which exhibits a particle size effect on the blood levels of griseofulvin has an equilibrium solubility of 0.024% in 0.05% aqueous lysolecithin solution which is about 70% higher than that in water (17). It seems that the higher the extent of interaction between the drug and biosurfactant, the greater the dependency of the drug concentration in the blood on the particle size of the drug.

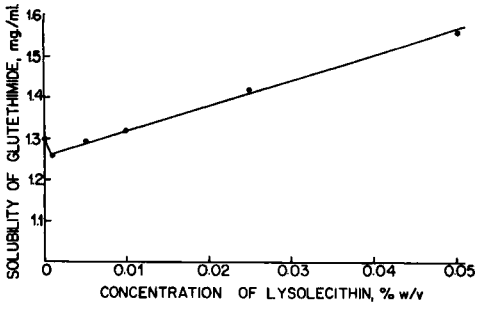

Fig. 6—Solubilization of glutethimide in aqueous lysolecithin solutions at 37°.

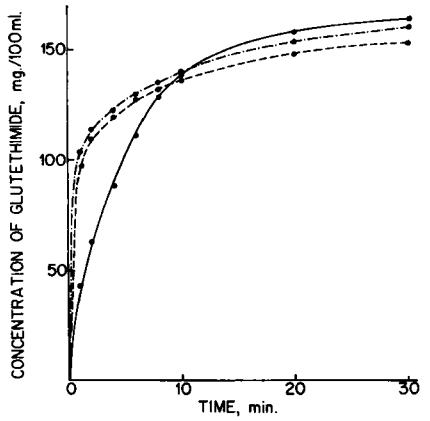

Fig. 7—Dissolution profiles of glutethmide in 0.1 N hydrochloric acid at 37°. Key: —, micronized powder; -·-, 100/120 mesh powder; ——, 20/40 mesh powder.

Since glutethimide is available commercially as a solid dosage form, the effect of particle size on the dissolution rate of glutethimide in 0.1 *N* hydrochloric acid at 37° was investigated. The results are graphically shown in Fig. 7. As observed previously for the dissolution rate in water and lysolecithin, the micronized glutethimide does not appear to go into solution faster than 100/120 mesh sample. However, the dissolution rate is increased significantly at the first 5-min. period of the dissolution run when the micronized or 100/120 mesh glutethimide is used in place of the 20/40 mesh material.

The physicochemical approach for investigating the nature of the interactions of water-insoluble drugs with normal constituents of the biological system is believed to contribute significantly to a greater understanding of the complexity of absorption, transport, interaction, and excretion of the drug in the living system. There exist certain physiologic surfactants either as normal constituents of the biological fluids or resulting from the discharge into a particular compartment or compartments of the biological system. Typical examples of these are lecithin, lysolecithin, and bile salts (35–40) which appear in the pancreas, duodenal fluid, and blood stream. As a result of the findings in this investigation and those from previous reports (17) the presence of naturally occurring physiologic surfactants, such as lysolecithin in the biological system can possibly increase the extent as well as the rate of dissolution of water-insoluble medicinal agents prior to the absorption process.

Presently under study is the influence of other physiologic surfactants, such as α-cephalin, phosphatidyl ethanolamine, and phophatidyl serine as well as phosphatidyl inositol on the dissolution properties of poorly soluble drugs, and information being obtained will be presented in a subsequent report.

CONCLUSION AND SUMMARY

The interdependence of physiologic surfactant and drug particle size on the extent and the rate of dissolution of diuretic Compound A, griseofulvin, and glutethimide was investigated. The presence of lysolecithin is generally shown to exhibit micellar solubilizing properties on the drugs investigated. From the dissolution profiles of these relatively water-insoluble drugs, the aqueous lysolecithin solution caused significant enhancement of the extent as well as the rate of solution. However, reduction of particle size through micronization does not necessarily increase the *in vitro* dissolution rate. A plausible explanation for this occurrence is the electrostatic charge that develops on the solids after milling resulting in the development of aggregates which can be larger in size than the unmilled drug.

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Keyphrases

Dissolution—water-insoluble drugs
Surfactant, physiologic—dissolution, water-insoluble drug
Particle size—dissolution, water-insoluble drug
Interdependence, surfactant, particle size—dissolution
Photomicrographs—drug particles

Determination of Vitamin E in Multivitamin Products by Gas-Liquid Chromatography

By F. P. MAHN, V. VISWANATHAN, C. PLINTON, A. MENYHARTH, and B. Z. SENKOWSKI

The determination of vitamin E in multivitamin preparations has presented considerable analytical problems. Reports on the gas-liquid chromatography of this vitamin have shown that a linear response with concentration can be obtained. This investigation deals primarily with the quantitative analysis of alpha-tocopheryl acetate utilizing a hydrogen-flame detector. The influence of gas flow rates on detector response and retention times is demonstrated. The conditions for the assay of vitamin E in multivitamin preparations are outlined. A suitable internal standard employed in this study was dotriacontane. The precision of the proposed procedure under the conditions studied is ± 2–3 percent.

SINCE A CONSIDERABLE number of multivitamin preparations contain vitamin E (alpha tocopherol or its esters), a specific and reproducible method of determination is desirable. A comprehensive review of the literature has been made by Kofler *et al.* (1) pertaining to existing methodology. The most popular procedure for the analysis of alpha tocopherol in vitamin preparations is the Emmerie-Engel (2) reaction utilizing the iron-bipyridyl complex. However, this method is susceptible to many interferences, especially vitamin A, which is present in most multivitamin products. In addition, the use of the colorimetric procedure requires a hydrolysis step to be carried out if esters of tocopherol are present. A publication by Fisher *et al.* (3) employed a hydrogenation step prior to colorimetry of alpha tocopherol in order to minimize the interference due to vitamin A. A review of titrimetric and colorimetric methods for the assay of vitamin E in pharmaceutical products was made by Lehman (4).

It would appear that the tremendous versatility of GLC should make this technique the one of choice for the quantitative determination of vitamin E. The majority of reports in the literature, concerning the GLC of vitamin E, emphasized the qualitative separation of the various isomers of the tocopherols (1, 5, 6, 7, 8). Most of the investigators reported that the best separations of the tocopherols were achieved using columns prepared with SE-30 on diatomaceous earth.[1] In this laboratory good results were obtained using a column containing 10% SE-30 on Aeropak 30, 100/120 mesh. However, the silanization of the diatomite aggregates[2] did not significantly change the results of the vitamin E assay, when this high percentage of liquid phase was used. Libby and Sheppard (9) have reported on the quantitative response of alpha, gamma, and delta tocopherol as well as alpha tocopheryl acetate, utilizing an argon ionization detector. Pillsbury *et al.* (10) in a later report, have applied GLC to vitamin E in pharmaceuticals and quantitatively analyzed for vitamin E, utilizing a hydrogen-flame detector. Comparison was also made with the Emmerie-Engel method.

This investigation deals primarily with the quantitative determination of alpha tocopheryl acetate by GLC, as carried out in this laboratory for quite some time on a number of different multivitamin preparations. In order to compensate for column characteristics, instrumental variations, and sample introduction technique, an internal standard was employed. Dotriacontane (11), a C-32 hydrocarbon was used as an internal standard in this study. It should be mentioned that a C-33 or C-34 hydrocarbon would have been preferable, but were not available commercially. An aluminum oxide column was used in the sample preparation in order to remove some interferences derived from excipients in the various formulations.

Received July 2, 1968, from the Analytical Research Laboratory, Hoffmann-La Roche, Inc., Nutley, NJ 07110

Accepted for publication August 27, 1968.

This paper in substance, was presented at the Metropolitan Regional Meeting of the American Chemical Society on February 1, 1965.

The authors wish to thank Mrs. C. Yao and Miss G. Capitano for performing some of the experiments, Dr. E. G. Wollish for his encouragement and advice, and Miss S. Andriola for assistance in the preparation of the drawings.

[1] Silanized Gas-Chrom P, Applied Science Laboratories, State College, Pa.; and Celite, Johns-Manville, New York, N. Y.

[2] Chromosorb W, Johns-Manville, New York, N. Y.

This method had previously been submitted, in 1965, to the United States Pharmacopeia for a preliminary collaborative trial.

EXPERIMENTAL

Operational Parameters—The instrument used for this work was a gas chromatograph, (Varian Aerograph model 600 or equivalent), equipped with a hydrogen flame-ionization detector. The column used was a copper coil, 121.9 cm. (4 ft.) long and 3 mm. i.d., packed with 10% SE-30 on Aeropak 30, 100/120 mesh. Prior to use, this packed column was flow conditioned at 280° for 16–20 hr. with a stream of nitrogen. The temperatures were: column, 245°; injector port, 275° with a Pyrex insert; and detector, 275°. The flow rates were: carrier gas, nitrogen, 40 ml./min.; detector gas, hydrogen, 20 ml./min.; and air 300 ml./min. All injections were made using a 10-μl. syringe (Hamilton) with the injection volume being approximately 5 μl. The instrument was operated at a range of 10 and attenuation 32×, or equivalent to result in approximately 50% response of the recorder scale. The recorder used was 0–1 mv. (Texas Instrument) with a pen response of 0.4 sec. and a chart speed of 12 in./hr. All peak areas were measured using either a disk integrator, digital integrator, or the peak height multiplied by the peak width at half height. Under the conditions stated the relative retention time of alpha tocopheryl acetate was 0.85 with respect to the internal standard, dotriacontane, which has a specific retention time of approximately 30 min. A sample chromatogram is shown in Fig. 1.

Reagents and Chemicals—Silicone gum rubber SE-30 and Aeropak 30, 100/120 mesh were obtained from Varian Aerograph. Benzene and tetrahydrofuran were obtained from Fisher Scientific Co. Solvent hexane (petroleum ether) was Merck grade, b.p. 30–60°. Alumina was Woelm neutral. Dotriacontane was purchased from Eastman Organic Chemicals.

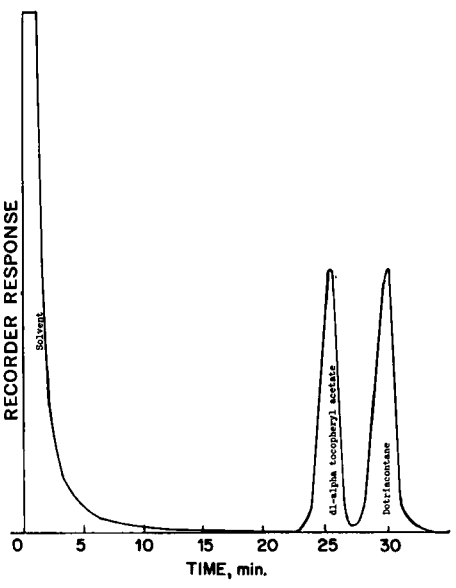

Fig. 1—Sample chromatogram.

Standard Preparation—*Internal Standard*—Accurately weigh 200 mg. of dotriacontane into a 100-ml. volumetric flask. Dissolve and dilute to volume with tetrahydrofuran. Mix well (internal standard stock solution).

Reference Standard—Accurately weigh 100 mg. of reference *dl*-alpha tocopheryl acetate into a 50-ml. amber volumetric flask. Dissolve and dilute to volume with the internal standard stock solution (working standard solution). Standards of alpha tocopherol and alpha tocopheryl succinate are prepared in a similar manner.

Sample Preparation—*A. Multivitamin Tablets or Capsules*—Determine the average tablet weight or capsule fill. Grind the tablets using a mortar and pestle and weigh a quantity of the tablet mass, or capsule fill, equivalent to about 6 mg. of *dl*-alpha tocopheryl acetate, into a 50-ml. centrifuge tube. Add 25.0 ml. of benzene, stopper, and shake, or place into an ultrasonic vibrator bath for 20 min. Centrifuge at high speed for 5 min. Transfer 15.0 ml. of the benzene layer into a glass column, 1.5 cm. in diameter and 30 cm. in length, which has been packed with 8 g. of alumina (Woelm, neutral) deactivated with 10–12% water (w/w). Collect the eluate in a glass-stoppered 50-ml. conical flask. Further rinse the column with an additional 30 ml. of benzene in small portions, allowing each portion to drain to the surface of the alumina, and collect the combined eluates in the 50-ml. conical flask. Evaporate the solution to dryness on a steam bath under a flow of nitrogen. Dissolve the residue in 2.0 ml. of the internal standard stock solution (sample working solution).

B. Gelatin Capsules—In the case where gelatin capsules are encountered, 5 ml. of water is placed into the centrifuge tube with the sample capsules and warmed for 5–10 min. at 50° to dissolve the gelatin. The resulting mixture is cooled, 25.0 ml. of benzene is added, and the procedure described under *A* carried out. Capsules which would not dissolve in water were slit open and the contents extracted with benzene as described.

C. Injectables and Liquids—Injectables and liquid samples are diluted directly with tetrahydrofuran and water is added, if necessary, to obtain a clear homogeneous solution resulting in the alpha tocopheryl acetate concentration of 4 mg./ml. Mix 5.00 ml. of this solution with 5.00 ml. of the internal standard stock solution (sample working solution). If miscibility is poor or interference is encountered, then extraction with 25.0 ml. of benzene is necessary and the procedure described under *A* carried out.

It may be necessary in some formulations to extract several times with benzene in order to quantitatively remove the alpha tocopheryl acetate. In such instances the combined extracts should be concentrated to 25 ml. and subjected to the procedure described under *A*.

D. Multivitamin Cream—Into a 50-ml. glass-stoppered centrifuge tube weigh accurately a sample of the multivitamin cream equivalent to about 10 mg. of alpha tocopheryl acetate. Add 10 ml. of water and heat at 70–80° for 10 min. Cool to room temperature and add 3 g. of sodium chloride. Extract the sample in the centrifuge tube with five 20-ml. portions of solvent hexane,

(Merck, b.p. 30–60°), shaking mechanically. Separate the layers by centrifugation. Collect the combined hexane extracts in a 125-ml. conical flask. Evaporate to a volume of approximately 10 ml. on a steam bath with the aid of a stream of nitrogen. Quantitatively transfer the hexane concentrate, with the aid of a small volume of additional hexane, onto a glass column, 1.5 cm. in diameter and 30 cm. in length which has been packed with 17 g. of alumina (Woelm, neutral) deactivated with 10–12% water (w/w). Adjust the elution rate at 4 ml./min., drain the solvent hexane to the surface of the alumina, and then elute with an additional 175 ml. of solvent hexane. Discard the first 25 ml. of solvent hexane, and collect the remaining eluate in a 250-ml. conical flask. Evaporate the collected hexane to dryness on a steam bath under a stream of nitrogen. Dissolve the residue in a sufficient volume of internal standard stock solution (5 ml.) to result in a concentration of alpha tocopheryl acetate of 2 mg./ml. (sample working solution).

E. Dry Powders—Weigh accurately a portion of the sample equivalent to 100 mg. of alpha tocopheryl acetate into a 50-ml. centrifuge tube. Add 5 ml. of 4*N* sulfuric acid and 5 ml. of 3A alcohol.[3] Swirl and heat in a hot water bath for 5 min. to disperse the beadlets. Cool to room temperature. Extract with 6 × 15 ml. of solvent hexane (Merck, low-boiling 30–60°), shaking mechanically for 5 min., centrifuging, and combining the hexane extracts in a 100-ml. volumetric flask. Dilute to volume with hexane and mix well. Pipet 10.0 ml. of the hexane solution into a small glass-stoppered flask. Evaporate this solution to dryness on a steam bath under nitrogen. The residue obtained is taken up in 5.00 ml. of internal standard stock solution (working sample).

Standard Calibration and Sample Analysis—Chromatography of several 5-μl. injections of working standard solution is required in order to condition the column and to determine the instrument sensitivity and peak retention times.

Five-microliter volumes of the working standard and sample solutions, equivalent to approximately 10 mcg. of alpha tocopheryl acetate are alternately injected into the instrument with the described operational parameters. After the elution of the dotriacontane peak the instrument is ready for the following injection. Duplicate samples and standards are chromatographed and the respective areas are determined from the integrator or by the peak height multiplied by the peak width at half height.

Calculations—Assay for alpha tocopheryl acetate in the preparation.

Determination of response factor for alpha tocopheryl acetate (RF_E).

$$RF_E = \frac{A_E\,(\text{std.}) \times C_D}{A_D\,(\text{std.}) \times C_E}$$

where:

A_E = peak area of tocopheryl acetate in the working standard.
A_D = peak area of dotriacontane in the working standard.
C_D = mg. of dotriacontane per ml. of working standard.
C_E = mg. of tocopheryl acetate per ml. of working standard.

The equation for mg. of alpha tocopheryl acetate per gram of sample is:

$$\frac{A_E\,(\text{spl.}) \times C_D \times F}{A_D\,(\text{spl.}) \times RF_E \times \text{sample weight (g.)}}$$

where:

A = peak area in the working sample.
RF_E = response factor as described above.
C_D = mg. of dotriacontane per ml. of working sample.
F = dilution factor of sample (per ml. of working sample).

Milligrams of alpha tocopheryl acetate per tablet, capsule, *etc.* = mg. of alpha tocopheryl acetate per gram × average unit weight.

RESULTS AND DISCUSSION

It was found necessary to saturate the column daily with the form of vitamin E to be determined in order to attain reproducible results. This conditioning effect also was described by Libby and Sheppard (9) in their initial report. Tocopheryl acetate required minimal daily conditioning to attain constant detector response. Tocopherol and tocopheryl succinate required longer conditioning times as well as periodic checking throughout the day.

In the course of this study a high ratio of liquid phase to inert support was employed in order to overcome any major loss of vitamin E by adsorption, thereby causing low results. Aeropak 30, with a mesh range of 100/120, increased column efficiency, and minimized adsorption.

Since no significant difference was observed in the results obtained with Pyrex or metal columns, copper tubing was selected for this work. Aging of the packed column will be evident by the resolution of the peaks. Usually a new column should be prepared when this resolution becomes poor, or after approximately 1 month of continuous use.

Several solvents were investigated for the dissolution of vitamin E prior to gas chromatography. It was found that acetone and chloroform resulted in poor reproducibility of response per unit quantity of vitamin E. This effect could be attributed to column adsorption. Tetrahydrofuran as well as *n*-hexane (9) appeared to minimize this result. Since the use of water was contemplated in some sample preparations, tetrahydrofuran, because of its water miscibility, was chosen as the solvent.

The effect of carrier gas flow rate with retention time was investigated and a linear relationship obtained. The equation calculated by the method of least squares is $\log t_r = -0.796 \log R_f + 2.38$, where t_r is the retention time in minutes and R_f is the flow rate in ml. per min. The effect of column temperature on retention time also resulted is a straight line following the equation $\log t_r = -0.0160\,Tc + 5.33$, where Tc is the column temperature in °C. Theoretical considerations of retention volume with absolute temperature and

[3] Five parts methanol and 100 parts alcohol, v/v.

TABLE I—DETECTION LIMITS AND RETENTION TIME[a]

Compd.	Limit of Detection, mcg.	Approximate Retention Time, min.	Relative Retention Time
Alpha tocopheryl acetate	0.4	25.5	0.85
Alpha tocopherol	1.2	21.3	0.71
Alpha tocopheryl succinate	1.2	22.5	0.75
Vitamin D_2 (calciferol)	1–2	20.4	0.68
C-32		30.0	1.00

[a] Column: 10% SE-30 on Aeropak 30; length 121.9 cm. (4 ft.), temperature 245°. Flow rate: N_2 = 40 ml./min.; H_2 = 20 ml./min.; air = 300 ml./min.

column loading are discussed in a paper by Sawyer and Barr (12). The detector response per approximately 10.8 mcg. of alpha tocopheryl acetate with the change in hydrogen flow rate at constant air and carrier flow was obtained. The optimum hydrogen flow to carrier gas was in the ratio of 1:2.

In this investigation the level of detectability of the various forms of vitamin E studied, as well as their relative retention times with respect to dotriacontane, are listed in Table I.

The retention times of alpha tocopherol and alpha tocopheryl succinate are quite similar under these conditions. The identification of these forms should be made using the Emmerie-Engel test while gas chromatography would serve as the quantitative assay.

The possibility of vitamin D interference in the determination of vitamin E also was investigated. With the use of a hydrogen-flame detector the ratio of vitamin E and vitamin D, per injection, which would affect the assay results by 5%, are shown in Table II. Since most multivitamin pharmaceutical products, with both vitamin E and D, contain up to 15 mg. of vitamin E per 400 units (10 mcg.) of vitamin D, no interference from vitamin D in the gas chromatographic method would be expected.

The linearity of response with alpha tocopherol, alpha tocopheryl acetate, and alpha tocopheryl succinate is shown in Fig. 2. As can be seen, the linearity of response for alpha tocopherol and alpha tocopheryl succinate is not as good at the lower concentrations. For these two forms it is desirable to work at the 10–15-mcg. range for best linearity. Since the alpha tocopherol and alpha tocopheryl succinate are usually present at higher levels than alpha tocopheryl acetate, an alumina column was not found necessary for tablets and capsules containing these forms of Vitamin E.

TABLE II—EFFECT OF VITAMIN D ON VITAMIN E RESPONSE PER INJECTION

Vitamin E, 10 mcg.	Level of Vitamin D to Cause a 5% Positive Error in Vitamin E Results, mcg.
Alpha tocopheryl acetate	8
Alpha tocopherol	1–2
Alpha tocopheryl succinate	1–2

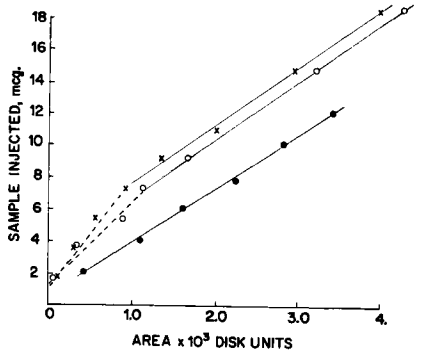

Fig. 2—Linearity of response with concentration. Column: 10% SE-30 on Aeropak 30, length 121.9 cm. (4 ft.); temperature 265°; 5 µl. injected; attenuation 32×. Key: ●, alpha tocopheryl acetate; ○, alpha tocopherol; ×, alpha tocopheryl succinate.

TABLE III—DETERMINATION OF VITAMIN E IN MULTIVITAMIN PRODUCTS

Sample	Label Claim	Found
Tablet		
A	2.00 mg./tablet	2.36 mg./tablet
B	1.00 mg./tablet	1.01 mg./tablet
C	2.72 mg./tablet	3.36 mg./tablet
Liquid	3.33 mg./ml.	3.76 mg./ml.
Cream	5.0 mg./g.	5.60 mg./g.
Capsule		
A	5.00 mg./capsule	5.14 mg./capsule
B	15.0 mg./capsule added	15.2 mg./capsule

A number of products containing tocopheryl acetate were assayed and the results presented in Table III. The area of the chromatographic peaks in a majority of samples was determined with the use of a disk integrator. Where applicable this technique of area measurement was found most suitable. The precision obtained with replicate determinations on manufactured multivitamin tablets of known composition was 101 ± 2.4%.

SUMMARY

A gas chromatographic method for alpha tocopheryl acetate has been developed which, in addition to saving considerable analytical time, also adds to the specificity of the assay for this vitamin.

Subsequent to the preparation of this manuscript a similar procedure was reported (13).

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Keyphrases

Vitamin E analysis—multivitamin products
Dotriacontane—internal standard
GLC—analysis

Technical Articles

Powder Flow Studies IV

Uniformity of Flow: Instrumentation and Applications

By GERALD GOLD, RONALD N. DUVALL, BLAZE T. PALERMO, and JAMES G. SLATER

Use of the recording powder flowmeter for the qualitative evaluation of nonuniform flowing formulations and additional instrumentation for the quantitative measurement of the variation are described. This additional refinement prints out the time, in hundredths of a minute, for preselected weight increments of powder to flow from a hopper. The variation in time is a measure of the uniformity of flow. To illustrate the utility of the instrument, two formulations having similar average flow rates but differing in their uniformity of flow were tableted. The nonuniform flowing formulation tableted on a single-rotary press had a higher coefficient of intertablet weight variation and did not conform to USP standards. Both formulations were tableted on a double-rotary press equipped with induced die feed and low coefficients of variation were obtained in both instances indicating that the die feed mechanism was effective in minimizing intertablet weight variation of an irregularly flowing formulation.

FLUCTUATING FLOW properties of tablet and capsule formulations have been generally overlooked in studying such formulations or at best, evaluated only very subjectively. This has been due, quite largely, to the fact that the two general methods used to evaluate flow of powders, angle of repose measurement, and timed delivery through an orifice, do not distinguish irregularly flowing materials from those which flow smoothly and consistently. For example, Gunsel and Lachman (1) in comparing the flow of formulations prepared with conventionally processed and spray-dried lactose using timed delivery through a funnel found that the formulation having the fastest flow rate exhibited the highest intertablet weight variation.

Another method used to evaluate flow is based on tablet weight variation as actually obtained during tableting trials. Augsburger and Shangraw (2) utilized this method in investigating the effect of various silica-type glidants on the fluidity of direct compression formulations. Increased tablet weight, along with decreased weight variation, indicates improved flowability. This method does give a true picture of the situation under production conditions, but it is time consuming, requiring numerous weighings, and expensive since large quantities of raw material are usually required. Knoechel *et al.* (3) used an instrumented rotary tablet press to evaluate the relative flowability of tableting materials. However, the use of either of these techniques is

Received June 17, 1968, from the Pharmaceutical Research and Development Laboratory, Consumer Products Division, Miles Laboratories, Inc., Elkhart, IN 46514
Accepted for publication August 8, 1968.
Presented to the Industrial Pharmaceutical Technology Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.
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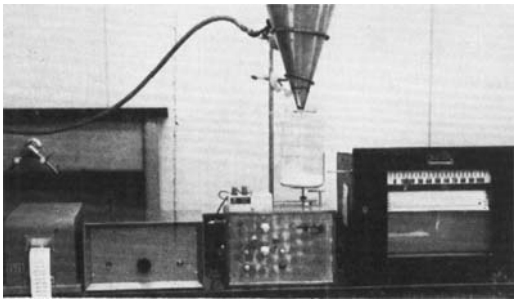

Fig. 1—Photograph of instrumentation showing time interval recorder, attenuator, strain gauge balance, hopper, and strip-chart recorder.

not very practical during the preliminary product development stage.

With the development of more potent drugs, it is becoming increasingly important to prepare dosage forms exhibiting minimum weight variation. Research in this area has been hindered at least in part, by the lack of suitable instrumentation for the direct measurement of variation in flow. This paper describes the use of the recording powder flowmeter (4) for the qualitative evaluation of nonuniform flowing formulations and describes additional instrumentation for the quantitative measurement of the variation in flow. Another objective was to determine the relationship between uniformity of flow and tablet weight variation as it is influenced by certain variables in the tableting process.

EXPERIMENTAL

Instrumentation—The recording powder flowmeter (RPF) consisting of a hopper, strain gauge balance, and recorder described in detail in the first report of this series (4) was redesigned to contain four strain gauges of which two were bonded to the upper surface and two to the under surface of a 0.47 × 0.95 × 25.4-cm. cantilever beam. The instrumentation and electrical diagram are shown in Figs. 1 and 2, respectively. Also included was a transistorized power supply containing a variable span and a zero control potentiometer for balancing the voltage across the two arms of the bridge circuit. It is important in constructing the platform of the cantilever beam that weight applied to the platform be transmitted to a central point on the beam. Otherwise, powder falling on different areas of the platform will cause the millivolt response to fluctuate. The range of the strip-chart recorder was 0–2 mv. for 25.4 cm. full scale and a chart speed of 0.33 cm./sec.

The RPF was further modified by the addition of a microswitch circuit, an attenuator, and a time interval recorder[1] in order to quantitatively measure variation in flow. Span and zero controls on the balance were adjusted so that a selected weight increment such as 50 g. caused full-scale deflection on the strip-chart recorder. At full-scale the pen-drive drum engages the microswitch thereby energizing both the time interval recorder and stepping coil relay of the attenuator. The span potentiometer of the attenuator was then adjusted so that each step in the stepping relay resulted in a voltage of equal magnitude, but opposite polarity to the output of the strain gauge balance, thereby returning the pen carriage to its original position. The time interval recorder prints out the time in hundredths of a minute for each consecutive weight increment and the variation in time is a measure of the uniformity of powder flow.

The instrumentation may be readily used for either qualitative or quantitative measurements by turning the on-off switch of the attenuator and time interval recorder and adjusting the span and zero controls.

Measurement of Powder Flow—Flow measurements were made by allowing 1 kg. of material to flow through a stainless steel conical hopper of 20 cm. top diameter, 30 cm. in length, and interchangeable orifices of 4.76, 6.35, 7.93, 9.52, and 12.70 mm. To study poorly flowing materials, a pressure of 10 p.s.i. was used as described in a previous publication (4).

RESULTS AND DISCUSSION

Application of the RPF for the qualitative evaluation of nonuniform flowing formulations is illustrated in Fig. 3. The flow patterns for aspirin granules[2] and anhydrous dicalcium phosphate (<100 mesh) are indicated by A and E, respectively, and various combinations by B, C, and D. The straight line tracing obtained for aspirin granules indicates uniform flow and the irregular tracing obtained for dicalcium phosphate indicates nonuniform flow. The closer the tracing is to the abscissa, the faster the flow rate. Aspirin had a flow rate of 10.53 g./sec. and dicalcium phosphate 2.38 g./sec. The addition of 25% dicalcium phosphate (Tracing B) increased the flow rate and did not alter uniformity of flow. In contrast, 50% dicalcium phosphate (Tracing C) did not change the flow rate but caused irregular flow. Increasing the concentration of dicalcium phosphate to 75% (Tracing D) resulted in both a slower flow rate and irregular flow. The pulsating characteristic of Formulation D may be measured. From Points a to b in Line D the rate of flow was 9.72 g./sec. and from b to c 3.17 g./sec. representing alternating periods of flooding and starving. The phenomenon of increased flow up to a maximum followed by a decrease on the addition of the finer dicalcium phosphate to the 12/50 mesh aspirin granules is consistent with previous findings in this laboratory (5) relative to the effects of added fines to granules.

To illustrate the utility of the RPF, two formulations having similar flow rates, but differing in uniformity of flow were studied. Figure 4 illustates the flow patterns of both formulations. Formulation A consisted of 32.5% anhydrous dicalcium phosphate, 33% microcrystalline cellulose,[3] 33% aspirin granules,[2] and 1.5% magnesium stearate and had an

[1] Model ET-100, Simplex Time Recorder Co., Gardner, Mass.

[2] Marketed as Aspirin, 10% starch granulation, white, 12–50 mesh, by the Monsanto Co., St. Louis, Mo.

[3] Marketed as Avicel by the FMC Corp., American Viscose Div., Marcus Hook, Pa.

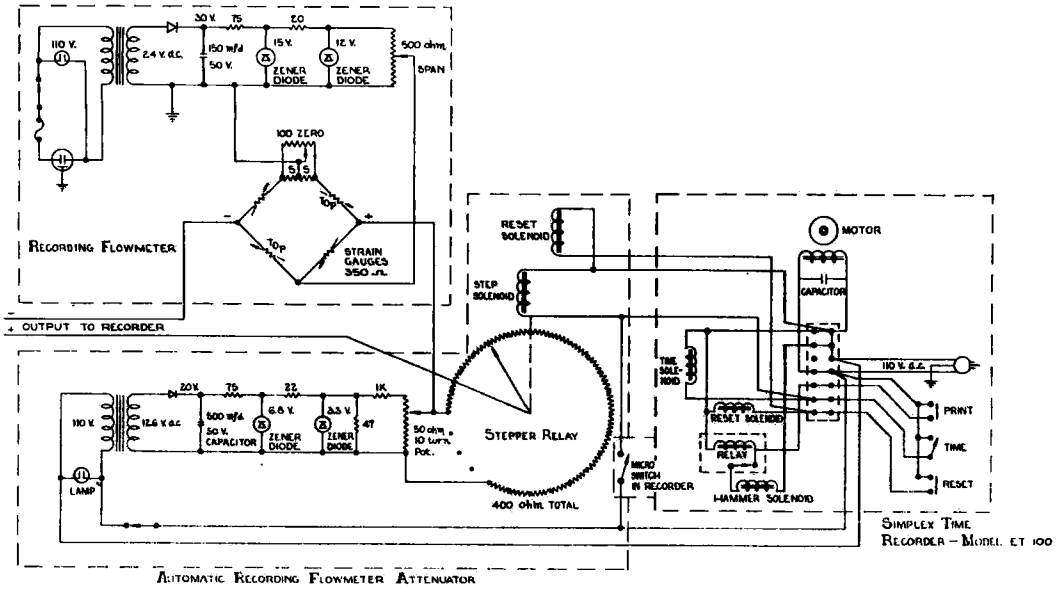

Fig. 2—Electrical diagram for the strain gauge balance, attenuator, and time interval recorder.

irregular flow characterized by flooding and starving. Formulation B consisted of 68.5% sorbitol granules, 30% mannitol powder, and 1.5% magnesium stearate and exhibited uniform flow.

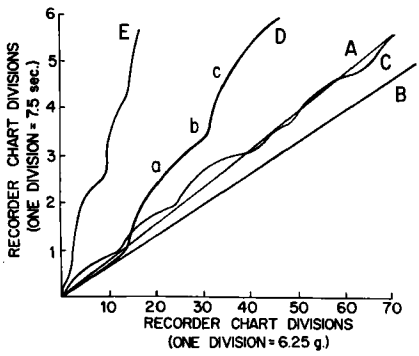

Fig. 3—Flowmeter tracings of formulations containing aspirin granules and anhydrous dicalcium phosphate powder. Key: A, ASA 100/DCP, 0%; B, 75/25; C, 50/50; D, 25/75; and E, 0/100.

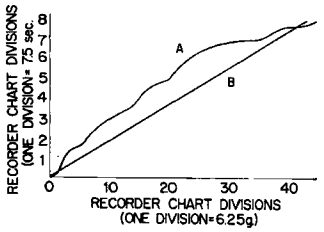

Fig. 4—Flowmeter tracings comparing the flow patterns of two formulations. Key: A, anhydrous dicalcium phosphate, 32.5%; microcrystalline cellulose, 33%; aspirin granules, 33%; and magnesium stearate, 1.5%. B, sorbitol granules, 68.5%; mannitol, 30%; and magnesium stearate, 1.5%.

Formulations A and B were tableted on the single-rotary press (Colton 216) using 10.3-mm. ($^{13}/_{32}$ in.) tooling. Due to the irregular flow of Formulation A the quantity of powder in the feed frame varied. When powder surged through the hopper the feed frame filled and in many instances overflowed; whereas, scanty flow resulted in a feed frame which was nearly empty. Comparative data obtained at three press speeds are given in Table I. The irregularly flowing Formulation A had a higher coefficient of variation at all press speeds and did not conform to the official USP weight variation test. In contrast, the formulation with uniform flow had a lower coefficient of variation and passed the official test.

Both formulations were also tableted on a double-rotary press (Colton 247) using 7.91-mm. ($^{5}/_{16}$ in.) tooling and equipped with an induced die feed. The data obtained are given in Table II. The coefficient of variation for both formulations was low and tablets from each formulation had intertablet weight variation well within the official limits. The induced die feed was, therefore, effective in minimizing intertablet weight variation of this irregularly flowing formulation. These experiments indicate irregular flow is associated with higher intertablet weight variation which may be minimized by appropriate mechanical flowing aides.

The RPF is capable of identifying nonunform flowing materials and is a useful tool in formulating a uniform flowing mixture. However, a definitive study of the factors affecting the uniformity of flow requires an instrument capable of quantitatively measuring the variation in flow. Such an instrument was constructed and the modified RPF measures the time interval in hundredths of a minute for consecutive weight increments of powder to flow, and the variation in time is a measure of the uniformity of flow. Measurements were made on the flow of 25/40 mesh glass beads, aspirin granules, and spray-dried lactose through different size orifices and the data are summarized in Table III. Since the magni-

TABLE I—UNIFORMITY OF TABLET WEIGHTS USING SINGLE-ROTARY TABLET PRESS[a]

Formula	Tab./min.	Av. Wt., mg.	*SD* mg.	*CV*, %	USP XVII
A	548	554	21.5	3.89	Failed
B	548	510	7.6	1.49	Passed
A	687	548	19.7	3.59	Failed
B	687	506	3.5	0.71	Passed
A	863	512	17.4	3.4	Failed
B	863	486	11.8	2.4	Passed

[a] Colton 216, 16 stations, $^{13}/_{32}$-in. diameter stand concave.

TABLE II—UNIFORMITY OF TABLET WEIGHTS USING DOUBLE-ROTARY PRESS[a] WITH INDUCED FEEDING[b]

Formula	Tab./min.	Agitator Setting	Av. Wt., mg.	*SD*, mg.	*CV*, %	USP XVII
A	3,200	50 low	193.3	3.93	2.03	Passed
B	3,200	50 low	160.1	2.68	1.67	Passed
A	3,200	50 high	196.3	3.89	1.99	Passed
B	3,200	50 high	156.9	2.85	1.82	Passed
A	4,000	50 high	190.5	3.45	1.81	Passed
B	4,000	50 high	155.5	3.03	1.95	Passed

[a] Colton 247, 49 stations, $^{5}/_{16}$-in. [b] Model 245.

TABLE III—INTERVAL TIME MEASUREMENTS[a] OF 10 CONSECUTIVE 50-g. INCREMENTS OF 25/40 MESH GLASS BEADS, ASPIRIN GRANULES, AND SPRAY-DRIED LACTOSE

Material	Hopper Orifice mm.	10^{-2} min. Mean	*SD*	*SD* Range	*CV*, %
Glass beads	4.76	28.6	0.64	0.42–0.70	2.24
	6.35	13.2	0.43	0.21–0.67	3.08
	7.93	6.7	0.24	0.21–0.47	4.86
Aspirin granules	7.93	24.7	0.89	0.74–1.22	3.65
	9.52	14.43	0.54	0.39–0.78	3.78
	12.70	6.13	0.23	0.00–0.45	3.75
Spray-dried lactose	4.76	58.8	0.98	0.77–1.25	1.67
	6.35	30.5	0.79	0.53–1.60	2.59
	7.93	17.0	0.61	0.35–0.74	3.59

[a] Based on six determinations.

tude of the standard deviation is related to the magnitude of the interval time measurement, such measurements should be approximtely the same for a valid comparison. For formulations having different flow rates this may be accomplished by varying the size of the orifice and weight increment. For this study, application of the instrument was limited to the comparison of formulations having similar flow rates.

Formulations A and B (Fig. 4) which had previously been evaluated qualitatively were also evaluated quantitatively using the time interval print out. From a plot of increment time measurements in Fig. 5, it is readily evident that Formulation A exhibited greater variation in flow. Formulation A had a *SD* of 8.85×10^{-2} min. and a *CV* of 63.6% as compared to a *SD* of 1.19×10^{-2} min. and *CV* of 7.53% for B. This is in agreement with the qualitative results and illustrates the utility of the instrumentation for quantitative measurements of the variation in flow.

Another application for quantitative measurement is illustrated by a practical example encountered in this laboratory. Qualitative analysis of a poorly flowing formulation indicated that one of the fine powders comprising 12% of the formulation was responsible for the poor flow. This powder was less than 40 mesh and was being offered in a 16/40 mesh granular form. Average flow rates of the formulations containing the powder and the granular form were found to be comparable. However, a plot of increment time measurements (Fig. 6) showed that Formulation A exhibited much greater variation. Formulation A had a *SD* of 1.99×10^{-2} min. and a *CV* of 26.39% as compared to a *SD* of 0.56×10^{-2} min. and a *CV* of 7.47% for Formulation B. Both

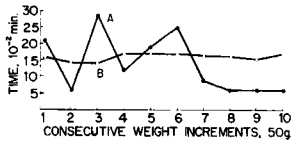

Fig. 5—Increment time measurements comparing the variation in flow of two formulations. Key: A, anhydrous dicalcium phosphate, 32.5%; microcrystalline cellulose, 33%; aspirin granules, 33%; and magnesium stearate, 1.5%. B, sorbitol granules, 68.5%; mannitol, 30%; and magnesium stearate, 1.5%.

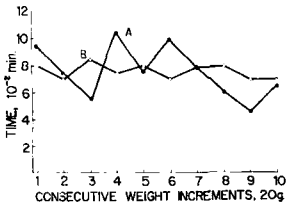

Fig. 6—Increment time measurements showing the effect of particle size of an ingredient comprising 12% of a powder mixture on the uniformity of flow. Key: A, < 40 mesh; B, 16–40 mesh.

formulations were subsequently tableted on the single-rotary press. Tablet weight data using the F test at 95% confidence limits indicated the more uniform flowing formulation had significantly less tablet weight variation.

SUMMARY

Instrumentation has been described for the qualitative and quantitative evaluation of the uniformity of flow of powders through a hopper orifice. For the qualitative evaluation, a recording powder flowometer was utilized in which a recorder tracing identifies fluctuating or inconsistently flowing materials. For quantitative measurement, a print out of the time for preselected weight increments of powder to flow is obtained and the variation in time is a measure of the uniformity of flow. Examples were presented to illustrate the utility of the instrumentation in identifying and evaluating nonuniform flow.

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Keyphrases

Powder flow studies
Instrumentation—powder flow
Flow, powders—uniformity
Tablet weight variation—powder flow effect
Diagram—powder flowmeter

Notes

LSD Analogs II. *N*-[2-(3-Pyridyl)ethyl]-β-alanine Derivatives

By KENNETH J. LISKA and ANJANEYULU S. TADEPALLI

As a continuation of the study of structure-activity relationships in LSD analogs, the ethyl esters and *N,N*-diethylamides of *N*-[2-(3-pyridyl)ethyl]-β-alanine and *N*-methyl-*N*-[2-(3-pyridyl)ethyl]-β-alanine were prepared and evaluated for antiserotonin activity in the isolated rat fundus. All four compounds and one of the intermediates were found to be devoid of activity. SAR implications are discussed.

THE THEORY OF a biochemical etiology of mental illness, especially of schizophrenia, expounded by Woolley (1) and others, has not gained general acceptance. Yet complete rejection of this theory is not justified, for it appears that insufficient data have been accumulated on all aspects of the problem. Among the various kinds of data needed are those on structure-activity relationships in the various chemical types of psychotomimetics. With these data, it might be possible to draw conclusions regarding the mechanism of action of both natural and unnatural neurochemicals and neurohormones.

The aim of this investigation was to continue the preparation of relatives of lysergic acid diethylamide (LSD). In part I (2), serotonin inhibition was utilized as an index of LSD-like activity, and three β-alanine derivatives were found to possess significant pharmacological properties. In the present work, four additional β-alanine derivatives have been prepared and evaluated, again utilizing serotonin inhibitory activity as the criterion.

Woolley believed that serotonin was a key substance in any consideration of a biochemical etiology of schizophrenia, and it is true that all of the psychotomimetic indoles antagonize serotonin (3). Past criticisms of the use of serotonin inhibition as an index of LSD-like activity were based in part on the

Received June 17, 1968, from the Departments of Pharmaceutical Chemistry and Pharmacology, School of Pharmacy University of Pittsburgh, Pittsburgh, PA 15213
Accepted for publication August 6, 1968.
Supported in part by grant I-41, Health Research and Services Foundation, Pittsburgh, Pa.
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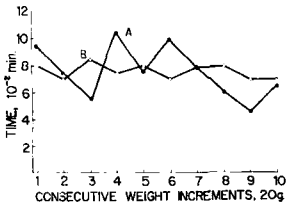

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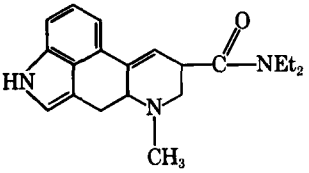
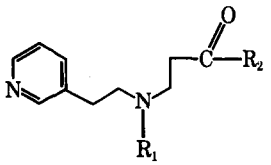
failure of 2-bromolysergic acid diethylamide (BOL) as a psychotomimetic even though it was a serotonin inhibitor. This criticism appears to be less valid when it is realized that substances such as BOL which antagonize serotonin in smooth muscle preparations need not necessarily have the same actions as LSD on the central nervous system. Thus, as Crossland points out (3), the sedative effect of serotonin, injected into cerebral ventricles, is antagonized by LSD but not by bromo-LSD, and there is evidence that bromo-LSD may not reach LSD-sensitive areas as easily as does LSD itself (3).

DISCUSSION

The relationship between LSD and this new type of β-alanine derivative is apparent from a consideration of their structures:



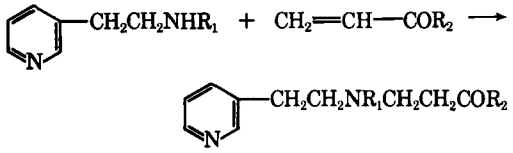
LSD



β-Alanine Derivatives

In place of the pyrrole ring in LSD, the analogs possess a pyridine ring. The simple ester (R_2 = OEt) and the *N,N*-diethylamide (R_2 = NEt_2) were prepared in both the N–H (R_1 = H) and the *N*-methyl (R_1 = CH_3) series.

Addition of primary or secondary amines to acrylic acid derivatives was utilized in all four preparations:



Hydrogenation of 3-pyridineacetonitrile in the presence of alcoholic ammonia afforded 3-(2-aminoethyl)pyridine (Compound I in *Experimental* section). Without the presence of ammonia to suppress secondary amine formation, the yield of the product was reduced by half. Even under optimum conditions, an average yield of 8% of the secondary amine, di-2-(3-pyridyl)ethyl amine (Compound II in *Experimental*) was obtained.

Formylation of the primary amine followed by lithium aluminum hydride reduction of the formaamide gave 3-(2-methylaminoethyl)pyridine (Compound IV). Yields of the *N*-methyl amine were poor owing to the insolubility of the formyl derivative in ether. The formylation was accomplished using formic acid and the primary amine, followed by heating to dehydrate; in each reaction, the simple formate salt could be isolated and recrystallized from *n*-butyl alcohol. It was found to be extremely hygroscopic.

TABLE I—EFFECT OF COMPOUNDS IV, VI, VII, VIII, AND IX IN RESPONSE OF ISOLATED RAT FUNDUS TO SEROTONIN

Compd.	Dose, mcg./ml.	No. of Expt.	Potentiation, %[a] Mean ± *SE*
IV[b]	50	5	117.9 + 6.1
	100	5	130.3 ± 5.2
VI	50	6	159.0 ± 14.15
	100	6	174.0 ± 14.53
VII	50	5	144.0 ± 17.6
	100	5	163.2 ± 23.2
VIII	50	1	121.0
	100	1	110.5
IX	50	1	127.8
	100	1	141.7

[a] Control response to serotonin is taken as 100%. [b] *p* values: Compd. IV, $p > 0.05$; Compd. VI, $p < 0.01$; Compd. VII, $p < 0.05$.

The addition of the primary and secondary amines to ethyl acrylate and to *N,N*-diethylacrylamide proceeded well without the inclusion of any glacial acetic acid. No yield, however, was obtained when 3-(2-aminoethyl)pyridine was allowed to react with acrylamide *per se*. The failure of this specific type of addition has been noted before, and it is assumed that while the desired compound may form initially, it readily decomposes under the conditions of isolation by distillation. All of the new compounds reported herein are water soluble; the tendency to form hygroscopic salts was pronounced.

The pharmacological procedures used were those described in the first paper in this series (2). The results, as summarized in Table I indicate that contrary to expectation, all of the compounds tested were devoid of serotonin inhibitory activity on the isolated rat fundus. Indeed, a slight but significant potentiating action was demonstrated for compounds VI and VII. The active serotonin inhibitors in Series I (2) possessed a group of high electron density on the aromatic ring, whereas the present compounds do not and are devoid of serotonin inhibitory activity. Thus it appears that one of the structural requirements for serotonin inhibition in these β-alanine derivatives, is the presence of groups of high electron density on the N-aromatic portion of the molecule.

EXPERIMENTAL[1]

3(2-Aminoethyl)pyridine (I)—W-2 Raney nickel reduction of commercial 3-pyridine acetonitrile, following the work of Robison and Robison (4), but using absolute alcoholic ammonia in place of aqueous, afforded 3-(2-aminoethyl)pyridine in 71% yield. n_D^{24}1.5379, b.p. 126–128° (24 mm.) [Lit. (4) 114–119° (15 mm.)]; picrate, from ethanol, m.p. 213.5–215° dec. (lit. 213–214° dec.); diHCl, from alcohol, m.p. 207.5–208.5° (lit. 206–207°). Primary amine NH (str.) 3413, 3333 cm.$^{-1}$.

Di-2-(3-pyridyl)ethylamine (II)—This secondary amine was isolated as a by-product in the preparation of Compound I by distillation of the pot residue. It was obtained in an average 8% yield as a yellow, mobile oil, b.p. 174–180° (0.1 mm.), n_D^{28} 1.5631. Secondary amine NH (str.) 3356 cm.$^{-1}$.

[1] M.p. and b.p. are not corrected. M.p.'s were determined in a Thiele apparatus. Analyses by Galbraith Labs. IR data from liquid films taken on an Infracord.

Anal.—Calcd. for $C_{14}H_{17}N_3$: C, 73.97; H, 7.54; N, 18.49. Found: C, 74.10; H, 7.59; N, 18.67.

N-[2-(3-Pyridyl)ethyl]formamide (III)—To Compound I (18.9 g., 0.15 mole), formic acid (7.2 g., 0.15 mole) was added dropwise with stirring in an open beaker. The solid formate salt which resulted was heated on a hotplate until most of the water was driven off as evidenced by cessation of fuming. The 22 g. crude yield was distilled directly, and after discarding a forerun, the fraction b.p. 170—172° (0.7 mm.) was collected as a colorless oil, n_D^{25} 1.5468, amide CO (str.) 1681 cm.$^{-1}$. Obtained 17.9 g. (77%).

Anal.—Calcd. for $C_8H_{10}N_2O$: C, 63.98; H, 6.71; N, 18.66. Found: C, 63.91; H, 6.70; N, 18.67.

3-(2-Methylaminoethyl)pyridine (IV)—Reduction of the formyl derivative (III) was accomplished using $LiAlH_4$, following the general procedure of Baumgarten *et al.* (5), and the work-up given by Mićović and Mihailović (6). The liquid formamide was added dropwise and the reaction mixture was stirred 4 hr. after addition was complete. Repeated ether extractions were found to be essential in the work-up owing to the water solubility of the product. In a typical reaction, the yield was 38% of a colorless oil, b.p. 124–128° (24 mm.), n_D^{29} 1.5185. Secondary amine NH (str.) 3356 cm.$^{-1}$.

Anal.—Calcd. for $C_8H_{12}N_2$: C, 70.55; H, 8.88; N, 20.57. Found: C, 70.49; H, 9.05; N, 20.67. Picrate, from absolute ethanol, m.p. 190–190.5°

N,N-Diethylacrylamide (V)—This was prepared according to the literature (7, 8), b.p. 90–95° (16 mm.), n_D^{26} 1.4655 [lit. 95° (19 mm.), n_D^{20} 1.4672].

N-[2-(3-Pyridyl)ethyl]-β-alanine Ethyl Ester (VI)—Commercial ethyl acrylate (14.4 g., 0.14 mole) was added with swirling to the primary amine (I) (17.6 g., 0.14 mole). The mixture was refluxed gently for 3 hr. and allowed to stand for 20 hr., after which it was distilled directly. After discarding a 1-g. forerun, an amber oil was collected, b.p. 160–170° (2–2.5 mm.), 13.7 g. (43%). n_D^{23} 1.5100, carbonyl CO (str.) 1725 cm.$^{-1}$.

Anal.—Calcd. for $C_{12}H_{18}N_2O_2$: C, 64.83; H, 8.16, N, 12.60. Found: C, 64.72; H, 8.24; N, 12.65.

Picrate, from alcohol, m.p. 138.5–140.5°. The HCl salt could not be obtained crystalline.

3-[2-(3-Pyridyl)ethylamino]-N',N'-diethylpropionamide (VII)—*N,N*-Diethylacrylamide (V) (6.35 g., 0.05 mole) was added dropwise to the primary amine (I). The mixture, protected with a drying tube, was heated under reflux on a steam bath for 4 hr. and then allowed to stand for 16 hr. Distillation yielded a main fraction, 7.9 g., b.p. 155–162° (0.08 mm.), which was redistilled to give 4.5 g. (36%) of a colorless oil, b.p. 160–170° (0.5 mm.). n_D^{29} 1.5083, tertiary amide CO (str.) 1625 cm.$^{-1}$.

Anal.—Calcd. for $C_{14}H_{23}N_3O$: C, 67.43; H, 9.30, N, 16.85. Found: C, 67.49; H, 9.17; N, 17.02.

The HCl salt was prepared crystalline but was very hygroscopic. The picrate crystallized after 4 days standing and was recrystallized from absolute alcohol, m.p. 102–103°, hygroscopic.

N-Methyl-N-[2-(3-pyridyl)ethyl]-β-alanine Ethyl Ester (VIII)—Commercial ethyl acrylate (3.2 g., 0.03 mole) was added with swirling to the secondary amine (IV) (4.2 g., 0.03 mole), and the mixture heated, as in the previous preparation, for 5 hr. and then allowed to stand overnight. Direct distillation gave a main fraction, b.p. 142–147° (0.2 mm.), which was redistilled to give 3.8 g. (52%) of yellow oil, b.p. 141–145° (0.1 mm.). n_D^{29} 1.4938, carbonyl CO (str.) 1727 cm.$^{-1}$.

Anal.—Calcd. for $C_{13}H_{20}N_2O_2$: C, 66.07; H, 8.53; N, 11.85. Found: C, 65.89; H, 8.55; N, 11.79. Picrate, from absolute ethanol, m.p. 143–145°.

3-[N-Methyl-N-2-(3-pyridyl)ethylamino]-N',N'-diethylpropionamide (IX)—*N,N*-Diethylacrylamide (2.8 g., 0.02 mole) was added to the secondary amine (IV) (3.0 g., 0.02 mole) as described in the preparation of VII. The reaction mixture was distilled directly, and after discarding a 2-g. forerun, collected 2.8 g. (48%) of a pale yellow oil, b.p. 170–177° (0.08 mm.). n_D^{25} 1.5089, tertiary amide CO (str.) 1655 cm.$^{-1}$.

Anal.—Calcd. for $C_{15}H_{25}N_3O$: C, 68.40; H, 9.57; N, 15.95. Found: C, 68.20; H, 9.77; N, 15.85. The picrate could not be obtained in a crystalline state.

PHARMACOLOGY

All experimental procedures were the same as for the previous series (2), except for the following. A Grass polygraph model No. 7WC8PA was used instead of a kymograph; the sensitivity of the polygraph was maintained constant for all experiments. The minimum amount of serotonin required to give a response of 7–10 mm. was determined to be 0.5–1.0 ng./ml. of bathing solution.

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Keyphrases

LSD analogs—synthesis
Structure-activity relationship—LSD analogs
Antiserotonin activity—LSD analogs
IR spectrophotometry—identity
Refractive index—identity

Gas Chromatographic Analysis of the Essential Oil from *Achillea millefolium* L.

By ELWINA A. BEJNAROWICZ and STANISLAUS J. SMOLENSKI

Gas chromatography with a differential flame-ionization detector was used with polyethylene glycol adipate on Chromosorb W AW 60/80 column. Thirteen components of the essential oil were identified and quantitatively determined.

IN THE PAST 200 years the essential oil from *Achillea* species has been a subject of interest to many investigators. The earliest recorded production of the volatile oil was in 1719 by Hoffman (1), whereas Bley (2) is officially considered to be the first one to produce the oil. Many investigators directed their work towards the study of azulene in the essential oil since it is responsible for the blue coloration of the oil (2–7). Oswiecimska (8) has reported that the presence of azulene or azulene precursors in the various species of *Achillea* is related to the number of chromosomes. Oswiecimska has found that the tetraploid plants produce azulene, whereas the hexaploid and octoploid plants do not. The authors have concentrated their work on *Achillea millefolium L.* which is the hexaploid variety and therefore it is azulene free (9).

EXPERIMENTAL

Plant Material Used—The plants used were a clone which has been started from seeds of a wild-growing specimen in Bemis Woods, Forest Preserves, Chicago, Ill. The seeds were germinated in the greenhouse of the University of Illinois Drug and Horticultural Experiment Station, Downers Grove, Ill. Later generations were obtained by vegetative propagation of shoots, thus assuring a pure strain for all observations.

Stems, leaves, and inflorescences were determined for the yield of the volatile oil and it was found that the anthodia (flower heads) produce the highest amounts of the oil. The following yields of oil were obtained per 100 g. of air-dried plant parts: Stems—0.18 ml; leaves—0.41 ml.; and anthodia—1.67 ml. The essential oil free of azulene is light yellow in color.

Extraction—The essential oil was obtained by steam distillation using a Clevenger apparatus. One hundred grams of anthodia were placed in a 5000-ml. round-bottom flask and 2000 ml. of water was added. The distillation was completed in 3 hr. From 1400 g. of anthodia 23.4 ml. of essential oil was obtained.

The distillate was saturated with anhydrous NaCl and then taken up in ether. The ether extract was then washed with an aqueous solution of $NaHCO_3$ and this was followed with an aqueous solution of NaOH and finally washed with distilled water until the extract was neutral. The ether was distilled off under reduced pressure at 32°, and the essential oil was stored over anhydrous Na_2SO_4 and refrigerated.

Apparatus—The oil was analyzed using a gas chromatograph (Perkin-Elmer model 881), with a differential flame-ionization detector. A recorder (Sargent SR) was employed, which was equipped with disk chart integrator model 204. Helium was used as a carrier gas, and the flow rate was maintained at 15 ml./min., which the chart speed of the recorder was 1.27 cm./min. and in the course of analysis was changed to 2.54 cm./min. as indicated on the chromatogram (Fig. 1). The gas chromatograph was equipped with automatic temperature programming.

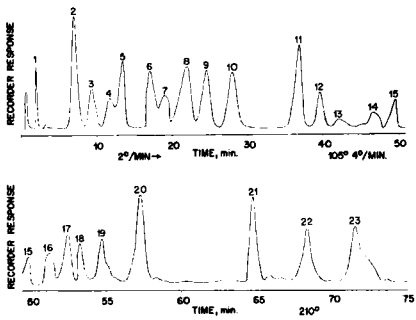

Fig. 1—Gas chromatographic separation of components of Achillea *oil on polyethylene glycol adipate. The attenuation settings are as follows: ×1K—1, 2, 8, 11, 16, 18; ×2K—3, 4, 5, 19; ×200—6, 7, 20–23; ×500—9, 10, 12–14; ×10K—15; ×5K—17. (For operating conditions see Table II.)*

Column Preparation—Two 152.4 cm. (6 ft.) columns were packed with 15% polyethylene glycol adipate as the stationary liquid phase. Polyethylene glycol adipate[1] was supported on Chromosorb W AW 60/80 mesh. One column served as reference and another as sensing. Columns were made of glass, with 0.71 cm. (0.28 in.) o.d. and 0.191 cm. (0.075 in.) i.d. and were cylindrically coiled.

Operating Conditions—Dual detector was used. A 4.5-μl. sample was used. Sample was injected at 55°. The temperature was kept constant for 15 min. After that time the programming was started at a rate of 2°/min. which was changed to 4°/min. when a temperature of 110° was reached. With the elution of Component 15 the chart speed was changed from 1.27 cm. to 2.54 cm./min. Maximum temperature at which 15% polyethylene glycol adipate column was operated was 210°.

Oil Sample and Standard Compounds—For purposes of identification, the retention times and relative retention times were compared with a known pure standard sample. This provided a tentative identification of most of the compounds. Table I lists the retention times, and relative retention times of standard compounds.

Received April 12, 1968, from the Department of Pharmacognosy and Pharmacology, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612
Accepted for publication August 9, 1968.

[1] Reoplex 400.

Table I—Retention Times and Relative Retention Times of Standard Compounds on Polyethylene Glycol Adipate at a Flow Rate of 15 ml./min.[a]

Standard Compd.	Retention Times, min.	Relative Retention Times
Menthol	2.0	0.20
(+)-α-Pinene	7.0	0.70
DL-Camphene	9.8	1.00
(−)-β-Pinene	12.3	1.21
L-Limonene	13.8	1.40
Cineole	21.9	2.30
p-Cymene	36.5	3.80
L-Borneol	41.8	4.40
Isovaleric acid	46.9	4.90
1-(−)-Camphor	49.5	5.20
Isobutyl acetate	51.9	5.41
Furfuryl alcohol	54.8	5.71
Terpineol	59.4	6.21

[a] DL-Camphene = 1.00

This procedure was followed by enrichment method in which known compounds were added individually to the sample chromatographed and compared in each instance to the oil alone. In each instance, on addition of standard compound, the height of the tentatively identified peak was increased. Table II lists relative retention times of individual components as compared with standard compounds.

Table II—Comparison of Relative Retention Times of the Components of *Achillea* Oil with Standard Compounds[a]

Peak No.	Compd.	Unknown	Known
1	Menthol	0.20	0.20
2	(+)-α-Pinene	0.70	0.70
3	DL-Camphene	1.00	1.00
4	(−)-β-Pinene	1.21	1.21
5	L-Limonene	1.40	1.4
6	Unidentified	1.80	—
7	Unidentified	2.01	—
8	Cineole	2.30	2.30
9	Unidentified	2.60	—
10	Unidentified	2.91	—
11	*p*-Cymene	3.81	3.80
12	Unidentified	4.11	—
13	L-Borneol	4.40	4.40
14	Isovaleric acid	4.90	4.90
15	1-(−)-Camphor	5.20	5.20
16	Isobutyl acetate	5.50	5.41
17	Furfuryl alcohol	5.80	5.71
18	Unidentified	5.91	—
19	Terpineol	6.21	6.21
20	Unidentified	6.71	—
21	Unidentified	8.31	—
22	Unidentified	9.10	—
23	Unidentified	9.71	—

[a] Stationary phase—polyethylene glycol adipate; helium flow rate—15 ml./min.; DL-Camphene = 1.00.

Table III—Percent Yield of Components of *Achillea* Oil

Component	Percent
Menthol	1.04
(+)-α-Pinene	8.21
DL-Camphene	5.98
(−)-β-Pinene	5.49
L-Limonene	10.70
Cineole	9.70
p-Cymene	7.30
L-Borneol	0.41
Isovaleric acid	0.81
1-(−)-Camphor	20.61
Isobutyl acetate	1.41
Furfuryl alcohol	11.41
Terpineol	4.30
Unidentified	12.63
Total	100.00

The percentage yield of each tentatively identified component was determined and is presented in Table III.

DISCUSSION

The gas chromatographic analysis indicates the presence of 23 compounds of which 13 were tentatively identified. The remaining 10 components are present in about 12.5% of the total amount of compounds. An analysis of *Achillea fragrantissima* was made by Shalaby and Richter (11) and it revealed the presence of 17 compounds of which identifications were indicated in 14 cases. It has been found that *A. millefolium* has 11 compounds not found in *A. fragrantissima* and that the latter has 12 compounds not detected in *A. millefolium*.

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Keyphrases

Achillea millefolium essential oil—analysis
Oil, essential, *A. millefolium*—constituents
GLC—separation, identity

Pharmacokinetics of Accumulation

By J. M. VAN ROSSUM

The rate and degree of drug accumulation following multiple fixed interval dosage schedules is calculated from the average plasma water concentration of drug per dosage interval. The degree of accumulation is determined by the dose, the distribution volume, the biological half-life, and the dosage interval. The rate of accumulation is mainly determined by the biological half-life. Accumulation to 90 percent of the plateau is reached after a time interval of 3.3 times the biological half-life.

CHRONIC ADMINISTRATION of a drug to patients may lead to a gradual increase in blood levels until finally a steady-state condition is reached. Accumulation occurs if drug intake exceeds elimination which may be the case when a drug is repeatedly administered in fixed doses at fixed intervals (3, 9–11). Accumulation is most evident for drugs which are slowly eliminated such as bromides and digitoxin (1).

In case of multiple dosing of drugs it is of importance to know the rate with which drug accumulation occurs and the plasma level that is attained at the plateau. The average plasma concentration at the plateau may be calculated by an equation presented by Wagner (9). The rate of drug accumulation has not been calculated.

In this paper the kinetics of drug accumulation with respect to both the rate and the degree of accumulation is presented.

MATHEMATICS OF DRUG ACCUMULATION

Most drugs are administered by oral or rectal routes. Assuming the operation of first-order kinetics, the drug concentration in the plasma water after administration of a single dose of a drug (A) may be given in the following equation (3, 8):

$$C_A = C_{Am}\,(e^{-t/\tau_2} - e^{-t/\tau_1}) \qquad \text{(Eq. 1)}$$

Here C_A is the plasma water concentration at the time t after administration, τ_1 and τ_2 are time constants for absorption and elimination, while C_{Am} is a constant depending on the dose Q_A^0, the volume of distribution V_1, and the time constants [$C_{Am} = Q_A^0 \cdot \tau_2/(\tau_2 - \tau_1)V_1$].

The biological half-life is directly related to the time constant for elimination ($t_{1/2} = 0.693 \cdot \tau_2$).

During administration of a drug on a multiple fixed dosage schedule such that a fixed dose Q_A^0 is given after a fixed time interval Δt, the plasma water concentration may be presented by the following equation (3):

$$C_{A(j)} = C_{Am}\left(\frac{1 - e^{-j\cdot\Delta t/\tau_2}}{1 - e^{-\Delta t/\tau_2}} \cdot e^{-t/\tau_2} - \frac{1 - e^{-j\cdot\Delta t/\tau_1}}{1 - e^{-\Delta t/\tau_1}} \cdot e^{-t/\tau_1}\right) \qquad \text{(Eq. 2)}$$

Here $C_{A(j)}$ is the plasma water concentration in the j'th interval at the time t after administration of the j'th dose ($j = 1$ for the first interval). Equation 2 has been called the accumulation equation (6).

The plasma water concentration of a theoretical drug A administered on a fixed multiple-dosage schedule as calculated with Eq. 2 is given in Fig. 1. It is obvious that for large values of j Eq. 2 acquires a limit which is the concentration for each interval at the steady-state situation:

$$C_{A(pl)} = C_{Am}[e^{-t/\tau_2}/(1 - e^{-j\cdot\Delta t/\tau_2}) - e^{-t/\tau_1}/(1 - e^{-j\cdot\Delta t/\tau_1})] \qquad \text{(Eq. 3)}$$

Here $C_{A(pl)}$ is the plateau plasma water concentration at the time t after administration of a dose. The plasma water concentration at the plateau therefore oscillates between a minimum value (when $t = 0$) and a maximum value (3, 6). (See also Fig. 1.)

The rate of accumulation could be calculated from Eqs. 2 and 3 by computing the time ($j.\Delta t$) at which the plateau is reached for 90 or 95%. The combination of Eqs. 2 and 3 leads however to complicated relations. A simple relation may be obtained from equations for the average plasma water concentration over each Δt interval.

The average plasma water concentration in the j'th interval may be obtained by integration of Eq. 2 for each Δt interval and division of the result by Δt, as shown by the following equation:

$$\bar{C}_{A(j)} = \frac{1}{\Delta t} \cdot \int_0^{\Delta t} C_{A(j)}\,dt = \frac{C_{Am}}{\Delta t}\,[\tau_2(1 - e^{-j\cdot\Delta t/\tau_2}) - \tau_1(1 - e^{-j\cdot\Delta t/\tau_1})] \qquad \text{(Eq. 4)}$$

Here $\bar{C}_{A(j)}$ is the average plasma water concentration in the j'th interval. The average plasma water concentration of two theoretical drugs administered on a fixed multiple-dosage schedule as calculated with Eq. 4 is given in Fig. 2. It may be seen that the plateau is reached earlier when the half-life is shorter.

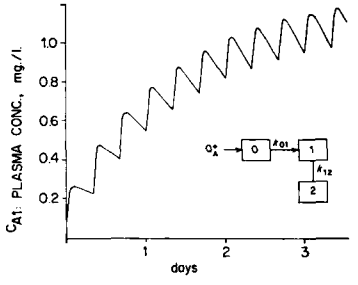

Fig. 1—An accumulation curve as calculated with Eq. 2 of the plasma water concentration following multiple dosing of a drug A ($\tau_1 = V_0/k_{01}$ *and* $\tau_2 = V_1/k_{12}$). *Dosage regimen, t.i.d.:* $\Delta t = 8$ *hr. Dose: 10 mg.;* $t_{1/2} = 24$ *hr.* $V_0 = 6$ *l.;* $V_1 = 36$ *l.;* $k_{01} = 12$ *l./hr.;* $k_{12} = 1.04$ *l./hr.;* $\tau_1 = 0.5$ *hr.;* $\tau_2 = 34.6$ *hr.*

Received April 26, 1968, from the Department of Pharmacology, University of Nijmegen, the Netherlands.
Accepted for publication July 31, 1968.
Supported by a grant of the Netherlands' Organization of Pure Scientific Research (Z.W.O. nr. 542-21).

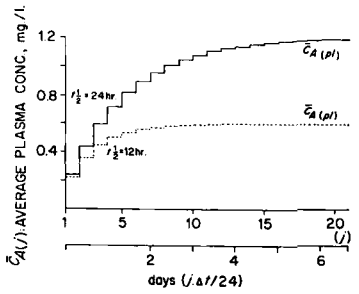

Fig. 2—Average plasma water concentration calculated with Eq. 4 following multiple dosing of two drugs with half-lives of 24 and 12 hr., respectively. The accumulation plateau is directly proportional to the biological half-life while the rate of accumulation is inversely proportional to $t_{1/2}$. *In this case* $\tau_1 = 0.2$ *hr. Dosage regimen, t.i.d.:* $\Delta t = 8$ *hr. Dose: 10 mg.*

Equation 4 reduces to the following equation for large j values, thus representing the average plasma water concentration at the plateau:

$$\bar{C}_{A(pl)} = \frac{C_{Am}}{\Delta t}(\tau_2 - \tau_1) = 1.44\,\frac{Q_A^0}{V_1}\cdot\frac{t_{1/2}}{\Delta t} \quad \text{(Eq. 5)}$$

This equation, which represents the degree of accumulation, is identical to that presented by Wagner *et al.* (9) and is discussed in detail by Krüger-Thiemer (6).

The rate of accumulation may be calculated from a combination of Eqs. 4 and 5:

$$\bar{C}_{A(j)} = \bar{C}_{A(pl)}\cdot\left(1 - \frac{\tau_2}{\tau_2 - \tau_1}\,e^{-j\,\cdot\,\Delta t/\tau_2} + \frac{\tau_1}{\tau_2 - \tau_1}\,e^{-j\,\cdot\,\Delta t/\tau_1}\right) \quad \text{(Eq. 6)}$$

The second exponential term of Eq. 6 is small with respect to the first one if $\tau_2 > \tau_1$. This implies that in such cases Eq. 6 reduces to the following equation:

$$\bar{C}_{A(j)} = \bar{C}_{A(pl)}\left(1 - \frac{\tau_2}{\tau_2 - \tau_1}\,e^{-j\,\cdot\,\Delta t/\tau_2}\right) \quad \text{(Eq. 7)}$$

It may be seen from this equation that the rate of accumulation largely is determined by the time constant for elimination and therefore by the biological half-life, and is dependent of the type of dosage regimen. See also Fig. 2.

It follows from Eq. 7 that the logarithm of the accumulation deficit, $\log\,[1 - \bar{C}_{A(j)}/\bar{C}_{A(pl)}]$, is linearly related to the time $(j\cdot\Delta t)$ after the start of the dosage schedule as may be seen from the following equation:

$$\log\,[1 - \bar{C}_{A(j)}/\bar{C}_{A(pl)}] = -0.301\,j\,\cdot\,\Delta t/t_{1/2} + \log\,[\tau_2/(\tau_2 - \tau_1)] \quad \text{(Eq. 8)}$$

The slope of the straight line representing the logarithm of the accumulation deficit as a function of time $(j\cdot\Delta t)$ is merely determined by the half-life. This relationship as calculated from Eq. 8 for two theoretical drugs is given in Fig. 3. It may be seen from this figure and Eq. 8 that the rate of accumulation does not depend on the dosage interval, but only depends on the $t_{1/2}$ value.

The rate of accumulation may be expressed in terms of a time constant for accumulation or as an accumulation half-life ($tc_{1/2}$). The latter may be obtained from Eq. 8 by computation of $j\cdot\Delta t$ for the case that $\bar{C}_{A(j)}/\bar{C}_{A(pl)}$ equals 0.5 (7):

$$tc_{1/2} = t_{1/2}[1 + 3.30\,\log\,\tau_2/(\tau_2 - \tau_1)] \quad \text{(Eq. 9)}$$

The accumulation half-life therefore is independent of the dosage interval and is directly proportional to $t_{1/2}$, it approaches to $t_{1/2}$ for $\tau_2 \gg \tau_1$ or $\tau_1 \to 0$. This is generally the case for drugs with long half-lives ($t_{1/2} > 12$ hr.) and always so in case of intravenous administration. This approximation does not hold for sustained-release preparations.

The plateau plasma concentration is practically reached after such a time interval $(j\cdot\Delta t)$ since $C_{A(j)}$ equals 0.9 or 0.95 of $C_{A(pl)}$. For clinical purposes it may be stated that the plateau is reached after a time equal to 3–4 times the biological half-life. The accumulation half-life, the number of doses, and the time for accumulation to 90% of the plateau as related to the biological half-life and the dosage interval are given in Table I.

RESULTS AND DISCUSSION

The gradual increase of the plasma concentration of desipramine following a fixed multiple-dosage schedule of 25 mg. t.i.d. in a single patient is given in Fig. 4 (4). In this patient the accumulation plateau is reached after 5 days. The logarithm of the accumulation deficit as a function of time results in a straight line from which the biological half-life may be calculated (see Fig. 5). The $t_{1/2}$ in this patient is 33 hr. From the data for different patients receiving the same drug (4) it follows that the accumulation plateau is reached sooner when the half-life is shorter. Variation in the biological half-life for humans and animals thus results in a variation of the plateau level as well as the rate of accumulation.

Since many drugs may cause an induction of drug metabolism (2) the half-life of a drug may decrease during the drug treatment. Consequently elimination may occur at a higher rate after cessation of chronic dosing.

For most drugs the biological half-life merely determines the accumulation rate. If excessive plasma protein binding occurs the apparent half-life is shorter when the plasma concentration is higher (5). This implies that deviation may occur from the equations derived above.

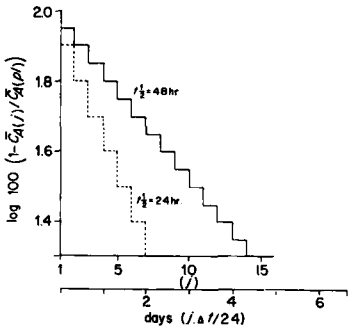

Fig. 3—Logarithm of the accumulation deficit as function of time for two drugs as calculated with Eq. 8. The slope of the straight line is inversely proportional to the biological half-life. Dosage regimen, t.i.d.: $\Delta t = 8$ *hr. Dose: 10 mg.*

TABLE I—THE RELATIONSHIP BETWEEN THE BIOLOGICAL HALF-LIFE, THE DOSAGE INTERVAL, THE RATE OF ACCUMULATION AND THE AVERAGE PLASMA CONCENTRATION AT EQUILIBRIUM FOLLOWING CHRONIC DRUG ADMINISTRATION[a]

Dosage Interval Δt, hr.	Biological Half-life $t_{1/2}$, hr.	Accumulation Half-time $t_{c1/2}$, hr.	Av. Plateau Concn. $\bar{C}_{Apl}$, mg./l.	Number of Doses for 90% of Plateau Value N	Time for Reaching a Plasma Concentration of 90% of the Plateau Value Days	hr.	min.
1	3	3.79	1.20	11		10	45
2	3	3.79	0.60	5		10	45
1	6	6.75	2.40	21		20	40
2	6	6.75	1.20	10		20	40
3	6	6.75	0.80	7		20	40
4	6	6.75	0.60	5		20	40
4	12	12.74	1.20	10	1	16	35
6	12	12.74	0.80	7	1	16	35
8	12	12.74	0.60	5	1	16	35
4	24	24.73	2.40	20	3	8	
6	24	24.73	1.60	13	3	8	
8	24	24.73	1.20	10	3	8	
12	24	24.73	0.80	7	3	8	
6	48 (2d)	48.72	3.21	27	6	16	
8	48 (2d)	48.72	2.40	20	6	16	
12	48 (2d)	48.72	1.60	13	6	16	
24	48 (2d)	48.72	0.80	7	6	16	
8	96 (4d)	96.72	4.81	40	13	7	
12	96 (4d)	96.72	3.21	27	13	7	
24	96 (4d)	96.72	1.60	13	13	7	
12	144 (6d)	144.72	4.81	40	19	23	
24	144 (6d)	144.72	2.40	20	19	23	
24	192 (8d)	192.72	3.21	27	26	14	
24	288 (12d)	288.71	4.81	40	39	22	

[a] The initial and maintenance dose has been fixed at 10 mg., the volume of distribution at 36 l., and the time constant for resorption $\tau_1 = 0.72$ hr.

The calculation of the average plateau plasma concentration by Eq. 5 is based on the assumption of irreversible first-order processes for absorption and elimination. This equation is not valid if absorption process is a reversible first-order process with clearance constants k_{01} and k_{10}. The time constants τ_1 and τ_2 then are no longer inversely proportional to k_{01} and k_{12} but also dependent on k_{10}. The constant C_{Am} in that case is also different $[C_{Am} = Q_A^0/(\tau_2 - \tau_1)k_{12}]$. The average plasma water concentration at the plateau is then given by the following equation:

$$\bar{C}_{A(pl)} = Q_A^0/k_{12} \cdot \Delta t \qquad \text{(Eq. 10)}$$

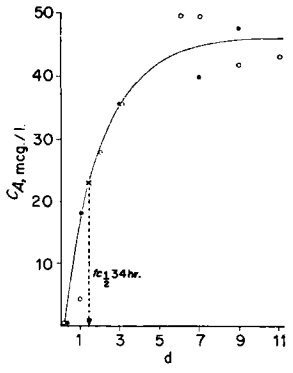

Fig. 4—Accumulation curve of desipramine following chronic administration of 25 mg. 3 times a day to a patient. Data after Hammer et al. *(4). The curve is the result of two experiments. The second experiment (●) is made 5 weeks after the first (○). Accumulation to 90% of the plateau is reached within 5 days. Desipramine, 25 mg. Dosage regimen, t.i.d.: Δt = 8 hr.*

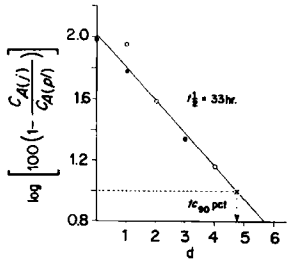

Fig. 5—Logarithm of the accumulation deficit of desipramine as a function of time. Data after Hammer et al. *(4). From the straight line the half-life of the accumulation is found to be 33 hr. for this patient.*

The clearance constant, k_{12}, may be obtained from plasma curves after intravenous administration of a drug. The equation for the accumulation rate remains valid, however in case of reversible absorption also.

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Keyphrases

Drug accumulation—pharmacokinetics
Pharmacokinetic equations—drug accumulation
Accumulation plateau—drug, multiple doses

Gas Chromatographic Determination of Hexachlorophene in Blood and Urine

By R. S. BROWNING, JR., JOHN GREGO, and H. P. WARRINGTON, JR.*

A rapid and convenient gas chromatographic technique has been developed for the determination of traces of hexachlorophene in human blood and urine. After a simple extraction, the hexachlorophene is acetylated. The acetyl derivative chromatographs well on conventional columns at a temperature within the operating range of a tritium foil electron capture detector.

ALTHOUGH HEXACHLOROPHENE (2,2′-methylene bis(3,4,6-trichlorophenol) is widely used in cosmetic preparations and pharmaceuticals, few methods are available for the determination of very low levels in blood or other tissue. Johnston and Porcaro (1) have described a method in which the hexachlorophene, after a relatively complex extraction procedure, is measured by colorimetry with 4-amino antipyrine as reagent. Using their method, as little as 20 mcg. of hexachlorophene can be measured in up to 100 g. of tissue. Wit and Van Genderen (2) used a similar technique in studying the recovery of hexachlorophene from the urine, feces, and milk of cattle dosed with the drug. Recovery data were reported for samples of various sizes, from a fraction of a gram for feces to 25 ml. for milk, containing 10–110 mcg. of drug. In another part of the same study, radiochemical assay methods were used to determine hexachlorophene in the excreta of rats and rabbits dosed with labeled drug.

Porcaro (3) has also described a gas chromatographic procedure for the determination of various bisphenols, including hexachlorophene. The minimum measurable quantity was claimed to be 5–10 mcg. He mentioned, but did not investigate, the potential increase in sensitivity which could result from the use of an electron capture detector. More recently, Wisniewski (4) has reported a gas chromatographic method for the determination of hexachlorophene in soap. In contrast to Porcaro's approach, in which the unmodified phenol was injected on a short, specialized column with a relatively high liquid loading, Wisniewski first prepared the trimethylsilyl derivative in order to reduce tailing and sample loss on the more conventional column he used. He, too, used a flame-ionization detector.

On columns available to the authors, using temperatures and liquid phases compatible with the use of a tritium foil electron capture detector, direct injection of hexachlorophene was unsatisfactory. Instrument response to samples in the subnanogram range was erratic. This variability was largely explained when it was definitely established that fractional amounts of hexachlorophene samples were held in the instrument. Derivatives which could eliminate this problem were therefore investigated. In this laboratory, silyl derivatives were not wholly satisfactory. Detector response at low levels continued to be somewhat erratic, and the retention times for the derivatives were undesirably long. Acetylation, however, proved to be rapid, simple, and reproducible, and the diacetyl derivative chromatographed reliably on several substrates, including a silicone gum (SE-30), a silicone oil (OV-17), and an organosilicone polymer (EGSP-Z).

On the basis of these results, a sensitive gas chromatographic method for the determination of hexachlorophene in extracts from whole blood and urine has now been developed. Using the procedure described here, it is easy to measure hexachlorophene at levels down to 0.05 mcg./ml.

EXPERIMENTAL

Reagents—USP grade hexachlorophene obtained from Winthrop Laboratories was used without further purification. All other materials were reagent grade, obtained from recognized suppliers.

Apparatus—All the glassware used was cleaned before use by soaking in a 1:1 mixture of 6 *N* HCl and methanol for about 0.5 hr., then rinsing with distilled water. Glassware was oven-dried. An F and M (Avondale, Pa.) model 400 gas chromatograph equipped with a tritium foil electron capture detector was used for the chromatography. The 121.9-cm. (4-ft.) long 0.635-cm. (0.25-in.) diameter column was packed with 1% EGSP-Z on 100/120 mesh Gas-Chrom Q (Applied Science Labs, Inc., State College, Pa.). The oven temperature was 205–210°,

Received June 24, 1968, from the Sterling-Winthrop Research Institute, Rensselaer, NY 12144
Accepted for publication September 3, 1968.
The authors are indebted to E. L. Pratt for his assistance in preparing certain of the standards used in this work.
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TABLE I—RECOVERY OF HEXACHLOROPHENE FROM BLOOD

Added, p.p.m.	Recovered, p.p.m.
0[a]	0.031
0[a]	0.006
0.050	0.036
0.060	0.051
0.100	0.079
0.120	0.108
0.200	0.162
0.240	0.192
0.300	0.236
0.360	0.238

[a] Blood blanks from two separate donors. All other tabulated values are corrected for the appropriate blank.

the detector temperature 215°, and the injection port temperature 275°. Helium was used as the carrier gas, and the pulse type detector was purged with 10% methane in argon. The pulse setting was 50 μsec.

Procedure—As soon as blood samples were drawn, they were well mixed with finely ground potassium oxalate, 0.7 mg./ml., to prevent clotting. Exactly 3 ml. of the oxalated whole blood was then transferred to a 15-ml. glass-stoppered centrifuge tube, 1 ml. of 0.4*M* pH 7.5 phosphate buffer added (the buffer was prepared by mixing 1 vol. of 0.4 *M* NaH_2PO_4 with 10 vol. of 0.4 *M* Na_2HPO_4), and the contents mixed well. The tube was then immersed at an angle, to prevent breakage, in a dry ice–acetone bath adjusted to about −20°, until the contents were solidly frozen. If the bath temperature is too low, or if tubes are supported vertically, some breakage may result. The tube contents were then allowed to thaw at room temperature. When the contents were liquid, exactly 10 ml. of ethyl acetate was added, the tube stoppered, and the contents mixed vigorously for 3 min. The tube was then centrifuged at moderate speed. Exactly 5 ml. of the clear ethyl acetate layer was transferred to a graduated 15-ml. tube, which was then immersed in a water bath held at 80°. The ethyl acetate was evaporated, under a stream of nitrogen, to 0.5–1.0 ml. The tube was then removed from the bath, 0.1 ml. of a 1:1 mixture of acetic anhydride and pyridine added, and the contents mixed well, after which the tube was placed in a second bath at 60° for 10 min. After removing from the bath, tube and contents were cooled to room temperature and the volume adjusted to 1.1 ml. with ethyl acetate. Aliquot portions of exactly 3 μl. of the well-mixed solution were then injected into the chromatograph.

Urine samples were handled similarly, but were not frozen.

Hexachlorophene standards, containing from 0 to 0.75 mcg. in 5-ml. portions of ethyl acetate, were evaporated to 0.5–1.0 ml. and treated in the same way as the sample extracts.

RESULTS

When amounts of hexachlorophene ranging from 0.05 to 0.36 p.p.m. were added to whole blood, the mean absolute recovery (least squares slope) was 81.6%. The recovery of similar amounts from urine was 84.3%. In no case was the relative standard deviation of the slope greater than 3.1%. The data for blood are reported in Table I and for urine in Table II. In each case the reported value, from which the recoveries above were calculated, is the average of duplicate assays. The estimate of the standard deviation of the assay pairs (blood) was 0.013. Variation in the volume of sample injected into the instrument is undoubtedly the major source of error, and this is not excessive. The relative standard deviation of four replicate injections was 2.3%.

TABLE II—RECOVERY OF HEXACHLOROPHENE FROM URINE

Added, p.p.m.	Recovered, p.p.m.
0	0
0.12	0.101
0.24	0.186
0.48	0.406

DISCUSSION

The peak found in the blank blood samples has not yet been positively identified, but all the present evidence indicates that it is, in fact, caused by hexachlorophene present in the samples. The retention times of hexachlorophene and the unknown peak correspond on both the EGSP-Z and on an SE-30 column, and in each case, before and after acetylation. No such peak was found in monkey, rabbit, or dog blood, nor in human urine.

The procedure has adequate accuracy at the level discussed. Greater precision can be achieved, if necessary, by running additional samples, without greatly increasing analytical time. The total elapsed time for an analysis is approximately 2 hr. Of this, the gas chromatographic step takes about 10 min.

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Keyphrases

Hexachlorophene analysis—blood, urine
GLC—analysis
Tritium foil electron capture detector—GLC analysis

Catharanthus Alkaloids XXI. Isolation of Lochnerinine, One of the Cytotoxic Principles of *C. pusillus*

By M. TIN-WA, H. H. S. FONG, R. N. BLOMSTER, and N. R. FARNSWORTH

Catharanthus pusillus **alkaloid fractions were found to be active against Eagle's 9 KB carcinoma of the nasopharynx in cell culture. Chromatographic separation of one of the active fractions resulted in the isolation of lochnerinine, and the activity of this alkaloid against the 9 KB carcinoma has been confirmed. Gradient pH fractionation of another active alkaloid fraction yielded ajmalicine and vindorosine, which were not cytotoxic.**

An apocynaceous plant indigenous to India is *Catharanthus pusillus*. Prior phytochemical investigations in these laboratories have yielded the alkaloids ajmalicine and vindorosine (1). Majumdar and Paul have reported the isolation of two amorphous alkaloids having cardiotoxic properties, which they named pusiline and pusilinine, in addition to three unidentified sterols (2). Battersby and Kapil isolated a neutral substance from a weakly basic alkaloid fraction of this plant in a yield of $1 \times 10^{-4}\%$, which was identified as *N*-benzoyl-L-phenylalaninol (3). No other phytochemical studies on *C. pusillus* have been reported in the literature.

In a continuing study of *Catharanthus* species for new antitumor agents, the authors have examined the alkaloids of *C. pusillus* leaves, utilizing different procedures than in previous studies from their laboratories (4). This study has led to the confirmation of ajmalicine and vindorosine in this plant, and reports for the first time the isolation of the cytotoxic alkaloid lochnerinine from *C. pusillus*.

EXPERIMENTAL

Plant Material—The *Catharanthus pusillus* (Murr.) G. Don (*Apocynaceae*) leaves used in this study were obtained from Drugland Corporation, Delhi, India, and a voucher specimen has been identified by one of the authors (N.R.F.), and has been deposited in the herbarium of the Department of Pharmacognosy, School of Pharmacy, University of Pittsburgh.

Preparation of Alkaloid Fractions—A sample of coarsely milled leaves (19.75 kg.) was extracted continuously for 24 hr. in a Lloyd extractor with skellysolve B. This extract was concentrated and removed from the extractor and a fresh charge of solvent was added. The procedure was repeated four times. Pooling of the four concentrates was followed by reduction of the volume to 2.0 l. To this concentrate was added 1.6 l. of 2 *N* HCl, and the mixture was distilled *in vacuo* to remove the skellysolve B, cooled, and filtered. A residue on the filter pad was dissolved in benzene and re-extracted into 2 *N* HCl as described above, and the two acid filtrates were combined (*ca.* 3 l.). This extract was decolorized with charcoal and filtered through a diatomaceous earth[1] filter pad. Ice was added to the clear filtrate and ammonium hydroxide solution was added in small portions with continuous stirring until the solution was alkaline to litmus paper. In order to extract the alkaloids from this basic solution, it was shaken several times with ethylene dichloride, and the ethylene dichloride extracts were dried over anhydrous sodium sulfate, filtered, and taken to dryness *in vacuo* to yield 6.7 g. of crude, weakly basic alkaloids (Fraction I).

The skellysolve B-extracted *C. pusillus* leaves were air dried, macerated with methanol, and exhaustively extracted by repeated maceration and percolation. The combined percolates (*ca.* 400 l.) were concentrated to 20 l. and then extracted into 20 l. of a 2% tartaric acid solution, residual methanol being removed *in vacuo*, and the acid solution filtered. Three 20-l. volumes of benzene were used to extract the combined filtrates, and the benzene extracts were pooled, dried over anhydrous sodium sulfate, filtered, and concentrated to give 43 g. of Fraction II (acid benzene solubles).

Residual benzene was removed from the aqueous acid layer of post-Fraction II *in vacuo*, and the resulting solution was chilled and rendered alkaline with ammonium hydroxide solution. This alkaline solution was then extracted with three 20-l. volumes of ethylene dichloride. The ethylene dichloride extracts were pooled, dried over anhydrous sodium sulfate, filtered, and were taken to dryness to yield 50 g. of crude tertiary alkaloids. This fraction was further divided into the nonphenolic part (Fraction III), and the phenolic part (Fraction IV) by passing it through a column of ion-exchange resin (IRA-400) (500 g.), which was pretreated with 5% sodium hydroxide solution, and washed with distilled water until the effluent was neutral to litmus paper. Fraction III (37 g.) was obtained by eluting the resin column containing the crude tertiary alkaloids with methanol (*ca.* 16 l.), and Fraction IV (6 g.) was removed from the column by elution with 0.1% HCl in methanol (*ca.* 4 l.).

The quaternary alkaloids (Fraction V) were obtained as chlorides from the aqueous alkaline solution after the removal of the total tertiary bases. This was accomplished according to the method described by Hogg *et al.* (5), to yield 29 g. of Fraction V. The mother liquor remaining after removal of Fraction V was termed Fraction VI. A scheme showing the method of preparation of the alkaloid fractions is presented in Scheme I.

Cytotoxicity Evaluation of Alkaloid Fractions—Fractions I–VI were evaluated for cytotoxicity against Eagle's 9 KB carcinoma of the nasopharynx in cell culture according to established protocols,[2]

Received June 25, 1968, from the Department of Pharmacognosy, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15213

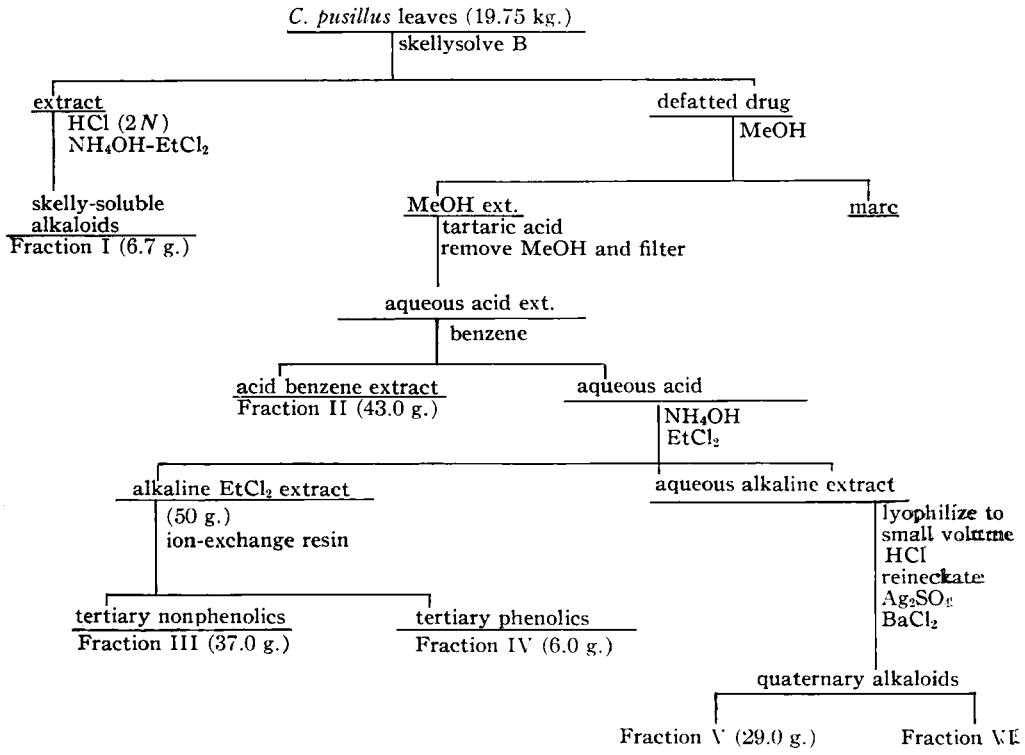
Accepted for publication August 22, 1968.

This investigation was supported, in part, by research grants CA-08228 and FR-05455, from the National Institutes of Health, U. S. Department of Health, Education and Welfare, Bethesda, Md., and the Jane Olmstead Thaw Fund, University of Pittsburgh.

Previous paper: Masoud, A. N., Farnsworth, N. R., Sciuchetti, L. A., Blomster, R. N., and Meer, W. A., *Lloydia*, **31**, 202(1968).

[1] Celite, Johns-Manville, New York, N. Y.

[2] For a description of the methods employed see *Cancer Chemotherapy Rept.*, **25**, 1(1962).



Scheme I

through the auspices of the Cancer Chemotherapy National Service Center, Bethesda, Md. The results are presented in Table I. Phytochemical work-up was made on the basis of active fractions being given first priority.

Chromatographic Separation of Fraction I—A glass chromatographic column (3 × 38 cm.) was slurry packed with 170 g. of alumina (Alcoa F-20) and benzene as previously described (4). The fraction (6.0 g.) was dissolved in a small volume of benzene and this solution was adsorbed to the top of the column, followed by the addition of benzene. Elution of the alkaloids was accomplished with benzene, followed by mixtures of benzene and chloroform, and finally with chloroform. Twenty-milliliter fractions were collected from the column, analyzed by means of TLC on silica gel G plates (6), and combined on the basis of the pattern of resolved alkaloids and chromogenic reactions after spraying each plate with the ceric ammonium sulfate reagent (6). The results of this chromatographic separation are presented in Table II.

TABLE I—CYTOTOXICITY OF *Catharanthus pusillus* FRACTIONS

Fraction	ED_{50} (mcg./ml.)[a]
I	2.6×10^{0}[b]
II	1.7×10^{1}
III	1.4×10^{0}[b]
IV	6.1×10^{0}[b]
V	1.0×10^{2}
VI	1.0×10^{2}

[a] An active fraction is one that exhibits $ED_{50} \leq 15.0$ mcg./ml. [b] Active cytotoxic fraction.

Isolation of Lochnerinine—Combined Fractions V–VI from the column appeared to contain only one alkaloid as evidenced by TLC. This alkaloid was suspected to be lochnerinine on the basis of its R_f value in three different solvent systems (6). The combined fractions were freed from solvent to yield a thick oil, which was dissolved in a small volume of methanol and hydriodic acid solution was added dropwise until an acidic solution resulted (pH *ca.* 3.0). Refrigeration of the solution for about 2 hr. resulted in the formation of fine yellow needles, which were removed by filtration. Recrystallization from methanol yielded 24.5 mg. of alkaloid, which when dried *in vacuo* at 100° for 24 hr., gave m.p. 207°. The UV and IR spectra, as well as TLC

TABLE II—CHROMATOGRAPHIC SEPARATION OF *C. pusillus* FRACTION I ALKALOIDS

Fraction No.[a]	Eluent
1–4	Benzene
5–6	Benzene
7–13	Benzene
14–48	Benzene
49–70	Benzene
71–154	Benzene
155–200	Benzene
201–240	Benzene–chloroform (3:1)
241–350	Benzene–chloroform (3:1)
351–380	Benzene–chloroform (1:1)
381–400	Benzene–chloroform (1:1)
401–449	Chloroform
450–480	Chloroform

[a] Each fraction was 20 ml. and all fractions contained alkaloids.

results (6), showed that the isolated alkaloid was identical with a reference sample of lochnerinine hydriodide which was prepared from authentic lochnerinine, isolated in these laboratories from *C. lanceus* (7).

Gradient pH Separation of Fraction III—Fraction III was subjected to a gradient pH separation according to the method of Svoboda (8), with only minor modifications. The fraction (32.0 g.) was dissolved in a minimum volume of ethylene dichloride and extracted into 1.5 l. of 0.2 *M* citric acid solution by heating on a steam bath and removal of the ethylene dichloride *in vacuo*. The resulting aqueous phase was decolorized with charcoal, and filtered. Extraction of this acidic filtrate (pH 2.7) was effected with three separate volumes of benzene and the aqueous layer was adjusted to pH 3.2 with ammonium hydroxide solution, followed by three benzene extractions. This procedure was continued with increases in pH by 0.5 units, with ammonium hydroxide solution, until a final pH of 9.2 was attained. Benzene extracts from each pH level were combined, taken to dryness, and adjusted to *ca.* 100 ml. with benzene. TLC (6) of each of the pH fractions served to point out those pH fractions to be combined prior to crystallization attempts. In this manner, the following groupings were made:

Group	pH	Wt., g.
III-A	2.7–3.2	3.42
III-B	3.7	1.84
III-C	4.2–5.2	3.55
III-D	5.7–7.2	4.73
III-E	7.9–9.2	1.25

These five groups were then each subjected to crystallization attempts using benzene, chloroform, acetone, ethylene dichloride, ethanol, or methanol.

Isolation of Vindorosine—Fine needles (250 mg.) were obtained from a benzene solution of Group II-A. An analytical sample was obtained following several recrystallizations from benzene. This sample was identical (m.p., UV, IR, TLC) with a reference sample of vindorosine.

Isolation of Ajmalicine—Colorless crystals (50 mg.) were obtained from an ethanol solution of Group III-B. Several crystallizations from ethanol afforded an analytical sample which was shown to be identical with reference ajmalicine.

Cytotoxicity of Lochnerinine—Ajmalicine and vindorosine were found to be inactive in the cytotoxicity test of the Cancer Chemotherapy National Service Center screening laboratories.[2] Lochnerinine, however, exhibited $ED_{50} < 1.0 \times 10^{-2}$ mcg./ml. aginst the 9 KB cell culture. A compound is considered an active cytotoxic agent if it has $ED_{50} \leq 1.0$ mcg./ml.[2] All three alkaloids were inactive when evaluated against the P-1534 leukemia in DBA/2 mice.[2]

DISCUSSION AND SUMMARY

Catharanthus pusillus leaves of Indian origin were studied in this investigation. The total crude alkaloids (0.6%) were separated into six fractions, and three of these were found to be cytotoxic against Eagle's 9 KB carcinoma of the nasopharynx in cell culture. Lochnerinine, an α-methyleneindoline base previously reported isolated only from the related *C. roseus* and *C. lanceus*, was isolated from the weakly basic alkaloid Fraction I by column chromatography, as the hydriodide salt, and was shown to be responsible for at least a part of the cytotoxicity of this fraction. Additional alkaloids could not be isolated from other chromatographic cuts of this fraction, each of which was extremely complex.

Fraction III, representing the nonphenolic tertiary bases, was subjected to a gradient pH separation and vindorosine was isolated in a yield of 0.2% of the total crude alkaloids, from the pH 2.7–3.2 fraction. Vindorosine has been isolated previously only from the related *C. roseus*. From the pH 3.7 fraction was isolated ajmalicine in a yield of 0.04% of the total crude alkaloids. This base also occurs in *C. roseus*, *C. lanceus*, and *C. trichophyllus*, as well as in many other apocynaceous plants.

The remaining pH fractions of Fraction III were complex and did not yield alkaloids by conventional crystallizing techniques. Neither vindorosine nor ajmalicine were found to be cytotoxic, and the active entity in Fraction III remains unknown.

As yet no attempt has been made to separate alkaloids from Fractions II, IV, or V.

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Keyphrases

Catharanthus alkaloids
Lochnerinine, *C. pusillus*—isolation, identification
Column chromatography—separation
TLC—separation, identification
Cytotoxicity—lochnerinine

Chromatographic Separation and Bioassay of the Gentamicin Complex

By NATHAN KANTOR and GEORGE SELZER

The gentamicin complex consists of three components, C_1, C_{1a}, and C_2, which are separated by paper chromatography using the lower phase of a solvent system of chloroform, methanol, and 17% ammonia (2:1:1). Two identical chromatograms are developed. On one, the positions of these components at R_f's of approximately 0.75, 0.35, and 0.50, respectively, are located by means of ninhydrin. On the other, corresponding areas are cut out, eluted from the paper with 0.1 *M* potassium phosphate buffer, pH 8.0, and assayed microbiologically. This procedure determines the relative amounts of the three gentamicin fractions, and is superior to the original official FDA procedure that measured only two fractions.

THE ISOLATION of gentamicin, an antibiotic complex from *Micromonospora purpurea*, was reported in 1963 (1, 2). Initially, this complex was considered to be a mixture of two fractions (C_1 and C_2). The invariance quotient of these two fractions is determined by acetylating the gentamicin sample with ^{14}C-labeled acetic anhydride, separating the two acetyl derivatives by paper chromatography, and measuring the relative amounts of the two fractions by radioautography (3). Further work (4), utilizing the technique of descending paper chromatography with a solvent system composed of chloroform, methanol, and 17% ammonia, revealed that C_1 consists of two fractions, C_1 and C_{1a}, making three fractions altogether. The exact structures of the gentamicin fractions are unknown, but they are amino-sugar antibiotics resembling the neomycin-kanamycin-streptomycin groups.

In order to characterize various batches of gentamicin with a view toward assuring compositional similarity of different batches, an analytical procedure for the gentamicin complex was developed, in which paper chromatography is used to separate the fractions and microbiological assay to measure the activity of each fraction.

EXPERIMENTAL

Materials—Reagent grade solvents and chemicals. Chromatographic paper, Whatman No. 2, 14.25 × 46 cm. Rule an origin line 9 cm. from the top of the paper and mark two dots on the origin line 4 cm. from each edge of the paper.

Apparatus—A standard cylindrical chromatography jar (30.5 cm. diameter, 61 cm. high) with a tightly fitting, ground-glass contact top and equipped for descending chromatography.

Solvent System—Equilibrate 200 ml. of chloroform and 100 ml. of methanol with 100 ml. of 17% ammonia [ammonium hydroxide + water (3 + 2)] by shaking in a separator. Without allowing the phases to separate, add the entire mixture to the chromatography jar and allow 24 hr. for saturation. Prepare another equilibration mixture as above but allow the phases to separate and use the lower phase only as the chromatographic solvent.

Ninhydrin Reagent—Prepare a modified Barrollier reagent (5) as follows: To 1 g. of ninhydrin and 0.1 g. of cadmium acetate, add 3 ml. of water and 1.5 ml. of glacial acetic acid, and shake. Add 100 ml. of *n*-propanol and shake until solution is complete. Store this solution in a brown bottle under refrigeration.

Procedure—Prepare an aqueous solution containing 40 mg. of the sample/ml. Apply 5 µl. to each of the two dots on the chromatographic paper and allow to dry. In like manner, prepare another paper. Place the papers in separate troughs in the chromatographic chamber, and fill the two troughs with the chromatographic solvent. Chromatograph until the solvent front reaches the bottom of the paper (approximately 3.5 hr.). Remove the papers and dry them in a hood for 30 min. Cut each paper in half lengthwise; spray one half with ninhydrin reagent, and place it in a drying oven at 100° for 1 min.

The gentamicin fractions appear as reddish zones; the zone farthest from the origin is C_1, the zone nearest is C_{1a}, and the middle zone is C_2. Using this half as a guide, cut the other half of the paper into segments representing, respectively, the C_1, C_2, and C_{1a} fractions. Cut each segment thus obtained into small strips and put the strips into a separate 125-ml. glass-stoppered flask. To each flask add 50 ml. of 0.1 *M* potassium phosphate buffer, pH 8.0, and swirl the flasks mechanically for 30 min. Decant each solution into a test tube and allow the paper to settle. Pipet 4.0 ml. of the clear solution into a 25-ml. volumetric flask and make to volume with the same buffer. Assay these solutions by the microbiological plate assay, using *Staphylococcus epidermidis* as the test organism (6).

Computations—The percentage of each fraction is calculated as follows:

$$\% C_1 = \frac{A_1}{504} \times \frac{100}{B}$$

$$\% C_{1a} = \frac{A_{1a}}{626} \times \frac{100}{B}$$

$$\% C_2 = \frac{A_2}{656} \times \frac{100}{B}$$

where:

A_1 = concentration of the assayed C_1 solution in mcg./ml.

A_{1a} = concentration of the assayed C_{1a} solution in mcg./ml.

A_2 = concentration of the assayed C_2 solution in mcg./ml.

$$B = \frac{A_1}{504} + \frac{A_{1a}}{626} + \frac{A_2}{56}$$

504, 626, and 656 are the activities of C_1 sulfate, C_{1a} sulfate, and C_2 sulfate, respectively, compared to the gentamycin sulfate standard.

Received July 5, 1968, from the Chemistry Branch, Division of Antibiotics and Insulin Certification, Bureau of Science, Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Washington, DC 20204

Accepted for publication August 22, 1968.

The authors thank Schering Corp. for samples of the three gentamicin fractions. The assistance rendered by the Microbiological Branch of the Division of Antibiotics and Insulin Certification, Food and Drug Administration, is gratefully acknowledged.

TABLE I—COMPARISON OF KNOWN AND ASSAYED VALUES OF THE GENTAMICIN FRACTIONS

Gentamicin Fraction	% Present	% Found ± SD
C_1	37.8	37.2 ± 2.04
C_{1a}	33.3	32.7 ± 2.17
C_2	28.9	30.0 ± 2.26

RESULTS AND DISCUSSION

A synthetic mixture of the three gentamicin fractions was prepared and assayed six times. The average recoveries for the fractions ranged from 98 to 104% (Table I).

Seven production lots of gentamicin sulfate were analyzed. The results (Table II) show a pattern of relative fractional composition which is rather consistent for this type of sample.

A differential microbiological assay of the gentamicin complex has been suggested which involves the use of a bioautographic procedure (7). Microgram and submicrogram amounts of antibiotic are spotted on narrow strips of paper for chromatographing. The chromatographed papers are placed on a seeded agar plate for the determination of antibacterial activity in which the sizes of the zones of inhibition produced by the sample are compared with those of the standard. A serious problem that may occur is the production of spurious zones caused by contamination, atmospheric and otherwise. This could be minimized by meticulous handling in a clean environment.

In the procedure described in the experimental section of this paper, any such possible interference is reduced more than 300-fold because microgram amounts of antibiotic are placed on the paper, and they are eluted and diluted even more to obtain concentrations suitable for microbiological assay. Another feature of this new procedure is that there is no need for reference standards of the fractions, since the dose-response curves for the three gentamicin components have the same slopes (4). Once the positions of the three fractions have been located on the chromatogram, the relative proportions are determined by microbiological assay using a single reference standard of gentamicin sulfate rather than three individual standards.

The solvent system of chloroform–methanol–17% ammonia (2:1:1) was used by Ikekawa *et al.* (8) for the separation of various antibiotics by TLC. Gentamicin was not included, as it was unknown at that time. Moreover, the upper layer of this solvent system was used, which is unsatisfactory for the separation of the gentamicin fractions. The lower layer must be used in the procedure described here.

The chromatography tank must be completely saturated for a satisfactory and rapid separation.

TABLE II—ANALYSIS OF PRODUCTION LOTS OF GENTAMICIN SULFATE

Lot No.	% C_1 ± SD	% C_{1a} ± SD	% C_2 ± SD
1	37.0 ± 1.73	27.0 ± 2.13	36.1 ± 0.96
2	34.0 ± 2.41	34.3 ± 4.20	31.7 ± 3.66
3	39.5 ± 2.26	25.2 ± 1.66	35.3 ± 1.80
4	40.7 ± 1.56	26.4 ± 1.19	32.9 ± 1.80
5	29.7 ± 1.40	32.8 ± 3.00	37.5 ± 2.36
6	38.3 ± 3.29	20.6 ± 1.89	41.1 ± 2.38
7	40.7 ± 2.14	21.0 ± 2.21	38.4 ± 2.81

The appearance of two phases on the paper is an indication that the tank is not completely saturated. It may be possible to obtain an acceptable chromatographic separation under this condition but the time of development would be increased so that the separation and elution could not be finished in one day.

Other papers such as Whatman No. 1 and S&S 589 Blue R were not as satisfactory; the time of development was longer and the separation of the fractions not as consistently good.

An attempt to assay the three fractions colorimetrically after a ninhydrin spray by determining the absorbances on a densitometer proved unsatisfactory. The intensities of the colors varied with time and with the fraction. Also, trace amounts of ammonia in the atmosphere caused the paper to gradually turn pink, thus interfering with the base line.

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Keyphrases

Gentamicin complex—separation, bioassay
Paper chromatography—separation, identification
Ninhydrin—color reagent
Microbiological analysis—gentamicin complex

Colorimetric Method for Determination of 7-Aminocephalosporanic Acid (7-ACA) and Related Compounds

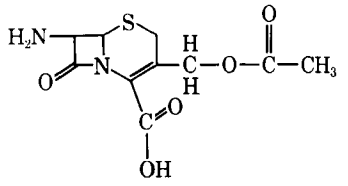
By L. P. MARRELLI

A reproducible colorimetric method is described for the determination of 7-aminocephalosporanic acid (7-ACA) and related compounds.

A NUMBER OF ACYL derivatives of 7-aminocephalosporanic acid (7-ACA) (I) and their antibacterial activities have been described (1–4). The D-phenylglycine derivative of 7-ACA, known generically as cephaloglycin[1] (II), has a broad spectrum of antibacterial activity. To determine the extent of acylation in the production of cephaloglycin, an assay was needed for small concentrations of 7-ACA. Methodology for the assay of 6-aminopenicillanic acid (6-APA) (III) in penicillin derivatives was investigated; however, the methods encountered had two basic drawbacks: (*a*) specificity (separation techniques utilized) and (*b*) sensitivity.

This paper describes a chemical method which permits the direct determination of small amounts of 7-ACA (0.2–1.5%) in cephaloglycin. At lower concentrations of 7-ACA (0.04%), a 25% error may be incurred. Optimum conditions were established for the rapid development of the 7-ACA-ninhydrin chromophore. Essentially no color formation was obtained with either cephaloglycin or phenylglycine under the same conditions.

7-Aminodesacetoxycephalosporanic acid (7-ADCA) (IV) and 6-aminopenicillanic acid (6-APA), both having an α-amino group adjacent to a β-lactam ring in common with 7-ACA, were found to respond in a similar manner. Likewise, 7-ADCA could be determined directly in small amounts (0.4–1.5%) in cephalexin[2] (V). At lower concentrations of 7-ADCA (0.1%), a 25% error may be incurred.



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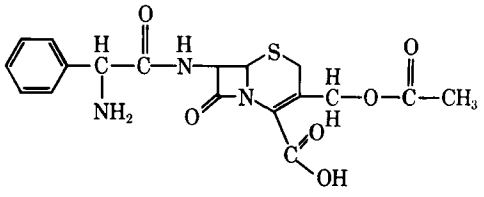

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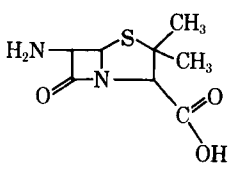
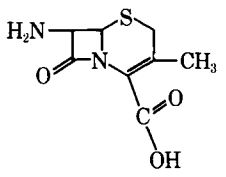
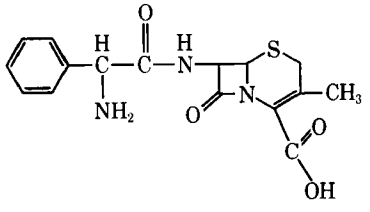


III



IV



V

EXPERIMENTAL

Apparatus and Reagents—A spectrophotometer (Beckman model DU) with 1-cm. glass cells was used in this laboratory, but any suitable spectrophotometer may be used. (*a*) Buffer solution—2.0% w/v citric acid ($H_3C_6H_5O_7 \cdot H_2O$) in 0.8 *M* sodium hydroxide; (*b*) formic acid—C.P., 98–100%; (*c*) ninhydrin solution—5.0% w/v ninhydrin (certified triketohydrindene hydrate, Fisher Scientific Co.) in methylcellosolve (peroxide-free).

Recommended Procedure—*Method I—Determination of 7-ACA*—Accurately weigh approximately 7 mg. (±0.01 mg.) of sample and 7-ACA reference standard, and quantitatively transfer each to a 100-ml. volumetric flask. Dissolve the contents in 5.0 ml. of formic acid, dilute to volume with distilled H_2O, and mix thoroughly. Prepare a reference blank solution in a 100-ml. volumetric flask by diluting 5.0 ml. of formic acid to volume with distilled H_2O and mixing well. Add by transfer pipet to a test tube in the following order: 2.0 ml. of each prepared solution, 1.0 ml. of buffer solution, and 0.50 ml. of ninhydrin solution. Mix thoroughly, and after a 9-min. waiting period, determine the absorbance of each solution at 407 mμ on a suitable spectrophotometer, using 1.0-cm. glass cells and the reference blank solution in the reference cell.

Calculations:

$$\frac{\text{abs.}_{407}\ \text{sample} \times \text{mg. standard}}{\text{abs.}_{407}\ \text{standard} \times \text{mg. sample}} \times 100 = \%\ \text{7-ACA}$$

Method II—7-ACA in Cephaloglycin—Accurately weigh approximately 100 mg. (±0.1 mg.) of cephaloglycin sample, and quantitatively transfer to a 10-ml. volumetric flask. Dissolve the contents in 0.50 ml. of formic acid, dilute to volume with distilled H_2O, and mix thoroughly. Prepare a reference standard stock solution by dissolving 70 mg. of 7-ACA reference standard in 50 ml. of formic acid

Received June 12, 1968, from the Analytical Development-Physical Department, Eli Lilly and Co., Indianapolis, IN 46206

Accepted for publication August 19, 1968.

The author wishes to express his indebtedness to Dr. E. M. Van Heyningen and Dr. L. D. Hatfield for providing the compounds under investigation, and also to Mr. T. E. Cole for the amino acid analyzer assays provided.

[1] Cephaloglycin is the generic name given to 7-(D-α-aminophenylacetamido) cephalosporanic acid.

[2] Cephalexin is the generic name given to 7-(D-α-aminophenylacetamido) desacetoxy cephalosporanic acid.

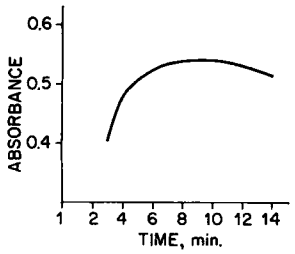


Fig. 1—Rate of color formation of 7-ACA chromophore.

and mixing well. Add 0.50 ml. of this stock solution to a 10-ml. volumetric flask, dilute to volume with distilled H_2O, and mix thoroughly. Prepare a reference blank solution in a 10-ml. volumetric flask by diluting 0.50 ml. of formic acid to volume with distilled H_2O and mixing well. Continue the assay as outlined in Method I, and calculate accordingly.

DISCUSSION

Standard Curve—A plot of the absorbance *versus* concentration was found to be linear and to pass through the origin, obeying Beer's law from 4–40 mcg./ml.

Stability of the Color—The color formation increases uniformly and rapidly, reaching a maximum in 9 min., then decreasing (Fig. 1). The yellow chromophore described exhibits a peak at 407 mμ; a secondary peak is also present at 570 mμ (Fig. 2).

Influence of Reagents—The color intensity was found to increase with a corresponding increase in pH, reaching a maximum at pH 4.5, then decreasing. An increase in pH not only influenced the rate of color development but also shifted the maximum absorbance peak to a higher wavelength (415 mμ). The intensity of the secondary peak (570 mμ) decreased with a pH increase. A pH 3 buffered system was selected, since cephaloglycin was found to be somewhat reactive at a higher pH. Both of the final concentrations of ninhydrin and methylcellosolve were found to influence the color formation. Optimum color formation was obtained when the final concentration of methylcellosolve (containing 50 mg./ml. of ninhydrin) was set at 14.3% v/v. Temperature fluctuations of ±3°, as well as light, did not affect the reaction rate. The effect of the 7-ACA standing in formic acid was found to be insignificant (0–3 min.).

Interferences—Several compounds were assayed by this procedure to determine the extent of color formation, as shown in Table I.

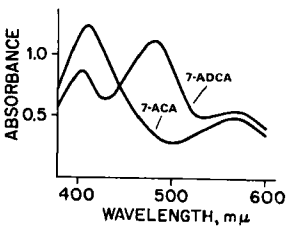


Fig. 2—Cary recording spectrophotometric curves of chromophores.

TABLE I—ABSORPTIVITY (a) AT 407 mμ

Compd.	a
7-ACA	15.3
7-ADCA	11.0
6-APA	5.4
Cephaloglycin	0.0018
Cephalexin	0.0035
Phenylglycine	0.33
NH_4Cl	0.16

Reproducibility—The following standard deviations were obtained on the methods outlined in Methods I and II of the *Procedure:* Method I—$2s = \pm 3.7\%$, $n = 6$; Method II—$2s = \pm 4.2\%$, $n = 6$.

A sample of cephaloglycin was assayed for 7-ACA by the colorimetric method, as well as by the amino acid analyzer. Agreement was obtained as follows: amino acid analyzer—1.35%; colorimetric method —1.42%.

7-ADCA and 6-APA—Essentially the same procedure was found to produce characteristic chromophores with 6-APA and 7-ADCA (Fig. 2); however, the rates of formation differed. In addition, the 7-ADCA color curve exhibited a third peak at 480 mμ which proved to be more suited to the direct determination of 7-ADCA in cephalexin. Maximum color development for the 7-ADCA chromophore was obtained in 18 min. at 407 mμ and in 50 min. at 480 mμ. The 6-APA chromophore reached maximum color development in 45 min. at 407 mμ.

The absorptivity for Compound 7-ADCA was 12.2 at 407 mμ and 11.5 at 480 mμ; for Compound cephalexin $a = 0.0065$ at 407 mμ and $a = 0.0035$ at 480 mμ; for Compound 6-APA $a = 9.55$ at 407 mμ.

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Keyphrases

7-Aminocephalosporanic acid (7-ACA), related compounds—analysis
Cephaloglycin—7-ACA determination
Ninhydrin—color reagent
Colorimetric analysis—spectrophotometer

Antiviral Activity of Selected *Catharanthus* Alkaloids

By N. R. FARNSWORTH, G. H. SVOBODA,* and R. N. BLOMSTER

Thirty-six alkaloids isolated either from *Catharanthus roseus* or *C. lanceus* were evaluated for *in vitro* activity against vaccinia and polio type III viruses. Nine of these alkaloids were effective as antiviral agents, with pericalline being the most effective.

In recent years, investigators have directed their attention toward the plant kingdom as a source of antiviral agents, and a number of plant extracts have been found to inhibit a variety of viruses in tissue culture as well as through *in vivo* experiments (1–11).

Although the authors were primarily interested in *Catharanthus* species as a source of antitumor alkaloids, the presence of compounds with other types of biological activity was not precluded. Thus, all isolates which were available in sufficient quantity were evaluated for *in vitro* antiviral activity against the vaccinia and polio type III viruses.

Prior to this investigation, Johnson had reported briefly on the *in vivo* antiviral activity of leurocristine against the mengo virus (12). To illustrate the specificity of this antiviral agent, it was shown that vincaleukoblastine was completely devoid of activity against the mengo virus *in vivo*, and these two alkaloids differ only slightly in their structures (leurocristine = N—CHO, vincaleukoblastine = N—CH_3).

A search of the literature has revealed that this is the first report to appear on the *in vitro* evaluation of alkaloids as antiviral agents.

EXPERIMENTAL

Materials—The following alkaloids, isolated either from *C. roseus* or *C. lanceus*, were utilized as test compounds in the study: pericalline (hydrochloride) (13, 14), perivine (hydrochloride) (15, 16), periformyline (17), leurosivine (sulfate) (14), leurocristine (sulfate) (18), vincaleukoblastine (sulfate) (16), perividine (19), vindolinine (dihydrochloride) (16, 20), carosine (21), leurosidine (sulfate) (18), desacetyl VLB (sulfate) (22), vindoline (hydrochloride) (15, 23), ajmalicine (13, 16), leurosine (sulfate) (15, 16), neoleurocristine (21), vincarodine (21), vinosidine (14, 23, 24), virosine (16), ammorosine (14), ammocalline (14, 25), catharine (20, 26, 27), carosidine (21), cavincine (sulfate) (14), lochrovicine (28), lochrovidine (28), lochrovine (28), lochnerine (acetate) (16), mitraphylline (14), neoleurosidine (21), perimivine (13, 28), sitsirikine (sulfate) (29), tetrahydroalstonine (16, 17), vinaphamine (22), vincolidine (28), and vincoline (25, 28).

Solutions of each alkaloid were prepared in a suitable solvent (benzene for the free bases and ethanol for the salts), and measured amounts were transferred to suitable filter paper disks, and the solvent was allowed to evaporate spontaneously. Disks were each impregnated with 1000, 500, 250, or 125-mcg. amounts of the separate alkaloids.

Test Procedure—The agar diffusion method based on reports by Herrmann (29) and Siminoff (30) was used. Green monkey kidney cells (BSC_1) were maintained in medium 199, containing 5% calf serum and penicillin (50,000 units/ml.). Vaccinia VI Lindeman and Type III poliomyelitis viruses were separately maintained in the above medium, and when used in the test had a titer at between log 10^5 and 10^7 viral particles/ml.

Interpretation of the Test—Activity (A) values represent the size (mm.) of the zone where protection of the cells from virus damage was observed. Morphology (M) values represent the degree of protection as shown in Table I.

RESULTS AND DISCUSSION

Of 36 alkaloids evaluated, nine showed a degree of antiviral activity which varied from moderate to marked (pericalline, perivine, periformyline, leurosivine, leurocristine, vincaleukoblastine, perividine, vindolinine, carosine) (see Table II). Leurosidine (sulfate), desacetyl VLB(sulfate), and vincarodine exhibited minimal activity. The following alkaloids were inactive at the concentrations tested: vindoline (HCl), ajmalicine, virosine, leurosine (sulfate), neoleurocristine, vinosidine, ammorosine, ammocalline, catharine, carosidine, cavincine, lochrovicine, lochrovidine, lochrovine, lochnerinine, mitraphylline, neoleurosidine, perimivine, sitsirikine, tetrahydroalstonine, vinaphamine, vincathicine, vincolidine, and vincoline.

In all instances, with the exception of perivine, activity was specific for either a DNA virus (vaccinia), or a RNA virus (polio III). It is difficult at this time to draw any conclusions regarding the structure-activity relationship of these alkaloids, since 17 of the 36 evaluated are of unknown structure. Monomeric as well as dimeric alkaloids were active.

Pericalline, the most active of the test compounds, is an unusual indole alkaloid in that it contains no

Table I—Interpretation of Morphology (M) Values

M Value	Description
4+	Dark staining areas that show healthy cells with no visual virus or drug damage upon microscopic examination.
3+	Stained area, not dark, upon microscopic examination show no virus damage but do not appear to be very healthy.
2+	Healthy cells with moderate amount of virus breakthrough.
1+	Healthy cells with an increased virus breakthrough.
—	No viable cells.

Received July 11, 1968, from the Department of Pharmacognosy, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15213, and the Eli Lilly and Co.,* Indianapolis, IN 46206

Accepted for publication September 6, 1968.

This investigation was supported, in part, by research grants CA-08228 and FR-05455 from the National Institutes of Health, United States Public Health Service, Bethesda, Md., and from the Jane Olmstead Thaw Fund, University of Pittsburgh. The antiviral screening reported in this paper was conduced at the Eli Lilly and Co.

Previous paper: Tin-Wa, M., Fong, H. H. S., Blomster, R. N., and Farnsworth, N. R., *J. Pharm. Sci.*, in press.

TABLE II—ANTIVIRAL ACTIVITY OF *Catharanthus* ALKALOIDS

Alkaloid	Form	Concn., mcg./ml.	Vaccinia A[a]	Vaccinia M	Polio III A	Polio III M
Pericalline	Hydrochloride	1000	0	0	25	4+
		500	0	0	25	3+
		250	0	0	20	3+
		125	0	0	0	0
Perivine	Hydrochloride	1000	14	2+	23	4+
		500	10	0	15	2+
		250	0	0	0	0
Periformyline	Base	1000	0	0	30	2+
		500	0	0	30	2+
		250	0	0	25	2+
		125	0	0	20	2+
Leurosivine	Sulfate	1000	30	2+	0	0
		500	23	2+	0	0
		250	22	2+	0	0
		125	20	2+	0	0
Leurocristine	Sulfate	1000	>50	2+	0	0
		500	40	2+	0	0
		250	35	2+	0	0
		125	28	2+	0	0
Vincaleukoblastine	Sulfate	1000	33	2+	0	0
		500	30	2+	0	0
		250	27	1+	0	0
		125	24	1+	0	0
Perividine	Base	1000	0	0	22	2+
		500	0	0	0	0
Vindolinine	Dihydrochloride	1000	0	0	20	2+
		500	0	0	20	2+
		250	0	0	15	2+
		125	0	0	0	0
Carosine	Base	1000	0	0	30	2+
		500	0	0	25	2+
		250	0	0	15	2+
		125	0	0	10	2+
Leurosidine	Sulfate	1000	20	1+	0	0
		500	15	1+	0	0
		250	0	0	0	0
Desacetyl VLB	Sulfate	1000	15	1+	0	0
		500	9	1+	0	0
		250	0	0	0	0
Vincarodine	Base	1000	0	0	20	1+
		500	0	0	0	0

[a] A, activity; M, morphology; 0, no activity. See the text and Table I for explanations.

oxygen in the molecule. On the other hand, perivine, periformyline, and perividine are the only representatives of monomeric 2-acylindole bases tested, and all three were active. Leurosivine, leurocristine, vincaleukoblastine, leurosidine, and leurosine, all dimeric indole alkaloids, are active tumor inhibitors, and with the exception of leurosine, they all showed antiviral activity.

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Keyphrases

Catharanthus alkaloids

Antiviral activity—*Catharanthus* alkaloids

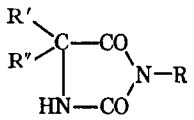
Paper disk method—antiviral testing

New Compounds: Derivatives of Some Imido Compounds Likely to Possess Therapeutic Activity

By S. EL-ZANFALLY, M. KHALIFA, and Y. M. ABOU-ZEID

The synthesis of certain *N*-alkylated derivatives of hydantoin, barbituric acid, succinimide, and glutarimide is described. The alkylation was achieved either by direct fusion of the imide with the halo compound in the presence of anhydrous potassium carbonate or by heating the sodio derivative of the imide with the halo compound in the presence of dimethylformamide as solvent. In the case of barbiturates, a dialkylated product was always obtained even when equimolecular amounts of reactants were used.

ALKYLATION of the imido nitrogen in hydantoins, barbiturates, succinimides, and glutarimide has recently become the subject of several patents. The vast majority of these *N*-substituted derivatives have been prepared as potent sedatives, analgesics, and hypnotics (1, 2). Motivated, in part, by the desire of producing other such new derivatives and in the light of what has been reported on the analgesic properties of *N*-substituted saccharin (3) and phthalimide (4), the authors decided to synthesize derivatives of hydantoins, barbiturates, succinimides, and glutarimide with the *N*-alkyl grouping containing the saccharino or phthalimido moieties.[1]



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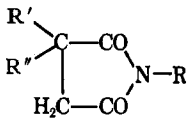
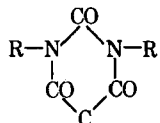


II

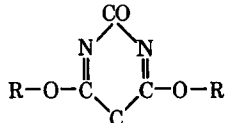
Derivatives having the above general formulas were synthesized by condensing the imido compound with β-substituted ethyl bromide. β-Saccharino ethyl bromide and ω-bromophenacetin were prepared according to reported procedures (5, 6). On the other hand β-phthalimido ethyl bromide was prepared from potassium phthalimide and ethylene bromide applying Gabriel's procedure after being modified by the use of dimethylformamide as solvent. As a result the dark colored reaction tars—frequently accompanying the Gabriel reaction—were completely excluded and a product of high purity was obtained. 5,5-Disubstituted hydantoins were synthesized from the appropriate ketones by decomposing the bisulfite addition products with potassium cyanide and heating the cyanohydrins, thus obtained, with ammonium carbonate (7). Glutarimide was obtained by fusing a mixture of glutaric anhydride and ammonium carbonate for 30 min. only instead of passing gaseous ammonia in the molten anhydride (8) or heating glutaric acid with concentrated ammonium hydroxide solution for 7 hr. (9). This modification, besides improving the yield, resulted in a considerable reduction in the reaction time.

Condensation of the imides with the halides was realized *via* two different procedures: (*a*) direct fusion of the starting materials in the presence of anhydrous potassium carbonate, and (*b*) heating the sodio derivative of the imide with the halo compound in the presence of dimethylformamide. Procedure *b*, which is essentially that described for the *N*-alkylation of saccharin, proved to be superior to *a* since through its application many of the compounds not obtainable by Procedure *a* were successfully prepared.

When sodium barbital was condensed with β-saccharino ethyl bromide according to Procedure *b*, two molecules of the latter condensed with one molecule of the former although equimolecular quantities of the reactants were used. For such a condensation product two structures, III*a* and III*b*,



III*a*



III*b*

are theoretically possible. A choice between the two structures was made possible by infrared analysis[2] because the observed frequencies of about 1776 and 1675 cm.$^{-1}$ may be ascribed to the 4- and 2-position carbonyls, respectively, of Structure III*a* (10, p. 221). On the other hand, the strong sharp band at 768 cm.$^{-1}$ may be assigned to the out-of-plane deformation vibration of the four adjacent ring hydrogen atoms (10, p. 77). Furthermore, the strong sharp bands at 1193 cm.$^{-1}$ and 1211 cm.$^{-1}$ may be attributed to the structure $Et_2C<$ by analogy with the structure $Me_2C<$ which absorbs at 1195 and 1210 cm.$^{-1}$, respectively (10, p. 26). Finally, it has been reported (11) that although the alkali salts of 5,5-dialkyl barbituric acids are known to exist in the enolic form, alkyl products prepared from them are known to occur only in the keto form; a fact which supports Structure III*a*.

EXPERIMENTAL

***N*-(β-Bromoethyl) Phthalimide**—This compound

Received January 30, 1968, from the Faculty of Pharmacy, Cario University, Cario, U.A.R.

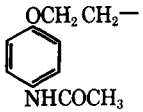
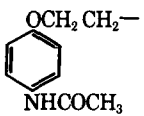
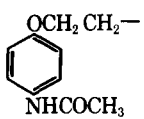
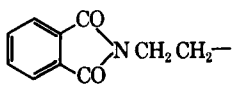
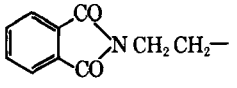
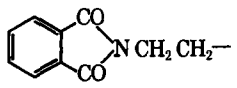
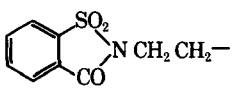
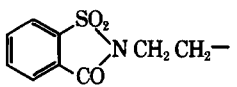
Accepted for publication May 2, 1968.

The authors express their appreciation to J. R. Geigy, S.A., Basle, Switzerland, and to Bayer, Leverkusen, Germany, for a donation of 1 kg. of dimethylformamide from each.

[1] The products at present are under preliminary screening for possible sedative and hypnotic action or any other possible pharmacological activity.

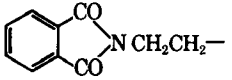
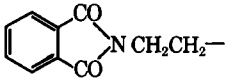
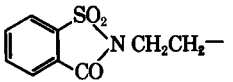
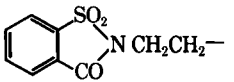
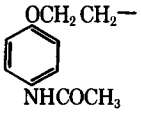
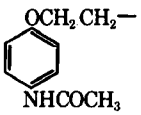
[2] The infrared spectra were determined on a 300-mg. KBr disk containing 1 mg. of sample. The spectra were run on a Perkin-Elmer model 21.

TABLE I—METHODS OF PREPARATION, SOLVENTS OF CRYSTALLIZATION, YIELDS, MELTING POINTS, AND ANALYSES FOR SOME *N*-SUBSTITUTED IMIDO COMPOUNDS

Chemical Name	R	R′	R″	Method of Preparation	Yield, %	Solvent of Crystallization[a]	M.p., °C.[b]	Formula	Anal.,[c] % Calcd.	Found
				***N*-Substituted Hydantoins (I)**						
3-[β-(*p*-Acetamidophenoxy) ethyl]-5,5-dimethyl-hydantoin		CH_3	CH_3	*b*	66	B	189–191	$C_{15}H_{19}N_3O_4$	N 13.77	N 13.27
3-[β-(*p*-Acetamidophenoxy) ethyl]-5,5-diphenyl-hydantoin		C_6H_5	C_6H_5	*b*	95	A	208–210	$C_{25}H_{23}N_3O_4$	C 69.92 H 5.36 N 9.49	C 70.49 H 5.71 N 9.50
3-[β-(*p*-Acetamidophenoxy) ethyl]-5-ethyl-5-methyl-hydantoin		C_2H_5	CH_3	*a*	70	C	174–175	$C_{16}H_{21}N_3O_4$	C 60.18 H 6.58 N 13.16	C 59.82 H 6.75 N 13.19
3-(β-Phthalimidoethyl)-5,5-dimethylhydantoin		CH_3	CH_3	*a*	42	D	189–190	$C_{15}H_{15}N_3O_4$	C 59.80 H 4.98 N 13.95	C 59.71 H 4.92 N 13.72
3-(β-Phthalimidoethyl)-5,5-diphenylhydantoin		C_6H_5	C_6H_5	*b*	70	A	236–237	$C_{25}H_{19}N_3O_4$	N 9.88	N 9.78
3-(β-Phthalimidoethyl)-5-ethyl-5-methylhydantoin		C_2H_5	CH_3	*a*	42	B	163–165	$C_{16}H_{17}N_3O_4$	C 60.95 H 5.39 N 13.33	C 60.63 H 5.94 N 13.27
3-(β-Saccharinoethyl)-5,5-dimethylhydantoin		CH_3	CH_3	*b*	43	A	200–201	$C_{14}H_{16}N_3O_5S$	C 49.85 H 4.45 N 12.46	C 49.89 H 5.00 N 12.15
3-(β-Saccharinoethyl)-5,5-diphenylhydantoin		C_6H_5	C_6H_5	*b*	90	A	238–240	$C_{24}H_{19}N_3O_5S$	N 9.11	N 8.82

(*Continued on next page.*)

TABLE I (*Continued.*)

Chemical Name	R	R′	R″	Method of Preparation	Yield, %	Solvent of Crystallization[a]	M.p., °C.[b]	Formula	Anal.,[c] % Calcd.	Found
				***N*-Substituted Succinimides (II)**						
N-(β-Phthalimidoethyl)-α-phenylsuccinimide		C_6H_5	H	*b*	38	D	143–145	$C_{20}H_{16}N_2O_4$	C 68.96 H 4.60 N 8.04	C 69.25 H 4.54 N 7.86
N-(β-Phthalimidoethyl)-succinimide		H	H	*a*	50	D	162–163	$C_{14}H_{12}N_2O_4$	C H N 10.29	C H N 10.14
N-(β-Saccharinoethyl)-α-phenylsuccinimide		C_6H_5	H	*b*	33	A	173–174	$C_{19}H_{16}N_2O_5S$	C 59.37 H 4.17 N 7.29	C 59.62 H 4.86 N 7.11
N-(β-Saccharinoethyl)-succinimide		H	H	*b* & *a*	40 60	D	219–220	$C_{13}H_{12}N_2O_5S$	C 50.65 H 3.89 N 9.09	C 50.76 H 3.95 N 9.43
N-(β-Saccharinoethyl)glutarimide				*a*	25	A	200–201	$C_{14}H_{14}N_2O_5S$	C H N 8.69 S 9.94	C H N 9.23 S 9.58
α-(*p*-Acetamidophenoxy)-β-saccharinoethane				*b*	43	A	163–164	$C_{17}H_{16}N_2O_5$	C 56.66 H 4.44 N 7.77	C 56.62 H 5.23 N 7.22
ω-Phthalimidoethyl salicylate				*b*	81	A	139–141	$C_{17}H_{13}NO_5$	C 65.59 H 4.18 N 4.50	C 65.34 H 4.84 N 4.58
ω-Saccharinoethyl salicylate				*b*	82	A	138–140	$C_{16}H_{13}NO_6S$	C 55.33 H 3.74 N 4.03	C 54.93 H 4.15 N 4.04
				***N*-Substituted Barbiturates (IIIa)**						
1,3-Bis[β-(*p*-Acetamidophenoxy)ethyl]-5,5-diethylbarbituric acid		C_2H_5	C_2H_5	*b*	50	B	165–167	$C_{28}H_{34}N_4O_7$	C 62.45 H 6.32 N 10.41	C 62.79 H 6.93 N 10.48
1,3-Bis[β-(*p*-Acetamidophenoxy)ethyl]-5-ethyl-5-phenylbarbituric acid		C_2H_5	C_6H_5	*b*	55	A	219–220	$C_{32}H_{34}N_4O_7$	C 65.53 H 5.79 N 9.56	C 65.02 H 6.17 N 9.56

(*Continued on next page.*)

TABLE I (*Continued.*)

Chemical Name	R	R′	R″	Method of Preparation	Yield, %	Solvent of Crystallization[b]	M.p., °C.[b]	Formula	Anal.,[c] % Calcd.	Found
1,3-Bis(β-Phthalimidoethyl)-5,5-diethylbarbituric acid		C_2H_5	C_2H_5	*b*	41	A	184–185	$C_{28}H_{28}N_4O_7$	C 63.39 H 4.90 N 10.56	C 62.89 H 4.99 N 10.54
1,3-Bis(β-Saccharinoethyl)-5,5-diethylbarbituric acid		C_2H_5	C_2H_5	*b*	65	B	187–188	$C_{28}H_{28}N_4O_8S_2$	C 51.82 H 4.32 N 9.30 S 10.60	C 51.89 H 4.39 N 9.72 S 9.61
1,3-Bis(β-Saccharinoethyl)-5-ethyl-5-phenylbarbituric acid		C_2H_5	C_6H_5	*b*	47	A	199	$C_{30}H_{28}N_4O_8S_2$	C 55.38 H 4.00 N 8.61 S 9.84	C 55.67 H 4.16 N 9.80 S 9.70

[a] A, ethyl alcohol; B, aqueous ethyl alcohol; C, water; D, dilute acetic acid. [b] All melting points are uncorrected. [c] The microanalyses were performed by Dr. C. Janssen, Beerse, Belgium, and by El-Nasr Company, U.A.R.

was prepared in 70% yield from potassium phthalimide and ethylene bromide applying Gabriel's procedure with the modification of using dimethylformamide as solvent. The heating and occasional shaking was continued for 2 hr. at 130°. The product crystallized from aqueous ethanol melted at 80–81° as reported (12).

Glutarimide—This compound was obtained in 75% yield by fusing glutaric anhydride with ammonium carbonate for 0.5 hr. Crystallization from acetone yielded a product melting at 150–151°. Reported yield and melting point are 63% and 145–146°, respectively (9).

Procedures for Final Compounds—The final compounds were obtained *via* two different procedures according to the following general directions.

Direct Fusion Method—The imide (1 mole), the halo compound (1 mole), and anhydrous potassium carbonate (0.5 mole) were mixed and heated gently to a state of quiet fusion in a small flask fitted with an air condenser. The heating and occasional shaking were continued for 2 hr. After being cooled, the hard brown glossy mass was dissolved in boiling diluted acetic acid and the product which separated on cooling was filtered and crystallized from the appropriate solvent (see Table I).

Dimethylformamide Method—A mixture of equimolecular amounts of the sodio derivative of the imide and the halo compound was suspended in dimethylformamide (about 5 ml. solvent per 1 g. of mixture) and heated under reflux at 120–130° for 2 hr. with occasional shaking. The product which separated on pouring the cold reaction mixture onto crushed ice was filtered, dried, and crystallized from the appropriate solvent (see Table I).

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Keyphrases

Alkylated imido derivatives—synthesis
Condensation reaction alkylation, imido derivatives
IR spectrophotometry—structure

Determination of Impurities in Phenacetin by Thin-Layer Chromatography II

Sir:

We reported earlier (1) that TLC can be applied to detect certain impurities in phenacetin. Additional experimentation in this laboratory prompted us to introduce a modification which, in addition to enhancing the extraction of the impurities, extends the upper limit of detection. The modified procedure is as follows:

(*a*) Control solution—Dissolve 10.0 mg. each of acetanilid, *p*-chloroacetanilid, and *p*-phenetidin in 100.0 ml. of absolute ether.

(*b*) Test and control preparations—Place 1.0 g. of finely powdered phenacetin into an 18- × 150-mm. test tube, add 3.0 ml. of absolute ether, and mark the volume. To a similar test tube, add 1.0 g. of finely powdered phenacetin reference standard and 3.0 ml. of the control solution, and mark the volume. To both test tubes, add 15 ml. of the ether and, by gently boiling on a steam bath, evaporate to the mark. Cool, adjust the volume to the mark with the ether, add 2.0 ml. of *n*-hexane, mix, and allow the particles to settle.

(*c*) Procedure—Perform TLC concurrently with the control preparation and the test preparation. Use a plate with a 0.25-mm. layer of Silica Gel GF,[1] a freshly prepared developing solvent consisting of 99 volumes of absolute ether and 1 volume of *n*-hexane, and a chromatographic chamber not lined with filter paper. Spot 50 µl. each of the supernatant obtained in the test preparation and in the control preparation, applying a continuous current of warm air in order to concentrate the spots and to completely evaporate the solvent. Allow the developing solvent to ascend at least 15 cm. above the spot points, remove the plate, allow it to air dry, and expose it to iodine vapor for 20 sec. Observe the chromatogram under short-wave UV light. (The spots from the control preparation appear in the following order above the principal spot of phenacetin: *p*-chloroacetanilid, acetanilid, and *p*-phenetidin.)

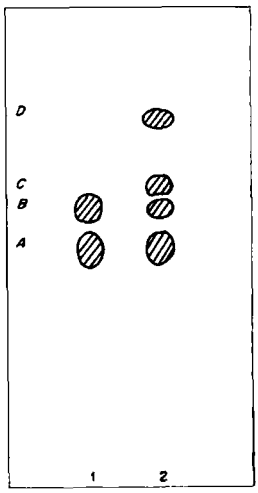

Fig. 1—TLC of: A, *phenacetin;* B, p-*chloroacetanilid;* C, *acetanilid;* D, p-*phenetidin. Key: 1* B, *30.0 mcg. (0.3%); 2* B, C, *and* D, *15.0 mcg. (0.15%) each.*

As a corroborative estimation of the *p*-chloroacetanilid, the plate should be exposed to short-wave UV radiation for about 5 min., and then inspected under a long-wave UV lamp: *p*-chloroacetanilid exhibits a bright blue fluorescence.

The significant differences between the original procedure and the modified procedure are as follows. (*a*) Original Procedure—Impurities are extracted by agitating 1 g. of phenacetin with 5.0 ml. of absolute ether for 5 min.; solvent in the final mixture is 5.0 ml. of ether; development is 10 to 15 cm. (*b*) Modified Procedure—Impurities are extracted by boiling 1 g. of phenacetin with an initial volume of 18 ml. of ether and continuing the warm extraction until the volume is reduced to 3.0 ml.; solvent in the final mixture is 3.0 ml. of absolute ether and 2.0 ml. of *n*-hexane; development is at least 15 cm.

The modifications were adapted basically for the following reasons.

1. The extraction technique provides for a more efficient dissolution of the impurities due to the initially larger volume of the solvent and to the applied heat.

2. Absolute ether–*n*-hexane (3:2) dissolves about 2.7 times less phenacetin than does absolute ether. Consequently, the area of the phenacetin spot becomes much smaller when the mixed solvent is applied. This reduces the pos-

[1] Among the adsorbents tested, Silica Gel GF_{254} (from E. Merck AG., Darmstadt, Germany) allowed for a relatively rapid development.

sible overlapping of the phenacetin and *p*-chloroacetanilid spots, and extends the upper limit of detection and estimation of the latter. A typical chromatogram is shown in Fig. 1.

3. By allowing the solvent front to ascend at least 15 cm. from the starting line, resolution of the spots is increased.

Summarizing our results, it was found that the proposed modified procedure provides an improved technique for identifying and estimating *p*-chloroacetanilid, acetanilid, and *p*-phenetidin in phenacetin with the aid of TLC.

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Staff review

NOTICES

Solvent Properties of Surfactant Solutions. Vol. 2 of Surfactant Science Series. Edited by Kozo SHINODA. Marcel Dekker, Inc., 95 Madison Ave., New York, NY 10016, 1967. ix + 365 pp. 15.5 × 23.5 cm. Price $18.50.

Directory of Government Agencies Safeguarding Consumer and Environment. 1st Annual Edition 1968. Serina Press, 70 Kennedy St., Alexandria, VA 22305. 140 pp. 15.5 × 23 cm. Price $4.95.

Les Methodes Statistiques en Pharmacie et en Chimie (Applications a la recherche, a la production et au controle). By JEAN PHILIPPE. Mason & Cie, 120 Boulevard Saint-Germain, Paris, France, 1967. x + 378 pp. 16.5 × 24 cm. Price 80F paperbound.

Pharmazeutisches Taschenbuch. Edited by HANS KAISER. Wissenschaftliche Verlagsgesellschaft MBH, Stuttgart, Germany, 1968. xii + 1126 pp. 13 × 17.5 cm. Price DM 75.

Bibliography of Vitamin E. 1965–1967. Compiled by WILMA F. KUJAWSKI. Distillation Products Industries, Rochester, NY 14603. 21.5 × 28 cm. approx. 140 pp. Single copies free to research workers in the field. Price $3.00.

Precis d'Anatomie et de Physiologie Humaines. Vol. II, 3rd ed. By Y. RAOUL, M. PIETTE, and R. MICHEL. Masson & Cie, 120 Boulevard Saint-Germain, Paris, France, 1968. vi + 316 pp. 16.5 × 21.5 cm. Price 60F.

Actualites Pharmacologiques. Edited by RENE HAZARD. Masson & Cie, 120 Boulevard Saint-Germain, Paris, France, 1967. 224 pp. 16 × 24 cm. Price 72F.

Einfuhrung in die Arzneibereitung. By FRITZ GSTIRNER. Wissenschaftliche Verlagsgesellschaft MBH, Stuttgart, Germany, 1968. xvi + 488 pp. 17.5 × 24.5 cm. Price DM 78,--.

Prostaglandins. Nobel Symposium 2. Edited by S. BERGSTROM and B. SAMUELSSON. Interscience Publishers, Inc., 605 Third Ave., New York, NY 10016, 1967. 299 pp. 16 × 24 cm. Price $18.50.

The Balkan Nephropathy. Ciba Foundation Study Group No. 30. Edited by G. E. W. WOLSTENHOLME and J. KNIGHT. Little, Brown and Co., 34 Beacon St., Boston, MA 02106, 1967. 123 pp. 12.5 × 19 cm. Price $3.50.

Cell Differentiation. Ciba Foundation Symposium. Edited by A. V. S. DE REUCK and J. KNIGHT. Little, Brown and Co., 34 Beacon St., Boston, MA 02106, 1967. 257 pp. 15.5 × 23.5 cm. Price $12.00.

Immunochemistry and the Biosynthesis of Antibodies. By F. HAUROWITZ. John Wiley & Sons, Inc., 605 Third Ave., New York, NY 10016, 1968. xvii + 301 pp. 15 × 23 cm. Price $10.95.

Nutrition and Infection. Ciba Foundation Study Group No. 31. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. Little, Brown and Co., 34 Beacon Street, Boston, MA 02106, 1968. 13 × 19 cm. vii + 144 pp. Price $3.50.

Pharmaceutical Chemistry. Part 1: Principles. By M. L. SCHROFF. National Book Centre, P-76, Dr. Sundari Mohan Ave., Calcutta-14, India, 1968. xviii + 628 pp. 15 × 22 cm. Price Rs 18/-.

Pyridoxal Catalysis: Enzymes and Model Systems. Edited by E. E. SNELL, A. E. BRAUNSTEIN, E. S. SEVERIN, and Y. M. TORCHINSKY. John Wiley & Sons, Inc., 605 Third Avenue, New York, NY 10016, 1968. xii + 770 pp. 16.5 × 23.5 cm. Price $18.95.

The Stereochemistry of Macromolecules. Vol. 3. Edited by A. D. KETLEY. Marcel Dekker, Inc., 95 Madison Avenue, New York, NY 10116, 1968. 16 × 24 cm. vii + 460 pp. Price $21.75.

Monoamine Oxidase Inhibitors: Relationship Between Pharmacological and Clinical Effects. Edited by J. CHEYMOL and J. R. BOISSIER. Pergamon Press Inc., 44-01 21st Street, Long Island City, New York, NY 11101, 1968. vii + 94 pp. 16 × 23.5 cm. Price $9.00.

Treatise on Analytical Chemistry. Part I: Theory and Practice. Vol. 8. Edited by I. M. KOLTHOFF and PHILIP J. ELVING. John Wiley & Sons, Inc., 605 Third Avenue, New York, NY 10016, 1968. xviii + 500 pp. (approx.) 17 × 24.5 cm. Price $19.00.

Systemic Mycoses. Ciba Foundation Symposium. Edited by G. E. W. WOLSTENHOLME and RUTH PORTER. Little Brown & Co., 34 Beacon St., Boston, MA 02106, 1968. 287 pp. 16 × 23.7 cm. Price $12.00.

Pharmaceutical Abstracts. Vol. IX, Nos. 1 and 2. Edited by HENRY M. BURLAGE. The University of Texas, College of Pharmacy, Austin, TX 78712, 1968. 21 × 28 cm. Price $4.30 each issue. Paperbound.

Index Nominum 1966, Supplementum 1967. Swiss Pharmaceutical Society, Zurich, Switzerland, 1968. 19 × 25 cm. 80 pp.

Indolalkaloide in Tabellen. By MANFRED HESSE. Springer-Verlag, Heidelberger Platz 3, Berlin, Germany, 1968. 249 pp. 21 × 29.7 cm. Price $17.50.

Growth of the Nervous System. A Ciba Foundation Symposium. Edited by G. E. W. WOLSTENHOLME and RUTH PORTER. Little, Brown and Co., 34 Beacon Street, Boston, MA 02106, 1968. xi + 295 pp. 16 × 23.7 cm. Price $12.00.

The Problems and Prospects of LSD. Edited by J. THOMAS UNGERLEIDER. Charles C. Thomas, 301-327 E. Lawrence Ave., Springfield, Illinois, 1968. xv + 109 pp. 16 × 23.5 cm. Price $5.00.

Molecular Basis of Virology. ACS Monograph 164. Edited by H. FRAENKEL-CONRAT. Reinhold Book Corp., 430 Park Avenue, New York, NY 10022, 1968. 16 × 23.5 cm. xiii + 642 pp. Price $18.00.

Fatty Acids and Their Industrial Applications. Edited by E. SCOTT PATTISON. Marcel Dekker, Inc., 95 Madison Avenue, New York, NY 10016, 1968. 16 × 23.5 cm. xii + 390 pp. Price $8.00.

Development of Safer and More Effective Drugs. Edited by SAMUEL W. GOLDSTEIN. American Pharmaceutical Association, 2215 Constitution Ave., N.W., Washington, DC 20037, 1968. 17 × 24 cm. x + 186 pp. Price $5.00 Paperbound and $6.00 Hardcover.

Principles of Physical Chemistry for Biology and Pharmacy. By L. SAUNDERS. Oxford University Press, 200 Madison Ave., New York, NY 10016, 1966. ix + 438 pp. 14.5 × 23 cm. Price $10.00.

Drug Responses in Man. Ciba Foundation Symposium. Edited by G. E. W. WOLSTENHOLME and R. PORTER. Little, Brown and Co., 34 Beacon St., Boston, MA 02106, 1967. 257 pp. 15.5 × 23.5 cm. Price $13.00.

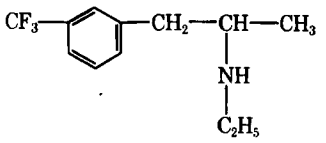
CITING CHEMICAL ABSTRACTS

In 1967, *Chemical Abstracts* began a new system which assigns a number to each abstract and a number to each page, instead of assigning a number to each column and a letter to each subdivision of the column as had been done in the past. Some confusion has arisen about the proper manner of citing a reference to an abstract which appears in a volume carrying the new numbering system. In the interest of clarifying the situation for our authors and readers, and of providing the greatest possible reference value, the *Journal of Pharmaceutical Sciences* has adopted a policy which permits the use of volume number, column number, column subdivision letter, and year for all *Chem. Abstr.* volumes through 1966, but beginning with the 1967 volume of *Chem. Abstr.*, the volume number, abstract number, and year should be cited. This revision in Journal policy will affect references to *Chemical Abstracts* only, and is supplemental to the information concerning references in the "Notice to Authors."

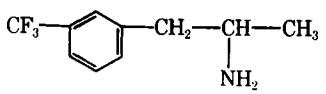
Errata

In the article titled "Fenfluramine Metabolism" (1), the following corrections should be made:

The structures of fenfluramine and its deethylated derivatives on page 1173 should be as follows:



Fenfluramine (7%)



Deethylated Fenfluramine (3%)

(1) Bruce, R. B., and Maynard, W. R., Jr., *J. Pharm. Sci.*, **57**, 1173(1968).

In the article titled "Studies on the Mechanism of the Mannich Reaction" (1), the following correction should be made:

On page 1098, column 2, structure XI should be IX.

(1) Nobles, W. L., and Potti, N. D., *J. Pharm. Sci.*, **57**, 1097(1968).

In the article titled "Crystal and Molecular Structure of 5-[1-(2′-Deoxy-α-D-ribofuranosyl)uracilyl] Disulfide" (1), the following corrections should be made:

In Tables I and III, pages 1294 and 1295, respectively, the heading y/b should be $-y/b$. The illustrations of the molecule and Table VI also reflect this error, in that they show the mirror image of the correct structure.

(1) Shefter, E., Kotick, M. P., and Bardos, T. J., *J. Pharm. Sci.*, **56**, 1293(1967).

In the article titled "Peyote Alkaloids II. Anhalotine, Lophotine, and Peyotine, the Quaternary Alkaloids of *Lophophora williamsii*" (1), the following corrections should be made:

1. On page 256, column 2, 5.95 (O—CH_2—O— - - -7,8) should read 5.95, 5.88 (O—CH_2—O— - - - 7,8).

2. On page 257, column 1 under *Anal.*, N, 5.5 should read N, 5.15.

3. On page 257, column 1 after "Specific rotation," $[\alpha]^{26}_{589} = -62°$; $[\alpha]^{26}_{436} = -111°$; $[\alpha]^{26}_{350} = -187°$ (c = 0.78, $CHCl_3$) should read $[\alpha]^{25}_{589} = -45°$; $[\alpha]^{25}_{436} = -83.7°$ (c = 0.82, $CHCl_3$).

(1) Kapadia, G. J., Shah, N. J., and Zalucky, T. B., *J. Pharm. Sci.*, **57**, 254(1968).

In the article titled "Stability Assay for Prazepam and Related Drugs" (1), the following correction should be made:

On page 1681, column 2, line 20, the sentence should read: "Methanol and a mixture of 3 vol. of concentrated sulfuric acid diluted to 100 vol. with absolute methanol (acid-methanol reagent) were the spectrophotometric solvents."

(1) Chafetz, L., and Gaglia, C. A., Jr., *J. Pharm. Sci.*, **56**, 1681(1967).

In the article titled "Nigerian Plants III. Phytochemical Screening for Alkaloids, Saponins, and Tannins" (1), the title should read:

"Nigerian Plants IV. Phytochemical Screening for Alkaloids, Saponins, and Tannins."

(1) Persinos, G. J., and Quimby, M. W., *J. Pharm. Sci.*, **56**, 1512(1967).

In the article titled, "Dissolution Kinetics of Benzoic Acid in High Concentrations of Surface-Active Agents" (1), the following correction should be made:

The term "poloxakol" should read "poloxalkol" throughout the article.

(1) Parrott, E. L., and Sharma, V. K., *J. Pharm. Sci.*, **56**, 1341(1967).

In the article titled "Comparative Study of a New Fluorometric Assay Mestranol Ether in Tablets with GLC and Colorimetric Assays" (1), the following correction should be made:

The title of the article should be changed to "Comparative Study of a New Fluorometric Assay of Mestranol in Tablets with GLC and Colorimetric Assays."

(1) Templeton, R. J., Arnett, W. A., and Jakovljevic, I. M., *J. Pharm. Sci.*, **57**, 1168(1968).

In the article titled "Micellar Solubilization of Testosterone I. In Aqueous Solutions of Polysorbates" (1), the following corrections should be made:

In Tables II and III, page 1123, under the "$\lambda_{max.}$, mμ" columns, decimal points should be inserted after every third figure in the four-figure values.

(1) Thakkar, A. L., and Hall, N. A., *J. Pharm. Sci.*, **56**, 1121(1967).

In the article titled "Selective Acid-Catalyzed Hydrolyses of Methoxysulfanilamidodiazines" (1), the following corrections should be made:

1. In Table II, page 1155 under Supporting Anal. Data for 2-Hydroxy-4-methoxy-6-sulfanilamidopyrimidine, should read: C, 44.59; H, 4.08; N, 18.91. Found: C, 45.08; H, 4.01; N, 19.18.
2. On page 1156, column 2, line 36, should read) [lit. (25), m.p. 105–107°].
3. On page 1156, column 2, line 43, should read [lit. (25), m.p. 175–176°].

(1) Venturella, V. S., *J. Pharm. Sci.*, **57**, 1151(1968).

In the article titled "Cactus Alkaloids IV. Macromerine from *Coryphantha runyonii*" (1), the following footnote should be added on page 515:

L. E. Below was the recipient of the National Merit Lundsford Richardson Award, 1967.

(1) Below, L. E., Leung, A. Y., McLaughlin, J. L., and Paul, A. G., *J. Pharm. Sci.*, **57**, 515(1968).

In the article titled "Conversion of Griseophenone A to (±)-Dehydrogriseofulvin in the Presence of Horseradish Peroxidase and Hydrogen Peroxide" (1), the following correction should be made:

On page 876 under "Discussion" methylacetylphloroglucinol (VII) (Scheme II) should read methylacetylphloroglucinol (VIII) (Scheme II).

(1) Segal, A., and Taylor, E. H., *J. Pharm. Sci.*, **57**, 874 (1968).

In the article titled "Computer Program for a Double Exponential Equation to Determine Biological Constants" (1), the following corrections should be made:

1. On page 171, column 1 of the computer program, line 57 should read: 281 XKD=XKA*R.
2. In column 2, line 77, the entry should read: 440 PAGE = PAGE + 1.

(1) Lowenthal, W., and Vitsky, B. L., *J. Pharm. Sci.*, **56**, 169(1967).

In the book notice for *The Human Adrenal Cortex: Its Function Throughout Life* [*J. Pharm. Sci.*, **57**, 356(1968)], the price should read $4.25 instead of 3.50.